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Research

3D X-ray analysis of the subterranean burrowing depth and pupal chamber size of *Laricobius* (Coleoptera: Derodontidae), a specialist predator of *Adelges tsugae* (Hemiptera: Adelgidae)

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The non-native hemlock woolly adelgid (HWA), *Adelges tsugae* Annand (Hemiptera: Adelgidae), has caused a significant decline of eastern hemlock, *Tsuga canadensis* L. (Pinales: Pinaceae), and Carolina hemlock, *Tsuga caroliniana* Engelmann (Pinales: Pinaceae), in eastern North America. Biological control of HWA has focused on the use of 2 *Laricobius* spp. (Coleoptera: Derodontidae), natural predators of HWA, which require arboreal and subterranean life phases to complete their development. In its subterranean phase, *Laricobius* spp. are subject to abiotic factors including soil compaction or soil-applied insecticides used to protect hemlock from HWA. This study used 3D X-ray microcomputed tomography (micro-CT) to identify the depth at which *Laricobius* spp. burrows during its subterranean lifecycle, characterize pupal chamber volume, and determine whether soil compaction had a significant effect on these variables. The mean burrowing depth in the soil of individuals was 27.0 mm ± 14.8 (SD) and 11.4 mm ± 11.8 (SD) at compaction levels of 0.36 and 0.54 g/cm^3, respectively. The mean pupal chamber volume was 11.15 mm^3 ± 2.8 (SD) and 7.65 mm^3 ± 3.5 (SD) in soil compacted at 0.36 and 0.54 g/cm^3, respectively. These data show that soil compaction influences burrowing depth and pupal chamber size for *Laricobius* spp. This information will help us better identify the effect of soil-applied insecticide residues on estivating *Laricobius* spp. and soil-applied insecticide residues in the field. Additionally, these results demonstrate the utility of 3D micro-CT in assessing subterranean insect activity in future studies.

Key words: Adelges tsugae, Derodontidae, biological control, X-ray imaging, micro-CT

Introduction

The hemlock woolly adelgid (HWA), *Adelges tsugae* Annand (Hemiptera: Adelgidae), is a non-native invasive pest on eastern hemlock, *Tsuga canadensis* L. (Pinales: Pinaceae), and Carolina hemlock, *Tsuga caroliniana* Engelmann (Pinales: Pinaceae). In eastern North America, HWA was first found near Richmond, VA, in 1951 (Gouger 1971) and has since spread to 21 other states and 1 Canadian province (Kantola et al. 2019, Foley et al. 2021). HWA uses specialized mouthparts to consume nutrients from storage parenchyma cells at the base of needles (Young et al. 1995), leading to a progression of symptoms starting with premature needle drop and branch dieback. If infestation levels are high and persist over multiple years, substantial host mortality occurs (McClure 1991).

The impacts of HWA can be mitigated using an integrated pest management (IPM) approach, which employs chemical treatments on specific infested trees, while releasing biological control agents on untreated infested trees within the same stand (Mayfield et al. 2020). Chemical applications of neonicotinoid insecticides (e.g., imidacloprid) are effective at reducing the impact of HWA and are mostly applied at or below the soil surface of infested trees (Coots et al. 2013, Eisenback et al. 2014). The pursuit for host-specific classical biological control agents for HWA began in the 1990s (Sasaji and McClure, 1997). To date, 8 species have been evaluated, approved, and released (Foley et al. 2021). Of these agents, *Laricobius nigrinus* Fender (Coleoptera: Derodontidae) and *Laricobius osakensis* Montgomery and

Shiyake (Coleoptera: Derodontidae) have been focal predators in the HWA biological control program over the past 2 decades (Salom et al. 2012, Foley et al. 2021).

