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Field-cage evaluation of the survival, feeding and reproduction of *Laricobius osakensis* (Coleoptera: Derodontidae), a predator of *Adelges tsugae* (Hemiptera: Adelgidae)

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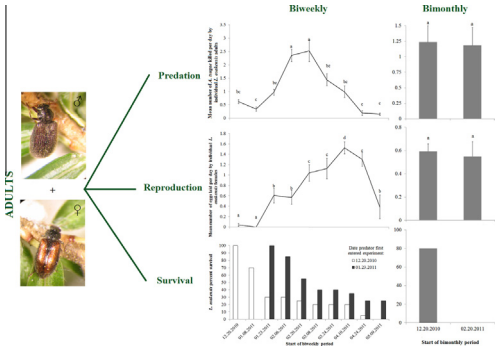
HIGHLIGHTS

- The predator was active and survival was >80% during winter (−18 °C low).
- Predation was high from December to March, when most terrestrial insects are dormant.
- Oviposition began in January and peaked in April.
- Larvae matured in 46–54 days and consumed an average of 39–43 ovisacs.
- *Laricobius osakensis* has potential to be an effective biological control agent of *Adelges tsugae*.

GRAPHICAL ABSTRACT



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ABSTRACT

The hemlock woolly adelgid, *Adelges tsugae* Annand, is a serious, non-native pest of hemlock in eastern North America. *Laricobius osakensis* Montgomery and Shiyake was identified as a key predator in Japan, where *A. tsugae* is native. Performance of adult and immature stages of *L. osakensis* was evaluated in sleeve cages on adelgid infested *Tsuga canadensis* (L.) Carrière in plant hardiness zones 5b and 6b in the mountains of southwestern Virginia, USA. Adults fed on the adelgid and laid eggs during all biweekly sample periods from December 2010 to May 2011, including winter when the temperature averaged below 0 °C. In cages with one adult pair (one male and one female), predation of *A. tsugae*/predator was 0.3–0.9/day during December and January, increased to 2.5/day in February, and then declined to 0.15/day in early May. The oviposition rate lagged the changes in feeding by 2–4 weeks, increasing from 0.02 eggs/day, from December to mid-January, to a peak of 1.5 eggs/day in early April, then declining to 0.4 eggs/day in late April. Mortality was 20% in cages left undisturbed for two months during the winter; even though temperatures were as low as −18 °C (cages examined biweekly had higher mortality, likely due to disturbance). In cages left undisturbed for two months during winter or early spring, 34 and 37 progeny were recovered, respectively. During each bimonthly period, a pair of adults and their progeny consumed 2.5 and 2.4 adult adelgids or ovisacs per day, respectively. Twenty-eight days after eggs were placed in the cages (on 11 March or 27 April 2012), 48% and 95% of the recovered larvae had reached maturity and each larva had destroyed 43 and 39 ovisacs, respectively. This research indicates that *L. osakensis* may have the potential to be an effective biological control agent of *A. tsugae* in most of the area where it is a pest in the eastern US.

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1. Introduction

Hemlock woolly adelgid, *Adelges tsugae* Annand (Hemiptera: Adelgidae) is an introduced pest from Japan that is threatening eastern (*Tsuga canadensis* (L.) Carrière) and Carolina (*Tsuga caroliniana* Engelmann) hemlock forests in the eastern United States (Havill et al., 2006; Orwig et al., 2002). This pest can colonize all hemlock species but can be fatal to eastern and Carolina hemlocks (Montgomery et al., 2009). It is relatively innocuous for Asian and western US hemlock species possibly due to the action of both host resistance and natural enemies (Havill and Montgomery, 2008; McClure and Cheah, 1999). Since its initial detection near Richmond, VA in the early 1950s, *A. tsugae* has spread throughout the eastern US, covering more than 50% of the geographic range of eastern hemlock in the US (USDA-FS, 2012).

Due to environmental concerns, operational problems, and the high cost involved in chemical control of *A. tsugae* in a forest settings, long-term sustainable efforts to control this pest have focused on biological control. A coordinated effort is being made to establish a complex of natural enemies, adapted to the various environmental conditions in which *A. tsugae* is present, that can reduce *A. tsugae* populations below damaging levels (Onken and Reardon, 2011). So far three species of predators have been released for the control of *A. tsugae*: *Sasajiscymnus tsugae* Sasaji and McClure (Coleoptera: Coccinellidae) in 1995, *Laricobius nigrinus* Fender (Coleoptera: Derodontidae) in 2003, and *Scymnus sinuanodulus* Yu and Yao (Coleoptera: Coccinellidae) in 2004 (Cheah et al., 2004). However, despite evidence of the establishment of *S. tsugae* and *L. nigrinus* in several locations, additional predators are needed since measurable impact has not yet been achieved by these predators (Davis et al., 2012; Hakeem et al., 2010).

Laricobius osakensis was found to be a key predator of *A. tsugae* in Japan (Lamb et al., 2011), the source of the introduction of *A. tsugae* to the eastern US (Havill et al., 2006). This predator is a highly-specialized natural enemy of *A. tsugae*, as it is only able to develop on this prey (Vieira et al., 2011). Emergence of *L. osakensis* adults starts in October and ends in December (Salom and Lamb, 2008). *L. osakensis* adult females start oviposition one to two weeks prior to the start of oviposition by *A. tsugae* (Vieira et al., 2013). *L. osakensis* larvae feed mostly on eggs but larger larvae (3rd and 4th instars) also feed and develop on *A. tsugae* nymphs (pers. obs.), and most larvae have already reached maturity and dropped to the soil to pupate by the end of May (Vieira et al., 2013). In laboratory studies, *L. osakensis* showed both a higher numerical response and functional response than *L. nigrinus* (Vieira et al., 2012). Overall, these studies indicate that *L. osakensis* has the potential to be a valuable addition to the natural enemy community previously released for the biological control of *A. tsugae*. *L. osakensis* has recently received regulatory approval for release from quarantine (USDA-APHIS-PPQ, 2010); thus, making pre-release evaluations in the field possible.

