Biocontrol Science and Technology, 2015
Vol. 25, No. 12, 1467–1482, http://dx.doi.org/10.1080/09583157.2015.1061099

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RESEARCH ARTICLE

Assessment of the potential for hybridisation between *Laricobius nigrinus* (Coleoptera: Derodontidae) and *Laricobius osakensis*, predators of the hemlock woolly adelgid (Hemiptera: Adelgidae)

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(*Received 28 February 2015; returned 1 June 2015; accepted 5 June 2015*)

In 2003, *Laricobius nigrinus* Fender was introduced into the eastern United States as a biological control agent of the hemlock woolly adelgid (*Adelges tsugae* Annand). Following its release, it was discovered that *L. nigrinus* was hybridising and producing viable progeny with *Laricobius rubidus* LeConte, a species native to eastern North America. Recently, *Laricobius osakensis* Montgomery and Shiyake was imported from Japan into the USA as a potential biological control agent of hemlock woolly adelgid. Hybridisation between *L. nigrinus* and *L. rubidus* led to interest in the outcome of interactions between *L. osakensis* and the other two *Laricobius* spp. The purpose of this study was to determine if *L. osakensis* could mate with *L. nigrinus*, if they could produce hybrid progeny, and whether mating interferes with reproductive output. *Laricobius* spp. were observed mating directly following emergence and found to be capable of producing sterile eggs in the absence of a mating event. Laboratory and confined field studies found no evidence that *L. osakensis* and *L. nigrinus* could produce hybrid progeny and the interaction between the two species did not result in a lower reproduction associated with interspecific mating attempts. Interbreeding should therefore not have an impact on biological control using these species. Fecundity experiments showed that *L. osakensis* produced eggs earlier in the season and at a higher rate than *L. nigrinus*, suggesting that *L. osakensis* may have the potential to be an even more successful biological control agent than *L. nigrinus*.

Keywords: hemlock woolly adelgid; *Laricobius*; hybridisation

1. Introduction

Laricobius nigrinus Fender is a predatory beetle native to the Pacific Northwest and western Canada where it feeds on hemlock woolly adelgid (*Adelges tsugae* Annand; hereafter HWA). In 2003, *L. nigrinus* was released at 22 sites in the eastern United States where HWA is invasive. The predator established at 13 of the 22 release sites and has been found to have a negative impact on HWA survival (Mausel et al., 2011; Mausel, Salom, Kok, & Fidgen, 2008). Recently, it was discovered that *L. nigrinus* can hybridise with *Laricobius rubidus* LeConte (Havill et al., 2012), a native eastern North American predator of pine bark adelgid (*Pineus strobi* Hartig).

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Laricobius nigrinus and *L. rubidus* are sister species (Davis et al., 2011; Montgomery, Shiyake, Havill, & Leschen, 2011), which have been shown to produce viable hybrid progeny (Havill et al., 2012).

Hybridisation between *L. nigrinus* and *L. rubidus* is a concern because of potential impacts to the biological control of HWA, as well as concerns regarding biodiversity (Havill et al., 2012; Hopper, Britch, & Wajnberg, 2006). For example, if hybrids have reduced fitness (Arnold, 1997; Goldson, McNeill, & Proffitt, 2003), they may have less of an impact on HWA populations than their parents. Alternatively, hybridisation could result in recombination that introduces advantageous alleles or new combinations that contribute to adaptive evolution (Arnold, 1997; Lewontin & Birch, 1966). This could result in hybrid progeny that are more fit than the parental species (Arnold, 1997) and potentially capable of having a greater impact on HWA populations. Either scenario could result in reduction of the distinctiveness of the parent species through genetic introgression at field sites where they are both present (Arnold, 1997).

The effects of hybridisation between *L. nigrinus* and *L. rubidus* are currently being studied. So far, introgression has been patchy and asymmetrical with many individuals that are still being identified as pure species (Havill et al., 2012). It is possible that a patchy distribution (a mosaic hybrid zone) of parentals and hybrids will result, since the preferred habitats of each *Laricobius* spp. [*Tsuga canadensis* (L.) Carrière/*Tsuga caroliniana* Englem. (hemlock) and *Pinus strobus* L.(white pine)] overlap throughout much of eastern North America (Havill et al., 2012). Sampling from 2010 to 2012 on both hemlock and white pine at 10 sites showed that *L. nigrinus* was more dominant on hemlock and *L. rubidus* was more dominant on white pine (Fischer et al., 2015). This may denote habitat preference and promote segregation between the two species, but a steady rate of hybridisation was maintained during the course of the study, suggesting that the two species remain in contact.

