Development of captive rearing and long-term handling protocols for larvae of the endangered Hine's emerald dragonfly (*Somatochlora hineana*).
Final Report

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Abstract:

We describe the results of a five year study whose goal was to develop protocols for successfully holding and rearing to adult stage, larvae of the federally-listed endangered Hine's emerald dragonfly (*Somatochlora hineana*). In addition, we have conducted research on methods for collecting eggs from the field and successfully rearing larvae from them. Rearing of mid-sized larvae began in the fall of 2003 with 33 larvae that were large enough to be positively identified as *S. hineana* (head width >2.0mm). Larvae were reared under conditions that closely mimicked wild conditions. This was done so that larvae they would grow and develop at a more or less natural rate, and emerge successfully at an appropriate time. A standard procedure for rearing larvae in individual containers was developed that has maintained survivorship of larvae handled in the captive program above 82%. We also developed a standardized, easily-used individual field cage design (S-cage) which allows larvae to be exposed to field conditions during the summer. Cage experiments were used to fine tune captive feeding levels, producing similar growth rates in captivity and in the wild. The sizes of captive larvae were comparable to those of wild larvae. An appropriate temperature regime for holding larvae over fall and winter was developed using data collected from crayfish burrows in Door County, Wisconsin. Cage related deaths and deaths in p-cups during the summer account for most fatalities. Larvae were first placed in emergence cages in 2004. In the following years, emergence cage design was improved upon and now a modified rectangular mesh crayfish trap ("Pagoda" style E-cage) is used as the standard. These devices are simple to construct and easily stored. Combination of results from 2005 and 2006 show that larvae held in the captive program for at least 1 winter still emerge at the same time of year as recently captured larvae when placed in E-cages for the summer. Emergence success continued for 2007 and 2008 and corresponded to flying period of adult *S. hineana.* The emergence period for larvae in this study was between June 19th and July 13th which compares favorably with previous field studies. Emergence success was between 95% and 100%. Overall survivorship of adults handled as part of the captive program ranged between 66.7% and 100%. The protocols developed as part of this study, although labor-intensive, have great promise and could be scaled up to provide large numbers of *S. hineana* larvae to be used to augment existing populations or re-establish populations in areas where the species has been extirpated.

Development of captive rearing and long-term handling protocols for larvae of the endangered Hine's emerald dragonfly (*Somatochlora hineana*).

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Background:

The Hine's emerald dragonfly (*Somatochlora hineana*) is a federally and state-listed endangered species that occurs at a number of scattered locations in the Midwestern United States and Canada. The aquatic larvae are unusual for dragonflies as they take 3-5 years to mature into the winged adult. Larvae generally occur in shallow, seasonally-intermittent streams and fens underlain primarily by dolomitic bedrock. These habitats are sensitive to changes in hydroperiod, contamination and human development. When the recovery plan for the Hine's emerald dragonfly (*Somatochlora hineana*) was drafted the development of captive rearing techniques were viewed as an important prerequisite for future plans to reintroduce the species into sites it formerly occupied, or to augment existing populations. Thus the development of captive rearing protocols was placed as a priority 2 task under Task 4.1 (Develop Captive Rearing Protocol).

Studies in 1997-2002 strongly indicated that there was an urgent need for the development of such protocols, so that it would be possible to remove *S. hineana* larvae and maintain them in captivity in the event of a natural or human-caused catastrophe. For example, strong declines in larval populations of the *S. hineana* were documented at one of the most important sites for the species as a whole (Soluk et al. 2000). The steep decline in the abundance and changes in the age structure of the population in the Lockport Prairie Nature Preserve in Illinois were hypothesized to be the result of changes in the hydrology of the preserve due to urban development and groundwater extraction (Soluk et al. 1998 and 2000, 2006). This area is one of the most important areas for the *S. hineana* in Illinois. Given these and other threats faced by *S. hineana* populations in Illinois and other sites, it was of central importance that consideration be given to developing protocols that would allow the temporary removal of some part of the population of *S. hineana* larvae until remediation techniques can be developed to restore their habitat.

In addition to the benefits of removal as a way of saving threatened individuals, captive rearing in the form of "head-starting" could also be used as a way to augment populations that may be in decline and provide individuals for reintroduction into restored habitat. Given the spatial and behavioral requirements of the adult stage of the Hine's emerald dragonfly, a breeding program wherein adults are held in captivity is not feasible. However, we may be able to benefit from some of the demographic features of dragonfly populations. In general, dragonfly larval mortality is extremely high during the first few larval instars. This natural mortality is caused by predation, cannibalism, and sometimes starvation. By collecting early instar larvae and raising them in predator free environments with abundant prey it should be possible to greatly increased survivorship

of these early stage individuals. Thus, we can generate individuals for reintroduction or augmentation of existing populations simply by "head-starting" individuals that were unlikely to survive otherwise.

There are some complicating factors, and successful rearing of Hines emerald dragonflies entails more than simply being able to keep some larvae alive in captivity. Any salvage or rearing program can only be deemed truly successful for conservation purposes, if it is able to demonstrate that larvae held in captivity can be reintroduced in such a way that they can successfully emerge as adults that survive to reproduce. This is sometimes a more difficult task with insects then might be presupposed. In insects, growth, development, and emergence into the adult stage are linked in complex ways. Unusually rapid growth, the lack of appropriate environmental cues for timing developmental events, or inappropriate diets may result in abnormalities that preclude successful emergence into the adult stage. The normal life cycle of *S. hineana* includes an extended larval growth and development period of 3 to 4 years, with both winter and summer periods of resting or diapauses (Soluk et al. 2000). For successful rearing of *S. hineana* larvae it may be necessary to replicate this slow growth rate and to simulate the extended periods of diapause in the burrows of crayfish. Unfortunately, holding or rearing larvae for periods as long as 3 to 4 years will be very time consuming and expensive, especially if large numbers of larvae are being held. The four main goals for this program were 1) to successfully hatch eggs and rear hatchlings with high survivorship, 2) to rear and hold larger larvae with minimal mortality, 3) to rear larvae in such manner that they emerge at the appropriate time of the year, and 4) to develop procedures for successfully accelerating growth rate (without creating developmental abnormalities) to reduce cost and effort required to rear *S. hineana* larvae in mass rearing programs.

This is a report of the results of the captive rearing program 2002 and 2008. Activities will be addressed in three parts: 1) egg hatching and hatchling rearing techniques, 2) techniques for holding and rearing of larvae between their second year and pre-emergence, and 3) techniques for promoting successful emergence. Progress towards all goals will be addressed, best rearing methods summarized, and recommendations presented. All studies were conducted in compliance with permits obtained from appropriate local, state, and national agencies (USFWS).

Methods and Results:

Egg collection and hatchling rearing

Egg hatching became part of the captive rearing program when *S. hineana* eggs were incidentally collected from a road-killed female in summer 2002. These eggs were stored in a cooler and transported to the laboratory. A few of the eggs hatched (n=12) before they were cooled for the winter. These hatchlings were held individually in the cells of a 96-well tissue culture plates partially filled with water from the *S. hineana* habitat. Larvae were fed daily with a number of prey items included oligochaetes, *Drosophila* maggots, chironomid larva, and ostracods, daphnids, rotifers, and brine shrimp nauplii. By January 2003, all 12 of the hatchlings born in 2002 had died. The remaining eggs were kept through the winter at 3ºC in the tissue culture wells (approximately 1-10 eggs in each well) and checked periodically for additional hatching. No additional hatches occurred in 2002.

In January of 2003, batches of eggs were separated and warmed to three differing temperatures: 8ºC, 12ºC and 16ºC. Eggs were checked daily for hatching. Egg hatching began when the temperature was raised at or above 12ºC. Twenty-six *S. hineana* larvae resulted. Hatchlings were collected with a pipette and placed into individual wells in 96 well tissue culture plates. Hatchlings were again fed a variety of different prey including chironomid larva, small oligochaetes, drosophila maggots, ostracods, daphnids, rotifers, and brine shrimp nauplii approximately every other day. Prey were offered to the dragonfly larva through a pipette and larvae were monitored through a dissecting microscope for 5-10 minutes after feeding to see if offered prey were consumed immediately. Any prey items remaining at the next feeding were noted. Of the 26 *S. hineana* hatchlings born that spring, 64% of hatchlings survived less than a month, a few lived for 2-3 months and 2 hatchlings survived for five months.