While laboratory assays can provide some indication of the potential impact of a natural enemy, field evaluations allow for the assessment of the efficacy of a natural enemy in more realistic conditions—they have to survive variable climatic conditions, deal with competitors and/or their own natural enemies (generalist predators), and be able to find their prey/host (O'Neil, 1989). The present study evaluates the survival, feeding, reproduction, and development of *L. osakensis* adults and larvae on *A. tsugae* populations in sleeve cages in Virginia, from late fall to mid-spring.

2. Materials and methods

2.1. Experimental subjects

The *L. osakensis* evaluated were progeny of beetles collected in October 2009 (adult trials) or 2010 (larval trials) from *Tsuga diversifolia* in Shiga Kogen, Japan, and then reared in a containment facility for at least one complete generation (Lamb et al., 2005a; Salom et al., 2012). Following emergence from aestivation in October-December, 2010, until they were used in the field trials, the F1 generation adults were maintained on field-collected *A. tsugae*-infested eastern hemlock twig cuttings in environmental chambers at 4 °C, 12:12 (L:D), and 65–75% RH until February, and then 9 °C, 12:12 (L:D), and 65–75% RH. Trials of larvae, conducted in spring 2012, were F2 progeny of *L. osakensis* collected in Japan in October 2010 and reared as stated above.

2.1.1. Adult sex determination

Adult *L. osakensis* exhibit some degree of sexual dimorphism—coloration ranges from coppery brown to black, but distinctive coppery brown individuals are usually females and dark brown or black individuals are usually males (Montgomery et al., 2011). To improve the accuracy of this approach, only individuals with pronounced colorings indicative of each sex were selected. From January to May, when *L. osakensis* was ovipositing in the laboratory, sex was verified by isolating individuals in $55 \times 20.3\ mm^2$ polystyrene Petri dishes with *A. tsugae*-infested twigs. Petri dishes were kept in an environmental chamber at 9 °C, 12:12 (L:D) and 75–87% RH for 3 days. After the trials, *A. tsugae* ovisacs were searched for predator eggs. If predator eggs were found, the individual was identified as a female, and in the absence of eggs, the individual was identified as a male, if it did not have female coloration.

2.1.2. Adult species confirmation

After field trials were started, it was discovered that the laboratory colony was a mixture of *L. osakensis* and a morphologically similar species, *Laricobius naganoensis* Leschen (Leschen, 2011). The two species can be distinguished by microscopic examination and dissection of male genitalia (Leschen, 2011). However, this method is not exact and does not allow for the identification of the species of the females; therefore, the identity of the beetles used in these experiments was confirmed using DNA sequence data. After use in the experiments, adult specimens were kept in dry storage or placed in 100% ethanol, until they were dissected under a microscope. The head, prothorax, elytra, and genitalia were kept as voucher specimens and the meso- and metathorax with membranous wings were used for the molecular analysis.

Genomic DNA was extracted using a Qiagen DNeasy Blood and Tissue Kit (Qiagen, California, USA) and PCR was used to amplify the partial cytochrome oxidase subunit I (COI). PCR was carried out following the protocol developed by Davis et al. (2011) with some modifications—15.5 μL sterile water, 4.8 μL $MgCl_2$ (25 mM), and 41 cycles. PCR products were purified using the QIAquick PCR purification kit (Qiagen Inc., Valencia, CA). Sequencing reactions were performed using the BigDye Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA) and analyzed on an Applied Biosystems 3730 automated sequencer. Edition and alignment of partial cytochrome oxidase subunit I (COI) sequences

Table 1
Voucher specimens for each study determined to species by sequence analysis. Each voucher is labeled with "10-LOG1-–followed by a unique sample number.

Sample number	Study	Species
01, 03–09, 11–12, 17–18, 22, 40–69	Adults (biweekly)	*Laricobius osakensis*
02	Adults (biweekly)	*Laricobius nigrinus*
23, 26–27, 30–33, 35–37	Adults (bimonthly)	*Laricobius osakensis*
F4, F7–F15, F22–F31	Larvae	*Laricobius osakensis*

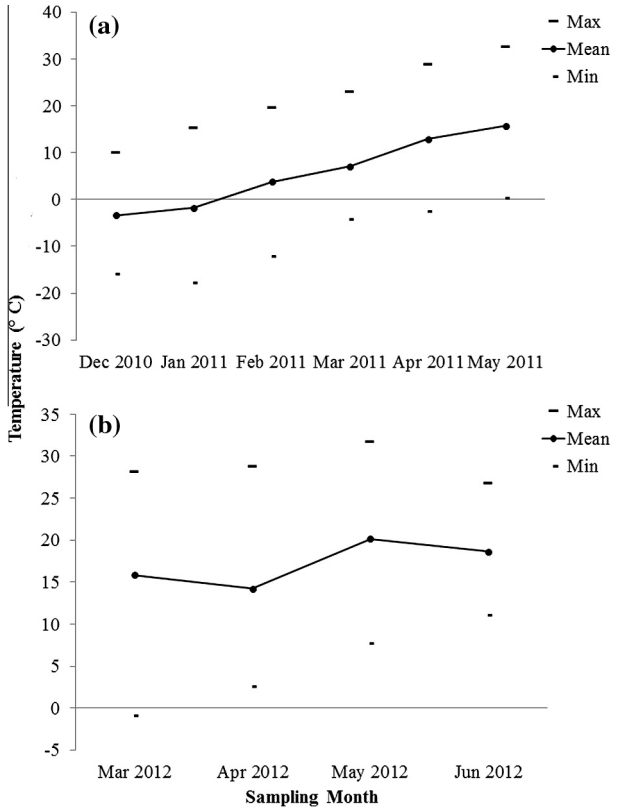

Fig. 1. Mean, minimum (min), and maximum (max) temperatures for each sample month at Saltville, VA (a) and Mountain Lake, VA (b).

were performed in Sequencher 4.2.2 (Gene Codes Corporation, Ann Arbor, MI).