In 2006, *Laricobius osakensis* Shiyake and Montgomery was imported from Japan into the USA as another potential biological control agent of HWA. *Laricobius osakensis* is native to the same region of Japan as the HWA strain accidentally introduced to the eastern United States; therefore, *L. osakensis* likely evolved with this HWA strain (Havill, Montgomery, Yu, Shiyake, & Caccone,2006; Lamb, Montgomery, Vieira, Shiyake, & Salom, 2011). In a study of comparative interactions among *Laricobius* spp., Story, Vieira, Salom, and Kok (2012) found that *L. osakensis* feeds more and has a greater fecundity than *L. nigrinus*. Additionally, *L. osakensis* may be better suited to the southern United States than *L. nigrinus*. In the southern United States, HWA breaks diapause later in the season (November) than in the north (October) due to the longer summers. *Laricobius osakensis* collected in the Kansai region of Japan emerge later in the season (November) (Lamb et al., 2011) than *L. nigrinus* (October) (Mausel et al., 2011), resulting in an improved alignment of the predator/prey lifecycles in the southern United States. For these reasons, the evaluation of *L. osakensis* for release into the field has been a priority in the biological control of HWA.

In contrast to *L. nigrinus* and *L. rubidus*, which are closely related sister species, *L. osakensis* is in a separate clade, together with other Asian species (Montgomery et al., 2011). The mean sequence divergence (K2P) between *L. osakensis* and *L. nigrinus* in the mitochondrial COI gene is 15.2%, whereas the distance between *L. nigrinus* and *L. rubidus* is 2.2% (Montgomery et al., 2011). In addition, there are major morphological differences between *L. osakensis* and the North American species, such as the presence of ocelli in *L. osakensis* but not in *L. nigrinus* or *L. rubidus* (Leschen, 2011).

Evolutionary divergence is likely to impact the propensity to hybridise. For example, *Heliconius* butterfly species that hybridise occasionally in the wild had mitochondrial DNA sequences that were at most 2–6% divergent, whereas no hybrids are found between species with >10% divergence (Mallet, McMillan, & Jiggins, 1998). We therefore did not expect that *L. osakensis* would hybridise with *L. nigrinus*.

Even if *L. osakensis* and *L. nigrinus* are unable to hybridise and produce viable progeny, it is possible that attempts at interbreeding could lead to substantial reduction in the population growth rate via incompatible interspecific crosses. For example, different species may recognise one another as mates and attempt copulation (Arnqvist, 1998), but if there is a large difference in genital morphology between species, interspecific copulation can cause genital injuries, which in turn can result in mortality of the copulating individuals and a reduction in the fitness of the population (Kubota & Sota, 1998). Differences in the male genitalia of *L. osakensis* and the two North American species have been observed (Montgomery et al., 2011).

In addition, reception of interspecific male seminal products could fail to stimulate the female, resulting in a decrease in oviposition and/or a decrease in the desire to remate (Eberhard, 1996). Males of some insect species have sperm removal/displacement devices (Ono, Siva-Jothy, & Kato, 1989; Waage, 1986) and/or sperm plugs (Aiken, 1992), which could result in the removal or denial of viable sperm from intraspecific mating before or following interspecific mating (Eberhard, 1996). Finally, infertile eggs could result from developmental failures in embryos between species that have diverged sufficiently (Eberhard, 1996). Any of these traits could result in lower reproductive output and, therefore, have an impact on biological control efforts.

The purpose of this study was to determine if *L. osakensis* could mate with *L. nigrinus*, and if so, whether mating produces hybrid progeny and/or interferes with reproductive output. Fitness components such as fecundity, fertility, viability, and mate choice were compared between intraspecific and interspecific mating pairs.

2. Materials and methods

2.1. *Fitness*

No-choice lab mating experiments were conducted in 2010 and 2011 to determine whether *L. osakensis* could mate with *L. nigrinus* and produce viable eggs. The assessment was based on measurements of three fitness components: fecundity (the number of eggs produced per cross), fertility (the number of prepupae produced per cross), and viability (the number of adults produced per cross). Treatments (hereafter: crosses) consisted of pairing *L. osakensis* with *L. osakensis* (Lo × Lo), *L. nigrinus* with *L. nigrinus* (Ln × Ln), and *L. nigrinus* with *L. osakensis* (Ln × Lo).

All beetles used in the experiment were lab-reared [for specifics on rearing methods, see (Salom, Kok, Lamb, & Jubb, 2012)], F_1, putatively virgin adults. Gender was not determined prior to pairing of adults because removing pupae from the soil and sexing them before adult emergence (Zilahi-Balogh, Humble, Kok, & Salom, 2006) results in high mortality (Salom, Kok, Lamb, Dellinger, & Story, 2009), and squeezing the abdomen of adults so that the genitalia are extruded (Shepherd, Montgomery, Sullivan, & Mayfield, 2014) could damage the reproductive system and/or sexual organs. There was a chance that beetles could mate intraspecifically in their rearing containers in the short time between emergence and before being collected for experiments

(collections were made every 12 h, the most frequent collections possible within the rearing system in place), and that paired adults would not all be ♂♀.

