# Evaluating the suitability of *Laricobius nigrinus* Fender [Coleoptera: Derodontidae] as a biological control agent for hemlock woolly adelgid, *Adelges tsugae* Annand [Hemiptera: Adelgidae]

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## Abstract

Hemlock woolly adelgid (HWA), *Adelges tsugae* Annand (Hemiptera: Adelgidae), is an introduced pest injurious to hemlock trees (*Tsuga* spp.) in the eastern United States. HWA currently infests hemlock in over 50% of its geographic range and has the potential to spread throughout its entire range. Since HWA populations in the eastern United States are not regulated effectively by natural enemies (Wallace and Hain 2000), classical biological control is the most promising option for controlling this pest in the forest setting. This work evaluates *Laricobius nigrinus* Fender (Coleoptera: Derodontidae), a predator associated with HWA in western North America, is being evaluated as a potential biological control agent for HWA (Cheah et al. 2004). Observations suggest that natural enemies may help keep HWA populations below injurious levels in the western United States (Mausel, pers. comm.).

Laboratory studies have revealed that *L. nigrinus* feed selectively on HWA and field studies in British Columbia, Canada have shown that its lifecycle is highly synchronous with HWA phenology. This predator is being further evaluated for its suitability as a biological control agent. In order to be a viable candidate, *L. nigrinus* must survive under natural field

conditions in the eastern United States, reduce the density of HWA, and it must be amenable to mass rearing.

The survival, reproductive capability and predator efficiency of *L. nigrinus* were assessed over 3 years in the field. In addition, the environmental factors regulating processes in the life cycle to develop and improve rearing procedures for this insect were identified. In the first year, adults survived from February – May, laid up to 41 eggs/beetle, and consumed approximately 4.3 adelgids per day. In the second season, *L. nigrinus* adults survived from November – April, laid up to 38 eggs/beetle and consumed approximately 4.5 adelgids per beetle throughout the study. In both seasons, adelgid populations were significantly lower on branches with predators than those without predators. This impact was demonstrated on both the winter and spring generation of HWA. The feasibility of caged field releases of *L. nigrinus* was determined in a third field study. An estimated 10, 000 *L. nigrinus* eggs were liberated in field cages in spring 2003. The density of adelgids in the subsequent (spring) generation of the adelgids was significantly lower on branches with larval activity than those with no predators. Despite extensive sampling, no $F_1$ adults were observed, however $F_2$ adults were recovered in the fall of 2004, 20 months after release.

The *L. nigrinus* life stages incurring high mortality during rearing were identified and factors affecting survival in the feeding and non-feeding life stages were investigated. Studies on the survival and feeding of adults, length of ovipositional period, density per cage, and survival of larvae were conducted. The effect of type of pupation medium, moisture level, disturbance, soil sterilization, temperature, and photoperiod on survival of the non-feeding stages and time of emergence from aestivation was examined. The most noteworthy finding, regarding rearing, is that aestivation can be extended by storing adults at high temperatures and long

daylength throughout the summer and decreasing the temperature and daylength in the fall. Based on the results of these studies, procedures for rearing *L. nigrinus* have been developed and it is currently being reared at two other institutions. To date, over 8, 000 adults produced at Virginia Tech have been released in 6 states and both $F_1$ and $F_2$ adults have been recovered from multiple locations.

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# Preface

There is some redundancy in the following chapters since each chapter was prepared as a "stand alone" paper for publication in peer-reviewed journals.

# Chapter 1

# Introduction and Literature Review

## Hemlock woolly adelgid in the eastern United States

Hemlock woolly adelgid (HWA), *Adelges tsugae* Annand (Hemiptera: Adelgidae) is an exotic insect that attacks and kills eastern hemlock, *Tsuga canadensis* (L.) Carriere and Carolina hemlock, *T. caroliniana* Engelmann trees in the eastern United States (Cheah et al. 2004). The adelgid poses a serious threat to eastern hemlocks since it inhibits the development of shoots and growth of new needles (McClure 1996), potentially causing complete mortality in affected stands in as little as four years after attack (McClure et al. 1996). All age classes of trees across the infested range have shown high susceptibility to this pest.

The geographic range of eastern hemlock extends from Nova Scotia, west to Minnesota and south to northern Alabama (Baumgras et al. 1999). HWA was first observed in the early 1950's in the landscape setting in Virginia and since the 1980's, has spread to fourteen states from Georgia to southern Maine, causing widespread mortality of eastern hemlock in Connecticut, New Jersey, Virginia, and Pennsylvania (Cheah et al. 2004). The rapid dispersal of this pest is facilitated by wind, birds, deer and humans (McClure 1990), enabling it to spread up to 25 km per year (Yorks et al. 1999). HWA outbreaks are facilitated by the absence of natural enemies (Cheah and McClure 1998) and their ability to survive low temperatures (Parker et al. 1999). HWA may have the potential to spread throughout most of the native range of eastern hemlock (McClure 1996), however, cold temperature may limit its

spread north (Skinner et al. 2003). Several management options are being investigated in an effort to slow the spread and decrease HWA populations below injurious levels.

Hemlock trees in eastern North America are a valuable resource for people and wildlife. Perhaps their highest value lies in the importance of the ecological niche they occupy as a component of eastern forests. Hemlock occupies about 800 000 ha of forest characterized as pure or mixed *T. canadensis* stands (Orwig and Foster 1998), providing important habitat and natural resources (Ward et al. 2004) throughout its geographic range. This late successional, shade-tolerant tree species creates a unique microclimate that provides critical habitat for many birds, mammals, amphibians, fish (Evans et al. 1996) and plant species (Ward et al. 2004). The dense canopy created by hemlock trees creates a distinctive microhabitat that is relatively cool in the summer and warm in the winter (Yamasaki et al. 1999). In addition, they maintain aquatic habitat ecosystem function by regulating stream flow and moderating water temperature (Evan et al. 2002). Large-scale mortality of hemlock will significantly change forest structure, composition, ecosystem function, and resource quality (Foster 1999), since it is the predominant species in much of its range in the northeastern U.S. forests (McWilliams and Schmidt 1999). They occur in rocky highlands, low lying wetlands, riparian habitats, and heavily populated suburban areas (Parker et al. 1999). Preservation of hemlock forests is critical because there are no other tree species that will replace the ecological role played by hemlock in the ecosystem (Evans et al. 2002).

Hemlock is also valued economically for its aesthetic appeal and commercial uses. In addition to being a favorite landscape species, hemlock is used commercially for pulp, paper, and sawlogs (McWilliams and Schmidt 1999), comprising 22 percent of the total volume of softwood growing stock in the northeastern U.S. (Ward et al. 2004). In addition, loss of

hemlocks will reduce property and park value (Battles et al. 1999). The economic and environmental impacts of HWA will become more significant as its infestation expands (Quimby 1996).

HWA is believed to originate from Asia, where it is an innocuous inhabitant of *Tsuga* spp. (Montgomery et al. 1999), and where populations are likely regulated by host resistance and natural enemies (McClure et al. 2000). In eastern North America, HWA populations reach lethal levels rapidly and importation of its natural enemies may be the most promising option for controlling HWA (Wallace and Hain 2000).

**Life Cycle of HWA**

HWA has a complex polymorphic life cycle that involves two asexual generations and one sexual generation annually (McClure 1987). The overwintering adults, the sistens generation, lay 50 – 300 eggs in woolly ovisacs (McClure et al. 2001). Sistens eggs are deposited over a long time period, from February to May. These eggs hatch into crawlers beginning in April and are active for one to two days (McClure 1987). Some of these nymphs develop wings, while the rest remain wingless. The wingless generation, the progrediens, remains on the host tree and they search for a suitable needle-base to settle and insert their stylets. The progrediens quickly develop through four instars, maturing in June. As adults, they lay approximately 25 eggs per ovisac during June and July (McClure 1989; Gray and Salom 1996). These eggs hatch into sistens crawlers that also settle at the base of young needles, feed for a short time, and then enter aestivation (summer diapause). Sistens nymphs aestivate for several months and resume feeding and development in October (McClure 1987; Gray and Salom 1996). They develop during the winter, reaching maturity by February, at which time the adult sistens begin laying eggs and complete the asexual cycle.

In eastern North America, the winged sexual generation, sexuparae, occasionally develop along side the progrediens. Sexuparae disperse to spruce trees and lay approximately 12 eggs per ovisac (McClure 1989). The progeny of sexuparae produced on spruce are called the sexuales. The sexuale nymphs hatch and begin to feed, however, they are unable to develop past the first instar (McClure 1989). This generation must develop on spruce, and fortunately, of the 15 (native and non-native) spruce (*Picea spp.*) species found in eastern North America, none are suitable as hosts (McClure 1992). Therefore, the production of sexuparae constitutes a mortality factor for HWA. The ratio of winged to wingless progeny produced by the sistens appears to be density-dependent (McClure 1991), i.e. the more sistens there are present on hemlock, the greater the proportion of their progeny will be sexuparae. The life cycle of HWA is similar in Asia and North America, however, the phenology and timing of their life stages and generations vary significantly with elevation, latitude, and weather conditions (McClure et al. 1999). In addition, the sexual generation has not been observed in western North America (Zilahi-Balogh 2003a). The development of HWA on hemlock in North America appears to have a wide temporal overlap of life stages (McClure 1987; Gray and Salom 1996).

The specific mechanism by which HWA causes mortality in hemlock is unknown. Following attachment, HWA nymphs insert their stylet bundle through the epidermal cells of a young hemlock shoot and penetrate the plant tissue. Feeding has only been observed on the parenchyma cells of the xylem rays, which serve as nutrient transfer and storage cells (Young et al. 1995). The severe damage caused by HWA may be caused by factors other than the depletion of photosynthates (McClure 1991) or predisposes the tree susceptible to other environmental stresses. Balsam woolly adelgid, *Adelges piceae* (Ratz.), an exotic adelgid

attacking *Abies* spp., has toxic saliva and cause injury to trees at relatively low populations (Hain 1989). However, this has not yet been demonstrated in HWA.

**Biological Control of HWA**

HWA can be effectively controlled by insecticides in urban settings (McClure 1987), however, these applications are not practical in the forest. Biological control may be the only viable option for reducing HWA populations in the forest setting. There are no known parasitoids that attack any member of the family Adelgidae (Montgomery and Lyon 1996). In the past few years, a complex of predacious insects (Cheah et al. 2004) and entomopathogenic fungi (Gouli et al. 1997) have been assessed for their potential as biological control agents against HWA. It is unlikely that a single species will control HWA. Management of this pest may require a complex of predators. One predator that may be an important part of this complex is *Laricobius nigrinus* Fender (Coleoptera: Derodontidae). *L. nigrinus* is a predacious beetle found in association with HWA in western North America, where western hemlock, *T. heterophylla* (Raf.) Sargent, is not typically injured by HWA (Furniss and Carolin 1977). Maintenance of HWA below damaging levels may be attributed to tree resistance and natural enemies in the West (Cheah and McClure 1996).

Derodontidae is a small family comprised of four genera that inhabit the temperate parts of both southern and northern hemispheres. *Laricobius* is the only genus in the family Derodontidae known to feed on adelgids (Lawrence and Hlavac 1979). There are four *Laricobius* species found in association with adelgids in North America, including: *L. erichsonii* Rosenhauer, *L. laticollis* Fall, *L. rubidus* LeConte and *L. nigrinus* Fender (Bright 1991). *L. erichsonii* was introduced into western and eastern North America as a biological control agent for balsam woolly adelgid (BWA), *Adelges piceae* Ratz. (Homoptera:

Adelgidae) (Brown and Clark 1962). This predator was found to significantly decrease the abundance of BWA, a species related to HWA, compared with the control treatment (Mitchell and Wright 1967). However, in retrospect the biological control program aimed at BWA has not been considered successful (Hain 1989). The adelgid on hemlock is a different system and there are hopes that the biological control program aimed at HWA will have a more positive outcome. *L. laticollis* and *L. rubidus* are native species found on Douglas-fir, *Pseudotsuga menziesii* Franco, in the West and eastern white pine, *Pinus strobus* L. in the East, respectively. Neither species has been well studied, but will likely get more attention in the future.

**Life Cycle of *Laricobius nigrinus***

Little was known about *L. nigrinus* prior to its study in the quarantine laboratory Virginia Tech, beginning in 1997. Field studies in British Columbia (Zilahi-Balogh et al. 2003a) and rearing *L. nigrinus* in the laboratory has revealed a univoltine life cycle that is synchronous with the life cycle of HWA sistens (Zilahi-Balogh et al. 2003b). The adults overwinter on the hemlock branches, feeding on HWA sistens. In late January or early February, adult *L. nigrinus* begin ovipositing single eggs directly in sistens ovisacs. Oviposition by *L. nigrinus* is synchronous with the oviposition period of sistens. After a few weeks, the eggs hatch into larvae within the woolly ovisac and feed on the nearby progrediens eggs. The first and second instars feed on progrediens eggs and the third and fourth instars feed on progrediens eggs and sistens. The duration for *L. nigrinus* development from egg to mature fourth instar larvae (pre-pupae) varies depending on temperature and food quality. The larvae develop at temperatures ranging between 9° and 21° C, however, pupal development does not occur at the higher temperatures of this range. The optimal temperature

for successful development from egg to adult is between 12° and 18° C (Zilahi-Balogh et al. 2003c).

After consuming a sufficient amount of prey to complete larval development, the pre-pupal stage is reached. This stage is a non-feeding fourth instar larva that migrates from the hemlock branches and seeks a pupation site in the soil. *L. nigrinus* remain as pre-pupae for about 10 days prior to pupation (Zilahi-Balogh *et al*. 2003b). They develop into pupae in cells in the ground, and after two weeks, metamorphose into adults. The adults remain in the soil and enter aestival diapause in early summer. In October, adult *L. nigrinus* emerges from the soil and migrate to the hemlock branches. This is approximately the same time that the HWA sistens are resuming development. Thus, both species enter and complete aestival diapause at approximately the same time in British Columbia (Zilahi-Balogh et al. 2003a).

*L. nigrinus* could play an important role as part of a complex of biological control agents aimed at regulating HWA abundance. This predator has several attributes that Huffaker and Kennett (1969) consider to be necessary for successful biological control agents including: feeding selectivity and synchrony with its host. In laboratory investigations, *L. nigrinus* was tested against other adelgid and non-adelgid species to determine ovipositional and feeding preferences. The host species tested included: balsam woolly adelgid, *Adelges piceae* (Ratzeburg), pine bark adelgid, *Pineus strobi* Hartig, eastern spruce gall adelgid, *Adelges abietis* (L.), giant conifer aphid, *Cinara spp*., green peach aphid, *Myzus persicae* (Sulzer) and the pine needle scale, *Chionaspis pinifoliae* (Fitch). *Laricobius nigrinus* larvae consumed significantly more eggs of HWA than eggs of balsam woolly adelgid or pine bark adelgid (Zilahi-Balogh et al. 2002). Larvae reared at 12°C and 12:12 (L:D) photoperiod consumed a mean of 226 HWA eggs to complete their development. Furthermore, *L. nigrinus*

preferred to oviposit in HWA sacs over other adelgid and non-adelgid species. In larval development tests, *L. nigrinus* completed development on HWA only, indicating that it is unable to survive on other prey species (Zilahi-Balogh et al. 2002).

*Laricobius nigrinus* possesses many favorable attributes that make it a good candidate for biological control on HWA in the eastern United States. This predator is highly prey-specific in feeding and oviposition, its life cycle and feeding stages are highly synchronized with the life cycle of HWA, females have a high fecundity, and adults are long-lived.

**Summer Diapause**

Summer diapause, or aestivation, is defined as "diapause induced before the height of summer, terminated and followed by reproductive, developmental, or feeding activities in autumn or winter" (Masaki 1980). According to the limited investigations conducted on the subject, summer diapause appears to be widespread geographically and is known to occur in twelve insect orders. The greatest proportion of known species to undergo summer diapause are beetles, and within this order, adults are the most common life stage to enter dormancy (Danks 1987). The primary functions of summer diapause are to synchronize the insect's life cycle with that of its host (Litsinger and Apple 1984) and may serve to synchronize the reproductive development among individuals in a population (Gomi and Takeda 1992, Zhu and Tanaka 2004) and bypass periods when food supply is low or absent (Wipking 1995).

Univoltine species that have a single dormant period that only occurs in summer, are likely to respond to the environment or a programmed sequence of physiological events to complete the dormant phase after the summer (Masaki 1980). In general, the environmental conditions regulating summer diapause are almost opposite the conditions that regulate winter diapause in relation to photoperiod and temperature (Beck 1980). Long daylength and high

temperatures tend to induce or maintain summer diapause, whereas short photoperiod and low temperature prevent or terminate it (Masaki 1980). Environmental factors can modify several distinct phases of development, including the onset of diapause (diapause induction), the processes after diapause begins (diapause development), and the rate of growth. These factors can act in one or both of two ways: as regulators that determine development directly, or as cues or environmental stimuli that act indirectly as seasonal signals (Danks 1999). Responses to environmental cues can change over time and may interact with one another; frequently, one of these cues suffices for completion of diapause development without the other. And many times, the second factor serves as a modifying factor.

Photoperiod is an important cue for diapause induction and is known to play a role in diapause termination, particularly in summer diapause (Tauber et al. 1986). Photoperiod is known to accelerate diapause development, primarily documented for lengthening days (Tauber and Tauber 1970, Hodek and Okuda 1997), but some insects are known to be influenced by shortening days (Jacobson 1960, Kato and Sakae 1981, Garcia et al. 1990, Gomi and Takeda 1992, Held and Spieth 1999, Zhu and Tanaka 2004 ). These photoperiodic responses usually depend on absolute photoperiods, that is on whether the photoperiod is short with respect to a critical photoperiod. However, some species monitor photoperiod quantitatively and the rate at which diapause comes to an end is directly related to the actual duration of the daylength.

In the long term, temperature is a highly reliable cue for seasonal position whether perceived absolutely or relatively, in summer or winter diapause. When temperature is used as a seasonal indicator, it is often correlated with photoperiod. Temperature varies day to day, but at any given site, clear seasonal patterns of monthly mean temperatures are retained.

Temperature is generally known to act in combination with photoperiodic control of diapause, however, it is known to be the sole factor in regulating summer diapause in some insects (Mansingh and Steele 1973, Nagell 1981, Gomi and Takeda 1992), particularly in those in a stable habitat, or one in which photoperiod is difficult to monitor (Danks 1987). Soil temperatures at 10 cm depth have much less pronounced daily fluctuations in temperature than air temperature fluctuations. Temperature levels would be more reliable in sheltered micro-sites such as soil, over long time periods. In the north-temperate insects that have been best studied, low temperatures commonly facilitate diapause development, but high temperatures are also effective in many species (Nakai and Takeda 1995, Xue et al. 2001).

Absolute photoperiods and levels of temperature chiefly govern the rate of diapause development, but some species monitor changes in these factors. The rate of diapause development can alter as daylength changes, accelerating as daylength changes after a solstice (Tauber and Tauber 1975, Butterfield 1976). In a few species, diapause development requires a change in absolute photoperiod, or a particular direction of change (Lutz 1974). Insects are also known to respond to changes in temperature during the diapause period, most documented in winter diapausing insects (Tauber and Tauber 1970, Wipking 1995), but a change in temperature is known to elicit a response in summer diapausing insects as well (Thiele 1969, Butler et al. 1985a, Nakai and Takeda 1995, Liu et al. 2004).

There are several ways temperature can be perceived. Since rates of metabolism are temperature-dependent, there is reason to suppose that temperatures can be measured relatively easily in poikilotherms (Hoffman 1969). For example, a given minimum temperature could be used as a seasonal index, as could the time spent at a minimum temperature, the time spent below a threshold temperature, or the number of days in

succession at an average temperature below a certain threshold (Bariola and Henneberry 1980). The summing of temperature, using information from a large number of cue events, would also be expected to be more reliable than a simple threshold response (Giesel 1976) and has been documented in the cranefly, *Tipula subnodicornis* Zetterstedt (Diptera: Tipulidae) (Butterfield 1976). In some cases, the number of photoperiodic signals or the duration of a particular temperature, controls diapause development (Danks 1987).

In many temperate regions, moisture level is typically not a reliable seasonal cue, however one region with a marked wet season is the Pacific Northwest and *L. nigrinus* may use it as a seasonal indicator. There are cases where development following the dormant period require moisture, particularly in species that come from habitats that are seasonally dry (Danks 1987).

In addition to sharing similar, but opposite environmental cues, summer diapause is much like winter diapause in most of its physiological and biochemical processes. Most insects that enter diapause for any length of time sequester substantial lipid and other reserves, like glycogen, in the fat body (Begon 1976), often at the expense of other body tissues. In adults, reproductive development is often arrested or reversed. Adult beetles have been documented to lose 10% fat reserve during diapause (Lambremont et al. 1964, Tombes 1971). *Hypera postica* (Coleoptera: Curculionidae) is inactive during aestivation and respiration rates fall to only one-fifth of fully active levels (Litsinger and Apple 1973). Dormancy for aestivating species is energetically expensive in terms of food reserves, and causes mortality if reserves are exhausted. Reserves are generally used more rapidly at higher temperatures, therefore more substantial reserves are required for maintenance during warm weather dormancies (Hodek and Cerkasov 1961), although some cold-climate insects maintain a

metabolism independent of temperature (Topp 2003). Summer dormancy in *L. nigrinus* is particularly interesting energetically, as individuals undergo metamorphosis and aestival diapause without feeding, a seemingly uncommon occurrence among beetles that undergo summer diapause.

**Justification for Research**

An important step in classical biological control is to survey and evaluate the effect of the imported natural enemy on the target pest. It is difficult to accurately predict the biological control potential of predaceous arthropods. Theoretical guidelines based on laboratory studies and mathematical models are not always useful to judge performance in nature (Bellows and Fisher 1999). The risks posed to the environment by highly host-specific predaceous arthropods are low, but not absent, therefore the values and weaknesses of the predator should be assessed to allow for a more knowledgeable decision on their release and utility as a biological control agent. To justify the tremendous effort put into rearing a biological control candidate, the potential benefit should be identified.

Due to the apparent lack of natural enemies of HWA in the eastern U. S. (Wallace and Hain 2000), classical biological control has become the most promising control option for this pest. Field studies in British Columbia and investigations in the laboratory have revealed that *L. nigrinus* displays several important attributes of a successful biological control agent. Two of these qualities include its strong prey specificity and its highly synchronous life cycle with HWA. Since most research to date has yielded positive results, it is necessary to conduct further research on the potential value of this predator. *L. nigrinus* was approved for field release by the USDA Animal Plant Health Inspection Service (APHIS), and the Virginia Department of Agriculture and Consumer Service (VDACS) in 2000. One critical step is to

study *L. nigrinus* under field conditions, where it is targeted for release, in order to determine its ability to survive and evaluate its impact on HWA populations in the eastern U.S.

Measuring the effectiveness or control power of a biological control candidate is an essential phase in a biological control program. The most convincing means of documenting the control value of a predator is by using check-methods (Huffaker and Kennett 1969). This methodology requires that the predator be excluded from prey on certain branches of the host plant, while including them on other branches (DeBach et al. 1949). The survival of the prey population can then be compared on the same tree, in the presence and absence of predators. A generally accepted method of determining the control value of a predator is the use of sleeve cages to include and exclude predators in association with their prey. Principal drawbacks of the sleeve-method are the possible modifications of the microclimate, interference with predator dispersal, and protection from wind, which normally may remove some individuals of the pest or predator species (DeBach et al. 1951).

One of the most important factors in selection of a natural enemy in classical biological control programs has been, and continues to be, availability and ease of a culturing technique for a candidate insect. Without successful rearing techniques, it is difficult to make progress in a biological control program. Colonies of *L. nigrinus* have been successfully maintained in quarantine for three years. As we begin to evaluate its suitability as a biological control agent in the field, large numbers need to be reared on a continuous basis. Currently, the number of individuals reared is limited by high mortality rates. The development of more efficient rearing techniques is necessary for the mass production of this insect.

In previous years, we used large, flat-bottomed Plexiglass® cages to rear *L. nigrinus*. Adults oviposited on HWA-infested twigs and these twigs were put in floral foam to stay

upright and moist. New infested twigs were added every week to feed the developing larvae. When the fourth instars reached maturity, they migrated down to the peat moss on the floor of the cage, where they would pupate and develop into adults. The peat moss from the cage bottom was collected and put in a Berlese funnel, where the adults burrowed through the peat moss and fell into collecting jars attached to the bottom of the funnel. There were several stages of this procedure that required many hours of labor, for little benefit. For example, when the pre-pupae migrated to the bottom of the cage, they got into the floral foam and died. There are, however, a few that develop into adults, so each block of floral foam was dissected in search of survivors. This process was time consuming and often resulted in 1 or 2 additional adults per cage. I propose to adjust several aspects of the rearing procedures described above, in an effort to reduce the amount of labor required in maintaining the *L. nigrinus* colony including: modified rearing cages and parafilm-wrapped floral foam.

The rearing procedure used in previous years did not allow us to determine at what stage in the life cycle of *L. nigrinus* mortality occurs. We could not see or account for any individuals between the egg and adult stage. We did not know how egg and larval densities affected survivorship. In addition, the environmental and physical conditions (i.e. temperature, photoperiod, moisture level, and type of pupation medium) used for pupation and adult summer aestivation were likely sub-optimal, since they were based on limited knowledge.

In recent years, *L. nigrinus* adults have emerged from aestivation over a long time period (July – December), the majority emerging in August. During the weeks following emergence, adults suffered high mortality because HWA was not present in the field until October. Instead adults were offered artificial food for Coccinellids, eastern spruce gall

adelgid and aestivating HWA nymphs during this time, but only ~5% of individuals emerging prior to Sept. 15th survived for 60 days. In addition, the long period in which adults emerged from the soil resulted in significantly more hours spent maintaining the colony.

A clearer understanding of the environmental/biological factors regulating the process of summer aestivation would increase our ability to rear this predator. If *L. nigrinus* adults remained in the soil until October (when food is available) and emerged synchronously, fewer hours would be spent collecting adults and the mortality suffered by *L. nigrinus* adults during the period following emergence would be greatly reduced. It is critical to determine the environmental factors regulating the duration of aestivation in *L. nigrinus* and use that information to modify our rearing of this predator to synchronize its emergence with prey availability in the field.

Possible seasonal cues vary in their reliability or predictability, in their frequency, and in their ease of detection by the insect. These properties may also vary according to whether absolute or relative cues are perceived. For example, the absolute day length indicates a seasonal position, but seasonal cues are also given by comparisons from day to day of the magnitude, and especially the direction of change in day length (Danks 1987). The environmental cues used to terminate diapause are primarily photoperiod and temperature.

Absolute photoperiods and levels of temperature chiefly govern the rate of diapause development, but some species monitor changes in these factors. In many cases, the number of photoperiodic signals or the duration of a particular temperature, controls diapause development (Danks 1987). Signals received during induction can modify subsequent requirements and alter the intensity of diapause. Duration of diapause also depends on genetic factors (Krysan 1982). Specific requirements of photoperiod, temperature, and other

cues to end diapause seem to be most marked in aestivation (Tauber and Tauber 1976) as opposed to cues used in winter diapause.

## Research Objectives

1. Assess field overwintering survival and reproduction of *Laricobius nigrinus* and assess its impact on HWA populations in Virginia (Chapters 2 & 3).
   a) Investigate adult survivorship and reproduction of *L. nigrinus* throughout the winter and spring in Virginia.
   b) Evaluate impact of *L. nigrinus* adults and larvae on HWA sistens and progrediens populations throughout the winter and spring in Virginia.
   c) Determine the ability of *L. nigrinus* to complete one generation in Virginia.

2. Improve the rearing procedures of *L. nigrinus* in the laboratory to facilitate field releases (Chapters 4 & 6).
   a) Quantify the mortality of each stage during the life cycle.
   b) Evaluate factors affecting adult survival, feeding, and larval production.
   c) Investigate the physical effect of density/container, type of pupation medium and soil moisture level on adult time of emergence and survival through summer aestivation.
   d) Modify rearing procedures to increase the number of individuals in the colony.

3. Determine factors that affect summer diapause in *L. nigrinus* (Chapter 5).
   a) Investigate the influence of genetic and environmental factors affecting number of adults emerging from soil and duration of summer diapause.
   b) Evaluate the effect of photoperiod, temperature, and soil moisture on the number of adults emerging from soil and duration of summer diapause.

# Chapter 2

## Field Survival and Reproduction of *Laricobius nigrinus* in Virginia

### Introduction

*L. nigrinus* is a winter-active predator and is unlikely to compete with other predators that are active later in the spring. Thus, we believe it to be a potentially complementary and important component of a developing predator complex for HWA. For *L. nigrinus* to be a viable biological control candidate, it must be able to survive under natural field conditions and help reduce HWA density below injurious levels. An essential phase in a classical biological control program is to survey for establishment of an agent in the field and evaluate its effect on the target pest (Bellows and Fisher 1999). Although environmental risks posed by predaceous arthropods are low, the efficacy of the predator should be evaluated to allow for an educated decision on its release and to justify the tremendous effort put into rearing it.

The most convincing means of documenting the effect of a predator is by using check methods, where the predator is excluded from prey on certain branches of the host plant, while including them on other branches (Huffaker and Kennett, 1969). The survival of the prey population can then be compared in the presence and absence of predators. Principal drawbacks of the sleeve-cage method are possible modifications of the microclimate, interference with predator dispersal, and protection from natural enemies wind (DeBach et al. 1951).

We report the results of two experiments conducted to determine the ability of *L. nigrinus* to survive in Virginia's climate and to evaluate its impact on HWA populations in sleeve cages. The specific objectives were to:

- quantify adult survivorship and reproduction of *L. nigrinus* throughout the winter and spring; and
- determine the impact of *L. nigrinus* adults and larvae on populations of sistens and progrediens.

## Materials and Methods

Study Sites

Field studies were conducted over two consecutive winters. The first experiment was conducted at three sites from February through June, 2001. The sites were located in Montgomery and Giles counties in Southwest Virginia. Site 1 was located at Poverty Creek [Universal Transverse Mercator (UTM): 17 4123129 N, 542429 E; (Lat/Long): 37°15 13 N, 80°31 17 W], Site 2 at Big Stoney Creek [UTM: 17 4140859 N, 533985 E; (Lat/Long): 37°24 50 N, 80° 36 57 W], and Site 3 was established at the Mountain Lake Nature Conservancy [UTM: 17 4134851 N, 541017 E; (Lat/Long): 37°21 34 N, 80°32 12 W]. The hemlocks at Site 1 and Site 2 were young, understory trees (Table 2.1). Site 1 was located near a bottomland stream in young forest dominated by eastern white pine, *Pinus strobus* L. Site 2 was located on a northwestern slope in a forest dominated by tulip poplar, *Liriodendron tulipifera* L., and eastern white pine, *P. strobus*. Hemlock trees at the high elevation site (Site 3) dominated the overstory vegetation.

