

## Research Article

# Laboratory Rearing of *Laricobius nigrinus* (Coleoptera: Derodontidae): A Predator of the Hemlock Woolly Adelgid (Hemiptera: Adelgidae)

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Coleopteran species are biological control agents of numerous invasive pests. *Laricobius nigrinus* (Coleoptera: Derodontidae), a predaceous, univoltine species, spends the summer aestivating but is active for the rest of the year. *Laricobius nigrinus* possesses many essential attributes for effective biological control of the hemlock woolly adelgid (Hemiptera: Adelgidae). The predator must be reared in large numbers for field releases. We describe some of the studies that led to the successful procedures currently used for mass rearing *L. nigrinus*.

## 1. Introduction

*Laricobius nigrinus* Fender (Coleoptera: Derodontidae) is a potential biological control agent of hemlock woolly adelgid (HWA), *Adelges tsugae* Annand (Hemiptera: Adelgidae), an exotic pest that attacks and kills hemlock trees (*Tsuga canadensis* L. (Carr.) and *T. caroliniana* Engelm.) in the eastern United States. Since its first release in 2003 [1], *L. nigrinus* has been established in the plant hardiness zones 7a, 6b, and 6a in the USA [2].

The ability to mass rear a biological control agent is fundamental in any classical biological control program. Delays in the program are often related to difficulties in laboratory rearing [3]. Biological control of HWA was hindered by poor success in mass rearing promising predators. Efficient rearing methods for producing large numbers of *L. nigrinus* is critical for it to be a viable biological control agent of HWA. HWA infests eastern hemlock in over 40% of its geographic range [4] and continues to spread [5], causing extensive damage and mortality of *Tsuga* spp. in the eastern USA [6, 7].

Laboratory rearing of *L. nigrinus* at Virginia Tech was initially constrained by high mortality rates and a lack of knowledge as to which life stages incur significant mortality because *L. nigrinus* has an obscure and complicated lifecycle.

Additionally, adults have no observable sexual dimorphism [8]. They emerge from the soil in the fall and feed on developing HWA nymphs throughout the winter [9]. In early spring, eggs are laid in adelgid ovisacs where the larvae hatch and develop through four instars feeding on HWA eggs [10]. Mature larvae drop to the soil, each forms a pupal cell, pupates, and enters aestivation as an adult for the summer.

*1.1. The Sequential Development of a Rearing Procedure from 2000 to 2010.* Initially, adults of predators were reared on HWA-infested twigs maintained in flat-bottom Plexiglass oviposition cages containing a layer of peat moss. Fourth instars, upon reaching maturity, dropped down to the layer of peat moss on the floor of the cage, where they pupated and developed into adults.

Two primary objectives that resulted from our initial rearing efforts were to

- (i) Identify the life stages of *L. nigrinus* that incur high developmental mortality in the laboratory,
- (ii) Improve production at each life stage where survival was low.

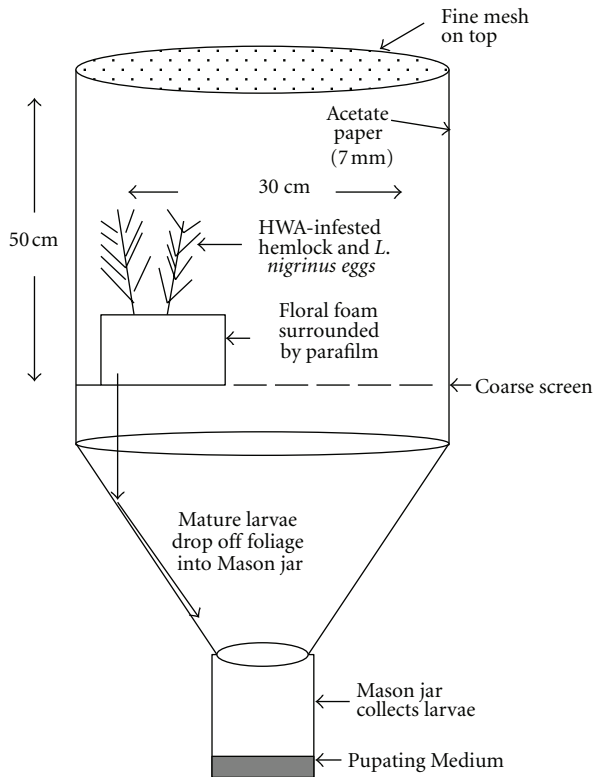


FIGURE 1: Diagram of the form and function of the *Laricobius nigrinus* larval rearing cage.

## 2. Methods and Materials

Studies were partitioned into two periods. Efforts focused on determining/enhancing survival of each life stage from 2000 to 2004. Emphasis on improving production numbers of *L. nigrinus* was the focus from 2005 to 2010.

