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Effect of food deprivation on hydrilla tip mining midge survival and subsequent development

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Abstract

Hydrilla, *Hydrilla verticillata* (L.f.) Royle (Hydrocharitaceae), is an invasive aquatic macrophyte found in fresh water. The introduction of hydrilla by the aquarium plant trade has led to its invasion throughout much of the southern US and its current listing as a federal noxious weed. Hydrilla employs many pathways of reproduction and once established can rapidly fill the water column, impeding recreation and negatively affecting the environment. Waterways infested with hydrilla typically experience matting of vegetation at the surface that results in less light penetration, and changes in dissolved oxygen levels, which disturb native species richness and diversity. Efforts to minimize hydrilla populations include using biological control agents such as the hydrilla tip mining midge, *Cricotopus lebetis* Sublette (Diptera: Chironomidae). The larvae of *C. lebetis* feed on the apical meristem of hydrilla tips, disabling further vertical growth and forcing growth into a branched horizontal direction. Currently, a colony of *C. lebetis* is being mass-reared to augment midge populations throughout hydrilla-infested waters. In order to maintain colony viability for effective releases, midge eggs must be collected and placed on hydrilla before larvae exhaust endogenous nutrient reserves. To understand the effects of larval starvation on survival and subsequent development to adult eclosion, neonates at 0, 1, 2, and 3 d post-hatch were studied with and without access to food. Midge survival and adult eclosion decreased significantly after continued starvation post-hatch. Larvae starved for 2 d post-hatch did not eclose. Highest survival to adult eclosion occurred when midge larvae were placed on hydrilla as soon as they hatched (48 h post-oviposition). This study highlights fundamental information necessary for efficient midge rearing for effective biological control of hydrilla.

Key Words: Chironomidae; *Cricotopus lebetis*; starvation; biological control; *Hydrilla verticillata*; nutritional stress

Resumen

La hidrilla, *Hydrilla verticillata* (L.f.) Royle (Hydrocharitaceae), es una macrofita acuática invasiva que se encuentra en el agua dulce. La introducción de hidrilla por el comercio de plantas de acuarios ha llevado a su invasión en gran parte del sur de los Estados Unidos y su inclusión actual como una hierba federal nociva. Hidrilla emplea muchas vías de reproducción y una vez establecido puede llenar rápidamente la columna de agua, impidiendo la recreación y afectando negativamente el medio ambiente. Los cursos de agua infestados con hidrilla típicamente experimentan una superficie opacada que da como resultado una menor penetración de la luz y cambios en los niveles de oxígeno disuelto, que alteran la riqueza y diversidad de especies nativas. Los esfuerzos para minimizar las poblaciones de hidrilla incluyen el uso de agentes de control biológico como la mosca chiquita que mina las puntas de las hojas de hidrilla, *Cricotopus lebetis* Sublette (Diptera: Chironomidae). Las larvas de *C. lebetis* se alimentan del meristemo apical de las puntas de hidrilla, incapacitan el crecimiento vertical adicional y forzan el crecimiento en una dirección horizontal ramificada. Actualmente, una colonia de *C. lebetis* está siendo criada en masa para aumentar las poblaciones de la mosca chiquita a lo largo de las aguas infestadas de hidrilla. Con el fin de mantener la viabilidad de las colonias para liberaciones efectivas, se deben recolectar los huevos de la mosca chiquita y colocarlos sobre la hidrilla antes de que las larvas agoten las reservas de nutrientes endógenos. Para comprender los efectos de la inanición de larvas sobre la sobrevivencia y el desarrollo después de la eclosión de los adultos, se estudiaron los neonatos a los 0, 1, 2 y 3 d después de la eclosión con y sin acceso a los alimentos. La sobrevivencia de la mosca chiquita y la eclosión adulta disminuyeron significativamente después de la inanición continua posterior a la eclosión. Las larvas no alimentadas durante 2 d después de la eclosión no eclosionaron. La mayor sobrevivencia a la eclosión adulta ocurrió cuando las larvas de la mosca chiquita fueron colocadas sobre la hidrilla tan pronto como nacieron (48 h después de la oviposición). Este estudio destaca la información fundamental necesaria para la cría eficiente de la mosca chiquita para un control biológico efectivo de la hidrilla.