All experimental subjects used in the trials were confirmed to be *L. osakensis*, except for one *L. nigrinus* used in the adult predators' evaluation (Table 1). The presence of the *L. nigrinus* specimen was probably a consequence of it being reared in the same facility as *L. osakensis*. It was not possible to trace and exclude the data represented by this specimen, but is too low to influence the results.

Vouchers for all specimens sequenced were placed in the Entomology Museum at Virginia Tech. These are labeled "10-LOSG1" (adult trials) or "11 LOSG1-F" (adult females that laid eggs for larval trials), followed by a two digit number. The label indicates the year the specimen emerged (10 or 11), the assumed species (LOS), the generation of the specimen in relation to the wild progenitors (G1), the sex of the specimen (F), and the specimen individual number.

2.2. Adult feeding, reproduction, and survival

2.2.1. Location and timing of experiments

Evaluation of *L. osakensis* adult feeding, reproduction and survival was conducted in Saltville, VA (36.886675°N, 81.855183°W), located in plant hardiness zone 6b (USDA-ARS, 2012), from 20 December 2010 to 9 May 2011. This site has an elevation of 636 m and was located on a southwestern slope. Eastern hemlock trees dominated the overstory vegetation at this site. Trees were selected for high levels of infestation and low lying branches that could be easily reached. The trees used for the experiments were 10–50 cm dbh (diameter at breast height), heavily infested, yet still producing new growth. Because the datalogger placed in the field was vandalized, temperature data (Fig. 1a) were retrieved from the closest meteorological station (Poor Valley, Saltville, VA, station code–KVASALTV2) at www.wunderground.com. The mean temperature increased month to month (Fig. 1a). The minimum and maximum temperatures registered during the sample period were −17.8 °C in January 2011 and 32.7 °C in May 2011, respectively. During the study, total precipitation was 586 mm, with several snow events. Monthly precipitation increased until March 2011 and decreased afterward.

At the beginning of the study on 20 December 2010, 2nd instar *A. tsugae* sistens were present in the field. By mid-February, *A. tsugae* was in the adult stage and had started ovipositing. By the end of April, the sistens adults had died and eggs and nymphs of the next generation (progrediens) were present. Observations in the laboratory and in the field in Japan indicate that all *L. osakensis* adults would have emerged from aestivation by the end of December and would be found on *A. tsugae* infested foliage, in diminishing numbers, until the beginning of May (Salom and Lamb, 2008; Vieira et al., 2013). The biweekly sample intervals thus capture the effects of weather and adelgid life history, while the first two-month sample interval covers the heart of winter to the beginning of *A. tsugae* egg laying and the second two-month covers the period of peak egg production by the sistens generation of *A. tsugae*.

2.2.2. Experimental procedure

At the beginning of each sample period, five trees were selected and pre-counts of *A. tsugae* were made on four branches of each tree. Starting from the tip of each branch, *A. tsugae* were counted on 62 cm of branch or less until a pre-set minimum prey amount or above was reached. The branch was marked with flagging tape where the count ended. The tree and branch codes as well as the number of *A. tsugae* ovisacs counted were registered on the flag. To ensure that the counted adelgids were not disturbed by the sleeve cage, it was secured to the branch above (proximal) of the flag at a point where the branch forked and was devoid of adelgid. On each tree, two branches were caged with a pair of predators inside (a male and a female *L. osakensis*) and the other two branches were caged without predators (control). Since it was not possible to find four branches with exactly the same number of *A. tsugae* on a tree, the two branches with the lowest and the two branches with the highest number of *A. tsugae* were grouped and the two treatments randomly assigned within the paired branches.

Cages were rectangular bags (72 × 55 cm) made of polyester fabric (white chiffon). The branches were vigorously shaken before the application of either treatment (with or without predators) to dislodge any native predators, such as spiders, that might be present. At the end of the sample period, the branch was cut proximal to the cage and taken to the laboratory to determine predator survival, reproduction, and feeding.

Predator feeding was based on the difference in the number of dead *A. tsugae* in cages with predators and cages without predators. Adult *L. osakensis* feeds by wounding its prey and sucking the hemolymph. Prey is not completely consumed usually and the dried cadavers of the prey remain (personal observation). Reproduction was based on the number of eggs and larvae recovered. For samples where both adults and larvae were present (second biweekly sample), feeding by larvae was estimated by

counting the number of disturbed ovisacs, which usually had all eggs consumed and only egg chorions remaining. Adult predators consume prey eggs and large predator larvae (3rd and 4th instar) can consume prey adults. However, adult predators do not consume all eggs in an ovisac like the larvae, and predator larvae do not consume prey adults without consuming all the eggs in the ovisac as well (Vieira et al., 2012).

2.2.3. Evaluation at biweekly intervals

For this study, only branches with 140–190 *A. tsugae* were used. Every two weeks the caged branches were clipped and taken to the laboratory, and cages (with predators and controls) were set up on new branches the following day. The number of surviving predators of each sex, predator eggs, and dead *A. tsugae* adults were counted. The adult beetles that survived were used in the new cages, and adult beetles were added to replace those that died. Specimens in the first sample period and those added in the second sample period were tracked (from cage to cage) during the entire trial.

2.2.4. Evaluation at bimonthly intervals

For this study, branches with an initial prey number of 400–485 *A. tsugae* were used. After 2 months, the caged branches were clipped and taken to the laboratory. The number and sex of live and dead predators, the number of dead and live *A. tsugae* adults, the number of disturbed and undisturbed ovisacs, and the number of immature predators (eggs and larvae) on the branches were counted. The following day, a new set of cages with predators and controls was set up in the field. As in the evaluation at biweekly intervals, surviving adult predators and additional beetles to replace the ones that died were used in the second bimonthly sample period.

