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Foraging depth of *Cricotopus lebetis* larvae

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ABSTRACT

Evidence from previous studies indicates that *Cricotopus lebetis* Sublette (Diptera: Chironomidae) might have value as an augmentative biological control agent for hydrilla [*Hydrilla verticillata* (L.f.) Royle]. Although several aspects of the insect's biology, impact on hydrilla, and host-finding behavior have been investigated extensively, it is not known how deep neonates of *C. lebetis* can swim or drift in the water column to locate and infest hydrilla. In Florida, hydrilla typically grows to depths of up to 3 m, with half of its biomass occurring within the upper 0.5 m of the water column. To determine the foraging depth of the insect, experiments were conducted in controlled greenhouse studies with hydrilla placed at known depths (0 m, 0.9 m, 1.8 m, and 2.7 m) and in Lake Istokopoga to investigate the foraging depth of *C. lebetis* under natural conditions. The greenhouse studies demonstrated *C. lebetis* can attack submersed hydrilla at depths ranging from 0 m to at least 2.7 m. Field studies demonstrated the ability of *C. lebetis* to attack hydrilla from the water surface level to the hydrosoil, which occurred at a depth of approximately 0.9 m. Our results showed most of the hydrilla in Florida's shallow lakes grows within a depth range accessible to larvae of *C. lebetis*.

Key Words: aquatic weed, biological control, hydrilla, invasive species.

INTRODUCTION

Hydrilla [*Hydrilla verticillata* (L.f.) Royle] is among the most destructive invasive aquatic plants in the United States and a major threat to biodiversity and ecosystem function in U.S. freshwater ecosystems (Langeland 1996, Dayan and Netherland 2005). The plant is included both on the federal list of noxious weeds (USDA APHIS 2017) and on the FDACS (Florida Department of Agriculture and Consumer Services) list of Class I Prohibited Aquatic Plants (FDACS 2017). Botanists speculate that the center of origin of the plant occurs somewhere in the warmer areas of Asia, ranging from China, Iran, Afghanistan, Pakistan, and India, to Southeast Asia (Cook and Lüönd 1982, Langeland 1996, Madeira et al. 1997, Zhu et al. 2015). Hydrilla was first reported in the United States in the 1950s near Tampa, Florida (Schmitz et al. 1991), where it was likely introduced via the aquatic plant trade (Schmitz et al. 1991, Madeira et al. 2007). The plant is now established in water bodies throughout Florida and other southeastern states, and to a lesser extent in the states of Texas, Arizona, California, and Washington (Langeland 1996, EDDMapS 2018). By 2016, it was estimated that hydrilla infested over 162,000 ha of public lakes and waterways in Florida (Schardt 2010, FWC 2016, EDDMapS 2018). Control costs during the past 5 yr averaged $8.5 million annually (Schardt 2016).

Hydrilla reproduces and spreads clonally, grows rapidly (Glomski and Netherland 2012), and competes aggressively for light and carbon, the two major resources that limit photosynthetic capacity of submersed plants (Bowes and Salvucci 1989, Santamaría 2002). Previous studies reported that hydrilla has advanced morphological, physiological, and reproductive adaptations; as a result, the plant has been named "the perfect aquatic weed" (Langeland 1996, Dayan and Netherland 2005). Hydrilla grows at a rate of 2.5 to 10 cm d^{-1} (Glomski and Netherland 2012) and exhibits a growth pattern characterized by the formation of dense surface mats. The mats form a monoculture that impedes recreational and commercial water-use activities (Pimentel et al. 2005, Bidigare et al. 2009), inhibits the growth of native macrophytes (van Dijk 1985, Chambers et al. 1993, Colon-Gaud et al. 2004) that narrows the foundation of native food webs (Kelly and Hawes 2005), and increases invasibility of the local ecosystems (Nicko and Muench 2004, Wilde et al. 2014). This phenomenon is referred to as invasional meltdown (Simberloff and Von Holle 1999).

Management options for hydrilla in the United States are limited by the development of hydrilla biotypes resistant to commonly used herbicides (Michel et al. 2004, Berger and MacDonald 2011, Netherland and Jones 2015) and high operational costs and nontarget effects of mechanical harvesters (Haller et al. 1980, Langeland 1996). A recent study found that a hydrilla biotype, currently dominating the Kissimmee Chain of Lakes, one of the largest water bodies in Florida (Netherland and Jones 2015), is resistant to fluridone, the herbicide used extensively to control hydrilla. Four host-specific insect species have been introduced into the United States for management of hydrilla. Three of the four species, *Bagous hydrillae* O'Brien (Coleoptera: Curculionidae) (Center et al. 2013), *Hydrellia pakistanae* Deonier (Diptera: Ephydridae) (Wheeler and Center 2001), and *H. balciunasi* Bock (Diptera: Ephydridae) (Grodowitz et al. 1997) have become established. However, the impact of these insect agents on hydrilla has been hampered by a range of factors, including low host-plant quality (Wheeler and Center 2001), attacks by native parasitoids (Coon et al.

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2014), and temperature extremes in winters and summers (Buckingham 1994, Wheeler and Center 2001). As a result, scientists have suggested that a complex of natural enemies will be required to manage hydrilla, and have proposed searching for new biological control agents (Buckingham et al. 1989, Wheeler and Center 2001, Cuda et al. 2008). One promising biocontrol agent is the hydrilla stem mining midge *Cricotopus lebetis* Sublette (Diptera: Chironomidae), which was discovered in 1992 mining the apical meristem of hydrilla in Crystal River, Citrus County, FL (Cuda et al. 2002). *Cricotopus lebetis* is an adventive insect (Epler et al. 2000) that is widely distributed in Florida water bodies (Stratman et al. 2014). Previous studies have reported occurrence of *C. lebetis* in Lake Rowell (Bradford County) (Cuda et al. 2011) and low field populations in Lake Tohopekaliga (Osceola County), Bulldozer Canal (Brevard County), and Lake Istokpoga (Highlands County) (Stratman et al. 2013a). Thermal tolerance tests demonstrated that larvae of *C. lebetis* developed optimally at water temperatures between 20 and 30 C and suffered mortality as water temperature dropped below 15 C or exceeded 32 C (Stratman et al. 2014). In Crystal River, FL, which has a relatively constant water temperature of 25 C year-round (Cuda et al. 2002), *C. lebetis* stunted the growth of hydrilla and prevented formation of dense surface mats (Cuda et al. 2002, Cuda et al. 2011). As a result, several research teams have suggested *C. lebetis* is a promising agent for augmentative control of hydrilla (Cuda et al. 2002, Cuda et al. 2011). Because *C. lebetis* is adventive in Florida, it is not subject to the extensive regulatory restrictions associated with classical biological control agents.