Laricobius nigrinus and *L. osakensis* are univoltine, cool-weather-active insects that occupy both arboreal and subterranean habitats, depending on their developmental stage, time of year, and host life cycle (Foley et al. 2021, 2022a). Synchronous with the life cycle of their prey, both adult and larval life stages consume HWA (Vieira et al. 2011, Foley et al. 2022a, 2022b). Adult *Laricobius* spp. oviposit into HWA sistens ovisacs between February and April, and once hatched, larvae feed on HWA eggs (Zilahi-Balogh et al. 2003b, Foley et al. 2022b). When matured to the pre-pupal stage, larvae drop from the trees to the soil surface and search for an optimal location to burrow into and undergo pupation (Zilahi-Balogh et al. 2003b, Foley et al. 2021). Once underground, *Laricobius* spp. pupate, eclose to the adult stage, and spend a prolonged period (>5 months) in estivation (Zilahi-Balogh et al. 2003a, b).

Soil conditions vary greatly within and between eastern and Carolina hemlock sites. Eastern hemlocks are commonly found in forest flats and coves where the soil is moist, acidic, receives good drainage (Godman and Lancaster 1990, Rentch et al. 2000, Orwig et al. 2002) and varies in soil compaction (A.P.H., personal observation). Carolina hemlocks are often found at higher elevations, along exposed ridges with rocky, dry, and acidic soil (Rentch et al. 2000). In the southern Appalachians, greater soil organic layer depth is associated with lower survivorship of *Laricobius* spp., and increases in soil moisture are associated with increased survivorship (Foley et al. 2022a). Survivorship of *Laricobius* spp. in the field was found to be ca. 50% less than observed in laboratory-rearing facilities (Foley et al. 2022a).

One challenge to survivorship for *Laricobius* spp. is the relatively high mortality (*L. nigrinus* 40% and *L. osakensis* 34%) observed during their subterranean life stage in the laboratory (Foley et al. 2021). One way in which *Laricobius* spp. provides protection for itself in preparation for undergoing pupation is by forming a cell or chamber around itself by compacting soil particles, within which the insect assumes a c-shape until adulthood (Clark and Brown 1958, Salom et al. 2012). After *Laricobius* spp. ecloses to adulthood (14 days), it remains in the soil until it emerges in the fall (Zilahi-Balogh et al. 2003a). It remains unclear how deep the larvae burrow into the soil to complete pupation, the volume of their pupal chambers, and whether there is any movement by the insects during their estivation stage. Findings from Foley et al. (2022a) suggest that compaction can be important to insect survival. Because systemic insecticides used to control HWA are applied to the soil surrounding infested trees, understanding the depth to which these insects burrow to complete their estivation will allow managers to select chemical application techniques that pose the least amount of risk to the beetle's survival. In addition, learning more about the pupal chambers of *L. nigrinus* and *L. osakensis* could reveal why there is such high mortality at this crucial life stage.

The subterranean nature of *Laricobius* spp. estivation makes it difficult to study and document these life stages. Destructive sampling methods have the potential to damage the insect's pupal chamber as well as lead to inaccurate burrowing depth measurements. The use of microcomputed tomography (micro-CT) provides investigators with a nondestructive technique to collect volume, relative density, and geometric data from concealed samples in 3D (Tracy et al. 2010, 2012, Herhold et al. 2019, Jansson et al. 2021). Micro-CT has been successfully used to track the activity and burrowing structures of subterranean life stages of insects such as wireworms (Coleoptera: Elateridae) (Booth et al. 2020). Using micro-CT imaging, wireworms were successfully identified within a soil microcosm along with their burrowing activities over a period of time and rendered into 3D images (Booth et al. 2020). The objective of this study was to use 3D reconstructions of scanned soil tubes to capture the depth at which *Laricobius* spp. burrow to complete estivation, the volume of their pupal chambers, whether they move as estivating adults, and test the effect of different soil compaction levels on these variables.