Dataloggers (Hobo®, Onset, Bourne, MA) were used to record temperatures at each site during both field seasons. Sensors were placed outside and within one sleeve cage at each site. The mean temperature recorded at the Mountain Lake Biological Research Station weather station (approximately 1 mile from Site 3) in February and March (2001) was 0.7°C, with maximum and minimum temperatures of 15° and –13.6°C, respectively. The mean temperature during April and May was 11.6°C, with maximum and minimum temperatures of 24.6° and –7.4°C, respectively. During the study, total precipitation was 39.4 cm with several snow and ice storm events. On average, Sites 1 and 2 experienced temperatures approximately 2°C warmer than Site 3. There was no difference in temperature measured inside and outside the sleeve cages. These two trends were also observed in the second field season.

The second year experiment was conducted from November 2001 through April 2002 at three sites in Giles County, Southwest Virginia. Two sites were located at Big Stoney Creek: Site 4 [UTM: 17 4142220 N, 543738 E; (Lat/Long): 37°25 32 N, 80°30 20 W] and Site 5 [17 4142097 N, 544395 E; (Lat/Long): 37°25 28 N, 80°29 53 W]. A third (Site 6) was located at Mountain Lake Nature Conservancy [UTM: 17 4136311 N, 542047 E; (Lat/Long): 37°22 21 N, 80°31 30 W]. The hemlocks used at Sites 4 and 5 were understory trees on northwest facing slopes (Table 1). The overstory vegetation at these sites was dominated by oaks, *Quercus* spp., and hickories, *Carya* spp. The hemlock trees used at Site 6 dominated the overstory vegetation.

The mean temperature measured at Mt. Lake Biological Research Station (Site 6) in November and December 2001 was 4.9°C with the highest temperature 20°C and the lowest –13.7°C. The maximum and minimum temperatures experienced in January and February

2002 were 15.8°C and –16°C, respectively, whereas the mean temperature during these months was -0.5°C. The mean temperature during March and April 2002 was 7.2°C with a maximum temperature of 31.1°C and minimum of –14.7°C. The total precipitation during the months of the study was 195 cm, and included several snow and ice storms.

1st Year Study

Two trees with high densities of HWA and accessible branches were chosen at each site. Twenty-seven branches, heavily infested with HWA, were selected from each tree. The total number of adelgids was determined by directly counting the ovisacs on all the twigs included on the terminal 45 cm of each branch. Each branch was then randomly assigned one of three treatments: caged branches with 2 female adult *L. nigrinus*; caged branches without predators; and uncaged branches without predators. The latter two treatments served as controls to determine the effect of cages on the survival of the adelgid. The adults used for these experiments were selected by isolating individual beetles and determining which individuals oviposited on supplied host material, since we are unable to use morphological characteristics to differentiate sex (Zilahi-Balogh 2001). The cages were sewn nylon mesh sleeves (# 110 mesh, Dynamesh, Chicago, IL) (open at one end), attached to the terminal end of each branch with 20 gauge, galvanized wire wrapped over rubber foam weatherseal (1.91 cm wide, 1.11 cm thick).

Using a randomized complete block experimental design, with each tree serving as a block, the effect of predators and time on adelgid populations was tested. There were six trees with nine replicates on each tree. One replicate of each treatment was removed from each tree at two-week intervals, and the total number of surviving predators, both adults and progeny, was counted using a microscope. In addition, the total number of surviving adelgids

on each branch was determined. Proportional data were arc-sine transformed and analyzed using a 2-factor analysis of variance (ANOVA) to determine if *L. nigrinus* had a significant impact on final adelgid densities or rate of decrease in adelgid densities (SAS Institute, 1992). All means were separated using Fisher's least significant difference (LSD) test.

2nd Year Study

One tree was chosen at each of the three sites. Nine branches, heavily infested with HWA, were selected from each tree, and the total numbers of adelgids were counted on all the twigs included on the terminal 45 cm of each branch. Each branch was then caged and randomly assigned one of three treatments: 5 adult *L. nigrinus* present throughout the study (permanent predators); 4 adult *L. nigrinus* replaced with lab-reared adults at each sample period (temporary predators); and no predators. We wanted to test *L. nigrinus* survival throughout the entire winter using the same individuals. The temporary predator treatment was added to collect feeding data, in case the permanent predators did not survive. Since the cage-effect on HWA density observed in the first year study was not significant, the uncaged control treatment was excluded in the second year.

At each sample period, all branches were removed and returned to the laboratory for closer examination. The number of surviving *L. nigrinus* adults on each branch was assessed and beetles were re-caged on new branches in the field the following day. The numbers of *L. nigrinus* progeny and surviving adelgids were counted using a microscope. Each sample period lasted 10 weeks, the first from November – January and the second from February – April.

This experiment was set up as a randomized block design with trees serving as blocks. A 2-factor ANOVA was used to determine if sample period or predator group had an effect

on predator survival or reproduction and if *L. nigrinus* had a significant impact on the decrease in number or proportion of total adelgids during each sample period (SAS Institute, 1992). Proportional data were arc-sine transformed to perform the analysis of variance and means were separated using Fisher's LSD test.

**Results and Discussion**

1st Year Study

*L. nigrinus* adults survived from February – May, and most oviposition occurred in March and April (Figure 2.1). Adult survival and oviposition activity declined over time, most likely because of a shortage of prey inside the cages and natural mortality. The total impact the 2 females and their progeny had on adelgid populations is illustrated in Figure 2.2, which compares the final density of the sistens and progrediens generations across treatments at each sample period. The density of sistens was significantly lower on branches exposed to predators than those without predators in every sample period ($F_{(2, 130)} = 46.67$, $p < 0.0001$). The density of progrediens was lower on branches with *L. nigrinus* than those without predators in every sample period, and significantly so in many sample periods ($F_{(2, 130)} = 12.53$, $p < 0.0001$) (Figure 2.2). Caged branches without predators tended to have higher adelgid densities than uncaged branches, but only significantly so in 3 sample periods. This suggests that cages may aid the survival of adelgids. Although *L. nigrinus* was not present after mid-May, adult and larval feeding on the sistens and the ovisacs they produced had a carry-over effect that lowered the density of the subsequent generation (progrediens). Sleeved control branches had higher densities of progrediens than control branches without sleeves (Fig. 2.2(B)) because hatching HWA eggs were protected from natural enemies and dispersal was

limited dues to the sleeve cages. The difference in the total decrease of adelgids on branches with and without predators was attributed to *L. nigrinus* and was used to calculate adult consumption (Table 2.2).

The branches exposed to *L. nigrinus* showed a significantly higher rate of decrease in sistens density than branches not exposed to predators ($F_{(2,80)} = 59.14$, $p < 0.0001$). In six of the first seven sample periods, branches caged with predators had a significantly higher rate of decrease in sistens density than those without.

2nd Year Study

We modified our field evaluation in the second season in an effort to determine whether *L. nigrinus* adults would survive the entire winter (beginning in November) in Virginia and to assess their feeding and oviposition activity while adequate prey were present. Data collected for permanent and temporary predators were pooled, since no differences were found between their survival ($F_{(1,31)} = 0.16$, $p = 0.6929$) or reproduction ($F_{(1,31)} = 0.02$, $p = 0.9557$). *L. nigrinus* survival and reproduction were significantly different between sample periods (Table 2.3).

The total decrease in sistens (original count – final count) was significantly greater on branches exposed to *L. nigrinus* adults than branches not exposed to predators in both sample periods ($F_{(1, 46)} = 17.10$, $p < 0.0001$). The mean decrease (± S.E.) in sistens from November to January was 1323 ± 85 and 704 ± 91 on branches with and without predators, respectively. From February to April, the mean decrease (± S.E.) in sistens was 1870 ± 105 and 962 ± 77 on branches with and without *L. nigrinus,* respectively.

Table 2.2 summarizes the predator activity collected during the two field seasons. The difference in the total decrease of sistens on branches with and without predators was

attributed solely to *L. nigrinus* and used to calculate adult consumption. In the first field season, we estimate that *L. nigrinus* females consumed an average of 4.3 ± 0.6 sistens per day from February through April. In the second field season, adults consumed an estimated average of 3.3 ± 1.6 sistens per day from November through January and 5.8 ± 2.1 sistens per day from February through April. The increased rate of consumption in the spring, compared to consumption in the fall, is likely due to warmer temperatures and the combination of *L. nigrinus* adult and their late instar progeny feeding. There were more prey on caged branches in the second year, which may have contributed to the observed higher rate of feeding as well.

The ovipositional activity of *L. nigrinus* was comparable in the first and second field seasons; the maximum number of progeny per branch was 164 and 152, respectively. The mean number of progeny produced per adult was higher in the second year (Table 2.2), but was likely due to a longer sample period and more adults per branch than in the previous year. In addition, HWA densities were higher than in the first year, which may have provided more oviposition sites for females in the second year.

Estimating the total number of adelgids eaten by each predator helps determine the potential feeding capacity of *L. nigrinus*, but provides little information on its relative impact on a specific population of adelgids. Figure 2.3 shows the total decrease in sistens as a proportion of the original number, and this may be a better measure of potential level of control by *L. nigrinus* adults. The proportional decrease in adelgid populations was significantly greater on branches with *L. nigrinus* adults than branches without *L. nigrinus* in both sample periods ($F_{(2, 46)} = 24.64$, $p < 0.0001$). The impact of *L. nigrinus* on adelgid populations was greater in the February - April sample period than in the November – January sample period ($F_{(2,46)} = 45.67$, $p < 0.0001$). This is likely attributable to increased

temperatures in the spring and perhaps the additive impact of late instar feeding on sistens. The cause of adelgid mortality on control branches was not determined but is probably due primarily to overwintering mortality.

One of the drawbacks of starting in November is that the adults could not be sexed because females were not yet ovipositing (Zilahi-Balogh et al. 2003b). Therefore, all data for the second year are reported in per beetle units, rather than per female units, as was done in the first year.

This study indicates that the activity period of *L. nigrinus* in Virginia is similar to its activity period in its native range (Zilahi-Balogh et al. 2003a) and is well synchronized with the life cycle of HWA in Virginia (Gray and Salom 1996). *L. nigrinus* adults were active and fed on sistens nymphs throughout the fall and winter and oviposited in sistens ovisacs in late winter to early spring.

*L. nigrinus* had a significant impact on adelgid populations in both the pre-oviposition period from November through January, and during the oviposition period from February through April. Of special interest is the early season impact (November - March), when no other known biotic mortality agents of HWA are present. Thus, the impact of *L. nigrinus* adults during the fall and winter should complement the actions of other predators, such as *Sasajiscymnus* (previously *Pseudoscymnus) tsugae* Sasaji and McClure and *Scymnus ningshanensis* Yu et Yao (Coleoptera: Coccinellidae), that become active later in the spring (Cheah and McClure 2000; Montgomery et al. 2002). *S. tsugae* adults are present on hemlocks year round, but only become active later in the spring, ovipositing when temperatures rise above 15°C, resulting in larval activity from May to September (Cheah and McClure 2000). Butin et al. (2003) found that the period of peak oviposition of *S. tsugae*

differs from year to year. The Chinese coccinellid, *S. ningshanensis*, a potential candidate for release, becomes active at temperatures above 7°C and oviposits for several weeks beginning in May (Montgomery et al. 2002; Butin et al. 2003). In a caged field study conducted from May to July, adelgid populations were reduced by *S. ningshanensis* but increased in cages with *S. tsugae* (Butin et al. 2003). Other studies indicated that adelgid populations have been reduced following *S. tsugae* release in the field (Cheah and McClure 1998).

*Laricobius* has been employed in a biological control program for a woolly adelgid in the past. *Laricobius erichsonii* Rosenhauer, a European species, is one of several predators that were introduced into the eastern and western U. S. for the control of balsam woolly adelgid (BWA), *Adelges piceae* (Ratz.). Clark and Brown (1958) caged *L. erichsonii* on fir (*Abies* spp.) trees to study the impact they had on BWA populations. *L. erichsonii* reduced adelgid populations by 61%, compared with control cages where BWA populations increased by 186% (Clark and Brown 1958). Between 1951 and 1955, 16,000 beetles were released in the northeastern US. Although *L. erichsonii* performed well in field cages and was recovered at most release sites in the following decade, no measurable control of BWA was recorded (Buffam 1962).

The biological control program for BWA is not considered to be successful (Schooley et al. 1984); however, it was conducted in a harsh environment with many predator species that were released prior to screening (Amman and Speers 1971). With such a diverse array of predator species released, there are many possible causes for their failure to establish. Few species were chosen for release based on their biological attributes. There were laboratory studies conducted on a few species released in North Carolina, and of 20+ species, only one hemipteran oviposited eggs on Fraser fir, *Abies fraseri* (Pursh) (Amman and Speers 1971).

Some species may have been in competition with each other or were merely unsuited to the environment. In addition, the number of individuals released was often low; for example, only 1700 *L. erichsonii* were released in North Carolina (Buffam 1962). Hemlock trees may have an advantage that balsam, *Abies balsamea* (L.), and Fraser fir did not have. Since they grow in a milder climate than the firs, the environment may be more conducive to predator establishment.

Adelgid predators can be difficult to recover from the field, but surveys to determine which BWA predators are currently established in eastern North America could add insight and may be useful for making management decisions related to the current HWA program. The importance of long-term monitoring following release is emphasized by Humble (1994), where, in western North America, establishment of *Aphidecta obliterata* on BWA was first documented more than 20 years after the initial release of small numbers of the coccinellid.

Identification and use of predators that provide an additive impact is the most promising way to reduce HWA populations below levels injurious to hemlock trees. A complex of predator species with ecologically distinct requirements will facilitate an additive predator impact and will likely be required to control HWA across the wide geographic range and variable environment in which hemlock grows. *L. nigrinus* may be especially important because it is active earlier in the year and ultimately reduces the density of the progrediens generation, which should enhance the ability of other predators to impact adelgid populations.

The data collected in this study demonstrate that *L. nigrinus* is a promising biological control agent for HWA. Since this predator can survive, reproduce, and impact adelgid populations in field cages, the next phase is to evaluate its impact and ability to establish following open releases.

Table 2.1. Summary of measurements describing the tree and site characteristics of experiments conducted in 2001 and 2002 field seasons.

| Approximate Parameter | 1st Year Study (2001) | | | 2nd Year Study (2002) | | |
|---|---|---|---|---|---|---|
| | Site 1 | Site 2 | Site 3 | Site 4 | Site 5 | Site 6 |
| Hemlock Height (m) | 4 | 8 | 20 | 12 | 12 | 15 |
| Diameter Breast Height (cm) | 10 | 24 | 65 | 27 | 30 | 64 |
| Elevation (m) | 602 | 720 | 1218 | 919 | 950 | 1213 |
| Slope Aspect[1] | 156.8° | 321.1° | 77.3° | 349.6° | 357.1° | 77.3° |
| Slope Grade[2] | 1.8° | 9.4° | 14.8° | 11.7° | 13.8° | 1.8° |

[1]Slope Aspect is measured in degrees, where 0° = north

[2]Slope Grade is measured in degrees, where 0° = no slope

Table 2.2. A summary of *L.nigrinus* feeding and reproduction estimates in the first and second field seasons.

| Sample Period | Sistens Consumed per Day per Adult (±SE) | Mean Progeny per Branch[3] (±SE) | Mean Progeny per Adult[3] (±SE) |
|---|---|---|---|
| 1st Year Study (2001) | | | |
| February – May | 4.3 ± 0.6[1] | 30.4 ± 5.3 | 19.5 ± 3.8[1] |
| 2nd Year Study (2002) | | | |
| November – January | 3.3 ±1.6[2] | 0.9 ± 0.3 | 0.22 ± 0.1[2] |
| February – April | 5.8 ± 2.1[2] | 93.2 ± 8.9 | 21.5 ± 1.9[2] |

[1]All adults are female, units are reported in /female *L. nigrinus*

[2]All adults are unsexed, thus units are reported in /adult *L. nigrinus*

[3]Progeny includes eggs and larvae

Table 2.3. The mean survival of *L. nigrinus* adults and mean number of progeny found per branch during each sample period.

| Sample Period | Adult Survival % (±SE) | Progeny per Branch[2] (±SE) |
|---|---|---|
| November – January | 88.9 ± 3.2 a[1] | 0.9 ± 0.3 a |
| February – April | 55.3 ± 6.9 b | 93.2 ± 8.9 b |

[1]Different letters within each column indicate significant differences between sample periods ($F_{(1, 31)} = 20.29$, $p < 0.001$ (adult survival), $F_{(1, 31)} = 86.79$, $p < 0.001$).

[2]Progeny includes eggs and larvae

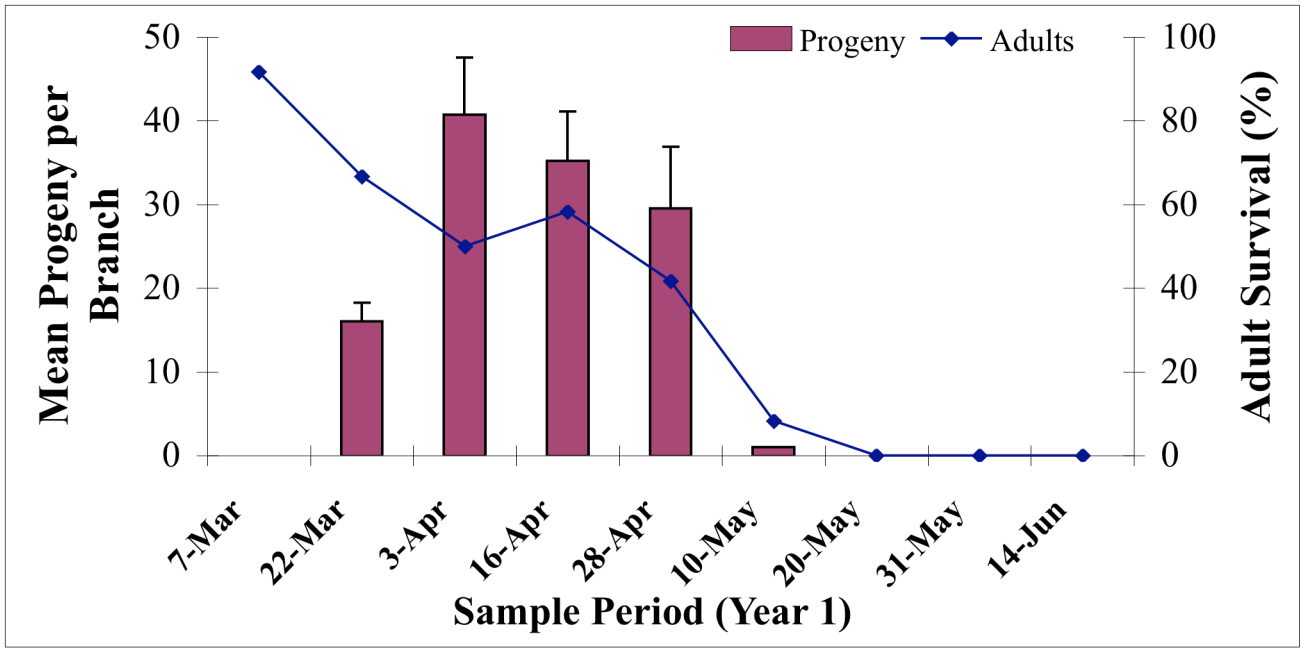



Figure 2.1. Percentage of *L. nigrinus* adults (maximum number per sample period is 12) and the mean number ($\overline{X}$ ± S.E.) of progeny found alive at each sample period (N = 54) in 2001. Adults were placed in sleeve cages on February 20, and sampled for the first time on March 7.

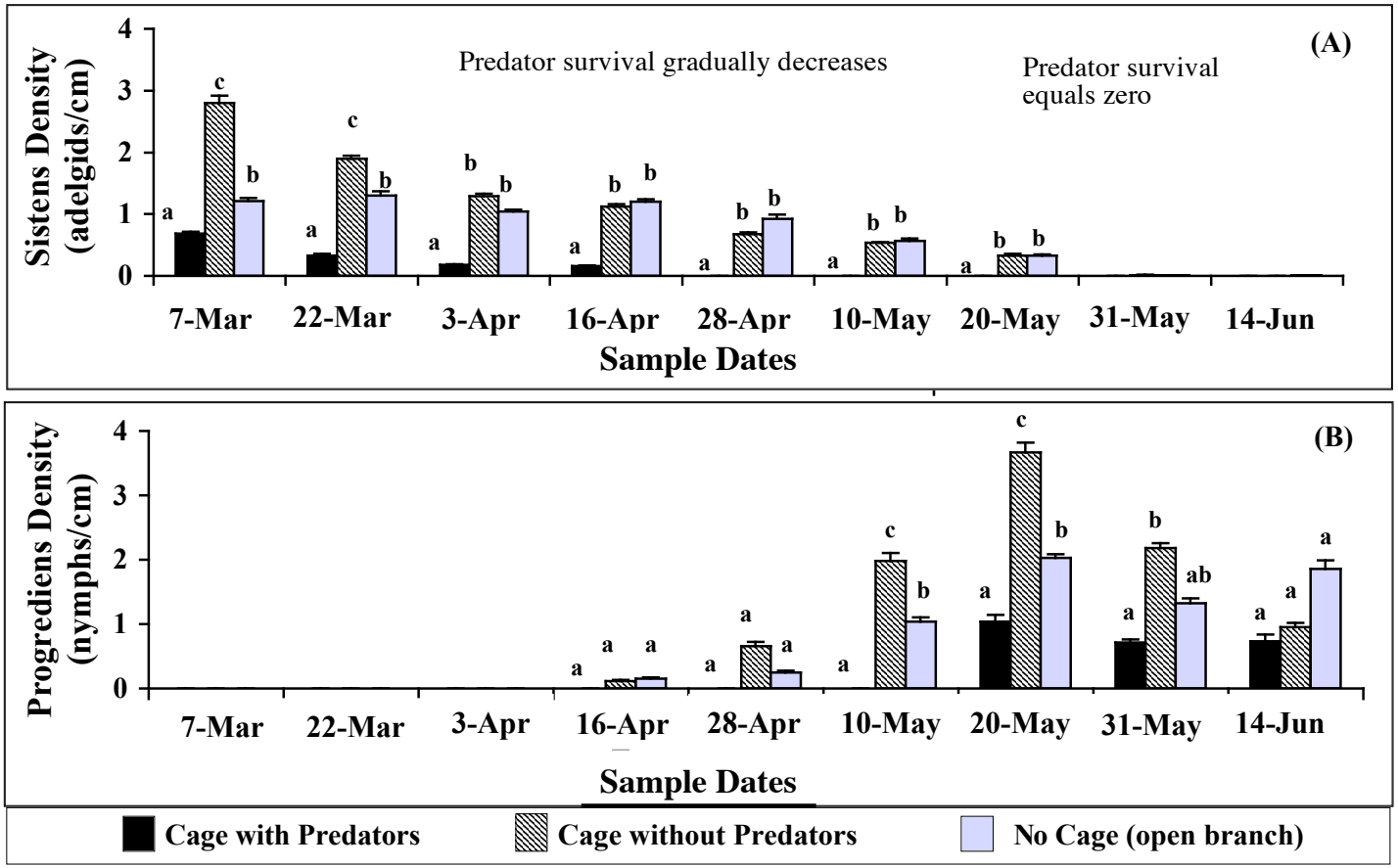



Figure 2.2 The mean final density of sistens (A) and progrediens (B) per cm branch ($\overline{X}$ ± S.E.) on sleeved hemlock branches with and without predators and unsleeved at each sample period. Mean densities significantly different at each sample period in (A) and (B) are shown by different letters, with means separated using Fisher's LSD in SAS ($F_{(2, 130)} = 46.67$, $p < 0.0001$ and $F_{(2, 130)} = 12.53$, $p < 0.0001$, respectively).

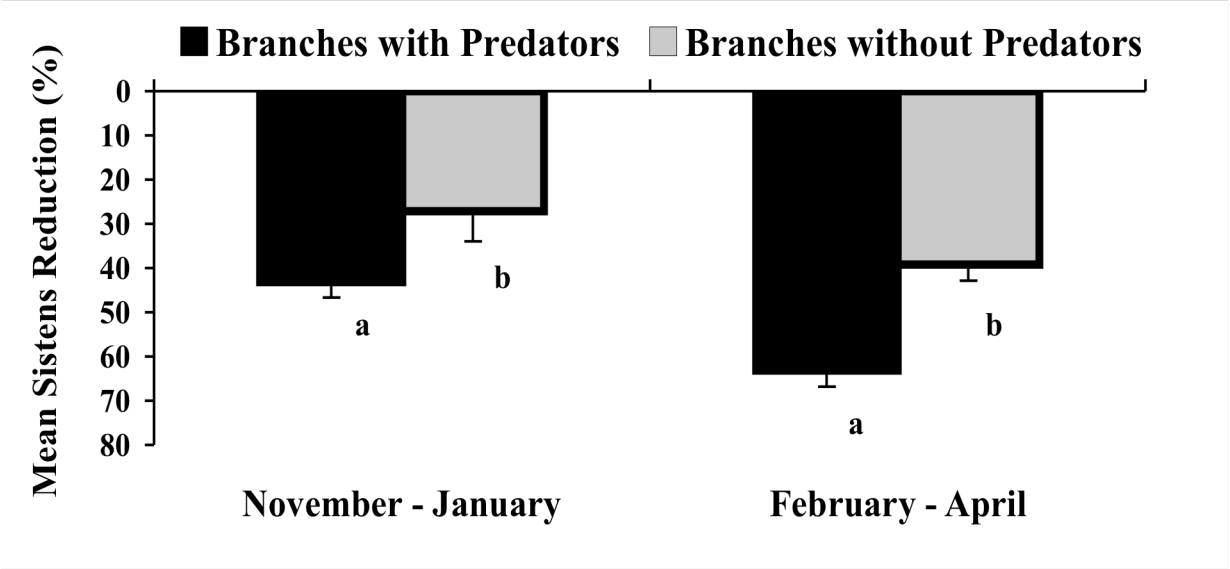



Figure 2.3. The mean percent reduction in sistens ($\overline{X}$ ± S.E.) per sample period on sleeved branches with and without *L. nigrinus*. Means with the same letter are not significantly different from each other ($F_{(2,\ 46)}$= 24.64, $p < 0.0001$). Sample period had a significant effect on mean percent reduction of sistens ($F_{(2,46)}$ = 45.67, $p < 0.0001$).

# Chapter 3

## Confined Field Release of *Laricobius nigrinus* in Virginia

### Introduction

*Laricobius nigrinus* Fender (Coleoptera: Derodontidae) is a predacious beetle found in close association with HWA on western hemlock in British Columbia (Zilahi-Balogh et al. 2003a), where hemlock trees are rarely injured by HWA in a natural forest setting (Furniss and Carolin 1977). Cheah and McClure (1996) suggested that, as in Asia, maintenance of HWA below damaging levels in western North America may be attributed to tree resistance and natural enemies. *L. nigrinus* is known to occur in Washington, Oregon, Idaho, and British Columbia (Fender 1945; Lawrence and Hlavac 1979). Field studies in British Columbia and investigations in the laboratory revealed that *L. nigrinus* possesses many qualities of successful biological control agents (Huffaker and Kennett 1969). It demonstrates strong host specificity (Zilahi-Balogh et al. 2002) and its life cycle is phenologically synchronous with that of HWA (Zilahi-Balogh et al. 2003a).

Field studies in Virginia reveal that *L. nigrinus* adults survive from fall through spring on caged branches and successfully reproduce in the target climate (Chapter 2). These predators have a significant impact on adelgid populations in both the pre-oviposition period from November through January and during the ovipostion period from February to May. Early season impact on HWA populations is of special interest because there are no known other biotic mortality agents acting on the sistens during this time, and *L. nigrinus* should

complement the actions of other HWA predators that become active later in the spring. Adult survival and oviposition appear to be higher in the field than in the lab, contributing to the urgency to begin field releases and expedite the establishment of *L. nigrinus* in eastern North America.

Investigation of release strategies, recovery techniques, and assessment of predator impact on HWA are important in evaluating field establishment of *L. nigrinus*. The optimal number of beetles released and timing of release required for successful establishment of *L. nigrinus* are not known. Techniques for rearing this predator have been developed (Chapter 6), but until recently, production of large numbers of beetles was constrained by high mortality rates, limiting the number of individuals available for experimental releases.

Our caged release study, reported in this Chapter, was conducted to test the feasibility of releasing low numbers of predators in the forest setting. A confined release was chosen to maximize the potential to recover predators by limiting adult dispersal, thereby concentrating their progeny on specific trees within a stand. In addition, this release strategy permits investigators to observe the biological development and behavior of a natural enemy in detail (DeBach and Bartlett 1964). Increased understanding of *L. nigrinus* behavior at different conspecific densities will aid in making decisions for large-scale field releases. The objectives of this study were to conduct the first field release of *L. nigrinus*, to determine the effect of *L. nigrinus* density on oviposition rate and location of eggs laid, and to measure the impact of *L. nigrinus* adult and larval feeding on adelgid densities.

## Methods and Materials

Study Site

This study was conducted in a mixed hardwood stand with a minor hemlock component near Big Stony Creek in Giles County, Virginia [Universal Transverse Mercator (UTM): 17 4142196 N, 543942 E; (Lat/Long): 37°25 31 N, 80°30 12 W] from March through June, 2003. This site was chosen for study because there were several dozen heavily infested understory hemlocks within the stand. The trees had accessible branches and the density of HWA in February was high enough that *L. nigrinus* would not become prey-limited within the sleeve cages. In addition, there were several understory hemlock trees in the stand that had low levels of HWA, and would provide adequate prey for $F_1$ *L. nigrinus* the following year. The site was on a northwest facing slope and the overstory vegetation was dominated by hardwoods, primarily *Quercus* spp., *Carya* spp. and *Liriodendron tulipifera* L.

Temperature was recorded throughout the study using a datalogger (Hobo®, Onset, Bourne, MA). A sensor was placed outside and within one cage to detect cage effect change in microclimate. The recorded temperatures and accumulated rainfall during each sample period are summarized in Table 3.1.

Four trees with high densities of HWA and accessible branches were selected within the stand. On each tree, the total number of adelgids was determined by directly counting ovisacs on the terminal 60 cm of 16 branches. Each branch was randomly assigned and caged with 0, 1, 2, or 3 *L. nigrinus* females. After 10 days, eight replicates of each treatment were randomly selected, removed, and returned to the lab, while the remaining eight replicates were left in the field. The above procedure was conducted in March, April and May, resulting

in three groups of adults caged on twelve trees, and data were collected 10 days after each group was caged.