Following emergence in Fall 2009, each beetle was placed in a separate Petri dish with HWA-infested branches. The number of potential mating pairs for each cross was based on the availability of adult beetles. Because of low emergence of *L. osakensis* at the Virginia Tech Insectary in fall 2009, 23 Lo × Lo, 31 Ln × Ln, and 54 Ln × Lo crosses were made. Beetles were paired in December 2009 and egg production was followed into spring 2010. In fall 2010, increased emergence of *L. osakensis* permitted pairing of 50 Lo × Lo, 52 Ln × Ln, and 100 Ln × Lo. Beetles were again placed in individual Petri dishes with HWA-infested branches following emergence; then, pairings were made in December 2010 with data collection proceeding into spring 2011.

Individual and paired beetles were kept in 50 × 9 mm polystyrene Petri dishes with ventilation holes cut into the top and covered with polyester mesh. The dishes were filled with HWA-infested hemlock branches and kept in a growth chamber under the conditions shown in Table 1. Beetles were fed once each week and placed in clean dishes. If one of the paired beetles in a dish died, both individuals were removed from the study and placed in separate microcentrifuge tubes with 95–100% ethanol.

2.1.1. *Fecundity*

Eggs on the branches in each of the Petri dishes were counted weekly to measure fecundity. Because gender was unknown at the outset of the study, individuals were sexed at the conclusion of the study to determine the make-up of pairs in each of the Petri dishes. The pairings were found to consist of ♂♀, ♀♀, or ♂♂ individuals. Pairs containing two males were eliminated from the study. Additional pairs were eliminated because of death or accidental loss of one or both adults in the pair, the presence of *L. naganoensis* which was accidently brought into the USA from Japan (see Fischer et al., 2014) or the presence of *L. rubidus*. As a result, 5 Lo × Lo, 11

Table 1. Temperature and photoperiod used in the 2010 and 2011 no-choice mating studies using *Laricobius* spp. (Salom et al., 2012).

	Adults	
	Temp°C (D/N)	Photoperiod (L : D) h
October	6/4	10.5 : 13.5
December	4/3	9.5 : 14.5
January	4/3	10 : 14
February	4/3	11 : 13
1 March	6/4	12 : 12
15 March	8/5	12 : 12
1 April	10/6	12 : 12
1 May	10/8	12 : 12
	Larvae/Prepupae	
March	13/13	12 : 12
May	19/19	12 : 12
October	13/13	12 : 12

Ln × Ln, and 8 Ln × Lo pairs were available for analysis in 2010, and 22 Lo × Lo, 18 Ln × Ln, and 24 Ln × Lo pairs in 2011. The dishes from each of the crosses for the two years (2010 and 2011) that were not eliminated were combined for analysis.

The number of eggs produced by ♂♀ and ♀♀ pairs in 2011 was compared among the three crosses because in 2010, a large number of eggs were observed in the dishes of Ln × Ln and Ln × Lo crosses containing two females (367 eggs from 20 ♀♀ pairs) compared with the number of eggs found within the dishes containing two males (11 eggs from 11 ♂♂ pairs). No comparison was made for the 2010 data because there was only one ♀♀ Lo × Lo pair, which died during the second week of the experiment without producing eggs.

2.1.2. *Fertility and viability*

To assess fertility, eggs were collectively placed in rearing funnels by cross (cross = Lo × Lo, Ln × Ln, and Ln × Lo). Approximately 250 and 150 eggs were placed in each funnel in 2010 and 2011, respectively. Funnels were checked daily and all prepupae produced were recorded and then placed in soil containers (50 per container) by cross to rear to adulthood. Viability was determined by counting all adults that emerged from the soil containers the following fall. The dates of emergence were recorded and the adults were placed in individual microcentrifuge tubes with ethanol.

2.1.3. *Sexing and identification*

All adults used in the no-choice lab mating experiment, and all adult progeny that emerged from soil containers were dissected following experimentation. The genitalia were extracted and mounted on slides and the heads and elytra were saved as vouchers and deposited at the Virginia Tech Insect Museum or the Yale Peabody Museum of Natural History. DNA was extracted from the thoraces, and the identity of beetles was determined using genetic analyses (described below).

2.2. *Fluorescence study*

In 2011 and 2012, fluorescent dye was used to determine whether eggs produced by crosses between *L. nigrinus* and *L. osakensis* had been fertilised. In 2011, a single egg was chosen from each of four Ln × Lo and four Ln × Ln mating pairs (eight eggs total). Additionally, five eggs were chosen from five *L. osakensis* virgins and three eggs from three *L. nigrinus* virgins. Virgins were female beetles that had been kept individually in dishes from the time of emergence. Eggs from virgin beetles were used as sterile (unfertilised) controls. In 2012, 11, 10, and 11 eggs were collected from Lo × Lo, Ln × Ln, and Ln × Lo pairs, respectively. All eggs used in this experiment were approximately one-week old.

Eggs were placed individually onto a slide and 5 µl of Prolong Gold with DAPI (4′, 6-Diamidino-2-Phenylindole, Dihydrochloride) was dropped onto the egg. DAPI fluoresces in contact with DNA (nuclei). A coverslip was placed onto the egg/dye and pressed to break open the egg and release its contents. The slide was then placed in the dark to incubate for 20 min, after which the egg was viewed and photographed using a Zeiss Axiovert 200 confocal microscope. We hypothesised

that unlike sterile eggs, fertilised eggs would exhibit fluorescing nuclei from either sperm or the dividing cells of the growing zygote.

