Stylet Bundle Morphology and Trophically Related Enzymes of the Hemlock Woolly Adelgid (Hemiptera: Adelgidae)

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ABSTRACT The hemlock woolly adelgid, *Adelges tsugae* Annand (Hemiptera: Adelgidae), is a pest of eastern and Carolina hemlocks (*Tsuga canadensis* (L.) Carrière and *Tsuga caroliniana* Engelmann, respectively) in the eastern United States and has already caused catastrophic changes to eastern forests. As one of the significant exotic forest pests, it is imperative that the basic biology of hemlock woolly adelgid be understood for use in novel and improved management techniques. Scanning and transmission electron microscopy and enzyme assays were used to elucidate the feeding biology of hemlock woolly adelgid and are discussed in the context of the pest–plant interactions and the implications for host plant resistance. Morphological characters indicate that hemlock woolly adelgids may use labial sensilla and neural canals within the mandibular stylets to guide their stylets through close-range host acceptance processes. Stylet bundle insertion is likely assisted by external sheath material that secures the stylet bundle to the plant surface and mandibular dentitions that may assist entry into or within plant tissues. In addition, results support the theory that extra-oral digestion is likely used by hemlock woolly adelgid, suggested by both a narrow food canal and the presence of four trophically related enzymes (a trypsin-like enzyme, an amylase-like enzyme, peroxidase, and polyphenol oxidase). The presence of these enzymes also has implications for causing a systemic response in host trees.

KEY WORDS *Adelges tsugae*, digestive enzyme, electron microscopy, feeding biology, hemlock

The hemlock woolly adelgid (*Adelges tsugae* Annand) (Hemiptera: Adelgidae) is an exotic invasive sucking insect causing extensive mortality to populations of eastern and Carolina hemlocks (*Tsuga canadensis* (L.) Carrière and *Tsuga caroliniana* Engelmann, respectively) in the eastern United States. After infestation, hemlocks decline in health, exhibited by needle drop, bud abortion, a lack of new growth, and a drought-like physiological reaction that has been likened with a hypersensitive response (Walker-Lane 2009, Radville et al. 2011). Tree death typically follows in 4–10 yr, and in most infested stands, 80–90% mortality occurs (Hale 2004, Townsend and Rieske-Kinney 2006).

The status of hemlock woolly adelgid as a significant pest of forest and ornamental hemlocks has provoked extensive research focused primarily on management. Despite being a focus of research for >20 yr, the basic biology of hemlock woolly adelgid remains poorly understood. Adelgids belong to the superfamily Aphidoidea (along with Aphididae and Phylloxeridae) and are commonly regarded as "aphid-like" (e.g., Pirone 1978, MacKenzie 2002, Speight 2007); because of this association, much of the basic biological understanding of hemlock woolly adelgid is based on the more comprehensive knowledge of aphid biology. This generalization occurs despite major biological differences (e.g., hemlock woolly adelgid are sessile, whereas aphids are motile; hemlock woolly adelgid feeds on xylem ray parenchyma cells, whereas aphids feed on phloem sap).

The feeding biology of hemlock woolly adelgid was originally investigated by Young et al. (1995), and this study constitutes most of what is known about hemlock woolly adelgid feeding. They described the stylet bundle insertion point and extensive stylet bundle length, a trait also found in the closely related balsam woolly adelgid (*Adelges piceae* (Ratzeburg); Forbes and Mullick 1970). A long stylet bundle is likely associated with a need to reach its feeding site and to secure itself to the host plant. Although lacking evidence with a cross-section image, Young et al. (1995) deduced that the stylet bundle morphology of hemlock woolly adelgid is similar to that of aphids and balsam woolly adelgid (Parrish 1967).

As members of Aphidoidea, hemlock woolly adelgid host plant penetration and salivary secretions are thought to be similar to that of aphids. Aphids pierce the plant cuticle with an alternating sawing motion of four stylets and produce two types of saliva: watery saliva and salivary sheath material (Miles 1959, 1968, 1972; Pollard 1973). The watery saliva may contain digestive enzymes that may help establish and main-

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tain feeding sites, suppress plant defenses, and induce changes in plant physiology (Cohen and Hendrix 1994, Miles 1999, Will et al. 2007, Mutti et al. 2008). The salivary sheath material is produced as a bead at the tip of the stylets that hardens upon extrusion, enclosing the stylets and acting to stabilize the labium during insertion, as a fulcrum for stylet maneuvering, to protect against host plant defenses, and enable stylet bundle reinsertion following a molt (Miles 1968, 1972, 1999; Cohen 1990).

Understanding digestive enzymes used by hemlock woolly adelgid is critical to further the body of knowledge regarding insect–plant interactions. It has been suggested that the injection of "toxic" saliva by hemlock woolly adelgid induces the systemic response in hemlocks (McClure 1995, Young et al. 1995, Shields et al. 1996), but there are no data to support that concept. Young et al. (1995) documented both saliva types in hemlock woolly adelgid using staining methods, but did not evaluate them for the presence of enzymes. They also suggest that pectinase, an enzyme significant in plant penetration, is present in the saliva of hemlock woolly adelgid, supported only by the predominantly intercellular stylet pathway within plant tissue and the indication of protein in the saliva via staining. Later, Kaur (2009) detected a weak presence of amylase in hemlock woolly adelgid homogenate but was unable to draw conclusions on the discovery.