Female adults used throughout the study were selected by isolating individuals, and determining those that oviposited, since morphological characteristics cannot be used to differentiate sex (Zilahi-Balogh 2001). The cages were sewn sleeves (45 cm wide, 60 cm long) (open at one end) using polyester fabric (white chiffon, Fabric.com), attached to the terminal end of each branch with 20-gauge wire wrapped over rubber weatherseal (1.91 cm wide, 1.11 cm thick).

*L. nigrinus* Survival and Oviposition

Adult survival was assessed on each of the 96 branches brought back to the lab. Each branch was cut into approximately 10 cm twigs and the numbers of predator eggs, adelgids, and adelgid ovisacs were determined on each twig. A randomized block design was used with time serving as blocks. The total number of predator eggs per branch and mean number of eggs laid per female were compared across treatments using a one-way analysis of variance (ANOVA). Means were separated using Fisher's least significant difference test (LSD). The average number of eggs per branch was used to estimate the number of predator eggs remaining in the field. Linear regressions describing the relationship between number of ovisacs per twig and the number of predator eggs found per twig were determined for each branch. A one-way ANOVA was used to determine the effect of predator density on this regression (SAS Institute 1992).

*L. nigrinus* Egg Release

The sleeves and female beetles from each of the branches remaining in the field were removed and re-caged onto different branches on the same tree. Adult survival was assessed as they were transferred to new branches at each sample period. Recovered adults were re-caged on new branches every 10 days for the duration of the study, resulting in open branches (previously sleeved) containing predator eggs. Dead adults were replaced with live ones from the lab. Overall adult survival was assessed every 10 days and the survival of the originally released beetles was measured throughout the study. The number of eggs in the field was estimated using the mean number of eggs found on branches brought to the lab. *L. nigrinus* progeny were left to develop on open branches.

Predator Impact on HWA Density

In late June, after *L. nigrinus* larvae had sufficient time to fully develop, two 10 cm branch terminals were randomly selected and removed from each branch that had been caged with March-released adults. The density of progrediens was determined on each branch sample and compared with control branches. The effect of predator density and sample period on final progrediens density was analyzed using a two factor ANOVA followed by Fisher's LSD (SAS Institute 1992).

Predator Recovery

Sampling was conducted weekly in September and October, 2003, in an effort to recover adult beetles. Several sampling methods were employed on release and non-release trees, including emergence traps and beat sheet sampling of accessible branches within the stand. Emergence traps included Lindgren traps (Pherotech, Surrey, BC) (baited and unbaited

with unmated males and females) and inverted funnels set out under the release trees in an attempt to intercept adults as they migrated back to the hemlock trees following summer aestivation. Bimonthly beat sheet sampling of release and non-release trees within the stand resumed in October 2004.

## Results

*L. nigrinus* Survival and Oviposition

Adults released in March, April, and May had similar survival throughout the study as approximately 13% of original beetles died during each sample period (Figure 3.1). Mean survival of all adults (both original and replaced) ranged from 76 - 88% per sample period. Oviposition was higher for adults released in March than those released in April or May (Table 3.2). Predator density significantly affected the total number of predator eggs found per branch ($F = 49.09$; $df = 2, 85$; $p < 0.0001$), however, this effect differed with time of release. In March, the number of eggs found per branch was significantly higher on branches with 3 females than branches with 1 female. This is not surprising because more eggs are expected from a higher number of females. However, in April and May, the total number of eggs found on branches with 2 females was not significantly different from those with 3 females. Adult oviposition may have been limited by prey availability in April and May since the abundance of HWA was lower in the latter two sample periods than in March (Table 3.1). Density of females on a branch influenced the number of eggs laid per individual ($F = 27.50$; $df = 2, 85$; $p < 0.0001$). Beetles caged in pairs laid more eggs ($\overline{X}$ ± S.E.) per female (31.3 ± 2.7) than beetles caged singly (22 ± 2.7) ($p = 0.0127$), whereas, those caged in groups of 3

laid the same number of eggs per female (26.8 ± 1.7) as beetles caged singly ($p = 0.1792$) or in pairs ($p = 0.2608$).

Regression analyses reveal a positive linear relationship between oviposition and the number of HWA ovisacs per twig, however, this relationship differs between branches with 1 and branches with 2 or 3 females (Figure 3.2). The slope is significantly greater on branches with 2 or 3 females than branches with 1 female in each release period ($F = 4.09$; $df = 2, 59$; $p = 0.018$). Branches caged with 2 females had the strongest relationship between number of eggs and number of ovisacs per twig ($R^2 = 0.470$), and weakest on branches caged with 1 female ($R^2 = 0.195$). The gentle slope on branches caged with only 1 female indicates that these adults were not limited by oviposition sites. Steeper slopes on branches caged with 2 or 3 predators suggest that females caged at higher densities are less selective when choosing oviposition sites and more likely to oviposit in regions without host material. This pattern is observed in each sample period, indicating that predator density and prey abundance influence *L. nigrinus* oviposition. This could also suggest that when more than one female is present, ovisacs were used up before the time period was over.

*L. nigrinus* Egg Release

Table 3.2 indicates the total number of field branches caged with *L. nigrinus* adults in March, April, and May and mean number of eggs observed per branch. The total size of the release was calculated by multiplying the number of field branches by the number of eggs per branch. The number of *L. nigrinus* progeny left in the field is estimated at 10, 344 (Table 2).

Predator Impact on HWA Density

The density of progrediens was significantly reduced on branches caged with predators for 10 days compared to branches without predators ($F = 90.15$; $df = 3, 157$; $p < 0.0001$) (Figure 3.3). The number of predators and the sample period in which adults were caged on branches did not significantly affect the final density of HWA on each branch.

Predator Recovery

*L. nigrinus* adults were not detected at the release site in fall 2003. In fall 2004, several adults were recovered from release and non-release trees using beat sheets. On October 21, 2004, 3 adults were recovered from release trees and 1 adult from a non-release tree and on November 17, 2004, 1 adult was recovered from a release tree and 1 adult from a non-release tree. Thus, a total of 6 $F_2$ adults were recovered 20 months after the study began.

**Discussion**

The confined release in spring 2003, using 150 *L. nigrinus* adult females, produced over 10, 000 predator eggs that were not confined. It represented the first field release of *L. nigrinus* in the eastern U.S. The recovery of *L. nigrinus* adults in fall 2004 indicates that these predators have persisted for two generations in Virginia. In addition, the released population of *L. nigrinus* has grown to a sufficient size to be detected using beat sheets since multiple adults have been discovered on more than one occasion in fall 2004.

Based on the results of this experiment, spring releases of predators should be conducted as early as March, since adult survival was similar throughout the spring and adults released later did not produce as many eggs. We speculate that adult releases should be

conducted even earlier than March since *L. nigrinus* oviposition begins as early as January in Virginia (Chapter 2).

This study also indicates that the number of eggs laid by adults is influenced by a shortage of prey, as HWA ovisac density influences the location and number of eggs laid. Females caged in pairs laid more eggs per adult than those caged with 3 females per branch. Eggs laid on branches with 3 females were less likely to be deposited in a viable ovisac than those laid on branches with only 1 or 2 predators. As prey abundance decreases, the number of eggs laid by *L. nigrinus* declines because of fewer suitable oviposition sites.

Since *L. nigrinus* adults are active from fall through spring, there is a wide temporal range in which releases can be made. Several observations support the notion that dispersal behavior of *L. nigrinus* adults differs between the fall and spring. In the lab, adults are active fliers and screened tents were made for feeding adults in the fall, but they rarely fly in the spring. Additionally, the only adult to escape while being transferred to a new branch in spring, was found on the same tree 10 days later during close inspection of branches. Based on these observations, we believe adults disperse farther distances in the fall than in the spring, and therefore, successful establishment may require different strategies for spring and fall releases.

Spring releases should focus on smaller numbers spread over a greater number of trees since it has been shown to persist even when released in small numbers. *L. nigrinus* adults are attracted to high densities of HWA and females do not need males during their oviposition period. Field releases of other adelgid predators have typically been at much higher densities, however, there are a few examples of successful establishment following low density releases (Humble 1994).

*Sasajiscymnus tsugae* Sasaji and McClure has been released at over 100 sites in 15 eastern states (Cheah et al. 2004), at densities ranging from 2400 to 10, 000 adults per site and up to 60 beetles per branch (McClure and Cheah 1999; McClure et al. 2000). Beat sheet sampling for *S. tsugae* in years following releases indicates this species will successfully overwinter, reproduce and disperse both laterally and vertically in the canopy (Cheah and McClure 2000; 2002). *Laricobius erichsonii* Rosenhauer, a European predator of the balsam woolly adelgid, was released at muliple sites in both eastern and western North America in the 1950's and 1960's. Between 50 and 2500 adults were released at each site in the Atlantic Provinces of Canada (Clark and Brown 1958) and between 1200 and 1700 per site in Washington and Oregon (Buffam 1962). Both confined and open releases of *L. erichsonii* were conducted and both methods resulted in successful establishment (Buffam 1962).

Environmental conditions to which *L. nigrinus* is subjected during their egg, larval, pre-pupal, and pupal stages may play a role in their establishment. Active adults seem to be resilient to extreme low temperatures and precipitation. However, eggs and larvae are susceptible to other predators and suffer high mortality at temperatures above 18°C (Zilahi-Balogh 2003c). Pre-pupae, pupae and aestivating adults are subject to ground predators and likely suffer high mortality rates in saturated soil after heavy rain.

Despite repeated sampling at regular intervals, *L. nigrinus* was not recovered in fall 2003, immediately following the spring release. There are several explanations for the lack of detection of $F_1$ predators. Populations of HWA crashed in the fall following this release and live adelgids were difficult to find in the lower canopy. The hemlock branches sampled with a beat sheet represented a small proportion of the total number of branches within the stand since only those within reach (< 2 m above ground) could be sampled. *L. nigrinus* density

may have been too low to detect in the fall following their release due to low survival or adult dispersal. In May and June 2003, the period in which *L. nigrinus* is pupating within soil, 684 mm of rainfall sustained ephemeral streams in the release site throughout the late spring and early summer and likely adversely affected *L. nigrinus* survival. The population of HWA was much lower in fall 2003, which may have forced adults to disperse in search of prey, perhaps to the upper canopy.

Repeated recovery of *L. nigrinus* $F_2$ adults from the lower canopy at the release site in fall 2004 suggests the field population is growing and may become established. A total of 6 adults were recovered from release and nearby non-release hemlock trees during 3 sample periods, indicating a detectable population of *L. nigrinus* at the study site.

*L. nigrinus* adult and larval feeding exerts a measurable impact on HWA populations. Although the density of progrediens was lower on branches that were caged with *L. nigrinus*, the average density of progrediens was >2/cm regardless of predator density (Fig. 2.3). Fluctuations in tree health and adelgid populations are typical following initial infestation (McClure 1991). The hemlock trees used in this study appeared to be healthy in January/February 2003, but few trees flushed new growth in spring 2003 and HWA populations crashed in fall 2003. The trees in the release stand did produce new growth in spring 2004 and HWA populations rebounded. It is premature to determine if *L. nigrinus* reduced HWA populations sufficiently or whether the decrease in HWA density occurred too late in the infestation's progression to rehabilitate tree health. While it is possible that site factors independent of HWA populations, such as canopy suppression or heavy rainfall, caused the observed decrease in HWA population and apparent decline in hemlock health the year following release, they are unlikely the primary causes. HWA population dynamics in

relation to hemlock health is not well understood, but it would be valuable to determine a physiological threshold that predators must reduce HWA below to prevent a decline in tree health.

Cheah et al. (2004) suggested that winter mortality of the sistens generation plays a significant role in adelgid population dynamics. Additionally, the presence of *L. nigrinus* throughout the year will complement abiotic mortality factors, and further decrease progrediens density since each adult feeds on approximately 270 developing sistens throughout the fall and winter, decreasing the number of adelgids that reproduce in the spring (Chapter 2).

One favorable attribute of *L. nigrinus* is their early season activity, which decreases HWA sistens density and avoids the active period of other predators. Thus, their actions are additive rather than competitive. The impact *L. nigrinus* exerts on HWA populations may not prevent a decline in tree health, but by decreasing the progrediens density by >50%, it will surely complement the actions of other HWA predators such as the Asian coccinellids: *S. tsugae*, and *Scymnus ningshanensis* Yu et Yao. *S. tsugae* has shown that it can significantly reduce the sistens generation following releases on progrediens (McClure et al. 1999). Butin et al. (2003) determined that *S. ningshanensis* had a significant impact on adelgid populations within sleeve cages. Interactions among *S. tsugae*, *Harmonia axyridus*, and *L. nigrinus* are currently being investigated (Flowers et al. 2005) and should prove useful in estimating competition among some of the predators in the developing natural enemy complex of HWA.

*Laricobius nigrinus* is considered a promising biological control agent for HWA because it has many favorable attributes and has demonstrated high survival and fecundity in Virginia forests. Our primary goal is to expedite the establishment of *L. nigrinus* by releasing

this species in newly infested forests on the expanding front of HWA. The recovery of adults 2 years after their release is encouraging because *L. nigrinus* survived despite the small size of the release and the harsh environmental conditions of that spring. In the early stages of field releases, when predator availability is low, it is critical to plan strategic releases by determining factors that influence establishment such as time of release, density of predators released, stage of HWA infestation within a stand, and environmental conditions following releases.

Time will determine whether or not biological control is a viable long-term solution to the hemlock woolly adelgid problem in eastern North America. Unfortunately, time is limited since hemlock mortality continues throughout the eastern U.S. *L. nigrinus* surviving 2 generations in Virginia marks a significant milestone in the effort to save *Tsuga canadensis* and *T. caroliniana*. *L. nigrinus* will continue to be released in stands throughout the eastern United States, constrained only by limitations in rearing capacity. Establishment of a population following an initial release of relatively few beetles bodes well for rapid establishment in the eastern U.S. The future of *Tsuga* spp. in the eastern states is uncertain, but no doubt brighter with the likelihood that widespread establishment of *L. nigrinus* is possible.

Table 3.1. Mean number of HWA sistens per branch at Big Stoney Creek, Virginia at the time of each release and average, maximum, and minimum temperatures and total rain recorded during each release period in 2003.

| Time of Release | Mean Sistens / Branch (±SE) | Temperature (°C)* | | | Total Rain Accumulation (mm)* |
|---|---|---|---|---|---|
| | | Average | Maximum | Minimum | |
| Mar 26 – Apr 6 | 703 ± 49.8 | 6.2 | 14.3 | -3.5 | 10.2 |
| Apr 23 – May 3 | 454 ± 26.6 | 11.4 | 20.6 | -1.2 | 39.1 |
| May 15 – May 25 | 98 ± 7.8 | 10.1 | 17.6 | 4.7 | 88.6 |

* Temperatures recorded with a datalogger within and outside sleeve cages were not different; rainfall was recorded at Mt. Lake Biological Research Station.

Table 3.2. Total predator eggs ($\overline{X}$ ± S.E.) found on branches caged with 1, 2, or 3 female *L. nigrinus* during each release period in March, April, and May that were used to estimate the number of eggs released at Big Stoney Creek, Virginia, in 2003.

| Time of Release | Total Eggs / Branch* | | | Replicates / Sample Period | Estimated Eggs Laid |
|---|---|---|---|---|---|
| | 1 Female | 2 Females | 3 Females | | |
| **Mar 26 – Apr 6** | 36 ± 3 c | 83 ± 4 b | 114 ± 5 a | 16 | 3728 ± 192 |
| **Apr 23 – May 3** | 14 ± 3 b | 41 ± 5 a | 39 ± 4 a | 32 | 3008 ± 378 |
| **May 15 – May 25** | 14 ± 2 b | 35 ± 2 a | 33 ± 3 a | 44 | 3608 ± 308 |
| | | | | | **10 344** |

***** Total *L. nigrinus* eggs left remaining on open branches in the field (no. of eggs multiplied by no. of replicates). Means sharing the same letter within each release period are not statistically different ($p < 0.05$; Fisher's LSD).

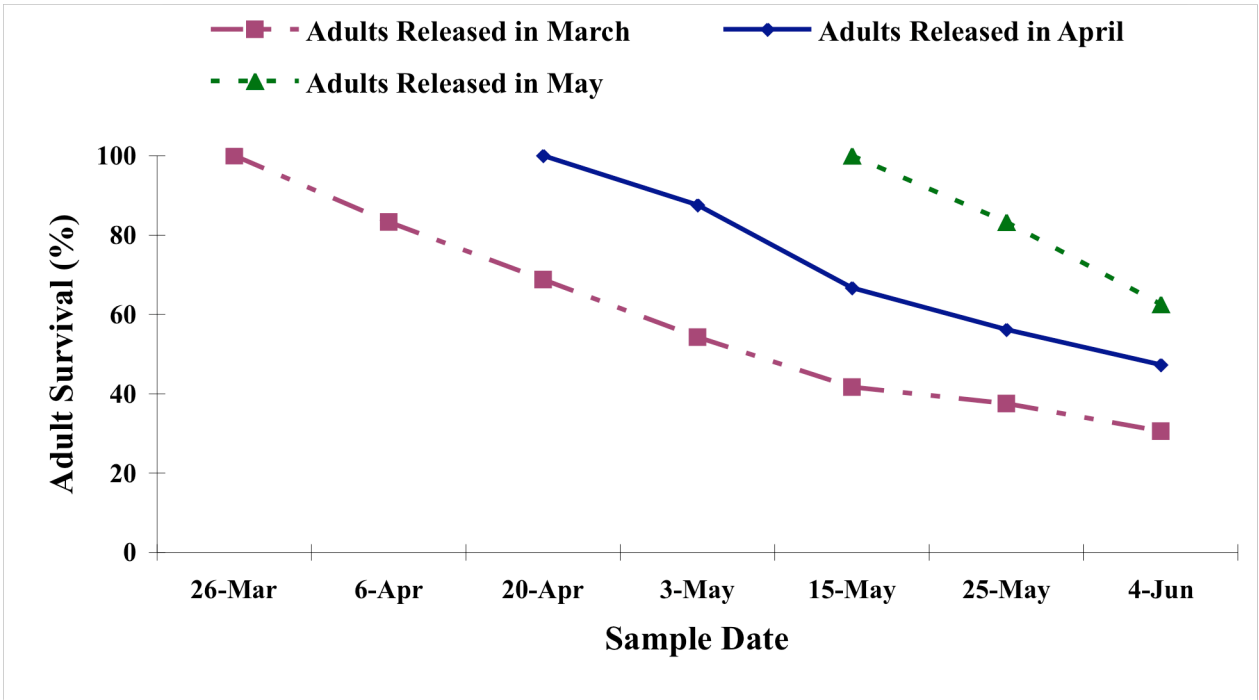



Figure 3.1. Percent survival of original *L. nigrinus* adults released in March, April, and May at each sample period near Big Stoney Creek, Virginia, in 2003.

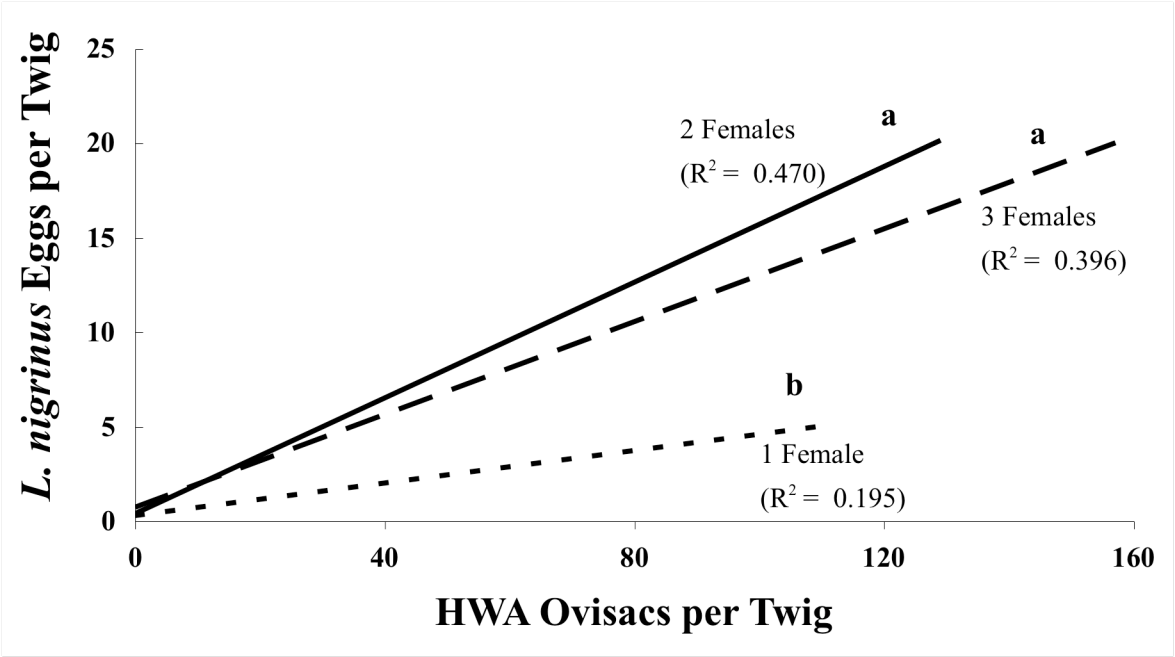



Figure 3.2. *L. nigrinus* oviposition in relation to the abundance of HWA ovisacs on branches with (dotted line) 1, (dashed line) 2, or (solid line) 3 *L. nigrinus* females at Big Stoney Creek, Virginia, in 2003; branches with 1 adult, $Y = 0.349 + 0.049X$ ($F = 230.19$, $p < 0.0001$); branches with 2 adults, $Y = 0.412 + 0.150X$ ($F = 732.47$, $p < 0.0001$); and branches with 3 adults, $Y = 0.632 + 0.135X$ ($F = 670.35$, $p < 0.0001$). Lines sharing the same letter do not have significantly different slopes ($p < 0.05$).

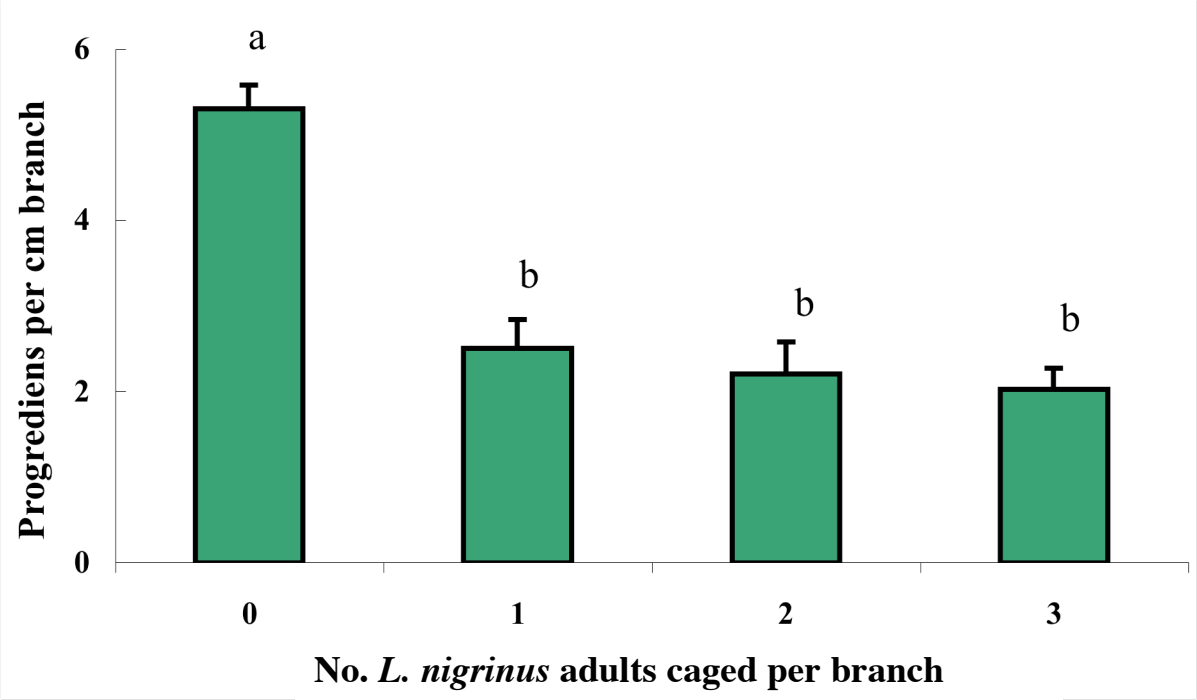



Figure 3.3. HWA density ($\overline{X}$ ± S.E.) on branches previously caged with 0, 1, 2, or 3 female *L. nigrinus* for 10 days at Big Stoney Creek, Virginia, in 2003. Means sharing the same letter are not significantly different ($p < 0.05$).

# Chapter 4

## Studies Related to the Development of Rearing Procedures for *L. nigrinus*

### Introduction

*Laricobius nigrinus* Fender (Coleoptera: Derodontidae) has been identified as a potential biological control agent for hemlock woolly adelgid (HWA), *Adelges tsugae* Annand (Hemiptera: Adelgidae), an exotic pest that attacks and kills hemlock trees (*Tsuga canadensis* and *T. caroliniana*) in the eastern United States. Establishment of *L. nigrinus* in the eastern U.S. is likely since caged field evaluations indicate *L. nigrinus* survival and reproduction in Virginia are high (Chapter 2) and $F_1$ and $F_2$ adults have been recovered from field release sites (Chapter 3, Mausel, unpublished data).

The ability to mass rear a natural enemy is fundamental in any classical biological control program, and delays in the program are often related to difficulties in laboratory rearing (Bellows and Fisher 1999). Biological control of HWA is no exception, and is currently hindered by poor success in mass rearing some promising predators. The development of efficient rearing methods for *L. nigrinus* is critical if they are to be considered a viable biological control option. This predator must be reared in large numbers on a continuous basis for field evaluations and mass releases. Time is of the essence as HWA currently infests Eastern hemlock in over 50% of its geographic range and threatens to spread throughout its entire native range in the eastern United States (Cheah et al. 2004).

A *L. nigrinus* colony has been maintained at Virginia Tech for several years, however, it has been constrained by high mortality rates and a lack of knowledge as to which life stages

incur significant mortality. *L. nigrinus* has an obscure and complicated lifecycle. It remains on hemlock branches from the fall through the spring and aestivates in the soil throughout the summer. Development of efficient rearing techniques is necessary for the mass production of this insect.

Our primary objectives were to:

a) identify the life stages incurring high mortality in the laboratory, and

b) optimize production of *L. nigrinus* at each life stage where low survival was observed.

To achieve this, accurate larval counts are essential, and a new rearing cage was devised that enabled counting of larvae reaching maturity and an estimate of pupal and aestivational success. Once stages suffering high mortality were determined several experiments were conducted between 2001 and 2005 to develop methods of rearing that reduced mortality at these stages. Source of adults was HWA-infested western hemlock near Victoria, BC, Canada (48.56°N, 123.43°W) in January 2001 and 2002, and November 2004.

The life cycle of *L. nigrinus* naturally splits into two distinct parts, feeding and non-feeding life stages. The feeding stage occurs on hemlock trees feeding on HWA (October - June), and in non-feeding stage ranges from pre-pupation to adults aestivating in the ground (April - October). Studies on factors affecting the feeding stages of *L. nigrinus* included: prey type and consumption, optimal storage temperature, density, feeding frequency, sex ratio, and lab-reared verses field-collected beetles. For the non-feeding stages, experiments focused on factors including: type of pupation medium and moisture level, disturbance, soil sterilization, density per container, type of pupation container, time of larval maturation, and environmental conditions during the summer.

**Methods and Materials**

All materials used within this chapter were used multiple times. For ease of reading, materials will be described upon their first mention and are the same materials in subsequent experiments unless otherwise stated. Refer to Figure 6.1 for images of each life stage of *L. nigrinus* and rearing conditions corresponding to each.

I. Survival of each Life Stage in 2001

In previous years, we used large, flat-bottomed Plexiglass® cages to rear *L. nigrinus*. Adults oviposit on HWA-infested twigs and these twigs were placed in floral foam to stay upright and moist. New infested twigs were added every week to feed the developing larvae. When the fourth instars reached maturity, they migrated down to the peat moss on the floor of the cage. There they pupated, and developed into adults.

A new larval rearing cage (Figure 4.1) was designed to intercept mature larvae as they dropped from the hemlock foliage. The top section of the new cage is an open-ended 30 cm diameter Dura-Lar™ acetate cylinder (0.007 thickness) (Grafix®, Cleveland, OH) with the top end covered with PeCap® polyester mesh (0.14 $mm^2$) (Sefar America Inc., Kansas City, MO). The base of the cage is a nonswirl galvanized steel funnel (McMaster-Carr Co., Atlanta, GA) with a 30 cm diameter, in which the acetate cylinder is placed. Hardware cloth (5x5 $mm^2$ mesh size) is cut to fit the inside of the funnels. The hardware cloth is placed inside the funnel base and rests where the funnel constricts. HWA-infested hemlock twigs with *L. nigrinus* eggs are inserted into fully water-saturated (Oasis® Deluxe) floral foam blocks (8x10x3 $cm^3$ bricks). Each floral foam block is wrapped in Parafilm M™ (Fisher Scientific,

Hampton NH) to help retain moisture and encourage mature larvae to continue searching for a pupation site (larvae were found inside unwrapped floral foam blocks in previous years).

Floral foam blocks containing hemlock branches with predator eggs were placed on the hardware cloth within the funnel cages. *L. nigrinus* eggs hatched and larvae developed on the hemlock foliage, feeding on HWA eggs within the funnel cages. When mature, the larvae dropped from the foliage and accumulated in the (Kerr®) 8 oz. Mason jars (Jarden Home Brands, Muncie, IN) attached beneath the funnel. The jars were spray-painted black and attached to the funnels by gluing the rims to the narrowest part of the funnel; this way Mason jars can be easily removed and reattached. Two teaspoons (30 ml) of steam-sterilized peat moss and 2 pieces of moistened filter paper (Whatman No. 1: 35 mm diam.) were placed in the Mason jars approximately three weeks after *L. nigrinus* eggs are added to the cage. After larvae completed feeding, they dropped from the hemlock foliage in search of a pupation site. Mason jars were checked daily for mature larvae and numbers from each funnel are counted.

Funnel cages were set up in custom-built racks and held in concrete-block rooms (cold rooms) cooled by air forced through water maintained at about 5°C, that are kept at a mean temperature of 13 ± 2°C with slight fluctuations influenced by outside air temperature, and a photoperiod that mimics that of natural conditions (increasing daylength from 12 to 14 h light over spring).

