Biology and Life History of Tetrastichus planipennisi (Hymenoptera: Eulophidae), a Larval Endoparasitoid of the Emerald Ash Borer (Coleoptera: Buprestidae)

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BIOLOGY AND LIFE HISTORY OF *TETRASTICHUS PLANIPENNISI* (HYMENOPTERA: EULOPHIDAE), A LARVAL ENDOPARASITOID OF THE EMERALD ASH BORER (COLEOPTERA: BUPRESTIDAE)

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ABSTRACT

Tetrastichus planipennisi (Hymenoptera: Eulophidae) is a gregarious larval endoparasitoid from China that is being released in the United States as a biocontrol agent of the emerald ash borer (*Agrilus planipennis* Fairmaire), an exotic beetle responsible for widespread ash mortality. The developmental time of immature stages, adult longevity, reproductive age, oviposition rate, and realized fecundity were determined in the laboratory under normal rearing conditions (25 ± 2 °C, 65 ± 10% RH, and L:D 16:8 hr photoperiod). It takes approximately four weeks to complete a single generation (from egg to adult) under normal rearing conditions. While male wasps lived for a median of 5 weeks, females lived significantly longer with a median survival time of 6 weeks and a maximum survival time of 9 weeks. Newly emerged females appeared to be as capable of producing progeny as older females, indicating that *T. planipennisi* may be synovigenic. The average number of progeny per reproductive female remained relatively constant through the first 6 weeks of the trial with each reproductively active female producing 23-26 progeny each week. Lifetime realized fecundity of the adults averaged 57 progeny per reproductively active female with a female-to-male sex ratio of approximately 3:1; the maximum number of progeny produced by a single female was 108. These results suggest that *T. planipennisi* may have several generations in the mid-Atlantic and Midwestern regions of United States, where normal growing seasons (with average temperature >25 °C) are normally four to five months (May-Sep). Because of the gregarious nature, long life span and oviposition period of adults, *T. planipennisi* is likely to have multiple overlapping generations and has the potential to be an effective biocontrol agent against EAB.

Key Words: *Agrilus planipennis*, parasitoid, fecundity, reproduction, development, synovigeny

RESUMEN

Tetrastichus planipennisi (Hymenoptera: Eulophidae) es un endoparasitoide larval, gregario, originario de la China, que está siendo liberado en los Estados Unidos como un agente de control biológico del barrenador del fresno (*Agrilus planipennis* Fairmaire), un escarabajo exótico responsable de la muerte generalizada del fresno. El tiempo de desarrollo de los estadios inmaduros, la longevidad de los adultos, la duración periodo reproductivo, la tasa de desarrollo, y la fecundidad total, fueron determinados en el laboratorio bajo condiciones normales de cría (25 ± 2 °C, 65 ±10% HR, y L:D 16:8 hr fotoperiodo). Tomó aproximadamente dos semanas para completar una generación (de huevo a adulto). Las avispas macho vivieron una media de 5 semanas, mientras que las hembras tuvieron una longevidad significativamente mayor, con un tiempo medio de sobrevivencia de 6 semanas y un tiempo máximo de sobrevivencia de 9 semanas. Las hembras recién emergidas fueron tan capaces de producir progenie como hembras maduras, indicando que *T. planipennisi* no es sinovigénica. El número promedio de progenie por hembra reproductiva permaneció relativamente constante durante las primeras seis semanas de los ensayos (23-26 individuos / hembra reproductiva/ semana). La fecundidad total promedio fue de 57 individuos por cada hembra reproductiva, con una relación de sexos aproximada (hembras: macho) de 3:1; el máximo de individuos producidos por una hembra fue de 108. Estos resultados sugieren que *T. planipennisi* puede completar múltiples generaciones en las regiones del medio-Atlántico y medio-Occidente de

los Estados Unidos, donde la temporada de cultivo (con una temperatura promedio >25 °C) usualmente es de cuatro a cinco meses (Mayo-Septiembre). Por su naturaleza gregaria, y por presentar periodos de vida y ovoposición largos, es probable que *T. planipennisi* tenga múltiples generaciones sobrepuestas y el potencial para ser un agente de control biológico efectivo contra el barrenador del fresno.