Palabras Clave: Chironomidae; *Cricotopus lebetis*; inanición; control biológico; *Hydrilla verticillata*; estrés nutricional

Hydrilla, *Hydrilla verticillata* (L.f.) Royle (Hydrocharitaceae), also commonly known as water thyme or Florida elodea, is an extremely successful macrophyte that has invaded several continents worldwide and is considered a nuisance species (Evans & Wilkie 2010). Its native origin is not certain but it probably originated in warmer regions of Asia (Cook & Lüönd 1982). In the Americas, scientists first discovered hydrilla in Florida in the 1950s (Schmitz et al. 1991), and by the 1960s it was found at 2 locations, a canal near Miami and in Crystal River (Blackburn et al. 1969). Likely introduced to the US through the aquarium plant trade (Schmitz et al. 1991), Florida's subtropical climate and extensive aquatic ecosystems, have allowed hydrilla to flourish and it now can be found in practically any body of fresh water including ponds, lakes, springs, and reservoirs (Cuda et al. 2011).

In the US, hydrilla is federally regulated and listed in the Noxious Weed Program under the Plant Protection Act, which makes it illegal to import or transport plant material between states without a permit (FLEPPC 2015). Hydrilla can exist as a rooted plant as well as a floating macrophyte, and often is problematic to boaters, swimmers, and an-

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glers because of its growth habit of matting at the surface (Coetzee et al. 2009). As hydrilla grows toward the surface, it branches profusely in the upper part of the water column and will eventually cover an extensive amount of surface area (Langeland 1996). Hydrilla's propensity to form mats causes navigational problems and can entangle boat propellers. Areas of dense hydrilla can potentially ensnare and impede swimmers from reaching the surface. Therefore, hydrilla can have negative economic and environmental impacts when it renders a body of water unavailable for its intended uses (Schmitz et al. 1991; Langeland 1996). Additionally, from a real-estate perspective, issues related to hydrilla in lakefront properties can be of concern to potential buyers. Recreational fishing also is affected by the surface mats created. However, for some systems hydrilla can be beneficial, because hydrilla provides a desirable ecosystem for some sport fish species.

In order to control invasive species such as hydrilla, several methods can be used, including but not limited to chemical, mechanical, physical, and biological control (Gillett-Kaufman et al. 2014). Historically, herbicides have been applied as a standalone method, but this strategy is problematic due to the development of resistance (Michel et al. 2004; Berger & MacDonald 2011; Giannotti 2013). In sensitive areas, mechanical control through harvesting or physical removal often is implemented. However, the creation of hydrilla fragments and rapid regrowth post-dispersal limits the use of mechanical harvesting on a large scale. In addition, the costs associated with the physical removal of hydrilla are prohibitively expensive. Consequently, an integrated management approach that combines multiple tools is advisable.

One potential tool to incorporate into a diversified approach is biological control. Natural enemies can selectively attack hydrilla and reduce its growth (Grodowitz et al. 2004; Doyle et al. 2007). Several biocontrol agents have been assessed for hydrilla management, including multiple insects and grass carp (Gillett-Kaufman et al. 2014). Although grass carp are highly effective at removing plant material, they are polyphagous and typically remove both native and invasive plants (Sutton et al. 2012).

The hydrilla tip mining midge, *Cricotopus lebetis* Sublette (Diptera: Chironomidae), was initially found in 1992 feeding on hydrilla in Crystal River, Florida, USA (Cuda et al. 2002, 2011). Later, it was discovered that *C. lebetis* had been established in southwest Florida since 1976 (Stratman et al. 2013a). In Crystal River, the hydrilla appeared to be "stunted" and later studies found that when larvae of the hydrilla tip mining midge hatch from the eggs, they mine into the apical meristems of hydrilla (Cuda et al. 2002, 2011). This action impedes the vertical growth of the plant due the loss of the apical meristem. Furthermore, the hydrilla will branch in a horizontal fashion and no longer reach the water surface (Cuda et al. 2002). Although hydrilla has several ways of spreading (Langeland 1996), the hydrilla tip mining midge is beneficial because it changes the architecture of the plants.

Augmentative biological control involves the release of large numbers of an agent to supplement the numbers already present, in expectation of a greatly increased effect (Frank 2000). The origin of the hydrilla tip mining midge and its distribution in Florida are unknown (Epler et al. 2000). However, *C. lebetis* has been detected occurring naturally in several lakes in Florida, albeit in low numbers (Stratman et al. 2013b). Augmentative releases of the hydrilla tip mining midge require mass rearing, as well as handling and storage of eggs or larvae prior to release, which can have negative effects on larval viability. For example, Baniszewski et al. (2015) found that refrigeration of *C. lebetis* eggs during storage reduced larval hatch rate after 7 d and adult eclosion after 2 d. The results of that study affected release protocols, and eggs are no longer refrigerated prior to release (JP Cuda, personal communication). However, it is unknown how long midge larvae can survive in storage even without cold stress, or how this might affect hydrilla management using this biological control agent. The aim of the current study was to determine how long larvae can survive when deprived of a food source prior to release, and how this starvation period affects subsequent development.