2.3. *Mate choice*

A mate choice experiment was conducted to determine whether encounters between *L. nigrinus* and *L. osakensis* would lower their fitness via a decrease in the production of progeny as a result of inviable interspecific mating. In March 2011, ≈120 *L. nigrinus* were placed together and ≈120 *L. osakensis* were placed together in separate containers with HWA-infested branches to allow the beetles to mate. A week later, beetles were individually placed in 50 × 9 mm polystyrene Petri dishes containing HWA-infested branches with ventilation holes cut into the top and covered with polyester mesh. The following week, the dishes were checked for eggs in order to distinguish males from females. The elytra of *L. osakensis* and *L. nigrinus* were then marked using non-toxic markers (Opaque Stix water-based pigmented marker) so that the two species could be distinguished from one another, and so that differential mate choice was not affected by the presence or absence of marker. The beetles were then separated into groups of four. Eight groups contained four *L. osakensis* (two ♂ and two ♀), eight contained four *L. nigrinus* (two ♂ and two ♀), and 16 groups contained two *L. nigrinus* (♂ and ♀) and two *L. osakensis* (♂ and ♀). Four days later, each group was placed in a separate 50.8 cm × 76.2 cm sleeve cage enclosing HWA-infested hemlock branches in Blacksburg, VA. The beetles remained in these cages for a week, after which the branches were cut and brought back to the lab. The adults were collected and placed in ethanol for dissection to confirm sex and for genetic analysis to confirm species. The number of HWA ovisacs on each branch was counted and the branches were placed in separate funnels with ≈25 additional HWA-infested hemlock branches to rear progeny to the prepupal stage. The progeny were collected, counted, and placed in ethanol for genetic analysis.

2.4. *Mate change*

The purpose of this experiment was to determine whether interspecific mating before intraspecific mating negatively affects the genitalia and/or the production of eggs. Sixty F_1 *L. osakensis* were marked with red and 60 F_1 *L. nigrinus* were marked with blue (Sharpie® water-based poster paint marker) in January 2012. The beetles had been kept in individual 50 × 9 mm polystyrene Petri dishes containing HWA-infested branches with ventilation holes cut into the top and covered with polyester mesh since the time of emergence. Two days later, 42 *L. nigrinus* were paired with 42 *L. osakensis*. Sixteen Ln × Ln and 20 Lo × Lo pairs were used as controls. The paired beetles were also kept in 50 × 9 mm Petri dishes with foliage infested with HWA adults in a growth chamber at the temperatures and photoperiods shown in Table 1. In March 2012, the interspecific pairs (Ln × Lo) were randomly re-mated, intraspecifically, as were the controls (Lo × Lo and Ln × Ln). Each week thereafter, the number of eggs oviposited per mating pair was counted.

All adults used in this experiment were dissected to confirm sex and species and were kept as voucher specimens. The genitalia were removed and mounted on slides to determine whether there was damage. The numbers of eggs produced from the pairs that were mated intra-intraspecifically were compared with the pairs that were mated inter-intraspecifically.

2.5. *Genetic analysis*

All adults used in our studies were identified to species using the partial cytochrome oxidase subunit I (COI) gene (Davis et al., 2011). Adults were identified to species because the *L. osakensis* and *L. nigrinus* colonies are reared in the same facility; therefore, it is possible that accidental mixing of species could occur. Additionally, because field-collected branches with HWA are used as food, it is possible that wild *L. rubidus* may be present within the colonies.

DNA was extracted using the DNAeasy kit (Qiagen Inc., Valencia, CA). Partial COI was amplified using primers LepF1 and LepR1 (Hebert, Penton, Burns, Janzen, & Hallwachs, 2004). PCR was performed in 30 µl reactions containing 3.0 µl 10× PCR Buffer, 2.4 µl dNTPs (10 mM), 4.8 µl $MgCl_2$ (25 mM), 1.0 µl BSA (10 mg/ml), 1.0 µl of each primer (10 mM), 0.3 µl Taq DNA polymerase (New England Biolabs, Ipswich, MA), and 1.0 µl DNA template. Thermocycling conditions were 95°C for 5 min followed by 35 cycles of 45 s at 95°C, 45 s at 48°C, and 1 min at 72°C, with a final extension of 72°C for 5 min. PCR products were purified using the QIAquick PCR purification kit (Qiagen Inc., Valencia, CA). Sequencing reactions were performed using the BigDye Terminator kit (Applied BioSystems, Foster City, CA) and analysed on an Applied BioSystems 3730xl automated sequencer. Sequences were aligned using the SeqMan Pro program of LASERGENE 8.0 software (DNASTAR; http://www.dnastar.com).