The emerald ash borer (EAB), *Agrilus planipennis* Fairmaire (Coleoptera: Buprestidae), has been found in 15 U.S. States and two Canadian Provinces and killed millions of North American ash (*Fraxinus* spp.) trees since its detection in 2002 in southern Michigan (Haack et al. 2002; Michigan State University 2011; Canadian Food Inspection Agency 2011). Within the next 10 years, the EAB infestation is expected to encompass 25 of the 48 contiguous U.S. states with projected costs on developed land alone exceeding $10 billion (upper estimates exceed $25 billion) (Kovacs et al. 2010). Regulatory efforts to contain this pest's spread via early detection, quarantine, and removal of infested ash trees, have had limited impact (Cappaert et al. 2005). Moreover, conventional chemical controls cannot be used to protect native ash trees in forested ecosystems because of prohibitive cost, general impracticality, and adverse effects on the environment (Poland & McCullough 2006). In contrast, classical biological control (the introduction of natural enemies from a pest's native range) may be a cost-effective, sustainable, and environmentally safe alternative. Consequently, the potential of biological control to curb or slow the spread of EAB must be fully explored and implemented (Bauer et al. 2010).

Tetrastichus planipennisi Yang is a gregarious parasitoid that is capable of attacking the second to fourth instars of EAB larvae in naturally infested ash trees (Liu et al. 2003; Liu and Bauer 2007; Yang et al. 2006; Ulyshen et al. 2010a). Following the safety testing with various non-target insects, it was introduced from China to the US for biological control of EAB (USDA APHIS 2007). In 2007, releases of *T. planipennisi* and 2 other EAB-biocontrol agents from China started in Michigan (Bauer et al. 2010; USDA APHIS 2007), and since then they have been released against EAB in 12 Midwestern and Mid-Atlantic States (Bauer et al. 2010; Gould et al. 2011). While the colonization, establishment, and potential efficacy of this parasitoid in suppressing EAB populations are currently being investigated in small scale research plots (Duan et al. 2010; Duan et al. 2011), more detailed understanding of *T. planipennisi* biology and life history is needed. Better knowledge in this regard will improve our ability to mass-rear this species in the laboratory and implement a large scale EAB biological control program in the US. This study investigates the development of immature stages of *T. planipennisi* as well as adult longevity, reproductive age, oviposition rate, and life-time fecundity realized using late instars of EAB as the host.

Materials and Methods

Parasitoids

Tetrastichus planipennisi used in this study were obtained from a laboratory colony established and maintained at the Beneficial Insect Introduction Research Laboratory (BIIRL) in Newark, Delaware. The BIIRL colony founders were collected in 2008 from parasitized EAB larvae harvested in the Liaoning province in northeastern mainland China; parasitoids used during these investigations were from the F1 to F5 generations of the BIIRL laboratory colony. Throughout these investigations we utilized only naive wasps (those with no previous exposure to the EAB host). Before being utilized in the bioassay procedures, parasitoids were housed in clear 2.4 liter polystyrene crisper boxes (Consolidated® Plastics Company, Stow, Ohio) and maintained in environmental chambers (Percival Scientific, Perry, Iowa) at 25 (±2) °C, with 65 ± 10% RH and 16:8 (L:D) hr photoperiod. The crisper boxes were ventilated with a series of 2.5-cm holes in the crisper box lids and walls; these were then covered with a fine (0.5 mm^2) plastic mesh. A water source was provided inside each crisper box via a 10-dram clear plastic vial (US Plastics, Lima, Ohio) fitted with a 10-cm braided cotton dental wick (Richmond Dental, Charlotte, North Carolina), and replaced once every week. A food source was offered to the parasitoids by streaking clover honey on the screen covering the ventilation holes.