The purpose of this research is to augment the current corpus of basic biological knowledge regarding feeding biology and host plant interactions of hemlock woolly adelgid. Specifically, the objectives were to 1) expand our understanding of hemlock woolly adelgid feeding behaviors using electron microscopy, 2) establish and corroborate stylet bundle cross-section morphology suggested by Young et al. (1995), and 3) identify trophically related enzymes used by hemlock woolly adelgid. An improved understanding of the interaction between hemlock woolly adelgid and its hemlock host will lead to a better comprehension of the host response, ultimately improving the work toward improved pest management techniques such as host plant resistance and induction of resistance by systemic chemicals.

Materials and Methods

Feeding Behaviors and Stylet Bundle Cross-Section Morphology. *Material Collection.* Branches of infested *T. canadensis* were field collected from locations in Crossnore, NC (36.0138, −81.9307) and Laurel Springs, NC (36.3970, −81.3339). Material was transported to Raleigh, NC, trimmed, and kept in water at 22°C and ≈65% humidity.

Scanning Electron Microscopy. All plant and insect material was fixed in 3.0% glutaraldehyde in 0.1 M Sorenson's buffer, pH 7.0, for 48 h. Samples were postfixed in 1% osmium tetroxide in the same buffer for 1 h at 4°C, then dehydrated in ethanol baths of 30, 50, 70, 95, and 100% (3×) for 1 h each at 4°C. Samples were critical point-dried, mounted, and coated with gold–palladium at a deposition thickness of 20–25Å on four sides and top. Samples were examined with a JEOL JSM-5900LV scanning electron microscope (JEOL, Tokyo, Japan) at an accelerating voltage of 15 or 20 kV. Measurements were taken with a planimeter on printed images. Images were modified for brightness and contrast by adjusting levels, sized, and placed together to produce figures using Adobe Photoshop 8.0 (Adobe Systems, San Jose, CA).

Transmission Electron Microscopy (TEM). Infested eastern hemlock material was sent via overnight mail to Charles A. Murphy (U.S. Department of Agriculture–Agricultural Research Service [USDA–ARS], Beltsville Agricultural Research Center, Beltsville, MD), who performed and imaged the TEM portion of this study. Tissue was fixed overnight at room temperature by immersion in a fixative comprising 3% glutaraldehyde and 2% paraformaldehyde in 0.05 M NaCacodylate buffer, pH 7.0. They were washed in a 0.1 M NaCacodylate buffer rinse, six times over 1 h, postfixed in NaCacodylate buffered 2% osmium tetroxide for 2 h at room temperature, dehydrated in an acetone series and slowly infiltrated with Spurrs low-viscosity embedding resin. Then, 90-nm gold sections of the tissue were cut on a Reichert/AO Ultracut microtome with a Diatome diamond knife and mounted onto 200-mesh Ni grids (Leica, Deerfield, IL). They were stained with 4% uranyl acetate and 3% lead citrate, and then viewed in a HT-7700 Hitachi microscope (Hitachi Corp., Tokyo, Japan) at 80 kV. Images were modified for brightness and contrast by adjusting levels, sized, and placed together to produce figures using Adobe Photoshop 8.0.

Trophically Related Enzymes of Hemlock Woolly Adelgid. *Material Collection for Enzyme Work.* Actively feeding second- and third-instar hemlock woolly adelgid nymphs used in these enzyme surveys were collected from live eastern hemlock material obtained from Laurel Springs, NC, in January 2011. The ovisac was teased off with a paintbrush and 10 individuals were stored per vial at −80°C. Similarly, neonate *Lygus lineolaris* (Palisot de Beauvois) (Hemiptera: Miridae) were stored 10 individuals per vial at −80°C for use as positive controls in these assays.

L. lineolaris is a major agricultural pest that feeds on at least 130 economically important plants, including cotton, cereals, fruits, vegetables, and alfalfa (Snodgrass et al. 1984, Young 1986, Butts and Lamb 1991). The use of this insect species for comparisons with hemlock woolly adelgid was useful given its accessibility, its similar biomass to hemlock woolly adelgid as a neonate, and its well-characterized use of digestive enzymes (Agusti and Cohen 2000). Trypsin-like protease has been partially characterized from their salivary gland complex, indicating that this enzyme is used extra-orally in a highly efficient feeding behavior (Zeng and Cohen 2000, Zeng et al. 2000). Many salivary enzymes are similar between homopterans and heteropterans (Miles 1964, 1965).

Sample Preparation. Means of obtaining watery saliva from hemipterans for analytical research can be done by several traditional methods, such as stimu-

lating feeding or probing (Adams and McAllan 1956, Corzo et al. 2001), extracting the salivary glands (Miles 1964), or collecting saliva directly from the mouthparts (Madhusudhan et al. 1994, Miles 1999). However, the sessile behavior and small size of hemlock woolly adelgid limit the application of these approaches. Baptist (1941) and Madhusudhan et al. (1994) suggest using salivary gland homogenate to perform tests in scenarios when collection of saliva is not plausible, but attempts to remove salivary glands from minute hemlock woolly adelgid bodies (crawlers are <0.5 mm in length [Young et al. 1995]) were unsuccessful. In some situations, whole-body homogenate is used to obtain watery saliva (Adams and McAllan 1958, Adams and Drew 1963). This is typically used for rapid enzyme assessment once the presence of an enzyme has been established (Miles 1999), but we used this approach to overcome the difficulties of isolating the saliva or salivary glands of hemlock woolly adelgid. Attempts to harvest saliva by providing starch and protein-containing substrate (Adams and McAllan 1958) were attempted, but eliciting feeding responses from hemlock woolly adelgid crawlers also proved impossible under laboratory conditions. Therefore, whole body extractions were resorted to for determination of trophic enzyme activity.

