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Cover Page Footnote

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Lessons Learned: Rearing the Crown-Boring Weevil, *Ceutorhynchus scrobicollis* (Coleoptera: Curculionidae), in Containment for Biological Control of Garlic Mustard (*Alliaria petiolata*)

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Abstract

In this paper, we describe lessons learned and protocols developed after a decade of rearing *Ceutorhynchus scrobicollis* Nerenscheimer and Wagner (Coleoptera: Curculionidae) in a Biosafety Level 2 containment facility. We have developed these protocols in anticipation of approval to release *C. scrobicollis* in North America for the biocontrol of garlic mustard. The rearing protocol tried to minimize the potential of attack by the adult parasitoid, *Perilitus consuetor* Nees (Hymenoptera: Braconidae), which may be present in field collected *C. scrobicollis* from Europe to prevent inadvertent introduction of parasitoids into North America.

All *C. scrobicollis* used for our quarantine rearing were field collected near Berlin, Germany. We have successfully reared *C. scrobicollis* on caged garlic mustard plants in a growth chamber by alternating temperatures and photoperiods to simulate those in its native range. In Germany, *C. scrobicollis* produces one generation per year and F1 adults emerge in late May. In containment, a new generation of adults emerged an average of 108 days after adults were placed on plants. We found the optimal time spent to collect F1 adults was four weeks after the appearance of the first F1 adult, with 95% of potential adults collected. Simulating a three-month summer aestivation period, followed by a week of fall, and three weeks of winter conditions resulted in optimum levels of oviposition in F1 females. Larvae first hatched 8- to-10 days after adults were placed on plants at 15/14 C day/night temperatures with a 9.5 hour photoperiod. We therefore recommend that *C. scrobicollis* adults are removed from garlic mustard rosettes after 8 days. This will maximize the period of female oviposition while minimizing the time when larvae are available for attack from *P. consuetor*.

Keywords: *Ceutorhynchus scrobicollis*, *Alliaria petiolata*, garlic mustard, biological control of weeds

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Garlic mustard (*Alliaria petiolate* (Bieb.) Cavara & Grande) is a biennial plant in the family Brassicaceae, native to Europe, where it has historically been valued for its medicinal and herbal properties (Grieve 1971). It was first recorded in North America in 1868 (Nuzzo 1993). Since its introduction, garlic mustard has spread to the Northeast, Midwest, and the Pacific Northwest, and is scattered throughout the remaining western United States (Nuzzo 1993). Garlic mustard is now recorded in 37 states in the U.S. and 5 Canadian provinces (UDSA NRCS 2019) and has the potential for wider distribution (Welk et al. 2002). The plant is listed as a noxious weed in eight states in the U.S. (USDA NRCS 2019).

With the capacity for abundant seed production, garlic mustard can rapidly colonize mesic forests to produce dense stands (Meekins and McCarthy 2002) (Fig. 1) and become more competitive than other woody understory species (Meekins and McCarthy 1999) which may reduce native plant diversity (Stinson et al. 2007). The invasion of garlic mustard into native plant communities can disrupt the mutual associations between native tree seedlings and arbuscular mycorrhizal or ectomycorrhizal fungi (Roberts and Anderson 2001, Stinson et al. 2006, Wolfe et al. 2008, Anderson et al. 2010) that are critical for tree growth and survival and can disrupt native legume-rhizobia mutualism (Portales-Reyes et al. 2015).

Figure 1. Garlic mustard invasion of a forested site, Anoka County, MN. May, 2008. (Photo: Steven Katovich, USDA Forest Service).

Garlic mustard thrives in the forest understory and grows among desirable native plants. Current garlic management strategies include eradicating new populations by the labor intensive and expensive methods of hand pulling or cutting. Application of herbicides can result in non-target injury to native plants. Implementation of a biological control program would provide affordable long-term management of garlic mustard without injury to native plant species (Becker et al. 2013).

Currently, three *Ceutorhynchus* Germar (Curculionidae) species are under investigation as potential biological control

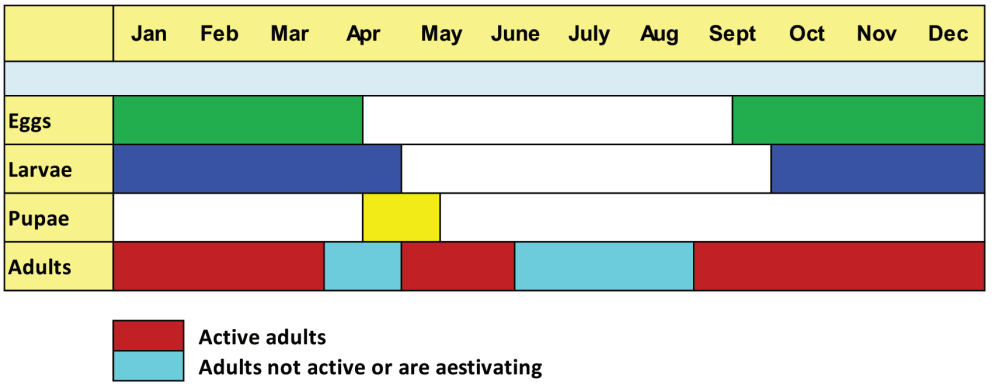


Figure 2. Life cycle of *Ceutorhynchus scrobicollis*. (Redrawn figure, original figure courtesy of Esther Gerber, formerly of CABI Switzerland). Colored bars indicate when life stages are present or active.

agents. Extensive host specificity testing with the crown-mining weevil, *Ceutorhynchus scrobicollis* Nerenscheimer and Wagner has been completed at CABI in Delémont, Switzerland and at a Biosecurity Level 2 (BSL 2) High Containment Facility at the University of Minnesota, St. Paul (Gerber et al. 2009). Results reported in Gerber et al. (2009) and our additional work since (unpublished data) confirm that *C. scrobicollis* is a highly specific herbivore. The USDA APHIS-PPQ Technical Advisory Group for Biocontrol of Weeds (TAG) has reviewed our host range test results and in February 2017 recommended *C. scrobicollis* for release in the U.S. The next steps in the approval process are to ensure compliance with the Endangered Species Act through U. S. Fish and Wildlife Service (USFWS) and the National Environmental Policy Act (NEPA) through the Animal Plant Inspection Service (APHIS) as well as tribal compliance. In Canada, a similar petition was approved in June 2018 and *C. scrobicollis* was subsequently released in the field.

