J. Aquat. Plant Manage. 49: 114-117

Overwintering biology of *Hydrellia pakistanae*, a biological control agent of hydrilla

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INTRODUCTION

Hydrellia pakistanae Deonier (Diptera: Ephydridae), the Asian leaf-mining fly, was first introduced into the United States as a host-specific biocontrol agent of the dioecious biotype of *Hydrilla verticillata* L.f. Royle (hydrilla) in 1987 (Buckingham and Grodowitz 2004). Female *H. pakistanae* oviposit on emergent hydrilla, and 3 larval instars mine submersed leaves of the plant before pupating at the base of a leaf. The life cycle of *H. pakistanae* from egg to adult is completed in approximately 23 d at 27 ± 1 C (Buckingham and Okrah 1993). Feeding by larvae reduces photosynthesis, thereby decreasing hydrilla biomass; additional indirect negative impacts include reduced tuber and turion production and fragment viability (Doyle et al. 2002, 2005, Grodowitz et al. 2003, Owens et al. 2006, 2008).

Since 1999, *H. pakistanae* has been mass-reared in earthen ponds at the US Army Corps of Engineers Lewisville Aquatic Ecosystem Research Facility (LAERF) in Lewisville, Texas (33°04'45"N, 96°57'30"W). Hydrilla containing *H. pakistanae* is harvested from these ponds and used for field releases. From 2000 to 2008 more than 28 million flies have been released at over 30 sites in 6 states with establishment (i.e., individuals present at sites 10 to 12 months after discontinuing releases) rates of greater than 78% (Harms et al. 2009).

During the winter months at LAERF as the hydrilla canopy senesces (Madsen and Owens 1998), *H. pakistanae* larvae and pupae are all but absent from the leaves of the hydrilla plant (Harms, unpubl. data). Despite this occurrence, fly populations recover each spring, both in rearing ponds at LAERF and at field sites around the country (Harms et al. 2009). In the rearing operation at LAERF, population estimates are only made, on average, from May until November, by counting the number of larvae in leaves of the plant. Although this provides estimates during the growing season, understanding the overwintering biology of *H. pakistanae* could clearly be important to predicting early season fly population dynamics and impacts to hydrilla, as well as understanding establishment failures. Although *H. pakistanae* has been used for biocontrol in the United States for more than 20 years, no one has yet examined the overwintering biology of the fly, likely because many of the early release sites were in southern locations that retain surfaced hydrilla and relatively warm temperatures year round.

Hydrellia pakistanae originates in Asia, and specimens have been collected from India, Pakistan, and China (Deonier 1993). The species has been collected as far north as Shier Li Piao Marsh, Heilongjiang Province, China (46°30'0"N; 125°12'0"E), where January temperatures average -19 C and winter can last from 5 to 8 months (http://www.britannica.com/EBchecked/topic/259646/Heilongjiang). This northern range suggests that *H. pakistanae* is able to survive cold temperatures and may possess a behavioral or genetic adaptation to deal with winter and absence of dense, surfaced hydrilla for ovipositing and feeding.

MATERIALS AND METHODS

During winter 2005-2006, qualitative observations were made at LAERF in an attempt to identify the overwintering biology of *H. pakistanae* (unpublished data). Based on data collected in 2005-2006, a quantitative study was undertaken during winter 2007-2008 to determine *H. pakistanae* overwintering life stage and location.

Data were collected from 2 *H. pakistanae* rearing ponds (Harms et al. 2009) at LAERF from November 2007 until July 2008. Because other *Hydrellia* spp. may overwinter as adults, larvae, or pupae (Deonier 1971), each life stage was examined with sampling methods that were relatively specific for a particular stage. Eggs were not examined because of their relative vulnerability and the lack of evidence of the egg as an overwintering stage (unpublished data).