### 2.1. Determining the Survival of Each Life Stage: 2000–2004.

A rearing cage was designed to intercept mature larvae as they dropped from the hemlock foliage (Figure 1). The top section of the cage is an open-ended 30 cm diameter Duralar acetate cylinder (0.018 cm thick) (Grafix, Cleveland, OH) with the top end covered with PeCap polyester mesh (0.14 mm<sup>2</sup>) (Sefar America Inc., Kansas City, MO). The base of the cage is a nonswirl galvanized steel funnel (McMaster-Carr Co., Atlanta, GA) with a top diameter of 30 cm in which the acetate cylinder is placed. Hardware cloth (5 × 5 mm<sup>2</sup> mesh size) is cut to fit the inside of the funnels. The hardware cloth is placed inside the funnel base as a coarse screen, resting where the funnel constricts. HWA-infested hemlock twigs with *L. nigrinus* eggs are inserted into water-saturated (Oasis Deluxe) floral foam blocks (8 × 10 × 3 cm<sup>3</sup> bricks). Each block is wrapped in Parafilm M (Fisher Scientific, Hampton NH) to retain moisture and prevent prepupae in search of a pupation site from entering the blocks.

Floral foam blocks containing hemlock branches with *L. nigrinus* eggs are placed on the hardware cloth within the funnel cages. When eggs hatch, the larvae develop in the

hemlock foliage, feeding on HWA eggs within the funnel cages. Mature larvae drop from the foliage and accumulate in the (Kerr) 8 oz. mason jars (Jarden Home Brands, Muncie, IN) attached beneath the funnel. The jars are spray-painted black and the covers cut open. The jar cover rims are attached to the bottom of the funnels with glue; this way mason jars can be easily removed and reattached. Two teaspoons of steam-sterilized peat moss and 2 pieces of moistened filter paper (Whatman No. 1: 35 mm diam.) are placed in the mason jars three weeks after adding *L. nigrinus* eggs to the cage. Larvae having ingested a sufficient amount of HWA eggs drop from the hemlock foliage in search of a pupation site. The Mason jars are checked daily and mature larvae counted.

Funnel cages were set up in custom-built racks and held in concrete block rooms maintained at 13° ± 2°C with fluorescent lights on timers programmed to provide a photoperiod that mimicked natural conditions (increasing day length from 12 to 14 h light over spring). A detailed description of the recommended rearing procedures during these months is provided by Lamb et al. [11].

### 2.2. Enhancing Survival for Each Life Stage

#### 2.2.1. Feeding Phase: Adults and Developing Larvae from October to June

(1) *Adult Survival at 4° and 13°C after Emergence from Aestivation.* Early emergence of adults had been a persistent issue, because of limited available HWA prey, resulting in high mortality. Techniques to minimize early emergence of aestivating *L. nigrinus* adults were developed by Lamb et al. [12], but some beetles continue to emerge before HWA break aestivation, making survival of early emerging beetles a continuing issue.

In 2002, in anticipation of early predator emergence, eggs laid by sistentes were stored in a refrigerator at 4°C from April to August. As adults emerged from aestivation before HWA, 70 of them were transferred to feeding containers with hemlock branches infested with aestivating HWA nymphs and one cold-stored branch with developing progredientes. This was not an ideal number of preys for the 70 adult beetles, but additional food was not available for the early emerging adults. There was sufficient HWA prey for only eight containers. All adults that emerged early (before HWA break aestivation) were fed every 12 days throughout September with HWA. One half of the containers was stored in environmental chambers at 4°C 12:12 (L:D) h, and the other half in a cold room at 13°C 12:12 (L:D) h. The number of adults surviving at each feeding period was recorded.

(2) *Adult Sex Ratio within the Colony.* Feeding containers were randomly selected from the colony, and individual adults were placed in separate 384 mL, clear plastic containers with a heavily HWA-infested hemlock twig (20–30 cm total linear length). After three days, each twig was examined for eggs with a microscope. Adults that did not lay eggs were

returned to separate containers with fresh host material and reexamined after another three days. Adults that still did not oviposit were considered to be males or unfertile females. In March and April 2003, adults from 21 containers were dissected and sexed ( $n = 699$ ).

(3) *Effect of Predator Egg Density on Survivorship to Mature Larvae.* A randomized block design experiment was set up with five densities of *L. nigrinus* eggs (10, 20, 30, 40, or 50) in funnel cages with an adequate amount of HWA prey. The five densities formed a block and two blocks were arranged in 10 funnel cages. There were two replicates with a total of 20 cages. The number of larvae that reached maturity and dropped to the mason jar below each funnel was counted. A one-way analysis of variance was used to determine whether egg density influenced larval survival ( $n = 20$ ).

(4) *Survival of "Immature" Larvae.* Immature larvae frequently drop early into collecting jars. They are usually smaller, darker in color, and less mobile than mature larvae and often still have white wool attached to their dorsal side. These larvae will search for prey if transferred back to a hemlock branch rather than drop off the branch as mature ones do. Immature larvae found in mason jars throughout the spring of 2002 and 2003 were transferred to fresh hemlock branches with HWA ovisacs and placed in one of nine "immature" funnel cages. The number of these larvae reaching maturity from each funnel cage was recorded and the overall survival rate calculated.