Materials and Methods

Twelve 70 × 26 mm clear plastic tubes were created from polyethylene terephthalate tubing, with a wall thickness of 0.5 mm fixed to 3-cm-diameter plastic caps (Thornton Plastics, Salt Lake City, UT), using water-resistant epoxy (Gorilla Glue, Inc). A soil mixture of 2:1 sifted peat moss: sand at 30–35% moisture measured by weight was used as the pupation medium. The soil compaction used in containers at the Virginia Tech Mass Rearing Facility (Salom et al. 2012, Foley et al. 2021) to mass produce *Laricobius* spp. is 0.36 g/cm^3; therefore, this value was used as a compaction treatment for 6 of the 12 experimental units. This compaction was achieved by first placing about 1.5 cm of pupation medium on the bottom of the container and pressing down firmly to create a dense bottom layer to collect any pooling moisture. The remaining soil was placed on top of the bottom layer and settled by lightly tapping the base of the tube on a benchtop (Salom et al. 2012). To achieve consistency, the tubes were weighed, and soil additions were adjusted by using a stainless-steel rod to compress the soil until desired compaction was achieved.

The second compaction treatment (0.54 g/cm^3) was applied to the other 6 experimental units. This higher compaction intensity was achieved by following the same procedure as before, but with increased pressure from the stainless-steel rod. Compaction consistency was achieved by using a soil penetrometer (Gilson Pocket Penetrometer HM-500, Global Gilson, Lewis Center, OH), to measure the unconfined compressive strength of the soil measured at a depth of 0.635 cm. Bulk density was calculated by dividing the mass of the dry soil (22.3 g for soil tubes with bulk density of 0.36 g/cm^3 and 28.3 g for soil tubes with a bulk density of 0.54 g/cm^3) by its volume (using the equation $V = \pi r^2 h$, where π is equal to 3.14, r = radius of the tube, and h = height of the tube).

Five pre-pupal, laboratory-reared *Laricobius* spp. larvae were placed into each tube (experimental unit) between 3 and 5 May 2021 for a total of 60 larvae. From 5 May to 6 July 2021, conditions were held at 13 °C, 12:12 (L:D), and 65% RH. From 7 July to 10 October 2021, temperatures were increased to 19 °C,14:10 (L:D), and 65% RH to mimic field conditions, and then back to 13 °C from 11 October to 11 November 2021 (Salom et al. 2012, Foley et al. 2021). Individual larvae were not differentiated between *L. nigrinus* and *L. osakensis* for this study due to the inability to differentiate between species in the larval stage.

Scanning Procedures

Tubes were separated into 6 pairs, with both compaction treatments represented in each pair. Each tube was scanned only once due to concerns about the effect of radiation on the insects. Based on the observed development of *Laricobius* spp., we divided the estivation period into six 30-day intervals to capture the insect at different life stages (Zilahi-Balogh et al. 2003a). Scans were done every 30 days starting in June 2021, with a new pair of tubes scanned at each sample date. Before scanning, each tube was covered with Parafilm M (Fisher Scientific, Hampton, NH) and secured to a rotating metal base using removable mounting putty. Using a SkyScan 1172