Two methods, floating soap traps and pond-edge debris Berlese funnel extraction, were used for collection of adult *H. pakistanae*. Soap traps consisted of a small plastic dish, surrounded by Styrofoam® (Dow Chemical), with weights attached at 2 opposite corners to stabilize the trap in the water. Once a month, from November 2007 to June 2008, 4 floating traps filled with water containing approximately 1 to 2 mL of liquid antibacterial hand soap were placed in each of 2 ponds. The soap acted to break the surface tension, trapping and drowning any surface-dwelling insects. The floating traps were left in the ponds for approximately 4 h mid-day (from 11:30 AM to 3:30 PM), and then all trapped insects were collected and preserved in 70% ethanol for later identification. Because durations were all within 30 min of one another, numbers are reported as an average of all traps in all ponds per sampling date.

Pond-edge debris (e.g., leaf litter and dead grass) was also collected once per month from the shore of each pond. Five samples collected randomly regardless of debris type or species were weighed and placed in Berlese funnels (60 watt bulbs) to extract invertebrates, including *H. pakistanae*. Plant samples were allowed to remain in Berlese funnels for 7 d

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until dried. Pond-edge debris sampling was discontinued in March when new growth was found along the edge of the pond and only minimal leaf litter or dead grass remained.

Five hydrilla stems (from the sediment to the apical tip) were randomly collected monthly from each pond and divided into 20 cm stem zones: 0 to 20 cm (apical tip), 20 to 40 cm, and >40 cm (nearest the roots). The leaves and stems of these sections were examined microscopically (7× to 10×), and immatures in the leaves were counted. In addition, stems were dissected and immatures in the stems were removed and preserved in 70% ethanol. Signs of damage to the stem were also recorded, but not quantified.

Ten additional apical stem pieces averaging 10 cm in length were collected monthly from each pond, and the stems were examined for *Hydrellia* larvae or pupae in the leaves. In addition, the percentage of leaves damaged by larval mining was recorded. Once examined, stems were placed in separate 1 L glass rearing containers fitted with mesh tops. The stems were incubated at 24 to 27 C and monitored daily for one month to estimate adult emergence. Such rearing was undertaken because it is difficult to identify *Hydrellia* species based on larval characteristics, and this provided verification of species present in the stems and leaves. All emerging insects were preserved in 70% ethanol for later identification.

Two native macrophytes that commonly show signs of leafmining were also examined to determine if they served as possible winter larval hosts. This was accomplished twice during the study, in March and April, by randomly hand collecting *Vallisneria americana* Michx. (wild celery) and *Potamogeton nodosus* Poiret (American pondweed) from each study pond and placing them in separate 4 L polycarbonate rearing containers at 24 to 27 C. On average, 3 containers per plant species were used. Containers were monitored daily for one month to record emergence. All adult flies that emerged were preserved in 70% ethanol for later identification.

Data were analyzed using STATISTICA version 8.0 (StatSoft®, Inc., 2007, Tulsa, OK) and included t-test, analysis of variance (ANOVA), and Neuman-Keuls (NK) for mean separation. Statements of significance refer to an alpha level 0.05 unless otherwise noted.

ANOVA was used to differentiate changes in number of *H. pakistanae* throughout the study, including adults in soap-trap collections and adults in pond-edge debris Berlese extractions. A 2-way ANOVA was used to compare *Hydrellia* sp. larval abundance for the duration of the study, using plant part (leaves vs. stem) and stem section (0 to 20, 20 to 40, and >40 cm) as the main effects. Neuman-Keuls post-hoc test was used to separate the significant samples into statistically different groups.

RESULTS AND DISCUSSION

High numbers of adults were collected by soap trap in November (>30 per trap) with only minimal numbers observed in December (~2 per trap). Following the December collection, no adults were observed from soap-trap collections during the months of January through March until a resurgence of adult numbers was noted in soap-trap collections in April (~6 per trap). This is likely related to higher water temperatures and regrowth of the hydrilla typically occurring in the spring.