Ceutorhynchus scrobicollis is native to central and eastern Europe and its range extends to the eastern Caucasus region and Ukraine (Colonnelli 2004, Rauth et al. 2011). In Europe, *C. scrobicollis* is only recorded from garlic mustard and field attack rates can reach 100%. Plant height, winter rosette survival and seed production are reduced as a result of *C. scrobicollis* attack (Gerber et al. 2007a, 2007b).

In the field, *C. scrobicollis* produces one generation per year. Oviposition begins in early fall, continues throughout the winter and ends in early spring (Gerber et al. 2009) (Fig. 2). Oviposition ceases if the mean daily temperature drops below –5°C (Gerber et al. 2009). Females lay the majority of eggs directly under the leaf epidermis and in leaf petioles of rosettes (Fig. 3), with a smaller number of eggs laid under the epidermis of the root/crown interface (Gerber et al. 2009).

Figure 3. *Ceutorhynchus scrobicollis* egg in margin of garlic mustard leaf. Eggs are deposited directly under the leaf epidermis. (Photo: Elizabeth Katovich, University of Minnesota).

Figure 4. *Ceutorhynchus scrobicollis* adult. Actual length, 3- to 4-mm. (Photo: Hariet Hinz and Esther Gerber, CABI Switzerland, Bugwood.org).

In the field, adult feeding is insignificant. Larvae progress through three instars, which can be distinguished by the diameter of the head-capsule. In Switzerland, first instar larvae are initially found in late September and by early November, third instar larvae are present. All three instars overwinter in roots and crowns of garlic mustard rosettes and the majority of damage to crowns is caused by larval tunneling. By late April, larvae exit garlic mustard roots and crowns to pupate in the soil. New adults (Fig. 4) emerge from early May to mid-June, feed briefly on garlic mustard leaves, then aestivate for the remainder of the summer (Gerber et al. 2009). Feeding and larval tunneling by *C. scrobicollis* can increase mortality of overwintering rosettes. Alternatively, primary shoots of rosettes can be killed, releasing crown buds from apical dominance. This may result in growth of secondary shoots that are thinner, shorter and produce fewer seeds (Gerber et al. 2007a, 2007b). In captivity, adults may live for one to two years, and up to three oviposition periods have been recorded (Gerber et al. 2009).

Perilitus consuetor Nees (Hymenoptera, Braconidae) has been identified as an endoparasitoid of *C. scrobicollis* adults (Haeselbarth, unpublished), which is its only host. Parasitism rates of up to 20% have been observed in field collected *C. scrobicollis* adults (Gerber et al. 2009). In Switzerland, *P. consuetor* pupae leave their hosts in May and adult parasitoids emerge by late May to mid-June. It is thought that *P. consuetor* adults attack *C. scrobicollis* in the spring or fall (Gerber et al. 2009). We wanted to prevent the inadvertent introduction of parasitoids into North America so while developing a rearing protocol we tried to minimize the potential of attack by the adult parasitoid, *P. consuetor,* which may be present in field collected *C. scrobicollis* from Europe.

Problems encountered with rearing biocontrol insects can become a major obstacle for a weed biological control program (De Clerck-Floate et al. 2008). In this paper we describe garlic mustard propagation methods and *C. scrobicollis* rearing protocols developed in our BSL 2 facility at the University of Minnesota in anticipation of permission to release *C. scrobicollis*. In addition, we conducted experiments to characterize *C. scrobicollis* development in containment to develop the most efficient and consistently reliable methods to rear *C. scrobicollis* from garlic mustard plants, informed and refined over a decade of experience. A method was also developed to screen for the endoparasitoid, *P. consuetor*, from new generations of *C. scrobicollis* to ensure that any potential parasitoids can be eliminated prior to field release in North America.

Materials, Methods, and Results

Garlic mustard propagation. Garlic mustard plants were propagated from seed to support *C. scrobicollis* rearing efforts. Whenever possible, garlic mustard seeds were stratified and plants propagated outdoors, so that they were phenologically similar to field grown plants. However, since *C. scrobicollis* was reared in containment, it was necessary to have a continuous supply of seedlings and plants year round, so seeds were also stratified at 4°C in a cold room and plants propagated in a greenhouse.

All seeds for garlic mustard propagation were collected from Silver View Park, in Mounds View, MN (Lat: 45° 06' 22" N, Long: 093º 13' 00" W). Seeds were cleaned and stored at 4°C. Garlic mustard seeds require cold stratification to break dormancy (Baskin and Baskin 1992). Field stratification consisted of planting seeds in plug trays filled with a standard commercial potting mix (LC8; 70–80% Canadian sphagnum peat moss, 20–25% perlite, 5–10% vermiculite; Sungro Horticulture, Agawam, MA). Trays were placed outside in November in St. Paul, MN and lightly mulched with straw to overwinter. Mulch was removed in early spring (April in Minnesota) when seedlings emerged.

Seeds were stratified in the lab by adding moistened sand to a 90 mm diameter × 15 mm deep plastic petri dish, adding a layer of seeds, then covering the seeds with additional moist sand. Petri dishes were sealed and placed in a refrigerator at 4°C (Baskin and Baskin 1992). After four months, seeds were removed and planted in a plug tray filled with the standard Sungro potting mix described previously. These methods ensured a continuous, year-round supply of seedlings.

Seedlings from both field and lab stratification methods were transplanted into 3.8 l pots containing a commercial rice hull growing mix (BM7; 35% bark: 20% rice hulls: 45% Berger Peat Moss mix, Saint-Modeste, Quebec, Canada). This soil mix was selected because it was purported to provide excellent drainage. We used this soil mix until it was discontinued by the manufacturer. We now use a 30% loam: 30% course sand: 40% peat moss mix provided by our greenhouse services.

Depending on the season, plants were grown outside in a shaded area, or in a greenhouse with a 16 h photoperiod and 21/18°C day/night temperatures. Plants were fertilized with a slow-release fertilizer containing macro- and micro-nutrients (Osmotcote Plus, 15-9-12 plus micronutrients, Scotts Company, Marysville. OH) at the recommended rate. Plants were watered only as needed and care was taken not to overwater plants as this promoted root and foliar diseases. Rosettes were a minimum of three months old when they were used for *C. scrobicollis* rearing.