Larvae of *C. lebetis* mine the apical meristem of hydrilla, stunting the growth of the plant (Cuda et al. 2002, Cuda et al. 2011). A survey in Crystal River, FL revealed the insect damaged up to 70% of the hydrilla apical meristems (Cuda et al. 2002). In a greenhouse study, larval feeding by *C. lebetis* reduced hydrilla biomass by more than 99% (Cuda et al. 2011). Larval feeding prevented the plant from "topping out" (Cuda et al. 2002, Cuda et al. 2011), a desired effect that minimizes the adverse impacts associated with the dense surface mats (Cuda et al. 2011). These findings generated interest in further investigating *C. lebetis* as a potential augmentative control agent of hydrilla (Cuda et al. 2002, Cuda et al. 2011, Stratman et al. 2013a,b, Stratman et al. 2014, Baniszewski et al. 2015, Mitchell et al. 2018).

Previous studies provided some insight into how *C. lebetis* detects and locates its host plant, hydrilla (Cuda et al. 2002, Lerner et al. 2008, Stratman et al. 2013b). Adults are terrestrial, whereas immatures are aquatic (Cuda et al. 2002). The adults swarm and mate aerially, and females oviposit on the water surface (Cuda et al. 2002). Lerner et al. (2008) showed that chironomid females identify water surfaces using polarized light. According to an oviposition study by Stratman et al. (2013b), *C. lebetis* females select oviposition sites near potential host plants and, in the absence of potential host plants, near available substrates. A laboratory study by Cuda et al. (2002) revealed that the egg masses of *C. lebetis* are sticky and negatively buoyant, and showed that as the egg masses sink, they attach either to host plants or substrates within the water column or continue to descend to the bottom of the water body. Evidence from these studies demonstrated that female oviposition behavior and egg mass properties increase the likelihood of neonates hatching near potential host plants. Neonates of *C. lebetis* located hydrilla randomly, but as the larvae matured, they acquired the ability to visually locate hydrilla (Stratman et al. 2013b). Chironomid larvae use three active modes of locomotion to move within the water column: swimming, crawling, and whole-body respiratory undulation (a sinusoidal wave action of the body bending in a head-to-tail direction) (Brackenbury 2000). Additionally, both the larval and pupal chironomid stages have negative buoyancy, so without active movement, both larvae and pupae free-fall in the water column (Brackenbury 2003). However, as the pupal stage nears the completion of development, pupae gradually gain buoyancy, which aids pupal ascendancy to the water surface in readiness for adult eclosion (Brackenbury 2000).

A knowledge gap exists in how deep neonates of *C. lebetis* can swim or fall in the water column to locate and attack hydrilla. Reports from previous studies suggest that herbivorous aquatic insects damage plants within certain depth ranges (Balciunas and Purcell 1991, Wheeler and Center 2001, Stout et al. 2002, Tindall et al. 2013). For example, some insect species, such as the weevil *Bagous affinis* Hustache (Coleoptera: Curculionidae), only attack hydrilla plants exposed during dry seasons and drawdowns (Bennett and Buckingham 1991). Knowledge of the foraging depths of biological control agents is important because hydrilla in Florida can grow to a depth of 15 m, but commonly grows at depths of up to 3 m (Langeland 1996). Approximately half of the hydrilla standing crop is composed of profusely branched stems occurring within the upper 0.5 m of the water column (Haller and Sutton 1975). Therefore, information on the foraging depth of *C. lebetis* can be used to better predict the efficacy of *C. lebetis* in controlling hydrilla populations that occur at various water depths. Previous studies in other systems have revealed that some chironomid species occur at relatively great depths (Linevich 1971). For example, a study in the world's deepest freshwater lake, Lake Baikal in Siberia, Russia, found a chironomid species, *Sergentia koschowi* Linevich (Diptera: Chironomidae), living at a record depth of 1,360 m (Linevich 1971). The objective of this study was to determine the foraging depth of *C. lebetis* larvae in the water column and the extent to which water depth limits the establishment and survival of *C. lebetis*. Experiments were conducted in controlled greenhouse studies on hydrilla placed at known depths and in a south Florida lake under natural conditions.

MATERIALS AND METHODS

Greenhouse experiments

Two experiments were conducted to investigate how deep larvae of *C. lebetis* can swim or free fall to locate hydrilla and the impact of water depth on the survival of *C. lebetis*. The objective was to generate information useful in designing mass-rearing facilities for *C. lebetis*, developing

field sampling and monitoring techniques, and predicting the efficacy of the insect in the management of hydrilla. Each experiment had four depth treatments: 0 m, 0.9 m, 1.8 m, and 2.7 m, and was replicated six times: twice in space and thrice in time. The two experiments were performed in 15.24-cm-diam extruded acrylic (Plexiglas) tubes[1] in four different lengths: 0.3 m, 0.9 m, 1.8 m, and 2.7 m. The 2.7-m Plexiglas tubes were formed by joining a 1.8-m Plexiglas tube and a 0.9-m Plexiglas tube using a flexible 15.24-cm-diam polyvinyl chloride (PVC) coupling.[2] The Plexiglas tubes all had a sealed and leakproof bottom and an open top. Plexiglas tanks have been used to rear hydrilla and *C. lebetis* in previous studies and are not toxic to hydrilla or developing larvae (Cuda et al. 2011). The experiments were conducted at the University of Florida (UF) Entomology and Nematology Department facilities (29.633882°N, 82.366733°W) in Gainesville, Alachua County, FL.