Emerald Ash Borer Larvae

EAB larvae used in all investigations were collected from EAB-infested ash trees in natural forests in central Michigan, Pennsylvania, and/or Maryland. The field-collection of EAB larvae involved removing bark from infested ash trees with a draw knife (Ben Meadows, Janesville, Wisconsin) and carefully removing the EAB larvae from the exposed feeding galleries. The field-collected EAB larvae were then placed into a 12-well cell culture plate (Corning, Ithaca, New York) lined with moistened Kim-wipes (Fisher Scientific, Pittsburgh, Pennsylvania) and held on ice for transport to our laboratory. All EAB larvae used in the study ranged in age from 3rd (L3) to

4th (L4) instars [including mature 4th instars, which are J-shaped (JL)], and are most acceptable to *T. planipennisi* (Ulyshen et al. 2010a).

Host Exposure to *T. planipennisi* for Parasitism

Most of our experiments required *T. planipennisi* adults to parasitize EAB larvae. We accomplished this by artificially inserting EAB larvae into *Fraxinus* spp. (ash) sticks prior to offering them to the parasitoids (Liu and Bauer 2007; Ulyshen et al. 2010a). Our exposure methods were modified according to the following: sticks of ≈10 cm in length and 1 cm in diam were freshly cut from green ash (*F. pennsylvanica*). The sticks were then washed with warm soapy water and surface sterilized in a 10% bleach bath for ½ hour; after which they were thoroughly rinsed under cold running tap water. The sticks were prepared to receive EAB larvae by shaving a thin bark-flap away from the longitudinal axis of the stick using a sharp utility knife; the bark flap remained attached to the midsection of the stick. We then fashioned a narrow U-shaped channel (4-5 mm deep × 5-7 mm wide) in the exposed wood using a #11 palm-handled wood veiner (Woodcraft Supply LLC, Parkersburg, West Virginia). An EAB larva was then placed in the channel, covered with the bark flap, and the bark flap was secured with thin bands of parafilm at each end of the flap. The number of EAB per stick varied according to the needs of the experimental design (typically from 1 to 5/stick). The sticks were placed, with the insertion-end up, either into cells of a 12-well tissue culture plate, or into 30 mL (1 oz.) PETE soufflè solocups® (Reliable Paper, Acworth, Georgia). To maintain moisture in the ash sticks, water was added either to the cells of the culture plate or to the soufflé cups so that the base of each stick was immersed in about ¼ cm of water. Parafilm was used to cover the surface of the plate or cup to prevent escape of water into the rearing arena and also to keep adult parasitoids from drowning. The EAB-infested ash sticks were then placed in a ventilated, polystyrene crisper box (17.6 × 12.6 × 10 cm), which served as the rearing arena. Next, we offered the infested ash sticks to mated *T. planipennisi* adults; none of the parasitoids had been previously exposed to EAB hosts. To ensure the parasitoids were mated, newly emerged wasps were held together immediately following emergence in some bioassays, or for at least 7 days prior to being used in other bioassays. The number of adult parasitoids and exposure durations in different experiments was dictated by the needs of the experimental design.

Developmental Time of Immature *T. planipennisi*

Developmental stages of immature *T. planipennisi* were observed by offering EAB larvae (L4 and JL) inserted into green ash sticks to *T. planipennisi* gravid females. Sticks containing EAB larvae were exposed to gravid *T. planipennisi* females in rearing arenas at a parasitoid: host ratio of 6:1 for a 24-h period. The exposed ash sticks were then transferred into 200-ml plastic cups secured with ventilated lids, and incubated in an environmental chamber at 25 ± 2 °C and 55-75% RH. To observe the developmental stages of the *T. planipennisi* larvae, the small strips of parafilm securing the insertion site bark-flap were carefully removed and the bark-flap peeled back to reveal parasitized host larvae in the gallery. When the parasitoid larvae broke out of the EAB host larva (after consuming the entire host body except the chitinous cuticle) and entered the wandering stage (just before pupation), they were carefully transferred to a 4-cm Petri Dish (B. D. Falcon, Falcon Lakes, New Jersey) lined with moist filter paper. Thereafter, we observed developmental stages of the larvae every 2-3 days until they reached the young (white) pupal stage, and then every day until adult emergence. We made observations on the development of 538 *T. planipennisi* larvae that survived to the adult stage from 13 parasitized EAB larvae.

