

**Biology and Host-Range Testing of *Laricobius kangdingensis* sp. n.  
(Coleoptera: Derodontidae), a Newly Discovered Predator of Hemlock  
Woolly Adelgid, *Adelges tsugae* Annand (Homoptera: Adelgidae)**

By

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**Abstract**

The biology and host-specificity of *Laricobius kangdingensis* sp. n. (Coleoptera: Derodontidae), a new predator of hemlock woolly adelgid (HWA), *Adelges tsugae* Annand (Homoptera: Adelgidae) were investigated and compared with that of a related predator of HWA, *L. nigrinus* Fender. Adults became active in June 2003; the males emerged first and in greater numbers than the females. The sex ratio of female to male was 1: 1.1. *Laricobius kangdingensis* sp. n. has four larval instars. The total number of eggs laid between 7 July 2003 and 7 August 2004 was 1327. Mean lifetime fecundity per female was  $196.4 \pm 53.4$  eggs and 97.9 eggs for the P<sub>1</sub> (parental gen.) and F<sub>1</sub> generations, respectively. In comparison, lifetime fecundity for F<sub>1</sub> *L. nigrinus* was  $100.8 \pm 89.6$  eggs.

Developmental rates were studied in fall 2003 and spring 2004. Combined with data gathered previously at 12 and 15°C in early 2003, the 6, 9, and 18°C data were used to determine lower temperature development thresholds for the egg (2.8°C), larval (1.6°C), and pre-pupal (5.8°C) stages. The egg and larval thresholds are lower than those observed for *L. nigrinus*. *Laricobius kangdingensis* sp. n. individuals completed development only at 12 and 15°C; however, these adults did not emerge from aestivation after pupation.

*Laricobius kangdingensis* sp. n. appears to be host-specific. In paired-choice feeding tests, adults consumed significantly more HWA than two other adelgid species [eastern spruce gall adelgid, *Adelges abietis* (L.), and pine bark adelgid, *Pineus strobi* (Hartig)], the pine needle scale, *Chionaspis pinifoliae* (Fitch), and the woolly alder aphid, *Paraprociphilus tessellatus* (Fitch). In paired-choice oviposition tests, females laid more eggs on HWA-infested eastern hemlock than on any other test prey. In no-choice host suitability experiments larvae fed on HWA; eastern spruce gall adelgid; the larch adelgid, *A. laricis* Vallot; the balsam woolly adelgid, *A. piceae* (Ratzeburg); pine bark adelgid; and pine needle scale, but not on woolly alder aphid or elongate hemlock scale, *Fiorinia externa* Ferris. Although no larvae completed development in any of the bioassays, 6 out of 15 larvae (40%) in the HWA treatment reached the pre-pupal stage. One larva out of 6 reached the pre-pupal stage on pine bark adelgid.

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# Chapter 1

## Introduction and Literature Review

### Impact of Hemlock Woolly Adelgid

Hemlock woolly adelgid (HWA), *Adelges tsugae* Annand, is a lethal pest of eastern hemlock, *Tsuga canadensis* (L.) Carriere, and Carolina hemlock, *T. caroliniana* Engelmann, and can kill trees within four to ten years of infestation (McClure et al. 2001, Cheah, et al. 2004). HWA feeds within xylem ray parenchyma cells that form a living network between the xylem, phloem, and pith (Young et al. 1995, Shields et al. 1996). This feeding activity depletes nutrient stores and disrupts water flow which leads to needle desiccation, destruction of buds, and defoliation (McClure 1995). Consequently, trees become more susceptible to other pests, diseases, and weather damage.

HWA, like other members of the superfamily Aphidoidea, is of Asian origin and was initially observed and described from western hemlock, *T. heterophylla* Raf. Sargent, in Oregon in the early 1920's (Annand 1924). The adelgid made its first appearance on the east coast of the United States 30 years later in Richmond, VA (McClure 1992, Gray and Salom 1996, Parker et al. 1999). Currently, HWA infests half the range of eastern hemlock (Knauer et al. 2002) and is spreading throughout the eastern U.S. at a rate of 17-25 km per year (Souto et al. 1996, McClure 2001). It is eventually expected to infest the entire natural range of hemlock in the U.S. from Maine to Georgia. Hemlock trees are economically valuable, and possess aesthetic beauty and ecological importance. The amplification and release of a complex of natural enemies seems to be the best option to save the hemlocks in forest landscapes since urban management techniques are not feasible in forest settings.

HWA is spread by way of wind, forest mammals, birds, and human activities (McClure 1990). Its survival in new infestation areas is facilitated by its cold-tolerance to temperatures as low as  $-30^{\circ}\text{C}$  (Parker et al. 1999). HWA may be able to adapt to colder temperatures and expand their range over time (Skinner et al. 2003).

HWA is present on western and mountain hemlocks, *T. heterophylla* and *T. mertensiana* (Bongard) Carriere; however, severe infestations occur only on stressed trees, particularly those in ornamental landscape settings (McClure 1992). Asian hemlocks also exhibit sub-lethal levels of adelgid infestation which many believe is due to host-resistance and the presence of effective natural enemies (Cheah and McClure 1996, McClure and Cheah 1999, del Tredici and Kitajima 2004).

Hemlocks are economically and ecologically valuable because they provide unique habitats for many birds, forest mammals, amphibians, reptiles, fish, and invertebrates, thus contributing to biodiversity in the forest (Quimby 1996, Cheah et al. 2004). Hemlocks are also commonly used as “living fences” in landscaping to block wind and noise. They are long-lived, shade-tolerant trees and are best suited to cool, humid areas of the forest understory. Hemlocks cool neighboring habitats with their thick crowns and dense foliage, benefiting aquatic organisms in the summer, while insulating woodland organisms from the cold in winter. As hemlocks die, they will likely be replaced in the forest by hardwood trees such as *Quercus* and *Betula* spp. (Mahan et al. 2004). This change will negatively impact wildlife species (such as the black-throated green warbler, *Dendroica virens*) that are dependent on hemlock for survival.

Hemlocks also play a role in long-term nutrient cycling and soil water chemistry. Tree mortality will result in greater nutrient loss rates and increased nitrogen turnover because nitrogen mineralization and nitrification is lower in hemlock stands than hardwood forests.

Yorks et al. (2003) reported that various ions including  $\text{NO}_3^-$  and  $\text{NH}_4^+$  will be leached from the soil, affecting the stand for many years afterward. Released nutrients can then move into waterways, negatively impacting countless aquatic organisms.

### **Hemlock Woolly Adelgid Biology**

Two generations of HWA, known as sistens and progrediens, are produced each year in the eastern United States. The sistens generation aestivates between July and October in Virginia (Gray and Salom 1996), reaches adulthood in the late winter/early spring, and is entirely female and parthenogenetic. These insects deposit 50 to 300 eggs in white, cottony ovisacs (McClure 1996) which protect the crawlers that hatch in March and April in Virginia into progrediens and sexuparae morphs (Gray and Salom 1996). Progrediens are apterous, stay on the hemlock host, and produce ovisacs in June and July that contain sistens eggs, which will hatch, settle, and enter aestival diapause. Sexuparae, a winged morph, are sometimes found developing with progrediens when the tree's resources are in rapid decline due to stress from a large HWA population (McClure 1992). The winged sexuparae fly off in search of a suitable spruce (*Picea* spp.) host on which to oviposit. Spruce is the ancestral host plant for adelgid species (Blackman and Eastop 1994, Zilahi-Balogh 2004). Although sexuparae lay eggs, termed sexuales, their progeny do not survive past the first instar in North America. Lack of a suitable spruce host and harsh winter temperatures are the most important sources of natural mortality of HWA in the eastern United States (McClure 1991) because established, native natural enemies have little impact (Montgomery and Lyon 1996, Wallace and Hain 2000).

Each HWA generation has three life stages, developing from an egg through four instars to an adult. The only stages that are potentially mobile are the eggs (attached to something

moving or being moved) and first instars, called crawlers (McClure 1987). The first few days after egg hatch, crawlers search for a suitable site to insert their long stylets (up to 3 times their body length or ~3 mm) at the base of young hemlock needles. HWA is unable to move once it has settled on the tree.

HWA can be managed in ornamental settings using insecticidal soaps, horticultural oils, and chemical pesticides such as imidacloprid (McClure et al. 2001, Cheah et al. 2004).

Unfortunately, pesticide usage can make trees the targets of secondary pests, e.g. mites (Raupp et al. 2004). In the forest, HWA is far more difficult to control. Sprays cannot be applied to the hemlocks due to their proximity to riparian habitats and patchy distribution (Quimby 1996).

Furthermore, applications of systemic insecticides are very labor intensive. Classical biological control using exotic natural enemies is likely the best weapon against HWA in our arsenal today.

### **Biological Control of HWA**

Biological control agents may include pathogens, predators, and parasitoids; however, there are no known parasitoids of adelgids (Zilahi-Balogh 2004), and native fungi and generalist predators have been unable to curtail the HWA infestation in the U.S (McClure 1987, Montgomery and Lyon 1996, Wallace and Hain 2000). Classical biological control studies were initiated in Japan, China, and Canada beginning in the early 1990's to find potential biological control agents of HWA (McClure 1992, Montgomery et al. 2000, Salom et al. 2001). McClure (1992) found that native hemlocks in Japan, *T. diversifolia* (Maxim.) Masters and *T. sieboldii* Carriere, are able to survive HWA attacks, most likely due to host-resistance and the prevalence of natural enemies such as *Sasajiscymnus tsugae* (Sasaji and McClure) (Coleoptera: Coccinellidae). McClure (1995) also described an oribatid mite, *Diapterobates humeralis*

Hermann (Oribatida: Ceratozetidae), that kills HWA by ingesting fibers of the ovisac, thus exposing the eggs to the environment and predators. It has been removed from the short list of potential biological control agents due to its long life-cycle, low fecundity, and because it is difficult to rear in the laboratory (Salom et al. 2001). Three species of *Scymnus* (Coleoptera: Coccinellidae) were imported from China for evaluation in Connecticut (McClure et al. 2001), and *Laricobius nigrinus* Fender (Coleoptera: Derodontidae) was imported from western North America into quarantine in Virginia (Zilahi-Balogh et al. 2002). Based on the favorable attributes of *L. nigrinus*, a search was initiated to identify more natural enemies of HWA in the native range of the pest. Two new *Laricobius* spp. were collected in southern China in 2002 (Cheah et al. 2004). Foreign exploration efforts will continue in Asia and the western U.S. to develop a diverse assemblage of biological control agents for control of HWA.