Aphids were a major problem encountered when propagating garlic mustard in the greenhouse. Secondary pests included the diamondback moth *(Plutella xylostella* L.). We avoided applying insecticides for pest control because they could adversely affect *C. scrobicollis*. To reduce insect problems, multiple garlic mustard potted plants were reared in the greenhouse inside large screened cages 2.4 × 0.9 × 0.9 m, length × width × height, respectively (Fig. 5). These cages consisted of frames built from PVC pipe designed to fit inside a greenhouse bench. "No-see-um" polyester netting was used to construct the screen cages that were placed over the PVC frames. The edges of the cages were secured by folding the netting underneath the frames. Ladybugs (*Hippodamia convergens* Guérin-Méneville) were purchased and placed into the screen cages for aphid control.

Field collection and rearing of *C. scrobicollis* in a containment facility. In our BSL 2 containment facility in Minnesota, all *C. scrobicollis* were reared in growth chambers (Model GR-48, Environmental Growth Chambers, Chagrin Falls, OH, 44022; Model E8, Conviron, Pembina, ND, 58271). Incandescent and fluorescent lighting provided an average light intensity of 250 µmol m^{-2} s^{-1}, similar to the shaded conditions in the outdoor propagating area. *Ceutorhynchus scrobicollis* were reared on individually potted garlic mustard plants covered with a screen cage made of "no-see-um" polyester netting. The netting was placed over wire loops stuck inside the pot at right angles, secured with elastic, and extended approximately 45 cm above the pot surface (Gerber et al. 2009). Plants were placed on plastic saucers and sub-irrigated.

Ceutorhynchus scrobicollis adults were field collected during their oviposition period in the fall, (usually October) in the vicinity of Berlin, Germany (52° 25' 8.6592"N, 13° 11' 13.4952"E). Adults were shipped to our BSL2 containment facility at the University of Minnesota. Shipment sizes varied, but always exceeded the 27 adult minimum, the number of individuals required to capture 99% of the genetic diversity at the Berlin collection site (Rauth et al. 2011). Collecting sufficient quantities to capture the entire genetic diversity of adults at a site increases the robustness of subsequent host range tests (Rauth et al. 2011). After arrival in our containment facility, adults were marked with different colored paint pens to easily dif-

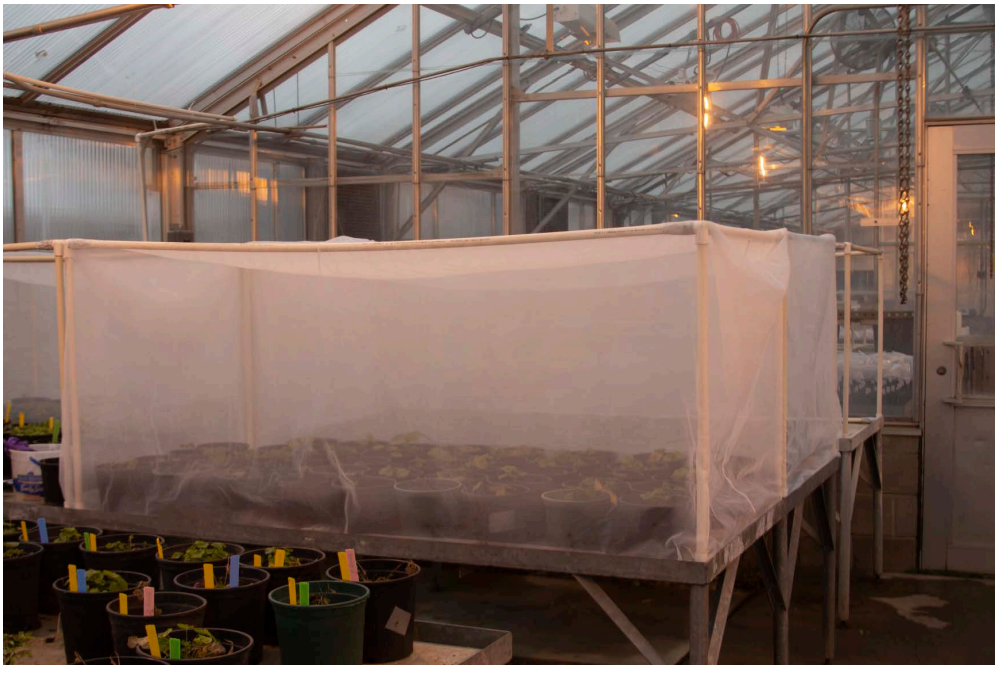


Figure 5. Screen cage used in greenhouse to reduce invertebrate pest problems when rearing garlic mustard plants. (Photo: Elizabeth Katovich, University of Minnesota).

ferentiate between sexes and to distinguish F1 adults (first generation adult progeny of a cohort) from their parents. Males can be distinguished from females by the concave indentation on their ventral abdomen (Gerber et al. 2009).

Upon arrival from Europe, all shipped adults were accounted for and allowed to feed on individually caged potted plants for a minimum of two weeks to allow them to acclimate to growth chamber conditions prior to beginning transfers for rearing. *Ceutorhynchus scrobicollis* are reported to lay the maximum number of eggs at 15°C (Gerber et al. 2002) so plants with insects were placed in a growth chamber at 15/14 °C day/night temperature regime with a 9.5 h photoperiod (Table 1). Photoperiods simulated average winter daylength at Berlin, Germany. (https://www.timeanddate.com/sun/germany/berlin).

In containment, female *C. scrobicollis* received in fall shipments from Berlin were depositing eggs when checked for oviposition after the two week acclimation period. Females continued to lay eggs for four- to five-months after the onset of oviposition. For rearing, we exploited this extended oviposition period by repeatedly transferring mating pairs to new garlic mustard plants

Table 1. Conditions in growth chambers to simulate seasons for the rearing of *Ceutorhynchus scrobicollis* in a Biosafety Level 2 High Containment Facility, University of Minnesota, St. Paul. 2003-2017.

	Temperature[a]		Photoperiod[b]	
Simulated season	Day	Night	Light period	Dark period
	(C)		(h)	
Fall	18	15	13.5	10.5
Winter	15	14	9.5	14.5
Spring	18	15	13.5	10.5
Summer	21	20	16.0	8.0

[a] Relative humidity was maintained at 60–70%.
[b] Light and dark photoperiod total of 24 hours.