2.3. Larval feeding, development, and survival

2.3.1. Location and timing of experiments

Evaluation of *L. osakensis* larval feeding and development was conducted at Mountain Lake, VA (37.3684589°N, 80.5217263°W, elevation 1218 m), located in plant hardiness zone 5b (USDA-ARS, 2012). Eastern hemlock trees also dominated the overstory vegetation at this site, which was located in proximity to a creek. Similar to the adult experiments, trees were selected for high levels of infestation and low lying branches that could be easily reached. The trees used for the experiments were medium size (30–75 cm dbh), heavily infested, but still fairly healthy. Temperature data were obtained from Mountain Lake Biological Research Station, about 1.5 km from the sample site. Temperature during the trials ranged from −0.9 °C on March 2012 to 31.8 °C on May 2012 with monthly means ranging from 14.2 °C and 20.2 °C (Fig. 1b). Total precipitation was 366 mm during the study.

There were two trials, one beginning 11 March 2012 and the other 27 April 2012, each lasting 4–6 weeks. The first trial began 1–2 weeks before *A. tsugae* sistens adults started laying eggs in the field and by the end of the trial adelgid eggs were still plentiful. The second trial began when the adelgid egg production was declining and by the end of the trial most of the eggs had hatched and 1st instar progrediens were present.

2.3.2. Experimental procedure

Evaluation of larval development and predation began with the placement in the field of *L. osakensis* eggs produced by ten predator adult females in the laboratory over a nine-day period and kept in storage at 4 °C for 23–31 days. Because of the unintended inclusion of *L. naganoensis* in the colony, it was necessary to store the eggs while the species of the egg's female parent was confirmed using molecular analysis, as previously outlined. For transfer to the field, the eggs were affixed to a bare hemlock twig by the wool material from an adelgid ovisac.

Adelgid ovisacs were counted on nine branches of five trees in the same manner as for the trials of adult predators, except that the number of ovisacs ranged from 150 to 177. Six branches were randomly caged with predator eggs and three without predator eggs (control). Branches on a tree with the most similar *A. tsugae* numbers were grouped in sets of three, with two branches in each group treated with predator eggs, and the remaining branch serving as the control.

For the predator treatments, a small twig with seven *L. osakensis* eggs of the same age (same day laid) was affixed with 34 gauge craft wire against the underside of a twig of the branch before it was enclosed in the cage.

Cages were harvested at different times because it was not certain when the larvae would mature—ideally cages would be harvested when 50% of the larvae were mature. One third of the cages were clipped and taken to the laboratory after three weeks or one month, and the remaining two thirds were removed from the field one week afterward. Counts were made on the number of dead and live larvae, with the state of larval development (immature or mature) noted, and the number of disturbed and undisturbed *A. tsugae* ovisacs. All disturbed ovisacs were counted; larval disturbance of ovisacs can be easily differentiated from those disturbed by other means. An ovisac disturbed by feeding of *Laricobius* larvae has more circular and looser wool covering, whereas an ovisac brushed by foliage or the cages has wool pulled from the ovisac. The number of disturbed ovisacs was used instead of the number of consumed ovisacs (all eggs consumed) because *A. tsugae* was ovipositing during the entire first sample period. Consequently, in this sample period, despite the larvae eating all the eggs before moving to a new ovisac, the *A. tsugae* adult left alive continued to lay eggs after the larvae left. The number of disturbed ovisacs was thus a better approximation of feeding.

For the second sample period, the number of live progrediens nymphs was also assessed. Recognition of mature *L. osakensis* larvae was based on size (>1.5 mm) and presence off the foliage on the bottom of the cage—mature larvae leave the foliage to pupate in the soil.

Larval developmental time (from the day egg was laid to larval maturity) was assessed only for the experiment set up on 11 March 2012, since the date of oviposition was known, and some of the larvae that reached the pre-pupal stage were still alive. It was not possible to assess development time for the experiments started on 27 April 2012 because temperatures were much higher during this period (Fig. 1b). For the latter experiment, when branches were recovered, larvae had already completed development and were all dead. Developmental time estimates include the time the eggs spent in cold storage in the laboratory (23–31 days).

2.4. Data analysis

Statistical treatments were performed in SAS (SAS_Institute_Inc., 2008) and JMP (SAS_Institute_Inc., 2009). All data were tested for normality and equality of variance.

Differences between the number of dead *A. tsugae* or disturbed ovisacs in cages with and without predators, for each sample period, were analyzed with a paired *t*-test. To compare differences in predation among sample periods, a predation rate (prey killed/day/predator) was calculated to account for the small differences in the duration of each sample interval and number of predators in the sample. It was presumed that the mortality due to factors other than predators would be the same in the cages with and without predators. Since sequential samples had new beetles added to replace those that did not survive from the previous sample, each sample was treated as unique rather than a measurement of the

same beetles. Differences in predation at different sample periods were determined with a one-way ANOVA followed by Tukey's HSD. The number of dead *A. tsugae* was also compared between cages with the same treatment (with or without predators) at different intervals in the two-month experiments using a one-way ANOVA.

Larval predation was calculated as the difference between the number of disturbed *A. tsugae* ovisacs in the cages with predator larvae and the number of disturbed *A. tsugae* ovisacs in cages without predator larvae. Larval predation for each sample period was compared with a paired *t*-test. The effect of larval predation on the following generation (progrediens) was determined as the difference between the number of live progredientes in cages with predator larvae and cages without predator larvae in the experiments set up on 27 April 2012, and analyzed with a paired *t*-test. In all analyses, the natural pairs were branches from the same tree with the same prey number that received the different treatments (control or predator adults/eggs). Cages seeded with *L. osakensis* eggs ($n = 62$) from which no larvae were recovered ($n = 17$) were excluded from the analysis.