Hydrilla was collected from ponds located at the UF Institute of Food and Agricultural Sciences (IFAS) Center for Aquatic and Invasive Plants (CAIP) (29.726796°N, 82.415235°W) in Gainesville, FL. *Cricotopus lebetis* were obtained from a laboratory colony founded from insects collected from Lake Istokpoga (27.351679°N, 81.288061°W), Highlands County, FL. The colony was reared following procedures described by Cuda et al. (2002) and was housed in laboratory facilities of the UF IFAS Entomology and Nematology Department located in Gainesville, FL.

The Plexiglas tubes were thoroughly cleaned with well water and air-dried prior to starting new experiments. The clean Plexiglas tubes were placed upright on the greenhouse floor, aligned along the side rails of the steel greenhouse benches (0.81 m height by 3.67 m length), and spaced about 10 cm from each other. Except for the 0.3-m-tall Plexiglass tubes, all other Plexiglas tubes were each separately secured with a bungee cord (0.9 m long) fastened onto the side rails of the greenhouse benches to prevent them from tipping over. The 2.7-m-tall Plexiglas tubes required additional support. Therefore, each was additionally tied onto a second steel rail, which was 2.1 m high above the greenhouse floor and running parallel to the side rails of the greenhouse benches and across the length of the greenhouse from one wall to another. The Plexiglas tubes were then filled with the same well water used in insect rearing, leaving 5 cm of air space at the top of each tube.

Bouquets of hydrilla were formed by pairing two bundles of insect-free hydrilla stem tips. Each bundle was comprised of 60 stem tips, each of which was 15 cm long. Pairing two bundles of 60 stem tips instead of one bundle of 120 stem tips ensured that bouquets remained healthy during the experiment. The two bundles were paired using a ribbon (40 cm length by 2 cm width) made from a black polyethylene shade cloth.[3] To create a bouquet, two untied bundles of 60 stem tips were placed 2 cm apart on a flat tray. One-half of the ribbon length (about 19 cm) was wrapped around the base of the first hydrilla bundle and the remaining half of the ribbon length was wrapped around the base of the second bundle of hydrilla.

A bouquet was lowered to the bottom of each Plexiglas tube using a twisted nylon twine anchored to the base of the bouquet. In the 0 m treatments, the tips of the bouquets were positioned just below the water surface in Plexiglas tubes, which were 0.3 m in length. In the rest of the treatments, the bouquets were placed at the bottom of the Plexiglas tubes, which were 0.9 m, 1.8 m, and 2.7 m in height, respectively. An additional treatment of 0 m, replicated four times, was set up in an environmental growth chamber maintained at 25 C and a 14 : 10 (light : dark [L : D]) photoperiod to provide a basis for detecting any potential anomalies in *C. lebetis* performance under the greenhouse conditions. To confirm that the clean stem tips used in the experiment were free of any insect contamination, an additional set of two 0.9 m Plexiglas tubes received bouquets of the same hydrilla source used for the experimental bouquets, but without receiving any insect treatment.

Greenhouse experiment I: Inoculation of neonate Cricotopus lebetis on the water surface. Greenhouse experiment I was conducted to investigate the ability of *C. lebetis* neonates to swim or drift from the water surface and locate host plants, mine into hydrilla, and impact plant growth at various water depths. Neonates ($n = 185 \pm 5$) were inoculated on the water surface of each of the 16 Plexiglas tubes containing hydrilla stem tips (described above). Because neonates of *C. lebetis* are active swimmers and difficult to count under a microscope, counting was done at the egg stage. Several 24-h-old egg masses of *C. lebetis* were randomly selected from the laboratory colony and analyzed for fertility and egg count at ×8 under a dissecting microscope.[4] Typically, an egg mass of *C. lebetis* contains 50 to 250 eggs. Fertilized eggs turn grayish brown 24 h after oviposition, whereas unfertilized eggs remain white (Cuda et al. 2002). Two or three egg masses that contained a total of 185 ± 5 fertilized eggs were selected and placed in 35 ml culture tubes[5] that contained 25 ml of well water.

The selected egg masses were maintained in an insect rearing room, set at 24 C and 14 : 10 L : D photoperiod, until larval eclosion. The 35 ml culture tubes containing the hatched neonates were immediately transferred to the greenhouse where the neonates were gently emptied onto the water surface of the Plexiglas tubes. The tops of the Plexiglas tubes were then covered with fine mesh cloths, clamped in place using Dixon® worm gear clamps.[6] Natural light in the greenhouse was supplemented by incandescent bulbs set at 14 : 10 L : D photoperiod.

Greenhouse experiment II: Inoculation of Cricotopus lebetis egg masses on the water surface. Greenhouse experiment II was conducted to investigate the effect of water depth on egg masses of *C. lebetis* that sink to the bottom of water bodies and the impact of water depth on the ability of the resultant larval stage to locate, develop in, and damage hydrilla stem tips staged at the aforementioned treatment depths. The experiment was conducted following the same procedure described in greenhouse experiment I, except in this case, the Plexiglas tubes holding the four treatments were each inoculated with fertilized eggs of *C. lebetis* ($n = 185 \pm 5$). Eggs were examined and counted under a dissecting microscope, placed in 35 ml culture tubes, and transferred to the greenhouse, where they were deposited on the water surface of the Plexiglas tubes with a medicine dropper. The top of

each Plexiglas tube was covered with a fine mesh cloth and clamped in place with a Dixon worm gear clamp.

Data collection and analyses for greenhouse experiments

In both experiments, the Plexiglas tubes were monitored daily for adult emergence, from day 12 until the end of adult emergence. Adult *C. lebetis* have been reported to eclose beginning on the 13th d after oviposition (Cuda et al. 2002). Emerged adults from each Plexiglas tube were collected daily using a mouth aspirator, sexed, and counted. Adult males and females isolated by Plexiglas tubes were placed in oviposition chambers that consisted of 35 ml glass culture tubes containing 25 to 30 ml of water and capped with a perforated plastic cap (Cuda et al. 2002). Oviposition chambers were checked after 24 h to recover oviposited egg masses. Fertile and infertile eggs in the egg masses were counted and recorded. After the last adult emerged in a Plexiglas tube, bouquets of hydrilla were recovered from the experimental tube.

