

Assessing the specificity of a herbivore on a plant of uncertain phylogenetic placing: *Listronotus elongatus* a herbivore of *Hydrocotyle ranunculoides*

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Received: 13 May 2016 / Accepted: 2 January 2017 / Published online: 5 January 2017
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Abstract *Hydrocotyle ranunculoides* (Araliaceae) is an aquatic plant native to the Americas and invasive in Europe. This work reports laboratory and garden studies on the host range of its main natural enemy, the weevil *Listronotus elongatus* (Hustache) (Coleoptera: Curculionidae). The genus *Hydrocotyle* is in a disputed phylogenetic situation such that both Apiaceae and Araliaceae must be included in the test list, but lacking a clear criterion of phylogenetic proximity. For this reason three testing instances were applied: no-choice on excised leaves, no choice on functional plants, and a multiple choice garden experiment. A large initial test-plant list was reduced to a smaller list adjusted by a feeding intensity criterion, survival curves and oviposition levels. Feeding was observed on the excised leaves of 21 out of 36 test plants, but as tests progressed to whole plants *L. elongatus* showed it could only develop and thrive on its natural host, and

possibly survive as adults on *Hydrocotyle modesta*. Results confirm that typical laboratory starvation tests can provide a dependable ranking of physiological acceptability of different plant species but severely overestimate the actual host range of a specialized herbivore. They also indicate that the phylogenetic criterion for test plant list construction must be approached cautiously if the phylogeny is uncertain or disputed.

Keywords Floating pennywort · *Listronotus elongatus* · Centrifugal phylogenetic method · Specificity tests · *Hydrocotyle ranunculoides*

Introduction

Water pennywort (*Hydrocotyle ranunculoides* L.f.) is a stoloniferous aquatic perennial that forms dense interwoven mats that float on stagnant or slow moving waters. It is native to the Americas, but has become invasive in the UK, other parts of Europe, and western Australia (Ruiz-Avila and Klemm 1996; Sheppard et al. 2006). Its rapid and thick growth can block watercourses, interfering with navigation, flood control, water oxygenation, and other recreational and ecological functions. Under suitable conditions it can double its biomass in three days (Ruiz-Avila and Klemm 1996).

Handling Editor: S. Raghu.

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Several specialized natural enemies have been identified in southern South America (Cordo et al. 1982; Cabrera Walsh et al. 2013). Among these, *Listronotus elongatus* (Hustache) (Coleoptera: Curculionidae) is the most common and widespread herbivore on the plant. It is also apparently the key herbivore behind the periodical collapse of *H. ranunculoides* patches in wetlands of the Paraná Delta in Argentina (Cabrera Walsh et al. 2013). Adults of this weevil feed on the foliage, and lay eggs inside the petioles, from where the larvae bore their way to the submerged stolons where they complete their development.

Cooperation between CABI-Europe and the FuE-DEI in Argentina (formerly USDA-ARS South American Biological Control Laboratory) began in September 2009, with the objective of studying the natural enemies and ecology of water pennywort in its native range, and select suitable biocontrol agents. Because of its characteristics, *L. elongatus* was the prime natural enemy considered, so its host range was assessed in the field and the laboratory. However, these studies presented an unusual problem in that the genus *Hydrocotyle* L., traditionally in the Apiaceae, has been quite recently placed in the Araliaceae (Apiales) (Plunkett et al. 2004), while other genera of the subfamily Hydrocotyloideae were kept in the Apiaceae, in two new subfamilies, Azorelloideae (mainly cushion plants) and Mackinlayoideae (found in Australasia) (Lowry et al. 2004; Konstantinova and Yembaturova 2010; Liu et al. 2012).

Within the Araliaceae, established intrafamilial relationships do not reflect clades resolved through molecular analyses (Lowry et al. 2004). Molecular evidence suggests that the Hydrocotyloideae are a basally branching, polyphyletic lineage within the Apiales, and that the genus *Hydrocotyle* L. should be considered a basal taxon for the Apiaceae and the Araliaceae (Lowry et al. 2001; Nicolas and Plunkett 2009), or even one subgroup within the Apiales that would include Apiaceae, Araliaceae, Pittosporaceae and Myodocarpaceae (Plunkett et al. 2004). Be as it may, *Hydrocotyle* L. was removed from its historical phyletic entourage, and is currently in an isolated clade with the Australasian genus *Trachymene* Rudge within the Araliaceae (Lowry et al. 2004; Nicolas and Plunkett 2009). Due to this indeterminate phylogeny, the so called “centrifugal phylogenetic method”, used to evaluate the specificity of a potential biocontrol