In 2004, the egg hatching activities were put on hold because there were no eggs available for hatching.

In 2005, egg collection was attempted by placing shallow plastic food trays filled with habitat water into the field in hopes of attracting gravid *S. hineana* females. Egg collection trays were laid out along streamlet systems in the Mud Lake Wildlife Area in Door County, Wisconsin in July and August, and at the Lockport Prairie Nature Preserve, Will County, Illinois, in July. Egg collection trays would be laid in the dry channel and checked after 24 hours. No eggs were collected from the trays laid out in Lockport Prairie, however, approximately 300 eggs presumptive *S. hineana* eggs were collected from the Mud Lake Wildlife Area. Eggs were stored at ambient temperatures and no hatching occurred in the summer or fall. Eggs were kept 4ºC throughout the winter months and checked periodically, but no hatching occurred.

Beginning in March 2006, the eggs collected in 2005 were warmed and hatching began when temperature was raised above 10ºC. Seventy-nine larvae were hatched in 2006 and initially reared in 24 well tissue culture plates. Hatchlings were fed newly-hatched

chironomid larvae according to the same schedule developed in 2003. None of these hatchlings survived more than two months.

Egg collections were made from the Mud Lake Wildlife Area in July and August 2006 using the technique described above. Approximately *S. hineana* 300 eggs were collected. Eggs were stored at ambient temperatures and no hatching occurred in the summer or fall. Eggs were kept 4ºC throughout the winter months and checked periodically, but no hatching occurred.

In March 2007, temperatures were raised and hatching began. A total of 218 larvae hatched, however, only 10 survived more than a few weeks. To try to minimize the mortality from possible water contamination issues associated with keeping the larvae in a small volume of water, hatchling housing was switched from microplate wells to the standard specimen containers that were used for larger captive larvae (see below). When a hatchling was found in the egg holding tray, it was extracted with a pipette, given its own container, specimen number, and a piece of 250 micron mesh nytex cloth. Hatchlings were checked at least every other day and fed small chironomid larvae.

Some of the eggs that had not hatched from the batch collected in 2005 were saved and then overwintered in the same way as the newer 2006 eggs. Some of these hatched in the spring of 2007. This indicates that *S. hineana* eggs can survive more than one winter season.

Egg collection continued during July and August 2007 using the technique described above. Approximately 300 *S. hineana* eggs were collected.

In 2008, egg warming began in January. First hatches occurred when the temperature reached approximately 13°C. All eggs were not warmed at the same time, rather batches of eggs were selected and warmed at regular intervals mimicking spring temperature rise. This was done to minimize the possibility of too many larvae hatching at one time, exceeding our ability to handle and care for them. Batches of eggs from the same egg collector were kept in 100ml plastic urine analysis cups (p-cups), rather than well tissue culture plates to minimize the possibility of eggs drying between checks. Eggs were monitored daily for hatching. A total of 404 hatchlings resulted from the egg hatching effort in 2008. Hatched larvae were fed approximately 20 brine shrimp daily until reaching approximately 8 weeks old. Because brine shrimp are hatched in salt water, shrimp nauplii had to be rinsed in fresh water before feeding them to the larval dragonflies. Only the nauplii (<24 hrs old) were used for feeding the smallest hatchlings and any uneaten nauplii (which die after a few hours in fresh water) were removed from the cups during subsequent feedings. Larger hatchlings were fed very small chironomids at a rate of five chironomids each day. By September, feeding of all hatchlings was decreased to five chironomids every other day. Success rate was greatly improved with this egg hatching methodology, with 104 hatched *S. hineana* surviving to be introduced into the captive rearing population. This represents an approximate 25.7% survival rate of hatchlings.

An alternative rearing method was attempted in 2007, wherein dragonflies were mass reared in small plastic shoe bins (approximately 800mm long and 30mm wide). Each bin (n=11) was prepared by adding water to a depth of approximately 6.0 cm and then adding a small amount of detritus to lightly cover the bottom. Detritus had been collected the previous fall from *S. hineana* habitat in Door County, Wisconsin, picked clean of large predators, and stored in food coolers at 3-4ºC during the winter. Initial startup cultures of *Paramecia*, *Euglena*, and pure culture of *Simocephalus* spp. (a small daphnid crustacean) were added and shoe bins were placed in an incubation chamber held at 14-16ºC. In March, 10 *S. hineana* hatchlings were added simultaneously to each bin and returned to the incubation chamber. Each bin was fed twice a week, once with a chironomid egg mass and once with *Simocephalus*. Roughly half of the volume of water was changed weekly and airstones provided aeration in each bin. Bins were picked for surviving larvae in the spring of 2008. Of the 110 hatchlings placed in bins in 2007, only eight were recovered. Bin mass rearing was attempted again in March 2008; however, no detritus from *S. hineana* habitat was available so leaf litter was used. Examination during the fall of 2008 indicted that no larvae survived in these bins.

Rearing of small to mid-sized larvae

This portion of the rearing program began in 2003 with larvae that were large enough to be positively identified as *S. hineana* (head width >2.0mm). The first 33 *S. hineana* larvae for the captive program were collected in the November of 2003 from the Mud Lake Wildlife Area in Door County, Wisconsin (Table 1). This area contains the largest known contiguous population of *S. hineana* and was unlikely to be impacted by removal of individuals into captivity.

The first goal of the captive program was to be able to maintain *S. hineana* larvae in captivity while mimicking wild conditions closely enough that they would grow at a more or less natural rate. Larvae were housed individually in small plastic containers ranging from 40-70mm in diameter. Water levels were kept shallow (15-50mm) to allow sufficient dissolved oxygen. Larvae were kept in coolers or incubator units on a temperature regime that roughly followed ambient temperatures recorded from crayfish burrows in the field. To simulate winter temperatures in crayfish burrows, *S. hineana* larvae were kept at 5-6ºC. Prey items were provided once a week during the spring and fall cool temperature periods, and approximately every other day during warm summer temperatures. Initially prey items were exclusively Chironomid larvae (cultured in the lab); however, during the spring and summer larvae were also fed leptophlebiid and baetid mayfly larvae along with other invertebrates. Larval feeding, growth, and general condition were recorded.

In the spring of 2004, 43 more *S. hineana* larvae were added from the Mud Lake Wildlife Area in Door County, Wisconsin (Table 1). Starting in spring 2004 larvae were also placed *in-situ* in specially constructed field cages. Field cages allow larvae to experience condition similar to wild larvae during the late spring and early summer when most growth occurs (Soluk et al. 2000). Before being placed in cages all larvae were measured and weighed. Two types of *in situ* field cages were used. "S-cages" were modified

aquatic plant planting baskets with approximately 2mm mesh bottom and sides and tops. "E-cages" were constructed of short black plastic trash cans with all sides and bottom removed and replaced with mesh 2.0 mm mesh. Cage tops extended above the water and were covered with screen. Cages were staked in place. Debris and vegetation collected directly from the larval habitat was placed in the cages to imitate natural conditions and attract prey species. Detritus was sorted carefully to remove any predators. Ten larvae were placed in "S" growth cages and 10 in "E" emergence cages (see discussion below). All 10 larvae in growth cages survived to the end of the experiment. All larvae were taken back into the lab and maintained in individual plastic cups as described above. Seven larvae collected for the captive program in 2004 were returned to their original location during the summer and three larvae died during the year (Table 2). The captive population numbered 60 larvae at the end of December. Survivorship of larvae handled in the captive program was 96.1% (Table 3).

In 2005, lab handling procedures in the first part of the year followed those of 2004, but feeding rate was modified after analysis of field versus lab growth experiments. These experiments were important for understanding whether being held in the laboratory was altering growth rates and condition of captive larvae when compared to wild populations. To accomplish this, growth rates of larvae in lab rearing and field conditions were compared, along with growth rate of wild vs. captive larvae. Twenty five of the larvae that had been held in the captive program over the winter of 2004-2005 were placed individually in field cages in larval habitat in the Mud Lake Wildlife area. Cages were of the same design as in 2004. They were placed in the field June 2-5, 2005, and remained in the site until the habitat dried in the second week of July. The other half of the captive larval population (24 larvae) were maintained in individual 100ml plastic urine analysis cups at the field station in Door Co., Wisconsin. These larvae were kept in a cooler at approximately 14-16ºC, and fed 1-2 prey items every second day. Prey items were collected from larval habitat or wetlands near the field stations and included chironomid larvae, mayfly larvae, stonefly larvae, amphipods and isopods. To obtain "wild" growth rates 12 *Somatochlora hineana* larvae were also collected from the Mud Lake Wildlife Area during May and June 2005. These larvae were placed in equal numbers in the field S-cages and in the laboratory rearing groups.