#### *Sasajiscymnus tsugae*

*Sasajiscymnus tsugae* was discovered by Dr. Mark McClure in 1992 in Japan during an expedition for natural enemies of HWA (Cheah and McClure 1998). It has several desirable characteristics of a biological control agent: high fecundity, ease of mass-rearing, bivoltinism, female longevity, and ingestion of all HWA life stages. The coccinellid is fairly host-specific, but will consume and develop on adelgid prey other than HWA. *Sasajiscymnus tsugae* reduced HWA densities in early field assessments by 47-87% (McClure et al. 2001) and is currently being mass-reared, released, and monitored annually. *Sasajiscymnus tsugae* adults were first released in 1995 in New England and in 1999 in Virginia. From 1995 to 2004, more than one million of these beetles were released in 15 states from Maine to South Carolina (Cheah et al. 2004). Although *S. tsugae* is occasionally recovered from release sites in Connecticut (Cheah and McClure 2002), it is rarely collected in Virginia.

Scymnus spp.

Three *Scymnus* spp. (*Neopullus*) (Coleoptera: Coccinellidae) were imported to the U.S. from China between 1996 and 1998 to determine their potential for biological control of HWA (Lu and Montgomery 2001). *Scymnus* adults are univoltine and mostly host-specific to adelgids, but will feed on aphids. They consume all HWA life-stages but larvae must consume HWA eggs to complete development. *Scymnus camptodromus* Yu et Liu has proven difficult to rear in culture due to its hibernal egg diapause. *Scymnus ningshanensis* Yu et Yao must over-winter in the adult stage before females can oviposit. Two species, *S. ningshanensis* and *S. sinuanodulus* Yu et Yao, are undergoing evaluation in the field and seem to help lower HWA numbers in field sleeve-cages; the former was released in the southern Appalachians in 2004 (Cheah et al. 2004).

Laricobius spp.

The derodontids, or tooth-neck fungus beetles, are commonly found in the cooler regions of the Northern and Southern hemispheres and are the most primitive of the Polyphagan beetles. While three of the four genera of Derodontidae are mycophagous, *Laricobius* spp. are predators of woolly adelgids throughout their holarctic distribution (Lawrence 1989). At least eleven species from the genus *Laricobius* have been documented (Zilahi-Balogh 2001), with newer discoveries made in southwestern China and Nepal (Háva and Jelínek 1999, Jelínek and Háva 2001). Two new species from China were discovered in 2002 and are being described: *L. baoxingensis* sp. n. and *L. kangdingensis* sp. n. (Cheah et al. 2004, Zilahi-Balogh, unpublished). Four species occur in North America: *L. laticollis* Fall, *L. erichsonii* Rosenhauer, *L. rubidus* LeConte, and *L. nigrinus* Fender. Little is known about *L. laticollis*.

*Laricobius erichsonii* is native to Europe and was introduced to North America in the 1950's and 1960's to control balsam woolly adelgid (BWA), *A. piceae* (Ratzeburg) (Schooley et

al. 1984). It became established but failed to control BWA, possibly due the high susceptibility of fir trees (*Abies* spp.) to light BWA infestations.

*Laricobius rubidus* is the only *Laricobius* species native to the eastern U.S. and attacks mainly pine bark adelgid, *Pineus strobi* (Hartig), but is increasingly found in association with HWA in eastern hemlock/white pine stands (Montgomery and Lyon 1996, Wallace and Hain 2000). *Laricobius rubidus* females prefer to oviposit in *P. strobi* ovisacs over HWA ovisacs; however, when given no choice, they will lay their eggs on HWA as often as *P. strobi*. Furthermore, *L. rubidus* can complete development on HWA which may lead to competition with *L. nigrinus* as it continues to be released and, hopefully, established in the eastern U.S. (Zilahi-Balogh et al. 2005).

*Laricobius nigrinus* is a small (< 3mm), shiny, pubescent, black beetle with elytra marked by rows of punctures (Fender 1945). It is native to the Pacific Northwest and feeds on HWA in Oregon, Washington, Idaho, and British Columbia (Lawrence and Hlavac 1979). It was first imported from B.C. to the quarantine facility at Virginia Tech in 1997 for study as a potential biocontrol agent of HWA. *Laricobius nigrinus* lays its eggs singly within HWA's woolly ovisacs from January to May. The larvae develop through four instars on hemlock and drop to the ground in late spring/early summer to pupate in the soil. Adults aestivate and become active in October, just as the sistens generation of HWA is breaking its aestival diapause (Zilahi-Balogh et al. 2003b) and begin laying eggs in January. In contrast, *S. tsugae* over-winters from October to March and does not reach its egg-laying peak until late spring (Cheah and McClure 2000). *Laricobius nigrinus* has a low development threshold temperature of about 3.7°C (Zilahi-Balogh 2001, Zilahi-Balogh et al. 2003b), which is similar to that of HWA sistens (Salom et al. 2002). However, the coccinellid will not develop below 9°C. The synchronized phenology

between *L. nigrinus* and HWA is encouraging as researchers continue to assess its potential as a biological control agent.

*Laricobius nigrinus* adults feed on immature and adult stages of HWA, while larvae feed principally on HWA eggs (Zilahi-Balogh et al. 2002). *Laricobius nigrinus* exhibits many other desirable characteristics such as a narrow host-range and preference for HWA, voracious feeding habits, completion of development only on a diet of HWA, high fecundity (up to 400 eggs/female), selective oviposition so larvae do not have to search for food, longevity, and good synchronization with HWA (Zilahi-Balogh et al. 2003). However, mass-rearing of this species is challenging since *L. nigrinus* is univoltine, requires cold temperatures, undergoes an aestival diapause in soil, and needs copious amounts of fresh HWA-infested foliage on which to feed. Unnatural lab conditions can also complicate the process by disrupting the synchronization of the predator and prey (Lamb 2005).

In September 2000, *L. nigrinus* was approved by APHIS for release in the field (Zilahi-Balogh et al. 2002). Field studies to evaluate its fecundity and effectiveness at controlling HWA were initiated in 2001 and showed that adults survived the winter while larvae caused 50% more HWA mortality than natural causes alone (Lamb et al. 2005a). In a subsequent study, larvae were left on the branches and sampled in 2003 and 2004 to determine establishment (Lamb et al. 2005b). Branches sampled from these field sites have yielded progeny (F<sub>2</sub> generation) that were reared to adulthood (n < 10) and identified as *L. nigrinus*. Open field releases of adults began in November and December 2003 at seven sites in the mid-Atlantic region. By March 2005, more than 9,400 beetles were released at 23 sites in eight states. Currently, four adults (F<sub>1</sub> generation) have been recovered from release sites in North Carolina and Virginia; 25 were found at a field insectary located at Kentland Farms near Blacksburg, VA in January 2005.

***Laricobius kangdingensis* sp. n.**

In April 2002 an expedition was made to Sichuan Province, China to collect predators from Chinese hemlock infested with HWA, particularly *Laricobius* species (Figure 1.1). Two new species from Baoxing and Kangding counties were collected, described, and named according to where they were found (Zilahi-Balogh unpublished). *Laricobius baoxingensis* sp. n. adults died without reproducing in the laboratory and no larvae were found on the adelgid-infested hemlock foliage [*T. chinensis* (Franchet) Pritzel] collected in China. However, *L. kangdingensis* sp. n. larvae (n = 23) collected behind the Luoxiba Deng Forestry Station completed development on eastern hemlock infested with HWA under quarantine at Virginia Tech in Blacksburg, VA.

*Laricobius kangdingensis* sp. n. males range in color from dark brown to black, while females are reddish-brown with a dark brown, vertical “stripe” on each elytron. This difference makes it fairly easy to determine the sex of adult beetles in the laboratory before the initiation of oviposition, unlike *L. nigrinus* which does not exhibit sexual dimorphism (Zilahi-Balogh 2001). Zilahi-Balogh (unpublished) described the *L. kangdingensis* sp. n. male holotype as being between 2.1 and 2.4 mm long, 1.0 mm wide, and having a pronotum 1.21-1.25 times wider than it is long. The pronotum is equally narrowed anteriorly and posteriorly, and is hardly emarginated before the posterior angles (Figure 1.2).



Fig 1.1. Map showing where *L. kangdingensis* sp. n. and *L. baoxingensis* sp. n. were found in China.

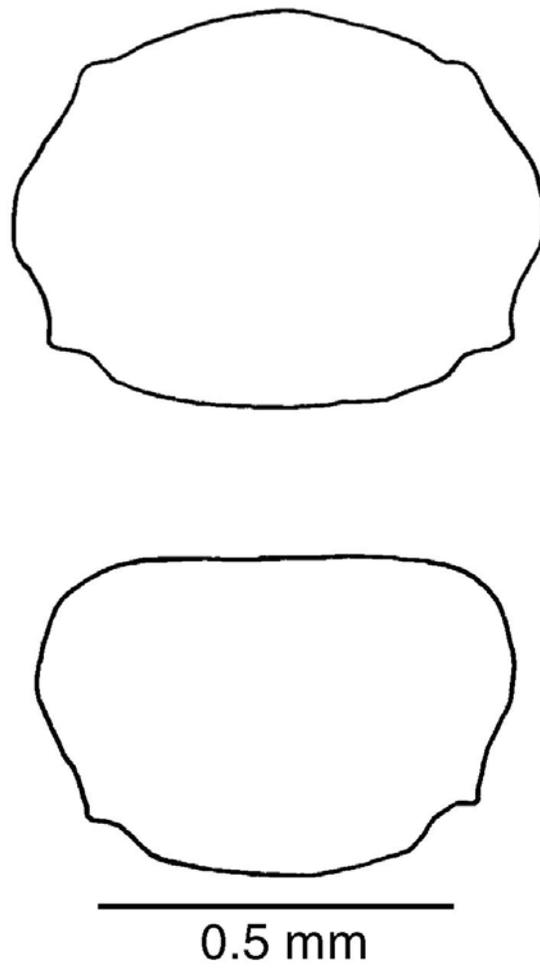


Fig 1.2. Comparison of the pronota of *L. nigrinus* (top) and *L. kangdingensis* sp. n. (bottom)

(Credit: Dr. G.M.G. Zilahi-Balogh)

## Research Objectives

The primary goal of this research was to establish and maintain a colony of *L. kangdingensis* sp. n. for the purpose of studying its biology and feeding preferences. Specific objectives included:

- 1) Studying reproductive biology and influence of temperature on the development of *L. kangdingensis* sp. n.
- 2) Evaluating host-specificity of *L. kangdingensis* sp. n.
- 3) Comparing biology, temperature-dependent development, and host-specificity of *L. kangdingensis* sp. n. with *L. nigrinus*

## Chapter 2

### **Biology and Temperature-dependent Development of *Laricobius kangdingensis* sp. n.**

#### **Introduction**

Hemlock woolly adelgid (HWA), *Adelges tsugae* Annand, is a serious threat to eastern and Carolina hemlock trees, *Tsuga canadensis* (L.) Carriere and *T. caroliniana* Engelmann, and has thrived in the eastern U.S. since the 1950's due to a lack of indigenous predators and high susceptibility of the tree to the pest (McClure et al. 2001, Cheah, et al. 2004). HWA feeds at the base of needles and depletes nutrient stores, causing needles to desiccate and drop (McClure 1995). Trees may die in as little as four years due to defoliation and an increased vulnerability to other pests, pathogens, or harsh environmental conditions (Ward et al. 2004).