In addition, pond-edge debris Berlese funnel extractions failed to detect adults with the exception of 10 adult *H. pakistanae* collected in December. In contrast, pond-edge debris often contained high numbers of parasitoid wasps, various arachnids, and isopods. Failure to detect adults in later winter months may indicate that low temperatures were too prolonged for adult survival and likely precludes debris-overwintering as a survival strategy under north Texas climatic conditions.

Overwintering behavior of most *Hydrellia* spp. is unknown. Based on soap-trap and pond-edge debris Berlese extraction data, *H. pakistanae* does not appear to overwinter in the adult life stage. Although unlikely, adults possibly overwinter farther from the waterbody than our sampling methods were able to detect. Baloch et al. (1980) reported that adults overwintered in Pakistan along the shoreline under dead plants, although evidence of that in the current study is lacking. Deonier (1971) did not name the species, but reported that other *Hydrellia* spp. overwinter as adults.

Hydrellia spp. immatures were detected through stem dissection in both leaves and stem mines during the course of this study. The highest numbers of larvae or pupae were observed in the 0 to 20 cm stem section, in the stem as opposed to leaves for the duration of the study (2-way ANOVA, main effects are stem section: $df = 2, 1611, F = 13.17, p < 0.05$; plant part: $df = 1, 1611, F = 8.73, p < 0.05$; stem section * plant part interaction: $df = 2, 1611, F = 1.99, p = 0.14$) (Figure 1a). The increased numbers of larvae in the stem was not expected because larvae are rarely observed in stem tissue during the growing season (unpublished data) and are usually found feeding and residing in leaf tissue (Baloch and Sana-Ullah 1973). In contrast, higher numbers of immatures in the upper portion of the plant was not considered unusual because observations over the last 15 years have indicated that most larvae are found in the leaves of upper stem sections near the water surface, which houses the largely branching canopy portion of hydrilla (Wheeler and Center 2001, Doyle et al. 2002). Because the majority of larvae or pupae were detected in the apical portion of the plant, further analyses were restricted to this plant section.

The total numbers of *Hydrellia* spp. larvae or pupae detected in both leaf mines and stem tunnels fluctuated over time (Figure 1b); larvae or pupae decreased from an average of 3.0 immatures per 20 cm (hydrilla) in November to a low of 0 immatures detected in April, then increased to a high of 3.2 immatures per 20 cm in May. Although 0 immatures were detected in the 0 to 20 cm stem section in April, one second instar larvae and 2 pupae were observed in the 20 to 40 cm stem section, and one second instar larva was detected in the >40 cm stem section. Although present in low numbers in April, subsequent population estimates increased in May, likely a result of mating and oviposition. Hence, although larval or pupae numbers are low during winter months, the data indicate that the larvae are overwintering in the upper portion of the stems by tunneling and boring within the stem tissue.

Highest numbers of immatures observed in the 0 to 20 cm stem sections during November 2007 through June 2008

