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Impact of refrigeration on eggs of the hydrilla tip mining midge *Cricotopus lebetis* (Diptera: Chironomidae): Larval hatch rate and subsequent development

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ABSTRACT

Hydrilla verticillata (L.f.) Royle, Hydrocharitaceae (hereafter hydrilla) is a federally listed noxious weed and one of the worst invasive aquatic plants in the United States, with millions of dollars spent annually to control infestations in all types of water bodies. Hydrilla invades natural and artificial ecosystems, producing surface mats that clog waterways, prevent sunlight penetration, and alter biodiversity. The adventive hydrilla tip mining midge (*Cricotopus lebetis* Sublette; Diptera: Chironomidae) is a potential biocontrol agent for hydrilla. During the larval stage, the hydrilla tip mining midge mines into the apical meristems of hydrilla, inhibiting vertical growth and restricting surface mat formation. The hydrilla tip mining midge is currently being mass-reared in a laboratory colony to augment established populations in Florida. Eggs often are refrigerated to delay development or for transportation to field release sites. Therefore, it is important to understand consequences of cold storage on hatch rates, development, and adult eclosion. Egg masses that were stored in a refrigerator at 5 C for 1, 2, 4, 7, 14, and 21 d were tested for larval hatch rate, development to pupation, and adult eclosion. Hatch rate and adult eclosion decreased significantly after 7 and 2 d refrigeration, respectively. Pupal mortality increased significantly after 2 d. Results of this study showed that cold storage of eggs destined for field release should be avoided. If storage is required, the number of eggs released should be increased to compensate for cold-induced mortality. This study will contribute to successful biocontrol of hydrilla by improving hydrilla tip mining midge rearing and release protocols.

Key words: biological control, Chironomidae, *Cricotopus lebetis*, hydrilla.

INTRODUCTION

Hydrilla [*Hydrilla verticillata* (L. F.) Royle] is a federally listed noxious weed and is listed as a Category I invasive plant by the Florida Exotic Pest Plant Council because it alters native plant communities (FLEPPC 2013). Hydrilla was introduced into Florida through the aquarium industry in the 1950s and has since thrived (Schmitz et al. 1991). Once established, hydrilla out-competes native plants for resources. When hydrilla "tops out," the surface mats block sunlight and the extensive biomass displaces native vegetation and removes nutrients from the water column. Surface mats produced by hydrilla also block waterways and prevent their use for recreation, flood control, and navigation. Management of hydrilla is difficult because of its ability to regenerate from fragments and its growth rate of up to 2.5 cm d^{-1} (Glomski and Netherland 2012). Therefore, although mechanical control of hydrilla can remove vast quantities of plant material, the cut plants will quickly grow back. Mechanical control methods may also contribute to the invasion process by creating fragments that may bud and root to create new plants. Additionally, there have been several documented cases of resistance to commonly used herbicides (Michel et al. 2004, Berger and MacDonald 2011, Giannotti 2013). These characteristics allow hydrilla to disrupt ecosystems and make it difficult to manage with conventional control methods (Haller and Sutton 1975, Puri et al. 2007). Incorporating novel biological control tools into an integrated pest management strategy could reduce the dependence on herbicides and provide a more sustainable solution to the hydrilla problem. Biological control methods of hydrilla that have been studied or associated with hydrilla include weevils (*Bagous* spp.), flies (*Hydrellia* spp.), a moth (*Parapoynx diminutalis* Snellen), and the Asian grass carp (*Ctenopharyngodon idella*). These species have failed to establish substantial populations in Florida ecosystems, such as the weevils; have a negligible impact on hydrilla control, such as the flies; or are generalist plant herbivores nonspecific to hydrilla, such as the moth and the Asian grass carp (Cuda 2009).

The hydrilla tip mining midge (*Cricotopus lebetis* Sublette; Diptera: Chironomidae), was discovered in 1992 in Kings Bay, Crystal River, FL, by U.S. Department of Agriculture (USDA) researchers who observed the presence of midge larvae along with stunted growth of hydrilla (Cuda et al. 2002). Since its discovery, the hydrilla tip mining midge has been studied as a potential biocontrol agent of hydrilla (Cuda et al. 2011). Larvae feed in the growing tips of the hydrilla, which kills the apical meristems and inhibits growth. The larval feeding damage does not kill the plant

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but changes its architecture. Larval feeding restricts vertical growth, eliminating the surface mats, which are the main problem with hydrilla as they are associated with restricted water flow, restricted sunlight penetration, and competition with native plants without detrimental effects to the ecosystem or wildlife (e.g., fish, waterfowl) that now use hydrilla for shelter and food (Cuda et al. 2002).