II. Rearing Procedures for each Life Stage

**A. Feeding Stages**

General Methods

Adults become positively phototactic and migrate to HWA-infested hemlocks upon completion of aestival diapause in autumn. The adults feed on developing HWA nymphs and

adults from this time until death in late spring. In early spring, adults begin laying eggs singly within the woolly ovisacs of HWA and oviposition continues as long as HWA eggs are provided, usually through late spring. Larvae develop through four instars by ingesting HWA eggs, and once sufficient feeding has taken place, mature larvae drop from the foliage in search of a pupation site.

In the laboratory, active adults are held in 3.78 L polyethylene terephthalate containers (General Bottle Supply Co., Los Angeles, CA) with moistened filter paper (15 cm diam.) cut to fit the bottom and two screen-covered holes to provide ventilation. One hole is in the lid (8 cm diam.), covered with polyester mesh, and the other is in the side, 3 cm from the bottom (3 cm diam.) and covered with metal mesh (0.10 $mm^2$ mesh size) (included with the funnels, McMaster-Carr Co., Atlanta, GA). Following emergence from summer diapause, adults are sprayed with a mist of distilled water every other day through the screened hole in the lid and if active before October, they are offered cold-stored progrediens, aestivating HWA nymphs, pine bark adelgid, and/or eastern spruce gall adelgid. However, survival of adults is low on these alternate prey items, and delaying emergence from aestivation until after October when HWA becomes available is a more desirable solution than finding alternative host material for early emerging adults (see Chapter 5).

Once HWA has completed diapause and nymphs are developing, adult containers are provided with one hemlock "bouquet" (a floral foam-filled film canister holding 15-20 heavily HWA-infested, 30 cm long hemlock twigs). The adult containers are stored in environmental growth chambers (Model I-36LL, Percival Scientific Inc., Des Moines, IA) programmed between 4 – 10°C, depending on the time of year (see Table 6.1). At each feeding, every adult is recovered from the hemlock bouquet in each container and transferred

to a fresh hemlock bouquet in a new container. During the oviposition period, the twigs from the old hemlock bouquets, containing the *L. nigrinus* eggs, are inserted into parafilm-wrapped floral foam and placed in funnel cages as described above.

Frequently, larvae that are not yet mature are found in the Mason jars and must be transferred back to HWA-infested hemlock and into funnel cages once again. We find immature larvae in the jars most often when larval density is too high, prey is insufficient, or when temperatures rise above 18°C. Since the recovery of immature larvae was labor intensive, determination of the survival of these individuals to adulthood was of interest.

Survival during the feeding stages is relatively high (70 – 100%) (Table 4.1). However, improvement is desired, leading to 10 experiments that look further at the basic biology and behavior of the predator, and attempts to determine optimal conditions for maximum production. Specifically, investigations examined adult feeding and survival post-emergence, adult sex ratio, storage density per container, feeding frequency, progeny production by lab-reared and field collected adults, and egg/larval survival.

1. *Adult survival at 4 and 13°C post-aestivation*

In anticipation of early predator emergence, eggs laid by sistens were stored in a refrigerator at 4°C from April to August, 2002. As adults emerged from aestivation ahead of schedule, 70 were transferred to each of 8 adult feeding containers, containing a bouquet of hemlock branches with diapausing HWA nymphs and one cold-stored branch with developing progrediens. Although this was not enough prey for the 70 adults, that was all that was available to provision the early emerging adults. All adults emerged on August 29, 2002 and every adult from each container was fed, or transferred to new hemlock foliage, every 12 days throughout September. Half the containers were stored in environmental chambers at 4°C

12:12 (L:D), while the other half were stored in the cold room at 13°C 12:12 (L:D). The number of adults surviving at each feeding period was recorded.

2. *Adult feeding on diapausing nymphs post-emergence in early September*

To determine whether adults will feed on diapausing HWA nymphs one week after emergence from aestivation, 25 adults were held separately and starved for 12 h at 4°C and 12:12 (L:D) photoperiod. Adults were held in 950 ml clear, polystryene containers with a polyester mesh-covered hole (10 cm diam.) in the lid and 2 layers of moistened filter paper (9 cm diam.). Each adult was then offered between 34 – 58 dormant 1st instars on 15 cm hemlock twigs for 13 days at 4°C and 12:12 (L:D) photoperiod. Adult survival was assessed and the remaining HWA nymphs were counted using a microscope. Consumption was calculated by subtracting the number of nymphs remaining from the original number offered.

3. *Male and female feeding rates during pre-oviposition period*

In November, approximately 8 weeks after emergence from aestivation, feeding tests were conducted to determine if consumption rates differed between males and females during the pre-oviposition period. On each of 50 HWA-infested hemlock twigs (16 - 20 cm linear length), HWA density was manipulated to have exactly 32 nymphs per twig. Sistens offered to adults were ~70% 3$^{rd}$ instars and ~30% 2$^{nd}$ instars. Twigs were from the same hemlock tree to reduce nutritional and developmental variation in the host.

Adult beetles were held separately in the same containers as described in experiment 2. After 15 h starvation at 12°C, the hemlock twigs with 32 HWA nymphs were randomly assigned to each container and adults were placed on each twig as twigs were introduced. Adults were held at 12°C, and after 48 h, each was removed from the twigs and the number of

live, intact HWA nymphs remaining were counted under the microscope. The number of sistens attacked was calculated by subtracting the number of live sistens, remaining on the twig, from the original 32 on each branch. Twenty-five adults of each sex that emerged from aestivation between August 20 – October 19, 2004 were tested, and the mean attack rate was compared using a 2-tailed t-test (Sall, 2001).

4. *Adult feeding rate at 4 and 12°C on various HWA densities during pre-oviposition period*

In December, approximately 10 weeks following emergence from aestivation, feeding tests were conducted on recently emerged adults to determine how consumption rates differ with temperature and host density during the pre-oviposition period. The same method as in Exp. 2 and 3 was used, with adults held separately for 12 h at 8°C prior to the experiment. Hemlock twigs (16 - 20 cm total linear length) were manipulated under the microscope to have exactly 3, 6, 12, 24, 36, or 48 HWA per twig. After 12 h starvation, each adult was randomly assigned a temperature and HWA density. Each adult was placed on the twig as it was introduced into each container. After 48 h at the assigned temperature, adults were removed from each container and the number of live, intact HWA remaining on each twig was determined using a microscope. Adult feeding rate was compared among temperatures at each adelgid density using a 2-factor analysis of variance (n = 60).

5. *Adult sex ratio within the colony*

To determine the adult sex ratio within the colony, containers were randomly selected from the colony and each adult was held in separate 384 ml, clear plastic containers with a heavily HWA-infested hemlock twig (20-30 cm total linear length). After 3 days, each twig was examined under a microscope for eggs. Adults that did not deposit eggs were placed

back in separate containers with fresh host material for another 3 days. Following the second test, adults that did not deposit eggs were considered to be males or unfertile females. In March and April, 2003, adults from 21 containers were sexed in this manner (n = 699).

6. *Comparing duration of oviposition periods*

In spring 2001, throughout the oviposition period, adults were arbitrarily maintained in adult containers in groups of 20 and were offered new prey at various frequencies. Oviposition duration (feeding frequency) ranged between 2, 3, 4, 5, 6, 7, 8, 9, 10 and 14 days for each group of adults. Twigs from each adult container were transferred to one corresponding funnel cage and the number of mature larvae dropping from each funnel was recorded. The number of larvae produced was compared to the number of days adults were allowed to oviposit.

7. *Adult density per oviposition container*

At the beginning of the oviposition period in January 2002, adults were held at densities of 10, 20, and 30 adults per container. Fresh prey was provided once a week by removing each adult and transferring it to fresh foliage in a new container. The branches from which adults were removed and transferred to corresponding funnel cages (one funnel cage per adult container). Following the third feeding period (February 18), adults stored at 10 per container were split in half and added to the groups of 20 and 30 per container to obtain densities of 25 and 35 adults per container. This was because adult storage at densities of 10 per container was impractical, proving to be an inefficient use of space.

Twigs from each container were transferred to one corresponding funnel cage weekly, except during weeks 8-10 (March 25 – April 8), when branches from each adult container

were split and transferred into 2 funnel cages because space and funnel cages were not limited. The larvae that matured from each funnel were tallied daily throughout the spring, and the number of larvae produced per beetle was calculated for adults stored at each density throughout the 12-week oviposition period.

8. *Progeny production of lab-reared and field-collected adults*

To determine whether laboratory rearing affects the fecundity of adults, lab-reared and field-collected beetles were maintained separately throughout the year. Field-collected beetles were obtained as adults from hemlock trees in Victoria, BC, Canada in January 2003. This study is similar to that described in experiment 7, except all adults were held at densities of 30 per container. Beginning in January 2003, branches from each group of adults (4,726 lab-reared and 663 field-collected) were transferred to corresponding funnel cages. Branches from 2 or more adult containers were transferred to one funnel cage because funnel cages were limited. The number of mature and immature larvae dropping from each funnel was recorded daily. The average number of larvae produced per beetle was calculated and compared between field-collected and lab-reared adults throughout the year.

9. *Survival from egg to mature larvae in funnel cages*

Suitable host material with 10, 20, 30, 40, or 50 *L. nigrinus* eggs were placed in each of 20 funnel cages with an adequate amount of prey. The numbers of larvae that reached maturity and dropped to the Mason jars below each funnel was quantified. This was a generalized randomized block design, with larval cohort serving as a block (2), with 2 replicates of each predator density per block. A one-way analysis of variance was used to determine whether the egg densities influenced larval survival (n = 20).

10. *Survival of "immature" larvae*

Immature larvae are usually smaller, darker in color, less mobile than mature larvae, and they 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 like mature larvae. 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 9 "immature" funnel cages. The number of mature larvae collected from each of these funnel cages was recorded and the overall survival rate was calculated.

The mature larvae collected from the "immature" funnel cages were transferred to corresponding pupation 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 larvae was calculated.

**Results**

I. Survival of each Life Stage in 2001

The new rearing cages (Figure 4.1) allowed us to quantify the number of larvae reaching maturity and estimate survival of pupae and aestivating adults, contributing to our understanding of *L. nigrinus* mortality at each life stage. The colony began with 350 field-collected adults, which produced 7500 larvae and 72% (5175) of these survived pupation. Adults suffered high mortality during aestivation and immediately following emergence, with only 200 adults surviving until the fall (Table 4.1).

There are several advantages to the funnel cage. *L. nigrinus* larvae develop within the funnel without additional maintenance such as adding foliage or searching for lost larvae

under the microscope as was done in previous years. Collection of *L. nigrinus* at this stage enables us to estimate egg and larval survival. By knowing the original number of *L. nigrinus* pre-pupae, survival through pupation and aestivation can be calculated. A third benefit to this cage is that during the spring, there is a consistent, easily 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 and require little maintenance during development.

There are two disadvantages to the funnel cages. Checking the jars daily for mature larvae is labor intensive and costly. Mature larvae must be transferred within 36 h of dropping, before larvae create a pupal cell and become immobile. In addition, if there is not an adequate amount of prey within the funnel cages, large numbers of immature larvae end up in the jars and must be transferred back to hemlock branches with HWA eggs. High numbers of immature larvae dramatically increase the time required to check the funnels jars.

II. Rearing Procedures for each Life Stage

1. *Adult survival at 4° and 13°C post-aestivation*

Adult survival was higher and more consistent following emergence from aestivation when held at 4°C than at 13°C (Figure 4.2). Mortality rate was high during the first 12 days; over 60% of adults stored at 13°C died compared with less than 13% mortality in adults held at 4°C. On Oct. 2, after three feedings, 5.7% of the adults held at 13° were alive, whereas 64.2% of the beetles held at 4°C post-emergence survived (total adults = 560).

2. *Adult feeding on diapausing nymphs post-emergence in early September*

Only 4 of the original 25 adults were alive at the end of the 13-day feeding test in September. Mean consumption rate ($\bar{X}$ ± S.D., n = 25) of the 4 live adults was 19.5 ± 6.4 nymphs, whereas the mean consumption of all adults (both alive and dead) was 23.8 ± 2.9 nymphs.

3. *Male and female feeding rates during the pre-oviposition period*

There was no adult mortality during the feeding test in November, 2004. On average ($\bar{X}$ ± S.E., n = 50), females consumed slightly more HWA nymphs per day (7.6 ± 0.4) than males (6.6 ± 0.4) in a 48 h period, however these rates did not differ statistically ($t_{48}$=1.74, p = 0.0879).

4. *Adult feeding rate at 4° and 12°C during the pre-oviposition period*

Adult mortality during the feeding test in December, 2004 was also zero. At both temperatures, adult feeding rate rose with increasing densities of prey ($F_{(4,59)}$ = 16.4, p < 0.0001) (Figure 4.3). In addition, adults consumed more HWA nymphs at 12° than at 4°C when offered 24 or 48 nymphs/twig ($F_{(1,59)}$ = 4.67, p = 0.036).

5. *Adult sex ratio within the colony*

Of the 699 adults sexed, 458 were ovipositing females. From 21 adult containers studied, the average ($\bar{X}$ ± S.D.) female to male ratio within a container during the peak oviposition period (March and April) was 1.91 ± 0.18:1 per container.

6. *Comparing duration of oviposition periods*

Adults fed frequently (every 2 - 3 days), produced as many progeny as adults fed every 2 weeks (Figure 4.4), indicating that longer ovipositional periods (feeding frequency) do not yield a greater number of mature larvae. The mean number of larvae per container ($\overline{X}$ ± S.D., n = 274) was highest (94.4 ± 54.3) when adults were fed every 6 days.

7. *Adult density per oviposition container*

The number of mature larvae produced per beetle was higher when adults were held at lower densities throughout most of the oviposition period (Figure 4.5). The number of progeny per beetle ($\overline{X}$ ± S.D., n = 274) increased when hemlock twigs containing *L. nigrinus* eggs were split into 2 funnels (eggs transferred to funnels between March 15 and April 15). In addition, there were more immature larvae collected from funnels with progeny produced by 35 adults (2,964) than from funnels with progeny produced by 25 adults (2,068), suggesting that maintenance of adults at higher densities is less than optimal.

8. *Progeny production between lab-reared and field-collected adults*

The number of progeny produced per beetle was higher in field-collected beetles than lab-reared beetles throughout most of the oviposition period (Figure 4.6). Peak oviposition was slightly earlier in field-collected than lab reared adults. In addition, the average number of immature larvae ($\overline{X}$ ± S.D., n = 580) produced by field-collected adults (3.90 per beetle) was ~5 times higher than those produced by lab-reared beetles (0.76 per beetle).

9. *Survival from egg to mature larvae in funnel cages*

Density of eggs per funnel cage (up to 50 individuals per cage) did not affect larval survival ($F_{4,19} = 0.90$, $p = 0.490$). The mean percentage of eggs ($\overline{X} \pm$ S.D., n = 20) that developed to larval maturity at all densities was 73.7 ± 15.4%.

10. *Survival of "immature" larvae*

There were 6116 immature larvae found in Mason jars throughout the spring of 2002. Of these, 3486 reached maturity after being transferred back to funnel cages with prey. Overall, 57% of the immature larvae collected from Mason jars survived and completed larval development.

In the spring of 2003, 8002 immature larvae were collected from the Mason jars and transferred back to funnel cages to complete development. Of these, 3905 larvae (48.8%) completed development and entered the soil for pupation and aestivation. In autumn, 1843 of these individuals emerged from aestivation, representing 23% of the larvae originally found to be immature.

**Discussion**

Survival was low when adults emerge from aestivation before October. For adults that emerged early, survival increased by maintaining them at 4°C. Since aestivating 1st instar sistens are not suitable food post-emergence, HWA eggs laid by sistens should be stored at 4°C throughout the summer to provide early emerging adults with developing progrediens nymphs.

At 12°C, males and females had statistically similar consumption rates during the pre-oviposition period; females consumed slightly more HWA nymphs than males, but rates

commonly overlapped. Attack rate is possibly related to the size of individuals as females are slightly larger than males (Zilahi-Balogh 2001). Adults fed at only marginally higher rates at 12°C than at 4°C during the pre-oviposition period. At both temperatures, adults fed at a higher rate as prey density increases and as temperature increases. To ensure maximum developmental potential, each adult should be provided with about 20 HWA nymphs/day in autumn.

Larval production was much higher in 2002 than 2003, even between field-collected adults stored at comparable densities per container. As observed in 2003, when *L. nigrinus* egg density per cage increases, larval production per beetle decreases and the number of immature larvae dropping into the Mason jars increases. Since larval survival is high in funnel cages at low predator densities, and no immature larvae are produced, larval production can be maximized by a low predator density per funnel cage.

There are several methods to minimize *L. nigrinus* egg density within funnel cages. The methods used to minimize predator density will depend on location-specific limiting factors such as amount of environmental chamber space or technical assistance. Adults can be stored at low densities (as low as 10 beetles/container) and should be fed as often as twice per week, however this often requires more labor and funnel cages than are available. A second method to reduce predator density in the funnel cages may be to split the branches from each adult container into two funnel cages, as was done in 2002. By minimizing larval density per funnel cage, more progeny per beetle will be produced and the number of immature larvae falling in the Mason jars will be reduced.

A regular infusion of field-collected beetles would be beneficial for the colony since field-collected adults seem to be more fecund than lab-reared beetles. In field studies

conducted in 2002 and 2003, fecundity measured in lab-reared beetles was similar to fecundity observed in field-collected beetles in 2001 (Chapters 1 and 2). Inbreeding depression can result in reduction of physiological fitness and reproductive capacity over time. This has been recognized by many biological control workers in the past and periodic supplementation of field-collected beetles is recommended by Etzel and Legner (1999).

**Methods and Materials**

**B. Non-feeding Life Stages**

Mature larvae burrow into the soil immediately upon dropping from the hemlock foliage. In the lab, these larvae were transferred from the Mason jars below the funnel cages to pupation containers for pupation and aestivation. These containers have 2 layers of filter paper to prevent pooling when the pupation medium is moistened with methyl paraben solution (0.42g/250 ml distilled water) throughout the summer to inhibit fungal growth. At least 5 cm of pupating medium is added to each pupation container. Pupation medium is made with a 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 (3x3 $mm^2$) and sphagnum moss is ground in an industrial blender and sifted through hardware cloth. The mixture is moistened and steam-sterilized for 12 h twice, separated by 24 h at room temperature. A variety of pupation containers are used, all are plastic with at least one polyester mesh-covered hole for ventilation. Larvae burrow into the pupation medium and after creating a cell within the soil, assume a c-shaped position and develop into pupae in approximately 14 days.

Pupation containers are kept in a cold room at 15 ± 2°C and 12:12 (L:D) photoperiod since this temperature is optimal for pupal development (Zilahi-Balogh et al. 2003c). 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, which may occur over a period of several months (August - December). Emerging adults are collected from the sides and lids of the pupation containers and transferred to adult containers.

11. *Pre-pupal survival, pupal sex ratio, and timing of male and female emergence from aestivation*

Sex cannot be determined in the adult stage, since genetalia retract into the body after eclosion (Zilahi-Balogh 2001). To determine the sex ratio of progeny, pupae are dug out of the soil and sexed using a microscope and external genetalia characters described by Zilahi-Balogh et al. (2002). In spring 2003, six pupation containers were randomly selected from the colony 3 weeks after larvae had entered the soil. Using a paintbrush, the pupation medium was sifted through and each pupa was 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° ± 2°C and 14:10 (L:D) photoperiod and watered weekly) throughout the summer and the number of adults emerging from each container in the fall were recorded daily.

In spring 2004, 28 pupation containers were selected to assess pupal survival and sex ratio. During that year, there was considerably more mold development in pupation

containers than in previous years, even though the methods used for preparing the pupation medium did not change between years. Pupation containers with high, medium and low levels of mold were selected three weeks after larvae had entered the soil. Levels of mold were assessed for each container with the naked eye using the following criteria: high levels had deformed surfaces, low containers had mold present in patches, and medium levels had mold consistently through it. For each container, the pupation medium was sifted and all surviving pupae were sexed and transferred to corresponding male and female containers with fresh pupation medium. These containers were maintained with the rest of the colony (15 ± 2°C and 12:12 (L:D) photoperiod until adult eclosion, 19°C, 16:8 (L:D) until September 27, 2004 and then changed to 13 ± 2°C, and 10:14 (L:D) photoperiod until emergence). Each container was watered weekly throughout the study period and the adults emerging from each container in the fall was recorded.

12. *Optimal container type for pupation and aestivation*

From March through June, the Mason jars below the funnels were checked daily and mature larvae were transferred to pupation/aestivation containers. To determine an ideal container for pupation and aestivation, a variety of containers was used for the colony in 2002 and 2003. The containers used varied in type of plastic, size, and the location of ventilation holes. Two types of plastic containers were used, clear (polystyrene and polyethylene terephthalate) and opaque (low density polyethylene). For each type of plastic container used, there were three sizes: small (473 ml), medium (950 ml), and large (1.82 L) size. Ventilation for some containers was created with a pair of 3 cm diameter, polyester mesh-covered holes in the sides of the containers and others have 8 cm diameter holes in the lids.

In 2002, mature larvae were transferred to all sizes of the clear plastic containers before opaque ones were used. The total number of mature larvae and depth of pupation medium was recorded for each container type. In the fall, the visible presence or absence of mold was recorded for each container and the number of adults emerging from each container was noted. The effect of container type, soil depth, mold and time of entry into pupation container on *L. nigrinus* survival during the period spent underground was determined.

In 2003, mature larvae were transferred to each of the nine container types in a systematic fashion, to ensure that all larval cohorts would be held in each container type. The total number of mature larvae and container type was recorded as each container was set up in the spring. In the fall, the adults emerging from each container were recorded and compared across container types.

13. *Effect of soil moisture and disturbance on pupal survival and adult emergence from aestivation*

The effect of moisture level and disturbance, within the pupation medium, on the survival of aestivating adults was investigated by placing 10 mature larvae in each of 48 clear polystryene containers (950 ml). Each container had 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; potting soil:peat moss) (Miracle-Gro®, Scotts Company, Marysville, OH) and were 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 every other day throughout the study (containers set up at same time using same method, but no larvae were added). Moisture

levels were maintained by adding the same amount of water to both the test containers and the control containers.

Half the containers at each moisture level were randomly selected to receive a "disturbance" treatment, in which the pupation medium was sifted through and the number of live individuals were assessed. The pupation medium was sifted twice; three and six weeks following entry into the soil. In this way, survival to the pupal stage could be assessed at each moisture level. Surviving individuals were put back in the pupation medium in the container and the total number of adults emerging from each container in the fall was recorded daily. The effect of moisture level and disturbance of pupae and aestivating adults on the total number of adults emerging was determined. This experiment was set up as a randomized complete block design with larval cohort serving as blocks (8). The effect of moisture and disturbance on pupal survival and adult emergence was determined with a 2-factor ANOVA using proc glm, means were separated using Fishers LSD (n = 48) (SAS Institute, 1992).

14. *Effect of pupation medium and moisture level on adult emergence from aestivation*

Four types of media were maintained at three moisture levels to test for suitability during *L. nigrinus* pupation and aestivation. The objective was to increase the duration of diapause or survivorship of *L. nigrinus* at the pupal and adult stages. The experiment was set up as a randomized block design with 8 replicates in each of 4 blocks. Larval cohort served as the block. The four types of pupation medium varied in concentration of ground sphagnum moss to peat moss to 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 2 layers of moistened filter paper and 4 cm of pupation medium. Batches of 5 mature larvae were added to each of the 96 containers in sequence, and repeated 4 times,

resulting in 20 genetically diverse larvae in each container. A third of each type of pupation medium was maintained at each 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. The moisture level of control containers was measured each week using a Lincoln® soil moisture meter and 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 8 weeks after entry into the pupation container and the pupation media was scooped out of containers and combed thoroughly for recently eclosed adults under the microscope. The survivorship and approximate depth at which each adult was found were recorded. The total number of adults and the time at which they emerged from each container was recorded daily from July 22 to December 11, 2002. The proportion of adults emerging and average time of emergence was compared across treatments using a 2-factor ANOVA in SAS and means were separated using Fishers LSD.

15. *Adult emergence in sterilized and unsterilized pupation medium*

Mature larvae were transferred to steam-sterilized and unsterilized pupation medium to determine the effect of sterilized medium on adult emergence from aestivation. The pupation medium used was a mixture of 2:2:1 [sphagnum:peat:sand], which was maintained at 45% saturation level. Two layers of moistened filter paper and 5 cm of pupation medium was added to each of 26 plastic containers (950 ml low density polyethylene), two of which served as moisture controls. Batches of 5 mature larvae were added to each of the 24 containers and repeated 8 times, resulting in 40 genetically diverse larvae per container. The

moisture level of each control container was measured and manipulated each week and the same amount of methyl paraben solution (by weight) was added to each of the test containers.

The total number of adults emerging from aestivation and the average duration of aestivation was determined for each container. This experiment was set up as a completely randomized design with 12 replicates. The proportion of adults emerging and average time of emergence was compared across treatments using a 1-way ANOVA in SAS.

16. *Optimal larval density per container*

Three levels of larval density per container were compared using a randomized block design with larval cohort serving as the block. For each block, 13 pupation 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. In order, 5, 10, or 15 mature larvae were added to each of 12 containers and repeated 6 times to produce densities of 30, 60, and 90 individuals/container. There were four replicates per block and a total of five blocks were set up on 5 consecutive days in May. The 13$^{th}$ container in each block served as a moisture control. Pupation medium was maintained at 45% saturation by monitoring and manipulating the moisture level of the control containers using the Lincoln® soil moisture meter and distilled water weekly. Equal volumes of methyl paraben solution were added to the test containers as the control containers using a scale. The total number of adults to emerge and duration of aestivation was determined for each container and compared across treatments using a 1-way ANOVA in SAS.

17. *Emergence from aestivation following storage in cold room verses outside*

Twenty mature larvae (batches of 5, repeated 4 times) were transferred from Mason jars to each of 32 organic planting containers (8 oz, Westsel Inc., Harrisonburg, VA). Each planter contained pupation medium mixed at 2:2:1 (sphagnum:peat:sand) added to a height of 8 cm. The open tops of each planter were covered with parafilm and held in place by rubber bands for 72 h to ensure larvae would burrow down into the pupation medium. Each planter was then put in a nylon mesh sleeve (#110 mesh, Dynamesh, Chicago, IL) and planted within a natural medium collected from a local conifer forest floor, in one of four perforated, metal trays with the sleeve openings pointing up. The ends of each sleeve were closed off with wire (20 gauge) and fastened to a wire (20 gauge) frame that held the sleeves upright.

Two trays were stored in the cold room (constant 14:10 (L:D), 15° ± 2°C) and two were planted into the ground at Price's Fork Research Station outside Blacksburg, VA, underneath white pine trees at a depth of 20 cm. The moisture level inside and outside the planters was measured once a week. Moisture level of the trays in the cold room was maintained at levels comparable with trays outside by adding distilled water weekly. Water was not added to trays outside. Beginning in July, the sleeve cages were checked for emerging adults every other day and the timing of each emerging adult was recorded. The time of emergence were compared between those stored in the cold room throughout the summer and those stored under natural field conditions. The number of beetles to emerge were not compared because adults emerging from the ground at Price's Fork had much less opportunity to be collected than those in the cold room.

## Results

### B. Non-feeding Life Stages

11. *Pre-pupal survival, pupal sex ratio, and timing of male and female emergence from aestivation*

The average percentage of larvae ($\overline{X}$ ± S.D., n = 6) that developed into pupae per pupation container was 58.7 ± 18.2% in 2003. Of the 183 pupae sexed, 95 were female and 88 were male, resulting in an average sex ratio ($\overline{X}$ ± S.D., n = 6) of 1.08 ± 0.51:1 F:M per adult container. Females and males had similar survival throughout aestivation (64.2 and 64.7%, respectively), however males emerged slightly earlier than females (Figure 4.7A).

In 2004, the percentage of larvae to develop into pupae was significantly affected by the presence of mold in the soil (Figure 4.8). Survival of pupae was dramatically higher in containers with light levels of mold (52%) than containers with heavy levels of mold (3%). Of the 933 pupae sexed, 515 were female and 418 were male. On average ($\overline{X}$ ± S.D., n = 28) the ratio of female to male pupae per container was 1.19 ± 0.74:1. Male survival through aestivation (40.9%) was slightly higher than female survival (35.7%), but the time at which they emerged was more synchronized than the previous year (Figure 4.7B). The difference in 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.

12. *Optimal container type for pupation and aestivation*

In 2002, the variation in percentage of adults emerging per container was very high (between 0 and 100%). The presence of mold seemed to affect the number of adults emerging in the fall. The mean percentage of adults emerging ($\overline{X}$ ± S.D.) from containers that were

mold-free at the end of the summer had 56.2 ± 21.2% of individuals emerge whereas, only 34.3 ± 22.6% of individuals emerged from containers with moldy soil (Figure 4.8).

Adult emergence decreased as the depth of soil increased ($F_{483}$ = 60.63, p< 0.0001) (Figure 4.9A). There appears to be a relationship between container type and adult emergence or larval cohort and adult emergence. Adult emergence was higher when stored in clear plastic containers than in stored in opaque containers (Figure 4.10A). However, since container type was not spread over larval cohorts, it is not possible to separate the effect of these two variables. The relationship between the number of adults emerging from each container following aestivation and larval cohort is significant ($F_{483}$ = 71.49, p < 0.0001) (Figure 4.11A); percent emergence decreases as larvae enter the soil later in the year.

In 2003, there was no relationship between adult emergence from aestivation and depth of soil ($F_{199}$ = 0.32, p = 0.572) (Figure 4.9B), however, containers did not have soil deeper than 50 mm. Mature larvae were transferred to 9 different types of pupation containers systematically throughout the spring to determine if container type affects the number of adults that are collected following emergence from aestivation in the fall. The average emergence ($\overline{X}$ ± S.D., n = 207) from the clear plastic containers was 59.7 ± 25.1%, whereas the average number of individuals to emerge from opaque plastic containers was 51.9 ± 30.9%. The average emergence ($\overline{X}$ ± S.D., n = 207) of individuals in containers with screened lids was 59.1 ± 31.3%, and 52.7 ± 26.9% without screened lids. Container type may influence adult emergence slightly (Figure 4.10B). The time at which larvae entered the pupation container again affected the percentage of adults emerging from aestivation ($F_{199}$ = 5.93. p < 0.0001); adult emergence decreases as larvae enter the soil later in the year (Figure 4.11B).