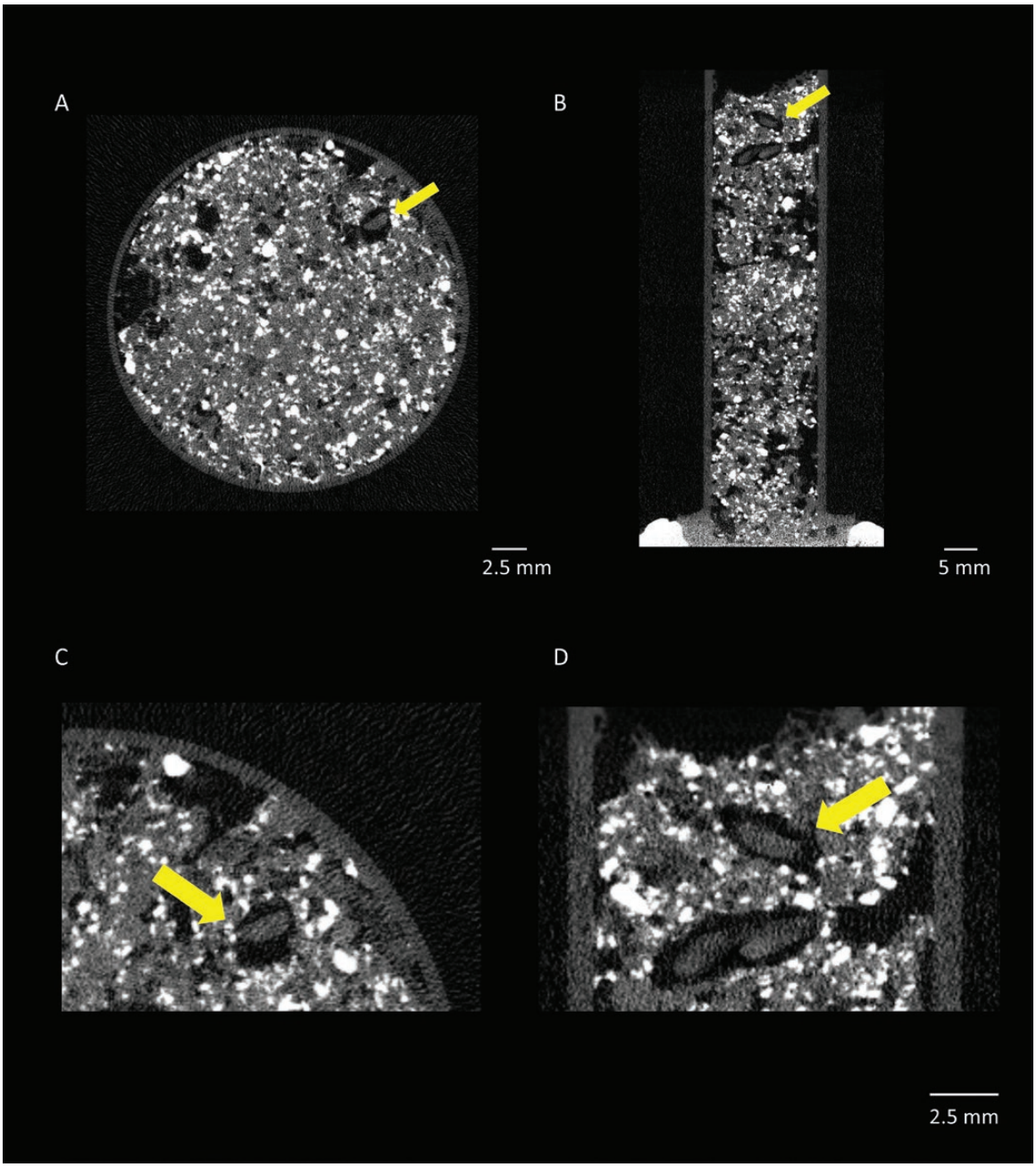

Fig. 1. Location of larvae in their pupal chambers within experimental soil containers. 3D Slicer software was used to visualize horizontal (A, C) and vertical (B, D) image slices from a reconstructed X-ray scan at day 48. Individual *Laricobius* spp. larvae are indicated using arrows. The original X-ray scans were obtained using a SkyScan 1172 Micro-CT machine set to 100 kV and 100 µA with a 0.5-mm aluminum filter.

micro-CT machine set to 100 kV and 100 µA with a 0.5-mm aluminum filter, each tube was scanned with a small pixel size of 17.4 µm for 9 h 48 min (Tracy et al. 2012). Due to the relatively large specimen size, each scan used an offset camera mode at 2× scanning size, enabling a larger field of view. This mode allowed us to capture the entire sample without decreased resolution.

Image Preparation and Analysis

For each sample, between 3,000 and 3,300 images were captured and uploaded into the SkyScan 1172 reconstruction software, NRecon (2020 Micro Photonics Inc.). Reconstruction adjustments included a misalignment compensation of –2.5, ring artifact reduction of 10, and beam-hardening correction of 55%. Using FIJI software (Schindelin et al. 2012), entire data sets were imported as an image stack (with typical dimensions of 1721 × 1706 px and 3160 images) and organized sequentially to examine the location of the insects within the scan. Notes were made about the general range of images that contained each of the 5 individual larvae per scan. Fiji was then used to simultaneously crop and adjust the brightness on all images within the stack before converting to an 8-bit image sequence and uploaded into the 3D Slicer program (Fedorov et al. 2012). Within 3D Slicer, the "Slicer Morph" module was used for data visualization and analysis. Image sequences could be viewed in both horizontal and vertical slices (Fig. 1). Utilizing these ranges of view, individual larvae and their surrounding pupal chambers were identified. The