The hydrilla tip mining midge is pale green in color and 3 to 4 mm long with black markings on the thorax and bands on the abdomen. Females have shorter antennae and wider abdomens than the males. Adult midges can live up to 3 d, but do not feed. Females lay 50 to 250 eggs in a gelatinous mass; eggs are synchronous in developing eyespots in 24 h and hatch from the egg mass within 48 h (Cuda et al. 2014). The larvae have a green body with a black band on the thorax and a light-colored head. The larvae can mine into the hydrilla apical meristems and create a tunnel 1 to 2 cm, which ultimately kills the hydrilla shoot. The duration of the larval stage ranges from 9 to 22 d, during which the larvae abscisses the hydrilla shoot to allow for its emergence after pupation. Pupation lasts for 24 to 48 h, after which an adult emerges. The entire life cycle of the midge ranges from 13 to 29 d, which allows for several generations yr^{-1} of the hydrilla tip mining midge in Florida (Cuda et al. 2014).

Following identification of the hydrilla tip mining midge (Epler et al. 2000) and its beneficial properties (Cuda et al. 2002, 2011), recent studies have focused on its host range and cold tolerance (Stratman et al. 2013a,b). Augmentive biological control is utilized when a nuisance pest, such as hydrilla, can be controlled by a biological agent found naturally in the same habitat range as the pest. If the pest species is widespread or highly invasive, augmentive control can utilize the naturally occurring enemy of the pest by supplementing natural populations. Therefore, for a successful augmentation program, it is essential to develop a mass rearing protocol to supplement natural enemy populations to have an effect on the pest species. Daily egg production can vary, ranging from no egg production to 10,000 eggs d^{-1}; therefore short-term cold storage of hydrilla tip mining midge eggs may be required prior to field release. Typically, hydrilla tip mining midge eggs are refrigerated to slow development until later use or during transport to the field. However, effects of refrigeration for various time intervals on the eggs or subsequent development are unknown. A better understanding of the effects of reduced temperatures on hydrilla tip mining midge development is essential to maximize potential biological control.

The aim of this study was to determine the effects of refrigeration at different time intervals on larval hatch rate and development through adult eclosion.

MATERIALS AND METHODS

The hydrilla tip mining midge was reared on hydrilla collected from the University of Florida Institute of Food and Agricultural Sciences (UF/IFAS) Center for Aquatic and Invasive Plants (29°43′35″N 82°25′4″W). The hydrilla tip mining midge was reared in a greenhouse at the UF/IFAS Entomology and Nematology Department at a temperature range of 21 to 38 C. Ambient light was supplemented with 243.84-cm florescent tubes (60 W, cool white) and round bulbs (40 W, 120 V, soft white) to maintain a 14 : 10-h (light : dark) photoperiod, as described by Cuda et al. (2002). Hydrilla initially was cleaned by low-pressure rinsing with well water to wash off all organisms that could be detrimental to the hydrilla tip mining midge colony. The hydrilla was sorted and tips approximately 12 cm in length were separated and rinsed again. Tips of hydrilla were then sonicated at 240 W and 117 V for 5 min to kill any remaining organisms.[1] Approximately 2,000 tips of cleaned and processed hydrilla were placed in a tray filled with aerated well water. The tray was set in a loosely fitted mesh enclosed cage (62 cm^3) and 1,200 to 1,500 eggs of the hydrilla tip mining midge were added. Trays were monitored daily for adult eclosion and to maintain water levels. As adults emerged, they were collected using a mouth aspirator tube and transferred to an oviposition chamber in the laboratory. The oviposition chamber was a 500-ml stopcock separatory funnel with 200-ml well water as described by Cuda et al. (2002). Adults were allowed to mate; females deposited egg masses, which were collected daily, in the oviposition chamber (Cuda et al. 2002).

Egg masses were collected from the oviposition chamber and immediately placed under a compound microscope to determine number of eggs (fecundity) and the number of viable eggs (fertility). Only egg masses with at least 100 eggs, with 90% fertility and that had not yet developed red eyespots were used. These egg masses were placed in a refrigerator at 5 C for time intervals of 1, 2, 4, 7, 14, and 21 d and then returned to room temperature (26 ± 2 C). Additionally, an egg mass was held at room temperature as a control. Egg masses typically only required 2 d for larvae to hatch completely (Cuda et al. 2002), but were allowed up to 3 d after removal from refrigeration to be evaluated for hatch rates, which were assessed by counting empty egg shells. Each treatment was assessed at least six times (range: six to eight times).