13. *Effect of soil moisture and disturbance on pupal survival and adult emergence from aestivation*

Moisture level did not affect the development of pupae ($F_{23} = 0.03$, $p = 0.9709$). The mean percentage of larvae ($\overline{X}$ ± S.E., n = 24) developing into pupae at all moisture levels was 69.1 ± 2.3%. The number of adults to emerge from aestivation in the fall was significantly affected by moisture level ($F_{(2, 42)} = 6.02$, $p = 0.0050$) and by the disturbance of sexing pupae ($F_{(1, 47)} = 4.08$, $p < 0.0498$). More adults emerged from containers held at 40% or 20% moisture level than at 5% (Figure 4.12). The disturbance caused by recovering individuals from the pupation medium resulted in a 10% reduction in adult emergence. The mean emergence ($\overline{X}$ ± S.E., n = 24) of undisturbed adults was 49 ± 2.3% whereas only 39 ± 3.0% of the individuals that were dug up from the pupation medium as pupae emerged as adults.

14. *Effect of pupation medium and moisture level on adult emergence from aestivation*

Mean pupal survival was 72.1 ± 3.5% ($\overline{X}$ ± S.E., n = 12), and was not affected by the compostion 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 significantly lower for individuals held in pure peat moss than those held in pure sphagnum moss or a mixture of the two mosses (Figure 4.13). The highest mean emergence ($\overline{X}$ ± S.E., n = 372) was observed from containers with 1:2 sphagnum:peat moss ratio (61.5 ± 0.8%) and the lowest was from containers with pure peat moss (52.8 ± 1.1%). Additionally, soil moisture affected the percentage of adults emerging from aestivation (Figure 4.14). Beetles stored in soil maintained at 30% moisture level emerged in greater numbers on average, than those held at higher moisture levels.

The timing of emergence from diapause was not affected by type of pupation medium ($F_{(3, 370)} = 0.30$, $p = 0.822$). On average ($\overline{X}$ ± S.E., n = 372), adults remained in the ground for 123.9 ± 0.3 days. Moisture level did affect time of emergence, adults stored in soil maintained at 30% or 45% saturation emerged before those held in soil maintained at 60% saturation ($F_{(11, 370)} = 22.51$, $p < 0.0001$). However, the duration of aestivation of adults stored at 60% moisture level (126.4 ± 0.2) was only 4 days longer, on average ($\overline{X}$ ± S.E.), compared with the duration at lower moisture levels (122.6 ± 0.3 days).

15. *Adult emergence in sterilized and unsterilized pupation medium*

Significantly more *L. nigrinus* adults emerged from aestivation ($\overline{X}$ ± S.E., n = 24) when stored in steam-sterilized pupation medium during pupation and aestivation (65.0 ± 2.7%) than those adults undergoing pupation and aestivation in unsterilized soil (49.8 ± 2.4%) ($F_{(1,22)} = 9.25$, $p = 0.006$). The timing of emergence from aestivation was not influenced by whether the pupation medium was sterilized or unsterilized ($F_{(1, 22)} = 3.36$, $p = 0.0806$); on average ($\overline{X}$ ± S.E.), adults remained in the soil for 138.8 ± 0.8 days.

16. *Optimal pre-pupal density per container*

The density of adults per container did not influence the percentage of adults emerging from aestivation ($\overline{X}$ ± S.E., n = 60) (32.2 ± 4.6%) or the duration of aestivation ($\overline{X}$ ± S.E., n = 60) (142.7 ± 0.6 days) ($F_{(4,53)} = 1.73$, $p = 0.1865$ and $F_{(4, 53)} = 0.02$, $p = 0.9836$, respectively).

17. *Emergence from aestivation following storage in cold room verses outside*

Adult emergence for beetles stored at 15 ± 2°C and 14:10 (L:D) photoperiod, in the lab, was much earlier and more spread out than those stored outside, under natural conditions

(Figure 4.15) (320 larvae placed in each condition). More adults were collected from trays stored in the cold room than outside, however, this is likely related to their location and the frequency at which adults may have been collected compared with those stored in the ground at Price's Fork. Attention should be focused on the peak emergence of individuals stored in the cold room at 15° ± 2°C and 14:10 (L:D) photoperiod occurred approximately 9 weeks before the peak emergence of adults stored outside. In addition, adult synchrony of emergence seems to increase under natural conditions since the period of adult emergence was shorter for adults stored outside than for those stored in the cold room.

**Discussion**

The sex ratio of pupae in mold-free pupation medium is equal and pupal survival was lower in 2003 and 2004 (~60%) than in previous years. Emergence of males occurred earlier than females when held at constant 15° ± 2°C and 14:10 (L:D) photoperiod, however this pattern was not observed in 2004 when adults were moved from summer conditions to cooler temperature in the fall. This may explain the high female:male ratio observed in ovipositing females that spring in 2003. Early emerging adults had the highest mortality, which may have been mostly male, since they emerge first after being held at 15°C throughout the summer. When maintained at a high temperature after adult eclosion and dropped to 13° ± 2°C and 12:12 (L:D) photoperiod in late September, the emergence period of adults was much shorter than the emergence period of adults the year before. The treatment of high temperature over the summer may help to synchronize adult emergence and shorten the period in which adults must be collected from pupation containers daily (see Chapter 5).

Moisture level, type of pupation medium, and sterilization of pupation medium influence the number of adults that emerged. Emergence is highest in moisture levels of 30 - 40% saturation, however a wide range of moisture level is tolerated. This is advantageous because of the wide range of precipitation from year to year. Adults have the highest emergence from a mixture of sphagnum and peat mosses that have been steam-sterilized twice. Although these factors affect emergence, larval cohort often accounts for much of the variation observed in emergence, suggesting there are unexplored factors involved. Two factors that were monitored but were not investigated in detail are mold in the pupation medium and time at which larvae entered the pupation medium.

The number of adults emerging from aestivation is strongly influenced by the level of mold in the pupation medium. Adult emergence from moldy pupation containers is very low, and mortality in these containers occurs after larvae enter the pupation containers, but before individuals eclose as adults. Dr. Richard Humber, USDA-ARS in Ithaca, NY, identified the fungus as a *Penicillium* spp. that is most likely a saprobe with the pupation medium. However, it is possible that is fungus is causing microconditions within the pupation medium that is lethal to pupating *L. nigrinus*. Modifications of the rearing procedures to limit mold formation have been implemented including: eliminating the use of filter paper, autoclaving the pupation medium, and watering with a mixture of methyl paraben and sorbic acid.

The time at which larvae enter the soil ranges over a period of 12 - 15 weeks, and seems 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. In all the experiments involving

mature larvae, larval cohort was a blocking factor and, in most cases, described the variation in adult emergence better than the factors being tested.

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 sistens and those maturing later in the season feed on eggs laid by progrediens. By late spring, two generations of HWA have fed on the hemlock trees, which may be depleted in resources by late spring, possibly affecting the nutrition of HWA. The nutritive chemical composition of host plants can affect the quality of phytophagous hosts, which has been known to affect their predators (Rice and Wilde 1989, Legaspi et al. 1996), particularly *S. tsugae* (Palmer and Sheppard 2002), another predator of HWA. For the colony, trees with the highest density of HWA are deliberately chosen to maximize the amount of prey collected that can be provided to *L. nigrinus*. However, hemlock trees of slightly lower infestation levels may be more suitable for colony rearing, if healthier trees do in fact influence *L. nigrinus* survival.

Clear plastic containers appear to be better than opaque plastic containers, however the differences are small. Experiments conducted in Chapter 5 indicate that *L. nigrinus* does monitor photoperiod while in aestivation and this is perhaps more detectable in clear plastic rather than opaque, however unlikely. The clear containers had screened lids whereas opaque containers had unscreened lids. The simplest explanation for more individuals recovered from clear containers is by the ease by which adults can rest on a screened lid rather than a smooth plastic lid. If adults have better traction and rest longer on screened lids, they will be more likely to be collected and included in the percent emergence.

Adults are positively phototactic when they emerge from aestivation and tend to walk along ledges, grooves, or seams in the plastic. Pupation containers should be modified to take

advantage of this behavior to reduce the labor required for collecting emerging adults. An ideal container should have plenty of ventilation to discourage mold growth and should have some device to collect adults as they emerge from the pupation medium.

The duration of aestivation can be extended by storing *L. nigrinus* in the ground outside rather than at constant cooler temperatures in the cold room throughout the summer. Factors that influence time of emergence are discussed in Chapter 5. In addition, the period in which adults were emerging from aestivation was shorter when adults were held outside than those held in the cold room. This pattern was also observed in male and female emergence in 2002 and 2004. In 2002, adults were maintained in the cold room and the emergence period of adults was much wider than that of adults held at high temperatures and moved to a lower temperature in autumn, as in 2004. These results explain the emergence pattern observed in the colony in 2002 and 2003; adults emerged for 5 months, beginning in July, whereas in 2004, adults only emerged for about 3, beginning in September.

Rearing *L. nigrinus* 2000 - 2005

Before 2001, a large square plexiglass® rearing cage was used for rearing all stages of *L. nigrinus*, except active adults. Since 2001, funnel cages have been used and 4 generations of *L. nigrinus* have been reared. The number of individuals at each life stage and the overall mortality incurred at each stage from 2000 to 2005 is shown in Table 4.1.

In 2002, the number of larvae produced was high, perhaps due to the low densities at which they were stored and *L. nigrinus* eggs from each adult container were transferred to only one funnel cage. Survival of individuals through aestivation improved, which may be

attributed to higher moisture levels and modified pupation medium. However, mortality following emergence from aestivation remained high.

In 2003, larval production per beetle decreased. This may be because branches containing *L. nigrinus* eggs from two or more adult containers were lumped into one funnel cage. Therefore the density of eggs per branch and larvae per funnel cage were higher and may have contributed to decreased production because of less food available per larva. *L. nigrinus* larvae are cannibalistic when prey is not available (pers. obs.). In addition, all adults were lab-reared whereas the ovipositing adults in 2002 were field collected and lab-reared beetles do not produce as many progeny as field-collected beetles. Survival through aestivation was slightly lower than 2002 and post-emergence mortality was reduced to 8% because developing progrediens nymphs were offered to early emerging adults. This was accomplished by storing hemlock containing HWA eggs laid by sistens at 4°C from April until needed in July – September. Approximately 6 truckloads of light-medium HWA-infested branches were collected from high elevation sites in April, when sistens had nearly completed laying eggs. Foliage was cut into 24 cm branches and inserted into water-filled tubs with floral foam and stored in walk-in coolers at 4°C. Water added to the tubs each week prevented branches from drying and were ready for use when needed in the late summer.

In 2004, larval production decreased dramatically, for which, there are several possible explanations. With so many active adults, space and technical support were limiting. Adults were maintained at high densities and branches from multiple adult containers were transferred to each funnel cage, resulting in very high predator densities in the funnel cages. The majority of the ovipositing adults (7300 of 8000) were $F_2$ lab-reared adults and data show that they produce fewer larvae than field-collected adults. In addition, due to an unusually

cold winter, heavily infested hemlocks were difficult to find because HWA experienced high mortality. In many cases, what appeared to be suitable host material was actually unsuitable beneath the white wool. Large numbers of immature larvae were observed in the Mason jars, an indication that the amount of prey was not adequate. Since *L. nigrinus* larvae cannibalistic at low prey densities, it is likely that some larvae were lost to hungry later instars in these funnels.

Emergence from aestivation was much lower during the 2004 autumn than in previous years. This is likely due to the high levels of mold in a large proportion of the colony. It seems that the steam-sterilized method was not as effective as it had been in previous years. This problem is being addressed by removing the filter paper from the containers and fresh materials are pressure-sterilized to create the pupating medium. In addition, sorbic acid has been added to the methyl paraben to create a stronger fungal inhibitor.

Of the adults that did emerge from aestivation, 99% were alive in October, after HWA resumed development in the field. Survival at this stage is attributed to an extension of aestivation induced by high temperature and long days. With the ability to manipulate timing of *L. nigrinus* emergence, adult survival has been very high immediately following emergence and throughout the fall months.

Improvement of the mass-rearing techniques used to produce *L. nigrinus* is essential for the progress of biological control of HWA. Although improvements have been made and methods produced enable many beetles to be produced, there is still much room for improvement. Success in colony rearing in the past two years has expedited the release of 8000 adults in 6 states in the eastern U.S. Continued success and increased field releases are anticipated as labs at Clemson University and the University of Tennessee have begun rearing

*L. nigrinus*. Specific methods recommended for rearing as a result of these experiments are described in Chapter 6.

Table 4.1. The number of individuals at each life stage of *L. nigrinus* in the laboratory colony from 2000 to 2004. The percent mortality experienced by each stage is shown in parentheses beneath each total.

| Life Stage | Total Number of Individuals and Mortality Rate (%) Per Life Stage | | | | |
|---|---|---|---|---|---|
| | **2000** | **2001** | **2002** | **2003** | **2004** |
| **Reproductive Adults (starting colony)** | 200[F] | 350[F] | 100[L]<br>1, 000[F] | 3, 000[L] | 7 ,000[L]<br>660[F] |
| **Mature Larvae to drop from foliage** | N/A | 7, 500<br>(28%) | 37 000<br>(~30%) | 30, 000<br>(30+%) | 27, 000<br>(30+%) |
| **Pupae** | N/A | 5, 175<br>(31%) | 25, 900<br>(27%) | 12, 300<br>(41%) | 7, 000[Mf] + 2, 200[M]<br>(43%), (94%) |
| **Adults emerging from aestivation** | 30<br>(85%) | 1, 867<br>(67%) | 21, 000<br>(19%) | 13, 000<br>(6%) | 8, 000<br>(13%) |
| **Adults Surviving as HWA breaks diapause in October** | 8<br>(74%) | 200<br>(89%) | 3, 700<br>(83%) | 12, 000<br>(8%) | 8, 000<br>(0%) |

[F] Adults collected in the field from western hemlock trees in Victoria, British Columbia

[L] Adults reared in the laboratory at Virginia Tech

[Mf] In pupation containers that were free of mold contamination

[M] In pupation containers contaminated with mold

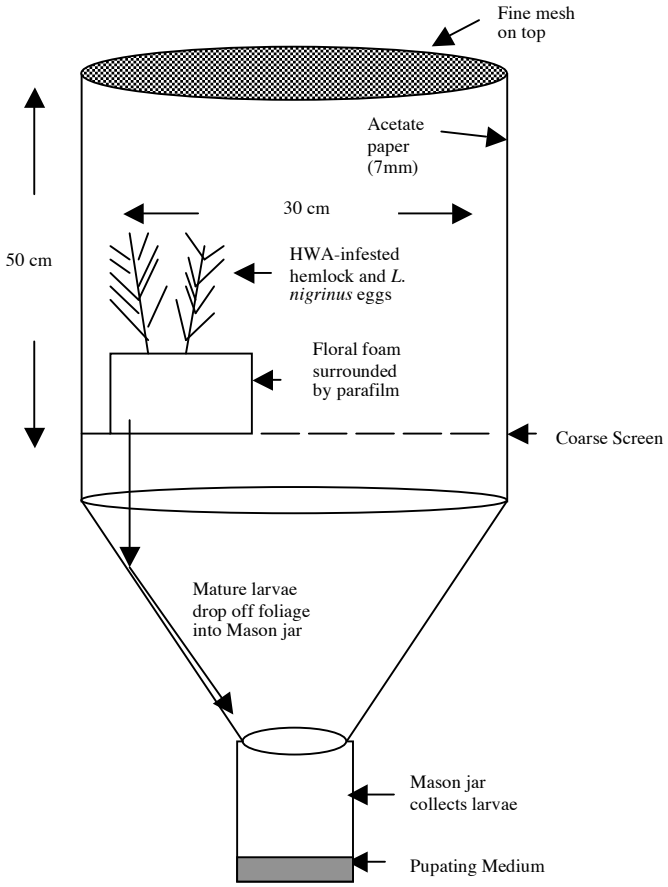



Figure 4.1. A diagram of the form and function of the *L. nigrinus* larval rearing cage.

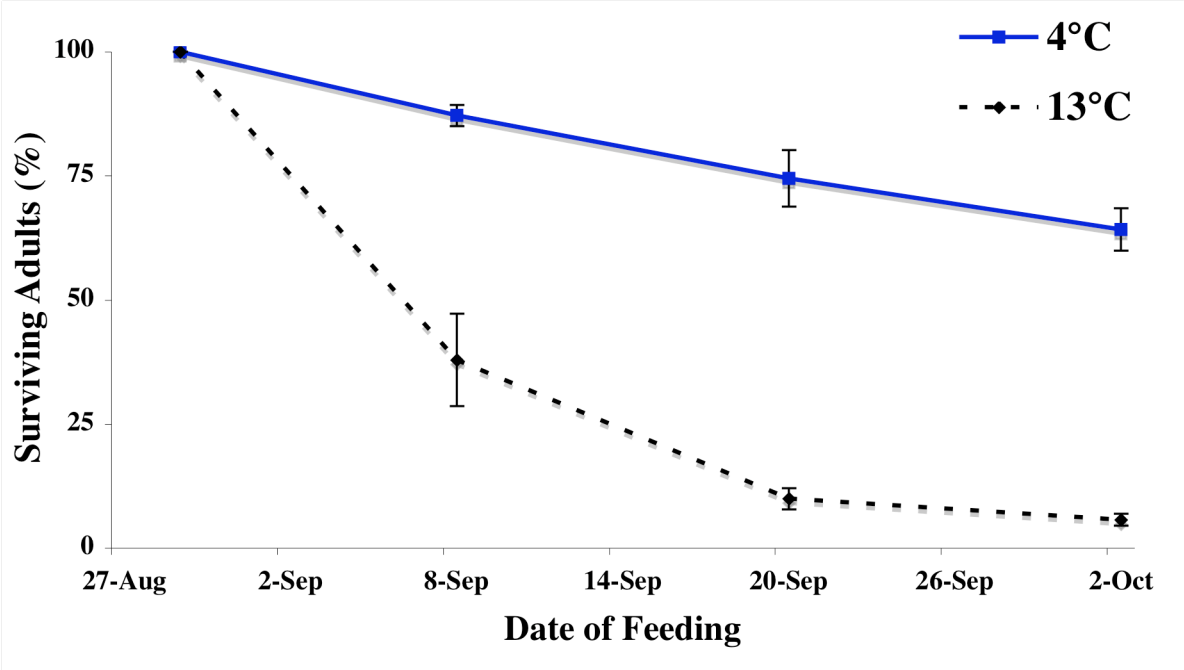



Figure 4.2. The mean percentage ($\overline{X}$ ± S.E., n = 8) of the original adults surviving at each feeding period when maintained at 13° and 4 °C in the weeks following emergence in 2003.

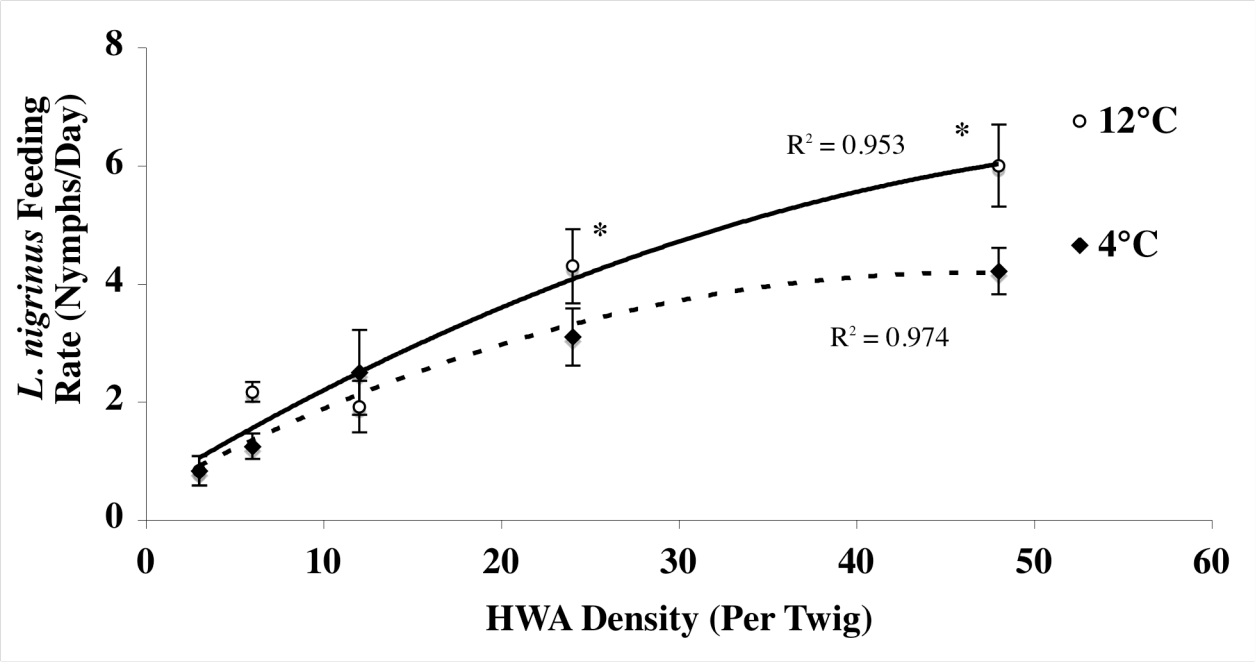



Figure 4.3. The mean feeding rate (2nd & 3rd instars/day) ( $\overline{X}$ ± S.E., n = 60) of adults at 12° (solid line, $Y = -0.0017x^2 + 0.1605x + 0.453$) and 4°C (broken line, $Y = -0.0014x^2 + 0.1817x + 0.5199$) in November, 2004. Feeding rate was influenced by prey density ($F_{(4,59)} = 16.4$, $p < 0.0001$); significantly higher consumption rates per density (*) are separated by Fisher's LSD ($p < 0.05$) ($F_{(1,59)} = 4.67$, $p = 0.036$).

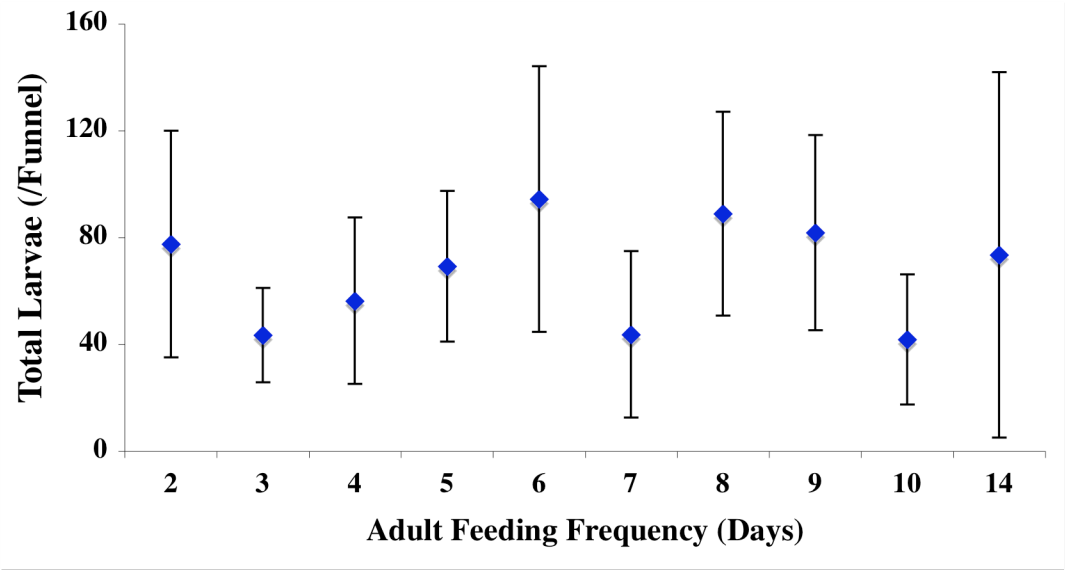



Figure 4.4. The relationship between the mean total mature larvae per funnel ($\overline{X}$ ± S.D., n = 274) and the frequency at which adults are fed. The frequency of feeding is equal to the oviposition period.

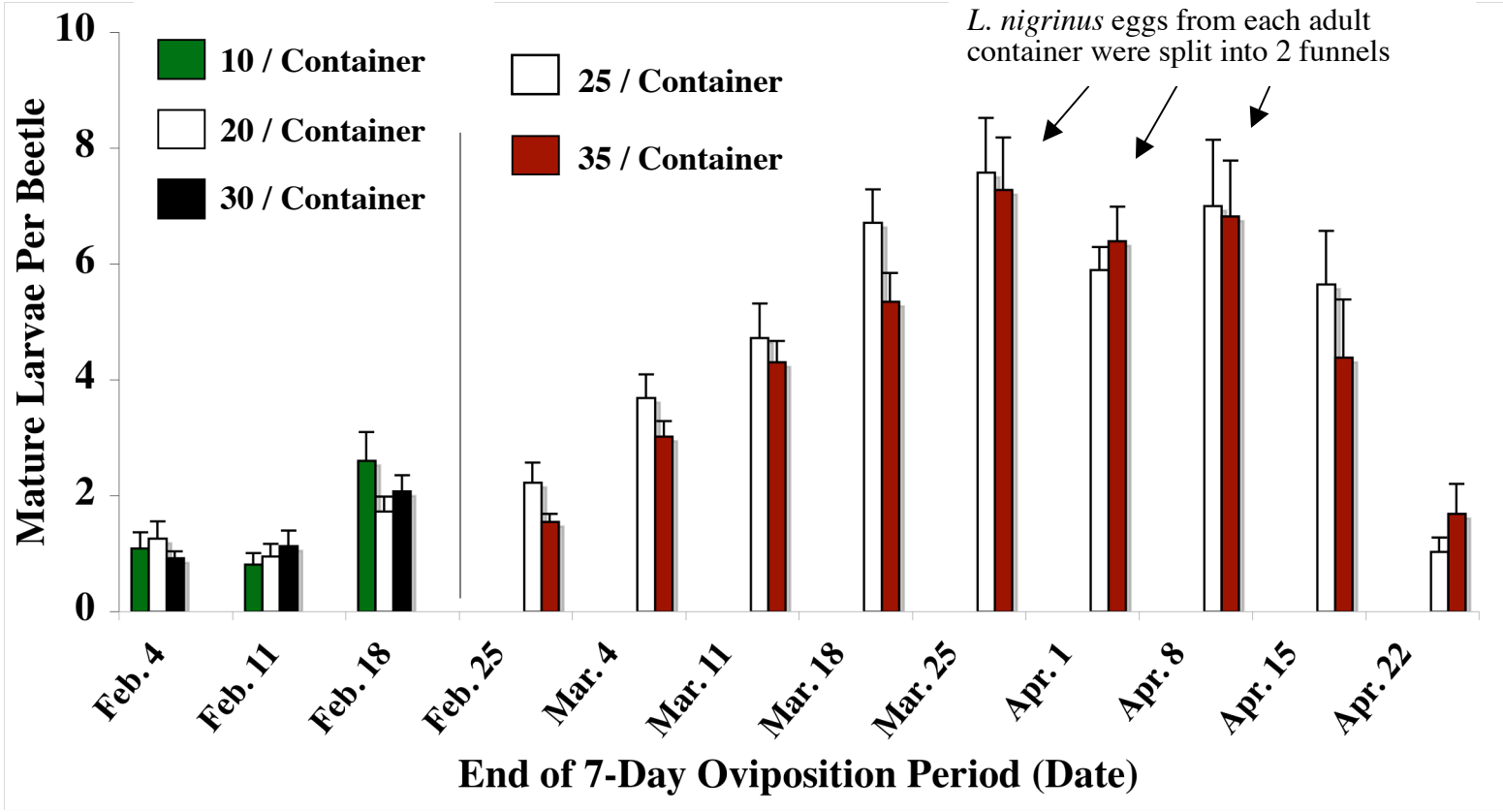



Figure 4.5. The mean number of mature larvae produced per beetle ($\overline{X}$ ± S.D.) when using different densities of adults throughout the oviposition period in spring 2002. In the first 3 feeding periods, bars represent 10, 20, and 30 beetles per container respectively, and after Feb. 18, white bars represent containers with 25 beetles and colored bars represent containers with 35 beetles.

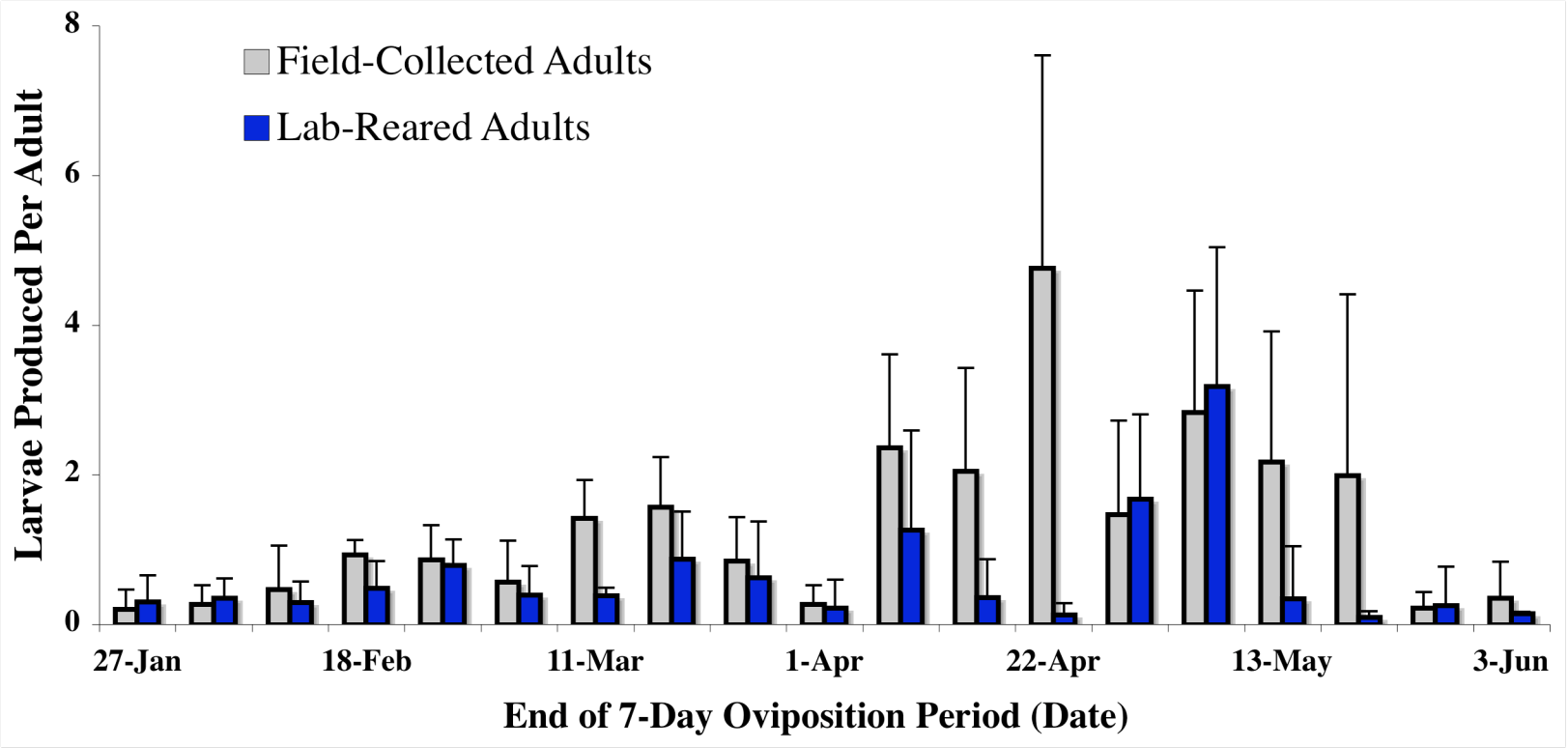



Figure 4.6. The mean number of larvae ($\overline{X}$ ± S.D.) produced per adult in field-collected and lab-reared beetles the oviposition period in spring 2004.