Efforts to identify natural enemies of HWA began in the early 1990's, but when native control agents were found to be generalists and in too small numbers to be effective (McClure 1987, Montgomery and Lyon 1996, Wallace and Hain 2000), the focus shifted to hemlock stands in Asia and western North America (McClure 1992, Montgomery et al. 2000). Three species of promising coleopteran predators [*Sasajiscymnus tsugae* (Sasaji and McClure), *Scymnus ningshanensis* Yu et Yao, and *Laricobius nigrinus* Fender] were subjected to laboratory and field testing and subsequently released in the eastern U.S.; they are currently being evaluated for their establishment and effectiveness against HWA in the field (Zilahi-Balogh 2002, Cheah et al. 2004, Lamb et al. 2005).

Despite the potential of the aforementioned insects, foreign exploration continued, and two new species of *Laricobius* were identified and collected in Sichuan Province in south-central

China in April 2002 (Cheah et al. 2004). *Laricobius baoxingensis* sp. n. adults were discovered in Baoxing County and brought back to the quarantine laboratory at Virginia Tech but failed to reproduce. Larvae of the other species, *L. kangdingensis* sp. n., that were collected in Kangding County completed development on HWA-infested eastern hemlock and provided the founding generation for a research colony.

Development time of immature stages of insects is temperature-dependent and can be used to predict development of insects in the field (Laudien 1973). A linear increase in development time is expected with a decrease in temperature; unless a lower temperature threshold is reached and development is retarded (Sanderson 1910, Sharov 1998). Development rate models enable the prediction of development time for each life-stage using variable temperature data and also help estimate temperature thresholds (Perdikis and Lykourkessis 2002). This information can be used to determine how well suited a biological control agent is for a particular region or host-prey. The range of eastern hemlocks stretches through several USDA plant hardiness zones with varying environmental temperatures and conditions (Skinner et al. 2003, Anonymous 2003). It is important to understand the development rate of *L. kangdingensis* sp. n. at a range of temperatures so that if and when it is released it can be matched with the most appropriate climate. Since *L. nigrinus* is already being released in the field, understanding the influence of temperature on the development of *L. kangdingensis* sp. n. is critical to ensuring that the predators' control efforts are collaborative, not competitive.

## Materials and Methods

### *Field-collected individuals (P1)*

In April 2002 an expedition was made by Dr. G.M.G. Zilahi-Balogh and Mr. Tom McAvoy to Sichuan Province, China to search for potential predators of the hemlock woolly adelgid (HWA). Twenty-three larvae of a new derodontid species, *Laricobius kangdingensis* sp. n., discovered behind the Luoxiba Deng Forestry Station in Kangding County, China (Lat./Long. 30° 03' 00" N / 102° 02' 00" E) were imported to the Virginia Tech Beneficial Insects Quarantine Lab and reared on HWA-infested eastern hemlock at 15°C, 12:12 (L:D), 75-87%RH.

Field-collected larvae were placed singly on HWA-infested hemlock twigs in 50 x 9 mm polystyrene Petri dishes (Falcon<sup>®</sup>) lined with two pieces of filter paper (Whatman No. 1) and moistened with methyl paraben (0.42 g / 250 ml de-ionized water) to inhibit fungal growth (Zilahi-Balogh 2001). The Petri dish lids were each ventilated with a 2 cm hole covered by PeCap<sup>®</sup> polyester mesh (0.14 mm<sup>2</sup>). Fresh HWA-infested foliage was added each time a dish was inspected every few days. The soil and filter paper were moistened every few days with methyl paraben.

Mature larvae were covered with sterilized soil medium (3:1:0.33 parts peat: sand: sphagnum) contained in 950 ml clear polystyrene containers equipped with a lid ventilated by a 10 cm diam hole covered with PeCap<sup>®</sup> polyester mesh (0.14 mm<sup>2</sup>) (Sefar America, Inc., Kansas City, MO) (Zilahi-Balogh et al. 2003b). Aestivation containers holding mature larvae were maintained in environmental chambers (Percival Scientific) at 15 °C and 12:12 (L:D). The moisture level was maintained between 30-40% by watering the soil 3-4 times per week (Zilahi-Balogh, unpublished). Containers were examined daily for adult activity.

Adult activity for this experiment was defined from the time all beetles emerged from the soil to death. Emerged adults were placed in oviposition containers, which were the same as the aestivation containers but lined with two pieces of filter paper (Whatman No. 1) moistened with methyl paraben instead of soil. HWA-infested clipped hemlock twigs were inserted in moistened floral foam (Oasis<sup>®</sup>) and placed upright on top of the filter paper. The oviposition containers were held at 5-8°C between September and December 2002, but were raised to 12 and 15°C on 11-13 December and 2-5 January to gather eggs for the temperature development study. Thereafter, oviposition containers were maintained at  $\approx 8^\circ\text{C}$  and 12:12 (L:D) to simulate natural conditions (Zilahi-Balogh, unpublished). Adults were initially held together in one container for mating, but females were placed individually in their own containers after oviposition was first observed. Two males were rotated among the females weekly. Oviposition and survivorship were monitored weekly.

#### *Laboratory-reared Colony Individuals (F<sub>1</sub>)*

Eggs laid by the parental generation (P<sub>1</sub>) adults in oviposition studies became part of the colony or were put into temperature-dependent development studies. Each week, colony eggs laid on HWA-infested hemlock twigs were counted and inserted in saturated floral foam blocks wrapped in Parafilm<sup>®</sup> M to maintain moisture in the branches. Larvae were reared in funnel-cages described by Lamb et al. (2002). Funnel-cages were kept in a cold-room maintained at 12-16°C, 12:12 (L:D). Mason jars affixed to the bottom of the cages were filled with  $\approx 5$  cm of soil medium and were checked for pupae 6-8 weeks after eggs were put in the funnel cages. Mason jars were spray-painted black to block out light. Pupae were separated by sex (Zilahi-Balogh 2001) and put into aestivation containers as previously described for field-collected pre-pupae.

Aestivation containers were checked daily for adult activity beginning in June 2003. Emerged adults were initially held at 8°C 12:12 (L:D), but moved to 4°C in mid-November 2003.

Oviposition containers were set-up as described for the P<sub>1</sub> generation. Oviposition and survivorship were monitored weekly.

HWA-infested eastern hemlock was collected from the Washington and Jefferson National Forest, less than 30 miles from Blacksburg, VA, in April 2003, stored at 4°C, and used to feed the F<sub>1</sub> generation of beetles upon emergence. Food was stored, because in the past, beetle emergence did not coincide with the presence of actively developing HWA in the field.

#### *Larval head-capsule measurements*

Dead larvae collected from temperature development and host-range studies in 2003 and 2004 were stored in 75% ethanol to measure head-capsule widths at a later time using a compound microscope equipped with a calibrated ocular micrometer. Dyar's ratio was calculated to determine the number of instars (Dyar 1890, Comstock 1920).

#### *Temperature-dependent Development*

Temperature-dependent development studies were set up in Petri dishes as described for field-collected larvae. Newly laid eggs (< 24 h) were removed from oviposition containers and affixed to HWA-infested hemlock (2-4 cm long) in environmental chambers held at 12 or 15°C, 12:12 (L:D) in early 2003, and 6, 9, or 18°C, 12:12 (L:D) in fall 2003 to spring 2004. Eggs were removed from the foliage, or left on the twigs, and then transferred to 50 mm Petri dishes lined with two layers of filter paper (Whatman no.1) moistened with methyl paraben. Eggs were randomly assigned to environmental chambers maintained at 6, 9, or 18°C, 12:12 (L:D) and

≈75% RH. Temperature-dependent development was previously studied at 12 and 15°C in 2003 (Zilahi-Balogh, unpublished); those data are presented here, as well. Eggs were monitored daily for eclosion and filter paper was moistened as necessary (2-3 times per week); larvae were initially examined every other day for molt to subsequent instars, but daily as ecdysis appeared imminent. Additional HWA-infested twigs were added at each inspection. Mature larvae (pre-pupae) found wandering off the food source in search of a pupation site were removed from the dish. The filter paper was replaced and soil medium was added to fill the dish. Pre-pupae were placed in the middle of the dish, covered with more soil, and monitored every one to two days for molt to the pupal stage. The soil was watered 2-3 times per week, as necessary. Beetles from the 12 and 15°C studies were moved to the larger aestivation containers (950 ml) held at 15°C, 12:12 (L:D), and were monitored for adult activity beginning in June 2003.

### *Data Analysis*

The Student t-test procedure (PROC TTEST) was used to test for differences between head-capsule measurements of *L. kangdingensis* sp. n. and *L. nigrinus*. Linear regression (PROC REG) was used to analyze median development rates (1/d) for each life-stage over the range of temperatures tested (SAS Institute, 1989). Development thresholds ( $D_{th}$ ) were calculated using the formula  $(-a/b)$ , where  $a$  represents the y-intercept and  $b$  is the slope of the line (Zilahi-Balogh et al. 2003b). The number of degree-days required for each life-stage to complete development was calculated using the formula  $(1/b)$ , where  $b$  is the slope of the regression equation (Zilahi-Balogh et al. 2003b). Degree-days, a measure of development rate that is independent of temperature, allow for prediction of development rate at various temperatures.

## Results

### *Field-collected generation (P<sub>1</sub>)*

Of the 23 larvae collected in China, 90% reached the pre-pupal stage (Zilahi-Balogh, unpublished data). A total of fifteen beetles emerged, ten of which (eight females and two males) provided the founding generation (P<sub>1</sub>) for our lab colony. Mean days ( $\pm$  SD) of adult activity and oviposition period was  $219.5 \pm 49.9$  d and  $184.5 \pm 49.9$  d, respectively (Zilahi-Balogh, unpublished data). Mean life-time fecundity per female ( $\pm$  SD) at  $\approx 8^\circ\text{C}$  (range: 5-15°C) was  $196.4 \pm 53.4$  eggs. The total number of eggs laid between November 2002 and July 2003 was 1554, of which 586 individuals (F<sub>1</sub>) reached the pupal stage (37% survival).