burrowing depth was measured from the lowest visible portion of the insect to the lowest visible point of the soil surface directly above it. Pupal chamber volumes were calculated by segmenting each pupal chamber, rendering them in 3D (Fig. 2), and using "Segment Statistics" within the 'Quantification' module of 3D Slicer. Segment Statistics utilized a binary labelmap representation of the pupal chamber segment to calculate volume in cubic millimeter (Fedorov et al. 2012).

Statistical Analysis

Data were tested for normality and homogeneity of variance using a Shapiro–Wilk test where $P < 0.05$. Data were log-transformed prior to analysis with a linear model to describe the effect of soil compaction and scanning interval on burrowing depth and pupal chamber volume. Analysis of variance (ANOVA) was then used to evaluate the effect of scanning interval and soil compaction on burrowing depth and pupal chamber volume with a cutoff of significance at 0.05 (version 3.3.2, R Core Team 2019).

Results

Individual *Laricobius* spp. and their pupal chambers were identified and rendered in 3D using micro-CT imaging and 3D image analysis software (Fig. 2). Burrowing depth was significantly different between compaction levels ($F = 23.6758$; df = 1, 45; $P = 0.00001436$) (Fig. 3). The mean burrowing depth in the soil of individuals was 27.0 mm ± 14.8 (SD) and 11.4 mm ± 11.8 (SD) at soil compaction levels of 0.36 and 0.54 g/cm^3, respectively. Mean pupal chamber volumes were significantly different between compaction levels ($F = 15.6626$; df = 1, 45; $P = 0.0002661$) (Fig. 4). The mean pupal chamber volume in soil was 11.15 mm^3 ± 2.8 (SD) and 7.65 mm^3 ± 3.5 (SD) at soil compaction levels of 0.36 and 0.54 g/cm^3, respectively. Neither burrowing depth ($F = 0.9187$; df = 1, 45; $P = 0.3429$)