To prepare hemlock woolly adelgid samples for the amylase-like enzyme, peroxidase, and polyphenol oxidase (PPO) assays, one vial containing 10 of the frozen hemlock woolly adelgid was thawed at room temperature and homogenized in 300 μl 1× phosphate buffered saline (PBS). To partially purify the homogenate, the sample was briefly centrifuged, and the resultant supernatant was filtered through a 3,000 MW centrifugal filter unit (Millipore Corporation, Billerica, MA) at 14,000 rpm for 15 min. The filter was inverted and inserted into a clean vial and centrifuged at 1,000 rpm for 2 min to recover products retained in the filter. The resulting concentrate was augmented with 100 μl of PBS to bring the volume to ≈130 μl.

Protease Assay. General protease activity was determined using the EnzChek Protease Assay Kit (Invitrogen, Eugene, OR). A casein substrate was prepared by mixing 0.2 ml of PBS to one vial of the lyophilized substrate. The solution was mixed, left at room temperature for 5 min, and added to 19.8 ml of 1× digestion buffer, resulting in a 10 μg ml^{-1} BODIPY casein substrate. An authentic trypsin standard was prepared by diluting trypsin (type II-S from porcine pancreas; Sigma-Aldrich, St. Louis, MO) in PBS at final concentrations of 2.32 mg ml^{-1}, 3.25 mg ml^{-1}, and 4.0 mg ml^{-1}. The positive control was derived from insects known to possess the enzymes in question, 1 and 10 *L. lineolaris* were thawed at room temperature and homogenized in 100 μl of PBS with a motorized pestle tissue grinder (*L. lineolaris* has confirmed trypsin-like protease activity [Zeng et al. 2002]). To prepare the hemlock woolly adelgid samples (crude extracts), 1 and 10 hemlock woolly adelgid were thawed and homogenized in 100 μl 1× PBS. In each well of a standard 96-well plate, 100 μl of the casein substrate was mixed with 10 μl of the standards and samples. Analyzed samples included three replications each of negative control, deionized water (diH_2O), trypsin standards, 1 and 10 *L. lineolaris* in 1× PBS, and 1 and 10 hemlock woolly adelgid in 1× PBS. Fluorescence, which is proportional to enzyme activity, was measured with a FilterMax F5 Multi-Mode Microplate Reader (Molecular Devices, Sunnyvale, CA) at an excitation wavelength of 485 nm and an emission wavelength of 535 nm at 37°C.

Amylase Assay. For determination of amylase-like activity, we used the EnzChek Ultra Amylase Assay Kit (Invitrogen, Eugene, OR). Starch substrate was prepared by mixing one vial of the lyophilized substrate with 50 mM sodium acetate buffer (pH 4.0). Solution was vortexed for 20 s and left at room temperature for 5 min with occasional mixing. Next, 900 μl of 1× PBS was added, the solution was mixed, and then kept in darkness at room temperature until used. An amylase standard curve was prepared by serially diluting α-amylase from *Aspergillus oryzae* (Moyashimon) (Sigma-Aldrich: Fluka BioChemika, Buchs, Switzerland). Final concentrations used in the assay were 2.5 mU ml^{-1}, 5 mU ml^{-1}, 10 mU ml^{-1}, and 20 mU ml^{-1}. For the positive control, 4 and 10 *L. lineolaris* were thawed and homogenized in 120 μl of 1× PBS with a motorized pestle tissue grinder (*L. lineolaris* has been documented to have α-amylase activity [Zeng and Cohen 2000]). In each well of a standard 96-well plate, 50 μl of the starch substrate was mixed with 50 μl of the sample. Analyzed samples included two replications each of: negative control (diH_2O), the amylase standard curve, 4 and 10 *L. lineolaris* in 1× PBS, and 10 purified hemlock woolly adelgid in 1× PBS. Fluorescence of the digestion products from the substrate was measured by the microplate reader at an excitation wavelength of 485 nm and an emission wavelength of 535 nm at 37°C.

Peroxidase Assay. Peroxidase assays were performed using the Amplex Red Hydrogen Peroxide/Peroxidase Assay Kit (Invitrogen, Eugene, OR). Hydrogen peroxide (H_2O_2) substrate was prepared by dissolving one vial of Amplex Red reagent in 60 μl of dimethylsulfoxide; then, 50 μl of this solution was mixed with 500 μl of 10 mM H_2O_2 and 4.45 ml of 1× reaction buffer. Peroxidase standard curve was prepared by serially diluting 10 U ml^{-1} horseradish peroxidase to final concentrations of 0.3 mU ml^{-1}, 0.6 mU ml^{-1}, 1.2 mU ml^{-1}, and 2.4 mU ml^{-1}. Although *L. lineolaris* has not been previously documented to produce peroxidase, the samples with these mirids were included to maintain consistency as a control. Four and ten *L. lineolaris* were thawed and homogenized in 120 μl of 1× PBS with a motorized pestle tissue grinder. In each well of a standard 96-well plate, 50 μl of the H_2O_2 substrate was mixed with 50 μl of the sample. Samples included two replications each of the negative control (diH_2O), the horseradish peroxidase standard curve, 4 and 10 *L. lineolaris* in 1× PBS, and purified hemlock woolly adelgid in 1× PBS. Fluorescence of the digestion products from the substrate was measured at an excitation wavelength of 535 nm and an emission wavelength of 595 nm at 37°C.