### *Laboratory-reared Colony Individuals (F<sub>1</sub>)*

Pupation containers were examined daily for *L. kangdingensis* sp. n. emergence beginning in summer 2003. Thirty-two percent of the pupae completed development and emerged as adults beginning on 2 June 2003, a full four months before HWA broke diapause in October in southwest Virginia (Figure 2.1). Males became active before females, mirroring observations of *L. nigrinus* (Zilahi-Balogh et al. 2003). A total of 190 individuals (87 males, 80 females, 23 unsexed) emerged by October 2003 and were initially held at 8°C. Approximately 10% of the emerged adults exhibited deformities in their elytra and/or wings, possibly due to inadequate nutrition or being disturbed as pupae. The pre-oviposition period was  $\approx 4$  weeks; females began laying eggs 7 July 2003. The HWA-infested foliage collected in April and maintained in a refrigerator at  $\approx 4^\circ\text{C}$  began to deteriorate quickly as the wool became moldy at the end of August. Subsequent oviposition was negatively impacted (Figure 2.2) and female mortality increased (Figure 2.3). Oviposition resumed in the colony (Figure 2.2) and female

mortality decreased (Figure 2.3) when the temperature was reduced to 4°C and beetles were placed on fresh foliage with developing HWA nymphs in October 2003. Mean lifetime fecundity per female was 97.9 eggs and 1327 eggs were laid between 7 July 2003 and 7 August 2004; however, this is not a true reflection of fecundity because oviposition was minimal when high quality food was unavailable.

#### *Larval head-capsule measurements*

Dyar (1890) noted that head-capsule widths of lepidopteran larvae followed a geometric progression in growth. Calculations of Dyar's ratio showed that *L. kangdingensis* sp. n. has four instars, like *L. nigrinus* (Table 2.1). Head capsule width measurements for all instars of *L. kangdingensis* sp. n., with the exception of the second instar, were significantly larger than those of *L. nigrinus* (Zilahi-Balogh et al. 2003a) (Table 2.1).

#### *Temperature-dependent development*

Development times for egg, larval, pre-pupal, and pupal stages are presented in Table 2.2. Data collected from experiments conducted in spring 2003 at 12 and 15°C (Zilahi-Balogh, unpublished data) are included. Egg development time decreased as temperature increased, although development time slightly increased at 12°C. Egg survival was optimal at 12°C and poorest at 18°C. Larval survival was best between 9 and 15°C. Larvae did not complete development at 18°C. Completion of pre-pupal development was highest at 15°C and lowest at 9°C; individuals failed to complete development at 6°C. Pupae did not survive at 9°C, but did complete development at 12 and 15°C; however, the adults failed to emerge from summer diapause with their undisturbed colony cohorts. Pupae in the 9°C developed into semi-adults; the

head and thoracic segments exhibited adult characteristics while the abdomen remained immature. This may have been due to inadequate nutrition or being disturbed while in a vulnerable life-stage.

Linear regressions of development rates for the egg, larval, and pre-pupal stages are shown in Figure 2.4. Parameter estimates for regression equations, development threshold, and degree-days are shown in Table 2.3. Egg development data gathered at 9°C was not used in the linear regression because of the unusually high mortality that occurred at that temperature. Minimum development temperature thresholds were estimated at 2.8°C, 1.6°C, and 5.8°C for egg, larval, and pre-pupal stages, respectively.

## **Discussion**

*L. kangdingensis* sp. n. has four larval instars, the same number reported for *L. erichsonii* (Clark and Brown 1958), *L. rubidus* (Clark and Brown 1960), and *L. nigrinus* (Zilahi-Balogh 2001). Head capsule widths are larger for *L. kangdingensis* sp. n. than *L. nigrinus*, which could prove useful in differentiating between the two species in their larval stages.

Based on survivorship in my studies and those conducted by Zilahi-Balogh (unpublished), optimal development time for all life-stages of *L. kangdingensis* sp. n. is between 12 and 15°C; a similar range was observed for *L. nigrinus* (Zilahi-Balogh et al. 2003b). However, mortality was unusually high at 9°C, perhaps due to poor food quality; further studies are needed at this temperature. Adults did mate and lay eggs at 4°C and larvae can reach the pre-pupal stage at that temperature, so the optimal temperature could in fact be lower than the 12-15°C range.

Zilahi-Balogh et al. (2003b) speculated that disturbance of the developing pre-pupae may be a significant mortality factor for *L. nigrinus* in the lab. This may also be the case with *L. kangdingensis* sp. n. because individuals in the 12 and 15°C studies completed development but failed to emerge from the soil with their undisturbed cohorts in fall of 2003. Unlike *L. nigrinus*, the egg development threshold for *L. kangdingensis* sp. n. is slightly lower than that of HWA (3.9°) which could potentially leave larvae without a food source in the field.

The coccinellid biological control agent, *S. tsugae*, has a minimum development threshold of 9.5°C (Cheah and McClure 2000). It is likely active later in the season than either *L. kangdingensis* sp. n. or *L. nigrinus*. Competitive interactions between *L. nigrinus*, *S. tsugae*, and *Harmonia axyridis* Pallas have been shown to be minimal in lab (Flowers et al. 2005) and field conditions (Flowers, unpublished) owing to the temporal separation of their activity periods so it stands to reason that *L. kangdingensis* sp. n. will not be affected by predators active later in the season. However, it could experience some congeneric interactions with *L. nigrinus* and *L. rubidus*, which may need to be investigated at a later time.

Table 2.1. Head capsule width measurements of *L. kangdingensis* sp. n. larvae

Instar	n	<i>L. kangdingensis</i> sp. n. head capsule widths (mm)			<i>L. nigrinus</i> head capsule widths (mm) <sup>2</sup>			P-value <sup>1</sup>
		Mean ± SD	Range	Dyar's Ratio	Mean ± SD	Range	Dyar's Ratio	
I	60	0.21 ± 0.01	0.17 - 0.23	-	0.20 ± 0.01	0.17 - 0.22	0.0000*	
II	4	0.28 ± 0.01	0.27 - 0.28	1.33	0.27 ± 0.01	0.24 - 0.30	0.53180	
III	8	0.41 ± 0.02	0.38 - 0.43	1.46	0.36 ± 0.02	0.31 - 0.40	0.0000*	
IV	43	0.50 ± 0.02	0.47 - 0.53	1.22	0.48 ± 0.03	0.41 - 0.55	0.0001*	

<sup>1</sup> T-tests (PROC TTEST) were used to compare *L. kangdingensis* sp. n. and *L. nigrinus* head capsule widths

<sup>2</sup> Zilahi-Balogh 2001

\* *L. kangdingensis* sp. n. significantly larger than *L. nigrinus* at  $P = 0.05$

Table 2.2. Survivorship and mean development time of *L. kangdingensis* sp. n. at different constant temperatures

Life Stage	Temp. (°C)	Initial # individuals	# surviving to the next life-stage	% survivorship	Mean ± SD (# days)	Median (# days)	Median rate (1/d)
Egg	6	118	86	72.9	37.3 ± 1.6	38.0	0.0263
	9	48	19	39.6	11.5 ± 1.8	12.0	0.0833
	12	61	56	91.8	12.9±0.7	13.0	0.0769
	15	65	51	78.5	9.0±0.5	9.0	0.1111
	18	17	1	5.9	8.0	8.0	0.1250
Larval	6	86	31	36.0	46.1 ± 6.3	45.0	0.0222
	9	19	12	63.2	38.0 ± 4.6	38.0	0.0263
	12	56	39	69.6	22.9±1.9	23.0	0.0435
	15	51	36	70.6	16.8±1.5	17.0	0.0588
	18	1	-	-	-	-	-
Pre-pupal	6	31	0	0.0	-	-	-
	9	12	3	25.0	44.3 ± 3.06	45.0	0.0222
	12	39	19	48.7	31.5±4.0	30.0	0.0333
	15	36	25	69.4	17.6±1.6	17.0	0.0588
	18	-	-	-	-	-	-
Pupal	6	-	-	-	-	-	-
	9	3	0	0.0	-	-	-
	12	19	11	57.9	30.4±1.2	30.0	0.0333
	15	25	14	56.0	20.4±0.8	20.0	0.0500
	18	-	-	-	-	-	-
Egg to Adult	6	-	-	-	-	-	-
	9	-	-	-	-	-	-
	12	11	9	81.8	97.2±5.1	98.0	0.0102
	15	14	13	92.9	62.5±2.6	63.0	0.0159
	18	-	-	-	-	-	-

Development studies at 12 and 15°C were conducted in spring 2003. All other studies were conducted between fall 2003 and early summer 2004.

Table 2.3. Estimates of regression equations, developmental threshold (Dth), and degree-day (DD) requirements for different *Laricobius kangdingensis* sp. n. life-stages

Life-stage	Temp. Range (°C)	Regression Equations <sup>1</sup>	P	r <sup>2</sup>	D <sub>th</sub> (°C)	<i>L. nigrinus</i>	
						DD (1/b)	DD (1/b) <sup>2</sup>
Egg	6.0 - 18.0	$y = -0.0238 + 0.0071x$	0.007	0.99	2.8	117.6	54.6
Larval	6.0 - 15.0	$y = -0.0067 + 0.0042x$	0.025	0.95	1.6	238.1	161.3
Pre-pupal	9.0 - 15.0	$y = -0.0351 + 0.0061x$	0.142	0.95	5.8	163.9	196.1
Pupal	12.0 - 15.0	-	-	-	-	-	212.8
Egg to Adult	12.0 - 15.0	-	-	-	-	-	666.7

<sup>1</sup> Regression model is  $y = a + bx$ , where y is development rate (1/days), and x is temperature (°C)

