# Biology and Larval Morphology of *Agrilus subcinctus* (Coleoptera: Buprestidae), with Comparisons to the Emerald Ash Borer, *Agrilus planipennis*

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**Abstract**

Emerald ash borer (EAB), *Agrilus planipennis* Fairmaire (Coleoptera: Buprestidae), is an exotic invasive pest of ash (*Fraxinus* spp.) trees that was first discovered in North America in 2002. There has been concern that surveyors could confuse immature stages of EAB with *A. subcinctus* Gory, an ash borer native to North America. We conducted studies in 2006-2009 in southern Michigan to determine biological and morphological characters for distinguishing immature *A. subcinctus* and EAB life stages. *Agrilus subcinctus* adults were captured on yellow sticky cards from late May through mid-August, with peak flight occurring in June. *Agrilus subcinctus* egg laying began in late May to early June. *Agrilus subcinctus* eggs were smaller than EAB eggs. *Agrilus subcinctus* eggs and immature stages were found only on dead ash twigs, while EAB primarily infests live stems and branches. We determined that *A. subcinctus* has four larval instars, with 4th instar *A. subcinctus* being similar in size to 2nd instar EAB. Shape of abdominal segments, pronotal groove, and urogomphi can be used to distinguish larvae of *A. subcinctus* from EAB. The following hymenopteran parasitoid species were reared from immature *A. subcinctus* stages: *Avetianella* sp. (Encyrtidae), *Ecphylus* sp. (Braconidae), *Eurytoma* sp. (Eurytomidae), near *Hadrotrichodes* (possible undescribed genus; Eulophidae), *Heterospilus* sp. (Braconidae), *Metapelma* sp. (Eupelmidae), and *Oodera* sp. (Pteromalidae).

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Emerald ash borer (EAB), *Agrilus planipennis* Fairmaire (Coleoptera: Buprestidae), is an exotic invasive pest of ash (*Fraxinus* spp.) trees that was first discovered in North America in 2002 (Haack et al. 2002). As of September 2010, EAB was found in 15 U.S. states and 2 Canadian provinces. Intensive surveys are conducted throughout the eastern United States to monitor spread and detect disjunct populations of EAB (Cappaert et al. 2005, Poland and McCullough 2006). Surveys for EAB include capturing adult beetles on green or purple sticky traps and girdled ash trees with sticky bands, as well as by debarking ash trees and inspecting for immature EAB life stages, primarily larvae that feed under the bark of ash trees.

Adult EAB have a coppery-green pronotum, emerald green elytra, and usually measure 7.5-13.5 mm in length (Yu 1992, Jendek 1994, http://www.emeraldashborer.info/files/agriscrn.pdf). The combination of emerald color and large size makes adult EAB relatively easy to distinguish from nearly all native North American *Agrilus* (ca. 178 spp., Nelson et al. 2008). Larvae of EAB and other *Agrilus* species can be distinguished from other Nearctic genera of

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Buprestidae by the presence of urogomphi (anal spines) protruding from the last abdominal segment (Peterson 1960). EAB usually infest the main trunk and larger branches of ash trees; however, we have found EAB in branches 1.5-2 cm in diameter (TRP, pers. observation).

*Agrilus subcinctus* Gory is a native borer that also feeds on ash and ranges throughout most of eastern North America and as far west as New Mexico (Bright 1987, Nelson et al. 2008). Adult *A. subcinctus* are usually less than 4 mm in length and are coppery brown with gray patterning on their elytra (http://www.emeraldashborer.info/files/agriscrn.pdf). The limited amount of information reported on the biology of *A. subcinctus* is somewhat confusing. Fisher (1928) believed poison ivy (*Toxicodendron* spp.) to be a larval host, given the number of adults collected from poison ivy foliage. Wellso et al. (1976) reported privet (*Ligustrum* sp.) as a host of *A. subcinctus.* Knull (1932) and Bright (1987) stated that *A. subcinctus* breeds in green ash (*F. pennsylvanica* Marsh.). Hespenheide (1973) collected adults from ash and found unidentified *Agrilus* larvae in ash twigs. MacRae (1991) reared *A. subcinctus* from dead ash twigs. Lelito et al. (2008) captured more *A. subcinctus* adults on sticky traps on ash trees at sites with high ash mortality compared to sites with low ash mortality.