In the laboratory, bouquets were disassembled, and the stem tips were examined under a dissecting microscope for signs of feeding damage caused by *C. lebetis*. Stems exhibiting feeding damage were counted and recorded. Length of each stem was measured using a meter stick to an accuracy of 0.01 m.[7] Data were analyzed with a generalized linear models procedure, SAS PROC GLIMMIX, using a distribution function appropriate for the response variable in question, i.e., binomial for proportions, Poisson for count data, and normal for plant length (SAS Institute, Inc. 2008). Overdispersion was deemed not to be an issue for binomial and count data because the χ^2/df ratio was less than 1.10 (Warton and Hui 2011). Estimated means and 95% confidence intervals were back-transformed using the ilink option, and *P* values for multiple comparisons were adjusted using simulation option in the above-named procedure.

Field experiments

Two field experiments were conducted between January and March in 2017 at Lake Istokpoga to investigate the foraging depth of *C. lebetis* in the water column under natural conditions. A previous study reported presence of *C. lebetis* in Lake Istokpoga (Stratman et al. 2013a). The study site area within Lake Istokpoga was approximately 0.9 m deep and was chosen because it had established populations of both hydrilla and *C. lebetis*.

Field experiment I: Use of sentinel stems to determine the foraging depth of Cricotopus lebetis. In field experiment I, 15 sentinel bouquets of hydrilla (hereafter referred to as bouquet) were made by pairing two bundles, each comprised of 60 hydrilla stem tips, following the procedure described in the greenhouse experiments. Each bouquet consisted of 120 hydrilla stem tips, which were insect-free, 15 cm in length, and previously collected from Lake Istokpoga. The bouquets were staged at three depths in Lake Istokpoga: 1) 0 m, surface; 2) 0.45 m, middepth; and 3) 0.9 m, bottom sections of the water column. They were held at the desired depths by fastening them with self-locking nylon cable ties (2 mm by 100 mm) to the corresponding points on a 184 cm in height by 1.5 cm diam, heavy-duty, plastic-coated steel garden stake[8] and hammering the garden stake 0.3 m into the lake sediment. The experiment had five replicates, which were spaced at least 100 m apart. After 14 d, the average development period for the larval stage of *C. lebetis*, the bouquets were retrieved from the lake and transported to the laboratory for further processing. Theoretically, the 14-day period allowed the bouquets to be exposed to attack by all larval instars. In the laboratory, each bouquet was disassembled, and the stem tips were examined under a dissecting microscope for presence of *C. lebetis* larvae and for signs of feeding damage. The experiment was repeated three times.

Field experiment II: Survey of naturally growing stems to determine the foraging depth of Cricotopus lebetis. Field experiment II was conducted to determine the foraging depth of naturally occurring *C. lebetis* within the water column in Lake Istokpoga. Using a boat, hydrilla beds were located either by visual inspection or by rake method (Johnson and Newman 2011), and stem tip samples were collected following a technique modified from Johnson and Newman (2011). One hundred hydrilla stem tip samples were collected from hydrilla plants growing within the following depth ranges: 1) 0 to 0.3 m, surface; 2) 0.3 to 0.6 m, middepth; and 3) 0.6 to 0.9 m, bottom sections, of the water column. This procedure was done at three randomly selected sites with hydrilla beds that were at least 100 m apart from each other. This experiment was repeated biweekly, four times.

Data collection and analyses for field experiments

In both experiments, the collected plant samples were bagged in separate 3.8 L plastic zipper-seal bags, which were placed in a portable ice chest and transported to the Weed Biological Control Laboratory at UF, Gainesville, FL, for further processing. In the laboratory, 100 stems were randomly selected from each bagged sample and examined under a dissecting microscope for *C. lebetis* larvae and mining damage. Data on the percentage of hydrilla meristems damaged by larvae of *C. lebetis* and on the abundance of *C. lebetis* per 100 hydrilla meristems were analyzed with a generalized linear models procedure using a binomial distribution family with a logit link (StataCorp 2015). Results were presented in terms of odds ratios. Odds ratio refers to the ratio of odds of an outcome in one treatment group relative to the odds of an outcome in a reference group (Rita and Komonen 2008). In this study, the 0 m treatment was designated as the reference group. Odds ratios provide direction and magnitude of differences between proportions (Rita and Komonen 2008, Warton and Hui 2011). An odds ratio less than one means that a treatment group has lower odds of an outcome compared to the reference group, an odds ratio of one means the treatment group and the reference group have the same odds of an outcome, and an odds ratio greater than one means the treatment group has higher odds of an outcome compared to the reference group. For all data analyses, the level of significance was $\alpha = 0.05$.