Test tubes (35 ml; $n = 40$) were set up with 30 ml of well water and a single hydrilla tip as described by Cuda et al. (2002). Hydrilla was prepared as described previously. Larval development from each egg mass was assessed by placing a randomly selected larva in each test tube. Larvae were removed from the Petri dish with a small glass pipette under ×2.5 magnification using a lighted dissecting microscope. Egg masses with less than 40 hatched larvae were not assessed for development. Each set of 40 test tubes was placed in an environmental chamber[2] set on a 14 : 10-h (light : dark) photoperiod and a temperature of 26 C. Test tubes were checked every 2 d after a 1 wk incubation period. Surviving larvae were recorded as observed in the test tubes and each test tube was marked with the stage of development. Monitoring of test tubes continued for the next 14 d (21 d total). Cuda et al. (2002) reported the larval stage of the life cycle is completed in 9 to 22 d.

A second experiment was set up as described previously, but only for eggs refrigerated for 1, 2, 3, and 4 d along with a control. Larvae, pupation, and adult eclosion were monitored daily; randomly selected samples also were monitored weekly for larval survival, visibility, development (by

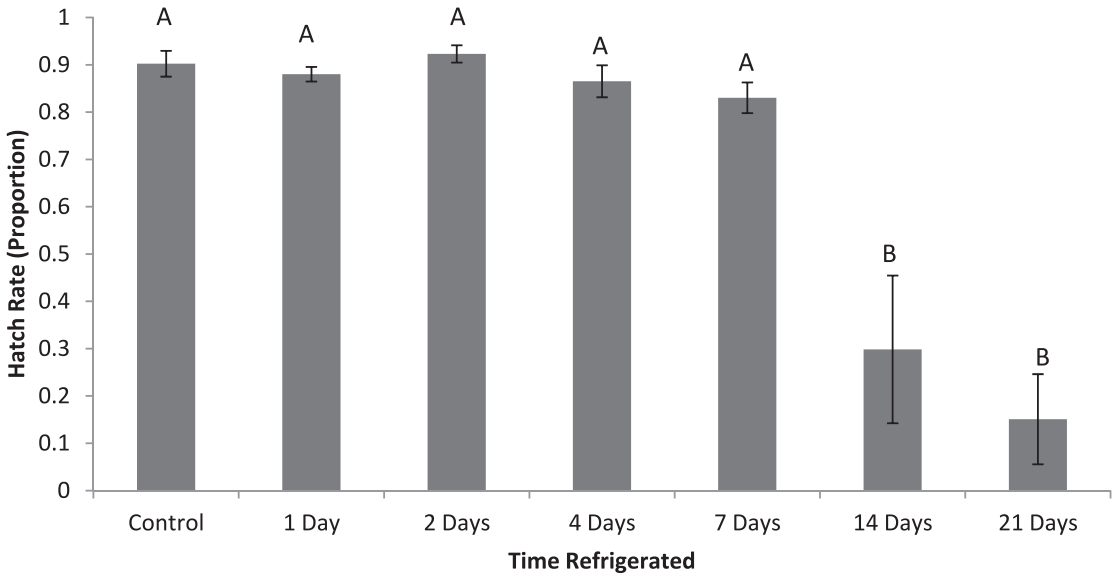

Figure 1. Hatch rate for the hydrilla tip mining midge larvae when eggs are refrigerated at 5 C for different time intervals. Hatch rate averages are calculated as a proportion of fertile eggs. Controls were held at room temperature prior to hatching. Means ± standard error are shown for each time interval refrigerated at 5 C. Significant differences in hatch rate between the number of days held at 5 C are indicated by different letters.

measuring size), and damage to hydrilla. All test tubes were observed daily for larvae visibility, relative size, position on hydrilla, and if they were mobile (eating or actively moving) or sedentary. Samples selected randomly using the RAND function in Excel[3] were selected weekly (day 7, day 14, and day 21 post–larval hatch) and hydrilla tips were dissected to evaluate larval survival, measure larvae size, and assess hydrilla for damage. Larval survival was determined by the presence of a motile larva in the water or removed from the dissected apical meristem of the hydrilla. Larval size was recorded by measuring larval length from cerci to the head with a calibrated millimeter microscope scale. Hydrilla damage was recorded as a hollow apical meristem with either a severed apical tip or apparent feeding damage on apical leaves of hydrilla. Death at pupation and adult eclosion also were recorded.