Several distinct developmental stages of immature *T. planipennisi* were categorized during our investigation as follows: (1) egg to newly hatching larva—no symptoms of parasitoid larvae visible through the host cuticle, (2) early-instar parasitoid larva we called a "swimmer" that was visible through the cuticle of the parasitized host larva above the dorsal vessel, (3) late-instar parasitoid larva that consumed and filled the interior of the host body, giving the cuticle a lumpy, and braided appearance we called the "braided stage", (4) "free-living" last-instar larva that entered the wandering stage and broke free of the host cuticle prior to pupation, 5) young pupae that were unmelanized and white with the compound eyes gradually turning pink after several d, and (6) mature pupae that were fully melanized and were evenly dark, and (7) adult that emerged from the mature pupa.

Adult Reproductive Age

To investigate if *T. planipennisi* successfully parasitized EAB larvae upon emergence as adults, we offered 50 EAB (5 EAB/tick × 5 replications × 2 treatments) larvae (L4) to 2 age cohorts of wasps: 1) < 24-hr old (since emergence) and 2) 5-d old. The EAB larvae were offered to the parasitoids for 1 d, after which the wasps were removed from the rearing arena and the exposed EAB larvae (in ash sticks) were returned to the environmental chamber to incubate at standard conditions [25 ± 2 °C; 55%-75% RH; 16:8 h (L: D) h)] for ≈2 weeks. At the end of the incubation period, the

ash sticks were dissected and EAB hosts were scored for parasitism by *T. planipennisi*.

Temporal patterns in *T. planipennisi* egg load were determined by iterative dissection of progressively older females. Fifty female wasps were randomly assigned to one of 5 age cohorts (5 age cohorts × 10 wasps/cohort): 1, 5, 10, 15 and 20 days after parasitoid emergence. The female wasps were placed into rearing arenas and held under the identical environmental conditions described previously without EAB hosts. At the end of the prescribed age treatment, each female wasp was dissected under a stereomicroscope and the number of mature eggs (i.e., fully elongated approximately 0.8 mm long × 0.1 mm in diameter) in the ovaries was recorded. The mean numbers of eggs (dependent variable) from the different age cohorts (factor) were compared using a one-way ANOVA; post-hoc testing was conducted using Tukey's HSD test.

Adult Longevity, Oviposition Rate, and Fecundity of *T. planipennisi*

To estimate longevity, oviposition rate, and lifetime realized fecundity of *T. planipennisi* adults, we monitored 30 pairs (one female × one male) of adult wasps from adult eclosion to death. Each wasp pair was provided with 3 (L4) EAB hosts inserted in green ash sticks (1 EAB/stick) using the method previously described. Three new EAB larvae were presented to each wasp pair every week; wasp mortality and survivorship data were recorded for each mating pair. The previously exposed EAB hosts were retained and incubated (inside ash sticks) in a controlled environment [(25 ± 2 °C, 55-75% RH, 16:8 h L:D in order to allow parasitoid larval development to progress. After approximately 2 weeks post-exposure, ash sticks containing the EAB hosts were dissected to determine the number of hosts successfully parasitized and the number and sex of parasitoid progeny per host.