Figure 6. Garlic mustard caged plants after *C. scrobicollis* attack (right), compared to a control without *C. scrobicollis* attack (left). The majority of damage to garlic mustard plants is the result of stem, root and crown tunneling by larvae of *C. scrobicollis*, and often results in dieback of above-ground tissue. Three pairs of *C. scrobicollis* were typically placed on each plant for two to three weeks. Shoots would normally regenerate from crown buds after above-ground shoot dieback. (Photo: Elizabeth Katovich, University of Minnesota).

every two to three weeks. The prolonged oviposition period, in combination with the addition and removal of adult pairs to new garlic mustard plants, allowed us to increase the number of plants with eggs for rearing F1 adult progeny. This protocol allowed us to maximize the number of reared F1 adults, despite relatively low adult emergence in growth chamber conditions.

For rearing, typically not more than three pairs of adults were placed on each caged garlic mustard plant. Gerber et al. (2007b) reported that more than four mating pairs reduced number of F1 progeny, as a result of intraspecific competition among larvae. All plants were numbered, and the dates and number of adults added to and removed from each plant were recorded. Adults were removed from plants by sifting through the top layer of soil and manually removing with forceps.

After oviposition, potted garlic mustard plants were maintained in winter conditions until first adult emergence was noted. During that time, rosette top-growth often died back after *C. scrobicollis* larvae mined roots and crowns (Fig. 6), frequently resulting in new lateral shoots arising from crown buds (Fig. 7). Since adults emerging from pupae in the soil litter can be very difficult to find, the first appearance of windowpane feeding on newly expanding garlic mustard leaves was our indicator of when F1 adults (adult progeny of a cohort) had emerged and could be collected. This characteristic "windowpane" was created in the leaf after adults grazed on the epidermis and mesophyll on one leaf surface while the other side remained intact (O`Day et al. 1998) (Fig. 8).

To continue their development, newly emerged F1 adults were removed and placed on new garlic mustard plants, approximately 6 to 8 adults per pot, for a minimum of two weeks in "spring" conditions (Table 1). F1 adults were then placed into three months of "summer" conditions (Table 1) for aestivation followed by one week of "fall" and three weeks of "winter" to facilitate the onset of oviposition. The number and date of F1 adults collected from each plant was

Figure 7. Regrowth from crown buds of a garlic mustard rosette after *C. scrobicollis* attack. (Photo: Elizabeth Katovich, University of Minnesota).

Figure 8. Adult *Ceutorhynchus scrobicollis* windowpane leaf feeding on caged garlic mustard plant. (Photo: Ghislaine Cortat, CABI Switzerland).

Figure 9. Emergence trap used to collect *Ceutorhynchus scrobicollis* adults. Weevils are attracted to the garlic mustard leaf in the syringe tube. Above-ground garlic mustard vegetation was removed following pupation of third-instar larvae but preceding adult emergence. (Photo: Elizabeth Katovich, University of Minnesota).

recorded. *Ceutorhynchus scrobicollis* live for up to three years (Gerber et al 2009), so if all adults are not found and removed in each cycle, different age structures will evolve on individual plants.

Adult *C. scrobicollis* collection and recovery with emergence traps. Finding newly emerging adults is tedious and time consuming, so we designed emergence traps to collect and recover these F1 adults. The emergence traps were modified from a design used by Skinner et al. (2004) and were created from polypropylene funnels (Nalgene™, Thermo Fisher Scientific, Inc., 168 Third Ave., Waltham, MA 02451) that measured 150 mm × 137 mm × 27 mm, top diameter of spout × total height × stem diameter, respectively. The exterior of the funnel was spray painted black leaving the stem unpainted. The inside of the funnel was scored with a narrow tip on a Dremel® rotary tool (Robert Bosch Tool Co., Mount Prospect, Il. 60056) so that adult weevils could crawl up into the funnel. The traps were sized so that when inverted, the mouth of the funnel fit snuggly inside the pot resting on the soil surface covering the opening of each pot containing a garlic mustard plant (Fig. 9).

To attract adults emerging from the soil to the emergence trap, a freshly harvested garlic mustard leaf was placed inside a syringe container (2.5 cm dia × 10.2 cm long polypropylene syringe container for 12 cc Monoject® syringe, without a needle, luer-lock tip, Monoject®. Sherwood Medical Co. St. Louis, MO 63103) and placed over the stem of the funnel. A freshly harvested garlic mustard leaf was kept in place with a 5 mm length cut from a tube of foam pipe insulation for a 1.27 cm diameter pipe, the circumference of the cut piece shortened to a diameter that fit snugly in the syringe container. This arrangement prevented leaves from falling out of the syringe container. Before installing the emergence trap, any remaining green garlic mustard rosette leaves or stems left after larval development were removed from the pot. Since we are working in a quarantine facility, the entire trap was covered with "No-see-um" polyester netting screen cage and secured with elastic around the top of the pot (not shown in Fig. 9). Pots were returned to the growth chamber in winter conditions. During the adult emergence period, traps were checked every two to three days and fresh garlic mustard leaves were placed in the collecting vial, at which time collected adults were removed from the trap and placed onto new garlic mustard plants to feed.

On average, 78% of F1 adults were recovered from traps over the period of adult emergence. This percentage is similar to the average weevil recovery rate from screened plants at CABI in Switzerland. Although not all F1 adults were collected in the emergence traps, it is clearly a more efficient collection method than the alternative of hand sifting through the soil and leaf litter of each individual plant.

Generation time and number of adults produced in two successive cohorts of *C. scrobicollis*. Two successive *C. scrobicollis* cohorts were followed to determine the number of days to emergence of F1 adults, total number of adult progeny produced per cohort, as well as average number of adults produced per plant. Cohort 1 adults were field collected in Germany and received in our containment facility and Cohort 2 was the F1 progeny of Cohort 1. Cohorts 1 and 2 F1 adult progeny were collected from 78 and 115 potted garlic mustard plants, respectively, after the repeated transfer of mating pairs

Table 2. Days to F1 emergence, total number of F1 adults per cohort and mean number of F1 adults per plant when reared in growth chambers in a Biosafety Level 2 High Containment facility, University of Minnesota, St. Paul. 2011–2012.