All progeny from the no-choice lab mating, mate choice, and mate change experiments were genetically identified using the partial *wingless* gene (Montgomery et al., 2011). This nuclear gene was used in the place of the mitochondrial COI gene for progeny because it would be able to detect F_1 hybrids, while COI only determines the maternal identity. Partial *wingless* was amplified using the primers Wg578 and WgAbR (Wild & Maddison, 2008). PCR was performed in 30 µl reactions containing 3.0 µl 5× PCR Buffer, 2.4 µl dNTPs (10 mM), 2.4 µl $MgCl_2$ (25 mM), 1.0 µl BSA (10 mg/ml), 1.0 µl of each primer (10 mM), 0.3 µl Taq DNA polymerase (New England Biolabs, Ipswich, MA), and 1.0 µl DNA template. Thermocycling conditions were 95°C for 5 min followed by 35 cycles of 45 s at 95°C, 45 s at 54°C, and 1 min at 72°C, with a final extension of 72°C for 5 min. Sequencing reactions were performed using the BigDye Terminator kit (Applied Biosystems, Foster City, CA) and analysed on an Applied BioSystems 3730xl automated sequencer (Applied Biosystems). Sequences were edited using Sequencer 4.2.2 (Gene Codes Corporation, Ann Arbor, MI) and aligned using MUSCLE 3.6 (Edgar, 2004).

2.6. *Statistical analysis*

2.6.1. *Fitness: fecundity*

The mean number of eggs produced per week was calculated for each of the beetle pairs that survived to the end of the observation period [hereafter: total weekly mean (13 weeks in 2010 and 19 weeks in 2011)]. The data (mean eggs/week) for the two years were then combined, tested for normality, and analysed using a one-way analysis of variance (ANOVA) followed by multiple comparison with Fisher's LSD (Zar, 2010) to test for significant differences in overall fecundity among the three crosses (Lo × Lo, Ln × Ln, Ln × Lo). JMP Pro 10 (SAS Institute, 1989–2007) and a significance level of $\alpha = 0.05$ were used for all analyses, unless otherwise stated.

The temporal patterns of oviposition for the three crosses (Lo × Lo, Ln × Ln, Ln × Lo) were examined using only the 2011 data because three weeks of data were missing for March 2010 at the time of peak oviposition. The mean number of eggs per pair (Petri dish) was calculated and plotted against time (19 weeks). Differences in the patterns of oviposition were assessed by fitting the relationship of percent cumulative mean egg production at each week to a Weibull function (Dodson, 2006; Wagner, Wu, Sharpe, & Coulson, 1984).

$$f(x) = 100(1 - e^{-(x/\alpha)^{\beta}}), \tag{1}$$

where $f(x)$ is the percent cumulative mean number of eggs at each week (x), α is a rate parameter, which represents the time at which 63.2% of cumulative mean egg production occurred (Dodson, 2006), and β describes the shape of the curve. The fit of the data to the Weibull function was carried out by nonlinear least squares regression in TableCurve 5.01 (SYSTAT Software, 2002).

Chi-square analyses were used to compare the total weekly mean number of eggs per dish in 2011 for ♂♀ and ♀♀ pairs among the three crosses (i.e., Lo × Lo, Ln × Ln, and Ln × Lo).

2.6.2. *Fitness: fertility and viability*

Chi-square analysis was used to determine if there were significant differences in the percent development of prepupae from eggs, and adults from prepupae among three crosses.

2.6.3. *Mate choice and mate change*

The ratio of the number of larvae per sleeve cage to the number of HWA ovisacs per branch was calculated, and an ANOVA was used to determine if this ratio differed among the three crosses (Lo × Lo, Ln × Ln, and Ln × Lo). The ratio of prepupae to ovisacs was used as the response variable because there is a positive numeric response to prey abundance in *Laricobius* spp. (Vieira., Salom, & Kok, 2012).

Chi-square analysis was used to compare the total weekly mean number of eggs per dish between remated intra-intraspecific pairs and re-mated inter-intraspecific pairs.

3. Results

Laricobius osakensis and *L. nigrinus* were observed attempting to mate with each other immediately following pairing. Neither species appeared to have a species preference when choosing a mate. Not only did different species of *Laricobius* attempt to mate, but males also attempted to mount and copulate with other males.

In 2010, eggs were found in the dishes of mating pairs of Ln × Lo as early as the third week after pairing. In 2011, oviposition began in the first week of observation (28 December 2010 to 4 January 2011).

3.1. *Fitness*

3.1.1. *Fecundity*

There was a significant difference among all three crosses in the mean number of eggs produced per week ($F = 24.63$; df = 2, 89; $p < .0001$). Lo × Lo pairs produced the highest mean (±SE) number of eggs per week with 7.62 (±0.54), followed by Ln × Ln pairs with 5.03 (±0.51). The interspecific cross, Ln × Lo, produced the fewest mean (±SE) number of eggs per week with 2.48 (±0.49). The pattern of egg production over time in 2011 is shown in Figure 1(a).