Materials and Methods

PLANTS AND INSECTS

Hydrilla (*H. verticillata*) used in the study was collected from ponds located at the University of Florida, Institute of Food and Agricultural Sciences, Center for Aquatic and Invasive Plants (UF/IFAS CAIP; 29.7263°N, 82.4177°E). The hydrilla was sorted, keeping about 12 cm of hydrilla from the apical meristem (hereafter described as "hydrilla tips"). Hydrilla tips were washed to eliminate snails, moths and other non-target organisms that might feed on the hydrilla, affecting the experiment. The hydrilla tips were stored in a 17 L container with well water aerated with a 20 L aquarium aeration pump until used for rearing or experiments.

The hydrilla tip mining midge (*C. lebetis*) was reared in greenhouses located at the UF/IFAS Entomology and Nematology Department. The greenhouse was maintained at a temperature range of 21 to 38 °C. Ambient light was supplemented with 243.8 cm (8 ft) florescent tubes (60 watt, cool white) and round bulbs (40 watt, 120 volt, soft white) to maintain a 14:10 h L:D photoperiod, as described by Cuda et al. (2002). Between 800 and 1,800 eggs were placed into trays containing processed hydrilla tips in aerated well water. After approximately 12 d, trays were placed into white mesh cages (61 × 61 × 61 cm). Adults began emerging 14 to 21 d from the time the eggs were laid. During this period, adults were collected using a filtered aspirator.

In a laboratory room, adults were placed into a 500 mL separatory funnel using the aspirator. The funnel was filled with 300 mL of filtered hydrilla-treated well water to induce oviposition. Hydrilla-treated well water was obtained by placing several hydrilla tips in a 3.8 L (1 gal) glass jar. To maximize fecundity, an approximately 1:1 ratio of males to females was placed in the funnel. Rearing room conditions were maintained at around 23 °C, 21% RH, and a 14:10 h L:D photoperiod. After 24 h, eggs were collected by opening the stopcock and draining the water from the separatory funnel to collect the egg masses (Cuda et al. 2002). Fecundity and fertility of eggs in each mass were recorded by viewing under a dissecting microscope. Fecundity was determined by counting the total number of eggs. Fertility was calculated by counting the number of pigmented eggs and dividing this by the total number of eggs (multiplied by 100 for percentage fertility). Typically, each egg mass contains approximately 150 eggs (Cuda et al. 2002). Egg masses then were placed into trays filled with hydrilla tips in the greenhouse or used in experiments.

INFLUENCE OF FOOD DEPRIVATION ON MIDGE SURVIVAL

An experiment was designed to establish how long midge larvae could survive after hatching without access to a food source. Three egg masses were collected and fecundity and fertility of eggs were assessed. Only egg masses containing 80 to 100 fertile eggs were selected and placed in 60 mm diameter Petri dishes. Forty-eight hours post-oviposition, larvae began to hatch and 60 larvae were individually placed into the wells of a 96-well plate. Larvae from different egg masses were placed in different 96-well plates. Using a compound microscope and a disposable glass pipette with a rubber pipette filler, a single larva was transferred from the Petri dish to each well. Well water was added to each well, filling to just below the top. Each 96-well plate was placed in an open 0.94 L (1 qt) zipper-type plastic bag (17.7 × 20.3

cm), which contained a damp paper towel to prevent excessive evaporation of well water from the wells. The 96-well plates were stored at approximately 24 °C. Each well was checked under a compound microscope daily for 2, 3, 4, and 5 d post-oviposition. Each larva was inspected for any movement. If no movement was observed, the larva was stimulated by moving the water or gently prodding with a dissection needle. If the larva still displayed no movement, it was considered dead. Each experiment consisted of 3 replicates containing larvae from different egg masses and the experiment was repeated 3 times across a period of several wk and different generations (n = 9).