Oviposition was analyzed as a function of sample period using the GLIMMIX procedure in SAS assuming a Poisson distribution (SAS_Institute_Inc., 2008). The adjusted Tukey–Kramer HSD test was used to determine significant differences in oviposition between sample periods.

Survival of adult predators was recorded for each individual as a binomial response (alive = 1, dead = 0) and was analyzed by sample period and sex using the GLIMMIX procedure (SAS_Institute_Inc., 2008). The adjusted Tukey–Kramer HSD was used to determine significant differences in survival between sample periods and sexes. Survival of adult predators first used in the first and third sample periods was also recorded as a binomial response and compared for each sample period using a Chi-square test.

3. Results

3.1. Adult feeding, reproduction, and survival

3.1.1. Evaluation of adults at biweekly intervals

Cages with a pair of *L. osakensis* adults had significantly more dead *A. tsugae* than cages without the predators in all biweekly sample periods from 20 December 2010 to 9 May 2011 ($t = 10.03$, $DF = 87$, $P < 0.0001$) (Table 2). The predation varied according to the sample period, being greater in February and beginning of March 2011, and lesser in mid-January and April 2011 (Table 2).

Oviposition started by the end of January, had its peak by the end of March, and decreased thereafter (Fig. 2). A maximum of 35 eggs were laid by a female during a biweekly period.

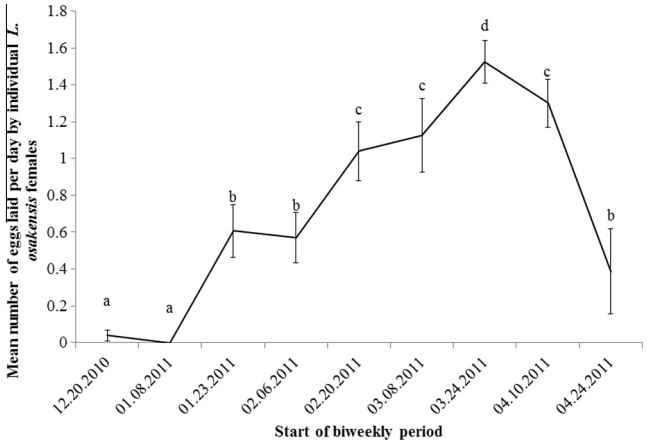

Fig. 2. Number of eggs laid by individual *L. osakensis* females per day (±SE) in sleeve cages in the field during each biweekly interval in Saltville, VA. Different letters indicate biweekly intervals with significantly different oviposition rates at *P*-value <0.05 using Tukey Kramer HSD test.

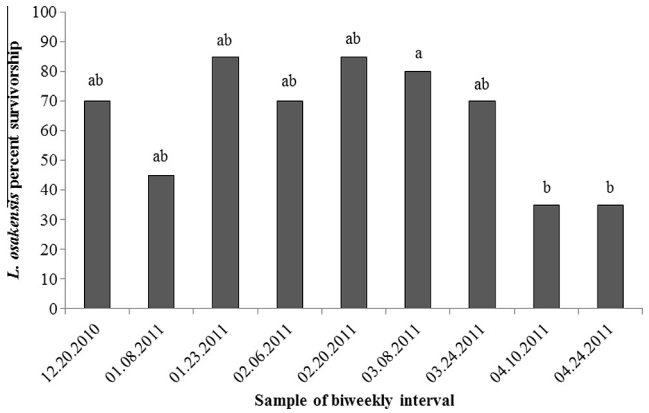

Fig. 3. Survival of *L. osakensis* adults during each biweekly interval in sleeve cages in Saltville, VA. Different letters indicate biweekly intervals with significantly different survival rates at *P*-value <0.05 using Tukey Kramer HSD test.

Survival of *L. osakensis* was examined two ways—survival of all beetles (both re-used and replaced beetles) at each sample period and survival of a set of individuals throughout the experiment. In the evaluation of survival at each sample period, no difference was found on the survival of *L. osakensis* males and females ($F(1,162) = 0.28$, $P = 0.5957$), but some differences were found between the sample periods ($F(8,162) = 4.38$, $P < 0.0001$). Paired comparisons indicated that survival was fairly consistent across

Table 2
Comparison of the number of dead *A. tsugae* in cages with and without (control) *L. osakensis* adults at the end of each biweekly interval (*t* statistic, degrees of freedom (*DF*) and *P*-value), and biweekly predation rates.

Sample interval (mm.dd.yy)	Dead *A. tsugae* (mean ± SE)					Predation rate[a] (prey/day/predator)
	Cages with predators	Cages without predators	*t*	*DF*	*P*-value	
12.20.10–01.07.11	51.7 ± 2.5	29.4 ± 1.4	8.39	9	<0.0001	0.6 ± 0.1 bc
01.08.11–01.22.11	69.7 ± 3.8	60.0 ± 4.2	4.28	9	0.002	0.3 ± 0.1 c
01.23.11–02.05.11	79.4 ± 3.6	54.2 ± 4.0	7.80	9	<0.0001	1.0 ± 0.1 bc
02.06.11–02.19.11	122.9 ± 10.6	61.8 ± 7.0	10.02	9	<0.0001	2.3 ± 0.2 a
02.20.11–03.07.11	129.2 ± 9.9	53.6 ± 9.2	6.35	9	<0.0001	2.5 ± 0.4 a
03.08.11–03.23.11	89.8 ± 6.7	46.6 ± 4.2	6.38	9	0.0001	1.4 ± 0.2 b
03.24.11–04.09.11	105.5 ± 7.3	73.6 ± 6.6	4.48	9	0.0015	1.0 ± 0.2 bc
04.10.11–04.23.11	155.0 ± 9.0	150.0 ± 9.6	2.27	9	0.0496	0.2 ± 0.1 c
04.24.11–05.09.11	159.0 ± 5.7	154.5 ± 5.4	3.72	7	0.0075	0.1 ± 0.0 c

[a] Predation rates with different letters were significantly different at *P*-value <0.05 using Tukey Kramer HSD test.