The survivorship of female and male *T. planipennisi* was recorded weekly, and analyzed using a product-limit survival estimate based on the Kaplan-Meier survival platform (SAS Institute 2009). Median survival time in wk and 95% confidence intervals (CI) were also estimated for both sexes using the survival analysis procedure. Lifetime realized fecundity was estimated using the total number of progeny (±SEM) produced by each female wasp over her lifetime, and oviposition rates were estimated using the mean numbers of progeny (±SEM) produced per week by each female wasp. Because *T. planipennisi* is not polyembryonic (JJD unpublished data), the number of parasitoid offspring (late-instar larvae/pupae) is a reasonable estimate of egg production or fecundity. Statistical analyses and calculations were carried out with JMP Version 8.02 (SAS Institute 2009).

Results

Developmental Time of Immature *T. planipennisi*

Under our laboratory conditions, *T. planipennisi* developmental stages from egg to late instar (braided stage) larvae were completed 7 d after oviposition and the entire host body (except chitinous cuticle, head capsules, and urogomphi) was consumed. Immediately following this time, 100% of the parasitoid larvae were in the wandering stage (free-living phase), seeking pupation sites within the host gallery (Fig. 1). By d 14, 50% of the parasitoid larvae had entered the young (white) pupal stage while 50% remained in the wandering stage. No larvae in the wandering stage remained after the 18th d. All *T. planipennisi* larvae had completed the mature (dark) pupae stage of development by d 22, and 100% of the larvae (N = 538) had developed into adults (AD) by d 27 (Fig. 1).

Adult Reproductive Age

Adult *T. planipennisi* ovaries contained 20-30 mature eggs on average, with no significant differences among the age groups studied (Turkey-Kramer HSD tests, all P > 0.05) (Fig. 2). Data from the host exposure assay (Fig. 3) showed no significant differences in the number of progeny produced by 1- and 5-d-old females (independent sample t-test, t = 2.01174, df =47, P = 0.3900). These results demonstrate that newly emerged females of *T. planipennisi* are as capable of reproducing as older females and thus may be synovigenic.

Adult Longevity, Oviposition Rate, and Fecundity of *T. planipennisi*

While adults of *T. planipennisi* did not live more than 10 wk during this study (Fig. 4), females survived significantly longer than males (χ^2 = 5.0259, df = 1, P = 0.0250). The median survival time of male wasps was 5 wk (95% CI: 4-6 wk) with a maximum of 8 wk while the median survival time of females was 6 wk (95% CI: 6-8 wk) with a maximum of 9 wk (Fig. 4).

Approximately 20% of the female wasps produced progeny within the first week of emergence; however, the percent of females producing progeny peaked after 3 wk with ~60% of females producing progeny, then declining thereafter (Fig. 5). All female wasps in our study ceased production of progeny at wk 8 (post-emergence). The average rate of progeny production (oviposition) by each reproductive female *T. planipennisi* ranged from 23 to 27 progeny every wk for the first 6 wk after adult emergence, and reduced sharply to 10 progeny at wk 7 (Fig. 5). Over the entire life span, each reproductive female *T. planipennisi* (n = 24)