All larvae were weighed and their head width and total length measured before cages were placed in the field, on June 23rd and 24th, and when cages were pulled. Percent increase in HW was calculated for each larvae using the following equation:

$$\%\Delta HW = (HW_{final}-HW_{initial})/HW_{initial}$$

If the same instar was present on two measurement dates, then those head widths and total lengths were averaged. A factorial ANOVA using feeding method and status (newly collected or held over the winter) as fixed factors was conducted. Very small larvae (HW < 2.00mm), which grow faster than larger larvae (Satyshur 2008), were not used in the analysis because they were only kept in the laboratory in 2005 due to risk of them escaping from field cages. ANOVA analysis indicated that growth rate of larvae in field cages was significantly higher than that of larvae being fed in captivity (F=21.60, df

= 1, $p<0.001$) (Table 4, Figure 1). Status (overwintered in captivity, or newly collected) did not affect larval growth under either feeding regime (F=2.56, df=1, p=0.116). This indicated that feeding rate of captive larvae needed to be increased, and thus quantity of prey offered during summer feeding in 2005 was increased.

In 2005, 9 larvae emerged, 10 died, 5 disappeared from cages and 27 larvae were added from Mud Lake North Wildlife Area (Table 2). Smaller larvae were collected to be used for more extensive cage experiments planned for 2006. In December there were 63 larvae in captivity. Overall survivorship of larvae handled in the captive program was 82.8% for 2005 (Table 3).

In 2006, protocols were standardized for housing and rearing captive larvae both in the lab and field cages. Larval individual container housing was standardized and all containers used for housing larvae were 100ml plastic urine analysis cups (Figure 2). All larvae were provided with a long rectangle of plastic screen which extended just above the water surface. Water was obtained from the habitat in Mud Lake Wildlife Area or from the well at the Door County field station. Water depth was maintained between 15mm and 35mm deep in the cups depending on larval size.

The size of the captive population was increased by retention of larvae from additional sites in Illinois and Wisconsin (Table 1) in 2006. All larvae from sites other than the Mud lake North Wildlife Area were only retained when they were too small to be positively identified as *S. hineana*. Previously these tiny larvae had been released without positive identification. The success of the rearing program meant that these larvae could be raised to identifiable size with minimum risk of fatality. They then could be released back into the habitat from which they had been collected.

To refine the thermal regime for holding larvae in the autumn through spring, when larvae are primarily found in burrows (Soluk et al. 2000, Pintor and Soluk 2006), temperatures recorded from 9 to 10 crayfish burrows in the Mud Lake Wildlife Area were examined. Average 24 hour temperature was calculated for the first and 15th day of each month. To simulate winter temperatures, larvae were held between 3-5°C until April 11th. Thereafter, temperature was raised by approximately 2 degrees every week until larvae were transported to the field station at the end of May. If larvae were not placed in field cages they are kept in a cooler equipped with a cooling unit between 16°C and 18°C. Fall cooling began on September 15th with temperature lowered from 16°C to 14°C. Temperature was dropped 2 degrees on the first and 15th of each following month until reaching 4°C on December 1st.

Although feeding was suspended during the winter, *S. hineana* larvae were still checked once a week. When *S. hineana* larvae were brought out of winter conditions in the spring, feeding was begun at a rate of once per week. Feeding rate was increased to once every 2 days in the summer, and larvae were fed approximately twice as much prey per feeding as in spring. Feeding was reduced in fall to the same rate as spring and was continued until the end of November. To provide a more varied prey base Amphipods which occur in S. hineana habitats were cultured and offered as alternative prey for larvae. These

amphipods were readily consumed by *S. hineana* larvae and have the advantage of higher tolerances for cool temperatures during late fall and early spring feeding.

New field cages were also developed in 2006. Non-emergent small field cages for larvae ("S-cages") were constructed from cylindrical PVC mesh tubes 220-260mm in length fitted around 76mm (3in.) diameter PVC pipe end frames covered with cloth mesh (Figure 3). This design allowed cages to fit easily in the shallow streamlets while still providing ample room for larvae. "S-cages", were made from mesh of two sizes (1.8 or 4.5 mm) in order to accommodate larvae as small as instar F-5 (about 1.8mm head width). Larval head width was used to determine size of mesh that prohibited escape. Larvae were placed in the largest mesh size possible to maximize movement of prey into cages. The construction and deployment of these cages is discussed by Satyshur (2008).

The majority (28) of the captive *S. hineana* larvae that were not expected to emerge (instar F-2 and below) were placed in "S-cages" in Mud Lake Wildlife Area during the late spring and early summer of 2006. Cages were placed in the field in late May and early June. To mimic streamlet conditions, vegetation was collected from the cage site and placed in cages. In addition to the larvae from the captive population, 3 recently collected larvae were also placed S-cages. Water level at cages was monitored on a daily basis. All larvae were weighed before, after, and during their time in the field and their condition noted. Larvae were brought back into captivity when the habitat dried on July 22, 2006. Wild-caught larvae used in S-cages were returned to their original collection location at the end of the summer.

At the start of the 2006 year there were 63 *S. hineana* larvae held in captivity (Table 1). Three larval *S. hineana* nymph deaths occurred in cages. As indicated above small larvae that were possibly *S. hineana* were added to the captive population from several locations, in 2006 (Table 1). Mud Lake Wildlife Area, contributed 27 potential *S. hineana* larvae. Seven larvae were also brought in from Cedarburg Bog near Milwaukee, WI, and 12 larvae were added from the Mink River Preserve in Door County, WI. Three locations in Illinois: Lockport Prairie, Long Run Seep and Waterfall Glen, contributed 22, 3, and 1 larva respectively. Of the total of 72 potential *S. hineana* larvae brought into captivity, 58 had been positively identified as *S. hineana* by the end of the year, 7 were probable *S. hineana*, 6 remained potential, and 1 was probably not *S. hineana*. Of the 71 new captive larvae, 11 died, however, only 5 were confirmed as *S. hineana*. Of the original captive population 3 *S. hineana* larvae did not survive the summer. At the end of December the captive population consisted of 95 *S. hineana* and 7 probable *S. hineana* larvae (Table 2). Survivorship was calculated as in previous years and equals 94.5% (Table 3).

Head width and total length of captive larvae that had been held at least one winter and from recently collected larvae from both 2005 and 2006 were plotted to compare their instar sizes and growth trajectories (Figure 4). Captive larvae did fall within the sizes found in wild populations, showing that captive program is not producing larger or smaller larvae than would be naturally found.

Captive rearing activities in 2007 included improvements to field cage design and provision of larvae for both genetic and habitat requirement studies. Containers, and feeding regime in 2007 followed the standardized methods developed in 2006. Temperature regime also followed that of 2006 except that the fall cooling began later. On November 9, 2007 temperature was dropped to 10°C and henceforth dropped regularly until reaching 5°C on December 1. Two facilities were available to hold larvae in during the summer, the TNC site in Door Co. and the "Fish Farm" facilities near Lemont, IL. The addition of this new facility in Illinois reduced the amount of stressful travel necessary and made it possible to hold larger numbers of larvae from Chicago sites for genetic and other studies.

We used the same standardized field cage design and methods developed in 2006 but expanded it to accommodate smaller larvae and ensure that larvae had no chance of escaping. S-cage were made from mesh of 4 sizes in order to accommodate larvae as small 1.4mm head width. For very small larvae a sleeve of fine plastic mesh (openings 1.4mm) was inserted into the coarsest mesh tube. Also, a finer cross stitching mesh was used to build a third stiff mesh tubing size for a total of 4 different sized mesh cages for small larvae.