	Days to F1 emergence		Total numbers F1 adults per plant		
Cohort[a]	Mean	range	mean	range	Total number of adults
Cohort 1	106.1	77-144	4.5	1–16	347
Cohort 2	109.6	75-162	4.7	1–31	539

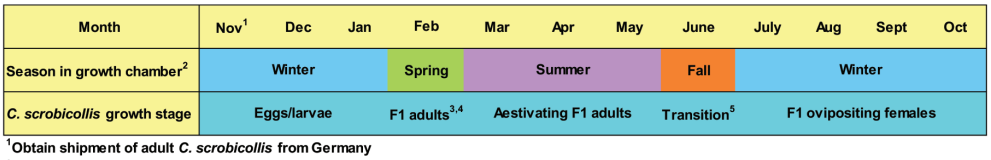
[a] There were 78 individual potted garlic mustard plants monitored for emergence of Cohort 1, and 115 individual plants for Cohort 2. Cohort 1 was the F1 offspring of *C. scrobicollis* field collected in Germany, received and reared in our BSL 2 High Containment facility. Cohort 2 adults were the F1 progeny of the Cohort 1 adults reared in containment.

during *C. scrobicollis* oviposition in our BSL2 facility (as described previously).

For Cohort 1, F1 adult progeny emerged after an average of 106 days (n = 78, SE = 1.9) from the time parents were placed on plants, ranging from 77 to 144 days (Table 2). An average of 4.4, F1 adults emerged from each plant (n = 78 plants, SE = 0.4) with a range of 1 to 16 adults per plant. We reared 347 F1 progeny. For Cohort 2, adults emerged after an average of 110 days (n = 115 plants, SE = 1.5) ranging from 75 to 162 days (Table 2). An average of 4.7 adults emerged per plant (n = 115, SE = 0.5), with a range of 1 to 31 adults per plant. We reared 539 F2 progeny. Generation time from parents to F1 adults averaged 108 days between the two cohorts (Fig. 10). In field reared F1 adults in Delémont, Switzerland, generation time varies, depending on whether eggs are laid in fall, winter or early spring. F1 adults emerge in late May to early June.

Adult emergence in this study was lower than the average of 6.2 adults per plant, recorded for our host range tests conducted earlier under similar conditions, but were highly variable (Katovich, unpublished). The number of *C. scrobicollis* adults recovered from each plant is also lower than reported by Gerber et al. (2009), with an average of 9.2 adults emerging when 2 to 3 females were placed on caged plants for 2- to 4-weeks. Differences in adult emergence between common garden experiments (Gerber et al. 2009) and our results in containment could be the result of less than optimal conditions in growth chambers compared to field settings. Regardless, the repeated transfer method used during oviposition ensured we obtained an increase in *C. scrobicollis* through the multiplier effect of numerous plants exposed to ovipositing females.

Minimizing the number of weeks required to collect *C. scrobicollis* F1 adults. F1 adults emerge over a period of time, so collecting adults from caged plants becomes a laborious, time consuming process. To determine the optimum number of weeks required to collect the majority of F1 adults from caged plants, F1 progeny from a single cohort were collected over the course of their entire emergence period. In this cohort of 71 plants, all parent *C. scrobicollis* had been removed within two to three weeks after initial placement on each plant, which ensured that eggs were of similar age. After F1 progeny were first found, funnel traps were placed on plants and checked every two to three days. Emerging F1 adults were removed and numbers recorded. Traps were checked until adults were no longer found



[1]Obtain shipment of adult *C. scrobicollis* from Germany

[2]Please refer to manuscript for temperature and photoperiod parameters for each growth chamber "season"

[3]Generation time from eggs to adults an average of 108 days in growth chamber.

[4]Paratisoid-free F1 adults may be kept in spring conditions until field release.

[5]Transition between adult aestivation and female oviposition

Figure 10. Development of *Ceutorhynchus scrobicollis* when reared in a growth chamber. St. Paul, MN.

Table 3. Duration of emergence of *Ceutorhynchus scrobicollis* F1 adults from potted garlic mustard plants when reared in growth chambers in a Biosafety Level 2 High Containment Facility. University of Minnesota, St. Paul. 2012.

Weeks after emergence of first F1 observed[a]	Number of F1 adults	Weekly F1 emergence (%)	Cumulative F1 emergence (%)
	(%)	(%)	
1	214	57.7	57.7
2	30	8.1	65.8
3	37	10.0	75.8
4	71	19.1	94.9
5	14	3.8	98.7
6	5	1.3	100.0
Total	371	100.0	

[a]Weekly emergence from 71 individual potted garlic mustard plants.

for two successive collections for each pot. At the end of the collection period, the base of each plant, as well as the surface soil and litter layer, were searched for F1 adults that were added to the number of F1's collected from funnel traps.

All F1 adults had emerged from pots within six weeks after progeny were first recorded and by week four, 95% of adults had emerged (Table 3). Based on these results, we recommend checking plants for four weeks after the first F1 adult is noted. After five or six weeks post emergence, searching for the remaining 5% of F1 adults is not an optimum use of time and labor. Although we determined the length of F1emergence in only one cohort of *C. scrobicollis*, these time periods provide guidelines for future rearing efforts.

Results of the following experiments helped optimize *C. scrobicollis* development and rearing in containment.

Experiment 1. Soil medium providing optimum emergence of ***C. scrobicollis.*** We encountered problems with F1 adult emergence in our growth chambers when using a commercial rice hull soil mix (described previously). Third instar larvae of *C. scrobicollis* exit garlic mustard crowns and use the surrounding soil to build soil pupal chambers. Since the crowns had extensive larval tunneling, we hypothesized that few larvae or pupae were surviving in the soil to emerge as adults. A study was designed to determine the best soil mix to ensure pupa survival and maximize adult emergence. Two treatments tested were 1) a standard rice hull potting mix used to propagate garlic mustard (described previously) and 2) addition of approximately 4 cm of a standard greenhouse soil mix (silt loam: sand: manure: peat, 1:1:1:1, v/v/v/v) covering the soil of the potted garlic mustard plant. Each treatment was replicated 11 times and randomly assigned to a single caged plant as a replicate. Three pairs of marked *C. scrobicollis* adults were placed on each plant for approximately two weeks and were then removed. Plants were maintained in a growth chamber as described previously and number of adults emerging from each plant was recorded.

The addition of greenhouse soil mix to the top of pots resulted in an average of ten F1 adults per pot versus two adults per pot with the commercial rice hull mix. We now routinely add the 1:1:1:1 greenhouse soil mix to the top of the potting mixture. Adding a soil mix with a lower percentage of peat to the top of each pot, while sub-irrigating, could allow the larvae to pupate in drier, warmer soil. Larvae may also prefer the greenhouse soil mix for their pupal chambers rather than the peat-based potting mix.