agent, could not be strictly applied to put together a test plant list for *L. elongatus*. The centrifugal phylogenetic method involves a hierarchical order in the exposure of the candidate biocontrol agent to the plant list, from more to less related plant species (Wapshere 1974; Briese 2005; Sheppard et al. 2005). It often also includes species in associated niches, and crops or protected species that are sometimes remotely related to the target plant, and may consider oviposition or other plant uses, as well as feeding (Heard and van Klinken 1998; Cabrera Walsh et al. 2014). Briese (2003) has warned of the need to consider phylogenies before taxonomy, because the latter does not necessarily reflect the former. Whatever the procedure to build this test-plant list is, the objective is to obtain a sound, significant group of plants that will challenge the feeding range of a potential agent in such a way that no surprise host shifts or host range expansions will be observed in the field after release (Marohasy 1996; Sheppard et al. 2005).

Consequently, it was necessary to include both Apiaceae and Araliaceae *sensu lato* in the test list, but lacking any established criterion to order the plant list according to phylogenetic proximity. The Apiales includes more than 3700 species, so it would be impossible to test all, or even a significant part of the species in the group (<http://taxateca.com>). For this reason three testing instances were applied. For the first step excised foliage was exposed to the adult weevils in petri dishes, because this kind of test is quick, easy, and takes up little rearing chamber space. Through these tests the large initial test-plant list was reduced to a smaller list that was more intensely tested. This intense testing included comparing functional plants to excised foliage and an open garden test, in which the insects were allowed to access the test plants on their own volition (see Briese et al. 2002, for a similar use of insects without enclosure). The novelty of the procedure is that it involved a widely phylogenetic criterion, but adjusted by a feeding intensity criterion. This criterion inevitably involved a degree of arbitration (Cruttwell McFadyen 1998). This type of arbitration is almost always needed in feeding preference tests because feeding results are seldom clear-cut. If specificity were defined as zero feeding on any plant species other than the target (even under a starvation setup in the laboratory), it should be considered very rare indeed. However, if specificity were defined as negligible use of another host (for feeding

or oviposition), then it can be considered quite common. Again, however, the border between negligible and significant non-host feeding is debatable and arbitrated. In the step-by-step procedure described in this work the arbitration process existed, but it was justified by the results of the testing sequence.

Materials and methods

Specificity studies on *L. elongatus* were approached in a three-stage process, beginning with a simple no-choice (or starvation) test using cut foliage test on 35 plant species, devised to select a smaller group of the test plants that underwent substantial feeding. This test was followed by a whole-plant against cut-leaf no-choice test on these selected species, in order to condense further the test-plant list and detect probable artificial results on cut leaves. Oviposition, eclosion and larval development were also recorded in these two tests. Finally, a multiple choice garden test was performed on a yet further reduced, and final, selection of test plants.

Test plant list

There are 20 species of Araliaceae (aside from the 13 species of *Hydrocotyle*), and 136 species of Apiaceae cited for Argentina (Gamerro and Zuloaga 1998, Instituto de Botánica Darwinion, <http://www.darwin.edu.ar/>). It was beyond the capacity of the laboratory to test or even acquire every plant species. Given the nature of our problem (an uncertain phylogenetic placing of the natural host plant) the construction of the list was based on biological and opportunistic considerations, as well as being limited only to plants present in Argentina (Table 1). Because *L. elongatus* is being studied for release in Europe, the list included every cultivated Apiaceae present in the country, every one of which is of Eurasian origin. It also included most European wild Apiaceae present in the country. The list included most of the Araliaceae in Argentina, with at least one species of every genus present. A biological criterion was also used to discard some test plants, such as woody plants in the Azorelloideae, because the mining larva could never bore into species of this group, even in the unlikely event of encounter with these desert plants. Also, as many native Apiaceae as could be found during preliminary

field trips were collected opportunistically for planting in flower pots in the laboratory gardens. Finally, *Pittosporum tobira* (Japanese pittosporum), one of the only two species of Pittosporaceae found in Argentina (the other is *P. tenuifolium* Gaertn.), was included in the test list so as to test at least one species in another of the Apiales families corresponding to the arrangement proposed by Plunkett et al. (2004). Myodocarpaceae were not included because they are not found in South America.