<sup>2</sup> Zilahi-Balogh 2001

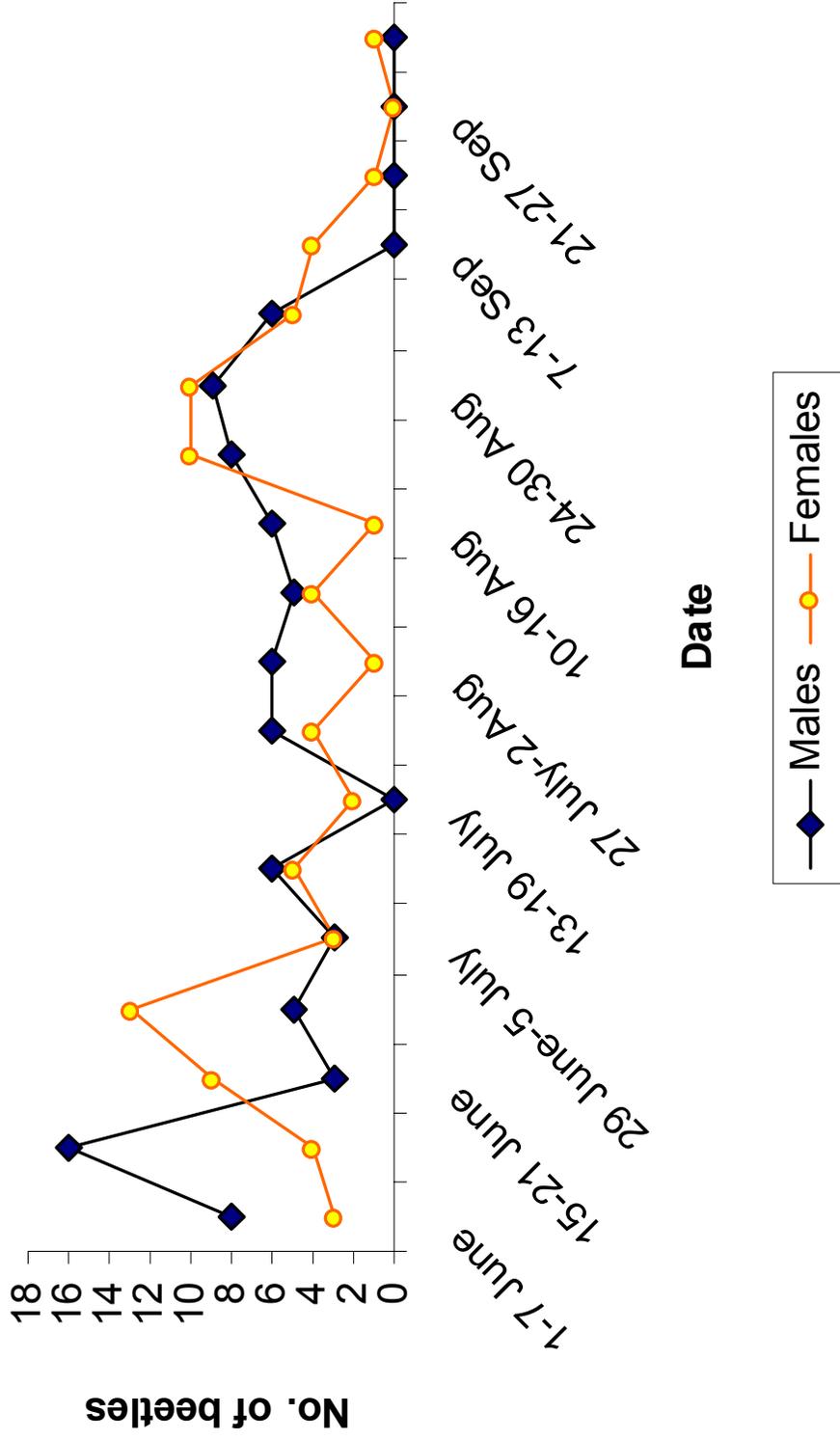


Figure 2.1. Emergence of the *L. kangdingensis* sp. n. F<sub>1</sub> generation in 2003.

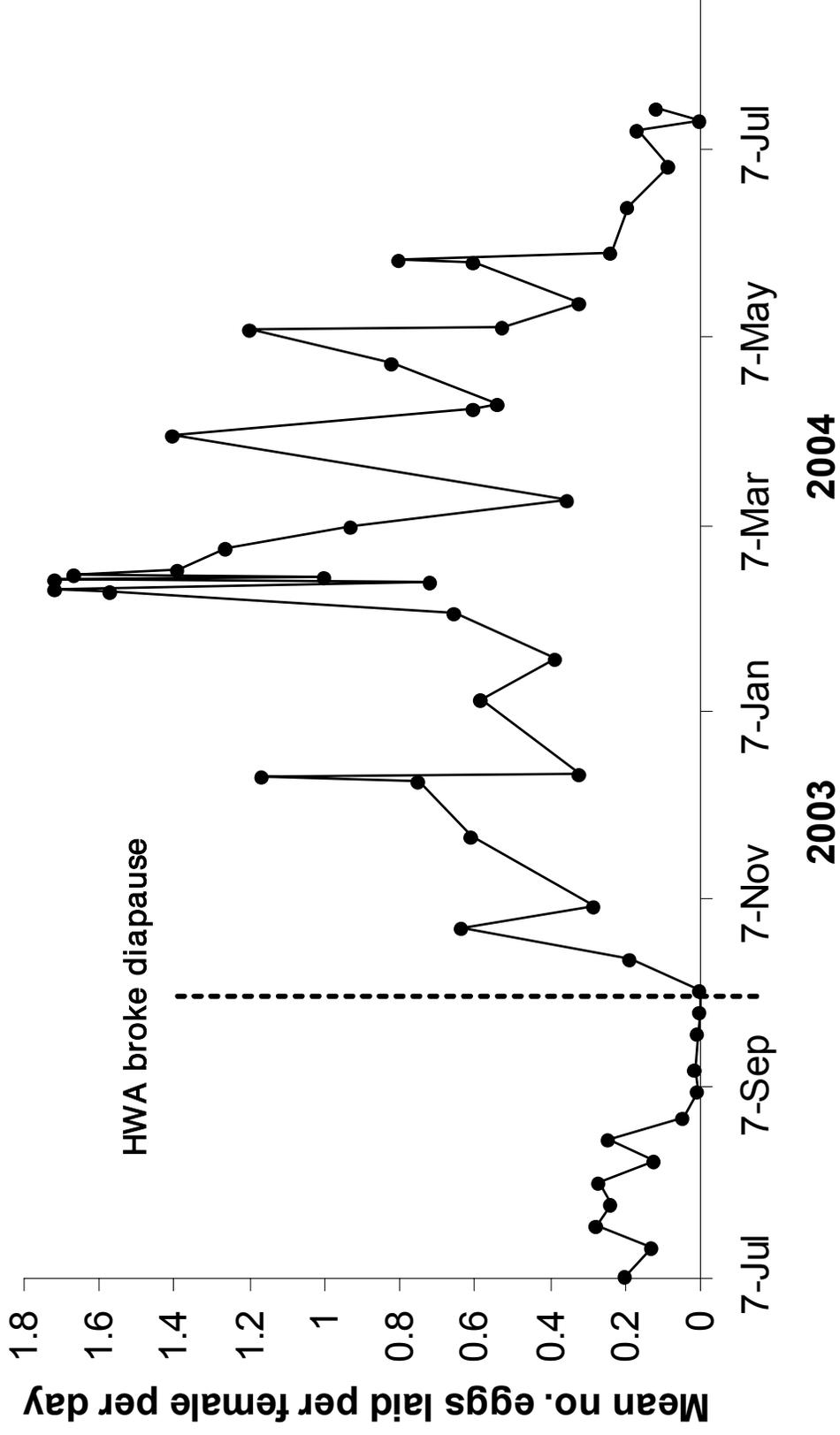


Fig. 2.2. Mean number of eggs laid per female per day from 7 July 2003 to 24 August 2004.

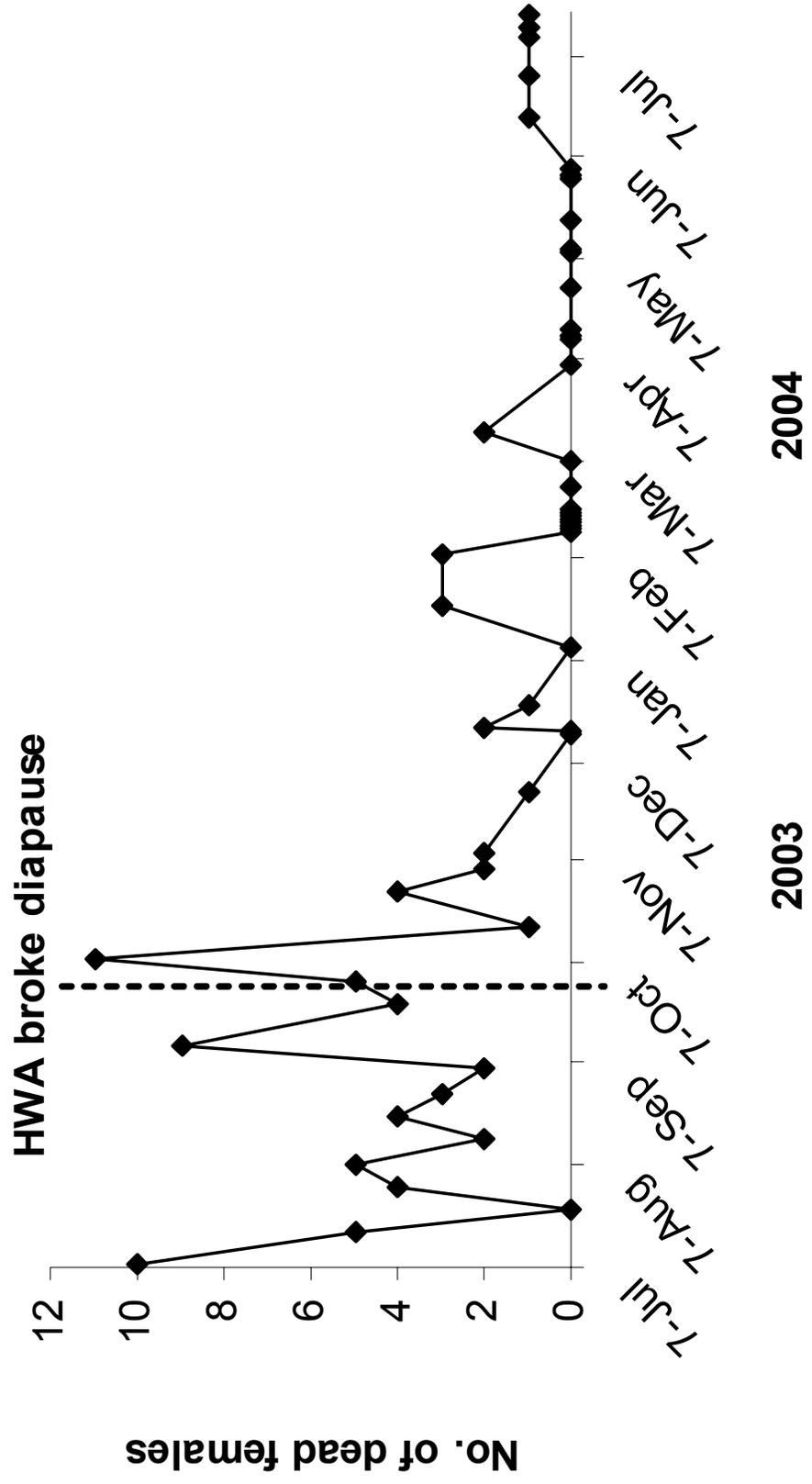


Fig 2.3. Female mortality from 4 June 2003 to 24 August 2004 (when the last female died).