Maximum egg production for all three crosses occurred between observation weeks 13 and 15 and was greater for Ln × Ln than Lo × Lo. The overall mean egg production per week was significantly greater for Lo × Lo than Ln × Ln or Ln × Lo (Figure 1(a)). The fit of the data to the Weibull function showed that egg production by Lo × Lo occurred at a significantly greater rate than for Ln × Ln and Ln × Lo pairs (Figure 1(b)). Based on the non-overlapping 95% confidence intervals, Lo × Lo pairs produced 63.2% of the cumulative mean number of eggs significantly

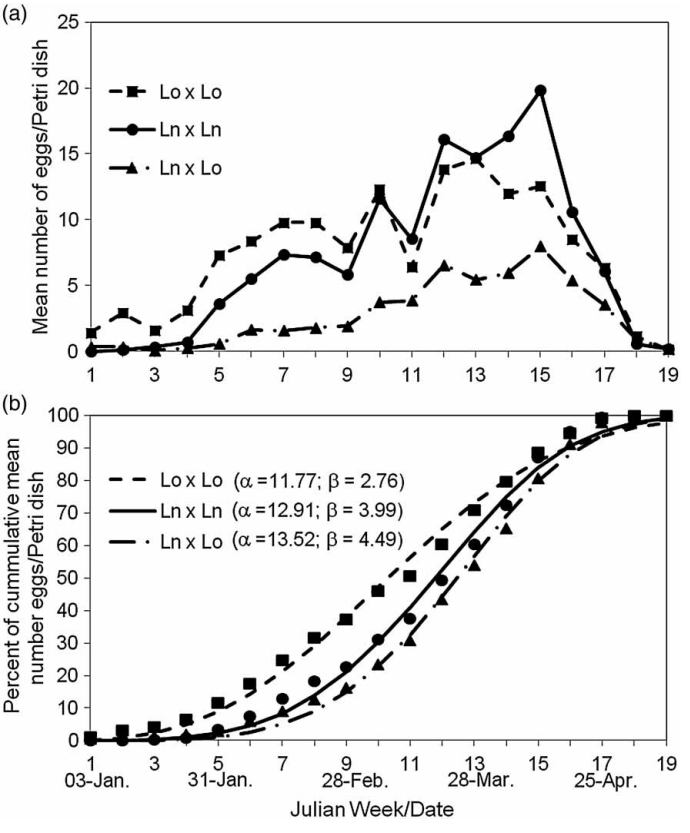

Figure 1. Oviposition patterns in a no-choice lab mating study for three crosses of *Laricobius* spp. during 19 weeks of observation in 2011. (a) Mean number of eggs per dish (per pair) over time; (b) relationship between percent cumulative mean number of eggs per dish and week (symbols) and fit of the data to the Weibull function (lines). Lo is *Laricobius osakensis*; Ln is *L. nigrinus*.

earlier ($\alpha = 11.77$; 95% CI = 11.47–12.07 weeks) than Ln × Ln pairs ($\alpha = 12.91$; 95% CI = 12.67–13.15 weeks) and Ln × Lo pairs ($\alpha = 13.52$; 95% CI = 13.33–13.72 weeks).

Comparison of the number of eggs produced by ♂♀ and ♀♀ pairs showed that there was a significant difference in the total weekly mean number of eggs produced per dish in 2011 ($\chi^2 = 30.995$; df = 2; $p < .0001$). The Lo × Lo and Ln × Ln ♂♀ pairs produced a greater percentage of eggs than their ♀♀ counterparts (56.50: 43.50 and 72.80: 27.20, respectively), while the opposite was true for Ln × Lo pairs (40.75: 59.25) (Figure 2).

3.1.2. *Fertility and Viability*

In both 2010 and 2011, prepupae began dropping from rearing funnels in March. Prepupae continued to drop through June for the Lo × Lo and Ln × Ln crosses in 2010 and 2011, but stopped at the end of April for the Ln × Lo cross in 2010 and at the end of May in 2011. In 2011, two prepupae that developed from the Ln × Lo cross were placed in ethanol for genetic analysis and were determined to be pure *L. osakensis.*

There was a significant difference among the three crosses in the percent of prepupae that developed from eggs ($\chi^2 = 2685.592$, df = 2, $p < .0001$; Figure 3). A significantly greater percent of prepupae developed from Lo × Lo and Ln × Ln crosses than from Ln × Lo (41.85, 47.62, and 3.09, respectively). The analysis also showed that a significantly greater percent of Ln × Ln eggs developed to the prepupal stage compared with Lo × Lo eggs ($\chi^2 = 92.138$, df = 1, $p < .0001$; Figure 3).

In 2010, adult emergence began with one adult beetle from the Ln × Lo cross on 13 September. Emergence of adults continued through January 2011. In 2011, emergence began with two adults from the Ln × Ln cross and one adult from the Lo × Lo cross on 24 October. Emergence of adults continued through December 2011.