In order to obtain more generalized growth rate information non-emergent *S. hineana* larvae (instar F-2 and below) were placed into field S-cages at two sites in addition to the Mud Lake Wildlife Area, Between May 12 and May 30, 2007, 19 larvae were placed in the Mud Lake Wildlife Area, 9 were placed in the Mink River Preserve (Door County, Wisconsin), and 12 in Lockport Prairie Nature Preserve (Will County, Illinois). All larvae in S-cages were from the captive population and larvae used in a particular system originated from that same area. All larvae were weighed before, after, and during their time in the field and their condition noted. Larvae were brought back into captivity when the habitats dried. In Wisconsin this occurred on June 15, 2007. This is more than a month earlier than last year. Cages in Lockport Prairie were pulled when the habitat dried up on June 12, 2007.

Studies on genetics of *S. hineana* populations were begun this year. The captive program contributed to this work by allowing safe temporary holding of larvae in captivity and providing exuvia and fecal pellets of larvae for genetic analysis. As part of the genetics study, a system of assigning individual identification numbers and a Microsoft Access database of both living and dead specimens was developed. From 2006 onward all larvae receive a 3+digit unique identifier number called a specimen number upon collection. All parts of a specimen that are preserved, as well as all collection, release and measurement data, are associated with that specimens' number.

At the start of 2007 there were 102 *S. hineana* larvae held in captivity at 4-6ºC (Table 1). All very small larvae collected in 2006 that survived to be identified were found to be *S. hineana*. We had two deaths in S-cages in Wisconsin and 4 in Illinois. Several captive larvae were released this year. All 6 larvae from Cedarburg bog and 6 larvae from Mud Lake North were released in June. Total number of captive larvae returned to their original collection locations this year was 28 (Table 2). Mud Lake Wildlife Area

contributed 45 larvae, and Lockport Prairie contributed 2 larvae. Larvae from Lockport were kept in order to rear them for identification. We also had two hatchlings from individual egg hatching studies and 8 larvae from detritus bin hatchling rearing studies survive and these were transferred to the captive population. Of the 57 new captive larvae, 1 died. Of the original captive *S. hineana* larvae 17 died, 6 emerged and 28 were released and one larvae's fate is unknown. At the end of December the captive population consisted of 106 *S. hineana.* Survivorship was calculated as before and equals 89.9% for 2007 (Table 3).

In 2008 many captive larvae were released in anticipation of high survivorship in the hatchling program. Larvae were also provided for genetic and habitat quality experiments. A new method of measuring larvae was developed and a review of survivorship to date and calculation of growth rate were conducted.

Containers, cages, temperature regime and feeding regime were the same as the standardized methods developed in 2006-2007. Water from the Fish Farm or bottled spring water were used in p-cups in addition to water from habitat areas in Door Co.

A subset of smaller larvae was warmed earlier for a study to determine the feasibility of extracting genetic material from fecal pellets. The 35 *S. hineana* larvae used in this study ranged in head width from 2.25mm to 4.64mm and represented a life stage unlikely to be affected by deviation from normal temperature regime. These larvae were moved to a temperature of 8°C on March 11, 2008 and warmed approximately 2 degrees every week. The genetics results are still being analyzed. As in 2007, the captive program also continued to help the genetic studies by providing exuvia, fecal pellets throughout the year and safe temporary holding of larvae.

During summer 2008, larvae were placed in field cages or held at the University of South Dakota (USD) and the Fish Hatchery facilities in Waterfall Glen Forest Preserve. Modifications made in the past year to the Fish Hatchery facilities made it possible to hold and rear large numbers of larvae with the same standardized protocols as used at USD. The facility in northeast Illinois is a significant asset to the captive rearing program as it allows the safe holding of larvae for genetic and other studies and reduces the danger of deaths during field season.

Thirty three captive *S. hineana* larvae were placed in S-cages in the field between May 30 and June 15, 2008. Twenty of the smaller *S. hineana* larvae were placed in S-cages for an experiment testing habitat quality between streamlets and adjacent larger wetlands in the Mud Lake Wildlife Area. The thirteen additional larvae were placed in S-cages at MLN to document growth rates in high quality habitat. All larvae in S-cages were from the captive population and larvae used in a particular area originated from that same area. All larvae were weighed before, after, and during their time in the field and their condition noted.

We had two deaths and three presumed deaths (either escaped or disappeared) in S-cages in at MLN (Table 2). The 28 surviving S-cage larvae were released at the end of the

summer. Several other larvae from the captive population were released this year: two from Long Run Seep and nine from Lockport Prairie in Illinois; and three from the Mink River Preserve, and 2 from the Mud Lake Wildlife Area, in Wisconsin. Total number of captive larvae returned to their collection locations this year was 44.

In summer 2008, larvae brought back to USD for the captive population were collected from several locations. Sites in Wisconsin contributed 11 larvae: six from Mud Lake North, two larvae from Piel Creek, two from Kellner Fen and one from Cedarburg Bog. Piel creek, Kellner Fen and Cedarburg Bog represent newly confirmed breeding sites and larvae were brought back for collection of fecal pellets for genetic material. The two larvae from Kellner Fen and one from Cedarburg Bog were returned in October. The Piel Creek larvae could not be returned because the site was devoid of water. Sites in Illinois contributed 46 larvae: 44 from Lockport Prairie and two from Long Run Seep. The hatchling rearing was relatively successful. Of the 362 hatched eggs, 104 hatchlings survived through the summer and these were transferred to the captive population.

Of the 57 new captive larvae brought back to USD in the fall, one died (Table 1). Of the original 106 *S. hineana* larvae held captive at the beginning of 2008, 22 died or were presumed dead, 7 emerged, 44 were released and 34 are still part of the captive population. 144 hatchlings were transferred to the captive program in September. At the end of December 2008, the captive population consisted of 190 *S. hineana.* Survivorship of larger larvae in captive program this year was calculated by the same equation used for previous years and equaled 85.9% for 2008 (Table 3).

In 2007-2008, we developed a method for photographing and digitally measuring larvae. This method is an improvement over the use of calipers and microscope because it allow for a permanent record, is likely more accurate and is less risky for the larvae. The accuracy and consistency of the digital method was tested. For a smaller larvae (HW~1.65) measurement range between 5 different pictures was 0.045mm for HW and 0.351mm for TL. For one of the largest larvae, (HW approximately 6.99) measurement range between 5 different pictures was 0.207mm for HW and 1.572mm for TL. For both larvae, the range of measurements was within 3% of the larvae average HW and within 8% of average TL. The range was calculated for 3 separate measurements of the same picture for 10 different larvae from 2.60mm to 6.95mm HW. The range in HW measures was only 1.1% of the average HW and the range of TL measures was 0.7% of average TL. All measurements were done by 1 observer. According to these results, human error in measuring pictures is lower than between picture error. Comparisons between methods for HW of individual larvae show that methods are roughly comparable. Caliper method tended to record lower values than the digital method, especially for F-0 larvae. The variation in the digital method is much lower than that for the caliper method. This is a very positive result and has helped efforts to define size range for larval instars.

Rearing of large pre-emergent and emergent larvae

Larvae were first placed in emergence cages ("E-cages") in spring of 2004. Cages were constructed of short black plastic trash cans (200 x 300 mm on the bottom, 260 x 360 mm on the top, 390 mm tall) with all sides and bottom removed and replaced with 3.0 mm mesh. Cage tops extended about 20-30cm above the water and were covered with hardware cloth screening. Cages were staked in place. Debris and vegetation collected directly from the larval habitat was picked clean of all large predators and placed in the cages to imitate natural conditions and attract prey species into cages. Larvae chosen were those in the last 2 (F-0 and F-1) instars, which meant they were the most likely to emerge in that summer. Emergence cages were place in the field on May 25, 2004. Ten larvae were used in cages, four that had been held over the winter and 6 recently field caught larvae. Larval head width ranged from 5.1mm to 6.9mm, which was later determined represent the last two instars. Only 6 of the 10 larvae emerged in the summer of 2004, despite the fact that the cages were left out until August 4th, 22 days after the last emergence. All the larvae that emerged had begun the summer in their final instar. The four larvae that failed to emerge in 2006 had molted to the final larval instar during the spring or summer. For those *S. hineana* larvae that did emerge, emergence dates of larvae (Table 5) held over the winter corresponded closely to those of larvae collected in the spring. All larvae that attempted to emerge, did so successfully, except for one that emerged during a rain event and had partly deformed wings. The adult was left to rest outside the cage and its exact fate was unknown, it may have been taken by a predator or it may have ultimately flown off. For 2008, emergence success was defined as the number of *S. hineana* larvae emerging at right time of year in a functional state divided by the number of larvae placed in emergence cages. By this measure emergence success in 2004 was 60%, since 4 larvae did not emerge. Adult survivorship was defined as the number of adult deaths, divided by the number of adults that emerged. Adult survivorship in 2004 (Table 6) was 83% if we assume that the one adult that did not fly away when released, had died.