The mature larvae collected from the "immature" funnel cages were transferred to corresponding soil containers. The number of adults emerging from these containers was recorded and the emergence rate of adults that had left the hemlock foliage prematurely as immature larvae was calculated.

**2.2.2. Nonfeeding Phase.** In nature, mature larvae burrow into the soil immediately upon dropping from the hemlock foliage. In the lab, these larvae are transferred from the mason jars below the funnel cages to containers for pupation, eclosion, and adult aestivation. These containers have two layers of filter paper as a base lining to prevent pooling when the pupation medium is moistened with methyl paraben solution (0.42 g/250 mL distilled water) throughout the summer to inhibit fungal growth. To each pupation container is added at least 5 cm of pupating medium consisting of an equal mixture of peat moss (Premiere Horticulture Inc., Quakertown, PA), sphagnum moss (Mosser Lee Long Fiber, Westsel Inc., Harrisonburg, VA), and sand (Quikrete Play Sand, The Quikrete Product Line, Atlanta, GA). Peat moss is sifted through hardware cloth ( $3 \times 3 \text{ mm}^2$ ), and sphagnum moss is ground in an industrial blender and sifted through hardware cloth. The mixture is moistened and steam-sterilized twice for 12 h, separated by 24 h at room temperature, and placed in plastic pupation containers that have at least one polyester mesh-covered hole for ventilation. Larvae burrow into the pupation medium, create a cell

within the soil, assume a c-shaped position in the cell, and develop into pupae in approximately 14 days.

Pupation containers are kept at  $15^\circ \pm 2^\circ \text{C}$  and 12:12 (L:D) photoperiod for optimal pupal development [13]. Each container is maintained at ~30% saturation, receiving ~5–8 squirts of methyl paraben solution weekly. Pupation lasts approximately 14 days, and the newly eclosed adults remain under the soil surface, in aestival diapause, throughout the summer. This new generation of adults begins emerging from the soil in early fall. The pupation containers are checked daily for emerging adults over a period of several months (August–December). Emerging adults are transferred to adult containers.

(1) *Prepupal Survival, Pupal Sex Ratio, and Adult Emergence from Aestivation.* As sex cannot be determined in the adult stage because genitalia retract into the body after eclosion [14], sex ratio of progeny was obtained by microscopic examination of the external genitalia characters of pupae [15]. In spring 2003, six soil containers were randomly selected from the colony three weeks after larvae entered the soil. Using a paintbrush, the soil was sifted and each pupa sexed and transferred to a corresponding male or female container with fresh pupation medium. These containers were maintained with the rest of the colony in the cold room ( $15^\circ \pm 2^\circ \text{C}$  and 14:10 (L:D) photoperiod, watered weekly) throughout the summer. Adults emerging from each container in the fall were recorded daily.

In spring 2004, 28 soil containers were selected to assess pupal survival and sex ratio. During this year, there was considerably more mold development in soil containers than in previous years even though the same methods were used for preparing the pupation medium. Soil containers with high levels of mold (present on entire surface and throughout the pupation medium), medium (present on entire surface only) and low mold contamination (present on less than half the surface) were selected three weeks after larvae had entered the soil. For each container, the soil was sifted through and surviving pupae sexed and transferred to corresponding male and female containers with fresh pupation medium. These containers were maintained with the rest of the colony at  $15^\circ \pm 2^\circ \text{C}$ , 12:12 (L:D) photoperiod until adult eclosion,  $19^\circ \pm 2^\circ \text{C}$ , 16:8 (L:D) h until 27 September 2004, and then decreased to  $13^\circ \pm 2^\circ \text{C}$ , 10:14 (L:D) photoperiod until emergence from the pupation medium [12]. Each container was watered weekly throughout the study period and the adults emerging from each container in the fall were recorded.

(2) *Effect of Abiotic Factors on Pupal Survival/Adult Emergence from Aestivation*

(a) *Soil Moisture and Disturbance.* Ten mature larvae were placed in each of 48 clear polystyrene containers (950 mL) that has an 8 cm diameter ventilation hole in the lid and 2 layers of filter paper moistened with methyl paraben solution. Pupation medium was added to a height of 2 cm

in each container (3:2 mixture of potting soil:peat moss) (Miracle-Gro, Scotts Company, Marysville, OH) maintained at one of three moisture levels (% saturation): high (35–45%), medium (20–25%), or low (5–10%). A Lincoln soil moisture meter (Forestry Suppliers Inc., Jackson, MS) was used to measure the relative soil moisture level in control containers (set up at same time with no larvae added) every other day throughout the study. Moisture levels were maintained by adding the same amount of water to test containers as the control containers.

For each moisture level, half of the containers was randomly selected and disturbed by sifting the pupation medium and counting the number of live individuals. The soil was sifted twice, three and six weeks following larval entry into the soil to determine survival to the pupal stage. Surviving individuals were put back in the soil in the container, and the total number of adults emerging from each container in the fall was recorded daily. This experiment was set up as a randomized complete block design with larval cohort serving as blocks (8). The effects of moisture and disturbance on pupal survival and adult emergence were determined with a 2-factor ANOVA using *proc glm* in SAS; means were separated using Fishers LSD ( $n = 48$ ).