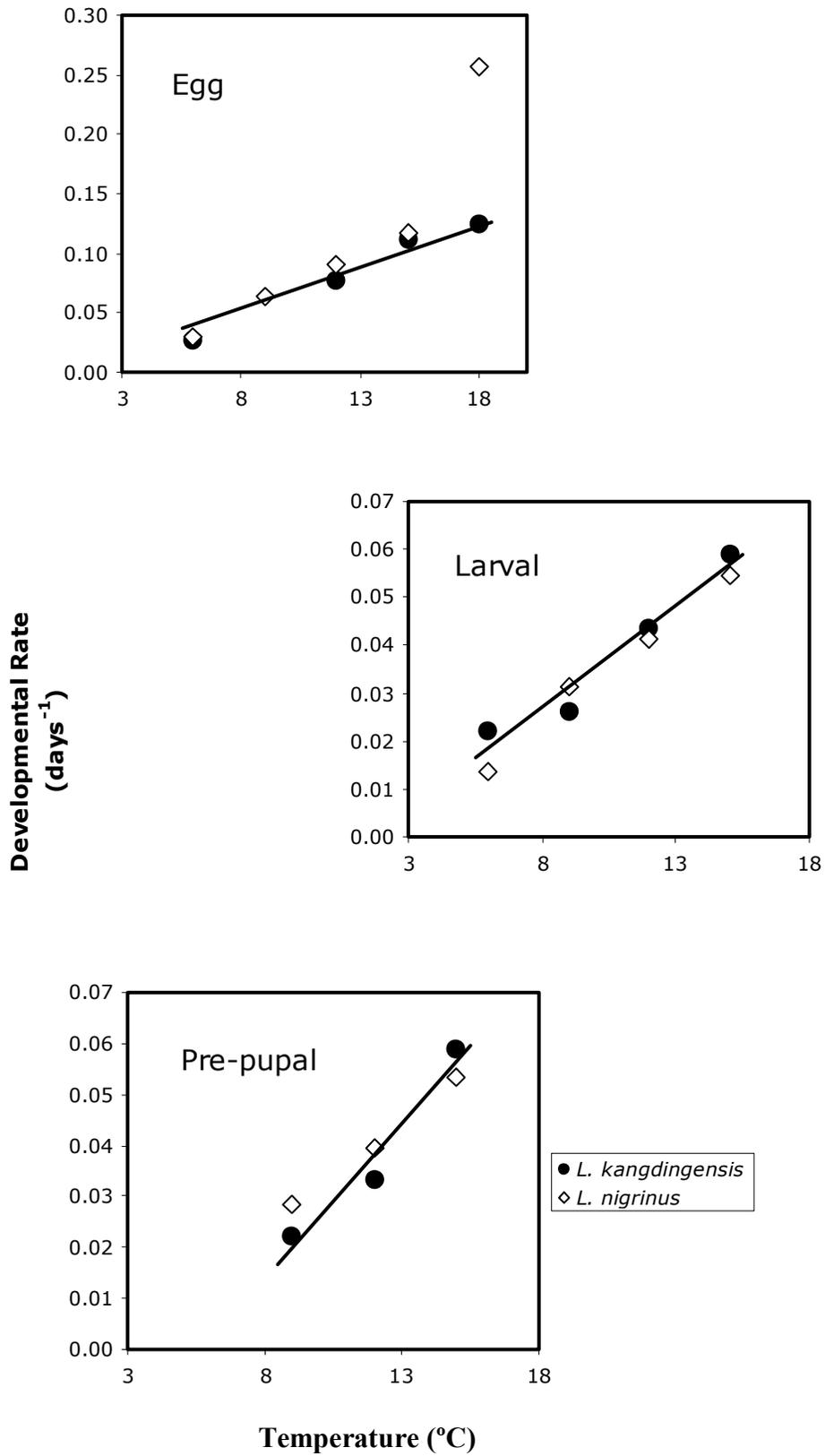


Figure 2.4. Linear relationship between median developmental rate and temperature for *L. kangdingensis* sp. n. life stages (circles). *L. nigrinus* median developmental rates (diamonds) are also shown for comparison.

## Chapter 3

### Host-specificity of *Laricobius kangdingensis* sp. n.

#### Introduction

Hemlock woolly adelgid (HWA), *Adelges tsugae* Annand, is a homopteran pest accidentally imported from Asia that can kill eastern and Carolina hemlock trees in as few as four years (McClure et al. 2001, Cheah et al. 2004). Half of the trees' range (from Nova Scotia in the north, westward to Minnesota, and stretching south to Alabama) is infested and the insect is spreading at up to 25 km per year via humans, deer, birds, and wind (Souto et al. 1996, McClure 2001, Knauer et al. 2002). HWA feeding leads to depletion of the tree's nutrient stores, followed by needle-drop and death if populations continue to expand year after year (McClure 1995).

There are two HWA generations per year, one of which (sistens) undergoes an aestival diapause and resumes development in the fall. Sistens feed and develop throughout the winter, laying eggs in March in Virginia that will hatch and become one of two morphs, progrediens or sexuparae (Gray and Salom 1996). Progrediens are apterous, parthenogenetic, and remain on the host-tree laying sistens eggs that will hatch into crawlers. Sexuparae are winged and disperse hemlocks in search of a suitable spruce host (*Picea* spp.) on which to reproduce sexually. Sexuparae produce offspring (sexuales) that do not complete development in North America, which is a significant mortality factor for this pest (McClure 1991).

The Hemlock Woolly Adelgid Working Group was convened in 1993 to address the HWA problem and direct research to find biological control agents to help control its spread and destruction (Reardon and Bullard 1996). Surveys of natural enemies in the eastern United States found no predators impacting HWA (Montgomery and Lyon 1996, Wallace and Hain 2000), and

no parasitoids are known to attack members of the adelgid family (Zilahi-Balogh 2004). Expeditions to find potential biological control agents were made in Asia and the Pacific Northwest where hemlocks are native and not as susceptible to HWA attack as are eastern hemlocks. Three coleopteran predators have been released in the field and their effectiveness is being evaluated (Cheah et al. 2004). One of these beetles, *Laricobius nigrinus* Fender (Coleoptera: Derodontidae), is especially promising because it is well-synchronized with HWA, feeds on it ravenously when other predators are absent, and has a high fecundity. Field cage studies showed that *L. nigrinus* both survives and reproduces in the field; most importantly, it has a significant impact on HWA populations (Lamb et al. 2005).

Three of the four genera in the family Derodontidae are mycophagous, but the members of the genus *Laricobius* feed solely on woolly adelgids (Zilahi-Balogh 2004). With this in mind, an expedition was made to China in 2002 to search for novel species of *Laricobius*, as well as other potential predators of HWA. Two new *Laricobius* species were discovered, one of which survived to establish a research colony.

In order to be considered for release from quarantine status, biological control agents must be tested in the laboratory to determine their host preferences. While phytophagous agents have been the subject of rigorous testing for decades because they may attack crops or other desirable plants, entomophagous species have not been intensely scrutinized until recently. Researchers have learned that non-target effects on native insects do occur and need to be considered before entomophagous agents are released. One example concerns the tachinid *Compsilura concinnata* (Meigen) that was originally imported into the U.S. in 1906 to control the gypsy moth. It is a generalist parasitoid which was released despite the knowledge that it has a host-range greater than 180 species. Unfortunately, *C. concinnata* negatively impacts native

saturniid moths (Elkinton and Boettner 2004). Despite the lack of strict protocols for agents that prey on invertebrates, great care must be taken voluntarily to determine host-ranges before biological control agents are released and become harmful.

There are several homopteran pests on which *L. kangdingensis* sp. n. may potentially encounter and consume following its release in the field:

Balsam woolly adelgid (BWA), *Adelges piceae* (Ratz.), is a pest of true fir (*Abies* spp.) trees in both ornamental and forest settings (Johnson and Lyon 1991). Millions of cubic meters of fir timber have been destroyed by BWA since its introduction from Asia or Europe in the early 1900's (Hain 1988). There are two generations per year in the Northeast. Adult females reproduce asexually, and like HWA, the crawlers are the only independently mobile stage. Swelling of the ends of tree limbs, or "gouting," occurs as a result of feeding by BWA. Heavy infestations are obvious, as the trunk becomes covered by the woolly ovisacs of the adults. Older, larger trees are usually attacked and killed first.

Pine bark adelgid (PBA), *Pineus strobi* (Hartig), reproduces continuously on pine, feeding on the phloem tissue under the bark surface (Johnson and Lyon 1991). PBA occurs throughout the range of the native eastern white pine, *Pinus strobus* L., and is also found on the exotic Scots pine, *Pinus sylvestris* L., and Austrian pine, *Pinus nigra* Arnold. Immature stages over-winter and resume feeding in the early spring. Upon reaching maturity, egg-laying begins. PBA is most common on the bark of older trees, but may be found on the new growth of seedlings and Christmas trees. The adelgid is often found in association with stressed trees, but is not usually fatal.

Eastern spruce gall adelgid (ESGA), *Adelges abietis* L., has spread throughout the northeastern U.S. since its introduction from Europe prior to 1900 (Anonymous 2005). It is a

primary pest of Norway spruce, *Picea abies* (L.) Karst., but may cause damage on other spruce species. Female adelgids over-winter and lay 100-200 eggs in woolly ovisacs upon reaching maturity in early spring. Nymphs feed at the base of needles, causing galls to form around them at the bottom of new shoots. The pineapple-like growths weigh branches down and cause them to break under physical stress, thus disfiguring the tree. The galls open in fall, freeing the adelgids inside to oviposit and repeat the life-cycle.

There are up to 11 different larch adelgid species that comprise the *A. laricis* Vallot complex, four of which occur in North America (Blackman and Eastop 1994, Zilahi-Balogh 2004). It is thought to be from alpine Europe, but researchers are not certain of its origin.