No-choice feeding and oviposition on excised leaves

Starvation tests were performed with *L. elongatus* adults collected in the field from the lower Paraná Delta (roughly S 34°12', W 58°53') in November 2010. Although the weevil could be reared in the laboratory, the rearing techniques were not developed enough, and required too much space to rear a cohort of more than 2000 weevils required to complete the tests. Five replicates of five weevils each were set up per plant species in 14-cm plastic Petri dishes (175 in all). One to five leaves, depending on their size, were cut with as much petiole or stem length as the size of the dishes could accommodate, wrapped at the cut end in ca. 50 cm of tissue paper dipped in Hoagland's nutrient solution. Preliminary tests had shown that leaves remained green and turgid in these conditions for at least a week. The dishes were kept in a rearing chamber with eight vertical TL-D 36 W/54-765 1SL/25 fluorescent lamps, piled haphazardly on the shelves. The rearing chamber had a 14:10 L:D photoperiod, $70 \pm 5\%$ RH, and 20 °C during the dark period and 25 °C during the light period. Every Petri dish was lined with a layer of five paper towels cut to size, and lightly sprinkled with distilled water. This lining served to keep moisture levels stable, afford refuge and water to non-feeding weevils, and prevent condensation in the dish that may trap the weevils or promote rotting of the leaves. There were two controls kept in equal conditions as described for the test plants: one with excised *H. ranunculoides* leaves—the standard against which all feeding levels were compared, and Petri dishes with the humid paper lining alone and no plant material.

Feeding was quantified by counting the number of feeding lesions, and their surface area (in mm²). For this the leaves were pressed under a sheet of

Table 1 Test-plant list for *Listronotus elongatus* showing feeding results

Species	Common name	Life form*	Feeding	Mean feeding (mm ² ± SE)
Apiaceae				
<i>Ammi majus</i>		Th	Negligible	
<i>A. viznaga</i>		Th	Negligible	
<i>Anethum graveolens</i>	dill	Th	Negligible	
<i>Apium graveolens</i>	celery	Th	Low	**
<i>Bowlesia incana</i>		He	0	
<i>Conium maculatum</i>	hemlock	Th	Negligible	
<i>Coriandrum sativum</i>	coriander	Th	Negligible	
<i>Cyclosporum leptophyllum</i>		Th	Negligible	
<i>Daucus carota</i>	carrot	Th	Negligible	
<i>Eryngium ebracteatum</i>		He	Low	9.64 ^d ± 2.59
<i>E. elegans</i>		He	0	
<i>E. horridum</i>		He	0	
<i>E. coronatum</i>		He	Negligible	
<i>E. mesopotamicum</i>		HI	0	
<i>Foeniculum vulgare</i>	fennel	Th	Negligible	
<i>Lilaeopsis carolinensis</i>		Hy	Low	21.98 ^c ± 3.89
<i>L. attenuata</i>		Hy	Negligible	
<i>Pastinaca sativa</i>	parsnip	Th	Negligible	
<i>Petroselinum crispum</i>	parsley	Th	Low	16.16 ^c ± 2.42
<i>Torilis anthriscus</i>		Th	Negligible	
Araliaceae				
<i>Aralia warmingiana</i>		Ph	0	
<i>Dendropanax affinis</i>		Ph	0	
<i>Fatsia japonica</i>	fatsi	Ph	0	
<i>Hedera helix</i>	ivy	Ep	0	
<i>Hydrocotyle bonariensis</i>	largeleaf pennywort	He	Substantial	117.23 ^b ± 14.9
<i>H. bowlesioides</i>		He	Substantial	117.10 ^b ± 15.17
<i>H. exigua</i>		He	Substantial	81.83 ^b ± 10.63
<i>H. modesta</i>		HI	Substantial	364.17 ^a ± 25.51
<i>H. ranunculoides</i>	water pennywort	Hy	Standard	377.86 ^a ± 34.3
<i>H. verticillata</i>		HI	Substantial	80.53 ^b ± 8.86
<i>Schefflera arboricola</i>	umbrella tree	Ph	0	
<i>S. elegantissima</i>		Ph	0	
<i>S. umbellifera</i>		Ph	0	
<i>Tetrapanax papyrifer</i>	rice-paper tree	Ph	0	
Pittosporaceae				
<i>Pittosporum tobira</i>	Japanese pittosporum	Ph	0	

Different letters indicate significant differences in feeding levels (Tukey's post-hoc multiple comparisons, $\alpha = 0.05$)

* Raunkiaer plant life-form: Ep, epiphyte; He, Hemicryptophytes; HI, helophyte; Hy, hydrophyte; Ph, phanerophyte; Th, therophyte

** Not compared statistically because most weevils died within thirteen days

transparent millimetre paper, and inspected under a dissection microscope. The area of each lesion was calculated by counting the number of 1-mm² squares

covered by it. Any feeding damage smaller than one whole 1-mm² square was recorded as 0.5 mm². The area of the damage to filiform or pinnately finely

dissected leaves (such as those of *Cyclosporum leptophyllum* (Pers.) Sprague ex Britton and Wilson, *Lilaeopsis attenuata* (Hook. and Arn.) Fernald, *Ammi visnaga* (L.) Lam., and *Foeniculum vulgare* Mill., Gard. Dict.) could not be measured because every lesion (bite) cut the leaf fragment off, preventing the measurement of the actual leaf tissue eaten, so we considered each lesion to be 0.5 mm². Area meter or photographic methods could not be applied because the feeding lesions did not consistently break through the leaves, nor could they be distinguished from other types of lesions through digital images.