Larval hatch rate was analyzed by using SAS 9.3.[4] Residual plots for each variable indicated that the data were normally distributed. The variables considered in the model were total egg mass fertility, time refrigerated, and the interaction between the two variables. Least square means with standard error were calculated at each time interval of cold temperature storage. Further development, including pupation and adult eclosion, was analyzed using one-way ANOVA with the variable time refrigerated. Daily larval visibility, survival, larval size, hydrilla damage, death at pupation, and adult eclosion were analyzed using one-way ANOVA with the variable time refrigerated.

RESULTS AND DISCUSSION

Time refrigerated had a significant effect on hatch rate ($t = 2.028$, df = 6, $P < 0.0001$). Larval hatch rate decreased significantly after egg masses were refrigerated for 7 d when compared to the controls (Figure 1; $t = 2.017$, df = 6, $P < 0.0001$). Proportion of egg mass fertility had no significant effect on proportion of hatch rate ($P > 0.05$).

Pupation, although not statistically significant, correlated negatively with longer refrigeration intervals (Figure 2; $t = 2.035$, df = 5, $P = 0.3880$). However, adult eclosion decreased significantly when eggs were refrigerated for only 2 d compared to the controls (Figure 3; $t = 2.035$, df = 5, $P = 0.0063$). Overall, refrigeration had a negative impact on hydrilla tip mining midge survival and development from larval hatch to adult eclosion.

The second experiment, which focused on larval development to adult eclosion, showed no significant effect of time refrigerated on larvae (Figure 4). Larval mobility did not differ by time refrigerated during the first week or the second week, indicating no impact of time refrigerated on behavior (Figure 4A). Larval survival was slightly higher in the first week compared to the second week, but not significantly so, and did not show an effect by time refrigerated (Figure 4B). Larval development, recorded as average larval length, did not show an effect by time refrigerated or from the first week to the second week of development (Figure 4C). Finally, damage to hydrilla was higher in the second week, as expected, but did not vary by time refrigerated (Figure 4D). However, although larvae did not show differences by time refrigerated, pupal mortality increased significantly after 2 d of refrigeration compared to the controls (Figure 5; $t = 2.145$, df = 4 $P = 0.0116$).

Although hatch rate was not significantly affected until after day 7, adult eclosion was clearly impacted by shorter periods at cold temperatures, and was significantly reduced by day 2. Therefore, cold storage may be an important limiting factor depending on the intended use of the egg mass. For instance, if only hydrilla tip mining midge larvae are required for experimental purposes, a longer storage time can be tolerated, but at the expense of a higher mortality rate in later stages. This was confirmed by the