Elongate hemlock scale (EHS), *Fiorinia externa* Ferris (Homoptera: Diaspididae), is a serious pest of eastern and Carolina hemlock on the east coast that was introduced from Japan (McClure 2002). It is more prevalent on and harmful to trees that are already stressed because of other pests or poor environmental conditions. Tree mortality is hastened when this pest occurs in conjunction with HWA. It occurs from Virginia to Massachusetts and west to Ohio. The scale insect feeds on the underside of hemlock needles, sucking fluids from the mesophyll cells via its long, stylet mouthparts. Winters are passed either in the egg stage, or as a mated female. Females typically lay  $\approx 20$  eggs under their yellow or brown scale armor. Eggs hatch in late spring in Maryland; a second generation of individuals hatch in early fall (Malinoski and Davidson 2005). Scale populations are typically kept in check in Japan by natural enemies such as *Aspidiotiphagus citrinus* Craw (Hymenoptera: Aphelinidae); however, this parasitoid is ineffective in the eastern U.S. (McClure 2002).

Pine needle scale (PNS), *Chionaspis pinifoliae* (Fitch), is a ubiquitous diaspidid pest of ornamental conifers, particularly Mugo pine, *Pinus mugo* Turra, and Scots pine (Johnson and

Lyon 1991). Like EHS, pine needle scale feeds on the mesophyll cells, causing defoliation and mortality with heavy infestations. After over-wintering in the egg stage under the female scale's armor, nymphs emerge in late spring.

Woolly alder aphids (WAA), *Paraprociophilus tessellatus* (Fitch) (Homoptera: Aphididae), are not economically important pests, but they are a vital food source for one of the few predaceous caterpillars, the immature stages of the Harvester butterfly, *Feniseca tarquinius* (Fabricius) (Lepidoptera: Lycaenidae) (Opler 1998). Adult harvester butterflies also rely on the aphids because they feed on the honeydew that is secreted. Great care must be taken to insure *L. kangdingensis* sp. n. does not pose any threat to the *P. tessellatus* population.

Host-specificity tests were conducted on *L. kangdingensis* sp. n. Specific objectives were to determine:

- (1) host-acceptance of various homopteran prey given to adult beetles in paired-choice feeding and oviposition tests and
- (2) host-suitability of various homopteran prey given to larvae in no-choice development tests.

## **Materials and Methods**

Host-specificity tests consisted of: (a) paired-choice adult feeding tests, (b) paired-choice oviposition tests, and (c) no-choice larval feeding/host suitability tests. Four prey species (eastern spruce gall adelgid, balsam woolly adelgid, woolly alder aphid, and pine needle scale) were tested against HWA controls in paired-choice adult feeding/oviposition tests. Eight test prey species were compared with HWA in the larval no-choice feeding tests. Brief descriptions of where and when the test prey spp. were collected follow:

BWA-infested Fraser firs [*Abies fraseri* (Pursh) Poir] were identified at a tree farm located in Plumtree, NC (Lat./Long. 36° 02' N / 82° 02' W) in Avery County, dug up, and brought back to the quarantine facilities at Virginia Tech in January 2004. The trees were potted and maintained in a greenhouse to encourage BWA development and oviposition for our studies.

PBA was collected several times between fall 2003 and spring 2004 from infested white pine trees located on the Virginia Tech campus and brought into the quarantine facilities to accelerate its development for our studies. The infested tree branches were put into plastic buckets filled with water and held at  $\approx 15^{\circ}\text{C}$  in the cold-room in the quarantine facility.

ESGA was collected in fall 2003 and spring 2004 from Norway spruce trees planted on the campus grounds of Virginia Tech. The foliage was collected after females had already laid eggs so the material was held at  $4^{\circ}\text{C}$  to slow development.

Larch adelgids (ELA) were collected for our studies in spring 2004 from infested European larch trees, *Larix decidua* Miller, located at the Blacksburg campus of Virginia Tech. As with ESGA, the foliage was collected after females had already laid eggs so the material was held at  $4^{\circ}\text{C}$  to slow development.

EHS-infested eastern hemlock branches were collected in High Point State Park, NJ (Lat./Long. 41° 33' N / 74° 73' W) and shipped from the Phillip Alampi Beneficial Insect Laboratory. The infested material contained adults with eggs and was held at  $4^{\circ}\text{C}$  to inhibit development.

PNS was collected from infested ornamental Mugo pines planted at the Horticultural Gardens on the Virginia Tech campus in Blacksburg, VA. Females had already laid eggs when the foliage was collected so the infested material was held at  $4^{\circ}\text{C}$  to inhibit development.

*P. tessellatus* was collected for our studies in fall 2003 and spring 2004 from infested hazel alder trees, *Alnus serrulata* (Ait.) Willd., growing around Pandapas Pond (Lat./Long. 37° 16' N / 80° 28' W) in the Jefferson and Washington National Forest in Montgomery Co., Virginia. The infested tree branches were put into plastic buckets filled with water and held at  $\approx$  15°C in the cold-room in the quarantine facility.

### Host-acceptance Tests

#### *Adult feeding and oviposition (a & b)*

In November 2003, adult beetles ( $n = 32$ ) were randomly assigned to one of four paired-choice test prey treatments: *P. strobi*, *A. abietis*, *P. tessellatus*, or *C. pinifoliae*. Each *L. kangdingensis* sp. n. adult was placed individually in 50 mm Petri dishes lined with two layers of moistened filter paper (Whatman No. 1). The choice assay consisted of one treatment (test prey with associated foliage) and one control twig (HWA on eastern hemlock). The densities of test and control prey were comparable—about 25-30 individuals. An exception was made for *P. tessellatus*; only ten nymphs (equivalent in size to sistens adults) were used in order to minimize the loss of individuals because they can move, unlike the adelgid and scale insects. The adults were maintained at 4°C, 12:12 (L:D),  $\approx$ 75% RH, and were removed from the bioassay after 72 h and placed on fresh HWA-infested hemlock foliage. The number of prey consumed and eggs laid on the treatment and control prey were then recorded.

## Host-suitability Tests

### *Larval feeding and development (c)*

*Laricobius kangdingensis* sp. n. eggs were removed from the oviposition study and maintained at 4°C to slow development while test-prey were brought into the quarantine facility in spring 2004 to synchronize their development. All test-prey (*P. strobi*, *A. abietis*, *A. laricis*, *A. piceae*, HWA, *F. externa*, and *C. pinifoliae*) were in the egg stage at the start of the experiment, except for *P. tessellatus* which was tested using immature nymphs roughly equivalent in size to HWA adults. Prey eggs/nymphs were displayed on their associated host plant; predator eggs were placed adjacent to the prey eggs/nymphs so that upon hatching food would be immediately available. Predator eggs were randomly assigned to one of eight no-choice treatments and held in 50 mm Petri dishes lined with two layers of moistened filter paper in an environmental chamber held at 6-9°C, 12:12 (L:D) and ≈75% RH. Eggs were monitored daily for eclosion and subsequent larval feeding.

### *Data Analysis*

Paired t-tests (PROC TTEST) were used to compare the number of test prey consumed with the HWA control in adult paired-choice feeding assays. Paired t-tests were also used to determine the preferred host for oviposition. Data were analyzed using SAS<sup>®</sup> (SAS Institute, 1989).

## **Results/Discussion**

### Host-acceptance Tests

#### *Adult Feeding and Oviposition (a & b)*

Adult *L. kangdingensis* sp. n. consumed significantly more HWA than *A. abietis* and *P. strobi* (Table 3.1). *C. pinifoliae* was not consumed in the study. Possible evidence of feeding was found in only one of the eight *P. tessellatus* replicates; two nymphs were missing but may have simply escaped. The data indicate that *L. kangdingensis* sp. n. prefers to feed on HWA and is not likely to negatively impact wild populations of *P. tessellatus*. This finding removes a major concern of adverse non-target effects because *P. tessellatus* is a food source for the carnivorous larval stage of the harvester butterfly.

There was no oviposition on *A. abietis*, *C. pinifoliae*, or *P. tessellatus* (Table 3.2). Only one egg was laid by a single female on *P. strobi*. Oviposition on HWA was observed in all four treatments with some females laying up to three eggs over the duration of the assay. These differences signify that *L. kangdingensis* sp. n. prefers to lay its eggs on HWA but not on non-adelgid hosts, and that it is quite selective on where it lays its eggs. The lack of statistical difference suggests the need for a larger sample size over a longer time period in future testing.

### Host-suitability Tests

#### *Larval feeding and development* (c)

*L. kangdingensis* sp. n. larvae did not survive past the first instar on *A. laricis*, *A. abietis*, *A. piceae*, *F. externa*, and *P. tessellatus* (Table 3.3). No feeding was observed on *F. externa* or *P. tessellatus*. One larva feeding on *C. pinifoliae* survived to the second instar. Larvae reached the pre-pupal stage only on *P. strobi* and HWA; however, no individuals completed development on any of the test prey (including HWA) in our tests. These findings suggest that none of the test prey is suitable for development of *L. kangdingensis* sp. n., except for PBA, and that it has a preference for HWA. Failure to emerge as adults was likely due to handling procedures and

rearing problems during the pre-pupal stage rather than lack of nutrients derived from HWA. Mold in the pupation medium (soil) was a major problem that negatively affected further development of the pre-pupae.

To resolve some of the rearing difficulties, pupae should be maintained at temperatures that simulate natural conditions to keep adults from emerging too early as they did in our colony. Lamb (2005) showed that *L. nigrinus* emergence is better synchronized with HWA by keeping them at higher temperatures initially, then moving them to cooler temperatures; this approach may work for *L. kangdingensis* sp. n., as well. The life cycle of HWA and *L. kangdingensis* sp. n. should be studied in their native range in China to determine what conditions are necessary to improve survival at each life stage.

The greatest mortality was observed in the pre-pupal stage while *L. kangdingensis* sp. n. was in soil which grew moldy over time despite various techniques to cleanse it, including steam-sterilization and autoclaving. The *L. nigrinus* colony at Virginia Tech used the same soil which became moldy and led to increased rates of mortality (Lamb, unpublished) but not all individuals were lost as was the case with our colony. For future colonies to flourish, care must be taken to ensure mold is not allowed to grow unabated in the laboratory.

## **Conclusions**

*Laricobius kangdingensis* sp. n. adults consumed significantly more HWA nymphs than any other prey, indicating a preference for HWA; however, more testing needs to be done using no-choice assays with more replicates and a wider range of test prey, including more adelgids. Zilahi-Balogh et al (2002) found that in no-choice assays *L. nigrinus* adults consumed significantly more HWA than *P. strobi*, but not *A. abietis*. *L. kangdingensis* sp. n. adults might

be more host-specific than *L. nigrinus*, but further studies are necessary to confirm that hypothesis.