(b) *Pupation Medium and Moisture Level.* Four types of media were maintained at three moisture levels (12 treatments) during *L. nigrinus* pupation and aestivation to determine optimal conditions for survivorship at the pupal and adult stages. The experiment was set up as a generalized randomized block design with eight replicates in each of four blocks with larval cohorts serving as the blocks. The four soil types varied in concentrations of ground sphagnum moss, peat moss, and sand (3:0:1, 2:1:1, 1:2:1, 0:3:1 (sphagnum:peat:sand)).

For each block, 96 plastic containers (384 mL) with ventilated lids (5 cm diam.) were set up with two layers of moistened filter paper and 4 cm of pupation medium. Five mature larvae were added to each container. This process was repeated four times with a total of 20 genetically diverse larvae in each container. A third of each soil type was maintained at one of the following moisture levels: 30, 45, and 60% saturation. A control container representing each of the 12 treatment combinations was set up at the same time as each block. Moisture level of control containers was measured each week using a Lincoln soil moisture meter. The same amount of methyl paraben solution (same weight) was added to each treatment and control container.

To estimate survival through pupation, one container from each treatment was randomly selected eight weeks after larval entry into the soil and the media were scooped out of containers and combed thoroughly for recently eclosed adults under the microscope. Survivorship and approximate depth were recorded for each recovered adult. Total number of adults and time of emergence for each container were recorded daily from July 22 to December 11, 2002. The proportion of adults emerging and average time of emergence were compared across treatments using a 2-factor ANOVA in SAS, and means were separated using Fishers LSD ( $P < 0.05$ ).

(c) *Optimal Density per Pupation Container.* Three levels of larval density per container were tested using a generalized randomized block design with larval cohort serving as the block. For each block, 13 plastic containers (950 mL low density polyethylene) were set up with 5 cm of pupation medium (2:2:1 sphagnum:peat:sand) and two layers of filter paper. Four replicates of five, 10, or 15 mature larvae were added to each container; the 13th container served as a moisture control. This was repeated six times to produce densities of 120, 240, and 360 individuals/larval density. In all, five blocks were set up on five consecutive days in May. Pupation medium was maintained weekly at 45% saturation by monitoring and manipulating the moisture level of the control containers using the Lincoln soil moisture meter and distilled water. Equal volumes of methyl paraben solution were added to the test containers as in the control containers using a balance scale. Adult emergence and duration of aestivation were determined for each container and compared across treatments using a 1-way ANOVA in SAS ( $P < 0.05$ ).

(d) *Assessing Importance of Sterilized Soil.* Sterilized soil showed increasing levels of mold in 2004 and 2005. An experiment to test the effects of soil type (soil mix versus forest soil) and sterilization using an autoclave was initiated in May 2006. Thirty mature *L. nigrinus* larvae were placed in each square container with either soil mix or forest soil that had been autoclaved or left unsterilized. Five containers were used for each treatment ( $n = 5$ ). Soil moisture was maintained at 20% to avoid excessive mold. Containers were kept at 15°C until beetles had entered the adult stage and then maintained at 19°C. Percentage emergence data were analyzed as a one-way CRD ANOVA using SAS with arcsine-square root transformation to stabilize variances.

2.3. *Rearing Procedures to Increase Production Numbers of L. nigrinus: 2005–2010.* The experimental studies from 2000 to 2004 improved rearing practices for 2005 to 2010. This included selection of the appropriate temperature, sex ratio, predator egg density, size of rearing containers, pupation medium, density of number of beetles, and abiotic factors that influence survival to adult emergence. Inclusion of field-collected beetles in the founding colony is important. Beginning in 2005, collections of adults from field populations in western USA were carried out annually and included into the rearing colony to provide hybrid vigor.

### 3. Results

3.1. *Survival of Each Life Stage: 2000–2004.* The numbers of larvae that reached maturity, pupating, and aestivating adults emerging were obtained for the first time in 2001. The colony began with 350 field-collected adults that produced 7,500 larvae, of which 69% pupated. Adults in aestivation and immediately following emergence from aestivation suffered high mortality, with 200 adults surviving in the fall (Table 1). In 2002, 1,000 field-collected adults added to the founding colony improved larval production that was much higher

TABLE 1: Survival of *Laricobius nigrinus* (Coleoptera: Derodontidae) at different life stages from laboratory rearing efforts between 2000 and 2004.

Life stage	Total number of individuals and mortality rate (%) per life stage				
	2000	2001	2002	2003	2004
Reproductive adults (starting colony)	200 <sup>F</sup>	350 <sup>F</sup>	100 <sup>L</sup> 1,000 <sup>F</sup>	3,000 <sup>L</sup>	7,000 <sup>L</sup> 660 <sup>F</sup>
Mature larvae drop from foliage	N/A	7,500 (28%)	37000 (~30%)	30,000 (30+%)	27,000 (30+%)
Pupae	N/A	5,175 (31%)	25,900 (27%)	12,300 (41%)	7,000 <sup>Mf</sup> + 2,200 <sup>M</sup> (43%), (94%)
Adults emerging from aestivation	30 (85%)	1,867 (36%)	21,000 (19%)	13,000 (6%)	8,000 (13%)
Adults surviving as HWA breaks aestivation in October	8 (74%)	200 (89%)	3,700 (83%)	12,000 (8%)	8,000 (0%)

<sup>F</sup> Adults collected in the field from western hemlock trees in Victoria, British Columbia.