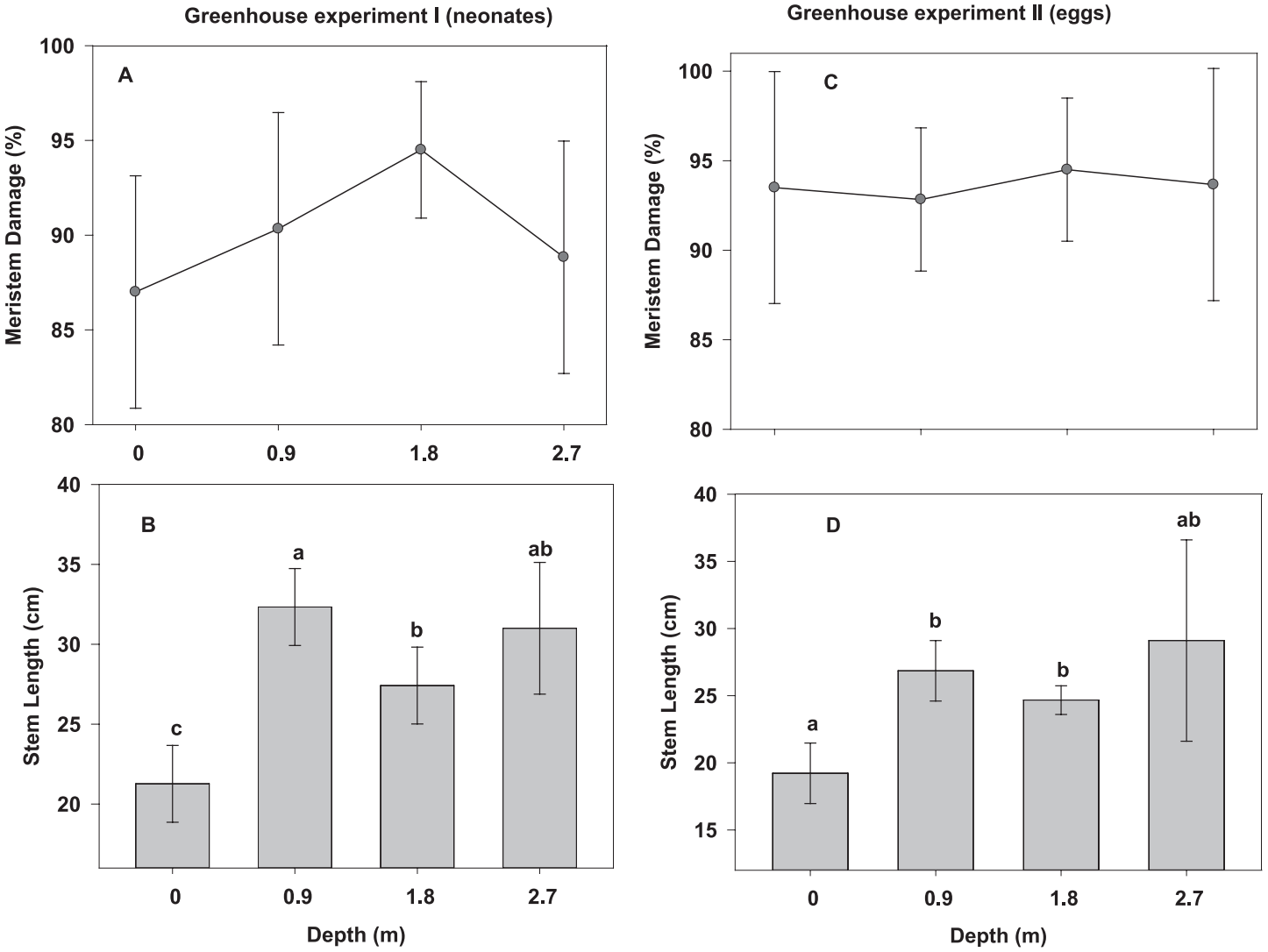

Figure 1. Impact of *Cricotopus lebetis* on hydrilla grown in Plexiglass tubes at different water depth treatments in greenhouse experiments I (*C. lebetis* inoculated as neonates) and II (*C. lebetis* inoculated as eggs). (A) and (C) Percentage (mean ± 95% CI) of damaged hydrilla meristems. (B) and (D) Length (mean ± 95% CI) of hydrilla stems. Different letters indicate means are statistically different at $\alpha = 0.05$.

RESULTS AND DISCUSSION

Greenhouse experiments

Greenhouse experiment I: Inoculation of neonate Cricotopus lebetis on the water surface. Adult emergence and larval feeding damage on the apical meristems of hydrilla (Figure 1) occurred at all four treatment depths. The proportion of adult emergence differed statistically among the treatments. Compared to 0 m treatment, the likelihood of *C. lebetis* adult emergence (survival) was 4.5 times greater in the 0.9 m treatment ($P = 0.0038$) and 2.4 times greater in the 1.8 m treatment ($P = 0.0459$), but not different in the 2.7 m treatment ($P = 0.8055327$). Sex ratio of the emerged adults, expressed here as proportion of males, did not differ statistically among the treatments. The sex ratio (male : female) were 1 : 1.33, 1 : 1.00, 1 : 1.08, and 1 : 1.04 for treatments 0 m, 0.9 m, 1.8 m, and 2.7 m, respectively. The mean number of egg masses oviposited by emerged females was significantly higher in the 0.9 m treatment than in all other treatments, but no statistical difference occurred among the rest of the treatments (Figure 2). Egg count per egg mass (mean ± SE) was 138.27 ± 9.32, 135.42 ± 5.30, 137.29 ± 7.67, 153.00 ± 21.08 in treatments 0 m, 0.9 m, 1.8 m, and 2.7 m, respectively. Adult emergence commenced on day 16 in all greenhouse treatments (Figure 3).

Mean percentage of apical meristems damaged by *C. lebetis* larvae exceeded 85% in all treatments, no matter the depth of hydrilla (Figure 1A). Among the four treatments, the mean stem length was statistically shortest at 0 m treatment, intermediate at the 1.8 m treatment, and longest at both 0.9 m and 2.7 m treatments (Figure 1B).

Greenhouse experiment II: Inoculation of Cricotopus lebetis egg masses on the water surface. At all treatments, egg masses of *C. lebetis* inoculated on the water surface were observed to sink to the bottom of the Plexiglas tubes, where the larvae