Feeding lesions were counted and measured twice a week, and the leaves replaced. As the inspection periods were not equal, they were summed to a weekly figure for statistical comparison. Feeding levels on plant species that showed weekly lesion averages (in mm²) $\geq 10\%$ the average feeding on *H. ranunculoides* were classified as “substantial”. Feeding levels $\leq 10\%$ $\geq 1\%$ were classified as “low”. Plants with feeding levels $\leq 1\%$ below the standard were considered “negligible”, and were not treated statistically. Plant species that had no feeding marks at all after four weeks were ruled out directly. Differences in weekly weevil feeding data per replicate (in mm²) were statistically analysed on the plant species that showed substantial to low feeding levels. Only the first eight weeks were taken because few test plants allowed weevil survival beyond that period. The data were log-transformed to verify normality, and compared with an ANOVA and Tukey’s post-hoc multiple comparisons (SYSTAT 2004).

In addition to feeding, mortality was accounted for during every inspection. Dead weevils were replaced by randomly selected adults from the garden colonies, so as to maintain equal numbers in each dish, but testing on each plant species was suspended once the total mortality on each test plant reached or exceeded 25. Through this method we maintained comparable feeding and oviposition pressure on each test plant, while obtaining comparable mortality curves. Dead weevils were not replaced in the no-food control. Mortality was followed throughout the experiment until the last of the weevils on *H. ranunculoides* died, but feeding was controlled only until the 25th weevil died on the second preferred test plant.

Mortality curves in weeks were estimated with a stratified Kaplan–Meier model, and compared with log-rank tests. The family-wise error rate was

controlled with Bonferroni adjustments by dividing the critical value by the number of pairwise comparisons (i.e. $0.05/36 = 0.00139$) (IBM SPSS 2010). Plant pairs with significant differences in survival time were grouped in different categories. However, as dead insects were replaced in order to maintain herbivory pressure, the survival model had to assume every dead weevil had been tested from the start. This assumption may lead to underestimate actual mortality of the initial test groups that fed on unacceptable/toxic test plants, and possibly overestimate it in the more accepted test plants, but since the age of the subject weevils at the onset of the test was unknown, this averaging effect was deemed acceptable.

Weekly oviposition was censused by counting the total number of eggs per week per Petri dish. Eggs were sought inside petioles, stems, leaf nerves, and the moist paper wrapping the petioles. The number of eggs per replicate (adding all the eggs of every revision of each Petri dish) was compared using plant species as factors with a Kruskal–Wallis test.

Despite there being an external sexual dimorphism in *L. elongatus* at the antennal insertion, telling the sex of living specimens through this trait was found to be time-consuming and unreliable, especially considering we had to test more than 2000 weevils. So 100 live weevils were frozen to kill them, dissected and sexed in order to estimate the expected sex ratio in our tests at the beginning. Then 100 dead weevils from the feeding tests were also dissected two weeks into the tests.

Excised versus functional plant feeding and oviposition

Hydrocotyle are creepers with the capability to root at each node. Each leaf with a node, root and a fraction of the stolon, can develop as a functional plant in any substrate where it can root and grow. Being easy to grow in Petri dishes, the four plant species that withstood the highest feeding and oviposition (*H. ranunculoides*, *H. modesta*, *H. bonariensis* and *H. bowlesioides*) were subjected to further testing in starvation conditions. Five Petri dishes per test plant, with five weevils each were prepared as described in the previous section. Another five dishes per test plant were prepared in a similar way, but with a section of a runner that included leaves and their corresponding nodes. The roots and stolon section were wrapped in

tissue paper soaked in Hoagland's solution, as in the previous experiment. These were considered functional plants, in the sense described above. The number of eggs laid, leaf-area eaten (in mm²), and mortality per Petri dish were registered for nine weeks, or until 25 weevils died, whatever came first.

Mortality curves were graphed with a stratified Kaplan–Meier model, to obtain survival curves for each treatment and the mean survival in weeks of the first 25 weevils to die in each Petri dish. The survival curves were inspected to verify they did not cross each other, and a Cox proportional hazard model was fitted comparing weeks until death data to evaluate the comparative effect of the treatment (functional or excised) and species (IBM SPSS 2010).

Weekly oviposition was censused by counting the total number of eggs per week per Petri dish. The number of eggs per replicate was tested for normality with Shapiro–Wilk tests (SYSTAT 2004). Oviposition was then compared with an ANOVA, followed by Bonferroni post-hoc comparisons (IBM SPSS 2010).