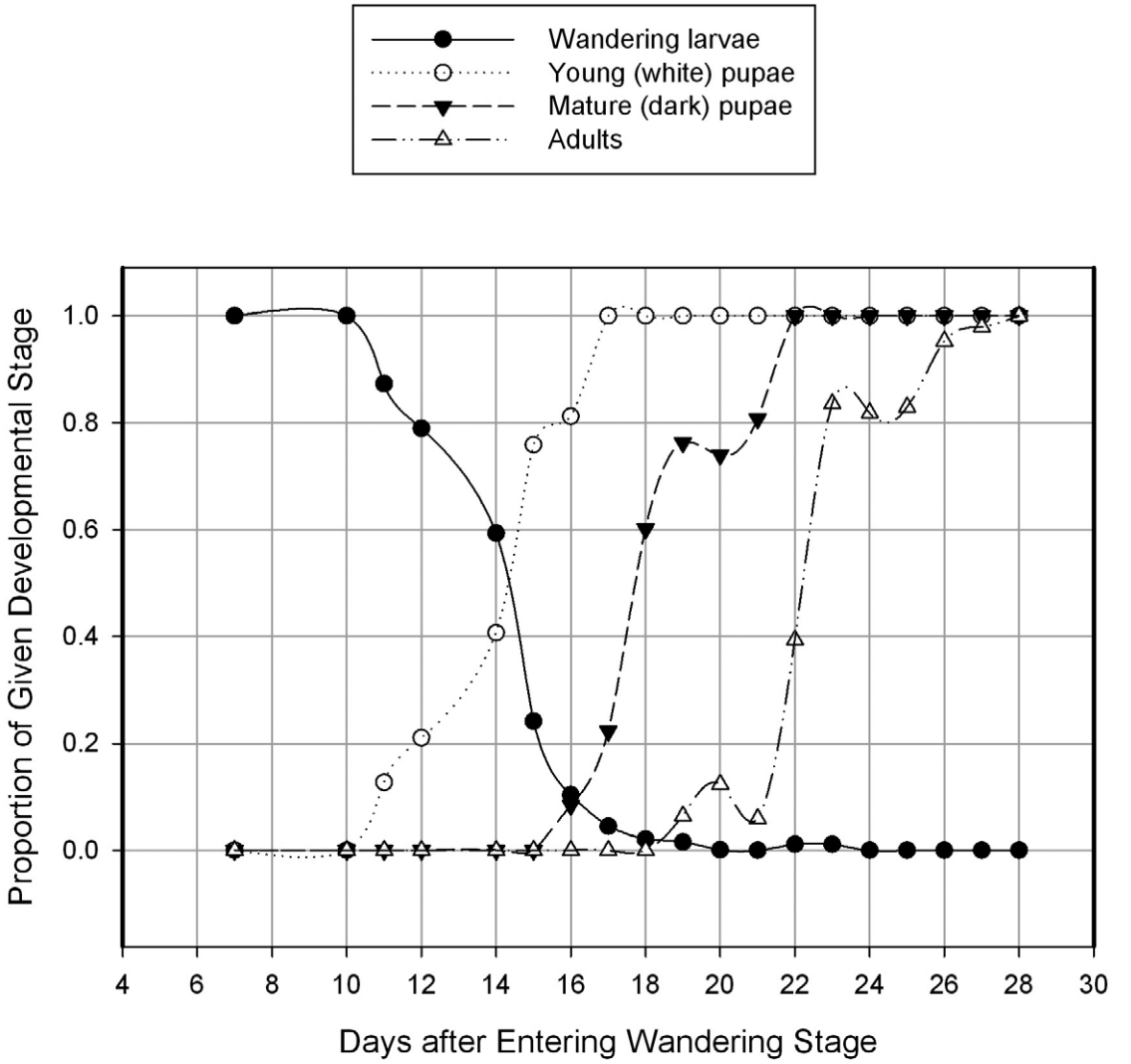

Fig. 1. Later developmental stages of immature *T. planipennisi* over time starting with the wandering stage of mature larvae. *Tetrastichus planipennisi* were reared at 25 ± 2 °C, 65 ± 10% RH, L:D16:8 hr photoperiod on EAB L4 larvae inserted into green ash sticks. The parasitoid larvae (n = 538) were examined observed every 2-3 days until they reached the young (white) pupal stage, and then every day until adult emergence.

produced a mean of 57 (±5.8 SEM) progeny with a minimum of 15 and maximum of 108 progeny. The sex ratio of all progenies produced during the course of the study was approximately 3:1 female to male. Over the entire course of the study, host utilization (parasitism) rate was approximately 13% and a total of 6 females (20%) failed to produce any progeny and were not included in the fecundity calculation.