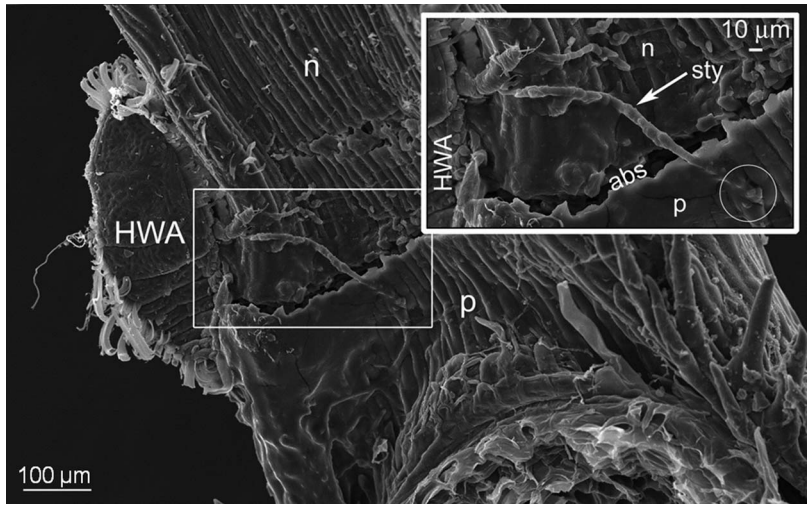

Fig. 1. Adaxial surface of needle petiole and pulvini with settled hemlock woolly adelgid; inset showing sheath-enclosed stylets traveling on the surface of the plant, traversing the abscission layer, and inserting into the pulvini. Insertion site is indicated by the circle. abs, abscission layer; HWA, hemlock woolly adelgid; n, needle; p, pulvini; sty, stylets.

PPO Assay. To determine the presence of PPO activity in hemlock woolly adelgids, we modified methods used by Lee et al. (2008). Samples were prepared by homogenizing 4, 10, and 10 *L. lineolaris* in separate vials in 120 μl of PBS with a motorized pestle tissue grinder. Two purified hemlock woolly adelgid samples were prepared, and then 50 μl of each sample were pipetted into wells, along with 10 μl of PBS or 1.0 mg ml^{-1} phenylthiourea (PTU), a PPO inhibitor. Samples included two replications each of diH_2O (with 10 μl of 1× PBS), four *L. lineolaris* (with 10 μl of PBS), 10 *L. lineolaris* (with 10 μl of PBS), 10 *L. lineolaris* (with 10 μl of PTU), 10 hemlock woolly adelgid (with 10 μl of PBS), 10 hemlock woolly adelgid (with 10 μl of PTU). Samples were incubated at 37°C for 20 min to allow inhibition of PPO by PTU. Then, 50 μl of 5 mM L-3,4-dihydroxyphenylalanine (as substrate) was added. Absorbance at 450 nm was measured from the substrate at 37°C.

Protein Quantification. To quantify the amount of protein in the samples, we performed a Bradford protein assay (Bradford 1974). Quick Start Bradford Dye Reagent, 1× (Bio-Rad Laboratories Inc., Hercules, CA) was prepared by diluting in a 1:4 ratio with diH_2O. The solution was filtered through a Whatman #1 filter to remove particles. Protein standards were prepared by serially diluting bovine serum albumin (Sigma-Aldrich, St. Louis, MO) in concentrations of 0.5, 0.25, 0.125, 0.063, 0.031, 0.016, and 0.008 mg ml^{-1}. Ten microliters of each standard and samples were pipetted into microplate wells. Two-hundred microliter diluted reagent was added, shaken for 5 s, and incubated at room temperature for 5 min. Absorbance at 595 nm was measured with a FilterMax F5 Multi-Mode Microplate Reader (Molecular Devices, Sunnyvale, CA) at room temperature.

Using the standard curve, the amount of protein for each sample was calculated. These were converted to units of activity per microgram of protein (as measured by the Bradford assay) per min. Data were analyzed for enzyme activity units at 1 h for amylase and PPO and at the asymptote of enzyme activity for trypsin-like enzyme (30 min) and peroxidase (15 min).

Results

Insertion Site and Sheath Material. The hemlock woolly adelgid stylet bundles were inserted on the adaxial side of hemlock needle, proximal to the twig with respect to the abscission layer, corroborating the findings of Young et al. (1995). Sheath material was observed outside plant tissue, extending from the tip of the labium to the insertion point, ≈200 μm (Fig. 1). Therefore, roughly 15–20% of the stylet bundle is outside the plant, with calculations based on stylets lengths of 1.04 ± 0.06 mm for crawlers, 1.11 ± 0.06 mm for established nymphs, and 1.27 ± 0.06 mm for established adults (as determined by Young et al. 1995). The sheath material adheres to the plant, beginning distal to the stem in reference to the abscission layer, then travels across the abscission layer to the insertion point on the pulvinus. Sheath material internal to the plant tissues was not observed using scanning electron microscopy.