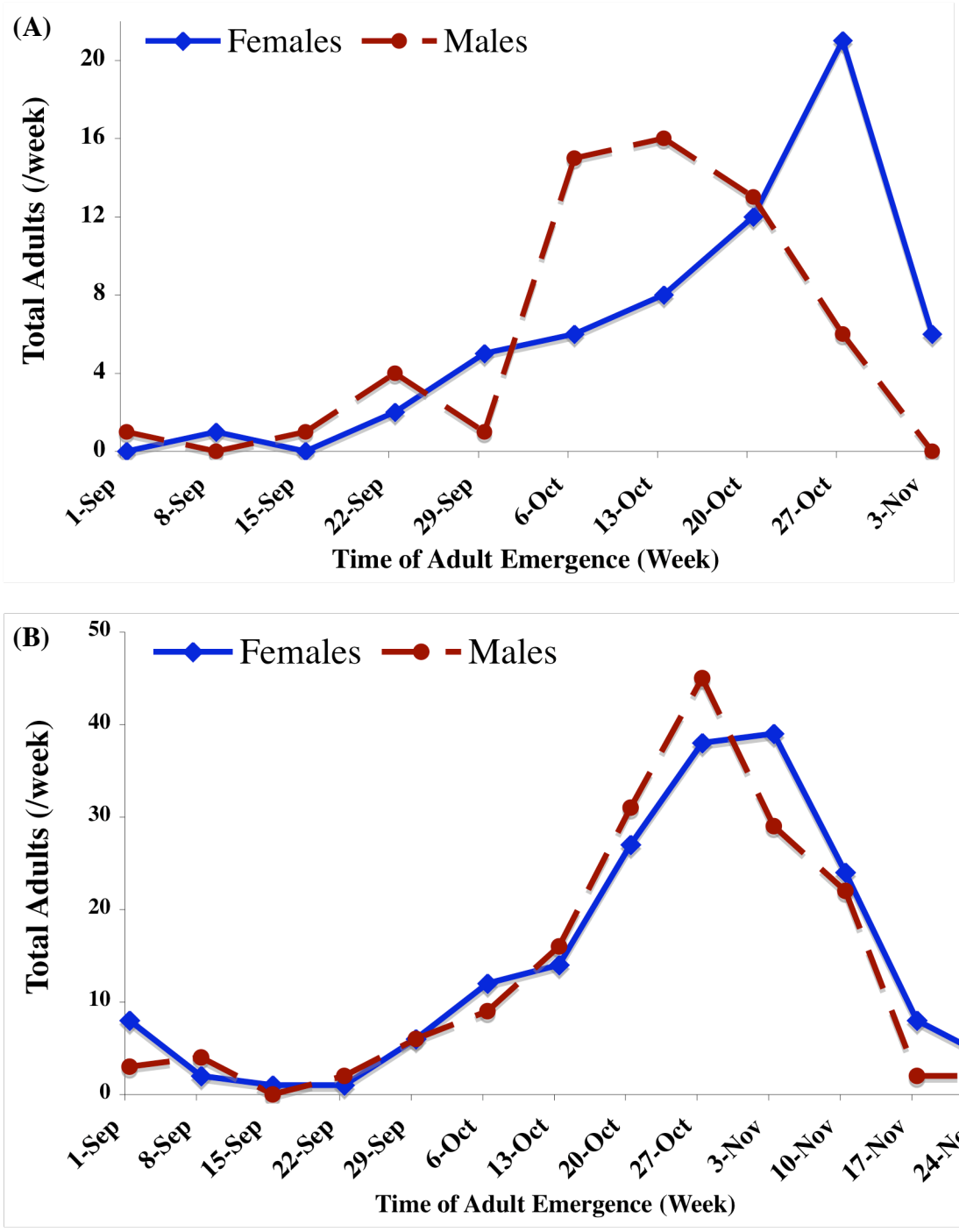



Figure 4.7. The total number of female (solid line) and male (broken line) adults emerging from aestivation each week in the fall of 2003 (A) and 2004 (B). Note the difference in scale between years. In 2003 only 6 pupation containers were assessed with 238 pupae sexed, whereas in 2004, 933 individuals from 28 pupation containers were sexed.

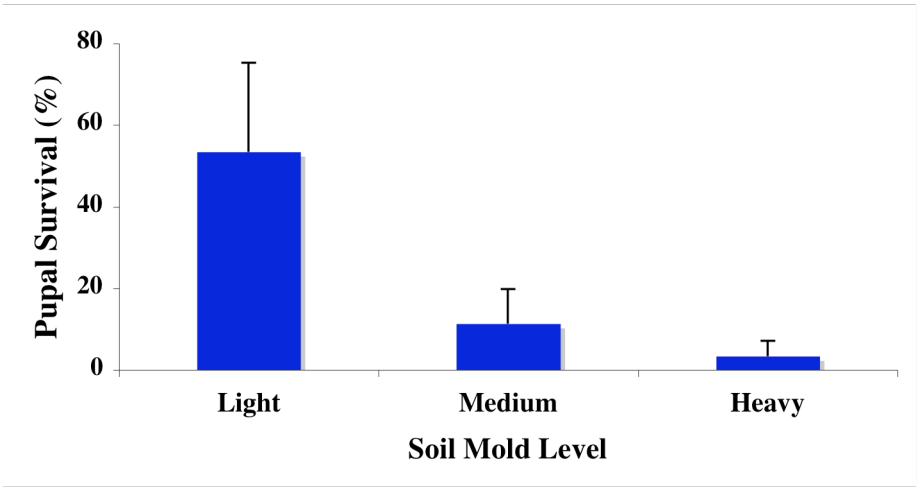
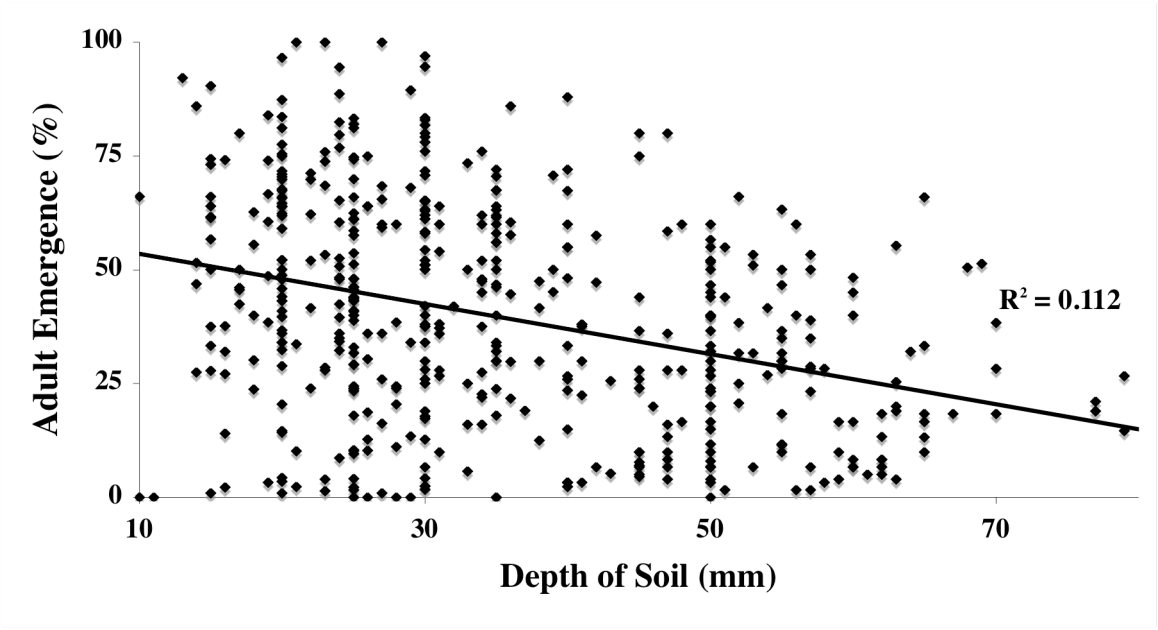



Figure 4.8. The percentage of pupae ($\overline{X}$ ± S.D.) found in aestivation containers with light, medium and heavy mold levels in spring 2004.

(A.)




(B.)

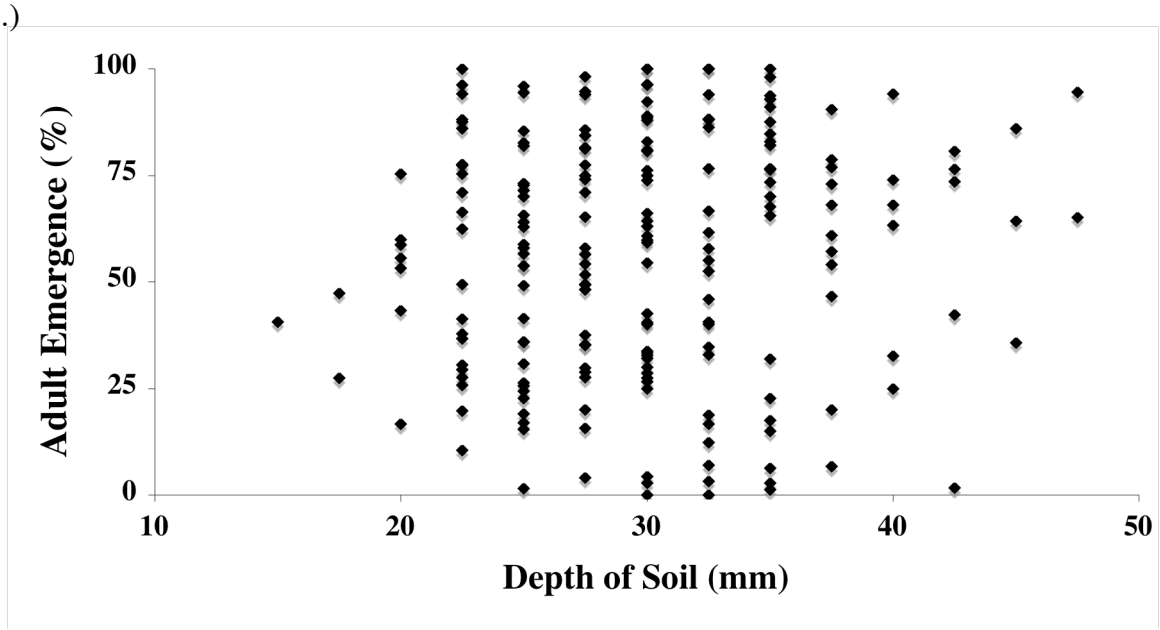



Figure 4.9. The percentage of individuals emerging from containers with varying depths of pupation medium in 2002 (A) and 2003 (B). In 2002, the percentage of adults that emerged decreased with increasing depths of pupation medium ($R^2 = 0.112$), $Y = -0.551x + 59.0$ ($F_{483} = 60.63$, $p < 0.0001$) and in 2003, soil depth did not affect adult emergence from aestivation ($R^2 = 0.001$), $Y = 0.167x + 51.5$ ($F_{199} = 0.32$, $p = 0.572$).

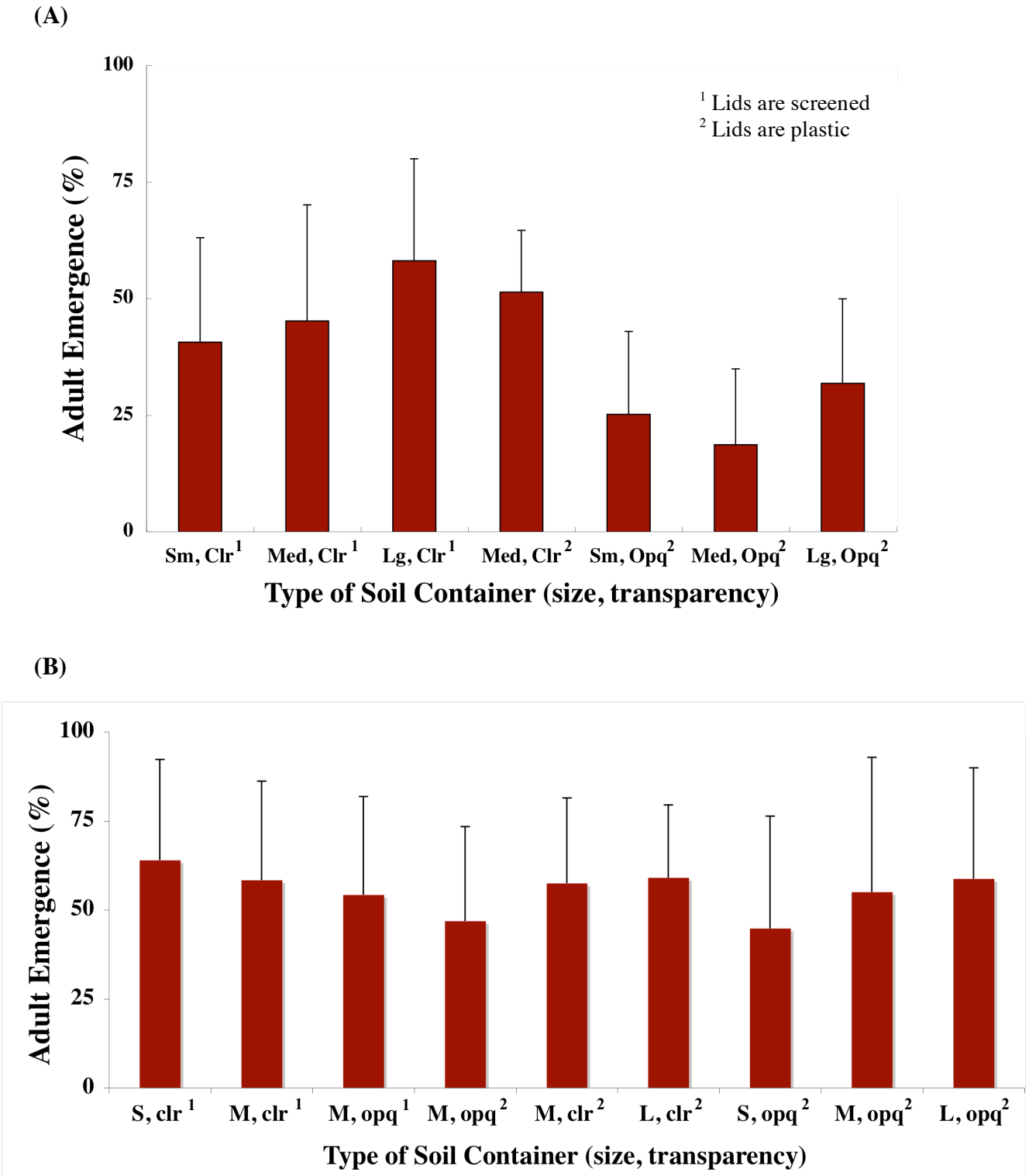
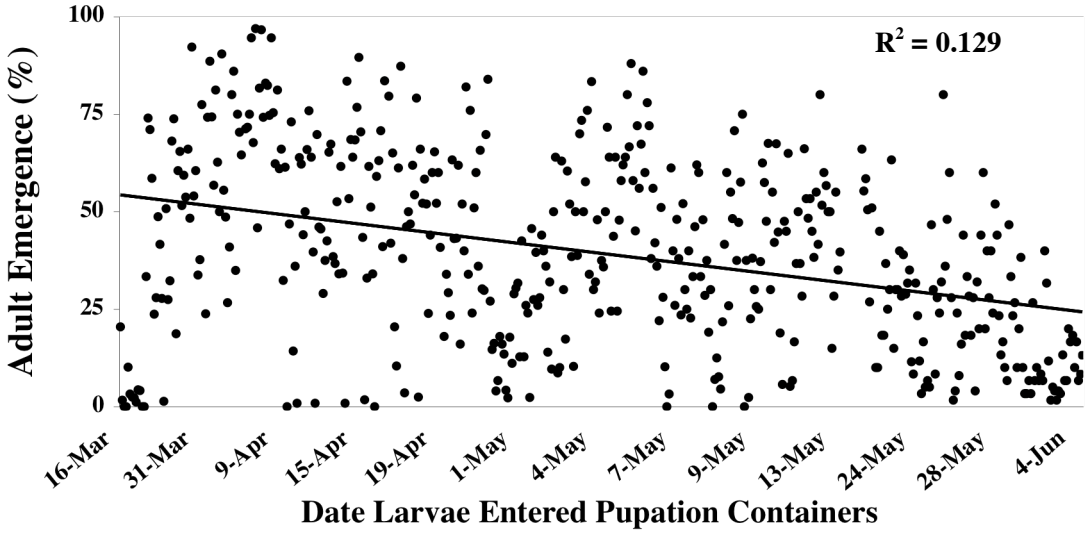



Figure 4.10. The percentage of individuals emerging from the soil as adults ( $\overline{X}$ ± S.D.) in relation to the type of container in which they were held in 2002 (A) (n = 484) and 2003 (B) (n = 207)(S = 473 ml, M = 950 ml, L = 1.82 L; clr = clear plastic, opq = opaque plastic, [1] = screened lids, [2] = plastic lids).

(A)




(B)

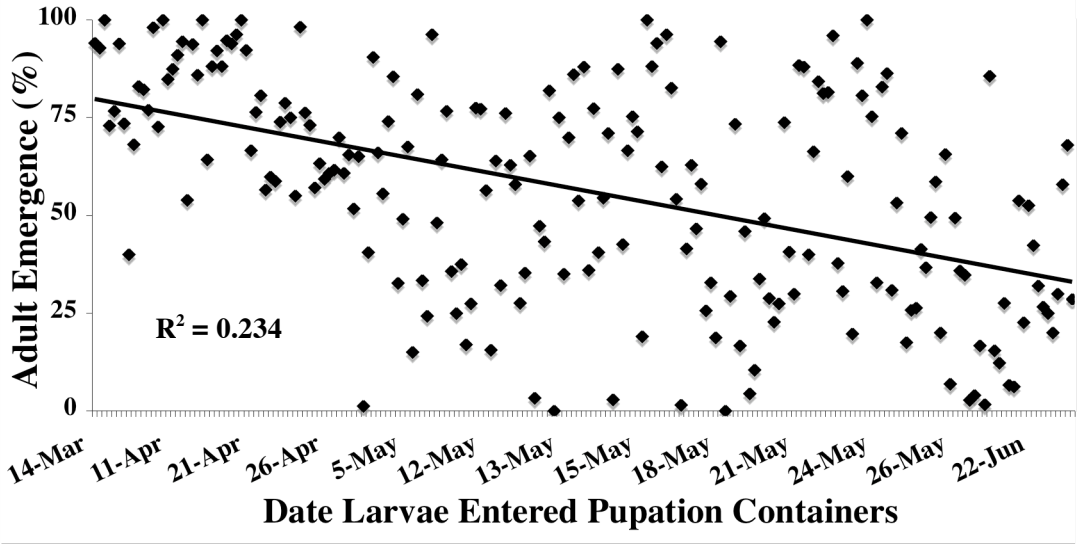



Figure 4.11. The percentage of adults emerging from each container following aestivation in relation to the time at which larvae entered the pupation containers in the 2002 (A) and 2003 (B) colonies. The percentage of adults that emerged decreased as larvae entered the pupation containers later in the year in 2002, $Y = -0.062x + 54.4$ ($F_{483} = 71.49$, $p < 0.0001$), and this relationship was more accentuated in 2003, $Y = -0.234x + 79.9$ ($F_{199} = 55.93$, $p < 0.0001$).

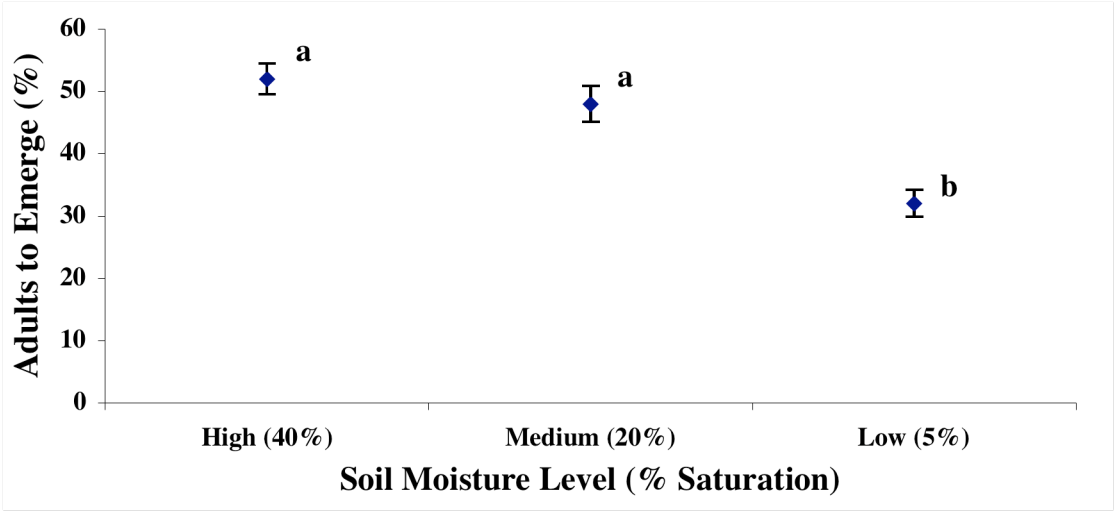



Figure 4.12. The mean percentage ($\overline{X}$ ± S.E., n = 24) of adults to emerge from aestivation when maintained at 40, 20, or 5% soil saturation during pupation and aestivation. Means with different letters are significantly different using analysis of variance followed by Fisher's LSD ($F_{(2, 47)}$= 6.02, p = 0.0050).

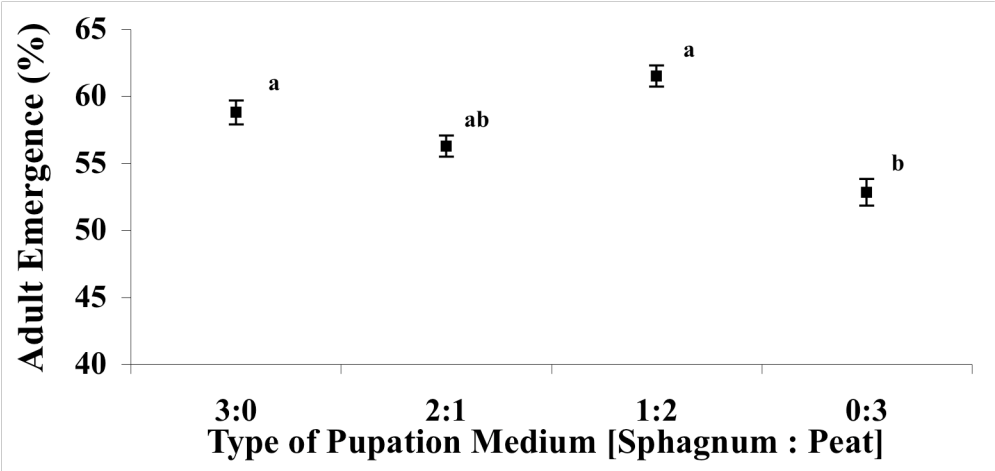



Figure 4.13. The mean percentage ( $\overline{X}$ ± S.E., n = 372) of adults emerging from aestivation when stored in four different ratios of sphagnum:peat during pupation and diapause. Means with different letters are statistically different ($F_{(3, 358)}$= 4.10, p = 0.0072).

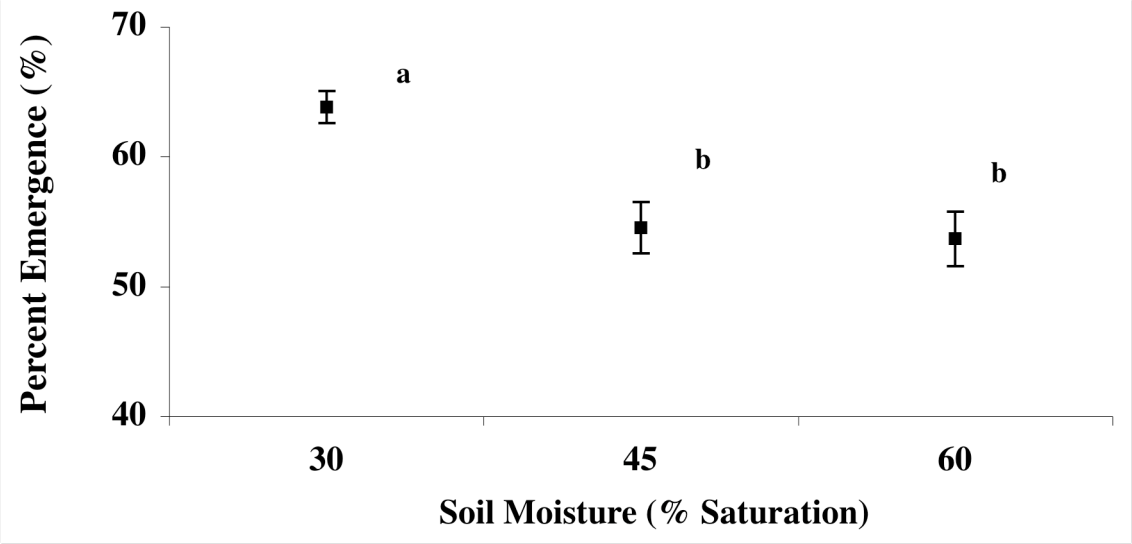



Figure 4.14. The mean percentage ($\overline{X}$ ± S.E., n = 372) of adults emerging from aestivation when maintained at 30, 45, and 60% soil saturation throughout pupation and diapause. Means with different letters are significantly different using an analysis of variance followed by Fisher's LSD ($F_{(2, 358)}$= 12.65, $p < 0.0001$).

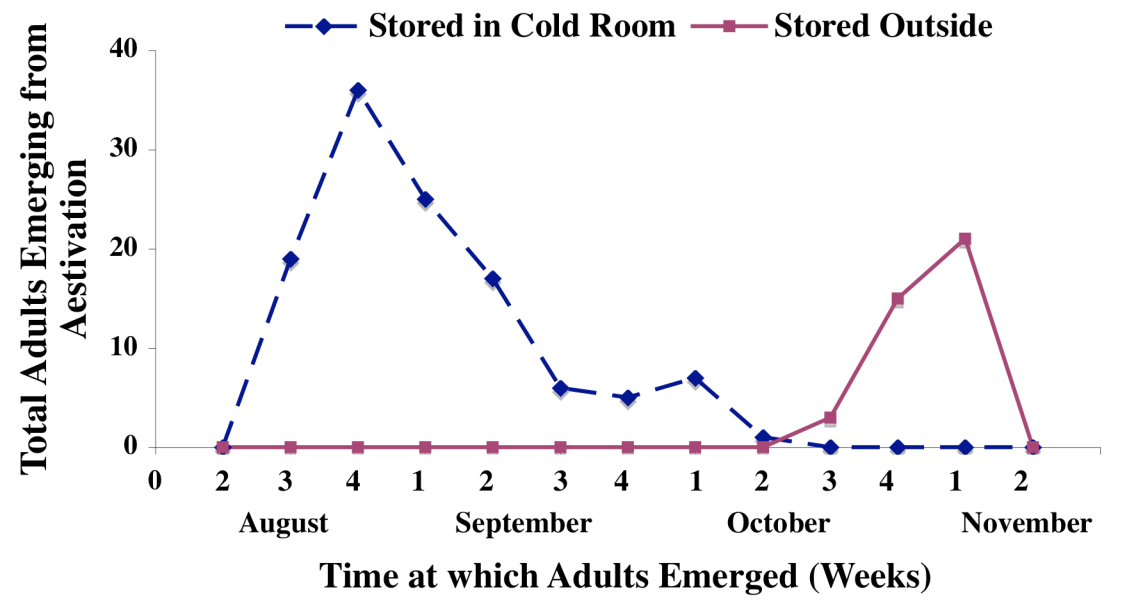



Figure 4.15. The number and timing of emergence from aestivation from early August to November when adults are maintained in the cold room (15° ± 2°C & 14:10 (L:D)) (broken line), and when maintained in the ground outside (solid line) throughout the summer.

# Chapter 5

## Factors that influence *L. nigrinus* Aestivation

### Introduction

Development and implementation of biological control agents is often highly dependent on critical timing of life stages of two or more species (Leppla and Fisher 1989, Chang et al. 1995). *Laricobius nigrinus* Fender (Coleoptera: Derodontidae) is a univoltine, derodontid beetle that undergoes diapause during the summer and is active throughout the rest of the year (Zilahi-Balogh et al. 2003a). This insect is a predator of the hemlock woolly adelgid (HWA), *Adelges tsugae* Annand (Hemiptera: Adelgidae), a lethal pest of hemlock trees in eastern North America, *Tsuga canadensis* (L.) Carriere and *T. caroliniana* Engelmann (McClure 1996).

*Laricobius nigrinus* feeds on HWA in the Pacific Northwest (Lawrence and Hlavac 1979, Zilahi-Balogh et al. 2003a), where western hemlock, *T. heterophylla* (Raf.) Sargent, is attacked but rarely killed by HWA (Furniss and Carolin 1977). *L. nigrinus* has many favorable attributes of a biological control agent (Zilahi-Balogh 2001) and, following a positive field evaluation (Chapter 2), over 20 releases of *L. nigrinus* have been made (Mausel, pers. comm.).

Once *L. nigrinus* was released from quarantine, research shifted to developing rearing methods for it. Successful rearing procedures were produced for all life stages of the predator (Chapter 4), except one step in the process was a perpetual problem. Adult survival through

diapause was low and their time of emergence, in the lab, was not synchronized with emergence of HWA in the field. These adults were emerging primarily from July – September, peaking in August; two months before their prey completes diapause in the field. Adults emerging from soil at this time were offered eastern spruce gall adelgid (until August when it is no longer present outside), aestivating 1st instar sistens, progrediens (lifecycle manipulated), and artificial ladybird beetle food of Wheast (Natural Planet, Bozeman, MT) and honey.