Emergence experiments were continued in 2005. The cage design was the same as in the previous year. All larvae that did not attempt to emerge in 2004 were put out again in 2005. Three of these four larvae emerged in 2005, one disappeared from its cage and was assumed to have died. These experiments showed that larvae may spend an entire year in their final instar (F-0) and that larval growth in the last 2 instars is slow. Nine additional larvae total were placed in emergence cages, 6 that had been held over the winter and 3 that had been collected in that 2005. All larvae emerged successfully; however, two died subsequent to emergence. Emergence dates of *S. hineana* held over the winter and those recently collected were within the same time period and also during a period when adult *S. hineana* were observed flying in the Mud Lake Wildlife Area. For 2005 emergence success was 100%, but adult survivorship was only 88.9%.

In 2006, emergence cage design was changed to "pagoda style" emergence cages (Figure 3) which were constructed from modified Promar® polyethylene small minnow traps, 254mm square by 457mm long. Funnel openings on either end where fish or crayfish would enter were closed with zip ties. Cages were stood on end and held up by a PVC

pipe frame so as to enclose enough area above water for larvae to emerge. Both recently collected and larvae held over the winter that could potentially emerge (instars F-0 and F-1), were placed in these "pagoda" cages.

The captive rearing was successful in that 19 final instar larvae (F-0) held for at least one winter emerged successfully in field enclosures and were released into the wild. Only one larva failed to emerge successfully and died due to a malformed front wing. Again, captive larval emergence exhibited essentially the same temporal distribution as wild-caught larvae, indicating success of the captive program. Emergence of F-0 larvae occurred between June 22nd and July 11th. One wild-caught F-1 larva emerged on July 22, 2006, the day the habitat dried up. This was the only larvae during captive emergence experiments that is known to have begun the summer as F-1 and emerged in the same year. For 2006, emergence success of larvae was 95%, and adult survivorship was 95%.

Larvae in the last two instars were again placed in "pagoda" emergence cages in 2007. Nine F-0 captive larvae, 1 F-1 captive larva and 1 wild-caught F-1 larva were placed in E-Cages in Mud Lake North, WI. Emergence cages were checked daily and any emerged adults immediately released. Emergence of F-0 larvae occurred between June 27 and July 6, 2007. Larval emergence exhibited essentially the same temporal distribution as in previous years and corresponded with the flying period of wild *S. hineana.* Six F-0 larvae emerged successfully, however, three emerged and then died. This yields an apparent emergence success of 66.7%. Only one of the emergent adults had improperly developed wings, the others appeared to have died after falling in the water post-emergence, so actual emergence success was likely closer to 90%. Adult survivorship remained at 66.7%. None of the captive or wild-caught F-1 larvae emerged, and they were brought back into captivity.

In 2008, final instar larvae were placed in "pagoda" emergence cages of the same standardized design previously used. Seven F-0 larvae from the captive population and 2 F-0 larvae collected in 2008 were placed in E-Cages at Mud Lake North (MLN), WI. Emergence cages were checked daily and any emerged adults immediately released.

Emergence of F-0 larvae occurred between July 9 and July 13, 2008. Larval emergence was slightly later this year than in previous years probably due to colder spring temperatures. Emergence did correspond with the flying period of wild *S. hineana*. The seven F-0 larvae emerged successfully and were released, while the two wild caught larvae did not attempt to emerge (one was subsequently released and the other brought back into captivity). In previous years it has been observed that larvae that begin the spring as instar F-1 (penultimate instar) will not emerge into adults that year. These two wild-caught larvae were found late enough in the season that they could have begun the spring as F-1, and therefore the fact that they did not emerge would not be surprising. All larvae known to have begun the spring in the final instar emerged, indicating apparent 100% emergence success. Adult survivorship in 2008 was also 100%

Overall Causes of Mortality

Monitoring causes of deaths in the captive program was important for improving survivorship and early detection of problems. The cause of death is not always readily apparent, but different housing methods and parts of the year have different stressors. Number of deaths during different periods of each year are presented in Figure 6. Cage related deaths and deaths in p-cups during the summer account for most deaths. Most disappearances or deaths in the field occurred when larvae were left in low water conditions. After cage designs were improved there was little chance that larvae escaped, and thus disappearances can be assumed to be deaths in 2007 and 2008. Fluctuating temperatures during transportation and inconsistent feeding regimes might have been a factor in deaths occurring during summer rearing.

Duration of larval stage

The duration of the larval stage in the captive program was calculated by determining the date and size of collection and date of emergence for captive *S. hineana* larvae between 2004 and 2008. A linear regression was run with initial head width as the independent variable and number of winters to emergence as the dependent variable. The regression was significant at $p<0.001$ and the Y-intercept was calculated to be 4.87 winters (Figure 7). Larvae in the collected for this part of the captive program do not include those with a head width less than ~0.9mm, so this time estimate includes the first part of larval life in natural conditions. We do not have enough evidence from sampling to tell precisely how duration of larval stage in captivity compares to that in the wild but this estimate of 4.87 winters to emergence is within the range predicted for wild *S. hineana* larvae (3-5 years to emergence) by Soluk et al 2000.

Discussion and Recommendations:

Egg handling

Egg collecting using egg traps provides a relatively non-destructive method of generating large numbers of *S. hineana* larvae for research, augmentation or reintroduction. One difficulty is that there is currently no easy way to separate eggs of *S. hineana* from those of other *Somatochlora* species, and possibly those of other odonates. Requiring either direct monitoring of the egg traps or hatching and rearing of larvae to an identifiable size. Alternative methods for collecting eggs include inducing females to lay their eggs in containers while holding them. We have used this technique with *Somatochlora* females including *S. hineana* (see above), dying after road injuries; however, results have been mixed. Such a technique might work with healthy females, but this is likely to be extremely stressful.

When eggs have been collected they should be stored at ambient temperature, any extended exposure to cold temperatures might induce premature hatching. Eggs should be cooled to 3-5ºC until hatching is desired. Batches of eggs from the same site should be kept in individual p-cups in order to minimize the possibility of eggs drying between checks. Egg masses should be monitored daily for hatching in fall and once spring warming begins. Hatching appears to begin shortly after the temperature is raised above the 10-14ºC threshold. Egg hatching is somewhat variable so eggs that do not hatch should continue to be monitored until mid-summer. Unhatched eggs should be overwintered and monitored again in subsequent years.

Hatchling rearing

Using individual p-cups has proven to be the most successful technique for rearing S. hineana larvae in the hatchling stage, When a hatchling is found in the egg cup, it should be given its own p-cup, individual specimen number and a piece of mesh nytex cloth. Hatched larvae should be fed approximately 20 brine shrimp daily until reaching approximately 8 weeks old. Because brine shrimp are hatched in salt water, their nauplii should first be rinsed in fresh water before feeding them to dragonflies. Only the nauplii (<24 hrs old) should be used for feeding the smallest hatchlings and any uneaten prey should be removed from the cups during subsequent feedings. Larger hatchlings should be fed very small chironomids at a rate of five chironomids each day. After 4-6 months hatchlings should be large enough (HW>~1.5mm) to be transferred to the mid size larvae rearing methods. Temperature regime should follow that of mid-sized larvae.

Although using individual p-cups has proven to be the most successful technique, it is a very labor intensive technique. The mass rearing method holds some potential as a much less labor intensive method to producing larvae. Each bin should be prepared using habitat water to a depth of approximately 65mm and enough detritus from *S. hineana* habitat should be added to lightly cover the bottom. Detritus should be picked clean of large predators and held at on the same temperature regime as larvae until needed. For final preparation, bins should be held in incubation chambers at 14-16ºC, and initial startup cultures of prey items should be added to the bins before hatchlings are

introduced. Prey used in these initial inoculations could include paramecia, euglena, and small daphnid crustaceans. We added 10 hatchlings simultaneously to each bin. The number was chosen so that larvae would not be overcrowded and potentially resort to cannibalism. Each bin should be fed twice a week, once a week with a chironomid egg mass and once a week with a dose of daphnia culture. Given that increased feeding has helped p-cup hatchlings the amount and frequency of prey added to bins may need to be increased. Water was changed (1/2 each time) weekly and aeration was maintained using individual airstones in each bin. Bins started around March should be picked by the end of September.