Although *A. subcinctus* and EAB adults differ substantially from one another in size and appearance, there has been concern that surveyors could confuse late instar *A. subcinctus* larvae with early instar EAB larvae, given that *A. subcinctus* and EAB both infest ash. Misidentification could have serious repercussions for EAB containment efforts and establishment of EAB quarantine zones. Our objectives were to study the biology and larval morphology of *A. subcinctus* and determine which characters were useful for differentiating immature stages of *A. subcinctus* from EAB.

## Materials and Methods

We studied *A. subcinctus* at three sites in Michigan during 2006-2009. We worked at an abandoned parking lot with green ash and white ash (*F. americana* L.) planted along the perimeter near Brighton, Livingston County, MI (Lat 42.54 N, Long -83.79 W) in 2006-2007; a natural stand of green ash at Kensington Metro Park, Oakland County, MI (Lat 42.54 N, Long -83.63 W) in 2006-2009; and a natural stand of green ash near Webberville, Ingham County, MI (Lat 42.66 N, Long -84.20 W) in 2007-2009. At all three study sites, EAB had killed most of the ash trees during 2004-2006. At each site, we documented the adult flight season of *A. subcinctus* by placing 6-12 yellow sticky cards (7.6 cm × 12.7 cm, Olson Products, Inc., Medina, OH) on 3-6 ash trees (2 cards per tree) per site to collect adults while they flew to foliage to feed. Sticky cards were suspended on live ash branches and sprouts 1-2 m above the ground with wire ties. We collected and replaced sticky cards approximately every 2 weeks from late spring (April-May) through midsummer (August), except in 2006 when the study began in July.

After adult flight began, live and dead ash twigs were visually inspected for *A. subcinctus* eggs. Because initially we found *A. subcinctus* eggs only on dead twigs of both green and white ash, we focused on dead twigs in most surveys. In 2006 and 2007, we collected sections of dead green and white ash twigs approximately every 2 weeks from late spring through late summer and on one or more occasions during late fall-early winter. Twigs were collected 1-4 m above the ground from 2 to 6 trees per site using pole-pruners as needed. We also collected sections of live ash twigs from the same trees if present. All twig sections were placed in labeled plastic bags and stored at 4-5ºC until they were inspected for *A. subcinctus* life stages in the laboratory. For each twig section, we recorded the number of eggs, larvae, prepupae, and pupae, as well as length and average diameter. Twig sections averaged 20 cm long and consisted primarily of 1- to 3-yr-old growth. To calculate twig moisture content,

we placed a subset of dead twig sections with current-year *A. subcinctus* eggs and live twig sections that lacked eggs in individual pre-weighed metal cans with tight fitting lids. We weighed the cans with twigs after we returned to the laboratory, then dried them at 100ºC for 24 hrs, and re-weighed them. We calculated percent moisture content of twigs using the formula: [(wet weight - dry weight)/wet weight] × 100.

*Agrilus subcinctus* larvae dissected from twig sections were preserved in 70% alcohol. Immature parasitoids found in association with *A. subcinctus* life stages were placed in vials and monitored for adult emergence. Adult parasitoids that emerged were preserved in 95% alcohol. Hymenopteran parasitoids were identified following Gibson et al. (1997) and Wharton et al. (1997). The dissected *A. subcinctus* larvae were prepared on slides using PVC mounting media. Larval peristoma width, i.e., the outer border of the mouthparts, and urogomphus length were measured at the same locations where Wang et al. (2005) measured EAB larvae using a compound microscope equipped with a micrometer. We used frequency distribution plots to determine the number of larval instars based on both of these variables, and validated these determinations by calculating Dyar's ratio and Crosby's ratio for each variable (Dyar 1890; Crosby 1973, 1974; Craig 1975; Loerch and Cameron 1983; Johnson and Williamson 2006). In practice, Crosby's ratios of less than 10% confirm that all instars have been accounted for (Crosby 1974, Craig 1975). We also measured the overall body length of a subset of intact *A. subcinctus* larvae for each instar.