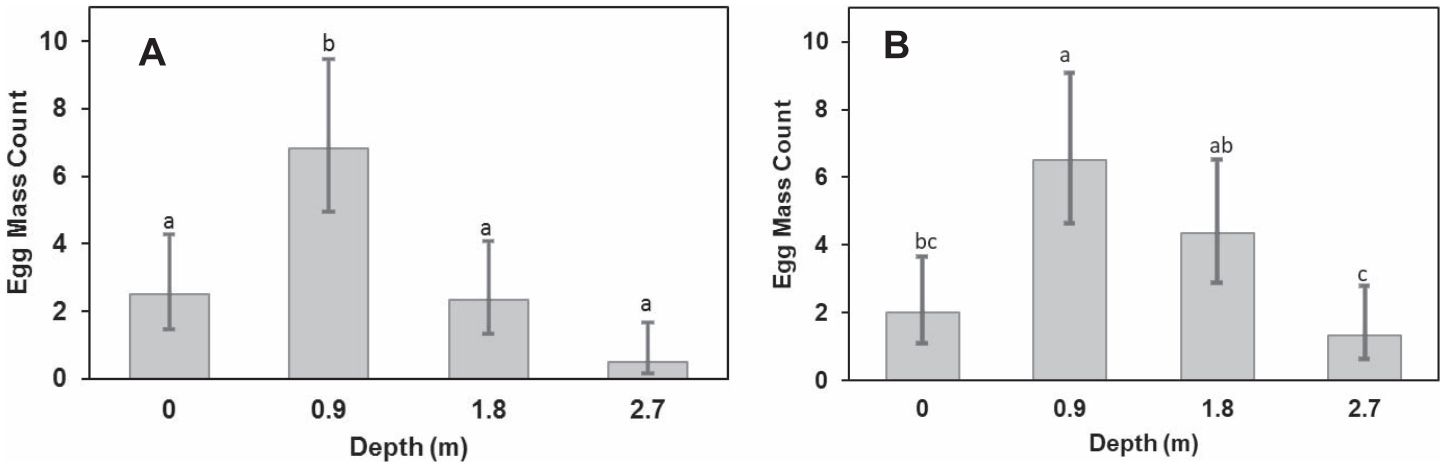

Figure 2. Number of egg masses (mean ± 95% CI) produced by *Cricotopus lebetis* that emerged from hydrilla grown in Plexiglass tubes at different water depth treatments in greenhouse experiments I (A) and II (B). Treatments were inoculated with *C. lebetis* neonates (experiment I) and egg masses (experiment II). Different letters indicate that means are statistically different at $\alpha = 0.05$.

hatched and attacked hydrilla (Figure 1). Results in this experiment exhibited trends similar to those observed in greenhouse experiment I. The likelihood of adult emergence was 2.1 times higher in the 0.9 m treatment compared to the 0 m treatment ($P = 0.0058$). No statistical difference was detected between the likelihoods of adult emergence in 1.8 m treatment ($P = 0.2029$) and 2.7 m treatment ($P = 0.5609$) compared to that in 0 m treatment. The sex ratio of the insect did not differ statistically among treatments. The male : female ratios were 1 : 1.17; 1 : 1.08; 1 : 1.22; and 1 : 0.92 in treatments 0 m, 0.9 m, 1.8 m, and 2.7 m, respectively. Production of egg masses by the emerged females was significantly higher in 0.9 m and 1.8 m treatments and lowest in 0 m and 2.7 m treatments (Figure 2). Egg count per egg mass (mean ± SE) was 145.25 ± 13.13; 162.67 ± 6.28; 151.27 ± 7.39; and 167.13 ± 12.69 in treatments 0 m, 0.9 m, 1.8 m, and 2.7 m, respectively.

The mean percentage of apical meristems damaged by *C. lebetis* larvae exceeded 90% in all four treatments and did not differ statistically among the four treatments (Figure 1C). Mean length of the hydrilla stems was significantly lowest in 0 m treatment compared to the 0.9 m and 1.8 m treatments (Figure 1D). No statistical difference was detected among the means of hydrilla stem length in 0.9 m, 1.8 m, and 2.7 m treatments (Figure 1D). Adult emergence in the greenhouse experiment commenced on day 16 in all treatments except in 0 m treatment, where adult emergence commenced on day 17 and lasted up to day 30 (Figure 3).

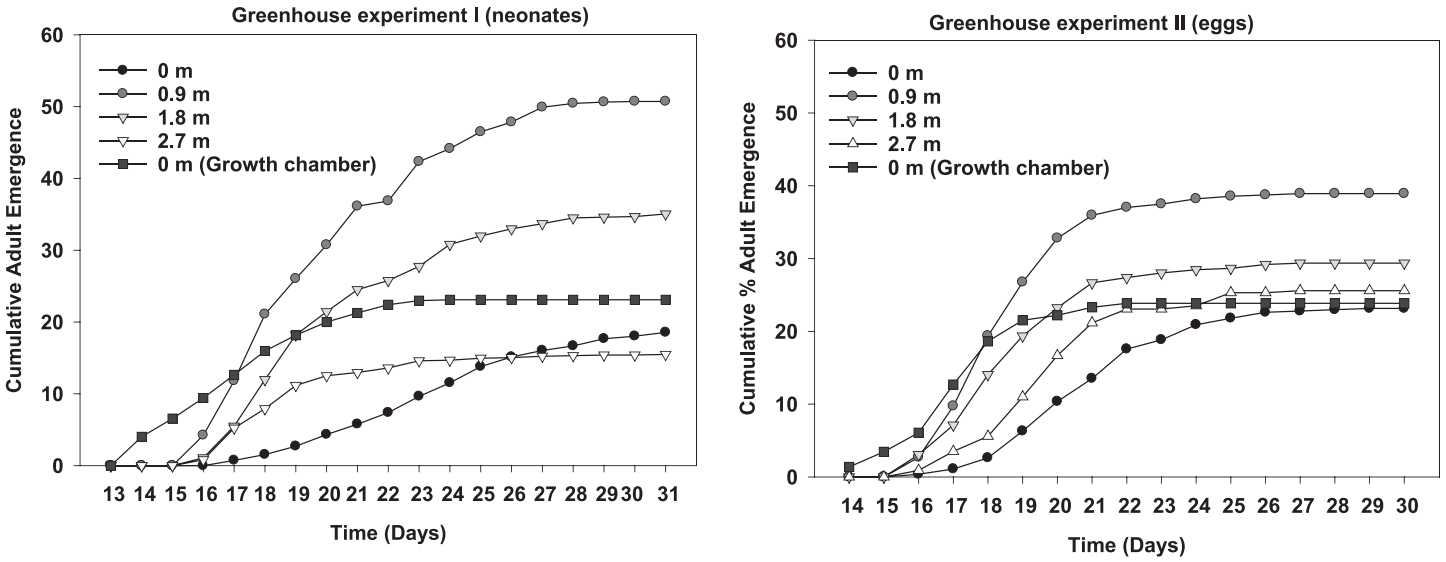

Figure 3. Cumulative percentage emergence pattern of adult *Cricotopus lebetis* following development of the insects in Plexiglas tubes on hydrilla at different depths. Treatments were inoculated with *C. lebetis* neonates (experiment I) and egg masses (experiment II). Error bars were not included to increase clarity of the graphical trends.