There was a significant difference among the three crosses in the percent of prepupae that developed to the adult stage ($\chi^2 = 8.340$, df = 2, $p = .0155$; Figure 3). The percent of prepupae that developed was significantly greater for the Lo × Lo and Ln × Ln crosses than for the Ln × Lo cross (24.95, 26.82, 15.79, respectively). There

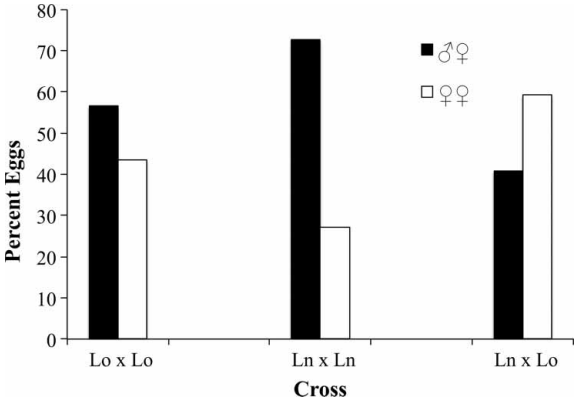

Figure 2. Results of contingency analysis showing the percent of the total weekly mean number of eggs produced per Petri dish between ♂♀ and ♀♀ pairs of the following three crosses: *L. osakensis* × *L. osakensis* (Lo × Lo), *L. nigrinus* × *L. nigrinus* (Ln × Ln), and *L. nigrinus* × *L. osakensis* (Ln × Lo).

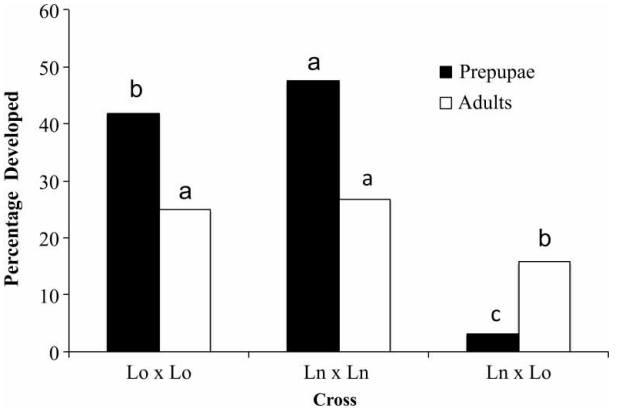

Figure 3. Percent development of prepupae from eggs and adults from prepupae among three crosses: *L. osakensis* × *L. osakensis* (Lo × Lo), *L. nigrinus* × *L. nigrinus* (Ln × Ln), and *L. nigrinus* × *L. osakensis* (Ln × Lo). Letters that are not the same denote significant differences within each life stage ($p < .05$). None of the prepupae or adults that developed from the Ln × Lo cross were found to be hybrids following genetic analysis.

was no significant difference in the percent of prepupae that developed to the adult stage between Lo × Lo and Ln × Ln crosses ($\chi^2 = 1.853$, df = 1, $p = .1734$).

Fifteen of the 16 adult beetles that emerged from the Ln × Lo cross were genetically analysed; eight were found to be pure *L. osakensis* and seven pure *L. nigrinus*. No hybrids were found.

3.2. *Fluorescence study*

Nine of 11 Lo × Lo eggs collected were from ♂♀ pairs with three of the eggs taken from the same dish. All of the nine eggs fluoresced nuclei; the remaining two eggs, which were from ♀♀ pairs, did not fluoresce nuclei. Eleven of the 14 Ln × Ln eggs were collected from ♂♀ pairs, with three of the eggs from the same dish. All of the 11 eggs fluoresced nuclei (e.g. Figure 4). The remaining three of the 14 eggs were from ♀♀ pairs; one fluoresced nuclei and the other two did not.

Of the 15 Ln × Lo eggs, two adults from two separate dishes could not be identified; therefore, three eggs, two from one dish and another from a separate dish, were dropped from the analysis. Four eggs were from ♂♀ pairs, two of which were from the same dish and eight eggs were from ♀♀ pairs. None of these eggs fluoresced nuclei e.g. Figure 4). None of the virgin eggs from *L. nigrinus* (three eggs) or *L. osakensis* (five eggs) fluoresced nuclei.

3.3. *Mate choice and mate change*

For the mate choice test, no significant difference was found in the ratio of prepupae to ovisacs between the intraspecific and interspecific crosses ($F = 0.9325$, df = 2, $p = .4051$). A sample of 118 prepupae from the Ln × Lo cross were identified using genetic analysis and none were found to be hybrids. Forty-three prepupae were identified as *L. nigrinus* and 75 were identified as *L. osakensis*.