We used a scanning electron microscope at the Michigan State University Center for Advanced Microscopy to photograph urogomphi of 4$^{th}$ instar *A. subcinctus* and 2$^{nd}$ instar EAB to inspect for morphological differences between the two species. These instars were selected because an average 4$^{th}$ instar *A. subcinctus* is similar in length to a 2$^{nd}$ instar EAB (see Results). Larvae were fixed at 4ºC for 1-2 hours in 4% glutaraldehyde buffered with 0.1 m sodium phosphate at pH 7.4. Following a brief rinse in the buffer, samples were dehydrated in an ethanol series (25%, 50%, 75%, and 95%) for 10-15 minutes at each gradation and with three 10-minute changes in 100% ethanol. Fixed larvae were mounted on aluminum stubs using high vacuum carbon tabs (SPI Supplies, West Chester, PA).

ANOVA was used to compare *A. subcinctus* egg densities between ash species, to compare twig moisture content between dead twigs with *A. subcinctus* and live twigs without *A. subcinctus*, and to compare *A. subcinctus* and EAB egg lengths and widths (PROC GLM; SAS Institute 2001).

## Results and Discussion

***Agrilus subcinctus* biology.** Overall, 410 dead ash twig sections (211 green ash; 199 white ash) with *A. subcinctus* life stages were dissected in 2006-2007. *Agrilus subcinctus* mean (± SE) egg density at the Brighton study site, our only site with both ash species present, was 0.45 ± 0.05 eggs per cm$^2$ of bark surface area for green ash (N = 72 twigs) and 0.37 ± 0.04 for white ash (N = 199); these two means did not differ significantly ($F$ = 1.51; df = 1, 269; $P$ = 0.2205). No *A. subcinctus* eggs or larvae were found on the 55 live green ash and 40 live white ash twig sections that were dissected. Mean diameter of dead twig sections with *A. subcinctus* life stages was 7.5 mm and ranged from 3.4 to 27.2 mm. Mean diameter of live twig sections dissected was 7.1 mm and ranged from 4.1 to 11.8 mm.

Mean (± SE) moisture content of dead twig sections (N = 26) with current-year *A. subcinctus* eggs was 18.5 ± 0.7 % and was significantly less than the moisture content of live twig sections (N = 6), which was 48.9 ± 2.5 % ($F$ = 260.28; df = 1, 30; $P$ = .0001). The large difference in moisture content between dead twigs with *A. subcinctus* eggs and live twigs suggests that *A. subcinctus* prefers

twigs that have been dead for some time, i.e., died during the previous growing season, rather than twigs that died during the current year. Given that we found *A. subcinctus* eggs only on dead ash twigs suggests that this beetle has little impact on live ash trees or seedlings. Moreover, no *A. subcinctus* have emerged from the more than 5,000 ash logs (8-30 cm diameter and 50-55 cm length) that we harvested from live and recently dead ash trees and used to rear EAB adults for research purposes in our laboratory at Michigan State University during 2004-2009 (TRP, pers. observation).