No-choice oviposition tests were also performed on growing plants rooted in hydrosol inside 3-l plastic jars with ventilated lids. We chose the species that received at least sporadic oviposition in the cut-leaf tests: *H. ranunculoides*, *H. modesta*, *H. bonariensis* and *H. verticillata*. Runners of between 50 and 60 cm were rolled into a spiral and planted in the hydrosol, and allowed to grow for four weeks. Three replicates per species were prepared. After that period four mated females were put into each jar and allowed to feed and oviposit for seven days. After that period they were taken out, so that the plants did not withstand excessive damage that might compromise their health and capability to support larval growth. The plants were incubated in the jars for three weeks until evidence of larval development could be observed. We recorded which species had eggs, and if larval development was observed.

Multiple-choice feeding test

The same four preferred test plants according to the no-choice tests were compared in a multiple-choice garden test: two trays of *H. modesta*, *H. bowlesioides* and *H. bonariensis* growing in 47 × 35-cm squat, perforated trays were placed in random positions in three canvas pools (2 × 1.4 m) with 5 cm of soil, and 5 cm of water, covered with a shading mesh. The trays

were placed on bricks to prevent them from being submerged in the water (they are not aquatic species). The pools had been previously planted with *H. ranunculoides*, which was then allowed to grow around the trays and fill the spaces between them for a month. The mesh was then removed to let an unknown number of *L. elongatus* adults spontaneously invade the pools from the neighbouring weevil populations that thrive in various ponds and pools in the vicinity. Two months later feeding levels on the three plant species were estimated by counting the number of leaves, damaged leaves, and feeding lesions in 30 randomly selected 10 × 10 cm squares delimited by a plastic frame (ten per plant species). The ten measurements per pool and plant were averaged and compared graphically.

Results

No-choice feeding and survival tests

Dissection of weevils yielded 51 males and 49 females among the euthanized weevils, and 52 females and 48 males among the dead ones. Given these ratios it was assumed that all tests had a 1:1 sex ratio, and all oviposition results to be comparable.

Of the 36 test plant species included in the no-choice tests, only the six species of *Hydrocotyle* tested had substantial feeding levels (as described above). Another four species had low feeding levels, and 12 had negligible feeding damage (less than one hundredth of the mean feeding on *H. ranunculoides*). Finally, 13 of the 35 plant species did not show even probing nibbles, including all the Araliaceae and *P. tobira* (Table 1). The six *Hydrocotyle* species and three of the low damage species were analysed statistically. Celery was not included in this analysis because weevils only lived 1.86 weeks (13 days) on it, giving very few data points, and feeding levels were marginal or negligible. There were significant differences in feeding levels within the group of nine species analysed ($F_{8,351} = 64.67$; $P < 0.001$). Feeding levels were significantly higher on *H. ranunculoides* and *H. modesta*. The remaining four *Hydrocotyle* species formed a second group, followed by a third and fourth group constituted by three species of Apiaceae outside the genus (Table 1).

The pairwise comparisons of the survival curves revealed that survival was significantly higher on *H.*

ranunculoides, with a mean of 12.56 weeks, followed by *H. modesta* and *H. verticillata* in a second group, with 9.2 weeks and 7.9 weeks, respectively. All other plant species had lower survival averages (Table 2). In sixth place, forming part of the fourth statistical group, with a mean survival of 4.7 weeks, was the no-food test group. This means survival was higher with water alone than for all the test groups that fed on species outside the *Hydrocotyle* genus. In fact the no-food had the longest surviving specimen next to the control (*H. ranunculoides*) and *H. modesta*, and the last specimen died after 14 weeks (98 days).

Only four plant species, all *Hydrocotyle* spp., were accepted for oviposition by *L. elongatus*. Oviposition was significantly higher on *H. ranunculoides* (total = 1741) than on *H. modesta* (total = 415) ($\chi^2_1 = 20.253$; $P < 0.001$; $N = 30$). *H. verticillata* and *H. bonariensis* were not tested because only four and three eggs, respectively, were laid in them in the course of the test.

Excised leaves versus functional plants

The excised leaves against functional plants comparisons were significantly different among treatments for every species. In the case of *H. ranunculoides* functional plants showed significantly higher feeding levels than excised leaves, and were the highest levels of all. This tendency was reversed for the other three species, as the excised leaves suffered significantly higher feeding than functional leaves (Table 3).