Soil physical properties, temperature, and moisture level affect insect behavior in the soil (Villani and Wright 1990) as well as pupal survival (Lapointe and Shapiro 1999). Lapointe and Shapriro (1999) document an increase in pupal mortality in the citrus root weevil, *Diaprepes abbreviates* L., at low or high soil moisture levels. Johnson et al. (2010) report that soil temperature as well as soil moisture affect clover root weevil, *Sitona lepidus* Gyllenhal, larval survival in soil. Our experiment was designed to develop a protocol to maximize the number of *C. scrobicollis* reared, not to address causes for differences in adult emergence between soil mixes. Future studies could examine the relationship among soil type, moisture level and temperature on adult emergence.

Experiment 2. Does transfer of *C. scrobicollis* from winter to spring conditions reduce development time? In their native range, *C. scrobicollis* larvae exit from garlic mustard crowns in April and adults emerge from the soil from mid-May to late-June. In growth chambers, F1 adults emerged in simulated continuous winter conditions. To determine whether adults reared

Table 4. Number of *Ceutorhynchus scrobicollis* eggs present in garlic mustard shoots after adults were placed in one, two or three month aestivation periods. Biosafety Level 2 Containment Facility. University of Minnesota, St. Paul, MN 2012.

Length of aestivation (months)	Length of fall/winter (weeks)	Total months	Number of eggs (total)	Number of eggs per shoot[a] (mean)	Feeding
1	1/3	2	3	0.6	+
2	1/3	3	2	0.4	+
3	1/3	4	69	13.8	+
			LDS (0.05)	2.1	

[a]With no aestivation period, an average of 1.7 eggs per shoot were present, which did not differ from 1 or 2 months of aestivation.

in containment would emerge earlier when placed in winter/spring instead of continuous winter conditions, we conducted a study with two treatments. In the first treatment, caged plants with insects were placed into winter conditions in a growth chamber for 2 months followed by 2 months of spring conditions. For the second treatment, caged plants with insects were kept in continuous winter conditions for 4 months. Four to five pairs of weevils were added to plants and F-1 adults were reared as described previously. The experiment was replicated four times, with each replication consisting of one caged plant with weevils added.

When caged plants were transferred from winter to spring conditions, adults emerged approximately one week earlier than when kept in continuous winter conditions (data not shown). An average of seven adults per plant emerged when transferred to spring conditions compared to two adults when maintained in continuous winter conditions. Although not statistically significant (Number of F1 adults: df = 1, $P = 0.28$; Days to F1 adult emergence: df = 1, $P = 0.44$), numerical treads indicate placement of caged plants into spring conditions, following two months of winter may increase the number of F1 adults and reduce the total F1 emergence time.

Experiment 3. Effect of summer aestivation interval on fall oviposition. In Europe, *C. scrobicollis* adults emerge from early-May to mid-June, feed on garlic mustard plants for a short time, then aestivate during the summer. Adults become active in September and begin to lay eggs in mid- to late-September (Gerber et al. 2009). We designed a study to determine the length of summer aestivation that induced females to deposit the greatest number of eggs in our simulated fall and winters when *C. scrobicollis* were reared in growth chambers.

Newly emerged F1 adults were placed onto garlic mustard plants, allowed to feed a minimum of two weeks in spring conditions (Table 1) and then placed into one of three summer aestivation treatments; three months (standard treatment), two months, or one month (Table 4). After completing the aestivation treatments, all caged plants were placed in fall conditions for one week, followed by winter conditions for three weeks. After the winter treatment, adults were removed from garlic mustard plants and placed into an oviposition test in winter conditions.

For the oviposition test, two females and one male (unless otherwise noted) were placed in a glass jar containing a garlic mustard leaf inserted into a piece of saturated florist foam and sealed with no-see-um cloth. After 2 to 3 days, leaves and petioles were dissected and checked for eggs. The number of eggs present per leaf was recorded. A minimum of four replications were completed, with each jar as a replication. Treatment means were separated with a Least Significant Difference test at the 0.05 level of significance.

After one month of summer aestivation, followed by 1 week of fall and three weeks of simulated winter conditions, all adults were feeding on plants, but only a total of three eggs were found out of five replications (Table 4). After two months of aestivation, adults were also actively feeding, but only two eggs were found out of five replications. Following the three-month aestivation period, a total of 69 eggs were found, an average of 13.8 eggs per leaf, a significantly higher number of eggs per leaf than the other aestivation periods (df = 3; $P = 0.002$). It should be noted that females laid a small number of eggs in an oviposition test after only two weeks spring treatment and without receiving an aestivation period

(1.7 eggs per leaf). From this we conclude that females are capable of depositing eggs after they emerge in the spring and prior to aestivation. In summary, the total number of eggs per leaf was highest with the standard three-month summer aestivation treatment, with a total length of time of four months before oviposition commenced (three months of aestivation followed by one week fall plus three weeks of winter).

Experiment 4. Length of *C. scrobicollis* egg stage. The possible presence of *P. consuetor* in field collected *C. scrobicollis* from Germany means that a minimum of one generation should be reared in a containment facility. This will ensure that any potential parasitoids can be eliminated prior to field release in North America. In our *C. scrobicollis* rearing protocol, we continually add mating pairs of adults to garlic mustard rosettes, then remove them after oviposition. *Perilitus* endoparasitoids are known to attack adult or larval hosts but not eggs (Obrycki et al.1985, Shaw 1988). Therefore, we wanted to isolate larvae from possibly parasitized adults to minimize the probability that *P. consuetor* could parasitize these larvae of the F1 generation. To accomplish this, it was critical to determine the length of the *C. scrobicollis* egg stage so that we could maximize the period of female oviposition while minimizing the risk of parasitoid attack to *C. scrobicollis* larvae or F1 adults.

To determine the length of time between *C. scrobicollis* oviposition and eclosion, *C. scrobicollis* adults were field collected near Berlin Germany in October 2018 and received into our containment facility. Plants and insects were maintained in a growth chamber simulating winter conditions of a 15/14 °C day/night temperature regime with a 9.5 h photoperiod (Table 1). This temperature regime was found to be optimum for *C. scrobicollis* oviposition (Gerber et al. 2002). After an acclimation period of approximately two weeks, four adult female and three- to- five male *C. scrobicollis* were placed in caged garlic mustard plants. Adults were added onto a total of eleven plants and the experiment was repeated in time.