In 2006 and 2007, adult *A. subcinctus* flight began before we first placed sticky cards in the field (Fig. 1). In 2008 and 2009, *A. subcinctus* adults were captured during late May through mid-August, with most adults captured in June. During the adult flight period, *A. subcinctus* adults were commonly found feeding on ash foliage and walking on both dead and live ash twigs. In 2007-2009 (2006 data collection began after most egg laying was complete), we first found *A. subcinctus* eggs on twigs in early June. Egg laying likely began before these dates given that site visits were made at 2-wk intervals each year. The first *A. subcinctus* eggs were found between 22 May and 6 June in 2007; 30 May and 9 June in 2008; and 29 May and 12 June in 2009. *Agrilus subcinctus* eggs were deposited on the bark surface of dead twigs and were easily visible. Newly laid eggs were light gray, but quickly turned glossy black as the embryo matured. *Agrilus subcinctus* eggs (N = 25) were significantly shorter (mean = 0.89 ± 0.01 mm; $F = 155.66$; df = 1, 43; $P = 0.0001$) and narrower (0.52 ± 0.01 mm; $F = 363.75$; df = 1, 43; $P = 0.0001$), compared with locally collected EAB eggs (N = 20) which averaged 1.22 ± 0.03 mm long and 0.95 ± 0.02 mm wide.

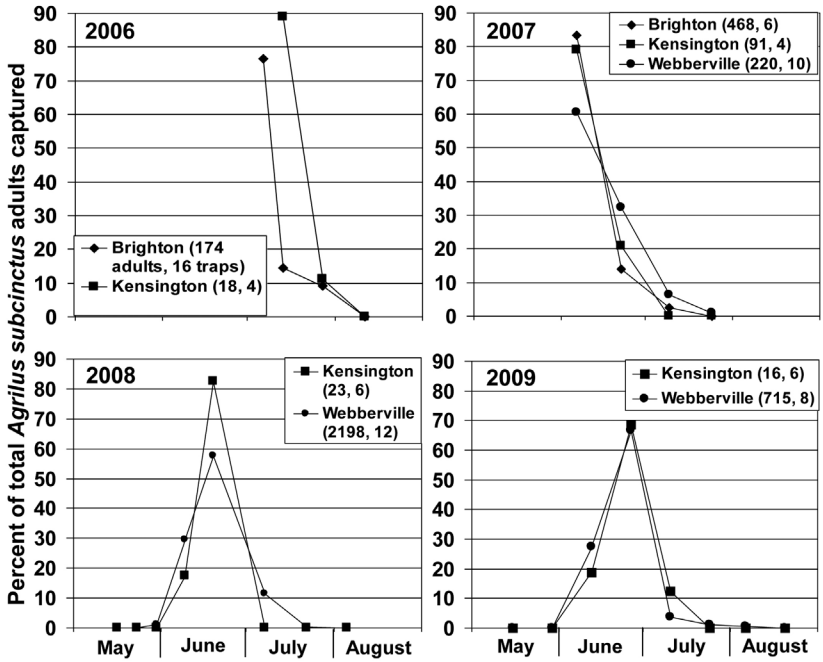


Fig. 1. Seasonal flight of *Agrilus subcinctus* in southern Michigan during 2006-2009, showing percent of all adults captured per collection date by field site and year. Total number of adults and number of sticky cards are given for each site and year. Collections were made with yellow sticky cards suspended from live ash (*Fraxinus* spp.) branches.

*Agrilus subcinctus* neonate larvae tunneled through the bark into the cambial region of twigs where they fed throughout summer and fall. We found *A. subcinctus* larvae to have four distinct instars (Table 1), which corresponds with the number of instars documented for other *Agrilus* such as *A. bilineatus* (Weber) (Coté and Allen 1980, Haack and Benjamin 1982), *A. anxius* Gory (Loerch and Cameron 1983), and *A. planipennis* (Cappaert et al. 2005, Wang et al. 2005). Larval galleries gently meandered with the wood grain along the length of the twig, but turned 180º at least once along their length. Mean *A. subcinctus* larval gallery length for all instars combined was 10.6 ± 1.1 cm (N = 5).