INFLUENCE OF FOOD DEPRIVATION ON DEVELOPMENT

A 2nd experiment was performed to test how food deprivation affected subsequent larval development, pupation, adult eclosion, and damage to the hydrilla tip. Three egg masses with a fertile egg count of 80 to 100 were placed into separate Petri dishes and stored at approximately 24 °C. After 48 h, when the larvae had hatched, the 1st set was placed into test tubes. Each test tube contained 1 rinsed and processed hydrilla tip with well water that completely submerged the tip. Using a compound microscope and a disposable glass pipette with a rubber pipette filler, larvae from the 1st egg mass were placed individually into 10 test tubes, and this was repeated for the 2 other egg masses. After successfully placing each larva into its test tube, the tubes were closed with a perforated plastic lid. The process was repeated every 24 h until no live larvae remained in the Petri dishes; all larvae were dead by 3 d without food. The racks containing the test tubes were stored at 26 °C with a 14:10 h L:D photoperiod in an environmental chamber (Lab-Line Biotronette Mark III, Lab-Line Instruments Inc., Melrose Park, Illinois, USA). Ten days post-hatch, data collection began for all 4 treatments (0, 1, 2, and 3 d without food). Observations of life stage (i.e., visible larvae, pupae, and eclosed adults) as well as number of damaged hydrilla tips were recorded daily until d 21 or adult eclosion ceased. The numbers of larvae and pupae were combined if both a larva and pupa were seen in the same tube at different times. Each experiment consisted of 3 replicates (different egg masses) of 10 tubes and the experiment was repeated 3 times (n = 9). Hydrilla was classified as damaged when mining was evident on the apical meristem.

STATISTICS

For the 1st experiment, the survival of starved larval midges over time (0–3 d post-hatch) was analyzed by fitting linear mixed models that consider the repeated nature of the data. Least significant difference (LSD) tests with the Bonferroni correction were obtained to compare mean mortalities over time ($\alpha = 0.05$). The analysis was completed in SAS version 9.3 (SAS 2008).

For the 2nd experiment, all variables were found not to follow a normal distribution by the Shapiro-Wilk W test, so the data were analyzed by the non-parametric Kruskal-Wallis test. Non-parametric means comparisons were performed using the Wilcoxon method to show statistical differences between larval starvation periods (0–3 d). For all tests, the significance level was set at $\alpha = 0.05$. All analyses were completed in JMP (Pro 11 software; SAS 2013).

Results

INFLUENCE OF FOOD DEPRIVATION ON MIDGE SURVIVAL

In the 1st experiment of this study, larvae were placed in 96-well plates to test for survival in the absence of a food source. Time (d) had a significant effect (F = 54; df = 3, 23.6; $P < 0.0001$) on the survival of larvae when they were kept in 96-well plates without nourishment (Fig. 1). The percentage of larvae alive decreased significantly each day, from 100% at the start of the experiment to 13.7% by 3 d. The interval that had the largest decrease in larval survival was from 2 to 3 d. The percentage of larvae alive decreased significantly (t = 9.06; df = 22.8; $P < 0.0001$) from 65.0% at 2 d to just 13.7% at 3 d.

INFLUENCE OF FOOD DEPRIVATION ON DEVELOPMENT

In the 2nd experiment of this study, starved larvae were placed in test tubes with hydrilla to determine if they were capable of continuing development. The number of combined surviving larvae and pupae over the course of the study, adult eclosion, and hydrilla tip damage all were significantly affected by the larval starvation period ($P < 0.0001$). All variables produced data with similar trends, and with the same statistical conclusions. Therefore, only the data for adult eclosion has been presented (Fig. 2).

The highest number of larvae and pupae (combined) observed throughout the experiment was from the 0 d of starvation treatment, with 5.22 midges (per 10 tubes; data not shown). There were significantly more larvae and pupae in the 0 d starvation treatment than for any of the other starvation periods (Chi-square = 25.65; df = 3; $P < 0.001$). There was also a significant decline in surviving larvae and pupae (combined) when increasing the starvation period up to 2 d, with only 0.22 midges per 10 tubes. There were no larvae or pupae observed if the larvae were starved for 3 d.

The highest number of eclosed adults observed throughout the experiment was from the 0 d of starvation treatment with 4.66 midges (Fig. 2). Significantly more adults eclosed from the 0 d of starvation than for any of the other starvation periods (Chi-square = 25.18; df = 3; $P < 0.001$). There was a significant decline in adult eclosion associated with increasing larval starvation period from 0 to 1 d and 1 to 2 d, with emergence of only 0.22 midges per 10 tubes by d 2. There was no adult eclosion observed if the larvae were starved for 3 d.