*L. nigrinus* mortality drops off dramatically for beetles emerging during or after October, at which time HWA is developing in the field. The premature emergence of adults in the lab has become the primary limiting factor in the mass propagation and ultimate release of *L. nigrinus* in the field. Recently, an abundance of hemlock branches with progrediens were stored in a cooler over the summer and offered to adults emerging early. By storing progrediens, *L. nigrinus* post-emergence mortality in the late summer was reduced. However, it would be more efficient and more practical if termination of diapause in *L. nigrinus* adults could be manipulated using environmental conditions. Increasing our understanding of the factors that influence the diapause process will improve our success in rearing.

Aestival diapause is a fascinating adaptation that has not been widely studied (Masaki 1980) and only a few temperate zone coleopterans with this type of lifecycle have been investigated. *Laricobius nigrinus* is a mid-latitude, specialist predator that is active throughout the fall, winter, and spring, and dormant throughout the summer months. Summer dormancy in *L. nigrinus* is particularly interesting energetically, as individuals undergo metamorphosis and aestival diapause without feeding, a seemingly uncommon occurrence among beetles that undergo summer diapause. In addition, there is increased interest in

understanding the genus *Laricobius* for their economic value as specialists on Adelgidae, a worldwide family of conifer tree pests (Lawrence and Hlavac 1979).

Since little was known about the summer diapause of *L. nigrinus*, three experiments were conducted throughout the spring, summer, and fall of 2003 to gain insight on the factors controlling adult survival and diapause completion. The influence of genetic and environmental factors on the duration of diapause were investigated. The environmental factors tested were: temperature, photoperiod, and moisture level of the pupation medium. The effect of constant environmental conditions and a change in these conditions were also investigated. Factorial combinations were used since interactions of seasonal cues are common, particularly photoperiod with temperature and temperature with moisture (Danks 1987). Sometimes up to four factors control dormancy (Morris 1967), presumably because cues acting in concert, ease seasonal detection and produce a more reliable response.

It can be informative to measure a species response to environmental cues throughout the entire process of diapause since many species respond to cues differently during induction than during post-diapause development. Most previous studies of diapause development simply measure the resumption of normal activity, without reference to any particular developmental stages of diapause. Similarly in this study, for practical reasons, we measure the total duration of diapause, from adult eclosion through diapause and post-diapause development, when adults re-emerged from the soil following the dormant period.

The objectives of this study were to determine whether:

- genetic or environmental factors affect the duration of diapause in *L. nigrinus* adults

- photoperiod, temperature, or moisture level affect the duration of diapause or the percentage of *L. nigrinus* adults to complete diapause
- a decrease in temperature or daylength following storage under summer conditions affects the duration of diapause or the percentage of *L. nigrinus* adults to complete diapause

The practical goal is to be able to manipulate the timing of *L. nigrinus* emergence from diapause to coincide with the time HWA completes diapause.

**Methods and Materials**

All individuals used in these experiments were progeny from one geographic strain of adults reared in the laboratory for 1 generation ($F_1$ adults). The beetles from which the colony was created, were collected from *T. heterophylla* in Victoria, BC, Canada (2 locations separated by ~5 km). In the laboratory *L. nigrinus* were reared using the procedures described in Chapter 6.

Each experiment was set up using a randomized block design with each block representing a cohort of predators. Each block consisted of a specific number of pupation containers, each an experimental unit. For each block, a series of polyethylene plastic containers (950 ml) (Rez-tech Corp., Kent, OH) with three mesh-covered holes (2.5 cm diameter) (0.14 $mm^2$ mesh size, Sefar America Inc., Kansas City, MO) were washed and weighed. Homogeneous pupation medium (106 g) was added to each container to a height of approximately 3 cm.

The pupation medium was created by mixing sifted peat, ground sphagnum moss, and sand at a 2:2:1 ratio and steam-sterilizing the mixture for two 24 h periods separated by 36 h

at room temperature (Chapter 4). Using a Lincoln® soil moisture meter (Forestry Suppliers, Inc., Jackson MS), distilled water was added to the mixture until the appropriate saturation level was reached and precisely 106 g of pupation medium was added to each container. The total weight of each container was calculated by the soil weight to the original container weight. The soil in each container was then maintained at the original moisture level by placing each container on a scale set at the original total weight of the container and adding distilled water until the original weight was attained. Every container was watered once a week throughout the entire study period, from set up in April/May 2003 through January, 2004.

Larvae were reared at 15°C and 12 h daylength on HWA-infested hemlock, in funnel cages, and were transferred to pupation containers as they dropped from the foliage at maturity (Chapter 6). During pupation, containers were stored at 15°C and 12 h daylength for 5 weeks to ensure all individuals had eclosed into adults. In experiment 1, all containers remained at 15°C and 12:12 (L:D) for the duration of the experiment. In experiments 2 and 3, following adult eclosion, containers were randomly assigned a temperature and daylength treatment and moved to the corresponding environmental chamber.

From July through January, each container was checked daily for emerging adults. The total number of adults that emerged and time of emergence was recorded for each container. In experiment 1, the results are reported as the total number of individuals to emerge per female. In experiments 2 and 3, every container had 20 individuals, therefore the results of these experiments are reported in percentage of adults emerging from each container. Data collected on the timing and emergence of *L. nigrinus* were analyzed using a

3-factor analysis of variance and means were separated using a Fisher's LSD test (SAS Institute, 1992).

Experiment 1

To investigate the influence of genetics, adult aestival survival and timing of emergence of individuals from the same female were measured and compared among progeny from other females. This experiment was set up as a randomized complete block design with cohort serving as a block. Fifteen adult females were maintained individually and fed an unlimited amount of prey weekly throughout the oviposition period (February – May). Each female served as a treatment. The eggs produced from each female were removed and transferred to separate corresponding funnel cages each week from January through June.

Blocks for this experiment consisted of 15 pupation containers and were set up every week while larvae were dropping from the hemlock foliage. When larvae were mature, the progeny from each female transferred to a corresponding pupation container. A total of seven blocks were set up throughout April and May, producing a total of 105 containers, each of which contain the number of larvae from each genotype to drop from foliage during each week. Aestivating adults were held at 15°C and 12:12 (L:D) daylength throughout the entire duration of the experiment; the conditions under which previous colonies had been held in the laboratory. Each container was watered weekly to maintain 30% saturation. Throughout the emergence period, each container was checked daily for adults resuming activity. The time that each individual adult emerged was recorded and variation between and within sibling groups was analyzed using a 1-factor analysis of variance (ANOVA). Differences in mean

total days for diapause completion were separated using Fisher's test of least significant difference ($p \leq 0.05$).

Experiment 2

A randomized block design, as a 3x3x2 factorial, was used to test the influence of temperature, soil moisture, and daylength on the number of adults to emerge and the duration of *L. nigrinus* aestivation. The factors were kept constant during the study period. Aestivating adults were held at one of three levels of temperature (10°, 15°, and 20°C), three levels of daylength (8, 12, and 16 h light), and two levels of soil moisture (30 and 45% saturation). Blocks were set up approximately every 2 weeks beginning in early April and there were 2 replicates per block.

Each block consisted of 36 containers, half of which were maintained at 30% soil saturation and the other half at 45% soil saturation. Mature larvae were transferred to each of the 36 containers in groups of 5 and repeated 4 times, resulting in 20 genetically diverse larvae in each container. Each container is treated as one experimental unit. The containers were randomly assigned 1 of the 9 temperature:daylength combinations following adult eclosion. Seven blocks were set up throughout April and May, producing a total of 252 containers. Due to unfortunate circumstances while watering, only 4 blocks remained at their assigned moisture level throughout the 9 months of the experiment, and of those, 126 containers remained undisturbed.

Five weeks after entering the soil, following adult eclosion, four containers from each block, 2 with soil maintained at 30% and 2 with soil maintained at 45% moisture level, were placed into one of the 9 growth chambers set at their assigned treatment condition (10°, 15°,

or 20° C) and (8, 12, or 16 h daylength). These containers remained in the environmental chambers for the duration of the experiment. Throughout the emergence period (July – January), each container was checked daily and emerging adults were collected. The percentage of adults emerging and the mean time of emergence were calculated for each container. The effect of these environmental conditions on adult emergence and timing of emergence was determined using a 3-factor ANOVA and means were separated using Fisher's test of least significant difference (SAS Institute 1992).

Experiment 3

A generalized randomized block design was used to investigate the effect of a change in temperature and/or daylength in late summer on the timing and number of *L. nigrinus* to emerge from diapause. The experiment was a 3x3x3 factorial testing a change in temperature, photoperiod, and time of change. Six blocks were set up, each representing a larval cohort. Each block consisted of 2 replicates, 54 containers were set up and maintained at 30% moisture level, and were set up approximately every 2 weeks beginning in early April. Adults were held in summer conditions (20°C and 16 h daylength) from eclosion to late summer, followed by a decrease in temperature and/or photoperiod at three different dates.

Mature larvae were transferred to each of the 54 containers in groups of 5 and repeated 4 times, resulting in 20 genetically diverse larvae in each container. Each container was randomly assigned one of 3 dates for the change in conditions (September 9, September 24, or October 7), one of 3 temperatures (10°, 15° or 20°C), and one of 3 daylengths (8, 12, or 16 h light). A total of seven blocks were set up throughout April and May, producing a total of 378 containers. Due to unfortunate watering circumstances in watering mentioned in

experiment 2, only 208 containers remained intact by the end of the experiment and used in the analysis.

Following adult eclosion all the containers were placed into one environmental chamber set at 20°C and 16:8 (L:D) daylength. Aestivating adults were stored at these conditions and watered weekly throughout the summer. On September 9, two containers from each block were transferred to the environmental chamber emulating one of the nine temperature/daylength treatment combinations assigned to each. This was repeated on September 24 and October 7. Throughout the emergence period (July – January), each container was checked daily for emerging adults. The percentage of adults emerging and mean time of emergence was calculated for each container. The effect of the tested environmental conditions on adult survival and timing of emergence was determined using a 3-way Anova using Proc GLM in SAS. Means were separated using Fisher's test of least significant difference and interacting factors were separated using the slice statement ($p \leq 0,05$).

**Results**

Experiment 1

The number of progeny produced and the total number of days each group spent in the soil was highly variable among females. The total number of progeny that emerged from the soil following aestivation ranged from 4 to 58 individuals per female. The variation in the number of days spent in the soil was greater among sibling groups than within groups (Figure 5.1). Although the difference in average length of time spent in the soil is less than 14 days, this does explain some of the variation observed in *L. nigrinus* emergence from aestivation.

Individual females (genotypes) have a substantial influence on the number of progeny emerging from aestivation. Since duration of diapause often varies by 60 days, environmental conditions likely have a greater influence on the time of emergence of *L. nigrinus* than genetically related factors, as shown in experiments 2 and 3.

Experiment 2

The percentage of adults that emerged from aestivation was significantly greater when the soil medium was maintained at a moisture level of 45% than 30%, with 50 and 40% of adults emerging, respectively ($F_{(1,107)} = 7.97$, $p = 0.005$). In addition, a greater percentage of adults emerged from aestivation when stored at 10°C (44.4 ± 3.3%) or 15°C (37.3 ± 3.4%) throughout the summer than those adults maintained at 20°C during aestivation (26.7 ± 4.0 %) ($F_{(2,\ 107)} = 4.12$, $p = 0.019$) (Table 5.1). Photoperiod did not have a statistically significant effect on adult survival ($F_{(2,107\ )} = 2.73$, $p = 0.07$), however, at each temperature, adult emergence was lower when stored in long daylengths (16 h) than when stored at shorter daylengths (8 or 12 h) (Table 5.1). In addition, there were no adults to emerge from aestivation following storage at 20C and long daylengths (16 h).

Soil moisture did not influence the time at which adults emerged from aestivation. Temperature had the greatest influence on the number of days spent in aestivation ($F_{(2,107\ )} = 379.59$, $p < 0.0001$). The total number of days in aestivation was longest when adults were maintained at 20°C, medium length at 15°C and shortest when adults were stored at 10°C. Daylength also had a significant effect on the number of days in aestivation ($F_{(2,107)} = 6.15$, $p = 0.003$). Completion of aestivation is significantly delayed if maintained at long daylength (16 h) rather than shorter daylengths (8 and 12 h), however, this effect was less pronounced than the effect of temperature (Table 5.2). Time of emergence seems to be delayed by increasing

temperature and daylength (summer conditions). No adults emerged from containers held at 20°C and 16 h daylength.

Experiment 3

The percentage of adults that emerged from aestivation was significantly higher when moved from 20°C and 16:8 (L:D) to either 10° or 15°C after mid-September ($F_{(2,178)} = 6.56$, $p = 0.002$). The percentage of *L. nigrinus* adults that emerged from aestivation when moved from 20° to 10° or 15°C and maintained at 30% moisture was 38.0% and 38.9% respectively, whereas only 28.7% of adults maintained at 20°C emerged from aestivation. The percentage of adults that emerged when moved from summer conditions to 10° or 15°C is only slightly less than those held at constant temperatures (Table 5.3). The late-season decrease in daylength and the time at which the temperature and/or daylength was decreased did not affect the percentage of adults that emerged ($F_{(2,178)} = 1.03$, $p = 0.359$ and $F_{(2,178)} = 1.11$, $p = 0.333$, respectively).

The timing of adult emergence from aestivation was influenced by a late-season decrease in temperature or daylength, and when the temperature and/or daylength were decreased. *L. nigrinus* emergence was significantly delayed when the adult storage temperature remained at 20°C (185 ± 0.9 days), compared to when temperature was decreased to 15° or 10°C (164 ± 1.2 and 166 ± 1.9 days, respectively) ($F_{(2,178)} = 179.08$, $p < 0.0001$) (Table 5.2). Although a decrease in temperature was the most influential factor determining the timing of *L. nigrinus* emergence, a decrease in daylength and when the environmental change occurred also affected the total days adults remained in the soil ($F_{(2,178)} = 12.51$, $p < 0.0001$, and $F_{(2,178)} = 57.88$, $p < 0.0001$, respectively). Both factors interacted independently with respect to temperature. At each temperature, adult emergence was further delayed by

decreasing daylength later or by postponing the date conditions change (Table 5.4). Depending on the temperature, daylength, and when temperature and daylength decreased, emergence from aestivation in *L. nigrinus* was delayed up to 60 days by storing adults at summer conditions (20°C 16:8 (L:D)) and moving them cooler temperatures compared to emergence of adults stored at constant environmental conditions (Table 5.4).

**Discussion**

As with many species that diapause in the summer, *L. nigrinus* exhibits considerable variation in the time they emerge from the soil, following aestivation. Individual organisms belonging to one population may vary in almost any trait, and genetic variation in the duration of diapause has been documented (Tauber and Tauber 1976, Ohashi et al. 2003). Individuals of some species characteristically differ widely in diapause intensity or in the level of signal required for a given response, and duration of diapause is largely governed by genetics (Fangsen and Kallenborn 1998, Takeda 1998). In other species, environmental conditions have a greater influence on the development of diapause (Ehlert et al. 1997). There was variation in the duration of aestivation among *L. nigrinus* siblings, but an even greater variance was observed in the duration of aestivation between sibling groups. This suggests that there is a genetic component that determines the duration of diapause or the response to environmental cues. Genetically-based variation is advantageous for two reasons: it enables *L. nigrinus* populations to evolve by responding to selection pressures and gradual emergence is a form of bet-hedging, and ensures some proportion of the population will survive in extreme conditions.

This genetically-based variation also explains, in part, the variation in emergence in the lab. The greatest difference in mean duration of aestivation among sibling groups was about 2 weeks, which, in some cases, would be a long enough delay to synchronize *L. nigrinus* emergence with completion of diapause in HWA. A high variation in emergence within one container (set up the same day) had previously been puzzling, but with 200 genetically different individuals per container, a 10-week emergence period no longer seems as obscure.

The high variation in duration of diapause observed among siblings has been observed in other insects (Helle 1968, Ineichen et al. 1979). Ring (1968) concluded that genetically determined differences explained the variation in diapause duration in *Lucilia caesar* L. (Diptera: Calliphoridae) and suggested that diapause development, diapause intensity, or temperature requirements for post-diapause development may also be under genetic control. This may be the case for *L. nigrinus*, however, experiments 2 and 3 provide data suggesting that environmental factors explain emergence patterns better than genetic differences.

Based on the response of *L. nigrinus* adults to a combination of environmental treatments, temperature appears to be the most important factor influencing time of emergence, with daylength being a modifying factor. When *L. nigrinus* was stored at constant temperatures throughout the dormant period, completion of diapause was delayed most by warmer temperatures. This effect was enhanced by exposure to longer daylengths. In most species, photoperiod is the primary factor determining duration of summer diapause (Garcia et al. 1990, Zhu and Tanaka 2004), however, the contribution of temperature is known to increase (Kostal and Hodek 1997, Takeda 1998), or even replace photoperiod, where seasonal changes in photoperiod are difficult to monitor as in the tropics, the arctic, or aquatic

environments (Mansingh and Steele 1973, Nagell 1981, Danks 1987). This is likely the case with *L. nigrinus*, as photoperiod is probably not easy to monitor from the soil. One soil-dwelling, temperate beetle, *Choleva elongata* Paykull (Coleoptera: Leiodidae), does not use exogenous cues at all, the duration of aestivation is genetically determined (Topp 2003).

When *L. nigrinus* is stored at high temperatures and long days throughout the dormant period, emergence is accelerated primarily by a change to cooler temperatures (10° and 15°C), and short days (8 h) accelerate emergence further. Several species have been found to also require a change in conditions to terminate diapause (Lutz 1974, Nakai and Takeda 1995, Schoeps et al. 1996). The summer diapause of the butterfly *Luehdorfia japonica* Leech (Lepidoptera: Papilionidae) is terminated by lowering temperatures, even under long days (Ishii and Hidaka 1982). Similarly, the summer diapause of *Pryeria sinica* Moore (Lepidoptera: Zygaenidae) can be shortened by cooling in long days, which at constant temperatures, maintains diapause (Ishii et al. 1983).

In many aestivating species, it is the lower temperatures of fall rather than the high temperatures of summer that favor diapause development (Hidaka et al. 1971, Butler et al. 1985a, Finch and Collier 1985). *Phyrrhalta humeralis* (Chen) (Coleoptera: Chrysomelidae) shows a similar response where completion of diapause is accelerated by lower temperatures and short days and delayed by higher temperatures, regardless of daylength (Nakai and Takeda 1995). High temperatures have been documented to delay aestivation completion in Dipterans, *Pegomyia bicolor* Wiedemann (Anthomyiidae) (Xue et al. 2001), and Lepidopterans (*Heliothis virescens* (Fabricius) (Noctuidae) (Butterfield 1976), and *Helicoverpa armigera* (Hubner) (Noctuidae) (Liu et al. 2004). In other species, *Bdellodes lapidaria* (Kramer) (Acari: Bdellidae) (Wallace 1971) and *Hypera brunneipennis* (Boheman)

(Coleoptera: Curculionidae) (Madubunyi 1978), diapause is completed faster at higher temperatures.

*L. nigrinus* adult emergence can be further manipulated by modifying the time at which the decrease in temperature and daylength occur. In a few species, diapause development depends on absolute photoperiod, while others require a particular direction of change in photoperiod (Lutz 1974). For example, in the chrysomelid *Galeruca tanaceti* (L.), development is accelerated when daylength decreases beyond a critical level (Siew 1966). Moisture level had no effect on the duration of diapause.

The lifecycle of *L. nigrinus* is unique in several respects, however our results are predictable when viewed within an ecological context. Since temperature is somewhat moderated in the soil (Danks 1987) and seasonal patterns are predictable and easily monitored, it makes sense that *L. nigrinus* would use it as a primary cue for controlling diapause. Photoperiod is likely a difficult cue to monitor from the soil. We did not test whether adults monitor temperature by accumulating "cool hours" or if they respond to a change in temperature below a critical threshold. It seems likely that they accumulate "cool hours" only when below a critical temperature. In other words, development is probably delayed above a critical threshold and resumes below this threshold.

Similarly, the precise role of photoperiod is not clear as adults may measure daylength itself or beyond a critical length (Nunes and Saunders 1999). If *L. nigrinus* is like most insects and a critical daylength is used, then it is likely shorter daylength than 12:12 (L:D). Adults may detect a decrease in daylength, but since adults moved to 12 h daylength completed aestivation at the same time as those that remained at 16 h days, it seems more likely that they measure daylength beyond a critical level rather than a directional change.

Using a critical daylength shorter than 12:12 L:D would ensure *L. nigrinus* remain in the soil beyond fall equinox since the earth cools down slowly in autumn and photoperiod shorten before temperatures drop (Danks 1987). This would be an ecologically appropriate time to emerge since HWA completes diapause in early October (Zilahi-Balogh et al. 2003a).

There is a distinction made between the three main phases of diapause: induction, diapause development, and completion and each are potentially cued by environmental signals. Induction was not investigated during this study, but previous work indicates that signals received during induction can modify subsequent requirements and alter the intensity of diapause (Bland 1971, Xue et al. 1997). In *Mamestra brassicae* (L.) (Lepidoptera: Noctuidae), higher temperatures during the induction period results in a more intense summer diapause (Danks 1987). If *L. nigrinus* had an induction period for aestivation and it was immediately following eclosion, then the pattern of expected emergence is similar to that found.

In some cases though, the dormant stage does not appear to be influenced by current or preceding environmental cues, and diapause is initiated under any condition. In other words, there is no sensitive stage for induction (Ehlert et al. 1997, Topp 2003). This appears to be the case with *L. nigrinus*, however it has not yet been investigated and needs confirmation. Ecologically, it would make sense for *L. nigrinus* to have an obligate diapause because it is univoltine and host specific on an ephemeral host. There would be no benefit to remaining active throughout the summer when its only host is dormant. Some beetle species, such as *Coccinella novemnotata* Herbst (Coccinellidae), are sensitive to inductive environmental cues for a week after eclosion (McMullen 1967). Other coccinellids, like *Chilocorus bipustulatus* (L.), are sensitive to induction in both the adult and larval stages

(Danks 1987), so information about previous conditions is passed on from stage to stage, or even generation to generation (McWatters and Saunders 1997, Xue et al. 1997).

To determine if diapause is obligate, *L. nigrinus* larvae should be reared under decreasing daylength and adults should be subjected to very cold temperatures upon eclosion. Some species that have summer diapause and are induced by long days and high temperatures are *Bombyx mori* L. (Hymenoptera: Bombycidae) (Beck 1980), *Phytomyza nigra* Meigen (Diptera: Agromyzidae) (Kamm 1977), and *Hypera brunneipennis* (Madubuyi 1978). Aestivation in *Adelges tsugae* is induced mainly by high temperatures but modified by photoperiod (Salom et al. 2001). Temperature is also known to induce summer dormancy in *Heliothis virescens* (Butler et al. 1985b), (Ohashi et al. 2003), and *Delia antiqua* (Meigen) (Diptera: Anthomyiidae) (Ishikawa et al. 2000).

In *L. nigrinus*, diapause induction was not investigated for two reasons. First, pupation of *L. nigrinus* is restricted to a narrow temperature range (15° C is optimal) (Zilahi-Balogh et al. 2005c) and adults enter aestivation immediately after eclosion. Experiments to prevent dormancy have been avoided simply because optimal survival is of primary importance. Secondly, conditions that would force *L nigrinus* to skip its dormant phase would be entirely out of ecological context and somewhat irrelevant for our purposes since we have no interest in preventing aestivation in *L. nigrinus*. Since HWA, *L. nigrinus'* sole prey, is also dormant throughout the summer and not available as a food source for laboratory colonies, it makes sense to allow adults to enter dormancy. Attention has focused on delaying *L. nigrinus* emergence from aestivation until HWA has resumed development in the field and is available for use in rearing the predators.

Once diapause has been induced, a critical temperature or accumulation of temperature is often a requirement for its completion, and usually, there is a requirement for temperatures within a certain range while the rate of diapause development may also depend on the actual temperature within this range (Wipking 1995, Xue et al. 2001). Once diapause development is complete, many species simply require conditions that allow post-diapause morphogenesis to take place, such as temperature above the threshold for flight activity. In other cases, development following the dormant period require moisture, particularly in species that come from habitats that are seasonally dry. This is usually a direct effect, and moisture stimulates or allows resumption of development in animals that have already completed diapause development. For example, the wheat gall midge, *Contarinia tritici* (Kirby) (Diptera: Cecidomyiidae), emerges in response to soil moisture following a temperature-dependent phase of diapause development (Danks 1987).

Moisture is not a factor governing diapause development in *L. nigrinus* but it may stimulate emergence (ends post-diapause), as more adults emerged at higher soil moisture levels. In the past, there has been a higher rate of emergence in the colony in the days after water was added to aestivation containers. The number of adults emerging from aestivation was also significantly influenced by temperature, which is likely correlated with the duration of aestivation. More adults emerged at lower temperatures because their diapause was shorter and therefore fewer individuals had exhausted their reserves.

These experiments were blocked by cohort, which accounted for some of the variation in the duration of aestivation and a very large amount of variation in the number of individuals to emerge from aestivation. Cohort is known to affect the duration of aestivation. Wade and Rummell (1978) found that earlier entry into dormancy sites by *Anthonomus*

*grandis* Boheman (Coleoptera: Curculionidae) gave earlier emergence the next year. The same pattern has been observed for *L. nigrinus* in the colony and in experiments. More importantly, the large influence of cohort on the number of individuals emerging from the soil is of greater interest due to mass rearing implications.

Variance in emergence of cohorts could be due to one or several factors. The survival of individuals through pupation and aestivation may vary, depending on the health or conditions they are subjected to during development. Or the variance in emergence may be artificial, an artifact of differences in type of pupation container. Since the stage of HWA varies throughout the period of larval activity (February - June), it may influence the number of adults emerging in the fall and is an area in need of further investigation. The stage of HWA has been found to greatly influence the behavior of *Sasajiscymnus tsugae,* a coccinellid predator of HWA (Palmer and Sheppard 2002).

In addition to changing food quality, blocks sometimes varied in container type due to constraints in the number of identical pupation containers. The blocks with the highest emergence were clear plastic and the lids had holes with screens. The screened lids may have allowed adults to rest after emergence, making them more visible and more likely to be collected and recorded than containers without screened lids (Chapter 4). This has implications for rearing and is an area in need of further investigation as much time is spent collecting emerging adults in autumn. Modification of pupation containers that take advantage of the positively phototactic behavior observed in emerging adults for easier collection would save time and perhaps increase the proportion of adults collected during emergence. It is also possible that the individuals in these blocks were better able to perceive

photoperiod compared to individuals in opaque plastic containers. If this is the case, photoperiod may be a more important seasonal cue than these results convey.

The perceived duration of a light signal depends on the response threshold of their light receptors. Unfortunately, light intensity was difficult to control for in this experiment and ranged from 796 – 2303 lum/$m^2$ across the nine environmental chambers. Differences in container type surely contributed to variation in light intensity experienced by individuals, however unless intensity itself is perceived, container type would not have affected the actual daylength perceived. Light intensity and quality has been documented as a cue used for dormancy control (Philogene 1982), and a common light intensity threshold is 1 lum/$m^2$ (Danks 1987). The light intensity threshold required for a response in *Grapholitha molesta* (Busck) (Lepidoptera: Tortricidae) is up to 32.3 lum/$m^2$, however the woodlouse *Armadilliduim vulgare* (Latreille) (Armadillidiidae) perceives light at 1.5 x $10^6$ lum/$m^2$ (Danks 1987). Caddisflies that aestivate in caves remain in the region of weak illumination, rather that entering total darkness, suggesting that the basic light-dark signal can be monitored precisely even in habitats where the signal is very faint (Malicky and Winkler 1974).

The remarkable extent of seasonal adaptations observed in insect populations reflect their interactions with a wide range of environmental conditions. Therefore, as one might expect, dormancies have multiple purposes, in particular, they serve not only to survive adverse seasons but also to ensure that activity and development take place at favorable times of year (Danks 2001). The dormancy period for *L. nigrinus* accomplishes several things: dormant stages are commonly protected by physical and behavioral adaptations to extreme weather and therefore survive the adverse conditions, it synchronizes the reproductive activity of *L. nigrinus* with that of its host to ensure adequate conditions for their progeny, and they

avoid periods when prey is absent. Like many parasites or herbivores, *L. nigrinus* is highly host-specific and goes dormant at the same time as its host, as observed with *Speyeria zerene* (Boisduval) (Lepidoptera: Nymphalidae) (Sims 1984) and *Athalia japonica* (Klug) (Hymenoptera: Tenthredinidae) (Nagasaka 1992).

Behavioral adaptations evolve in response to selective pressures and allow certain conditions to be avoided. Timing of development influences survival during extreme conditions and coincidence with food supplies. It is hard to determine what factors created selective pressure for summer dormancy to evolve and which ones are fortuitous results. However, summer dormancy also allows *L. nigrinus* to avoid periods when activity of natural enemies is high.

Responses to seasonal factors are often complex and can be programmed in advance genetically, or the genetic program can be modified by environmental cues. *L. nigrinus* likely uses temperature primarily and photoperiod as a modifying factor because in its habitat, they are easily detected and reliable season cues that will ensure adults complete diapause at the same time as their host.

If it had not been for the recovery of $F_1$ and $F_2$ individuals following field releases, there be some been concern for *L. nigrinus* diapause in eastern North America since average temperature and particularly precipitation patterns are quite different than those in the Pacific Northwest. However, *L. nigrinus* has survived the 2003 and 2004 summers in several states in the mid-eastern U.S. and has shown remarkable resilience to high moisture levels, even floods, at field release sites (Chapter 3), Mausel unpublished data, McDonald pers. comm.). In addition, *L. nigrinus* has displayed a relatively high plasticity in response to a wide range of environmental conditions in the lab, as some individuals emerged from every condition.

Since *L. nigrinus* emergence can be delayed in the laboratory, adult post-emergence mortality has decreased and field releases are increasing. *L. nigrinus* has now been released in 6 states, at over 21 locations and many more are planned in the coming months. In addition, since procedures for rearing have been worked out, several other groups have begun rearing this predator to increase the number of beetles available for release and therefore expedite their establishment in the eastern United States. With the help of other introduced predators, *L. nigrinus* may be able to reduce HWA populations to a level that is not injurious to trees.