Larval rearing

Our standardized protocol allows relatively high survivorship of larvae and successful emergence at the appropriate time of year. Overall, survivorship of larvae handled in the captive program was above 82%. Cage related deaths and deaths in p-cups during the summer accounting for most fatalities. For lab housing, place larvae individually into 100ml plastic urine analysis cups. All larvae should be provided with a long rectangle of plastic screen which extended just above the water's surface. Water should be obtained from *S. hineana* habitats, wells connected to the same groundwater sources, or bottled spring water should be used. Water should be maintained free of debris and feces and at levels between 15mm and 35mm deep (~20-45ml) in the cups depending on size of larva.

Larvae should be held between 3.4-5°C from the middle of December until April 8th. Thereafter, temperature is raised by approximately 2 degrees every week until reaching 14-16°C. These warm summer temperatures should be maintained as consistently as possible. Overheating may cause larval death directly, or may increase larval metabolism using up food resources. Fall cooling begins on September 15th and drops by 2 degrees every 2 weeks until reaching approximately 4°C in the first week of December.

At different points larvae have been reared in complete darkness or with a lighting schedule approximating the natural daylight in Door Co. Wisconsin and northeast Illinois. Larvae have grown and emerged successfully under both conditions.

In fall and spring at temperatures of 5-6°C, feeding 2 prey item 1x per week appears sufficient. At temperatures of 7-9°C, feed 2 prey 2x per week. At temps 10°C to 14°C feed 2 prey every other day, and for temperatures between 14-16°C feed 3-4 prey every other day. The phrase "one prey item" is defined as a suitable prey species of the largest size that a larva will attack. For chironomid larvae this is one larvae that when curled up is approximately the size of the dragonfly larvae's head capsule. Similar size reference can be used for other prey items, i.e. whole body size of prey equals head capsule size of dragonfly. Two smaller prey may be substituted so long as their combined size is that of one prey item.

When larvae are collected they should be given an individual identification number and a record sheet where the larva's number, collection location, date, and larval dimensions are recorded. This sheet should follow the larvae and additional changes in larvae

condition, molts, and fecal collected, and return to habitat or death should be recorded. Maintain appropriate copies of these sheets and data entry should be kept up to date.

Field growth cages (S-cages) may be used during summer months to grow larvae in field conditions. Extreme care should be taken that cages are placed in deep enough water, provided with appropriate vegetation and staked to remain in contact with the substrate to allow larvae to hide and forage as they would naturally. Very consistent and careful monitoring of water level around cages is necessary. Cages should be removed from the habitat when water level is less than 20mm deep in the cage. The caretaker must be aware that although the cage may appear to sufficiently submerged, muck can build up in the cage and significantly reducing water levels. If water conditions are acceptable, cages do not need to be opened more than once every two weeks

Adult emergence

Generally we have had high success with our emergence program especially using the "pagoda" style E-cages. The emergence period for larvae in this study was between June 19th and July 13th which is very similar to the range of emergence dates found in field surveys for *S. hineana* exuviae (Foster and Soluk 2004).

To minimize any possible mortality all F-1 and F-0 individuals should be assumed to have the capability to emerge in any particular year, although this study strongly indicates that only those individuals who start the year as F-0 are likely to emerge. The final instar of *S. hineana* is distinct from preceding instars, recognizable by its dark brown-almost black color and wing pads reaching or passing abdominal segment 6, headwidth greater than 6.0mm and total length greater than >20.0mm. Debris and vegetation should be collected from the habitat where the E-cages will be placed, picked clean of large predators, and placed in the bottoms of cages to attract prey and provide hiding places for *S. hineana* larvae. Cages should be staked so that the bottom is in close contact with substrate. Cages probably should not be in direct sunlight.

Emergence cages need to be checked daily between 9am and 11am. Any adults that are ready to fly should be immediately released. If the emerged adult has not hardened and cannot fly, then the handler should wait until it is ready. On rainy days it is probably better to get there early and also to look directly into each cage to see if the adult has fallen into the water. Adults may be rescued from water successfully if they are not left there too long.

Conclusions:

This study has been successful in establishing some clear protocols for successfully rearing the larval stages of the endangered Hine's emerald dragonfly from egg to adult. Interestingly, this study has also provided significant insights into the life history and ecology of this species. These insights and the techniques developed will greatly facilitate possible augmentation of threatened populations, and will make reintroductions of *S. hineana* into areas where it has been extirpated, a very viable option. This research is still ongoing and we expect to improve the rearing procedures especially with respect to shortening the larval period. We also expect to develop habitat evaluation procedures with caged larvae that will greatly improve our ability to assess habitat quality and suitability without the need for destructive sampling of habitats.

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Table 1: Collection location and number of larvae added to captive program each year.

Site or System	State	2003	2004	2005	2006	2007	2008
Mud Lake North Wildlife Area	WI	33	36	27	27	45	6
Cedarburg Bog	WI	0	0	0	7	0	1
Mink River Preserve	WI	0	0	0	12	0	0
Piel Creek	WI	0	0	0	0	0	2
Kelner Fen	WI	0	0	0	0	0	2
Lockport Prairie	IL	0	7	0	22	2	44
Long Run Seep	IL	0	0	0	3	0	2
Waterfall Glen	IL	0	0	0	1	0	0
Hatchling rearing	WI	0	0	0	0	10	104

Table 2: Number of *S. hineana* larvae handled each year and their fates.

year	# Jan-1	Released	emerged	Emerged but died	Deaths	Disappeared	Number added	Number Dec-31
2003	0	0	0	0	0	0	38	38
2004	33	7	6	0	3	0	43	60
2005	60	0	9	2	10	5	27	63
2006	63	0	19	1	6	1	65	102
2007	102	28	9	3	15	1	57	106
2008	106	47	7	0	20	3	161	190

Table 3: Captive rearing larval survivorship calculations for each year.

year	Total larvae handled	Total larval deaths	Larval survivorship
2003	38	0	100.0
2004	76	3	96.1
2005	87	15	82.8
2006	128	7	94.5
2007	159	16	89.9
2008	163	23	85.9

Table 4: Results for individual factors in ANOVA explaining % change in head width by feeding method (Food) and whether larvae were recently collected of had been held over the winter (Status).

Source	DF	Type III SS	Mean Square	F-Value	p-value
Food	1	0.31350586	0.31350586	21.60	<0.001
Status	1	0.03722280	0.03722280	2.56	0.116
Food*Status	1	0.01101214	0.01101214	0.76	0.388

Table 5: Emergence dates of recently collected and larvae held over at least one winter in the captive rearing program.

	First emergence	**Last emergence**
Captive 2004	7/11/2004	7/12/2004
Field collected 2004	6/29/2004	7/13/2004
Captive 2005	6/19/2005	7/3/2005
Field collected 2005	6/23/2005	6/29/2005
Captive 2006	6/24/2006	7/11/2006
Field collected 2006	6/22/2006	7/6/2006
Captive 2007	6/27/2007	7/13/2007
Captive 2008	7/9/2008	7/13/2008

Table 6: Captive Rearing emergent adult survivorship calculations for each year.