When *A. subcinctus* larvae reached maturity, they tunneled into the xylem or pith of twigs where they created individual chambers for pupation, terminated feeding, and began the prepupal stage. Pupation chambers were usually located in the xylem of larger diameter twigs and in the pith of smaller diameter twigs that we inspected. For example, dissected twigs 8mm in diameter or smaller had 88% of the pupation chambers located in the pith, while twigs larger than 8mm had only 15% of the pupation chambers in the pith. When pupation chambers were complete, mature larvae folded their bodies into a "V" shape about half-way along their length, and then gradually unfolded their bodies as they shortened to prepare for pupation.

*Agrilus subcinctus* overwintered as larvae, prepupae, and pupae (Fig. 2). The highest percentage of prepupae found in twigs was during September; most individuals pupated by late fall or early winter (November-December), with only a small percentage remaining as prepupae through the winter. In comparison, EAB has been found only to overwinter as prepupae or immature larvae. To our knowledge, *A. subcinctus* is the first *Agrilus* species reported to overwinter primarily in the pupal stage, although we should note that relatively few life-history studies have been completed on the nearly 3,000 *Agrilus* species found worldwide. *Agrilus subcinctus* immature larvae overwintered in the phloem of the dead twigs. Most *A. subcinctus* individuals that overwintered as immature larvae were 3rd and 4th instars (Table 2).

Some *A. subcinctus* larvae may require 2 years to complete development given that a few 2nd instar larvae were found in twigs collected in November 2006 and December 2007 and presumably overwintered as 2nd instars (Table 2). It is

Table 1. Range and mean (±SE) urogomphus length and peristoma width for *Agrilus subcinctus* larvae dissected from dead ash (*Fraxinus* spp.) twigs in southern Michigan in 2006-2007, including Dyar's ratio and Crosby's ratio.

| Variable measured | Instar | Size Range | Mean (±SE) | N | Dyar's ratio[1] | Crosby's ratio (%)[2] |
|---|---|---|---|---|---|---|
| Urogomphus | 1 | 0.050-0.106 | 0.079 ± 0.003 | 24 | | |
| length (mm) | 2 | 0.108-0.146 | 0.130 ± 0.004 | 13 | 1.639 | |
| | 3 | 0.151-0.254 | 0.202 ± 0.004 | 75 | 1.556 | -5.05 |
| | 4 | 0.259-0.392 | 0.312 ± 0.002 | 192 | 1.541 | -0.99 |
| Peristoma | 1 | 0.138-0.186 | 0.158 ± 0.001 | 55 | | |
| width (mm) | 2 | 0.188-0.251 | 0.227 ± 0.003 | 28 | 1.441 | |
| | 3 | 0.256-0.384 | 0.322 ± 0.004 | 93 | 1.416 | -1.73 |
| | 4 | 0.389-0.550 | 0.471 ± 0.003 | 170 | 1.463 | 3.29 |

[1]Calculated by dividing the mean for each instar by the mean of the previous instar for each structure measured.
[2]Calculated by dividing each Dyar's ratio by the Dyar's ratio of the previous instar for each structure measured. Crosby's ratios less than ±10% verify that all instars were accounted for (Crosby 1974, Craig 1975).