<sup>L</sup> Adults reared in the laboratory at Virginia Tech.

<sup>Mf</sup> In soil containers that were free of mold contamination.

<sup>M</sup> In soil containers contaminated with mold.

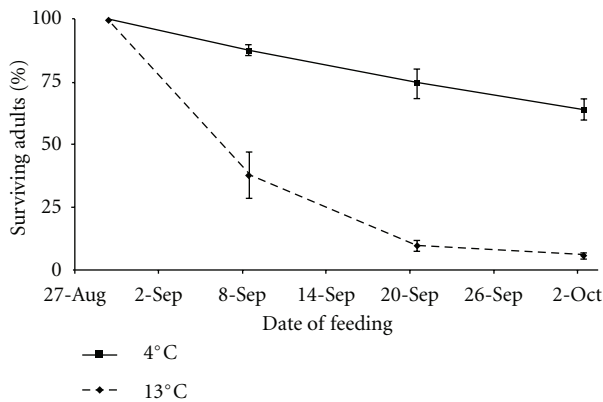


FIGURE 2: Mean percentage ( $\pm$ S.E.) of the original adults surviving at each feeding period maintained at 13° and 4°C in the weeks following emergence in 2003.

than in 2003 where only lab-reared beetles were used. Pupal survival was greater as well in 2002 compared with 2003 and 2004. Considerable pupal mortality in 2004 was likely attributed to contamination of the soil by mold.

### 3.2. Developing Rearing Procedures for Each Life Stage

#### 3.2.1. Feeding Phase

(1) *Adult Survival at 4° and 13°C after Emergence from Aestivation.* Adult survival was higher and more consistent following emergence from aestivation when held at 4°C than at 13°C (Figure 2). Mortality rate was high during the first 12 days when over 60% of adults stored at 13°C and 13% at 4°C died. On October 2, after three feedings, 5.7% of the adults held at 13°C and 64.2% of those at 4°C were still alive.

(2) *Adult Sex Ratio within the Colony.* Of the 699 adults sexed, 458 were ovipositing females. Mean female-to-male ratio ( $\bar{X} \pm$  S.D.) within a container during the peak oviposition period in March/April was 1.91 ( $\pm$ 0.18) : 1.

(3) *Effect of Predator Egg Density on Larval Survival.* Density of eggs per funnel cage (up to 50 individuals per cage) did not affect larval survival ( $F_{4,19} = 0.90$ ,  $P = 0.490$ ). Mean percentage of eggs ( $\bar{X} \pm$  S.D.) that hatched and newly eclosed first instar reaching larval maturity was  $73.7 \pm 15.4\%$ , indicating that higher densities of eggs can be used in funnels to maximize larval production.

(4) *Survival of “Immature” Larvae.* There were 6,116 immature larvae recovered in mason jars throughout the spring of 2002. Of these, 3,486 (57%) reached maturity after being transferred back to funnel cages with prey and completed larval development.

In the spring of 2003, 8,002 immature larvae were collected from the mason jars and transferred back to funnel cages to complete development. Of these, 3,905 larvae (48.8%) completed development, entering the soil for pupation and aestivation. In fall, 1,843 of these individuals emerged from aestivation, representing 23% of immature larvae reaching adulthood.

#### 3.2.2. Nonfeeding Phase

(1) *Prepupal Survival, Pupal Sex Ratio, and Timing of Adult Emergence from Aestivation.* In fall 2003, the mean percentage of larvae ( $\bar{X} \pm$  S.D.) that developed into pupae per soil container was  $58.7 \pm 18.2\%$ . Of the 183 pupae sexed, 95 were female and 88 were male, with sex ratio ( $\bar{X} \pm$  S.D.) of 1.08 : 1  $\pm$  0.51 : 1 F : M per adult container. Females and males had similar survival throughout aestivation (64.2 and 64.7%, resp.); however, males emerged earlier than females (Figure 3(a)).

In 2004, of the 933 pupae sexed, 515 were female and 418 were male. Ratio of female to male pupae per container was 1.19 : 1  $\pm$  0.74 : 1. Survival of males through aestivation (40.9%) was higher than females (35.7%), but the time at which they emerged was better synchronized than the previous year (Figure 3(b)). Different emergence between years is attributed to a change in storage conditions; in 2004, adults were maintained at a higher temperature (19°C) throughout the summer, based on findings reported in [12].