Table 2 Mortality of *Listronotus elongatus* on excised leaves: different letters indicate significant differences in survival curves (log-rank test, after Bonferroni correction, $\alpha = 0.05$)

Plant species	Mean survival time (weeks) \pm SE
<i>H. ranunculoides</i>	12.56 \pm 0.82 ^a
<i>H. modesta</i>	9.2 \pm 0.61 ^b
<i>H. verticillata</i>	7.88 \pm 0.59 ^{bc}
<i>H. bonariensis</i>	6.88 \pm 0.55 ^c
<i>H. exigua</i>	5.64 \pm 0.42 ^d
No food	4.72 \pm 0.56 ^{de}
<i>Lilaeopsis carolinensis</i>	4.34 \pm 0.38 ^{de}
<i>H. bowlesioides</i>	4.4 \pm 0.49 ^{de}
<i>Apium leptophyllum</i>	3.8 \pm 0.32 ^e

Overall comparisons: $\chi^2 = 136.06$, $df = 8$, $P < 0.001$

The survival curves revealed that survival was highest on excised *H. ranunculoides*, with a mean of 9.01 weeks, followed by functional *H. ranunculoides* and excised *H. modesta*, at 5.94 weeks and 5.42 weeks, respectively. All other plant species provided significantly lower life expectancies, but survival was higher on excised than on functional leaves for every species except *H. bowlesioides*, which showed the lowest survival for both excised and functional leaves. The overall test of the Cox proportional hazard model was significant ($\chi^2_4 = 214.5$; $P < 0.001$; $N = 231$). The Wald statistics for each factor, species and treatment, were also significant ($\chi^2_3 = 144.42$; $P < 0.001$; and $\chi^2_1 = 25.46$; $P < 0.001$, respectively). The model then provided three dummy variables generated to show the hazard ratios of the reference level (in this case *Hydrocotyle ranunculoides* hazard rate) to the hazard rates of the other three species: *H. bonariensis* (group 1), *H. bowlesioides* (group 2) and *H. modesta* (group 3), and one dummy variable for the excised to functional hazard ratio. The results indicate that the hazard ratios ($e^{(b)}$) were significant between *H. ranunculoides* and the other three species ($e^{(b)}$ group 1 = 12.63, $P < 0.001$; $e^{(b)}$ group 2 = 62.51, $P < 0.001$; $e^{(b)}$ group 3 = 2.83, $P < 0.001$) as well as between excised and functional plants ($e^{(b)} = 0.46$, $P < 0.001$). The value of $e^{(b)}$ shows the order of magnitude of the difference in hazard rates. This doubled from excised to functional plants within species, and it was roughly 60 times higher for *H. bowlesioides* than for *H. ranunculoides*, 12 times higher for *H. bonariensis* than *H. ranunculoides*, and more than twice for *H. modesta*.

Oviposition in functional *H. ranunculoides* petioles was higher than in any other treatment, and \approx ten times higher than in *H. modesta* (Fig. 1). Oviposition in *H. bonariensis* and *H. bowlesioides* was zero but for four eggs in one excised *H. bonariensis* petiole, so they were not included in the ANOVA. The ANOVA was significant ($F_{3,16} = 17.19$; $P < 0.001$), but ensuing Bonferroni post-hoc comparisons determined that the functional *H. ranunculoides* was significantly different from the other three tests, which were not significantly different among them.

The oviposition test on plants growing in hydrosol resulted in zero eggs in *H. bonariensis* and *H. verticillata*. *H. modesta* received several ovipositions,

Table 3 Results of no-choice feeding tests on excised and functional *Hydrocotyle* spp

Plant species	Treatment	Mean feeding (mm ² ± SE)	t-tests
<i>H. ranunculoides</i>	Excised	396.92 ± 22.07	$t_{88} = 5.06$; N = 90; P < 0.001
	Whole	654.80 ± 45.94	
<i>H. modesta</i>	Excised	553.29 ± 41.0	$t_{88} = 2.18$; N = 90; P = 0.032
	Whole	437.48 ± 33.7	
<i>H. bonariensis</i>	Excised	219.74 ± 28.98	$t_{68} = 3.5$; N = 70; P = 0.001
	Whole	97.63 ± 19.39	
<i>H. bowlesioides</i>	Excised	219.80 ± 23.23	$t_{38} = 4.03$; N = 40; P < 0.001
	Whole	91.55 ± 1.6	

All four species showed significantly different feeding levels between the functional and excised leaves, but the natural host plant was the only case in which feeding on whole plants exceeded that on excised plant tissue