The highest amount of tip damage observed throughout the experiment was from the 0 d of starvation treatment with 4.44 tips damaged (data not shown). Significantly more tips were damaged in the 0 d of starvation treatment than for all the other starvation periods (Chi-square = 25.27; df = 3; $P < 0.001$). There was a significant decline in tip damage associated with increasing larval starvation period from

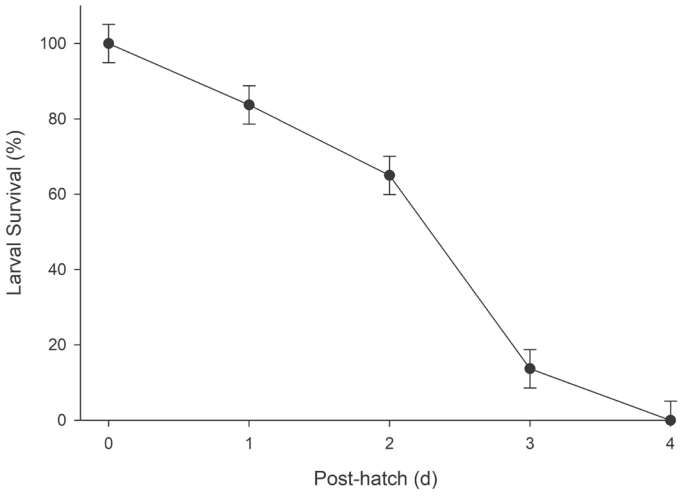

Fig. 1. Effect of food deprivation on larval survival of the hydrilla tip mining midge, *Cricotopus lebetis*. Number of larvae alive recorded for each day post-hatch in 96-well plates. Mean percentage survival ± standard error of the mean. The number of larvae alive decreased significantly each day ($P < 0.05$).

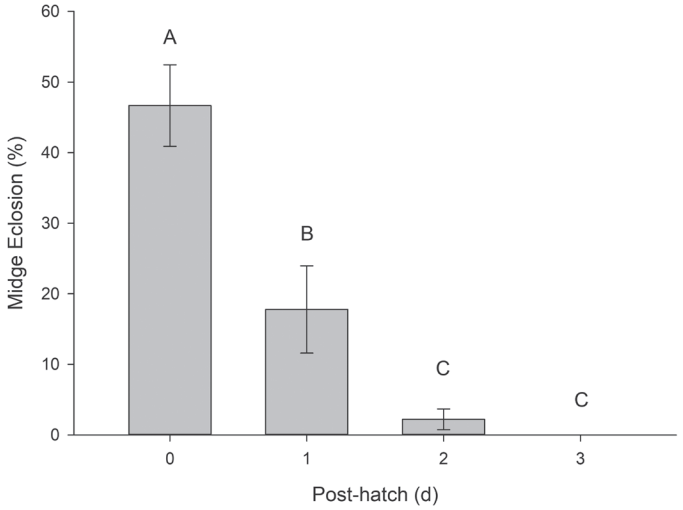

Fig. 2. Effect of starvation post-hatch on the eclosion of *Cricotopus lebetis* adults from hydrilla (*Hydrilla verticillata*) stems in test tubes. Midge eclosion was defined as observing an adult *C. lebetis* in the test tube. Bars represent mean percentage midge eclosion ± standard error of the mean. Statistical differences between the midge eclosion observed after different starvation periods post-hatch are indicated by different letters.

0 to 1 d and 1 to 2 d, with 0.22 tips damaged per 10 tubes by day 2. No tip damage was observed if the larvae were starved for 3 d.

Adult eclosion occurred in the 0 d of starvation tubes between 13 and 19 d post-inoculation (average 17 d); this range in eclosion was the same for those larvae that were starved for 1 d. The only 2 larvae to eclose after 2 d starvation did so after 20 d. No larvae eclosed following 3 d of starvation, as previously mentioned.

Discussion

Biological control is an important component of the integrated management of pest plants. In contrast to classical biological control, which involves the use of non-native species, augmentative biological control typically involves the supplementation of a native species up to a level that results in weed control below an acceptable threshold. In order for this to occur, the herbivore must be gathered or mass reared prior to release, and techniques to do so efficiently and economically need to be investigated. For example, previous studies with the hydrilla biological control agent *C. lebetis* found that storage of eggs or larvae in a refrigerator prior to release had a negative effect on the viability of the larvae (Baniszewski et al. 2015). The aim of the current study was to determine how long midges can survive without a food source prior to inoculation and how this starvation period affected their subsequent development.