After 48 hours, all adults were removed from plants. Starting at eight days after the adults were placed on garlic mustard plants, a single shoot was removed from five randomly selected plants, totalling 5 shoots per day. Shoots were dissected under a dissecting microscope and all eggs/larvae were counted, location on the petiole noted and egg/larvae development stage recorded. Shoots were sampled daily until 15 days after adults were placed onto plants. By this time, too few shoots remained on plants to continue the experiment and the majority of eggs had hatched.

There were no significant differences between the two trials of the experiment, so trials were combined (df = 1; $P = 0.75$). Initial eclosion was noted at 10 days after adults were placed on plants (Fig. 11). This means that eggs could have been 8 to 10 days old, since adults were removed from plants 48 hours after they were added. Since our goal is to minimize the probability that *P. consuetor* will parasitize larvae of the F1 generation, we will routinely remove adults from garlic mustard rosettes within 8 days after they are added, the point when eclosion was first observed. This will minimize the time when larvae are available for attack from *P. consuetor* if they happen to emerge from *C. scrobicollis* adults.

In containment, the majority, 56% of *C. scrobicollis* eggs were laid in the leaf petiole or at the base of the petiole while 44% of eggs were laid in the leaf blade. Similar results were found with larval location, 60% of larvae were located in the petiole or base of the petiole and 40% in the leaf blade, respectively. These results are similar to those reported by Gerber et al (2009) in field grown garlic mustard rosettes.

Conclusions

Ceutorhynchus scrobicollis can be successfully reared on caged garlic mustard plants in a growth chamber by alternating growth chamber temperatures and photoperiods to mimic natural conditions in its native range. In Germany, *C. scrobicollis* produces one generation per year and F1 adults emerge in late May. In containment, a new generation of adults emerged an average of 108 days after parent weevils were placed on plants. After emergence, F1 adults fed on garlic mustard rosettes for a minimum of two weeks before they were placed in a summer aestivation period. We found optimum oviposition after three-months of summer aestivation, followed with a week of fall, and three weeks of winter (four months total). In containment, we were able to produce a generation of *C. scrobicollis* every four months. However, the number of adults produced per plant was lower than that recorded for field reared plants at CABI in Delémont, Switzerland. Finally, we determined that the majority, 95% of potential F1 adults, were collected within the four week period after the first F1 adult appeared as described previously.

Lastly, the possible presence of the adult endoparasitoid, *P. consuetor*, in field collected adult *C. scrobicollis* from Europe will require that a minimum of one generation be reared in containment. This will

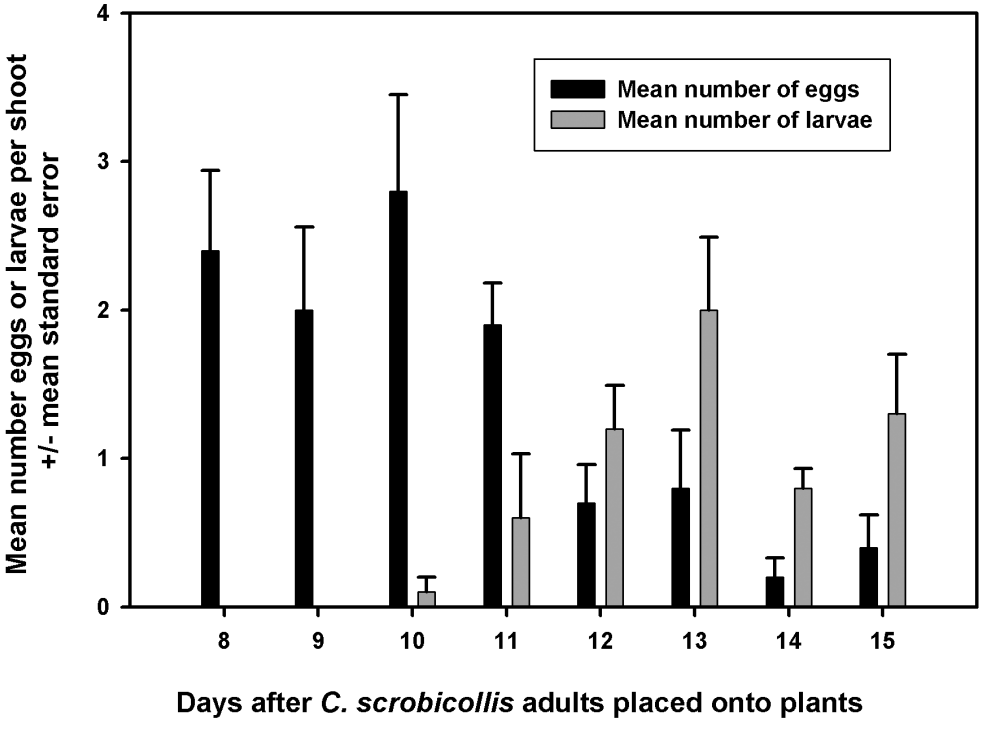


Figure 11. Number of *Ceutorhynchus scrobicollis* eggs and larvae in garlic mustard shoots when sampled from 8-to 15- days after adults were first placed onto rosettes. Plants were maintained at 15/14 °C day/night temperatures with a 9.5-hour photoperiod for the duration of the study. Bars represent mean number of eggs or larvae from combined trials ± SE.

ensure the elimination of potential parasitoids prior to field release of *C. scrobicollis* in North America. Larvae first hatched 8 to 10 days after adults were placed on plants at 15/14°C day/night temperatures with a 9.5-hour photoperiod. In our rearing protocol, we will routinely remove *C. scrobicollis* adults from garlic mustard rosettes within 8 days after they are added. This will maximize the period of female oviposition while minimizing the time when larvae are available for attack from *P. consuetor*, if they happen to emerge from adults.

The rearing methods we have described provide baseline guidance for *C. scrobicollis* rearing in containment. We acknowledge that mass rearing procedures will need to be developed for conditions outside of containment, should *C. scrobicollis* be approved for field release in the U.S.

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Literature Cited

Anderson, R. C., M. R. Anderson, J. T. Bauer, M. Slater, J. Herold, P. Baumhardt, and V. Borowicz. 2010. Effect of removal of garlic mustard (*Alliaria petiolata*, Brassicaceae)

on arbuscular mycorrhizal fungi inoculum potential in forest soils. Open Ecology Journal. 3: 41–47.

Baskin, J. M. and C. C. Baskin. 1992. Seed germination biology of the weedy biennial *Alliaria petiolata*. Natural Areas Journal. 12: 191–197.