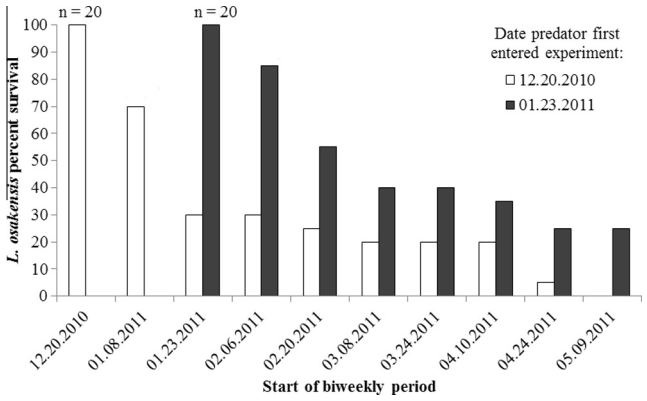

Fig. 4. Survival of adult *L. osakensis* first placed in the field on 20 December 2010 or 23 January 2011 for the biweekly sleeve cage studies, to the end of the experiment on 09 May 2011, in Saltville, VA.

the entire experimental period, but was significantly less at the end (sample periods starting on 10 and 24 April 2011) (35%) than on 08 March 2011 (80%) (Fig. 3). Survival was tracked for individuals first used in the first and third sample periods to the end of the experiment in May (Fig. 4). Some of these specimens survived almost the entire experimental period from December 2010 to May 2011. However, for both groups there is a large decline in survival in the two sample periods following the introduction into the field—70% and 45% mortality for individuals first used in 20 December 2010 and 23 January 2011, respectively. After the second group went through these two sample periods (one month) of higher mortality, survival in this (55%) and the first group (30%) was not statistically different (χ^2 (1, $N = 35$) = 0.89, $P = 0.3454$). Survival continued to diminish in following sample periods but to a lesser extent (Fig. 4).

3.1.2. Evaluation of adults at bimonthly intervals

In the bimonthly evaluation, cages with the *L. osakensis* adult pair had significantly fewer live and significantly more dead *A. tsugae* than in cages without predators in both bimonthly sample periods (Table 3). The number of dead *A. tsugae* adults was significantly greater in the second sample period than in the first sample period both in cages with (F (1, 18) = 16.36, $P = 0.0008$) and without (F (1, 18) = 10.97, $P = 0.0039$) predators.

The predation rate (prey killed/day/predator) was similar in the first (1.2 ± 0.3) and second (1.2 ± 0.3) sample periods (F (1, 18) = 0.05, $P = 0.83$). However, in the second sample, resources were exhausted in the cages with predators. There were no live adelgids and only two ovisacs with any eggs remained in all of the cages with predators.

Each cage with predators had an average of 37 eggs and 34 larvae at the end of the first and second sample period respectively. Individual females produced a maximum of 59 and 69 progeny in the first and second bimonthly periods, respectively. The average number of progeny produced per female/day was 0.6 ± 0.1 and 0.5 ± 0.1 in the first and second sample periods, respectively, which were not significantly different (F (1, 18) = 0.09, $P = 0.7691$).

Survival of the pair of adult beetles placed in the cages was significantly greater during the first than the second bimonthly period, where no adults survived (F (1, 36) = 76, $P < 0.0001$). The high mortality in the second sample period was likely related to exhaustion of available resources. During the first sample period, 70% and 90% of the males and females survived, respectively, but this difference was not significant (F (1, 36) = 0.34, $P = 0.5635$).

3.2. Larval feeding, development, and survival

Of the 210 eggs placed in the field at each sample period, an average of 21.4% and 28.0% hatched and developed for sample periods starting at 11 March 2012 and 27 April 2012, respectively. The low egg hatch is likely a consequence of handling and storage of the eggs in the laboratory. Of the larvae recovered from the 11 March 2012 trial, only 2 (6.7%) died before reaching maturity. All were still immature after 21 days, but 48.1% had reached maturity after 28 days in the field. For the 27 April 2012 trials, only 3 larvae (5.2%) did not reach maturity; all remaining larvae were mature and dead after 28 or 38 days in the field (Table 4). It took an average of 48.1 ± 0.7 days for *L. osakensis* to develop from egg (day egg was laid) to mature larvae in March/April 2012, with a minimum and a maximum of 46 and 54 days, respectively.

In the 11 March 2012 trials, after 21 days in the field, the larvae were still immature (probably 3rd or 4th instar) and each preyed upon an average of 23.0 ± 1.9 ovisacs. But after an additional week in the field (28 days total), most larvae were mature or close to maturity (probably 4th instar) and each preyed upon an average of 42.7 ± 2.6 ovisacs. For the second sample period, starting on 27 April 2012, after either 27 days or 38 days in the field all larvae were dead and mature, and 40.0 ± 3.2 or 38.7 ± 2.7 ovisacs on average were eaten, respectively (Table 4).

Cages with the *L. osakensis* larvae had a significantly greater number of disturbed *A. tsugae* ovisacs than cages without predators, in all sample periods ($t = -29.73$, $DF = 102$, $P < 0.0001$) (Table 5). Disturbed ovisacs were registered for both cages with and without predator larvae. However, it was evident that the disturbance in cages with predators was caused by predator larval activity. The disturbance of the ovisacs seen in the control cages was a result of some ovisacs rubbing against something, probably the fabric of the cage. Additionally, no other predators in any stage were found in the control cages. Predation was similar during all sample periods ($F = 0.0004$, $DF = 1$, $P = 0.98$). Cages with predator larvae had significantly fewer adult progrediens than cages without predators ($t = 10.27$, $DF = 21$, $P < 0.0001$), with an average of 50.6 ± 7.2 and 150.1 ± 9.2 progrediens, respectively.