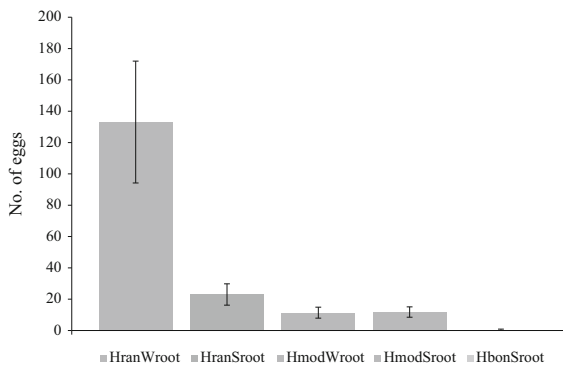


Fig. 1 Mean number of *L. elongatus* eggs (± SE) in the three plant species accepted for oviposition in the functional versus excised leaves test

but the number of eggs could not be counted in the dense mat without damaging the plants. After the incubation period, however, inspections revealed that at least seven eggs had eclosed and some larval development was observed, but every mined leaf died within one week, as did the larvae in them, so the test could not be followed through. *Hydrocotyle ranunculoides* had 27 live larvae at the time of inspection.

Multiple-choice feeding test

The number of feeding lesions and damaged leaves were much higher on *H. ranunculoides* than on *H. modesta*. The low number of replicates precluded the use of ANOVA to compare the results. Non-parametric rank statistics were not applied either because every *H. ranunculoides* case ranked higher than any *H. modesta* case, constituting significant differences by definition.

The number of damaged leaves was 3.2 times higher for *H. ranunculoides* (mean ± SE = 7.7 ± 0.73 vs. 2.3 ± 0.47). Yet, when these data were made relative to the number of leaves per samples (*H. ranunculoides* leaves are larger than *H. modesta* leaves, so fewer leaves per quadrat were sampled of the former) this tendency was multiplied tenfold (mean ± SE = 0.59 ± 0.07 vs. 0.065 ± 0.02). Finally, the number of lesions per quadrat was on average six times higher on *H. ranunculoides* than on *H. modesta* (mean ± SE = 25.2 ± 5.33 vs. 4.2 ± 0.85). *H. bonariensis* and *H. bowlesioides* did not receive any feeding at all in this experiment.

Discussion

There are several examples in nature of chemical herbivory deterrents functioning as attractants and feeding/oviposition stimulants for specialized insects (Nishida and Fukami 1990; Biere et al. 2004; Cabrera Walsh et al. 2008). In this situation the fact that the plant is able to produce and translocate chemical defences could actually increase the weevil's attraction for its host plant, but decrease its attraction to the other test plants. Alternatively, increased feeding on the *H. ranunculoides* ramets with roots may also respond merely to these being healthier and thus more palatable than the cut leaves. However, the first explanation seems more plausible for several motives: this effect should have been noticed in the other test species as well, but instead the opposite happened; plant material was provided in excess in every test; the

period between ramet replacement was quite brief (3–4 days); and, finally, no noticeable wilting was observed within the period between ramet replacement other than that produced by feeding itself.

Defence and/or attraction characteristics may be impaired in excised leaves from being separated from the rest of the plant. This is assumed to be due to the interruption of signalling and compound mobilization routes (Howe and Schaller 2008; Rasmann and Agrawal 2008). This in turn may modify herbivory patterns and/or feeding levels. It has been reported before that bioassays with excised plant tissues need to be interpreted cautiously because the chemical response of the plant can be modified by cutting the communication between the subject tissues and the rest of the plant (Olckers and Hulley 1994; Schmelz et al. 2001). For instance, production in the roots of direct anti-herbivore defences (nicotine) was induced by feeding damage in the leaves of *Nicotiana attenuata* (Baldwin 1999). On the other hand, it has also been reported that specificity tests on herbivores with excised tissues generally provide reliable results, especially with chewing insects (Palmer 1999). Furthermore, there is an additional advantage to excised-leaf tests in that they can override life-form differences among test plants that may prevent the subject organism from accessing the test plant. For instance, if *L. elongatus*, an aquatic weevil, were enclosed among the foliage of a tree, one could well be testing the weevil's climbing aptitudes, rather than its physiological fidelity to the target weed. Our results confirm both statements: that typical laboratory starvation tests can provide dependable results but the results typically indicate a broader than natural host range (Heard and van Klinken 1998; Hinz et al. 2014). Damage levels kept the same preference ranking within the *Hydrocotyle* genus, but feeding was significantly reduced on functional plants compared to cut leaves for the non-target species, whereas it increased significantly in the natural host.

Survival curves also provide significant evidence of the specificity of *L. elongatus*. Survival on *H. ranunculoides* was consistently and significantly higher than on any other test plant. The test weevils that had no food showed a remarkable survival time, with an average of 33 days, and a maximum of 98 days at 20–25 °C. This datum was instrumental to identify that some plants showed toxicity for *L. elongatus*: any survival curve beneath it can be considered to indicate

a species that is in fact toxic to *L. elongatus*. This in the sense that the test plant not only does not provide suitable nourishment, it actually causes higher mortality than having no food at all, even in very small amounts in the order of 1% of the normal feeding levels. Only four *Hydrocotyle* species, including the natural host, came significantly above this ad-hoc “toxicity” threshold. Oviposition levels in functional and rooted plants also validate the notion that *H. ranunculoides* is by far the preferred host of *L. elongatus*, and perhaps the only viable host.