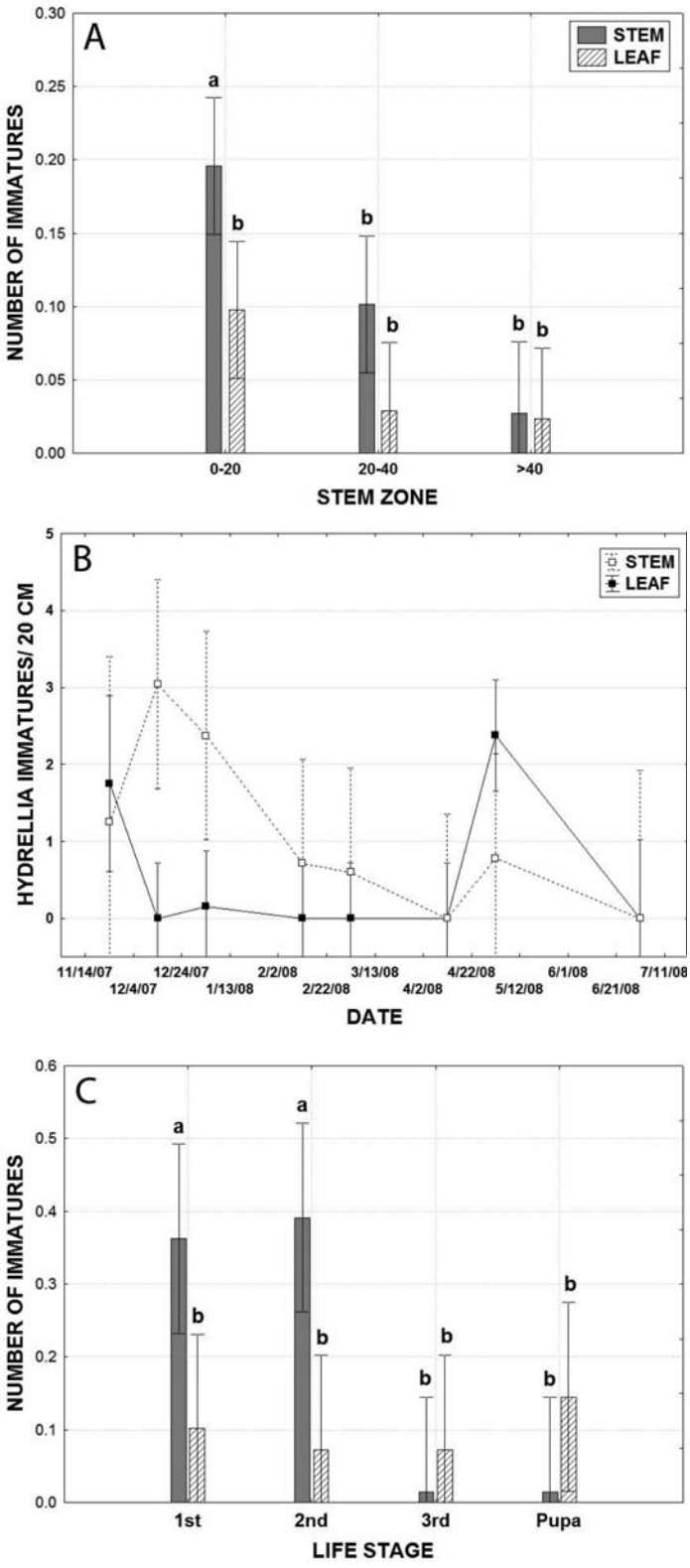

Figure 1: Numbers of *Hydrellia pakistanae* (A) in each stem section combining both those present in the leaves and stems for the duration of the study (note: the 0 to 20 cm stem section is closest to the water surface with following sections closer to the sediment), (B) averaged in stems and leaves over time, and (C) for each life stage and plant part for the 0 to 20 cm stem section. Vertical bars denote 0.95 confidence intervals and letters represent statistically distinct groups.

were first and second instars, with only minimal numbers of third instars and pupae (Figure 1b). Numbers of first and second instar larvae recorded per 20 cm were 4-fold greater in comparison to the combined numbers of third instar larvae and pupae per 20 cm. Within the stem, first and second instar *Hydrellia* spp. represented 95% of those recovered from mines. Third instar and pupae were rare, representing only 2% and 3%, respectively.

Adult *H. pakistanae* were reared from examined stems throughout the study. For example, 7 stems during the winter months had zero *Hydrellia* spp. larvae or pupae present in the leaves, but reared 12 adult *H. pakistanae*. These flies were likely overlooked in examination because they were mining the stem of the plant, which became obvious during plant dissections. No other species of *Hydrellia* were reared from hydrilla stems during the study, so it is unlikely that these larvae represented additional species, including the native species *H. bilobifera* Cresson and *H. discursa* Deonier.

Note that no *H. pakistanae* were reared from the native species *V. americana* or *P. nodosus*, potentially excluding them as winter hosts. Insects reared from those species included the native *H. bilobifera* and *H. discursa*, as well as *Trichopria columbiana* Ashmead (Hymenoptera: Diapriidae) and the lepidopteran parasitoid *Bassus* sp. (Hymenoptera: Braconidae). Because we were only able to sample native plants twice during the winter, it remains possible, though unlikely, that *H. pakistanae* spends a portion of the winter on native species.