Table 1. Summary of the logistic regression analysis examining the effects of water depth of the host plant hydrilla on the proportion (mean ± 95% CI) of damaged apical meristems sampled from sentinel bouquets placed at 0 m, 0.45 m, and 0.9 m below the water surface (Field experiment I) and naturally growing plants sampled from the surface, middepth, and bottom sections (Field experiment II) in Lake Istokpoga, Florida, 2017.

			95% CI for the Mean				95% CI for the Odds Ratio	
Experiment	Depth	Mean %	Lower	Upper	*P* value	Odds Ratio	Lower	Upper
Field experiment I	0.00	15.2	9.8	20.7				
	0.45	23.1	15.4	30.9	0.0180	1.7450	1.1009	2.7657
	0.90	14.4	5.7	23.0	0.8470	0.9309	0.4505	1.9238
Field experiment II	Surface	39.0	30.0	48.0				
	Middepth	42.0	34.0	50.0	0.5700	1.1490	0.7119	1.8540
	Bottom	45.0	38.0	52.0	0.1640	1.3578	0.8824	2.0894

Field experiments

Field experiment I: Use of sentinel stems to determine the foraging depth of Cricotopus lebetis. Apical meristems of the hydrilla sentinel stems, at all treatment depths, were infested and damaged by larvae of *C. lebetis*. Larvae of *C. lebetis* damaged 15.2% of the sentinel stems at 0 m treatment, 23.1% of stems placed at 0.45 m treatment, and 14.4% of stems at 0.9 m treatment (Table 1). The likelihood of larval damage on apical meristems of hydrilla was 74% higher at 0.45 m treatments ($P = 0.018$), compared to the 0 m treatment (Table 1). However, the likelihood of larval damage on the apical meristems of hydrilla at 0.9 m and 0 m treatments did not differ statistically ($P = 0.847$) (Table 1). The mean number of larvae of *C. lebetis* per apical meristem was 0.003 at 0 m treatment, 0.004 at 0.45 m treatment, and 0.002 at 0.9 m treatment. The likelihood of larval presence in 0.45 m treatment ($P = 0.3856$) and 0.9 m ($P = 0.1984$) treatment did not differ statistically from likelihood of larval presence in 0 m treatment.

Field experiment II: Survey of naturally growing stems to determine the foraging depth of Cricotopus lebetis. Larval damage to hydrilla apical meristems and occurrence of *C. lebetis* larvae was present at all Lake Istokpoga treatment depths. No statistical difference was detected in the proportion of damaged hydrilla apical meristems growing at the surface (39%), middepth (42%), or bottom sections (45%) of the water column (Table 1).

The mean number of larvae of *C. lebetis* per stem was 0.01 at the surface, 0.02 at middepth, and 0.02 at the bottom sections of the water column. The likelihood of larval presence at middepth ($P = 0.381$) and in the bottom section ($P = 0.395$) of the water column did not differ statistically from likelihood of larval presence at the surface section.

Taken together, results from the greenhouse study provided the first empirical evidence of larvae of *C. lebetis* attacking submersed hydrilla at depths ranging from 0 m to at least 2.7 m. Field studies conducted at Lake Istokopoga provided further evidence of the ability of *C. lebetis* to attack hydrilla from the water surface level to the hydrosoil, which occurred at a depth of approximately 0.9 m. Previous reports of the foraging depth of *C. lebetis* relied on anecdotal evidence (Cuda et al. 2002, Cuda et al. 2011). For example, Cuda et al. (2011) speculated that the established population of *C. lebetis* in Crystal River, FL was attacking hydrilla to depths of up to 0.7 m below the water surface. Results from the greenhouse experiments demonstrated that egg masses can sink to depths from 0 m to at least 2.7 m, where the neonates can successfully hatch, locate and attack their host plant, and complete their development. In addition, results from the greenhouse study indicated that larvae hatching at the water surface, which is likely to happen in natural environments when an oviposited egg mass becomes attached to substrates floating on the water surface (Cuda et al. 2002), are able to swim or drift, locate, and attack hydrilla to depths of at least 2.7 m.

In Florida, hydrilla commonly occurs at depths of up to 3 m (Langeland 1996) with half of the plant's biomass occurring within the upper 0.5 m of the water column (Haller and Sutton 1975). These growth habit observations suggest that most of the hydrilla in Florida grows within a depth range accessible to larvae of *C. lebetis*. Intense larval feeding damage to the apical meristem of hydrilla (over 85%) was observed in all greenhouse treatments. This finding indicated that *C. lebetis* can attack hydrilla not only within the upper 0.5 m of the water column where the dense surface mats occur, but also the sprouting hydrilla at the hydrosoil level. Higher rates of adult emergence and egg mass production observed at 0.9 m demonstrated that the insect can sustain a high population level in a depth range reported to contain half of the hydrilla biomass (Langeland 1996). Results from the field study in Lake Istokpoga provided additional evidence of the ability of *C. lebetis* to attack hydrilla wherever it occurred in the water column.

Percent adult emergence of *C. lebetis* observed in the greenhouse study (range, 16 to 51%) was comparable to that reported in previous laboratory studies. For instance, Cuda et al. (2002) reported less than 30% adult emergence of *C. lebetis* reared on the Florida strain of hydrilla, which is dioecious, whereas Stratman et al. (2013b) reported 56% adult emergence of *C. lebetis* reared on a similar biotype of hydrilla and a 100% adult emergence on monoecious hydrilla.