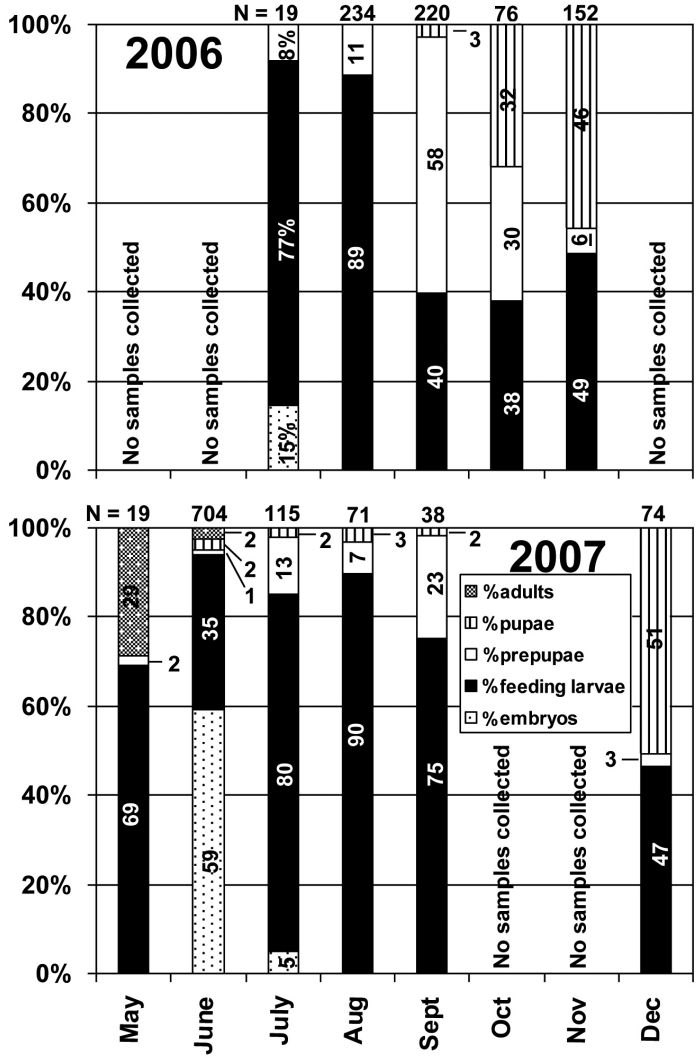


Fig. 2. Seasonal development of *Agrilus subcinctus* in southern Michigan in 2006-2007, showing percent of individuals found in dead ash (*Fraxinus* spp.) twigs by life stage for each month of collection. Total number of *A. subcinctus* life stages (N) found is given for each month and year.

Table 2. Frequency distribution of *Agrilus subcinctus* larvae by instar (based on peristoma width and urogomphus length) and collection month for larvae found feeding in the phloem of dead ash (*Fraxinus* spp.) twigs collected in southern Michigan in 2006-2007.

| Year | Collection month | Frequency distribution by instar (%) First | Second | Third | Fourth[1] | N |
|---|---|---|---|---|---|---|
| 2006 | June | 56 | 44 | 0 | 0 | 18 |
| | July | 75 | 13 | 9 | 3 | 64 |
| | August | 2 | 3 | 27 | 68 | 178 |
| | September | 1 | 3 | 24 | 71 | 94 |
| | October | 7 | 0 | 45 | 48 | 29 |
| | November | 0 | 9 | 40 | 51 | 45 |
| 2007 | May | 0 | 0 | 0 | 100 | 7 |
| | June | 67 | 0 | 0 | 33 | 3 |
| | July | 57 | 13 | 30 | 0 | 23 |
| | August | 0 | 0 | 11 | 89 | 9 |
| | September | 0 | 3 | 30 | 67 | 30 |
| | December | 0 | 4 | 43 | 52 | 23 |

[1]Does not include prepupae.

unlikely that these larvae would have had time to complete development and emerge as adults by the end of the adult flight period the following summer. The larvae that overwinter as 2nd instars likely represent the small number of prepupae and pupae that we found in twigs in late July and August after adult flight was complete and while most current-year larvae were still feeding in the phloem (Fig. 1, Table 2). Plasticity in larval development rates is common for wood-boring species, especially those that feed in dead woody tissues (Haack and Slansky 1987).

**Parasitoids.** Overall, *A. subcinctus* egg parasitism was 8.6% in 2006 (N = 3,414 eggs) and 9.4% in 2007 (N = 1,835). Only two adult egg parasitoid specimens were successfully reared in the laboratory and they were both identified as *Avetianella* sp. (Encyrtidae) (Table 3). Overall, larval parasitism was 2.6% in 2006 (N = 388 larvae) and 9.1% in 2007 (N = 320). We reared five species of hymenopteran parasitoids from *A. subcinctus* larvae, including *Ecphylus* sp. (Braconidae), *Eurytoma* sp. (Eurytomidae), near *Hadrotrichodes* (possible undescribed genus; Eulophidae), *Heterospilus* sp. (Braconidae), *Metapelma* sp. (Eupelmidae; Table 3). One parasitoid species, *Oodera* sp. (Pteromalidae), was reared from an *A. subcinctus* prepupae.