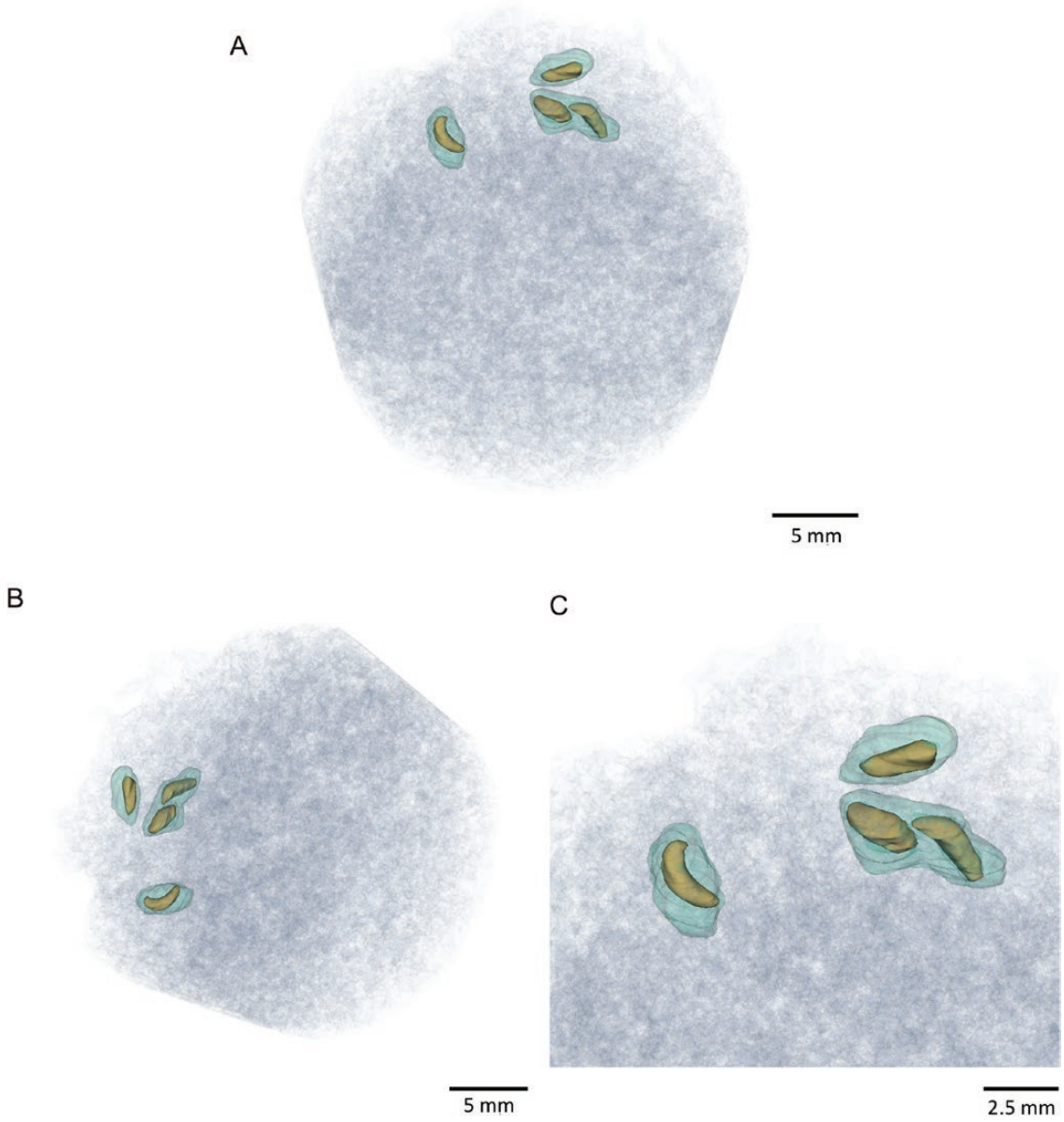

Fig. 2. A three-dimensional visualization of a portion of a specimen tube in anterior A), left B), and zoomed-in C) views with soil, pupal chambers, and *Laricobius* spp. rendered using 3D Slicer software.

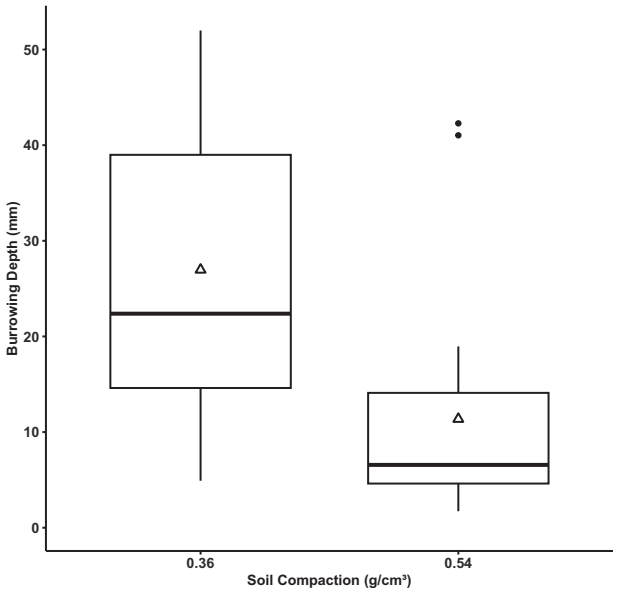

Fig. 3. Box plot depicting the burrowing depth (millimeter) of *Laricobius* spp. in relation to soil compaction (gram per cubic centimeter). The horizontal lines within the boxes represent the median burrowing depths, the triangles represent the means, and the lines above and below the boxes are the SE values. $N = 29$ for 0.36 g/cm^3 and $N = 19$ for 0.54 g/cm^3.