4. Discussion

Adult predation impact was evident throughout both biweekly and bimonthly experiments. It varied according to the sample

Table 3
Comparison of mean number of live *A. tsugae* adults, dead *A. tsugae* adults, and consumed *A. tsugae* ovisacs in cages, with and without (control) *L. osakensis* adults at the end of each bimonthly interval (*P*-value based on paired t-test, DF = 9).

Sample interval (mm.dd.yy)	Dependent variable	*A. tsugae* (mean ± SE)		*P*-value between treatments
		Cages with predators	Cages without predators	
12.20.10–02.20.11	Live adults	193.3 ± 26.1	348.8 ± 10.6	0.0010
	Dead adults	332.4 ± 27.5	176.9 ± 11.5	0.0010
	Ovisacs consumed	0 ± 0	0 ± 0	nd
02.20.11–04.23.11	Live adults	0 ± 0	146.6 ± 35.4	0.0025
	Dead adults	450.8 ± 8.9	304.2 ± 36.7	0.0025
	Ovisacs consumed	404.2 ± 45.8	0 ± 0	<0.0001

Table 4
Percentage of eggs that hatched, dead and alive immature and mature *L. osakensis* larvae recovered from sleeve cages at Mountain Lake, VA.

Sample interval (mm.dd.yy)	Eggs that hatched	Immature larvae (%)		Mature larvae (%)	
		Dead	Alive	Dead	Alive
03.11.12–04.01.12	15 (21.4%)	0	100	0	0
03.11.12–04.08.12	30 (21.4%)	7.4	44.5	22.2	25.9
04.27.12–05.23.12	18 (32.1%)	5.6	0	94.4	0
04.27.12–06.03.12	40 (26.0%)	5	0	95	0

Table 5
Comparison of the number of disturbed *A. tsugae* ovisacs in sleeve cages in the field with and without *L. osakensis* larvae (*t* statistic, degrees of freedom (*DF*) and *P*-value), and predation.

Sample interval (mm.dd.yy)	Disturbed *A. tsugae* ovisacs (mean ± SE)					Predation (prey/larva)[a]
	Cages with predators	Cages without predators (total)	*t*	*DF*	*P*-value	
03.11.12–04.01.12	53.6 ± 11.6	6.7 ± 0.4	4.07	7	0.0047	23.0 ± 1.9 a
03.11.12–04.08.12	89.2 ± 7.9	9.1 ± 0.3	10.37	14	<0.0001	42.8 ± 2.6 b
04.27.12–05.25.12	97.7 ± 14.3	10.7 ± 1.1	5.89	7	0.0006	40.0 ± 3.2 b
04.27.12–06.03.12	111.9 ± 9.1	10.4 ± 1.3	10.63	13	<0.0001	38.7 ± 2.7 b

[a] Predation rates with different letters were significantly different at *P*-value <0.05 using Tukey Kramer HSD test.

period in the biweekly experiments, probably as a function of variation in temperature, live prey availability, prey stage, and predator mortality. Temperature is usually a driving force for insects, with most terrestrial insects being inactive at temperatures below 4 °C (Schowalter, 2011), but both *L. osakensis* and *A. tsugae* are unusual in feeding during the winter months when temperatures are above freezing. Adult predation decreased due to lower temperatures in December through January (Fig. 1a). The low predation in the sample period starting on 08 January 2011 corresponded with very low temperatures experienced during that 15-day period. Predation peaked from February to the beginning of March, corresponding to an optimal range of temperatures, prey availability, and prey stage. During this time *A. tsugae* adults are fully developed, starting to oviposit, and their chance of survival is high. Temperature during these months (Fig. 1a) is likely optimal for both predator and prey survival and activity. The low predation in April and May has to be interpreted with caution since it can be an artifact of the type of prey assessed. The number of dead/alive *A. tsugae* adults is a measure of predation more reliable than the number of eggs or nymphs, since the number of adults does not vary from the beginning to the end of each sample period, and is also easier to assess. However, adult *L. osakensis* also feed on *A. tsugae* eggs and nymphs, leading to an underestimation of the total predator impact. This underestimation was especially noticeable in April and May, since most *A. tsugae* adults had died naturally but the eggs and eclosed nymphs remained a food source. The increase in predation from winter to spring was also observed in cage studies with *L. nigrinus* (Lamb et al., 2005b).

For the entire duration of both bimonthly evaluations, the predation rate of an adult beetle was 1.2 *A. tsugae* sistens adults/day, which is near the average rate of the biweekly data. The similarity in the predation rate in both bimonthly sample periods was likely more related to the impact measure selected (dead *A. tsugae* adults) and prey limitation than to a true lack of differences. Almost all *A. tsugae* adults, and all predator adults and larvae were dead by the end of the second sample period. This suggests that if sufficient prey were available predation would be much higher. Additionally, the higher natural mortality of *A. tsugae* adults at the end of the sistens generation life cycle, as seen in the cages without predators, also made it difficult to detect predator impact. The bimonthly predation rates found for *L. osakensis* seem much lower than the 3.3 and 5.8 adelgids/day/adult predator rates reported for *L. nigrinus* in sleeve cage studies at 90-day intervals for November–January and February–April, respectively (Lamb et al., 2005b). However, these rates do not account for natural mortality (mortality in control cages), attributing all mortality in cages with predators to predation. Accounting for natural mortality, predation rates for *L. nigrinus* were 1.2 and 2.0 adelgids/day/adult predator for November–January and February–April, respectively, similar to the bimonthly predation rates found for *L. osakensis* in this study. The discrepancy between the spring predation rate of *L. osakensis* (1.2 adelgids/day/adult predator) and *L. nigrinus* (2.0 adelgids/day/adult predator) was likely motivated by different prey availability in the two studies. The spring cage evaluation of *L. nigrinus* ended earlier and food was still plentiful, more than one-half of the initial number of adelgids remained, whereas the spring evaluation of *L. osakensis* ended later in the season and all adelgids were dead either from predation or natural mortality. Additionally, while the *L. nigrinus* studies lasted longer, the initial densities used were twice that of the ones used in this study. Given sufficient prey, *L. osakensis* predation in the second bimonthly period would likely match that of *L. nigrinus*. Similar predation rates were also previously found in laboratory studies (Vieira et al., 2012).