**Comparison of *Agrilus subcinctus* and EAB larvae.** Late instar *A. subcinctus* larvae are somewhat similar to early instar EAB larvae. Average urogomphus length, peristoma width, and overall body length of 4th instar *A. subcinctus* were similar to those of 2nd instar EAB larvae (Table 1, Fig. 3; Cappaert et al. 2005, Wang et al. 2005). There are, however, subtle morphological differences between larvae of the two species. One difference is the general shape of the larval abdominal segments, which are generally oval for *A. subcinctus* but more trapezoidal for EAB with each segment expanding from the anterior to posterior end (Fig. 3). The shape of the pronotal groove also varies considerably between these two agrilids. When viewed dorsally, the pronotal groove of *A. subcinctus* remains entire along its length, while the pronotal groove of EAB becomes bifurcate toward its distal end (Fig. 3). Also, the urogomphi, when viewed laterally, narrow abruptly approximately two-thirds along their

Table 3. Summary data for hymenopteran parasitoid species reared from *Agrilus subcinctus* immature stages dissected from dead ash (*Fraxinus* spp.) twigs in southern Michigan from 2006 to 2009, including parasitoid species, life stage attacked, date of field collection, Michigan counties where the collections were made, and number (or range) of parasitoid progeny found per life stage of the host.

| **Parasitoid species (Family)** | ***Agrilus subcinctus* life stage attacked** | **Date of field collection** | **Michigan counties** | **No. parasitoids found per life stage attacked (Range)** |
|---|---|---|---|---|
| *Avetianella* sp. (Encyrtidae) | Egg | 13 Jul 2006 | Livingston | 1 |
| Near *Hadrotrichodes* (possible undescribed genus; Eulophidae) | Larva | 19 Sep 2007 | Livingston | 9 |
| *Metapelma* sp. (Eupelmidae) | Larva | 11 May 2009 | Ingham | 1 |
| *Eurytoma* sp. (Eurytomidae) | Larva | 11 May 2009 | Ingham | 4 |
| *Ecphylus* sp. (Braconidae) | Larva | 8 Dec 2008 | Ingham | 2-3 |
| *Heterospilus* sp. (Braconidae) | Larva | 21 Sep 2006; 12 Dec 2007; 8 Dec 2008 | Ingham, Livingston | 1-2 |
| *Oodera* sp. (Pteromalidae) | Prepupa | 12 Dec 2007 | Livingston | 1 |

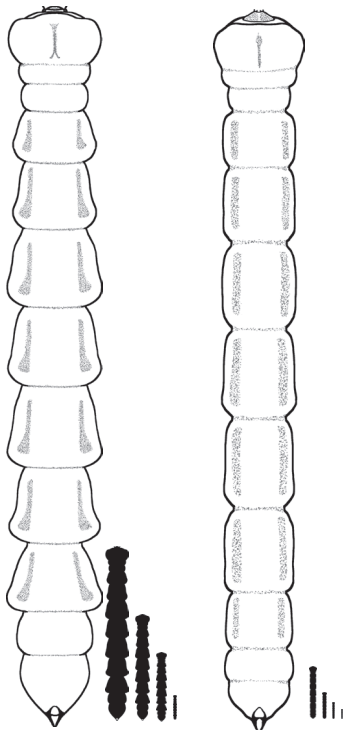

Fig. 3. General shape of the larval abdominal segments and pronotal groove (viewed dorsally) of a 2nd instar *Agrilus planipennis* larva (left) and a 4th instar *A. subcinctus* larva (right); and silhouettes that represent the average actual length of the four larval instars of *A. planipennis* and *A. subcinctus* based on measurements of larvae that were collected in Michigan, U.S.A.

length for *A. subcinctus*, while they taper gradually along their entire length for EAB (Fig. 4). In addition, the invaginations on the inner surface of *A. subcinctus* urogomphi are much more conspicuous than those on EAB urogomphi (Fig. 4).