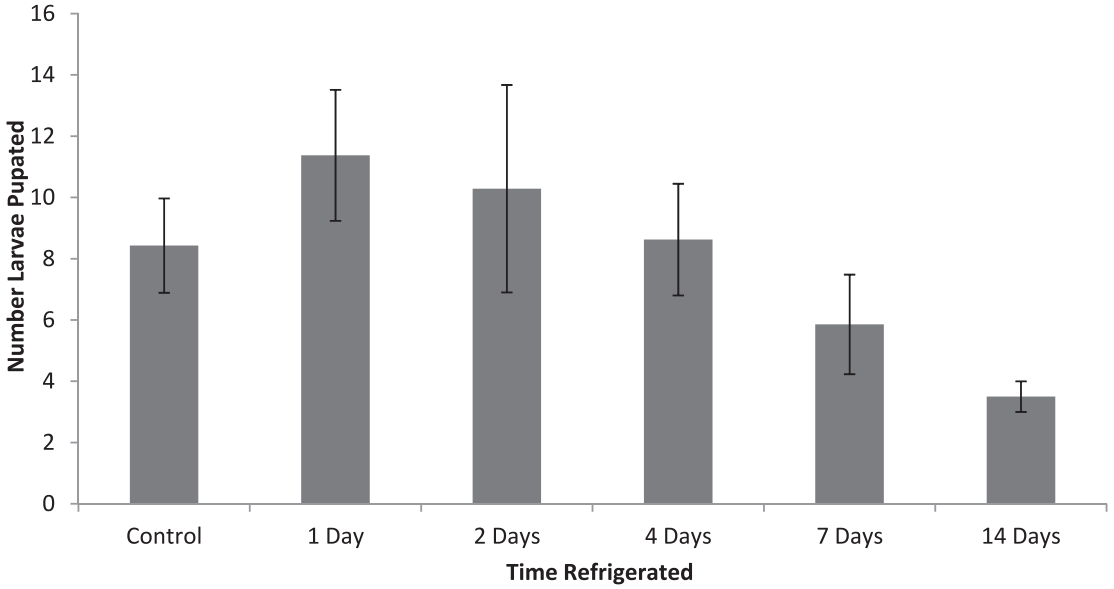

Figure 2. Number of hydrilla tip mining midge larvae pupated when eggs are refrigerated at 5 C for different time intervals. Only larvae that pupated by 15 d posthatch were counted. Controls were held at room temperature prior to hatching. Means ± standard error are shown.

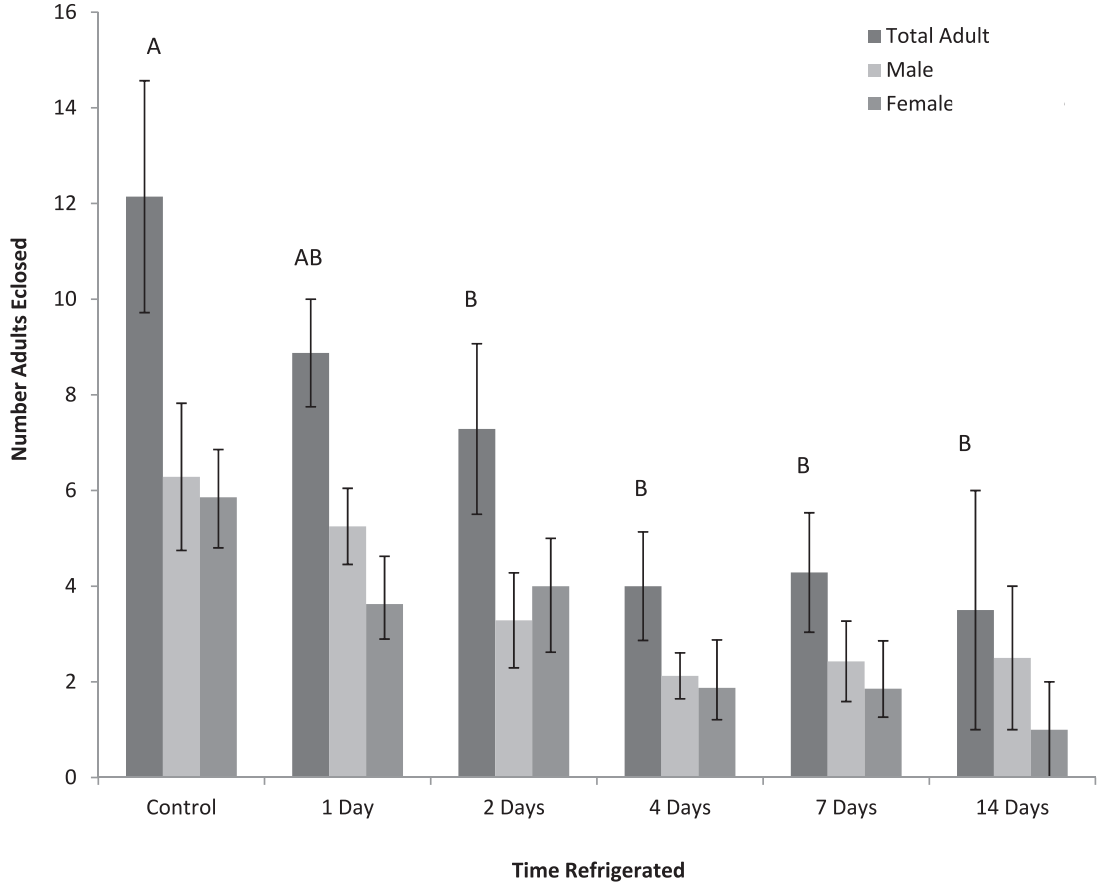

Figure 3. Number of hydrilla tip mining midge adults eclosed when refrigerated at 5 C as eggs for different time intervals. Average adult eclosion is shown for total adult eclosion and male and female eclosion by 21 d posthatch. Controls were held at room temperature prior to hatching. Means ± standard error are shown. Significant differences in total eclosion between the number of days refrigerated at 5 C are indicated by different letters.