Oviposition in the biweekly experiments showed a pattern similar to predation but with a delay. In early February there was a significant increase in predation. The following sample period showed a significant increase in oviposition. This increase also coincided with the onset of oviposition by *A. tsugae* sistens adults. Peak oviposition on 24 March 2011 was reached one month after peak predation on 20 February 2011. The delay between increases in predation and oviposition is likely related to adult female predators' nutritional requirements for oviposition as well as reproductive phenology. Since egg production is nutritionally costly for the females, it is likely they have to increase their food intake in preparation for egg production (Chapman et al., 1998). The lower predation at peak oviposition might be related to female behavior. In previous studies (Vieira et al., 2012), *L. osakensis* females did not feed on ovisacs in which they oviposited. If adult prey was limited, they would feed on adelgid eggs in other ovisacs and even on some of the eggs in the ovisacs they had laid an egg. With both behaviors, females left prey for their progeny.

The oviposition results of this study are comparable in many respects to those of similar cage studies with *L. nigrinus* (Lamb et al., 2005b). Oviposition of both *L. nigrinus* and *L. osakensis* varied throughout the season and peaked in mid-April. Both species laid some eggs before February, but *L. osakensis* produced 8 eggs/female

from December–January, whereas *L. nigrinus* produced only 0.2 eggs/adult from November–January. The start of oviposition by *L. osakensis* before the onset of *A. tsugae* sistentes oviposition has also been observed in predators' native range (Lamb et al., 2011). Total spring fecundity of *L. osakensis* (34 progeny/female) was higher than the 20 progeny/female by *L. nigrinus*. The higher oviposition by *L. osakensis* in relation to *L. nigrinus* reflects what would happen under similar conditions, since the range of temperatures in this study (−17.8 to 32.7 °C) and that with *L. nigrinus* (−16 to 32.7 °C) were similar. The average temperature in January and February was also lower in this study (−2.6 °C) than in the study with *L. nigrinus* (−0.5 °C). The higher oviposition of *L. osakensis* in relation to *L. nigrinus* was also seen in laboratory studies (Vieira et al., 2012).

The lack of differences in reproduction during the two sample periods in the bimonthly experiments was probably because of limited resources causing mortality of the adults in the second sample period. When the adults and progeny exhausted the food resources, greater reproduction would only reduce the chance of survival of the progeny. Additionally, all adults died in the second sample period, so none of the females oviposited during the entire sample period. That only eggs were found at the end of the first bimonthly period suggests that the eggs were laid near the end of the period and these developed slowly because of the cool temperatures (Fig. 1a). In contrast, only dead, mostly mature, larvae were found at the end of the second bimonthly period, suggesting that most of the eggs were laid by the predator during the early part of the period and these developed rapidly with the warmer temperatures (Fig. 1a). There are two possible reasons why the larvae died—the larvae ran out of food and died of starvation before reaching maturity or larvae reached maturity but died because the cage prevented them from entering the soil to complete development.

Survival of almost all of the adult specimens in the first bimonthly period indicates *L. osakensis* can withstand low winter temperatures at high elevations in Virginia's mountains. The sharp decrease in survival one month following initial introduction of the individuals into the field and the lower winter survival in the biweekly study than in the bimonthly study, indicate that the need for acclimation of the insects after being maintained at 4 °C (December–February) or 12 °C (March–May) in the laboratory. *L. osakensis* survival (80%) after 2 months in the field was similar to that found for *L. nigrinus* (88.9% November–January) (Lamb et al., 2005b). Since *L. osakensis* was exposed to −18 °C and *L. nigrinus* to −16 °C in the field cages (Lamb et al., 2005b), both species seem cold-adapted and able to survive equally cold winters in more northern latitudes at lower elevations (Mausel et al., 2010).

Larvae produced from eggs laid in the cages placed in the field on 20 February 2010 were almost all mature or dead before reaching maturity when the cages were collected on 23 April. In these cages, the impact from both predator adults and larvae was evident. Most *A. tsugae* adults in the cages with predators were dead with all the eggs consumed. In most cages, only 1 or 2 *A. tsugae* ovisacs had intact eggs.

Larvae in the cages in which laboratory eggs were seeded reached maturity in 48 days, with each larva feeding on >40 *A. tsugae* ovisacs. Thus, a larva feeds on about 1.3 ovisacs/day, which is about the predation rate of an adult over its lifetime. The significant increase in the predation from three to four weeks in the field shows the considerable activity of *L. osakensis* larvae in the 3rd and 4th instars. It was also clear that the predator activity, larval in this case, had an impact on the next prey generation. The larvae directly eliminate the eggs that would be part of the next generation. The carry-over effect of predator activity on the next generation (progrediens) was also observed for *L. nigrinus* (Lamb et al., 2005b, 2006). The development times from egg to mature larvae under field conditions are likely much shorter than the ones determined in this study, which were affected by the time the eggs spent in cold storage.

Overall, *L. osakensis* can survive, reproduce, develop, and impact *A. tsugae* populations in sleeve cages in plant hardiness zones 5b and 6b in Virginia. The earlier onset of oviposition and higher fecundity of *L. osakensis* are indications that *L. osakensis* has the potential to be a more effective biological control than *L. nigrinus*. Future experiments should focus on the performance of this predator in open releases, evaluating its impact, establishment, and dispersal.

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