Hespenheide (1969) found that smaller *Agrilus* species are usually associated with smaller-diameter host material, while larger *Agrilus* species infest larger-diameter host material. *Agrilus subcinctus* and EAB follow this general trend; however, there is some overlap in the size of host material that both species infest. The fact that *A. subcinctus* apparently infests only dead ash twigs and EAB rarely infests dead ash material greatly reduces the chance of confusing these two species while collecting in the field. Furthermore, the morphological differences between *A. subcinctus* and EAB larvae that we have described should help eliminate misidentification of these two species. However, in certain situations, molecular analyses may be required to distinguish the two species, such as when larvae are severely damaged to the extent that morphological characters cannot be examined.

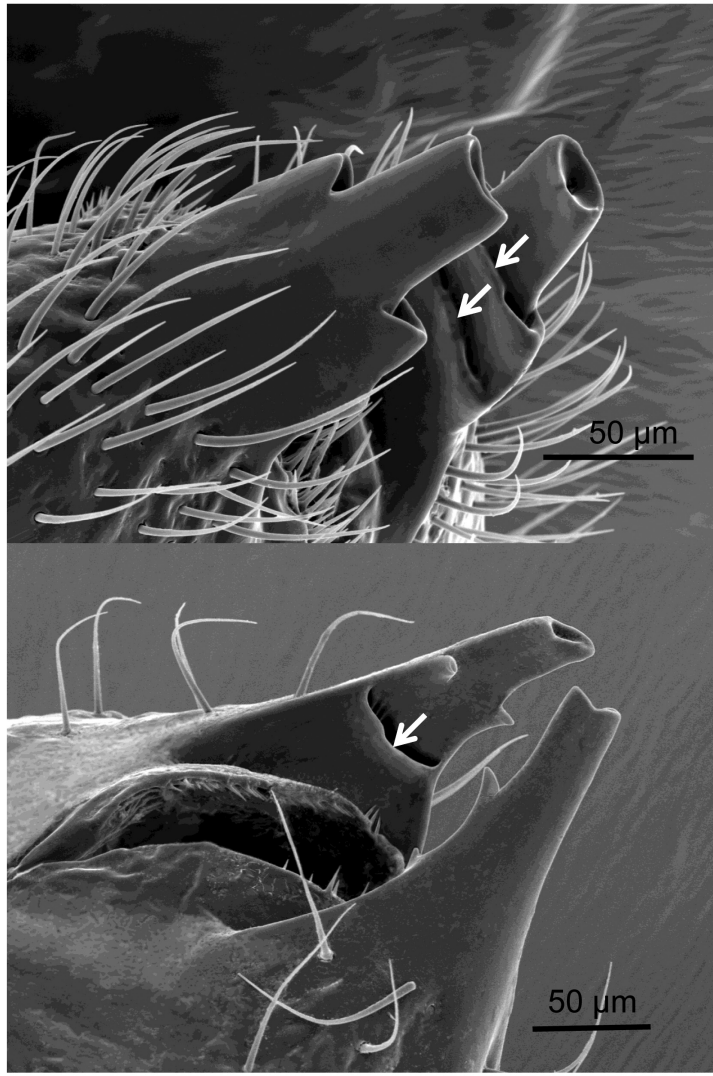


Fig. 4. Scanning electron micrographs of larval urogomphi of a 4th instar *Agrilus subcinctus* (top) and a 2nd instar *Agrilus planipennis* (bottom) with arrows pointing to invaginations on the inner surface of the urogomphi.

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