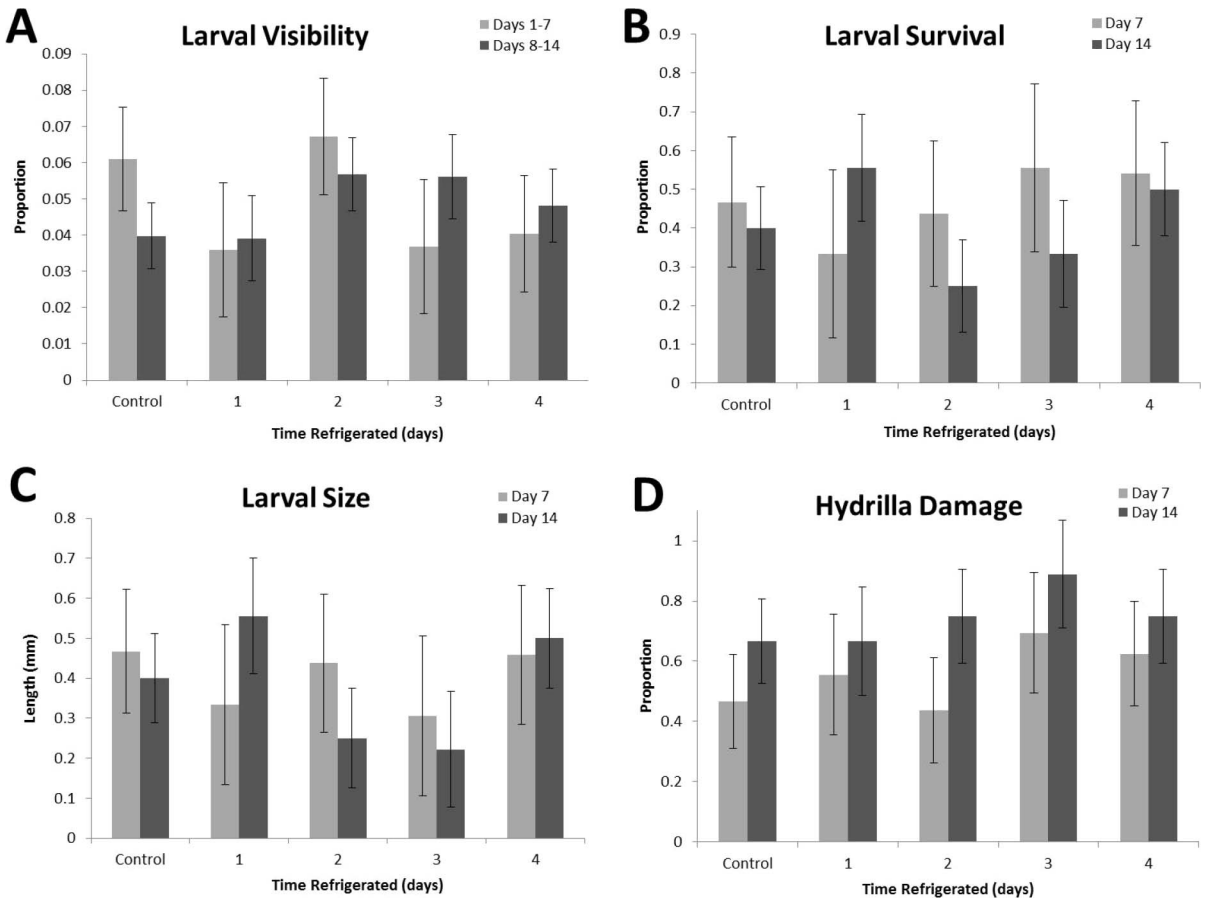

Figure 4. Development of hydrilla tip mining midge larvae, and damage to hydrilla when eggs are refrigerated at 5 C for different time intervals. Bars represent the following: (A) proportion of larvae visible, (B) proportion of larvae alive (survival), (C) development by measuring size of larvae (mm), and (D) proportion of larval damage to hydrilla. Controls were held at room temperature prior to hatching. Means ± standard error for day 7 and day 14 are shown.