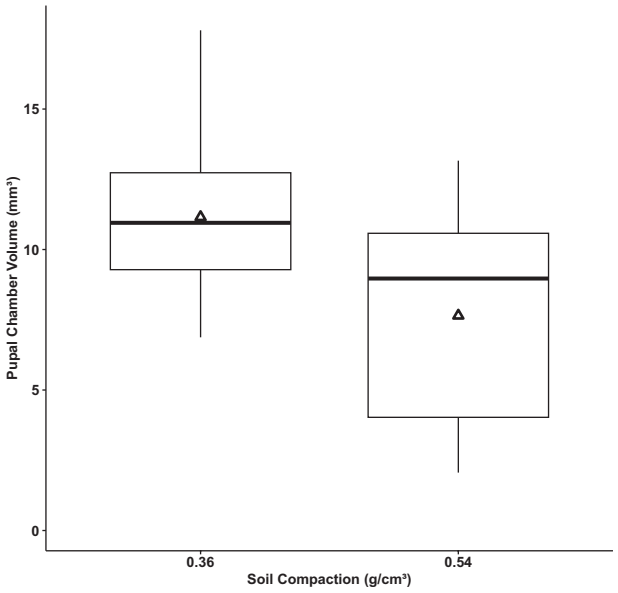

Fig. 4. Box plot depicting *Laricobius* spp. pupal chamber volume (cubic millimeter) in relation to soil compaction (gram per cubic centimeter). The horizontal lines within the boxes represent the median pupal chamber volume, the triangles represent the means, and the lines above and below the boxes are the SE values. $N = 29$ for 0.36 g/cm^3 and $N = 19$ for 0.54 g/cm^3.

nor pupal chamber volume ($F = 0.6714$; df = 1, 45, $P = 0.4168920$) varied over time. Additionally, there was not any evidence of empty pupal chambers or trails of movement in the soil. Both lines of evidence suggest that beetles do not move during estivation.

Discussion

This is the first study to document the impact soil compaction has on the subterranean burrowing depth and pupal chamber volume of *Laricobius* spp. On average, *Laricobius* spp. in containers with a soil compaction of 0.36 g/cm^3 burrowed 42.2% deeper than those in containers with a compaction of 0.54 g/cm^3. Similarly, pupal chamber volumes of *Laricobius* spp. were 68.6% smaller in tubes with the higher compaction soil treatment than those in containers with less compacted soil.

A previous study that assessed the impact of machinery-wheel-induced soil compaction on the western corn rootworm, *Diabrotica virgifera virgifera* LeConte (Coleoptera: Chrysomelidae), found that as compaction increased, subterranean survival of the western corn rootworm decreased (Ellsbury et al. 1994). Higher compacted soil created conditions that decreased the ability of western corn rootworm to move throughout the soil. Our findings are consistent with this study, as *Laricobius* spp. movement (as reflected in burrowing depth) was reduced by increased soil compaction.

Based on development studies of *L. nigrinus* and *L. osakensis*, estivation typically takes place between May and November. Developmental changes from larva to pre-pupa to pupa take place in less than 30 days when held at the conditions used in this study (Zilahi-Balogh et al. 2003a). Tubes were scanned only once at their assigned time interval due to the potential lethal impact radiation would have on the insects if repeatedly scanned over the duration of the experiment. Because neither burrowing depth nor pupal chamber volume differed over the time of sampling, and no other evidence of movement such as trails or empty chambers was observed, it is likely that the insects remain in place once they make their chambers.

This is the first study to measure the volume of *Laricobius* spp. pupal chamber. Laboratory studies of *L. nigrinus* development documented the presence of a pupal chamber surrounding the pupa (Salom et al. 2012) as well as body length at each of the 4 larval instars, but did not obtain length or chamber volume measurements during the pupal stage (Zilahi-Balogh et al. 2003a). From field studies of *Laricobius erichsonii* Rosen (Colepotera: Derodontidae), a biological control agent of the balsam woolly adelgid, *Adelges piceae* Ratzeburg (Hemiptera: Adelgidae), Clark and Brown (1958) reported that pupae were roughly 4.0 mm in length and 2.0–2.4 mm in width within the mineral soil, with burrowing depths ranging between 2.54 and 15.2 cm below host trees (Clark and Brown 1958). Unfortunately, no description was provided on how pupal chamber size was measured or how those soil depth observations were obtained. Furthermore, soil compaction was not reported with the documented burrowing depth for *L. erichsonii* of 2.54–15.2 cm (Clark and Brown 1958), making it difficult to draw comparative conclusions with our observed burrowing depths. In ongoing field studies, the unconfined compressive strength of the soil below hemlock trees varies substantially, ranging between 0.1 and 2.25 kg/cm^2 (A.P.H., personal observation), at hemlock sites growing near the Virginia Tech Beneficial Insects Containment Facility in Blacksburg, VA (measured using a Gilson Pocket Penetrometer HM-500, Global Gilson, Lewis Center, OH, at a depth of 0.635 cm).