Discussion

Results from our study show that *T. planipennisi* takes approximately 4 wk to complete a single generation (from egg to adult) under our laboratory rearing conditions (25 ± 2 °C, 65 ± 10% RH, L:D16:8 hr photoperiod). Additionally, adult female wasps had a median survival time of 6 wk with maximum progeny production occurring at wk 3. Considering the growing season (with daily temperatures averaging 25 °C) normally lasts less than 6 mo (from May to Oct) in the Mid-Atlantic States (less than 5 mo in the more northern reaches of the Midwestern United States), it is not unreasonable to suggest that *T. planipennisi* is likely to have multiple generations per year. In addition, females of *T. planipennisi* produce many progeny with a female to male sex ratio of ≈3:1

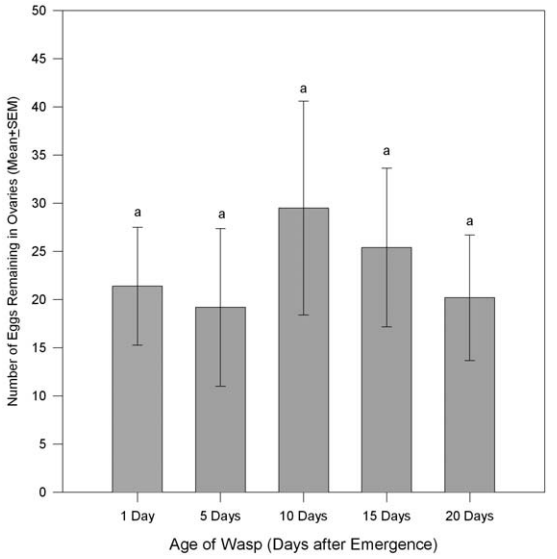

Fig. 2. Average number of mature eggs in ovaries of *Tetrastichus planipennisi* of different age groups (n = 10 for each group) after emergence (without access to host larvae). Bars labeled with the same letter indicate no significant differences among different age groups (Turkey-Kramer HSD tests, all $P > 0.05$).

over a typical life span. The extended adult longevity, multi-voltinism, high reproductive potential and female-biased progeny sex ratio of *T. planipennisi* are all life history traits that may be advantageous in establishing a self-sustaining population in North American EAB-infested forests.

Recent field studies demonstrated that *T. planipennisi* successfully established a presence with a consistent low level of EAB larval parasitism (1 to 5%) in several central Michigan release sites where approximately 3,000 female wasps were released (Duan et al. 2010, 2011). Although

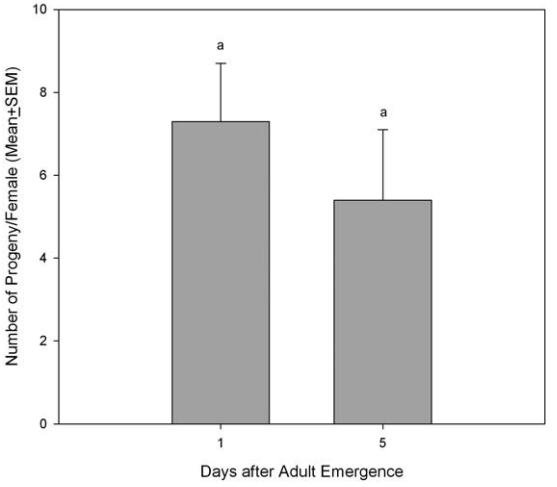

Fig. 3. Number of progeny produced on parasitized EAB hosts by newly emerging (<24 hr old) females (n = 25) and 5 d old females (n = 25). The EAB larvae were exposed to both age groups of parasitoids for 24 hr. Bars labeled with the same letter indicate no significant differences between the 2 age groups (independent sample t-test, t = 2.01174, df = 47, P = 0.3900).