Year	Total adults handled	Total adult deaths	Adult survivorship
2003	0	0	
2004	6	0	100.00
2005	9	2	77.78
2006	19	1	94.74
2007	9	3	66.67
2008	7	0	100.00

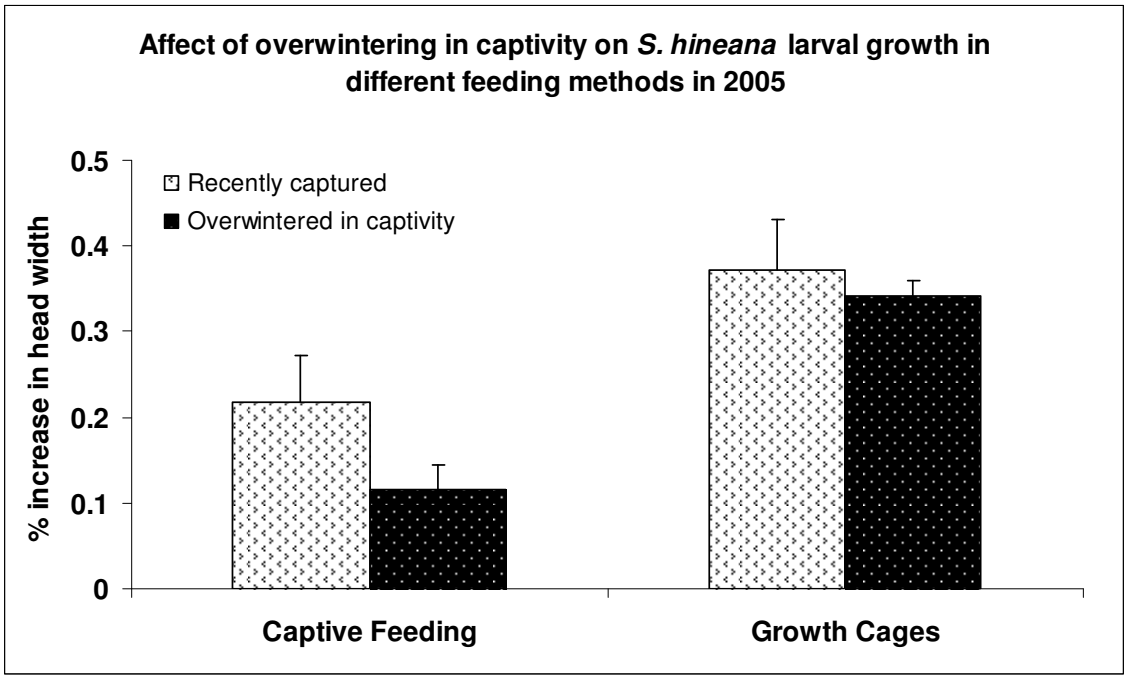

Figure 1: Comparison of growth rate of larvae in field cages and in the laboratory. Larvae that had overwintered and larvae recently collected from the field were used in both treatments. Bars indicate standard error. Larvae in each group were of comparable size.

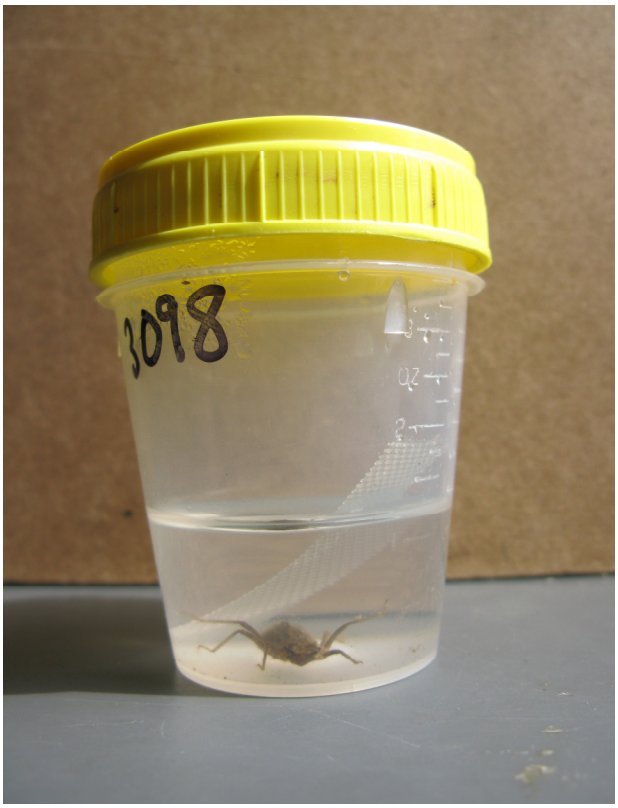

Figure 2: Standardized individual p-cup container used to house larvae at university laboratory and field station facilities.

Figure 3: Standardized small growth ("S-cages") (left photo) and emergence cage ("E-cages") (right photo) designs for housing larvae in the field, developed in 2006.

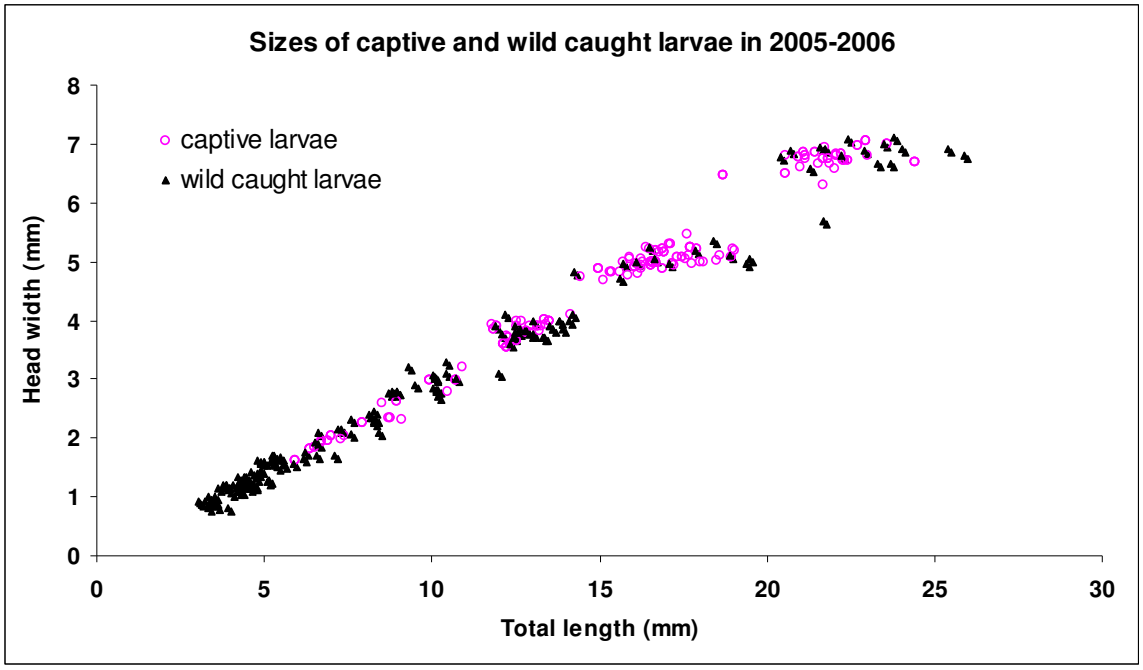

Figure 4: Sizes of *S. hineana* larvae held in captivity or collected from the wild in 2005and 2006 were approximately equal.

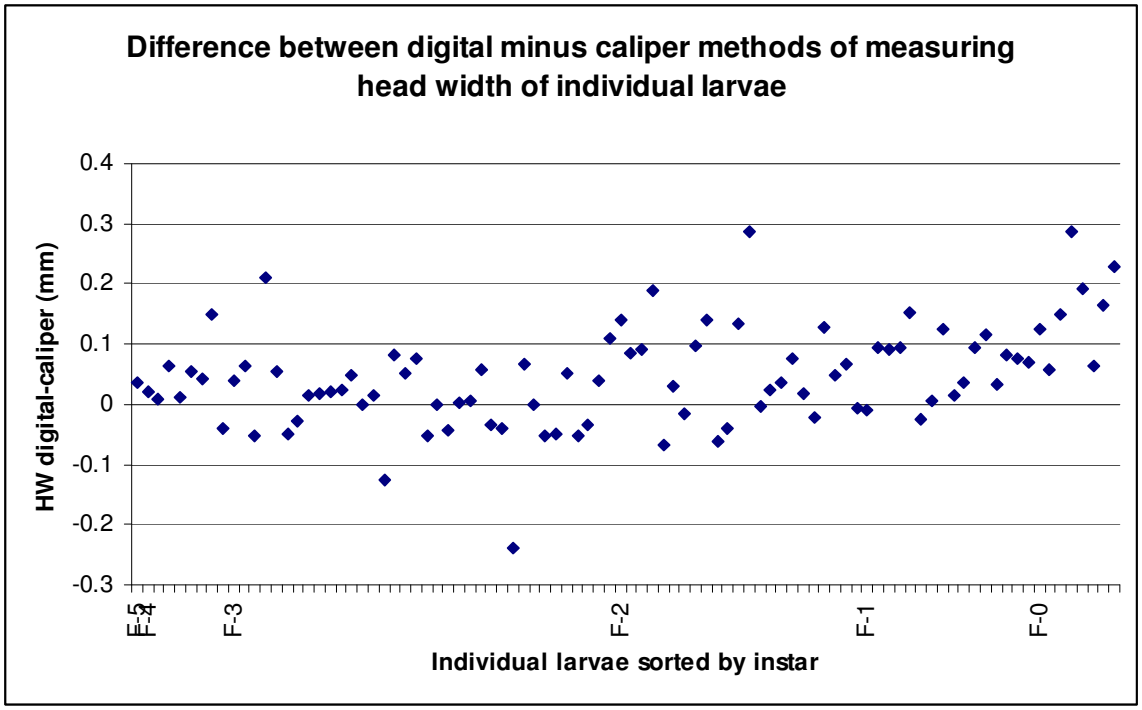

Figure 5: Comparison of individual larvae measurements made with caliper method and new digital method in winter 2007-2008.