In paired-choice oviposition tests, *L. kangdingensis* sp. n. was found to lay more eggs on adelgid compared with non-adelgid prey. This result is similar to what was found with *L. nigrinus* (Zilahi-Balogh et al. 2002). In order to find significant differences, future studies of *L. kangdingensis* sp. n. should include no-choice assays with more replicates over a longer time period or at higher temperatures to increase oviposition.

*Laricobius kangdingensis* sp. n. larvae had higher survival to the pre-pupal stage with HWA as a host, but unlike *L. nigrinus*, the pre-pupae did not complete development. This result was most likely due to mold infiltrating the pupation medium and producing toxic byproducts or causing adverse changes in the microclimate of the soil. Larvae did complete development on HWA in 12 and 15°C temperature development studies, so HWA is indeed a suitable host. *L. nigrinus* was found to reach the fourth instar on *P. strobi* and HWA, but also *A. piceae* (Zilahi-Balogh et al. 2002). Although *L. nigrinus* completed development only on HWA, potential larval hosts seem a bit narrower for *L. kangdingensis* sp. n. Developmental tests are the most important host-specificity tests because they reveal whether a biological control agent can complete development and reproduce on alternate hosts (Kok et al. 1992), thus negatively impacting native species. Further tests with more replicates are necessary to conclusively say *L. kangdingensis* sp. n. is a suitable biological control agent of HWA.

Table 3.1. Mean number ( $\pm$  SE) of prey consumed by adult *L. kangdingensis* sp. n. in a 3-day paired-choice test. <sup>1</sup>

Treatment	Mean number of prey consumed				t Statistic	df	P-value <sup>2</sup>
	n	Test prey $\pm$ SE	Control HWA $\pm$ SE	Difference $\pm$ SE			
<i>Adelges abietis</i> (ESGA)	8	3.60 $\pm$ 0.46	12.0 $\pm$ 0.93	8.40 $\pm$ 1.16	7.19	1, 6	0.0002*
<i>Pineus strobi</i> (PBA)	8	3.50 $\pm$ 1.49	10.0 $\pm$ 1.41	6.5 $\pm$ 2.35	2.77	1, 6	0.0276*
<i>Chionaspis pinifoliae</i> (PNS)	8	0	13.5 $\pm$ 1.86	13.5 $\pm$ 1.86	7.25	1, 6	0.0002*
<i>Paraprociophilus tessellatus</i> (WAA)	8	0.25 $\pm$ 0.25	8.0 $\pm$ 1.32	7.75 $\pm$ 1.29	6.00	1, 6	0.0005*

<sup>1</sup> Test conducted at 4°C and 12:12 (L:D) photoperiod, 6-9 November 2003.

<sup>2</sup> Paired t-tests (Proc ttest), \* significantly different at  $P = 0.05$

Table 3.2. Mean number ( $\pm$  SE) of eggs laid by adult *L. kangdingensis* sp. n. in a 3-day paired-choice test (Each treatment was paired with an HWA control).<sup>1</sup>

Treatment	n	Mean number of eggs laid			t Statistic	df	P-value <sup>2</sup>
		Test prey $\pm$ SE	HWA $\pm$ SE	Difference $\pm$ SE			
			Control				
<i>Adelges abietis</i> (ESGA)	8	0	1.00 $\pm$ 0.50	1.00 $\pm$ 0.38	1.32	1, 6	0.2275
<i>Pineus strobi</i> (PBA)	8	0.250 $\pm$ 0.18	1.25 $\pm$ 0.53	1.00 $\pm$ 0.38	1.32	1, 6	0.2275
<i>Chionaspis pinifoliae</i> (PNS)	8	0	1.40 $\pm$ 0.47	1.40 $\pm$ 0.44	1.99	1, 6	0.0875
<i>Paraproctiphilus tessellatus</i> (WAA)	8	0	0.75 $\pm$ 0.34	0.75 $\pm$ 0.26	1.43	1, 6	0.197

<sup>1</sup> Test conducted at 4°C and 12:12 (L:D) photoperiod, 6-9 November 2003.

<sup>2</sup> Paired t-tests (Proc ttest), \* significantly different at  $P = 0.05$

Table 3.3 Survival of *L. kangdingensis* sp. n. larvae in a no-choice host suitability test (Each treatment was paired with an HWA control).<sup>1</sup>

Test Prey	n	Feeding <sup>2</sup>	No. surviving to each life stage							
			L1	L2	L3	L4	Pre-pupal	Adult		
<b>Egg stage</b>										
<i>Adelges laricis</i> (ELA)	6	+	3	0	0	0	0	0	0	0
<i>Pineus strobi</i> (PBA)	6	+	2	1	1	1	1	1	1	0
<i>Adelges abietis</i> (ESGA)	17	+	9	0	0	0	0	0	0	0
<i>Adelges tsugae</i> (HWA)	15	+	11	10	10	9	6	6	0	0
<i>Adelges piceae</i> (BWA)	6	+	4	0	0	0	0	0	0	0
<i>Chionaspis pinifoliae</i> (PNS)	12	+	9	1	0	0	0	0	0	0
<i>Fiorinia externa</i> (EHS)	9	-	6	0	0	0	0	0	0	0
<b>Nymph stage</b>										
<i>Paraprociophilus tessellatus</i> (WAA)	8	-	5	0	0	0	0	0	0	0

<sup>1</sup> Test conducted at 6-9°C and 12:12 (L:D) photoperiod, March-June 2004.

<sup>2</sup> Larvae observed feeding ( + ) or not feeding ( - )

## Chapter 4

### Summary

Hemlock woolly adelgid (HWA) is an exotic homopteran pest of eastern and Carolina hemlocks in eastern North America. HWA attaches itself at the base of hemlock needles; as it feeds, it depletes nutrient stores and causes needles to desiccate and drop. If HWA populations are allowed to grow unimpeded the infestation can be fatal to the tree in as little as four years. Hemlocks are valuable ecologically, aesthetically, and economically. They are the most shade-tolerant trees in the eastern U.S. (Ward et al. 2004) and their dense, lush foliage offers an exceptional habitat for many woodland organisms. Aquatic vertebrates and invertebrates also benefit from the cooling effect hemlocks have on nearby streams. Furthermore, hemlocks are simply beautiful trees; they are popularly used in an ornamental setting for shade or as hedges at property perimeters. Additionally, hemlocks provide economic benefits to communities by serving as pulp, paper, lumber, or mulch. HWA is easily controlled by horticultural oils or systemic insecticides in nursery or backyard settings, but it is not feasible in a forest setting. Classical biological control efforts were initiated in the early 1990's to find natural enemies of HWA in its native region, Asia. Several potential biological control agents were identified from China and Japan, two of which are currently being evaluated in the field. A third candidate, *Laricobius nigrinus* Fender, was imported from the Pacific Northwest to Virginia Tech in Blacksburg, VA for research purposes. Based on its promising characteristics, an expedition was made to China in April 2002 to search for new agents, particularly those belonging to the genus *Laricobius*. *Laricobius kangdingensis* sp. n. was one of two species collected and brought back to the quarantine facilities at Virginia Tech.

The overall goal of this research was to establish and maintain a colony of *L. kangdingensis* sp. n. in order to study its biology and host-range preferences to determine if it could be a good control agent of HWA.

Chapter 2 describes observations on reproductive biology and temperature-dependent development of *L. kangdingensis* sp. n. at a range of constant temperatures. There are four larval instars. Females exhibit high fecundity ( $\approx 100$  eggs / female). Low temperature development thresholds are 2.8, 1.6, and 5.8°C for the egg, larval, and pre-pupal stages, respectively. Larvae completed development at 12 and 15 °C (Zilahi-Balogh, unpublished) but not at 6, 9, and 18°C in late 2003 and early 2004.

In Chapter 3, host-specificity of *L. kangdingensis* sp. n. was studied. In host-acceptance studies, adults were determined to be significantly host-specific in their feeding preferences and laid more eggs on HWA than other test prey. In host-suitability studies, larvae reached the pre-pupal stage on HWA and *P. strobi* but did not complete development on any host.

In order for *L. kangdingensis* sp. n. to be released from its quarantine status further testing will need to be done to more precisely define its biology and host range. Small numbers of individuals were used in these studies because the colony was declining and there was a desire to preserve life, if possible. Recommendations for areas of further study include:

#### Biology

- Larval feeding rates

#### Temperature-dependent Development

- Studies at 6, 9, and 18°C should be repeated because individuals in the studies described in Chapter 2 were plagued with moldy soil which may have impacted survival. Experiments conducted on F<sub>1</sub> individuals in 2003 at 12 and 15°C showed that *L.*

*kangdingensis* sp. n. can successfully complete development on a diet of HWA, so it must be determined why they did not succeed in my studies.

#### Host Acceptance: Adult feeding

-Preferences should be determined using no-choice assays at higher temperatures with more replicates and more adelgids as test prey, including *A. piceae* which was not available at the time of the study described in Chapter 3.

#### Host Acceptance: Oviposition

-As with adult feeding, oviposition preferences should be determined using no-choice assays with more replicates and more test prey at higher temperatures. Control experiments should be conducted to account for egg-dumping that may occur from being exposed to non-host prey for long periods. The duration of the study should also be increased from 72 h to allow enough time for more eggs to be laid.

#### Host Suitability: Larval survival

-The number of replicates needs to be increased in the no-choice larval survival/development studies because the results may be a better indicator of host preferences than oviposition or adult feeding (Van Driesche and Murray 2004). If results from host-acceptance and host-suitability experiments are negative, then it is clear that the test prey will not be a suitable host for *L. kangdingensis* sp. n.

#### Competitive Interactions

-It will be of great interest to see how the trio of *Laricobius* spp. interact in the laboratory and the field. Are certain species better suited to particular regions with varying environmental conditions?

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## **Vita**

Holly A. Gatton is the only child of Danny and Jan Gatton and was born in Washington, D.C. on October 24, 1979. In her junior year at Virginia Tech she took a course in insect biology taught by Dr. Rick Fell and discovered her love of insects. After graduating with her B.S. in Biology, she worked during the summer for Dr. S.M. Salom who would offer her an assistantship the following autumn. She was always fascinated by creepy critters, so Entomology seemed an appropriate discipline in which to specialize. Holly began working as a Project Manager for Dr. Mike Weaver in VT Pesticide Programs in May 2005.