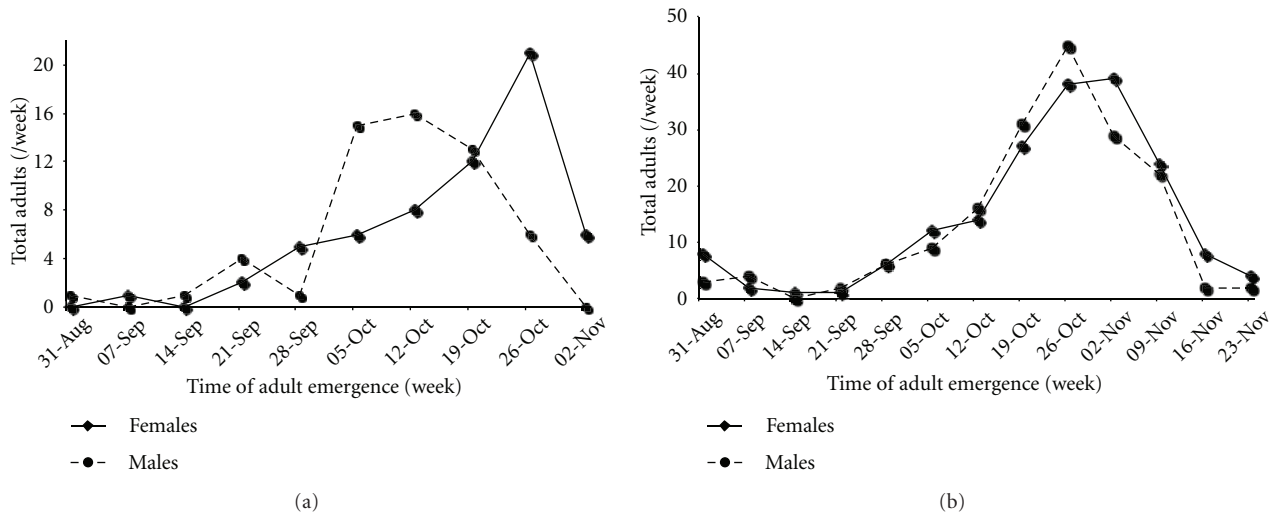


FIGURE 3: Weekly total number of female (solid line) and male (broken line) adults emerging from aestivation in fall 2003 (a) and 2004 (b). Note difference in scale: 2003 from 238 pupae sexed; 2004 from 933 pupae sexed.

## (2) Effect of Abiotic Factors on Pupal Survival/Adult Emergence from Aestivation

(a) *Soil Moisture and Disturbance on Pupal Survival.* Moisture level did not affect the development of pupae ( $F_{(1, 23)} = 0.03, P = 0.9709$ ). The mean percentage of larvae ( $\bar{X} \pm \text{S.E.}$ ) developing into pupae at all moisture levels was  $69.1 \pm 2.3\%$ . However, the number of adults emerging from aestivation in the fall was affected by moisture level ( $F_{(2, 47)} = 6.02, P = 0.0050$ ) and by disturbance of pupae ( $F_{(1, 47)} = 4.08, P < 0.0498$ ). More adults emerged from containers with 40% or 20% moisture than at 5% (Figure 4). Disturbance of the soil to recover individuals lowered adult emergence by 10%. Mean emergence ( $\bar{X} \pm \text{S.E.}$ ) of undisturbed pupae emerging as adults was  $49 \pm 2.3\%$  compared with  $39 \pm 3.0\%$  for disturbed pupae.

(b) *Pupation Medium and Moisture Level.* Mean pupal survival was  $72.1 \pm 3.5\%$  ( $\bar{X} \pm \text{S.E.}, n = 20$ ). It was not affected by the composition of medium ( $F_{(2, 6)} = 1.19, P = 0.348$ ) nor the moisture level within the range of 30–60% ( $F_{(3, 6)} = 0.329, P = 0.804$ ). However, the number of adults emerging from aestivation was lower for individuals held in pure peat moss than those in pure sphagnum moss or the 1:2 sphagnum:peat mixture (Figure 5). The latter mixture had the highest mean emergence ( $61.5 \pm 0.8\%$ ) and the lowest was from containers with pure peat moss ( $52.8 \pm 1.1\%$ ). Soil moisture did affect the percentage of adults emerging from aestivation (Figure 4, 2nd experiment). Beetles stored in soil with 30% moisture level emerged in greater numbers than those held at higher moisture levels.

The timing of emergence from diapause was not affected by soil type ( $F_{(3, 284)} = 0.30, P = 0.822$ ). Adults remained in the ground for  $123.9 \pm 0.25$  days. Moisture level affected