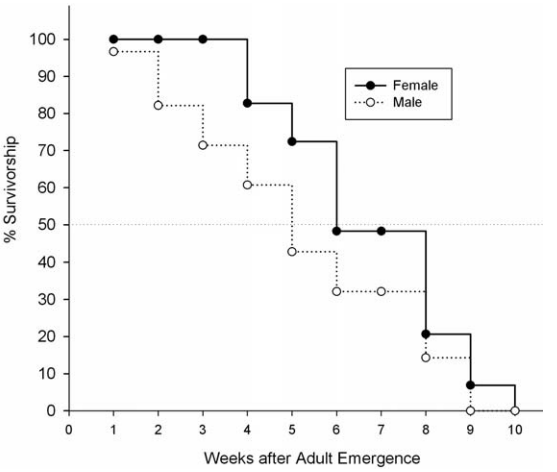

Fig. 4. Survivorship of adults of *Tetrastichus planipennisi* (n = 30 for both sexes) when continuously supplied with EAB larvae on a weekly basis for the duration of their lifespan. Female Tetrastichus survived significantly longer than the males (Log-rank χ^2 = 5.861, df = 1, P = 0.0159).

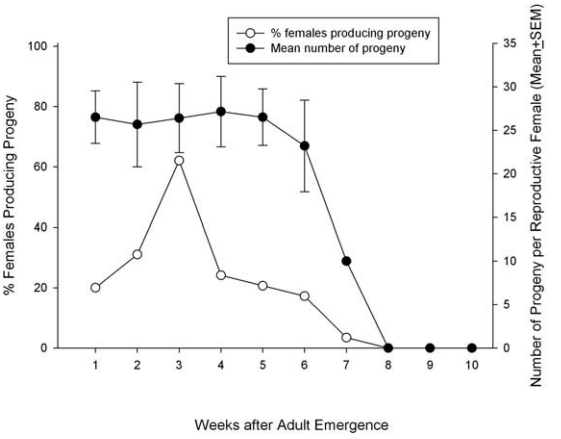

Fig. 5. Changes in female reproductive activity over time following adult eclosion (n = 30). Percent females producing progeny and the average number of progeny produced per female over time; each week non-reproducing females were excluded in the calculation of mean number of progeny produced weekly per female.

EAB parasitism by *T. planipennisi* reported in Michigan is much lower than parasitism rates typically seen in the native range of EAB in China (Liu et al. 2007), *T. planipennisi* is the most abundant species of hymenopteran parasitoid attacking EAB larvae in the Michigan release sites. *Tetrastichus planipennisi* accounted for 93% of all the hymenopteran parasitoid individuals collected immediately after field release in 2009 and 58% one year later in 2010 (Duan et al. 2011). The quick increase in *T. planipennisi* abundance relative to other hymenopteran parasitoids in the Michigan release sites was most likely due to the gregarious nature and high reproductive potential of *T. planipennisi*.

Field surveys conducted in the native range of *T. planipennisi* (China) as well as the new release areas in North America (Michigan, USA) indicate that immature stages of *T. planipennisi* occur throughout the year within the host galleries. Because immature stages take less time (4 wk) to complete development than the period a typical adult female survived in our laboratory study (median age = 8 weeks), it is very likely that *T. planipennisi* has 2 or more overlapping generations in the Mid Atlantic and Midwestern states of the continental US, where the EAB host typically has a semivoltine to univoltine breeding cycle. Further field or laboratory studies are needed to determine the threshold temperature for normal development and successful overwintering (Ulyshen et al. in press).

Previous observations also indicate that between 4 and 172 *T. planipennisi* offspring may be produced per EAB host in both field and laboratory settings with this number and the sex ratio depending largely upon the size of the host larvae at the time of parasitization (Liu et al. 2007; Ulyshen et al. 2010a; 2010b; 2011). Findings from the current study suggest that the average number of realized progeny per reproductive female remained relatively constant through the first 6 wk of the trial with each reproductively active female producing from 23 ~ 26 progeny each wk. Thus, our data suggest that large brood sizes of >30 progenies observed in the field (e.g., Liu et al. 2007) and in group rearing (Ulyshen et al. 2010) with multiple reproductive females are likely a combination of progeny from 2 or more parental females. This information needs to be considered when designing an efficient rearing program for mass production of *T. planipennisi* for EAB biocontrol programs.

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