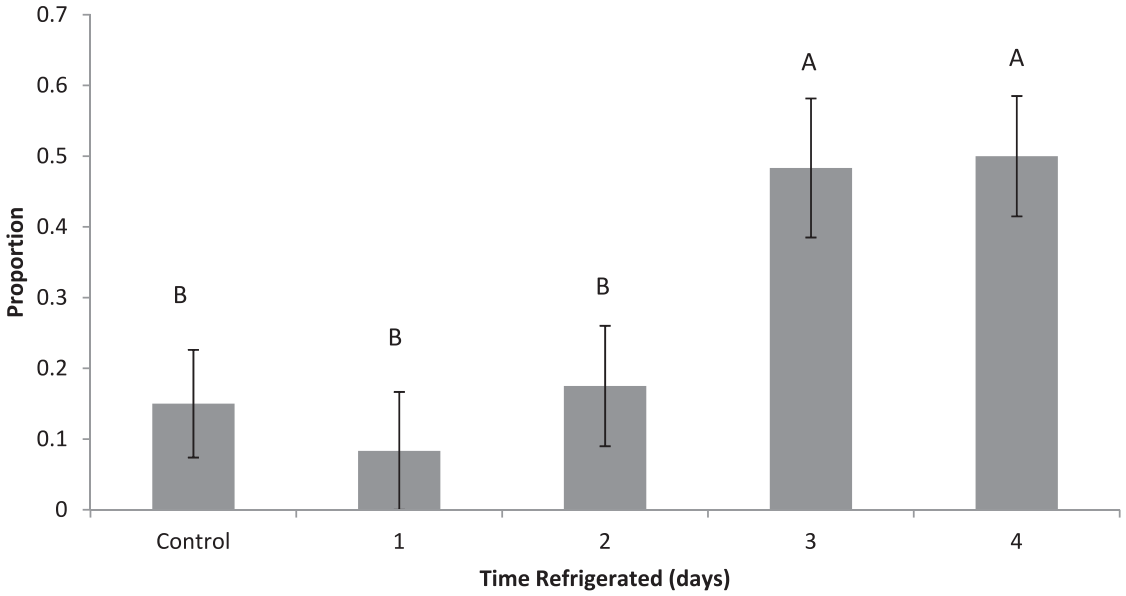

Figure 5. Proportion of hydrilla tip mining midge larvae that died at pupation when refrigerated at 5 C as eggs for different time intervals. Controls were held at room temperature prior to hatching. Mean numbers of larvae ± standard error that reached pupation and died before eclosing as adults are shown. Significant differences in eclosion between the number of days refrigerated at 5 C are indicated by different letters.

second experiment, which showed no significant effect of time refrigerated on larval survival, development, or impact on hydrilla. However, pupal mortality increased if egg masses were refrigerated for more than 2 d, which correlates with reduced adult eclosion after 2 d. Therefore, if the goal is to produce adults or an actively reproducing population, refrigeration is not recommended because even short periods of cold storage increase death at pupation and subsequent adult eclosion. These results can serve as guidelines to improve colony rearing and more efficient release of the hydrilla tip mining midge for biological control of hydrilla.

Larval hatch can be affected by extreme high and low temperatures, as seen in other dipteran species exposed to temperatures outside a normal distribution (Impoinvil et al. 2007, Baek et al. 2012). Although larval hatch of the hydrilla tip mining midge at different temperatures has not been assessed previously or in the current study, other studies have defined the temperature thresholds for larval development. Distribution of the hydrilla tip mining midge has been shown to be limited by temperatures below 10 C and above 30 C (Stratman et al. 2014). Studies with other closely related Chironomidae species indicate that the hydrilla tip mining midge is not likely to be further impacted through reduced larval hatch success within this 10 to 30 C temperature range. For example, the limits for larval hatching for *Glyptotendipes tokunagai* Sasa (Diptera: Chironomidae) was 10 to 40 C (Baek et al. 2012). Similarly, Impoinvil et al. (2007) studied larval hatch viability of the African malaria mosquito (*Anopheles gambiae* Giles). They found that 12 and 42 C reduced larval hatch, which was likely due to the temperatures imposing negative effects on embryonic development. Early embryonic development was impacted more severely by extreme temperatures than later embryonic development (Impoinvil et al. 2007). Both studies suggest an optimum temperature range of 25 to 30 C for larval hatching (Impoinvil et al. 2007, Baek et al. 2012).