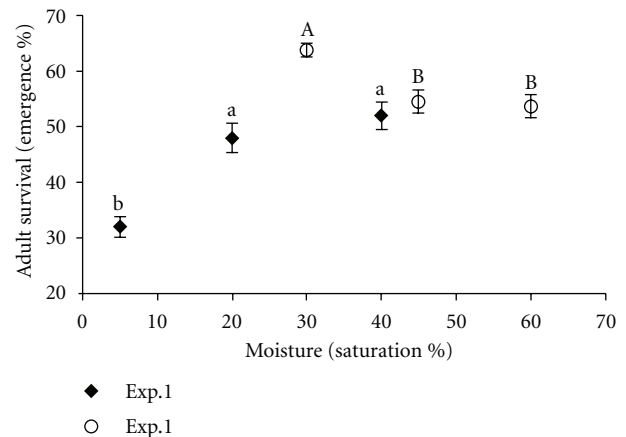


FIGURE 4: Mean percentage ( $\pm \text{S.E.}$ ) of adults emerging when maintained at 5, 20, and 40% soil saturation in Exp. 1 and at 30, 45, and 60% soil saturation in Exp. 2. Means with different lower case letters are significantly different (Exp. 1,  $n = 24$ ) and means with different upper case letters are significantly different (Exp. 2,  $n = 372$ ).

time of emergence as adults in soil maintained at 30 or 45% saturation emerged before those held at 60% saturation ( $F_{(11, 284)} = 22.51, P < 0.0001$ ). However, mean duration of aestivation of adults stored at 60% moisture level ( $126.4 \pm 0.24$ ) was only four days longer than the duration at lower moisture levels ( $122.6 \pm 0.25$ ).

(c) *Optimal Density per Pupation Container.* The density of adults per container (30, 60, and 90) did not influence the percentage of adults emerging from aestivation ( $32.2 \pm 4.57\%$ ) ( $F_{(4, 53)} = 1.73, P = 0.1865$ ) or the duration of aestivation ( $142.7 \pm 0.6$  days) ( $F_{(4, 53)} = 0.02, P = 0.9836$ ).

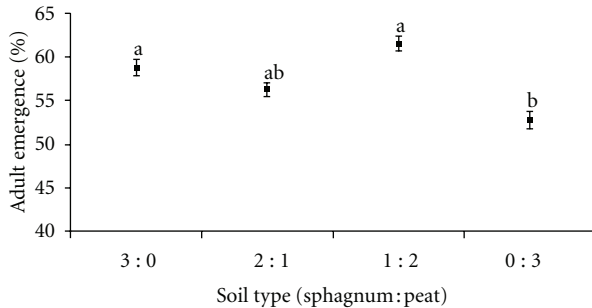


FIGURE 5: Mean percentage ( $\pm$ S.E.) of adults emerging from aestivation when stored in four different ratios of sphagnum:peat during pupation and aestivation. Means with different letters are statistically different ( $F_{(3, 284)} = 4.10, P = 0.0072$ ).

(d) *Assessing Importance of Sterilized Soil.* In fall of 2006 containers with unsterilized soil mix had higher mean emergence than the other treatments ( $67.3 \pm 4\%$ ) (Table 2). Emergence from containers with unsterilized forest soil exceeded all those containers with autoclaved soil ( $39.3 \pm 3\%$ ).

**3.3. Rearing Procedures to Increase Production Numbers of *L. nigrinus*: 2005–2010.** Production of *L. nigrinus* adults that successfully emerged after aestivation improved after 2006 (Table 3). In 2005 and 2006, the numbers of larvae produced dropped from about 31,000 in 2002–2004 (Table 2) to 17,042 (Table 3). Emerging adults dropped more drastically from 10,000 (Table 2) to 2,725 beetles (Table 3). Mold contamination of the soil medium was likely the main cause. The sterilizing study helped us determine that sterilizing the soil contributed to the onset of mold problems. By not sterilizing the soil, the mold problems disappeared. It was likely that beneficial microorganisms were being removed during sterilization allowing saprophytic fungi to flourish, but we did not investigate this further.

Beginning in 2007, we finally reached a stable point in rearing production. From 2007 to 2010, we produced an average of 19,036 adults per year (Table 3). There is still considerable variation from year to year due to environmental conditions. In 2009 our production dropped, due mostly to severe winter kill of HWA from low temperatures in February that year throughout the mid-Atlantic states, resulting in poor food availability for developing larvae. In contrast, HWA populations recovered in 2010. Food availability was adequate, and we obtained a remarkably high level of emergence, where 71% of the larvae survived pupation and aestivation.

#### 4. Discussion

The creation of the funnel cage contributed greatly to the assessment of survivorship as well as providing a functional way to rear larvae. *L. nigrinus* larvae developed within the funnel without additional maintenance, such as adding foliage or searching for lost larvae under the microscope,

as in previous years. Collection of *L. nigrinus* prepupae dropping from the foliage enabled us to determine egg and larval survival and allowed for the calculation of survivorship through *L. nigrinus* pupation and aestivation. A third benefit of using this cage is that, during spring, there is a consistent accessible supply of *L. nigrinus* larvae dropping from the foliage, allowing experiments to be set up with less effort and coordination since larvae do not have to be reared individually.

There are some challenges to using the funnel cages. Mature larvae must be transferred within 36 h of dropping, before they create a pupal cell and become immobile. Checking the jars daily for mature larvae is labor intensive and costly. Without adequate prey within the funnel cages, large numbers of immature larvae that end up in the jars must be transferred back to hemlock branches containing HWA eggs for them to survive. Also, high numbers of immature larvae increase the time required to check the funnel jars.