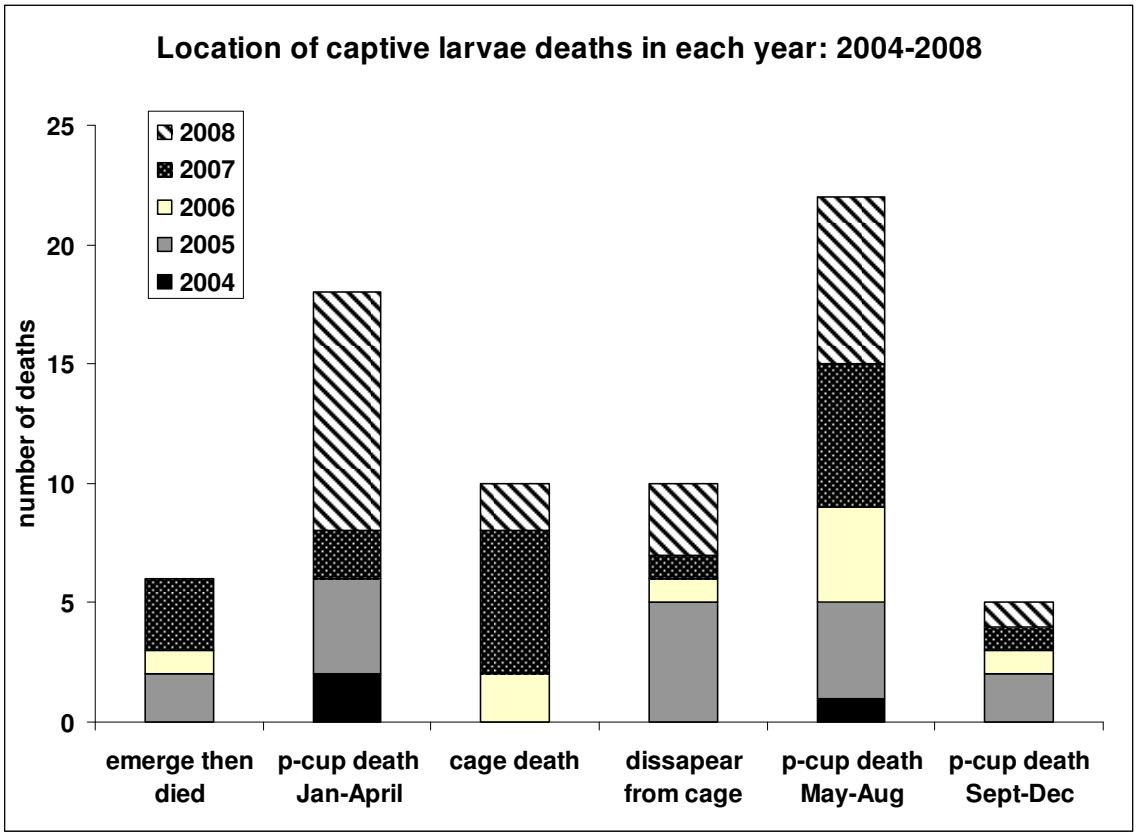

Figure 6: Number and location of larval deaths in the captive program. Cage related deaths and deaths in p-cups during the summer account for most deaths. After cage designs were standardized there is little chance that larvae escaped and disappearances can be assumed to be deaths in 2007, 2008.

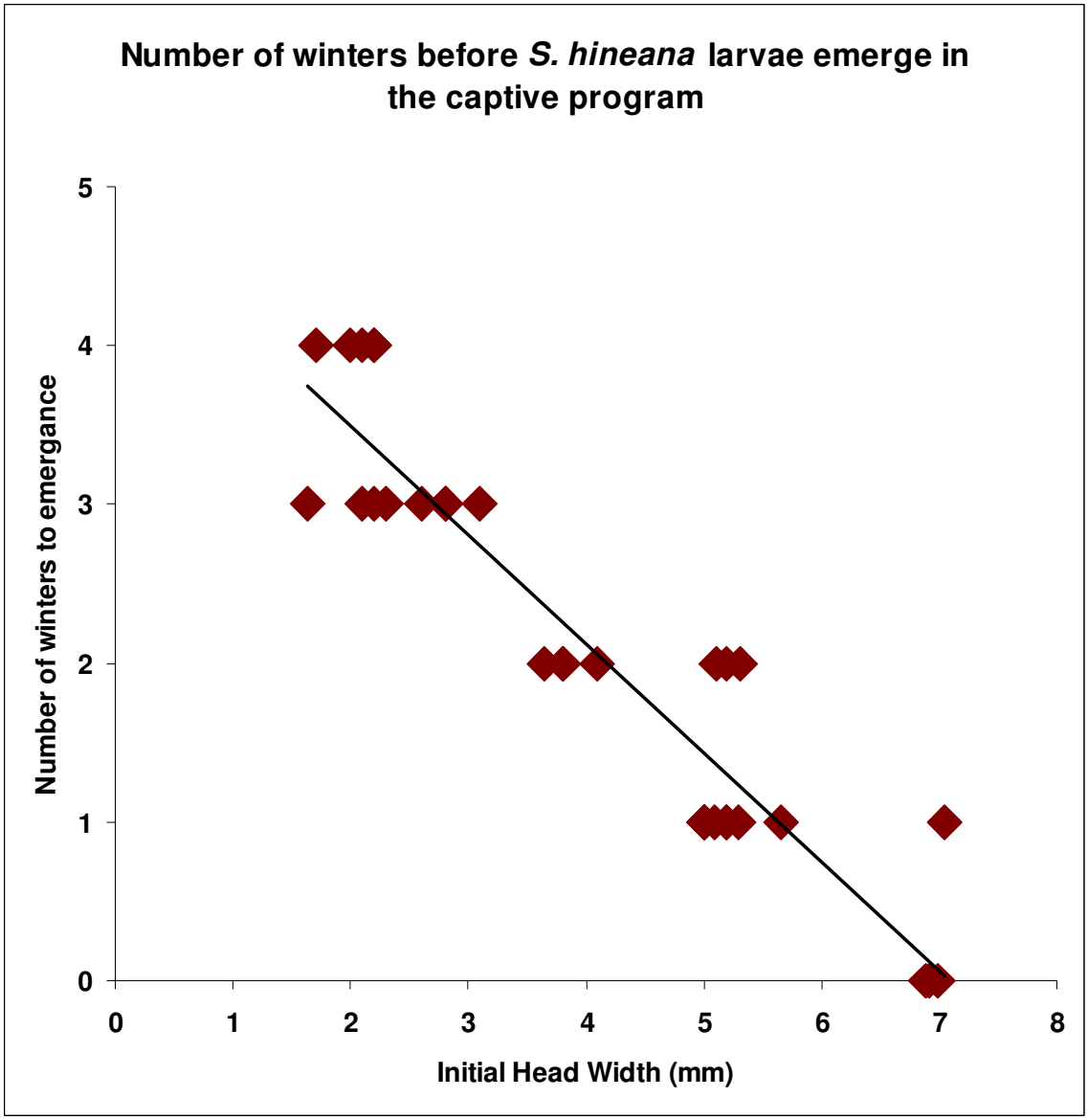

Figure 7: Initial HW at time of collection and number of winters larvae spent in captive program before emerging. SAS places the y-intercept at 4.87 winters. Larvae with HW 1.8 collected in May are likely young of the previous year.