Because adult *C. lebetis* do not feed, energy for mating and reproduction by the adults must be gained as larvae. Therefore, it is crucial that developing larvae have sufficient resources available to them for growth and future fitness. Consequently, adult *C. lebetis* maximize survival and development of their offspring by laying their eggs in gelatinous tubes that likely serve to stick the egg mass to nearby plant material (Cuda et al. 2002). When larvae hatch from the eggs, they are in close proximity to their food supply. When their life cycle is manipulated, such as it is in a laboratory colony, it is important to consider the effects of these changes on survival, development, and suitability as a biological control agent.

Not surprisingly, starving larvae post-hatch resulted in mortality. Two days after the eggs were laid, larvae started to hatch, and with every subsequent day without access to food, there was a significant decrease in survival. By d 4, all remaining larvae were dead. Larvae hatch with limited resources for growth and metabolism, and typically have just enough to keep them alive until they locate a food source. Without a food source, energy intake does not occur, and to maintain metabolic functions, available body reserves are used instead. This continues until the reserves are exhausted and mortality occurs.

In the 2nd experiment, providing a food source to these starved larvae did not prevent a significant decrease in survival. When larvae were placed on hydrilla tips 0, 1, 2, and 3 d post-hatch, by d 1 there was a significant decrease in the survival of larvae to eclosion as adults. A 3 d period of starvation resulted in no adult eclosion, and all larvae died during development. This result indicates that for *C. lebetis* larvae, even 1 d of starvation results in such a drain in reserves for growth and metabolism that 50% of larvae are not able to survive even when provided with a suitable food source.

In a predatory midge species, *Feltiella acarisuga* (Vallot) (Diptera: Cecidomyiidae), food deprivation time was found to have a significant effect on survival, with less than 40% survival after 4 d of starvation (Sawyer 1998). However, these larvae were fed prior to the experiment and so likely had increased resources compared to the neonates used in this study.

In addition to causing mortality directly, starvation of insects can have other effects on their development as well as indirect effects on their survival. Predatory midges, *F. acarisuga*, that were deprived of food had an increased development time with increasing food deprivation time as well as reduced pupal weights (Sawyer 1998). Studies with other insect species have found similar effects; starvation of common green lacewings, *Chrysoperla carnea* (Stephens) (Neuroptera: Chrysopidae) affected larval growth and development, and adult fecundity (Zheng et al. 1993a). Starved lady beetles have slower development, reduced size and weight, and lower reproductive output as adults (Hodek & Evans 2012).

In our study, larval development occurred in 13 to 19 d with a starvation period of 0 to 1 d; a 2 d starvation period resulted in high levels of mortality, but those few larvae that survived took 20 d to complete development. To test if development was delayed in midges starved for longer durations, observations were conducted in 3 replicates up to 31 d. However, no additional adult eclosion occurred after 20 d (data not shown).

Although a temporary deprivation of food can delay development, many insects studied demonstrate a period of increased feeding, which likely enables them to 'catch up' to undeprived individuals (Rollo 1984; Zheng et al. 1993b). For example, caddisfly larvae consumed more prey if starved for 0 to 72 h (Sangpradub & Giller 1994). This compensation usually occurs through 1 of 2 ways, either increased time until pupation or accelerated larval growth rate through more efficient food conversion (Hodek & Evans 2012). In our study, 1 d of starvation resulted in significant mortality, but those individuals that survived were able to develop within the same time frame as those that were not deprived of food after hatching (13–19 d). Similarly, predatory midges starved for 1 d were able to catch up with the fed controls (Sawyer 1998). However, in that example there were effects on development time after 1 d of starvation.

In order to maintain a laboratory colony for mass rearing *C. lebetis*, further research is needed to fully understand how certain environmental conditions affect midge development. Metabolic studies may help to explain how the midge will react under varying environmental conditions. Starvation, desiccation, and temperature are only a few stress factors to consider and further investigation concerning plant density preference, host-plant viability, and water chemistry parameters should be investigated for optimal rearing and release conditions.

When considering both experiments, increasing time post-hatch without food resulted in a substantial impact on larval survival as well as on midge development to adult eclosion. Significant mortality will occur in larvae kept without food for as little as 1 d and even those midges that do survive may be less able to invade hydrilla tips, cause damage, and develop to a pupa or adult. These results are important if the hydrilla tip mining midge is to be mass reared or if optimal survival is expected for experimental purposes. More importantly, if the midge is to establish in the field and be an effective biological control agent, it is crucial that released midges are of optimum viability.

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