These experiments reinforce the notion that starvation tests, while providing a correct ranking of physiological acceptability (Sheppard et al. 2005), can sorely overestimate the actual host range of a specialized herbivore. Laboratory results of the excised-leaf no-choice tests could indicate that *L. elongatus* may have several, albeit less preferred, alternative hosts. The multiple choice and functional plant tests, however, suggest that the only viable alternative host for *L. elongatus* adults may be *H. modesta*, although much less preferred than its natural host, and incapable of supporting larval development. *Hydrocotyle modesta* is only present in southern South America, and in Argentina has the same distribution as *H. ranunculoides* and grows in close proximity to it (Pedersen 2005). Note must be taken that *L. elongatus* was never collected on this species in the field, nor was *Listronotus*-like feeding damage ever observed on it.

There is a curious result in the significantly lower survival on functional plants than on excised leaves for the natural host, *H. ranunculoides*, and not only for the non-hosts. Several explanations come to mind, such as the accumulation of antifeedants having a toxic effect (Appel and Martin 1992), a higher metabolic rate on the functional plants due to increased feeding and an earlier unburdening of the egg load, or a combination of all these factors. Increased longevity compensating for oviposition delays have been observed in many insect species before (Kehat and Gordon 1975). In any case, specific experiments should be designed to discover the underlying cause. Another contrasting result is the general shorter survival periods in the second series of feeding tests as compared to the first feeding tests. This is easily explained by the fact that the former was begun several weeks after the first series, so the weevil population used in the tests were considerably older. This is no reason to question the validity of the tests in themselves, since each separate

series was simultaneous and was not compared to the other.

It is not unusual for phylogenetic rearrangements to cause genus or family level relocations. Within families with weedy species, some of the recent rearrangements may challenge past test plant lists, e.g.: Bombacaceae to Malvaceae, Plantaginales to Lamiales, Cuscutaceae to Convolvulaceae, Dipsacaceae and Valerianaceae to Caprifoliaceae, Naja-daceae to Hydrocharitaceae, Sonneratiaceae to Lythraceae, Hydrophyllaceae moved from the Solanales to the Boraginaceae (Boraginales); several genera of Euphorbiaceae moved to Phyllanthaceae (<https://florabase.dpaw.wa.gov.au/articles/sequence>). What can be disquieting, as in the case of this study, is that the new taxonomic arrangement may result in a less informative test plant list. The fact that none of the Araliaceae outside the genus *Hydrocotyle* were fed on at all is significant in two ways: for one it suggests that there are compounds in the Apiaceae that are recognized and elicit at least some feeding in the weevil which the Araliaceae and Pittosporaceae tested lack. It also raises some alarms as to the selection of test plants when the systematics of a group is not properly resolved. If the systematic upheaval surrounding the Hydrocotyloideae were ignored, Apiaceae may have never been tested, since testing outside the family of a target plant is not always done, except for cultivated or threatened plants (Briese 2005; Sheppard et al. 2005).

However this is not the first time that a phylogenetic approach does not present a good host range resolution. For instance, in *Ceutorhynchus cardariae* Korotyaev, a gall-inducing weevil considered as biocontrol agent for *Lepidium draba* L., phylogenetic distance of the test species to the target weed did not explain a significant amount of the variation in host preference or suitability. The authors suggested that other factors, such as secondary metabolite profiles and morphological characteristics had to be considered (Hinz et al. 2008).

So far, the accumulation of evidence indicates that *L. elongatus* is a specific and highly damaging candidate for the biocontrol of water pennywort. Some aspects would need further study, such as specificity tests on *Hydrocotyle*, notably *H. vulgaris* L., of the Old World and Australasian flora that could not be tested in South America.

The reliability of controlled tests for long term anticipation of host range in plant-herbivore

relationships is the core of weed biocontrol, and our reliance on phylogenetic relationships has served us well. However, caution must be used to put together a test list when available phylogenies are in any way controversial.

Acknowledgements We wish to thank Juan José Valla and Pablo Pica for several of the plant identifications, three anonymous referees and two editors of the journal for many, many important pieces of advice, and Octavio Bruzzone for statistical help. CABI-Europe financed most of the studies through a Cooperative Agreement of April 2012. Collecting permits to initiate the laboratory colonies were obtained from the DGRN of the Production Ministry of the Province of Entre Ríos, Argentina.

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