Table 5.1. The percentage of adults ($\overline{X}$ ± S.E., n = 20) that emerged from the soil when held at constant temperature and photoperiod from eclosion through to emergence following aestivation.

| **Conditions under which Adults were Maintained Throughout the Summer** | | **Mean Percent of Adults that Emerged from the Soil[1] (%)** | |
|---|---|---|---|
| **Temperature (°C)** | **Photoperiod (L:D)** | | |
| 10 | 8:16 | 53.3 ± 2.4 | a |
| | 12:12 | 50.3 ± 3.6 | a |
| | 16:8 | 38.5 ± 4.3 | a |
| 15 | 8:16 | 43.0 ± 2.3 | a |
| | 12:12 | 46.0 ± 1.9 | a |
| | 16:8 | 40.4 ± 2.1 | a |
| 20 | 8:16 | 43.0 ± 3.1 | b |
| | 12:12 | 39.3 ± 2.9 | b |
| | 16:8 | zero adults emerged | c |

[1] The percentage of adults emerging from aestivation was not significantly affected by photoperiod ($p > .06$), however there is a distinct pattern, where, at each temperature, more individuals tended to emerge when maintained at shorter daylengths than those adults maintained at longer daylengths throughout the summer. Adults held at temperatures with different letters were significantly different (Fishers LSD; $F_{(2, 107)} = 4.12$, $p = 0.019$).

Table 5.2. The number of days ($\overline{X}$ ± S.E.) *L. nigrinus* remained in the soil when maintained at constant temperature or daylength (Exp. 2) compared to duration of aestivation for adults held in summer conditions (20°C 16:8 (L:D)) and moved to cooler temperature (Exp. 3).

| **Adults stored at Constant Conditions** | | | | **Stored at Summer Cond. (20°, 16:8 L:D) and moved to assigned Temp. in Autumn** |
|---|---|---|---|---|
| **Temperature (°C)** | Mean Days in Soil at Constant Temperature[1] (± S.E.) | **Photoperiod (L:D)** | Mean Days in Soil at Constant Photoperiod[2] (± S.E.) | Mean Days in Soil[3] (± S.E.) |
| **10** | 129.1 ± 1.4 a | **8:16** | 159.7 ± 3.8 *a* | 166.0 ± 1.9 a |
| **15** | 160.6 ± 1.8 b | **12:12** | 162.7 ± 4.5 *a* | 164.7 ± 1.2 a |
| **20** | 197.3 ± 2.6 c | **16:8** | 199.3 ± 3.4 *b* | 185.4 ± 0.9 b |

[1] Means with different letters in this column are significantly different (Fisher's LSD; $F_{(2,107)} = 379.59$, $p < 0.0001$).

[2] Means with different letters in this column are significantly different (Fisher's LSD; ($F_{(2,107)} = 6.15$, $p = 0.003$). The mean duration of aestivation at 16:8 (L:D) h includes adults held at 10° and 15°C only, since no adults emerged from the 20° 16:8 (L:D) h treatment.

[3] Means with different letters in this column are significantly different (Fisher's LSD; $F_{(2,178)} = 179.08$, $p < 0.0001$).

Table 5.3. The percentage of adults ($\overline{X}$ ± S.E., n = 20) that emerged from aestivation when held at constant temperature and 30% or 45% moisture level (Exp. 2) or when transferred from summer conditions to cooler temperatures in autumn (Exp. 3).

| **Conditions under which Adults were Maintained** | | **Mean Percent (± S.E.) of Adults Emerging from Soil Following Aestivation (%)** | | |
|---|---|---|---|---|
| **Temperature (°C)** | **Soil Moisture (% Saturation)** | **Remained at Constant Conditions[1]** | | **Moved from 20°C, 16:8 L:D in Sept./Oct.[2]** |
| **10** | **30** | 41.8 ± 1.2 | a | 38.0 ± 2.3 a |
| | **45** | 56.0 ± 0.2 | | - |
| **15** | **30** | 40.5 ± 1.0 | a | 38.9 ± 1.9 a |
| | **45** | 44.6 ± 2.3 | | - |
| **20** | **30** | 22.8 ± 2.7 | b | 27.8 ± 2.0 b |
| | **45** | 32.0 ± 4.5 | | - |

[1] There was no interaction between the effects of temperature and moisture on percentage of adults emerging, therefore no statistical tests were performed but are presented for purpose of comparing experiments. Adults held at temperatures with different letters were significantly different (Fisher LSD; $F_{(2, 107)} = 4.12$, $p = 0.019$).

[2] Means with different letters in this column are significantly different (Fisher's LSD; ($F_{(2,178)} = 6.56$, $p = 0.002$).

Table 5.4. The mean number of days ($\overline{X}$ ± S.E.) adults remained in soil during aestivation when held at constant temperature and photoperiod (Exp. 2) throughout the summer or when held at summer conditions (20°C, 16:8 (L:D)) and transferred to cooler and/or shorter days on three different dates in autumn (Exp. 3).

| **Environmental Conditions** | | **Maintained at Constant Conditions[1] throughout Summer and Autumn** | **Maintained in Summer Cond. (20°C and16:8 L:D) and moved to New Cond. in Autumn** | | |
|---|---|---|---|---|---|
| | | | | **Change in Temperature[2]** | **Change in Photoperiod[3]** |
| **Temperature (°C)** | **Photoperiod (L:D)** | **Days in Soil** | **Date of Change** | **Days in Soil** | **Days in Soil** |
| **10** | **8:16** | 128.1 ± 2.2 a | **Sept. 9** | 154.8 ± 2.1 *a* | 159.9 ± 2.1 *a* |
| | **12:12** | 127.9 ± 3.0 a | **Sept. 24** | 167.0 ± 3.3 *bc* | 171.2 ± 3.2 *c* |
| | **16:8** | 134.0 ± 3.8 a | **Oct. 7** | 174.8 ± 3.2 *d* | 166.2 ± 3.2 *b* |
| **15** | **8:16** | 156.7 ± 4.1 b | **Sept. 9** | 158.2 ± 1.9 *a* | 160.5 ± 2.1 *a* |
| | **12:12** | 162.2 ± 4.4 b | **Sept. 24** | 164.3 ± 2.1 *b* | 165.4 ± 1.8 *b* |
| | **16:8** | 166.2 ± 5.4 b | **Oct. 7** | 169.8 ± 2.1 *c* | 166.4 ± 2.4 *b* |
| **20** | **8:16** | 194.8 ± 4.2 c | **Sept. 9** | 181.2 ± 1.7 *e* | 182.8 ± 1.2 *d* |
| | **12:12** | 203.7 ± 3.4 c | **Sept. 24** | 185.9 ± 1.4 *ef* | 187.3 ± 2.0 *d* |
| | **16:8** | - | **Oct. 7** | 187.2 ± 1.2 *f* | 185.0 ± 1.3 *d* |

[1] Constant temperature or photoperiod had a significant effect on the number of days *L. nigrinus* remained in the soil (Table 5.2).

[2] The effect of a decrease in temperature varied with the date at which conditions changed; different letters within the column indicate means were different (Fisher's LSD; $F_{(2,178)} = 57.88$, $p < 0.0001$).

[3] The effect of a decrease in temperature varied with decreasing daylength, different letters within this column indicate different means (Fisher's LSD; $F_{(2,178)} = 12.51$, $p < 0.0001$).

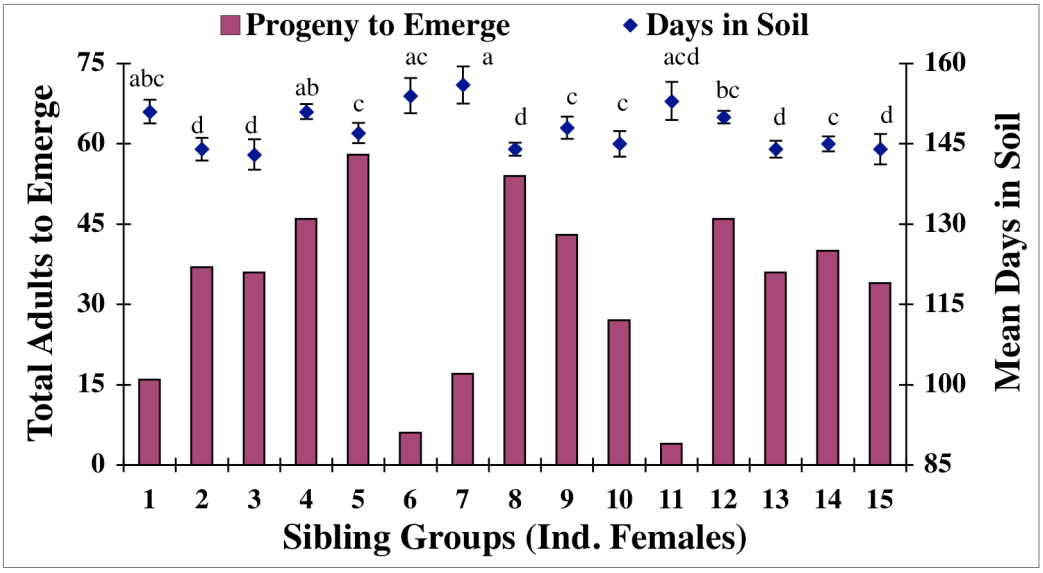



Figure 5.1. The total number of *L. nigrinus* adults to emerge from each sibling group following aestivation and the mean number of days ($\overline{X}$ ± S.E.) the progeny in each sibling group remained in the soil. Means with different letters are significantly different ($F_{(14,479)} = 1.80$, $p = 0.036$).

# Chapter 6

# Procedures for Rearing *Laricobius nigrinus*

## Introduction

In order for *L. nigrinus* to be a viable biological control candidate, it must be successfully mass reared for field releases. Our mass rearing efforts have focused on laboratory propagation and the use of field insectaries. Successful procedures for laboratory rearing *L. nigrinus* have been developed based on the results of four years of research (Chapter 5). Although there is room for improvement, the following methods have yielded several generations of the beetle. This chapter describes current procedures used for rearing each life stage in the laboratory at Virginia Tech.

Figure 6.1 illustrates the life cycle of *L. nigrinus* and the general rearing procedures coinciding with each life stage. References made to specific months indicate the average time of year HWA is available in particular stages in Blacksburg, VA. The exact time of year *L. nigrinus* begins and ends oviposition is influenced by the stage of the HWA provided to them. All materials used are described in Chapter 4, unless otherwise stated.

### I. Active *L. nigrinus* Adults

The general procedures for storing and maintaining adults are similar throughout the year, however there are minor changes in conditions between the pre-oviposition (October - January) and oviposition (February - June) periods (Table 6.1).

**Storage: (Adult Feeding/Oviposition Containers)**

- Adults are in 3.78 L containers with 2 holes for ventilation and moistened filter paper are cut to fit the bottom.
- Each container holds one hemlock "bouquet" (a floral foam-filled film canister holding 10-15 heavily HWA-infested hemlock branches (~20 cm long).
- Adults are stored in environmental chambers programmed to create conditions shown in Table 6.1.

**Maintenance:**

- At the end of each feeding period, every adult is recovered from its current container and transferred to a new container with a fresh hemlock bouquet (sprayed with distilled water).
- Containers are kept at 4°C immediately before adults are fed to reduce flight.
- Adult transfers are carried out under a nylon screen (Chaffon, Fabric.com, Marietta, GA), table-top cage to prevent flying adults from escaping.

(a) Pre-ovipositing Adults:

- If all adults are recovered from old containers, old hemlock branches from bouquet are discarded.
- If some individuals are not accounted for, old hemlock branches are placed in a large Plexiglass® box at room temperature and observed daily for missing adults.

(b) Ovipositing Adults:

- It is critical to provide heavily-infested foliage, with ovipositing HWA, to adults to ensure that early instars can access adelgid eggs. Adult *L. nigrinus* should be provided

with enough adelgids (preferably excess amounts, ~200 ovisacs per adult beetle) for themselves and the early instars of the progeny they produce. Host material provided to adults should contain HWA still ovipositing to ensure eggs are still available when larvae are feeding. Collecting host material from various elevations and storage at various temperatures can be advantageous when targeting this specific stage of HWA.

- To ensure predator eggs are deposited in optimal sites (singly, in full adelgid ovisacs) adults are fed twice a week and the number of adults per container is reduced (Table 6.1). These modifications also decrease larval competition during their development and increase synchrony of larval maturation.
- The hemlock branches that adults are removed from at each feeding contain the *L. nigrinus* eggs laid since the time of the last feeding. These branches are transferred to Parafilm-wrapped floral foam blocks and are inserted alternately with fresh hemlock branches infested with oviposting HWA. The additional fresh foliage provides host material for the more mobile 3$^{rd}$ and 4$^{th}$ instars. These blocks are placed into funnel cages (Chapter 4).

II. *L. nigrinus* Larvae (developing from February – June):

After a few weeks, the *L. nigrinus* eggs hatch within the woolly ovisac and early instars feed on the nearby adelgid eggs. Third and fourth instars are more mobile and feed on adelgid eggs and developing nymphs. The duration for *L. nigrinus* development from egg to mature fourth instar larvae (pre-pupae) varies depending on temperature and food quality. The larvae develop at temperatures ranging between 9° and 21° C, however, pupal development does not occur at the higher temperatures of this range. The optimal temperature

for successful development of larvae is likely between 12° and 18° C (Zilahi-Balogh *et al.* 2003b). After consuming a sufficient amount of prey (200+ eggs) to complete larval development, the pre-pupal stage is reached. This stage is a non-feeding fourth instar that drops from the hemlock branches and seeks a pupation site in the pupation medium. Larvae are reared in funnel cages, designed to take advantage of this "dropping/migrating" stage so mature larvae (prepupae) essentially collect themselves.

**Storage: (Larval Funnel Cages)**

- Hemlock branches with HWA and *L. nigrinus* eggs are inserted into Parafilm-wrapped floral foam blocks, intermixed with fresh HWA-infested branches, and placed into funnel cages. The Mason jars below each funnel cage contain about 2 teaspoons of moistened pupating medium.[1]
- The funnel cages are set up on racks in cold rooms that are maintained at about 13° ± 2°C and daylength increasing at a rate similar to natural conditions. At this temperature and with adequate prey, mature larvae begin collecting in the Mason jars below the funnels after 3-4 weeks. If the temperature increases above 18°C, development rate of larvae increases dramatically, causing funnels to yield both mature and immature larvae. It is possible that the higher temperatures cause the larvae to be more active, resulting in immature larvae collecting in the jars below the funnels. This situation can also be observed when larvae are not provided with an

[1] Pupation Medium:
- Southland peat moss (sifted through mesh screen with 0.5 cm openings), Mosser Lee long fiber sphagnum moss (ground finely), and Play sand. Mix materials (2:2:1 peat:sphagnum:sand) and add distilled water until mixture reaches ~50% saturation. Mixture is then sterilized using two treatments of heat and pressure for several hours or steam sterilization for 12+ hours separated by 24+ hours at room temperature.

adequate amount of HWA eggs within the funnel. Differences in appearance and behavior can separate mature and immature larvae.[2]

**Maintenance:**

- Funnels are set up immediately after adults are fed.
- The foliage in each funnel is sprayed with distilled water twice a month and soil in Mason jars below are moistened every couple days (in anticipation for maturing larvae).
- Several days before anticipated larval drop, the Mason jars below the funnels are checked by emptying the contents into a Petri dish and a paintbrush is used to sift through the materials.
- Mason jars are checked daily since mature larvae become settled for pupation if given enough time (~36 h) and mortality of immature larvae in the Mason jars is high unless immature larvae are returned to branches with prey promptly.
- Mature larvae (prepupae) are transferred, using a moistened paintbrush, to pupation/aestivation containers for the summer.
- Immature larvae are transferred to HWA-infested hemlock and placed back in a funnel cage.

[2] Mature larvae have a yellow ventral side and sclerotized dorsal side; typically are moving and are negatively phototactic; will drop off branch if placed back on HWA-infested hemlock branch. Immature larvae are smaller in size, dark in color, and often have white wool attached to their dorsal side; attach to objects and do not move; will not drop off branch if placed back onto HWA-infested hemlock branch.

III. Mature Larvae / Prepupae: (March – July)

Upon being transferred to pupation/aestivation containers, mature larvae burrow into the soil, form pupal cells, and remain in a c-shape within their pupal cells for 10-14 days before development into pupae. Within 36 h of dropping from the foliage, prepupae will settle into cells within the pupation media to pupate. It is important that individuals are transferred to a pupation/aestivation container before a pupal cell is formed since they have difficulty moving to a new site to pupate after they have settled.

**Storage: (Pupation/Aestivation Containers)**

- Quart-sized and half gallon-sized, clear and opaque, plastic containers with several screened holes for air movement. Each container contains at least 5 cm of pupating medium, the bottom layer is packed tightly and the upper layer loosely.
- Depending on the size of the container, between 50 and 200 larvae are placed in each container
- Mold within the pupation/aestivation containers has been a recurring problem. Sources of fungal spores are minimized; all containers and the pupation medium are sterilized; and an anti-fungal agent (methyl paraben) is applied to the soil surface weekly (5 g dry weight methyl paraben is added to 3000 ml of distilled water and mixed thoroughly).
- Pupation/aestivation containers are maintained at 15°C and long days (16 h).

**Maintenance:**

- Moisture level of pupation medium should be maintained at about 30-40% saturation by spraying distilled water in containers once a week.

IV. Pupae/Aestivating Adults: (late April through July)

The delicate pupae develop two weeks after burrowing in the soil. *L. nigrinus* pupae are bright yellow and require a narrow temperature regime during this time (Zilahi-Balogh *et al.* 2003c). At 15°C, pupation is complete in ~2 weeks and the newly-eclosed adults remain under the soil surface, and enter summer diapause (aestivation). Adults remain in the soil until they are cued to emerge by a decrease in temperature (Chapter 5). To prevent premature emergence, aestivating adults are stored at high temperatures until mid-September, at which time, the temperature is decreased and adults emerge several weeks later.

**Storage: (Pupation/Aestivation Containers)**

- Pupation/aestivation containers are kept in the large cold room at 15°C until pupation is complete. Once adults have eclosed, containers are moved to 19°C and long daylength (16h) until mid-September when the containers are moved to 13°C and decreasing daylength at a rate similar to natural conditions.

**Maintenance:**

- Pupation/aestivation containers are watered weekly to maintain about 30% soil saturation.
- Containers are checked every other day beginning in late August to recover adults emerging prematurely
- In September, after the temperature is decreased, containers are checked daily for emerging adults.

V. Emerging Adults: (Emerge September – December)

Adults complete aestivation and emerge from the soil in the fall, approximately 3 weeks after the temperature is decreased. Adults become active and climb up the sides and lids of the pupation/aestivation containers. Premature adult emergence is undesired because emerging adults require developing HWA nymphs to feed on, which do not emerge from diapause until October.

**Storage: (Feeding/Oviposition Containers)**

- Emerging adults are transferred from the lids and sides of the pupation/aestivation containers to adult feeding/oviposition containers with developing HWA nymphs and distilled water.
- Recently emergent adults are kept at 6°C and 12 h daylength. Temperature is lowered if prey is limited.

**Maintenance:**

- Pupation/aestivation containers are checked for emerging adults several times per day, particularly in the late afternoon and evening.
- Distilled water is sprayed through the screen lids of the feeding/oviposition containers several times per week to ensure adults have adequate water. When temperature is decreased (about November), the frequency of watering decreases.
- Active adults are either released in the field or maintained in feeding/oviposition containers according to the procedures outlined in Table 6.1.

**Discussion**

These rearing procedures have produced approximately 44, 000 $F_1$ adults during the past 4 years. Survival through pupation and aestivation has a greater influence on the number of $F_1$ adults produced, rather than size of the initial colony. In addition, field collected adults seem to be more fecund than lab-reared adults, therefore starter colonies should be larger (≥2000) if adults are lab-reared and smaller (500-1000) if adults are field-collected.

Several aspects of the current rearing procedures need further improvement. Most importantly, the preparation and maintenance of the pupation medium needs to be modified to reduce the high level pre-pupal and pupal mortality that occurs. In addition, pupation/aestivation containers should be modified to increase the ease and efficiency of adult recovery while they are emerging from the soil. Development of rearing containers that take advantage of the "migratory stage" of mature larvae immediately after dropping from the foliage would reduce the time spent checking funnel jars. The cage should allow larvae, needles, and debris to fall into one level and from there, enable the larvae to move themselves to a pupation/aestivation container, thereby preventing needles and debris from falling into the sterile pupating medium.

Table 6.1. Holding temperatures and day-length for *Laricobius nigrinus* adults at different times of their active period.

| Time of Year | Day:Night Temp. (°C)[1] | Daylength (h)[2] | Adults per Container | Frequency of Feeding | Destination of Old Hemlock Bouquets |
|---|---|---|---|---|---|
| Pre-oviposition (October-January) | 4:2 | 12 to 10 | 50 | Every 2 weeks | Large plexiglass box to recover lost adults |
| Oviposition (January-February) | 6:4 | 10 to 12 | 25 | Once per week | Inserted in floral foam and placed in funnel cages |
| Oviposition (March-June) | 10:8 | 12 to 16 | 25 | Twice per week | Inserted in floral foam and placed in 1+ funnel cage(s) |

[1] Increase in temperature should coincide with peak *L. nigrinus* oviposition and when HWA ovisacs are 50-75% full, not according to the calendar.

[2] Daylength is gradually increased or decreased over each period

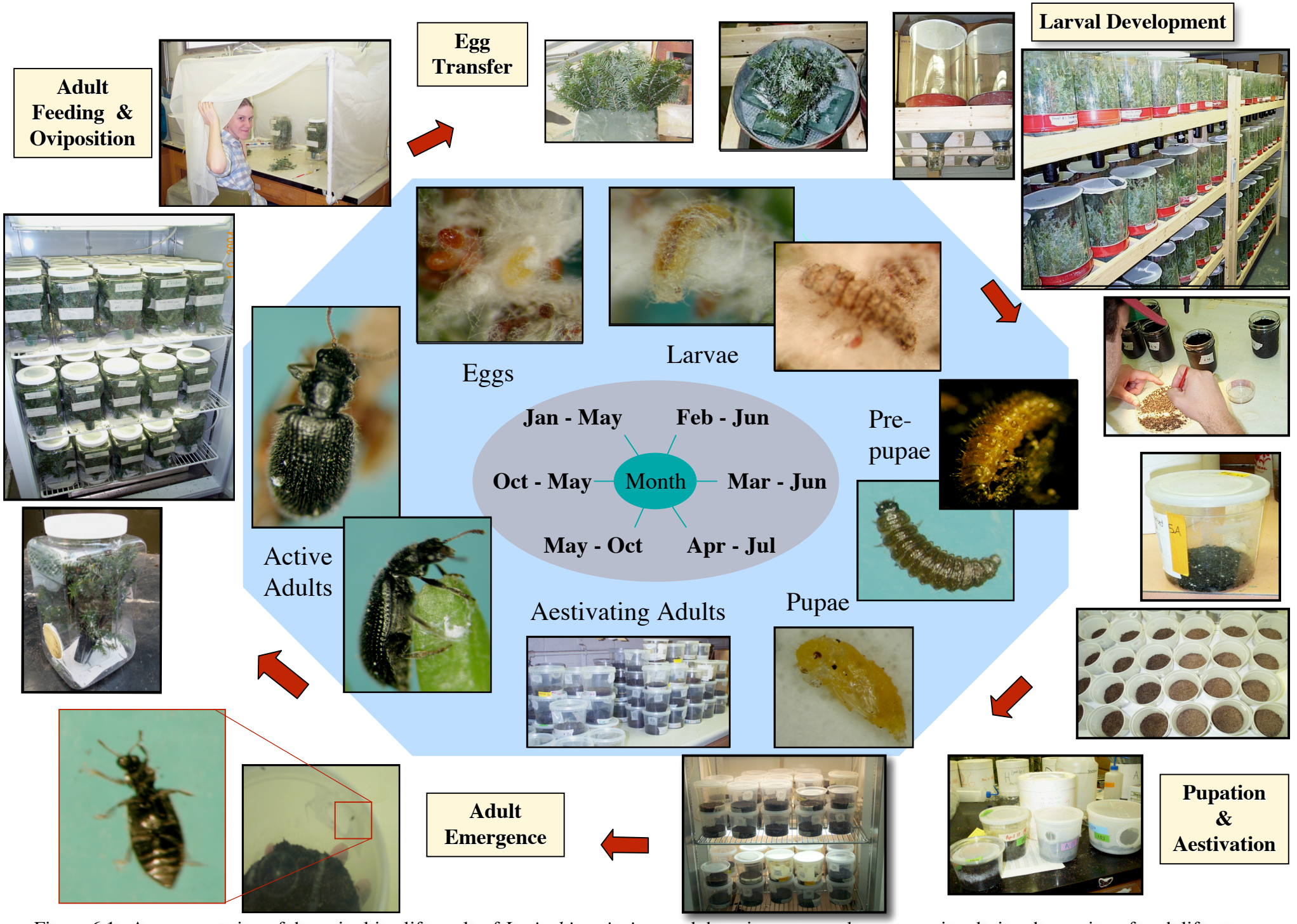



Figure 6.1. A representation of the univoltine life cycle of *Laricobius nigrinus* and the primary procedures occurring during the rearing of each life stage.

# Chapter 7

## Summary

*Laricobius nigrinus* (Fender) (Coleoptera: Derodontidae), has been approved for field release as a biological control agent against hemlock woolly adelgid, *Adelges tsugae*, Annand (Hemiptera: Adelgidae), a lethal pest of eastern hemlock trees. HWA is an exotic pest in the eastern U.S. that attacks and kills hemlock *Tsuga* spp. and is responsible for the widespread mortality of *Tsuga canadensis* in several eastern states. Preservation of hemlock trees is important both ecologically and economically. Due to constraints related to the use of chemicals, biological control is deemed the most viable option for controlling HWA in the forest setting.

The overall objective of this research was to evaluate *L. nigrinus* for its suitability as a biological control agent for HWA, *Adelges tsugae*, in the field in the eastern U.S., and to improve and establish rearing procedures for this predator.

Chapter 2 reports the survival and reproduction of *L. nigrinus* and the impact it has on HWA populations under natural conditions. Predator exclusion studies indicate adults survive the winter and spring in Virginia and lay up to 41 progeny per female. In two field seasons, the final density of sistens and progrediens was significantly lower on caged branches containing *L. nigrinus* than on caged and uncaged branches without predators.

Chapter 3 describes a caged study that was conducted to evaluate survival and oviposition of *L. nigrinus* adults, egg development in the field, and their impact on HWA density. In spring, groups of *L. nigrinus* females were caged on hemlock branches and moved after 10 days. Adult survival, total eggs laid, and oviposition location with respect to prey

abundance were determined. An estimated 10, 344 eggs left on open branches represented the first field release of *L. nigrinus* in the eastern U.S. *L. nigrinus* density affected the total eggs laid per female and oviposition site selection. Branches caged with *L. nigrinus* had lower densities of HWA than branches without predators. $F_2$ adults were recovered in fall 2004.

In Chapter 4, life stages of *L. nigrinus* suffering high developmental mortality within the colony and factors influencing the survival of each life stage were determined. The number of individuals and mortality rate of each life stage are reported from 2000-2004. Factors investigated on the feeding stages (adults and larvae) include: adult survival and feeding, length of ovipositional period, density per cage or container, and larval survival. Factors studied for effects on non-feeding stages (pupae and aestivating adults) were: soil moisture level, type of pupation medium, soil sterilization, soil depth, density per container, container type, and larval cohort. Results indicate that adult survival is higher when held at low temperatures and offered developing HWA nymphs in the fall. Larval production can be maximized by limiting the density of adults per container and larvae per funnel cage. Survival of the non-feeding life stages can be increased by using a sterilized mixture of sphagnum and peat moss maintained at 30-40% moisture level and by leaving individuals undisturbed.

Chapter 5 describes three experiments conducted to examine genetic and environmental factors influencing adult development and termination of diapause. The first study evaluated the influence of genetics on the number of adults that emerged and the duration of aestivation. The second was on aestival survival of *L. nigrinus* and emergence from diapause at 2 moisture levels and 9 constant temperature and photoperiod combinations. The third study examined the effect of a late-season change in temperature and day length on

adult survival and their time of emergence. Survival of *L. nigrinus* was highest when maintained at a high moisture level and cooler temperatures throughout the summer. Time of emergence was significantly delayed by increasing temperature and day length, and conversely, emergence can be hastened by decreasing temperature at desired emergence time. Temperature appears to be the primary factor influencing time of emergence, and day length a modifying factor.

In Chapter 6, the methods for rearing *L. nigrinus* that were developed based on the results of Chapters 4 and 5 are described in detail.

Based on these findings, it can be concluded that *L. nigrinus* is a promising candidate for biological control of HWA in the eastern United States because it:

a) has high survival and reproduction in Virginia;

b) has a significant impact on HWA populations within sleeve cages;

c) is able to survive several generations in Virginia;

d) can be reared in large numbers.

Rearing procedures for *L. nigrinus* have improved over the past four years based on the results of this research. With adult emergence from aestivation synchronized with HWA development, over 8, 000 *L. nigrinus* adults have been released in 6 eastern states. Two other institutions have begun rearing *L. nigrinus*, using the methods developed at Virginia Tech, to expedite field releases. *L. nigrinus* will continue to be released in threatened stands throughout the eastern United States. Establishment is anticipated since $F_1$ and $F_2$ adults have been recovered. However, reliable sampling methods, predator dispersal, and the impact it exerts on HWA density in the open field situation need to be determined.

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## Vita

Ashley Lamb was born in Calgary, Alberta, Canada on September 27, 1976. She grew up in a remote valley near Kimberley, British Columbia, spending much time outdoors, playing softball and skiing. Upon high school graduation, Ashley moved to Victoria, British Columbia to pursue an undergraduate degree in biology. As an undergrad, she worked for the Canadian Forest Service as a technician at the Pacific Forestry Center supervised by Dr. Lee Humble. It was there that she was inspired by entomological research and got involved with *Laricobius nigrinus*, as a field technician. Ashley collected field data on this predator in support of a biological control program at Virginia Tech. Following the completion of her undergraduate degree, Ashley moved to Virginia to pursue a graduate degree in forest entomology under the advisement of Drs. Scott Salom and Loke Kok. During her time at Virginia Tech, Ashley was actively involved in many departmental activities and events such as serving as President of the W. B. Alwood Entomological Society, participation in outreach programs, and serving on committees. As a student, she was the recipient of the Asa Fitch Memorial Scholarship (Eastern Branch ESA), the Gene A. and Ida Mae James Graduate Tuition Scholarship (College of Agriculture and Life Sciences), and the J. M. Grayson Scholarship Award (Entomology of Department, Virginia Tech). She is currently a member of the Entomological Society of America, the Entomological Society of British Columbia, and the International Organization for Biological Control.