The evidence supports the conclusion that, in north Texas, *H. pakistanae* survives winter months in immature life stages, predominantly as first and second instars in the upper portion of the plant closest to the water surface. This is similar to *H. bilobifera* and *H. trichaeta* Cresson, which can overwinter as larvae in pondweed (Deonier 1998). In addition, the normal *H. pakistanae* leaf-mining behavior is apparently altered for overwinter tunneling inside the hydrilla stem. The reasons for this behavior are unknown but several possibilities exist. Stem mining may afford the larvae protection at a time when slowed metabolism makes them more vulnerable to predation or parasitism. Some species of *Hydrellia* are provided more protection from parasitism than others based on mining and pupariation location within the host plant (Deonier 1998). More likely, at a time when the structural stability of the hydrilla plant is weakened during winter senescence (Madsen and Owens 1998), the stem probably provides relatively greater protection than the leaves.

Estimates of overwintering populations of *H. pakistanae* were previously made from leaf counts, but this study has shown that more accurate estimates can be made from combined leaf and stem analyses. For example, by examining only leaves for population estimates (as was the traditional method), we would have estimated 0.3 immatures per 20 cm hydrilla in the month of January. By combining leaf and stem examinations, we estimate 2.4 immatures per 20 cm hydrilla, a nearly 8-fold difference. The lowest population estimates of the study were in April, when zero larvae were found in the apical 20 cm of plants, but 0.2 immatures per 20 cm hydrilla were found in distal portions of the plant. This is likely either an error based on insufficient sampling or an effect of sample timing, because adult flies were found in substantial numbers in the ponds at this time. Re-

gardless, estimating establishment success in field release sites seems possible, even during the winter months, by continuous site monitoring and dissection of hydrilla stems to detect larvae.

Because adult flies begin to emerge in April and May, it is important to understand population numbers prior to emergence. In March, a population estimate of 2.8 immatures per 20 cm plant material was observed. While a seemingly low number, this equates to approximately 2.8 million immatures per hectare of hydrilla. This general estimate is determined by multiplying 2.8 immatures per apical 20 cm plant material by a plant density estimate of ~100 stems m^2 in the pond (M. Smart, per. comm.). When converted from square meters to hectares, we arrive at approximately 2.8 million immature *Hydrellia* per hectare. While the abundance of *Hydrellia* necessary for control is not clear, feeding damage of 15 to 35% has been shown to reduce photosynthesis to the point where it equals respiration, and damage levels of 70 to 90% reduces photosynthesis to the point where survival of the plant is unlikely (Doyle et al. 2002).

In summary, although north Texas populations of *H. pakistanae* decline dramatically in the winter months, larvae still inhabit plant tissue, primarily utilizing the stem as opposed to leaf tissue such as occurs in summer months. They overwinter as first and second instars, and adult emergence begins in early spring. As long as environmental conditions allow submersed stems of hydrilla to remain intact all winter, *H. pakistanae* should be able to survive winter conditions. This may preclude establishment and subsequent increase on the monoecious biotype of hydrilla, which overwinters as tubers and turions (Steward and Van 1987, Grodowitz et al. 2010), leaving little, if any, suitable habitat for *H. pakistanae*.

ACKNOWLEDGEMENTS

This work was funded by the US Army Corps of Engineers Aquatic Plant Control Research Program, under the leadership of the technical director, Alfred F. Cofrancesco. We would like to thank Dr. Wayne Mathis for verification of *Hydrellia* sp. as well as Drs. Lubomir Masner and Bob Kula for parasitoid identifications and Ms. Julie Nachtrieb, who aided in collections. We also thank the comments and suggestions of two anonymous reviewers. Permission to publish this information was granted by the Chief of Engineers.

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