Survival is low when adults emerge from aestivation before October. Maintaining them at  $4^{\circ}\text{C}$  increases their survival. Since aestivating 1st instar HWA sistentes are not suitable food for postemergence adult beetles, HWA eggs laid by sistentes in the previous spring should be stored at  $4^{\circ}\text{C}$  throughout the summer to provide early emerging adults with developing progredientes nymphs. The increase in larval production in 2002 is attributed to the almost exclusive use of field beetles for the starting colony. These were larger and apparently oviposited more eggs than lab-reared adults. Sex ratio of pupae in mold-free soil was close to 1:1. Emergence of males occurred earlier than females when held at constant  $15^{\circ} \pm 2^{\circ}\text{C}$ , 14:10 (L:D) photoperiod. This may explain the high female:male ratio observed in ovipositing females. When maintained at a high temperature ( $19^{\circ}\text{C}$ ) after adult eclosion and lowered to  $13^{\circ} \pm 2^{\circ}\text{C}$ , 12:12 (L:D) photoperiod in late September to stimulate emergence, the emergence period of adults was much shorter than the emergence period of adults the previous year [12].

Moisture level and soil type influence the number of adults that emerge. Emergence is the highest at moisture levels of 30–40% saturation although a wide range of moisture levels in the soil is tolerated. This is advantageous because precipitation varies from year to year. Adults have the highest emergence from a mixture of sphagnum and peat mosses. Although these factors affect emergence, larval cohort often accounts for much of the variation observed in emergence, suggesting there are unexplored factors involved.

The time at which larvae enter the soil ranges over a period of 12–15 weeks and appears to influence the number of adults emerging from aestivation. The same pattern is observed each year in the colony; larvae maturing early in the spring have a higher rate of adult emergence than those maturing later in the year. This pattern may be explained by variation in nutritional value of HWA over a season. Larvae maturing early in the season feed on eggs laid by sistentes and those maturing later in the season feed on eggs laid by progredientes. By late spring, two generations of HWA have fed on the hemlock branches, which may be depleted in resources, possibly affecting the nutrition of HWA. The

TABLE 2: Total number of *Laricobius nigrinus* larvae, emerged adults, and percent emergence in each treatment.

Container	Soil type	Autoclaved	<i>n</i>	Total larvae	Emerged adults	% Emergence $\pm$ SE <sup>A</sup>
Square	Mix	Yes	5	150	30	20.0 $\pm$ 4% c
Square	Mix	No	5	150	101	67.3 $\pm$ 4% a
Square	Forest	Yes	5	150	5	3.3 $\pm$ 1% d
Square	Forest	No	5	150	59	39.3 $\pm$ 3% b

<sup>A</sup>Means followed by a different letter are significantly different from one another (Tukeys,  $P \leq 0.05$ ).

TABLE 3: The number of *Laricobius nigrinus* in each life stage at the Virginia Tech Insectary from 2005 to 2010.

Year	Founding colony (reproductive adults)	Mature larvae	Adult : larvae	Emerging adults (after aestivation)
2005	713 <sup>a</sup>	19,593	1 : 27	3,430
2006	1,067 <sup>a</sup>	14,492	1 : 14	2,019
2007	1,231 <sup>b</sup>	40,978	1 : 33	15,294
2008	1,200 <sup>a</sup>	46,028	1 : 38	20,601
2009	1,030 <sup>a</sup> + 200 <sup>c</sup>	32,382	1 : 26	13,283
2010	870 <sup>a</sup> + 200 <sup>c</sup>	38,690	1 : 36	27,504

<sup>a</sup>Wild-caught adults from Washington, USA.

<sup>b</sup>Wild-caught adults from Washington, USA and Kentland Farm, Virginia, USA.

<sup>c</sup>Adult F<sub>1</sub> generation reared in the insectary at Virginia Tech.

nutritive chemical composition of host plants can affect the quality of phytophagous hosts and has been known to affect their predators [16, 17], particularly *S. tsugae* [18], a predator of HWA.

The experiments conducted between 2000 and 2004 led toward more consistent production of larvae and lower mortality at each individual stage. Consequently, the number of beetles produced is now more predictable and appears to be mostly a function of food quality (prey). In general, rearing beetle predators on a natural diet is enormously difficult when prey is not consistently available [19]. Artificial diets have been developed and tested but usually result in significantly greater mortality during development of the predator [20, 21]. Artificial diets can be used to augment predator rearing [22] and are currently being investigated for *L. nigrinus* [23], but much work is still needed. Therefore we continue to rely on the collection of preys from abundant populations on healthy host trees.

Developing a reliable mass rearing procedure was a critical objective addressed by these experiments. The results from the experiments carried out from 2000 to 2004 led to a detailed description of *L. nigrinus* rearing procedures documented in [11]. These have led to procedures being followed by rearing labs at Virginia Tech, Clemson University, and the University of Tennessee. The success in colony rearing to date has resulted in the release of more than 100,000 adult *L. nigrinus* at 267 locations in 13 states in the eastern USA [24]. The techniques developed here are also applicable to rearing other *Laricobius* species being considered for release [25, 26].

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