Labium and Stylet Bundle Morphology. The hemlock woolly adelgid labium possesses several short sensilla at the proximal end (Fig. 2A). Four stylets extend from the labium. The stylet bundle of hemlock woolly adelgid, like most other members of Hemiptera, is composed of four components: two interlocking maxillary stylets surrounded by two mandibular stylets. A food canal, salivary canal, and neural canals are evident in the stylet bundle cross-section observed with TEM (Fig. 3). The maxillary stylets interlock with

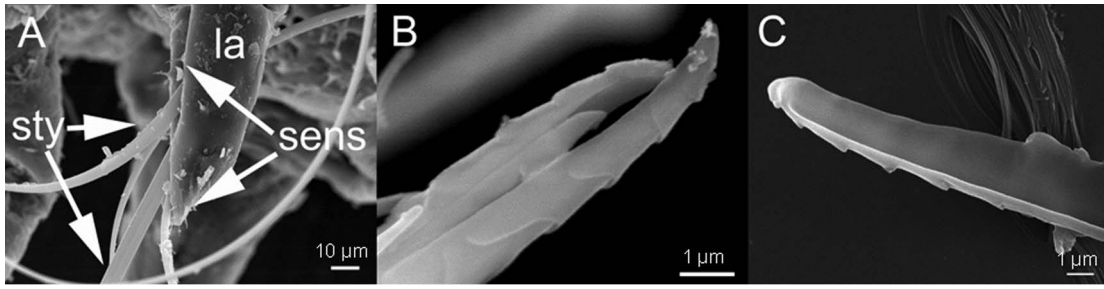

Fig. 2. Labium and stylet morphology. (A) Distal segment of labium, showing sensilla on labium and four stylets emerging. (B) Tip of stylet bundle, showing mandibular stylets enclosing over the interior maxillary stylets. (C) Tip of maxillary stylet with five distinct dentitions. la, labium; sens, sensilla; sty, stylet.

one another to form the food canal and salivary canal. The diameter of the food canal is 0.89 ± 0.14 μm, with a range of 0.48–1.0 μm (n = 11); the diameter of the salivary canal is 0.45 ± 0.08, with a range of 0.25–0.54 μm (n = 11). Because serial sections were taken, the distances of each cross-section from the labium tip were not determined, and therefore these measurements represent the stylet bundle at different points along the length of the stylet bundle. For 10 of the 11 images, cross-sections of the food canal ranged from 0.86 to 1.0 μm. The narrowest, outlying measurement was 0.48 μm. Similarly, 10 of the 11 measurements for the salivary canal cross-sections were 0.43–0.54 μm, with an outlier measuring 0.24 μm. The tip of the insect's mandibular stylet had at least five ridge-like dentitions, with the apex of each directed toward the labium of the insect (Fig. 2B and C).

Trophically Related Enzymes. In whole-body homogenates of hemlock woolly adelgid, all four of the enzymes tested (trypsin-like protease, amylase-like enzyme, peroxidase, and PPO) were present. There were clear differences in the enzymatic activities between hemlock woolly adelgid and *L. lineolaris.* The kinetic curve for hemlock woolly adelgid enzymatic activity for trypsin-like activity reached its asymptote at 30 min, whereas the *L. lineolaris* comparison continued to increase after >1 h (Fig. 4A). Trypsin-like activity in hemlock woolly adelgid (equivalents of 0.259 BAEE U μg protein^{-1} min^{-1}) was approximately four times lower than the values measured in *L. lineolaris* (equivalents of 0.8405 BAEE U μg protein^{-1} min^{-1}; Fig. 4B). Amylase-like activity in the sample with 10 hemlock woolly adelgid inclined more quickly, but did not reach as high a level as they did in *L. lineolaris* (Fig. 4C). *L. lineolaris* amylase-like activity was almost eight times higher (equivalents of 0.7748 mU μg protein^{-1} min^{-1}) than hemlock woolly adelgid (equivalents of 0.1088 mU μg protein^{-1} min^{-1}; Fig. 4D). At 15 min, hemlock woolly adelgid peroxidase enzyme activity reached an asymptote, whereas *L. lineolaris* continued to increase after 1 h (Fig. 5A). The amount present in hemlock woolly adelgid (equivalents of 0.2323 mU μg protein^{-1} min^{-1}) is almost twice as much relative to peroxidase of *L. lineolaris* (equivalents of 0.1283 mU μg protein^{-1} min^{-1}; Fig. 5B). Investigations into PPO-like activity reveal that hemlock woolly adelgid (without PTU) is approximately three times more active in hemlock woolly adelgid (equivalents of 0.0569 abs μg protein^{-1} min^{-1}) than *L. lineolaris* (equivalents of 0.017 abs μg protein^{-1} min^{-1}), which did not exhibit a kinetics curve and the sample that contained PTU (a PPO inhibitor) was almost 18 times less active than the sample without PTU (equivalents of 0.00323 abs μg protein^{-1} min^{-1}; Fig. 5C and B).

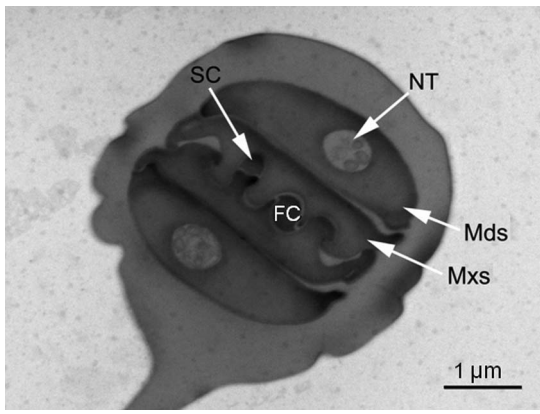

Fig. 3. Transmission electron micrograph of hemlock woolly adelgid, *A. tsugae*, stylet cross-section. FC, food canal; Mds, mandibular stylet; Mxs, maxillary stylet; NT, neurotubules of mandibles; SC, salivary canal.