Distribution and release of the hydrilla tip mining midge as a biological control agent of the invasive weed hydrilla can be effected by refrigeration and storage and needs to be considered in protocols of rearing and release in order to maximize the potential impact of hydrilla tip mining on hydrilla control efforts. Storage of hydrilla tip mining midge eggs in the refrigerator at 5 C is occurring within 24 h post oviposition; therefore early embryonic development may be impacted by this highly reduced temperature. Negative impacts on early embryonic development were likely the cause of the mortality in larval hatch rate. In order for there to be no negative impact on embryonic development, the egg must be able to tolerate cold temperatures. Water temperatures in south and central Florida (below 28°N latitude) rarely go below 14 C even during winter months (Beaver et al. 1981). Lakes in northern Florida may experience temperatures as low as 8 C in winter and other parts of southeastern United States may also experience much lower lake temperatures (Beaver et al. 1981). Water body temperature range is an important consideration for determining the potential distribution of the hydrilla tip mining midge and its host plant because biological control is most effective if the hydrilla tip mining midge is able to establish and to expand its population size (Stratman et al. 2014). Stratman et al. (2014) found that larval survival was reduced with prolonged exposure at 5 C; only 50% of larvae survived 8 d of exposure and no larvae survived after 32 d of exposure at 5 C. However, nearly 100% of larvae survived 16 d of exposure to 7.5 C, which indicates a potential range for the hydrilla tip mining midge in the southeastern United States through Florida, part of Georgia, Alabama, Mississippi, Louisiana, and Texas (Stratman et al. 2014). Therefore, it also is important to understand impacts of cold temperatures on egg masses and the effect on larval hatch success in addition to development to predict the potential ecological range of the hydrilla tip mining midge with its host, hydrilla. Hydrilla has been documented by the USDA's PLANTS database (USDA NRCS 2014) to occur as far north as Maine and Washington State in the United States. Consequently, hydrilla is not likely to be as limited as the hydrilla tip mining midge to warmer water temperatures and may be able to avoid this biocontrol agent if the insect cannot survive and overwinter in lakes that reach temperatures as low as 5 C. However, augmentative releases of the hydrilla tip mining midge can be conducted annually when water temperatures are more amenable to hydrilla tip mining midge survival.

One limitation of this study, when considering the ecological range of the hydrilla tip mining midge, is that we evaluated larval hatch rates at only one temperature. To better understand the impact of cold tolerance on eggs for predicting the ecological range of the hydrilla tip miner, future studies should test different temperatures such as Stratman et al. (2014) has already demonstrated with larval development. Although prolonged exposure time greater than 15 d at 7.5 C negatively impacted hydrilla tip mining midge larvae, any time at 5 C had a negative impact on larval survival (Stratman et al. 2014). Due to the difference in hydrilla tip mining midge larval survival with this slight increase in temperature, there is potential that egg hatch may also be less severely impacted at longer time intervals at a temperature even slightly higher than 5 C. Future work should focus on potential prolonged storage greater than 7 d, and determine if egg hatch could be delayed without detrimental effects to hatch success at a higher temperature such as 7.5 C.

When considering an integrated pest management program, it is the larvae of the hydrilla tip mining midge that are the damaging stage. Thus, care should be taken when refrigerating eggs to ensure the greatest hatch rate. Refrigeration of up to 7 d can be tolerated, but longer times may result in reduced hydrilla tip mining midge populations. In colony rearing where adults are needed to maintain high colony production, any refrigeration longer than a 2-d period decreases the number of eclosing adults and may reduce colony productivity. If refrigeration is unavoidable, then based on the results of this study, it is possible to adjust the number of eggs to be released according to the amount of time the hydrilla tip mining midge eggs have been refrigerated. It is possible that higher temperatures, such as between 5 and 10 C, may be of use in delaying egg hatch for field transportation, timing of

experiments, or other practical purposes, while maintaining the rate of adult eclosion.

Developing more effective mass rearing protocols for the hydrilla tip mining midge has a positive impact on aquatic ecosystems because it can improve prospects for biological control of the aquatic weed hydrilla by improving the ability to establish hydrilla tip mining populations. Incorporating biological control as part of an integrated pest management strategy will reduce reliance on herbicides as a stand-alone control method. Reduced reliance on herbicides is critical now that resistance of hydrilla to commonly used herbicides has been documented.

SOURCES OF MATERIALS

[1]Ultrasonic Cleaner B-52, Branson Cleaning Equipment Co., Parrott Dr., Shelton, CT 06484.

[2]Lab-Line Biotronette Mark III Environmental Chamber, Lab-Line Instruments Inc., 1999 N. Bloomingdale Ave., Melrose Park, IL 60160.

[3]Excel, Microsoft, 1 Microsoft Way, Redmond, WA 98052.

[4]SAS version 9.3, SAS Institute Inc., 100 SAS Campus Dr., Cary, NC 27513.

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