The X-ray tubes used in this experiment were 7 cm tall with roughly 6 cm of soil. Due to the physical limitations of the Skyscan 1172 X-ray machine, tubes could not be made any larger, restricting the ability to measure any insects that might burrow deeper than 6 cm if given the space. However, given the average range of burrowing depths (1.14–2.70 cm) and maximum burrowing depth of 5.2 cm obtained when held in soils less compacted than the low range of soil compaction intensity found in the field, there is no reason to expect *Laricobius* spp. would burrow deeper in a natural setting. The soil medium used in this study provided optimal subterranean conditions for *Laricobius* spp. estivation based on their historical mass production at Virginia Tech (Salom et al. 2012, Foley 2021). Therefore,

the results in this study should be regarded as a "best case scenario" for burrowing depth, and higher compacted and more variable soils would be expected to impact borrowing depth in field settings. Unrestricted burrowing potential is of particular interest when considering the level at which chemical residues from neonicotinoid (imidacloprid) treatments for HWA settle in the soil profile and if *Laricobius* are coming into direct contact with them. Depending on the application technique and formulation of imidacloprid, as well as site conditions, chemical residues have the potential to persist at different depths within the soil profile.

Common imidacloprid application techniques include burying CoreTect tablets (a slow-release pellet formulation of imidacloprid) 5–12 cm below the soil surface, soil drenches poured directly on the soil just below the organic layer, and soil injections typically applying imidacloprid at a depth of 7.6–20.0 cm (Steward et al. 1998, Benton and Cowles 2017). Future studies should assess whether insecticide residues are affecting survivorship based on the mode of application as well as the role of soil compaction and depth of imidacloprid residues. The methods used in this study show that it is possible to document the burrowing depth and pupal chamber volume of *Laricobius* spp. during estivation. Furthermore, we know that compaction significantly impacts this subterranean activity; however, we did not test the wide range of variability found in a field setting.

Our findings demonstrate that micro-CT imaging is a viable technique for documenting the subterranean activity of *Laricobius* spp. and other arthropods that are active in the top layers of soil. Understanding the assumed depth at which *Laricobius* spp. completes its estivation in relation to the depth at which imidacloprid residues persist in the soil profile will allow us to select the best application technique that poses the least risk to our biological control agents. Integrating these findings into our management tactics will strengthen our IPM strategy and biological control for HWA.

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Author Contributions

Ashleigh Hillen (Conceptualization-Equal, Data curation-Lead, Formal analysis-Equal, Investigation-Lead, Methodology-Equal, Visualization-Lead, Writing – original draft-Lead, Writing – review & editing-Equal), Jeremiah Foley (Conceptualization-Equal, Formal analysis-Equal, Validation-Equal, Visualization-Equal, Writing – original draft-Equal, Writing – review & editing-Equal, Mary Salcedo (Formal analysis-Equal, Methodology-Lead, Software-Lead, Validation-Equal, Writing – review & editing-Equal), Jake Socha (Conceptualization-Equal, Investigation-Equal, Methodology-Lead, Resources-Lead, Supervision-Equal, Validation-Equal, Writing – review & editing-Equal), Scott Salom (Conceptualization-Equal, Funding acquisition-Lead, Methodology-Equal, Project administration-Lead, Resources-Equal, Visualization-Equal, Writing – review & editing-Equal)

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