The dioecious hydrilla typically occurs in the southeastern States (EDDMapS 2018), where the climate is subtropical and suitable for establishment of *C. lebetis* (Stratman et al. 2014). Additionally, the biotype overwinters partly as stem shoots (Bowes et al. 1979, Harlan et al. 1985, Madsen and Owens 1998), which can host the larvae of *C. lebetis* through winter (November to late February or early March). Tubers and turions of the biotype from the previous growing season begin sprouting from May to October (Bowes et al. 1979, Madsen and Owens 1998). A study at

Crystal River, FL, revealed the field population of *C. lebetis* similarly begin peaking in May (Cuda et al. 2002), suggesting May would be an ideal month for initiating augmentative releases of *C. lebetis*, before the plant growth reaches the water surface. A survey of six water bodies in Florida revealed presence of *C. lebetis* in 50% of the surveyed water bodies (Stratman et al. 2013a). On the other hand, the monoecious hydrilla typically occurs in the northeastern states (EDDMapS 2018) in a temperate climate, where the water temperature in winter can drop below the survival threshold for egg (Baniszewski et al. 2015) and larval stages (Stratman et al. 2014) of *C. lebetis*. Additionally, the monoecious biotype exhibits an annual growth habit, undergoing a complete dieback in winter and overwintering as tubers and turions (Harlan et al. 1985), suggesting the biotype is unavailable to host *C. lebetis* during winter. The vegetative propagules sprout beginning late March to mid-April, when the hydrosoil temperature is between 11 and 13 C, and continues through August (Harlan et al. 1985). Thus, augmentative releases of *C. lebetis* will likely succeed later in the growing season when the water temperature is between 20 and 30 C, the optimal temperature range for larval development (Stratman et al. 2014).

Although the proportion of adult emergence in the two greenhouse studies varied extensively among treatments (range, 16% to 51% in experiment I and 23% to 40% in experiment II), the proportion of damage to the apical meristem did not vary (range, 87% to 94% in experiment I and 92% to 94% in experiment II). Larval density is positively correlated with the proportion of apical meristem damage (Cuda et al. 2011). Therefore, the high level of apical meristem damage observed in greenhouse treatments indicated that early instars occurred in high densities in all treatments but, as the insects developed, mortality occurred at varying rates among the treatments. A similar survival trend, relatively high and equal larval densities among treatments of different levels of abiotic stresses but varying rates of adult eclosion, was observed by Baniszewski et al. (2015). Their study reported that although subjecting the egg stage to refrigeration for 2 d did not impact the resultant larval stage, it disproportionately increased mortality of the pupal stage, significantly reducing adult eclosion.

The relatively low survival of *C. lebetis* in the 0 m treatments was not surprising. Photosynthetic and respiration activities by the hydrilla surface mats have been reported to cause drastic diurnal fluctuations of water quality, which imperil fauna inhabiting the impacted water (Van et al. 1976). Equally important, quality of hydrilla apical meristems in the 0 m treatment was likely poor due to the air/water interphase and lack of room for vertical elongation. This was evidenced by the 0 m treatment having the least stem elongation during the study. Other aquatic insects, such as the rice water weevil [*Lissorhoptrus oryzophilus* Kuschel (Coleoptera: Curculionidae)], avoid host plants growing at the surface level and prefer plants growing in deeper waters (Stout et al. 2002, Tindall et al. 2013). In the studies conducted at Lake Istokpoga, the proportion of damaged hydrilla apical meristems likely provided the better estimate of larval activity and abundance than the number of larvae observed in the stem samples. Previous studies hypothesize that sampling procedures of stem samples often captures the older larval instars but excludes most of the other life stages of *C. lebetis* and their resultant damage to the plants (Cuda et al. 2002).

The foraging depth of *C. lebetis* observed in the present study was greater than the reported foraging depths of other biological control agents of hydrilla. For example, *Hydrellia pakistanae* attacks the top 20 cm of the hydrilla canopy (Wheeler and Center 2001), and *Bagous affinis* only attacks hydrilla exposed during dry seasons or drawdowns (Bennett and Buckingham 1991). Field observations in Australia suggested that *B. hydrillae* attacked hydrilla within the upper 1 m of the water surface (Balciunas and Purcell 1991). This suggests, in theory, that *C. lebetis* can be used in combination with the previously released insect agents to increase their impact on hydrilla.

Knowing the foraging range of *C. lebetis* will be useful in designing mass rearing facilities for *C. lebetis,* which has been recommended for augmentative control of hydrilla (Cuda et al. 2002, Stratman et al. 2013b). In addition, this information can be used in designing field sampling and monitoring techniques and in predicting the efficacy of the insect in the management of hydrilla. Additionally, results from greenhouse experiments confirm that both neonates and the egg stage can be used to effectively inoculate release sites.

Although this study did not elucidate the maximum foraging depth of the insect, it does provide empirical evidence that *C. lebetis* has the capacity to reach and infest hydrilla along the entire water column of the shallow lakes and rivers in which it is growing in Florida. Additional research should focus on the effect of larval feeding damage on the growth pattern of hydrilla under field conditions and on the potential impact of larval feeding on hydrilla sprouting from tubers or turions.

SOURCES OF MATERIALS

[1]Extruded acrylic (Plexiglas) tubes; estreetplastics.com, Royse City, TX.

[2]Flexible polyvinyl chloride (PVC) coupling; American Valve, Inc., Greensboro, NC.

[3]Black polyethylene shade cloth; Sunblocker® Premium, FarmTek, Dyersville, IA.

[4]Dissecting microscope; Zeiss Stemi DV4; Carl Zeiss, Berlin, Germany.

[5]Culture tubes; Fisherbrand®, Fisher Scientific, Hampton, NH.[6]Dixon® worm gear clamps; Dixon Valve and Coupling Co., Chestertown, MD.

[7]Meter stick; Johnson Level and Tool Co., Mequon, WI.

[8]Heavy-duty, plastic-coated steel garden stake; Gardener's Blue Ribbon®, Lititz, PA.

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