Discussion

As members of Sternorrhyncha, hemlock woolly adelgid produce durable and recognizable stylet sheaths that provide evidences of feeding choices of hemlock woolly adelgid (Miles 1968, Pollard 1973, Backus 1988, Young et al. 1995). The bead-like appearance of the hemlock woolly adelgid salivary sheath outside the plant tissue (Fig. 1) is similar to descriptions of aphid salivary sheaths in unconfined spaces (Miles 1987). In addition, the stylet bundle travels outside plant tissue and appears to adhere to the plant surface, which may help prevent the stylet bundle from slipping during insertion (Pollard 1973).

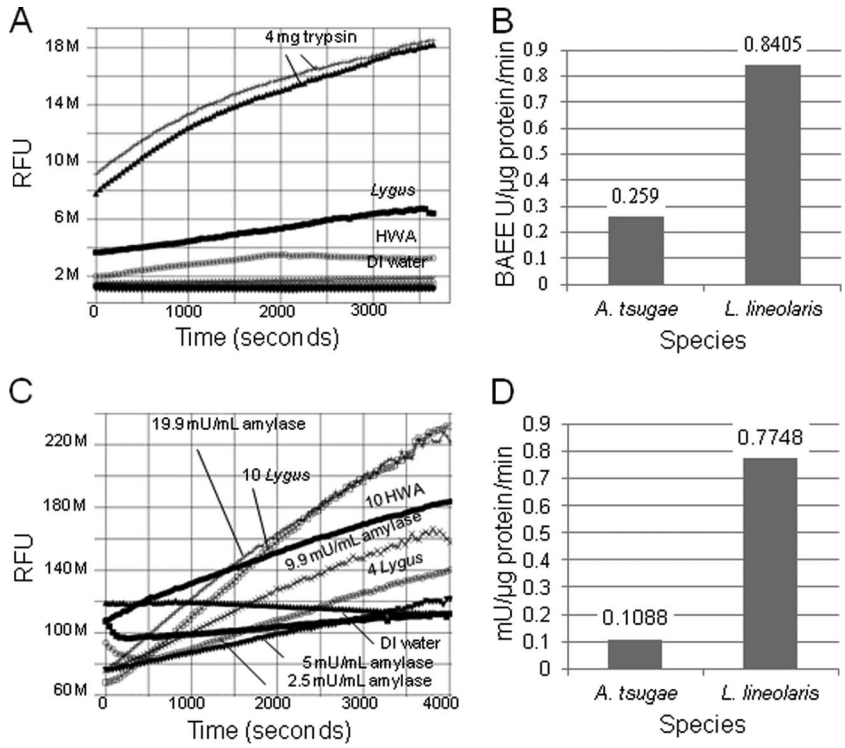

Fig. 4. (A) Enzyme kinetics of trypsin-like assay. (B) Trypsin-like activity per microgram of protein per minute of *A. tsugae* and *L. lineolaris.* (C) Enzyme kinetics of amylase-like enzyme assay. (D) Amylase-like activity per microgram of protein per minute of *A. tsugae* and *L. lineolaris.*

As passive dispersers, hemlock woolly adelgid crawlers do not use long-range host searching and must rely on close-range host acceptance processes to determine a suitable stylet bundle insertion site. As in other insects, this is likely facilitated by mechanoreceptors, gustatory receptors, or chemoreceptors (Backus 1988). The general appearance and placement of hemlock woolly adelgid labial sensilla (Fig. 2A) are similar to the apical labial sensilla of aphids, which are used strictly as mechanoreceptors during host acceptance (Wensler 1977, Tjallingii 1978). Given the need of a close-range sensory system, it is logical to suggest that the apical labial sensilla of hemlock woolly adelgid are used in a similar way.

The TEM cross-section image of the hemlock woolly adelgid stylet bundle (Fig. 3) confirms the suggestion made by Young et al. (1995) that the stylet bundle is made of two mandibular and two maxillary stylets, similar to that of the balsam woolly adelgid (Forbes and Mullick 1970) and other hemipterans (Mittler 1957, Parrish 1967, Cobben 1979, Klingauf 1987, Cohen 1990, Miles 1999). The interlocking maxillary stylets form a salivary and food canal, with diameters comparable to those found in aphids (Klingauf 1987). The narrowest measurements likely represent a more distal portion of the stylet bundle, as the food and salivary canals in aphids are tapered (Pollard 1973). The narrowest diameter of the food canal also imposes limitations on the size of particles that can be ingested: clearly, the largest particle that can be ingested must be smaller than the diameter of the food canal.