Becker, R., E. Gerber, H. L. Hinz, E. Katovich, B. Panke, R. Reardon, M. Renz, and L. Van Riper. 2013. Biology and biological control of garlic mustard. Forest Health Enterprise Team. USDA-Forest Service. FHTET-2012-05. 61 pages.

Colonnelli, E. 2004. Catalogue of the Ceutorhynchinae of the World. Argania editio S. C. P. Barcelona, Spain. 124 pages.

De Clerck-Floate, R., H. Hinz, T. Heard, M. Julien, T. Wardill, and C. Cook. 2008. Preliminary results of a survey on the role of arthropod rearing in classical weed biological control. In: Proceedings of the XII International Symposium on Biological Control of Weeds. M. H. Julien, R. Sforza, M. C. Bon, H. C. Evans, P. E. Hatcher, H. L. Hinz, and G. B Rector. (Eds.), pp 528–534. CAB International: Wallingford. UK.

Gerber, E., H. L. Hinz, M. Schat, G. Cortat, N. Guazzone, and L. Lovis. 2002. Biological control of garlic mustard, *Alliaria petiolata*. Annual Report. CABI-BioScience: Switzerland.

Gerber, E., H. L. Hinz, and B. Blossey. 2007a. Interaction of specialist root and shoot herbivores of *Alliaria petiolata* and their impact on plant performance and reproduction. Ecological Entomology. 32: 357–365.

Gerber, E., H. L. Hinz, and B. Blossey. 2007b. Impact of the belowground herbivore and potential biological control agent, *Ceutorhynchus scrobicollis* on *Alliaria petiolata* performance. Biological Control. 42: 355–364.

Gerber, E., G. Cortat, H. L. Hinz, B. Blossey, E. Katovich, and L. Skinner. 2009. Biology and host specificity of *Ceutorhynchus scrobicollis* (Curculionidae; Coleoptera), a root-crown mining weevil proposed as biological control agent against *Alliaria petiolata* in North America. Biocontrol Science and Technology. 19: 117–138.

Grieve, M. 1971. A Modern Herbal. Dover, NY.

Johnson, S. N., P. J. Gregory, J. W. McNicol, Y. Oodally, X. X. Zhang and P. J. Murray. 2010. Effects of soil conditions and drought on egg hatching and larval survival of the clover root weevil (*Sitona lepidus*). Applied Soil Ecology 44: 75–79.

Lapointe, S. L. and Shaprio, J. P. 1999. Effect of soil moisture on development of *Diaprepes abbreviatus* (Coleoptera: Curculionidae). Florida Entomologist. 82: 291–299.

Meekins, J. F. and B. C. McCarthy. 1999. Competitive ability of *Alliaria petiolata* (garlic mustard, Brassicaceae) an invasive, nonindigenous forest herb. International Journal of Plant Science. 160: 743–752.

Meekins J. F. and B. C. McCarthy. 2002. Effect of population density on the demography of an invasive plant (*Alliaria petiolata*, Brassicaceae) populations in a southeastern Ohio forest. American Midland Naturalist. 147: 256–278.

Nuzzo, V. 1993. Distribution and spread of the invasive biennial *Alliaria petiolata* (garlic mustard) in North America. In B.N. McKnight (Eds.) *Biology pollution: the control and impact of invasive exotic species.* Indiana Academy of Science. Indianapolis, IN.

Obrycki J. J., M.J. Tauber, and C.A. Tauber. 1985. *Perlitis*-Coccinellae (Hymenoptera, Braconidae) Parasitization and development in relation to host-stage attacked. Annals of the Entomological Society of America 78, 852–854.

O'Day, M., A. Becker, A. Keaster, L. Kabrick, and K. Steffey. 1998. Corn insect pests. A diagnostic guide. University of Missouri Extension Manual. No. 166.

Portales-Reyes C., T. Van Doornik, E. H. Schultheis, and T. Suwa. 2015. A novel impact of a novel weapon: Allelochemicals in *Alliaria petiolata* disrupt the legume-rhizobia mutualism. Biological Invasions. 17: 2779–2791.

Rauth, S. J., H. L. Hinz, E. Gerber, and R. A. Hafbauer. 2011. The benefits of pre-release population genetics: A case study using *Ceutorhynchus scrobicollis*, a candidate agent of garlic mustard, *Alliaria petiolata*. Biological Control. 56: 67–75.

Roberts, K. J. and R. C. Anderson. 2001. Effect of garlic mustard [*Alliaria petiolata* (Beib. Cavara & Grand)] extracts on plants and arbuscular mycorrhizal (am) fungi. American Midland Naturalist. 146: 146–152.

Shaw, S.R. 1988. Euphorine Phylogeny—The Evolution of Diversity in host-utilization by parasitoid wasps (Hymenoptera, Braconidae). Ecological Entomology 13, 323–335.

Skinner, L. C., D. W. Ragsdale, R. W. Hansen, M. A. Chandler, and R. D. Moon. 2004. Temperature-dependent development of overwintering and *A. nigriscutis* (Coleoptera: Chrysomelidae): Two flea beetles introduced for the biological control of leafy spurge, *Euphorbia esula*. Environmental Entomology. 33(2): 147–154.

Stinson, K.A., S. A. Campbell, R. J. Powell, B. E. Wolfe, R. M. Callaway, G. E. Thelen, S. G. Hallett, D. Prati, and J. N. Klironomos. 2006. Invasive plant suppresses the growth of native tree seedlings by disruption

belowground mutualisms. PLOS Biology. 4: 1–5.

Stinson, K., S. Kaufman, L. Durbin, and F. Lowenstein. 2007. Impacts of garlic mustard invasion on a forest understory community. Northeastern Naturalist. 14: 73–88.

USDA, NRCS. The PLANTS Database (http://plants.usda.gov), October 2019). National Plant Data Team, Greensboro, NC 27401-4901 USA.

Villani, M. G. and R. J. Wright. 1990. Environmental influences on soil macroarthropod behavior in agricultural systems. Annual Review of Entomology 35: 249–269.

Welk, E., D. Schubert, and M. H. Hoffmann. 2002. Present and potential distribution of invasive garlic mustard (*Alliaria petiolata*) in North America. Diversity and Distribution. 8: 219–233.

Wolfe, B. E., V. L. Rodgers, K. A. Stinson, and A. Pringle. 2008. The invasive plant *Alliaria petiolata* (garlic mustard) inhibits ectomycorrhizal fungi in its introduced range. Journal of Ecology. 96: 777–783.