The dimensions of the stylet bundle, including the length and the diameter of the food and salivary canals determine physical constraints that hemlock woolly adelgid must face in feeding. These constraints are determined by Poiseuille's Law (Nobel 1983) and applied to aphid feeding by Mittler (1967), to other members of Hemiptera by Cohen (1995), and to insects in general by Kingsolver and Daniel (1993). Poiseuille's Law predicts the relationship between sucking force and 1) stylet length, 2) stylet diameter, and 3) fluid viscosity necessary for flow. Based on this law, smaller insects, such as hemlock woolly adelgid, must generate higher pressure differences to overcome the resistance of the feeding apparatus (Novotny and Wilson 1997). Given the diameter and length measurements of the stylet bundle and the small body size of hemlock woolly adelgid, it is apparent that a pressure differential (or sucking force) must be used to overcome the feeding limitations for hemlock woolly adelgid. This has implications for the use of digestive enzymes by hemlock woolly adelgid, which would decrease the viscosity of materials in the food canal, allowing for greater ease of ingestion of food particles. This is a critical step in digestion by some insects, as highly viscous liquids would be dif-

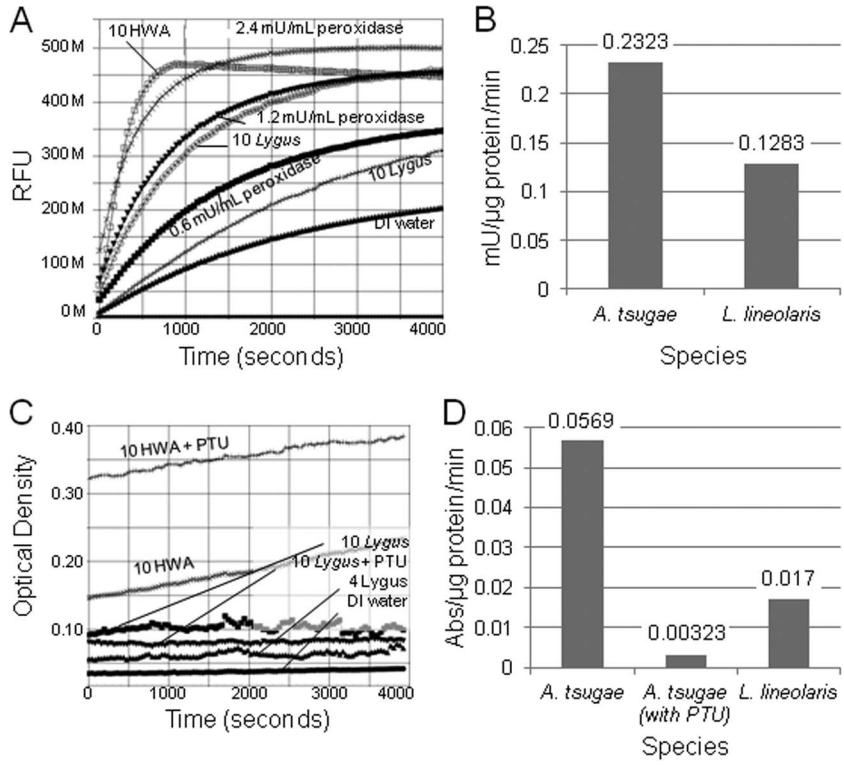

Fig. 5. (A) Enzyme kinetics of peroxidase assay. (B) Peroxidase activity per microgram of protein per minute of *A. tsugae* and *L. lineolaris.* (C) Enzyme kinetics of PPO assay. (D) PPO activity per microgram of protein per minute for *A. tsugae*, *A. tsugae* incubated with PTU for 20 min, and *L. lineolaris.*

ficult to transport in a long and narrow food canal (Mittler 1967; Cohen 1998, 2000).

These results also indicate the presence of neural canals in the mandibular stylets, representative of a sensory function. In related insects, the neural canals of the mandibular stylets detect the movement and position of the stylet bundle (e.g., Forbes 1966, Parrish 1967, Backus and McLean 1982, Leopold et al. 2003). Neural canals are not present in the maxillary stylets of hemlock woolly adelgid, demonstrating that hemlock woolly adelgid uses single stylet innervation, which optimizes sensation for the mandibular stylets-ahead probing method, typical of all sternorrhynchous homopterans (Backus 1988). In addition to a lack of stylet tip sensilla (Fig. 2B and C), evidence of neural canals indicates that hemlock woolly adelgid likely uses their dendrites as proprioreceptors.

Although the method of hemlock woolly adelgid stylet bundle penetration into host tissues is unknown, the ridge-like apical dentitions of the mandibular stylets (Fig. 2B and C) suggest a behavior similar to that of aphids, which use an alternating sawing motion of their stylets (Pollard 1973), and adds further evidence for a mandibular stylets-ahead probing method. Alternatively, these serrations may be used to secure the insect to the plant, as a fulcrum for maxillary stylet movements (Cobben 1978, Cohen 2000, Boyd et al. 2002), or to mechanically disintegrate their food source, as observed in predaceous heteropterans (Cohen 1995). Dentitions are observed in other members of Hemiptera (Cohen 1990, Boyd et al. 2002, Leopold et al. 2003) and are not as deeply serrated and occur in fewer numbers in phytophagous members of the group (Cobben 1978, Cohen 1990). The shallow dentitions seen in hemlock woolly adelgid may indicate that limited mechanical maceration occurs, suggesting the use of chemical maceration of plant tissues (perhaps by the injection of extra-oral digestive enzymes).

Although the four trophically related enzymes detected here could be present in the salivary gland complex, midgut, or other compartments of the insect (because hemlock woolly adelgid homogenate was used rather than isolated saliva), their occurrence is nevertheless revealing of hemlock woolly adelgid feeding habits. The presence of a trypsin-like enzyme (Fig. 4) indicates that hemlock woolly adelgid digests proteins either extra-orally or within the alimentary canal, rather than depending on free amino acids, as is the case for sap-feeding insects. Protease activity is common in the salivary glands, gut, or filter chamber of many phytophagous insects (Agusti and Cohen 2000), including many aphids (e.g., Bramstedt 1948, Srivastava and Auclair 1963). In addition to a digestive role, if trypsin-like protease enzymes are used extra-orally by hemlock woolly adelgid, then therein lies the ability to digest structural proteins of the plant (Hori 1970, 1971), enhancing stylet maneuvering within plant tissues and enabling access to internal feeding

sites. Evidence of amylase-like enzymes, macerating enzymes that break down plant starches, provides supporting evidence to the previously established notion that hemlock woolly adelgid feeds on xylem ray parenchyma cells (Young et al. 1995). As the primary food source for hemlock woolly adelgid, the starches of parenchyma cells must be digested by amylase-like enzymes to convert them to sugars (van der Maarel et al. 2002), a dietary need for all aphids (Srivastava 1987). Moreover, because plant starch granule sizes vary based on host plant (e.g., granules of examined agriculture crops vary between 2 and 100 μm in diameter [Robyt 1998]), the size of the starch granule being digested is a prohibitive factor for ingestion of intact granules. Because hemlock woolly adelgid stylets are long and narrow (and subject to Poiseuille's Law) and the food canal is <1 μm in diameter (Fig. 3), we suggest extra-oral digestion is occurring to facilitate the ingestion of starches and sugars. The observed PPO activity may be characteristic of hemlock woolly adelgid stylet sheath material, based upon the fact that *L. lineolaris* does not secrete a stylet sheath (Smith 1926, Miles 1972) and that PPO is readily detected in the sheath material of many aphids (Miles 1965; Miles and Slowiak 1970; Urbanska et al. 1994, 1998). While it is possible that these enzymes from hemlock woolly adelgid homogenate originate in part or wholly from microorganisms within hemlock woolly adelgid, there is considerable precedence of each of these enzymes being of insect origin based on their presence in closely related species.

The presence of these enzymes also has several implications for biochemical interactions occurring between hemlock woolly adelgid and its host plant. When herbivorous insects feed, some plants respond through the systemic expression of protease or amylase inhibitors (Garcia-Olmedo et al. 1987, Dietrich et al. 1999, Ryan 2000). If this is occurring in the hemlock woolly adelgid–hemlock system, then resistant hemlocks must successfully execute the physiological processes of the pathway, effectively inhibiting protease or amylase activity of hemlock woolly adelgid. This interaction occurs in the closely related grape phylloxera, *Viteus vitifoliae* (Fitch), whose cathepsin-like protease enzymes are inactivated by resistant grape vines (Blagowestschenski 1955). In some cases, inhibitors are bifunctional, capable of inhibiting protease and amylase activity (Ryan 1990). Because of these implications, there is potential for the development of a transgenic hemlock with an enzyme inhibitor(s), similar to the transgenic crops developed for resistance to aphids (Rahbé et al. 2003, Azzouz et al. 2005).

The presence of oxidases (peroxidase and PPO) suggests a detoxification role against plant defenses if it is present in the saliva (Miles 1999). Generally, peroxidases may counteract H_2O_2, but the action of salivary PPO on plant phenolics has been shown to generate H_2O_2 (Madhusudhan and Miles 1998). Therefore, the oxidase activity reported here could either counter or induce the elevated H_2O_2 levels exhibited in infested hemlocks (Radville et al. 2011). Alternatively, because peroxidases require H_2O_2 as an electron acceptor (Miles 1999), it is possible that increases of H_2O_2 in infested hemlocks are enhancing the activity of injected salivary peroxidases, further assisting in the detoxification of plant defenses. Oxidases can also cause systemic damage in host tissues (Madhusudhan and Miles 1998). The PPO detected in these studies may not be entirely salivary in origin, as insect hemolymph also has PPO activity used in immunity responses to foreign bodies (Ourth and Renis 1993). However, we suggest that the PPO detected in hemlock woolly adelgid homogenate primarily originates from the salivary gland complex of hemlock woolly adelgid with minimal hemolymph-derived PPO, based on the relatively higher levels of PPO activity in hemlock woolly adelgid (without PTU) to *L. lineolaris* (Fig. 5D) and the fact that PPO occurs in the sheath material and watery saliva of many members of Aphidoidea (Miles 1965, Madhusudhan and Miles 1998, Urbanska et al. 1998). These implications should be further substantiated through the challenging task of isolating hemlock woolly adelgid saliva. In addition, further characterization of these enzymes will be useful in understanding the biochemical feeding of this pest.

In conclusion, these studies indicate that 1) sheath material is produced both outside and inside of plant tissue and has a number of possible functions, namely, to assist tissue penetration and for stylet stabilization; 2) sensilla are present on the labial tip and may serve as receptors that aid in host acceptance processes; 3) hemlock woolly adelgid uses separate food and salivary canals to feed, and the length and diameter of the food canal indicates that either great force is used or extra-oral digestion is used; 4) neural canals in the mandibular stylets indicate single stylet innervation is occurring, likely used in proprioreception; 5) dentitions are present on the mandibular stylets and may be used to assist stylet bundle insertion, anchor hemlock woolly adelgid to the stem, and to mechanically macerate plant tissue before ingestion; and 6) at least four trophically related enzymes are used by hemlock woolly adelgid, providing support that hemlock woolly adelgid uses extra-oral digestion, maybe inducing a systemic response, and infers potential mechanisms of resistance in resistant hemlocks. In light of their ascent as one of the most significant forest pests in the eastern United States, it has become increasingly important that the basic biology of hemlock woolly adelgid be studied and understood for use in novel and improved management techniques. As these results indicate, there is a great deal regarding the basic biology of hemlock woolly adelgid that is deserving of future research attention.

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