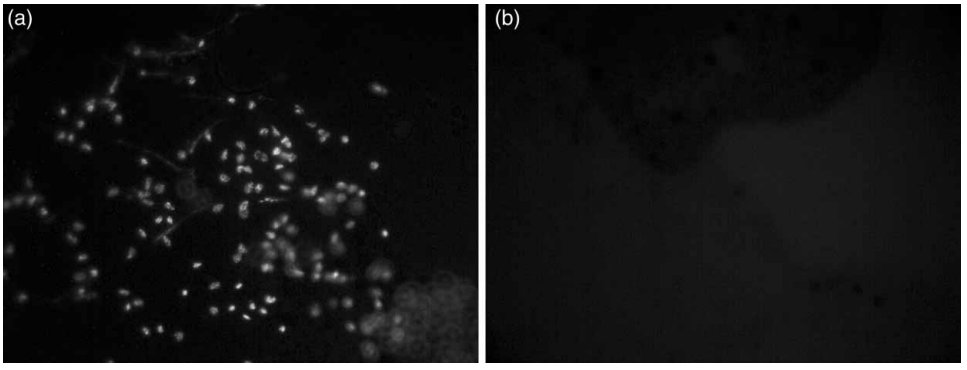

Figure 4. Content of an egg produced by (a) a mating pair of *L. nigrinus* and (b) a mating pair of *L. nigrinus* and *L. osakensis*. Eggs were stained with Prolong Gold with DAPI (4′, 6-Diamidino-2-Phenylindole, Dihydrochloride) to fluoresce nuclei.

By the end of the mate change experiment, data from six Lo × Lo (intra-intraspecific), four Ln × Ln (intra-intraspecific), four Lo × Lo (inter-intraspecific), and three Ln × Ln (inter-intraspecific) replicates were used for statistical analysis due to mortality. Three weeks (from 28 March to 18 April) of egg count data were available for analysis for the same reason. There was a significant difference between the intra-intraspecific and the inter-intraspecific remated crosses ($\chi^2 = 4.570$, df = 1, $p = .0325$) in the total weekly mean number of eggs per dish. The Lo × Lo inter-intraspecific re-mated cross produced a greater proportion of eggs than the Lo × Lo intra-intraspecific cross (64.06: 35.94). The Ln × Ln crosses showed the opposite trend, with the intra-intraspecific cross producing a much greater proportion of eggs than the inter-intraspecific cross (87.1: 12.9). There was no evidence of broken or torn genitalia in the male specimens that had been remated. Damage was observed in the female samples, but it was not possible to determine whether this occurred during dissection, rather than as a result of mating.

4. Discussion

4.1. *Fitness*

The interspecific ♂♀ Ln × Lo cross produced significantly fewer eggs, prepupae, and adults compared with the intraspecific ♂♀ crosses. It is likely that the eggs produced by interspecific ♂♀ mating pairs were not fertilised, but were the result of contamination. Contamination could have occurred through the collection of HWA-infested branches containing *L. rubidus* and/or via intraspecific mating in soil containers prior to collection for experimentation. It is also possible that these eggs were sterile and produced by females. This can be deduced because interspecific ♀♀ pairs produced more eggs than interspecific ♂♀ pairs, while the opposite was true for the intraspecific pairs (Figure 2). Females of some insect species that produce sterile eggs have a reduced oviposition rate until after they have copulated (Ridley, 1988). Once copulated, products from male accessory glands and testes can act as allohormones, increasing female oviposition rates (Brent, Fasnacht, & Judd, 2011). Therefore, intraspecific ♂♀ pairs would produce more eggs than the ♀♀ pairs because the ♂♀ mating

pairs are producing fertile eggs. More eggs would be produced by interspecific ♀♀ pairs than the ♂♀ interspecific pairs because there are two females producing sterile eggs, instead of one.

None of the prepupae or adults produced by the interspecific cross were found to be hybrids. This raises the question: why were non-hybrid prepupae and adults found in the Ln × Lo crosses? Initially, it was thought that these prepupae were wild *L. rubidus* that had been brought in on HWA-infested branches, but none of the progeny was identified as *L. rubidus*. In 2010, there were two accidental intraspecific crosses among the interspecific pairs. It is possible that the prepupae that developed from the Ln × Lo cross in 2010 were from the accidental intraspecific crosses. However, in 2011, a greater number of prepupae developed from the Ln × Lo cross and there were no accidental intraspecific crosses found that year. For this reason, we suspect that *Laricobius* spp. mate as soon as they make contact with one another following emergence from the soil. On one occasion, two *L. nigrinus* were observed mating in a soil container directly following emergence. Although these two beetles were not used in our mating experiments, other pre-mated adults may have inadvertently been included. Because *Laricobius* spp. females can store sperm following copulation, the offspring that resulted from the interspecific cross may be from intraspecific mating that occurred in the soil containers before being used in experiments. If this is the case, it suggests that *Laricobius* spp. females can hold sperm in their spermatheca for at least two months, since the adult beetles had been collected in fall 2010. While intraspecific mating in the soil containers prior to their collection complicated our experiments, there was no procedural way to ensure that all newly emerged adults were virgins *a priori* to the studies.

4.2. *Fluorescence*

The lack of stained nuclei in the virgin eggs and the presence of nuclei fluorescing in mated *Laricobius* spp. eggs suggest that these nuclei are from the dividing cells of zygotes. We found that all 11 eggs from Ln × Ln and all nine eggs from Lo × Lo fluoresced nuclei, while the four eggs from Ln × Lo did not. There was one ♀♀ Ln × Ln egg that fluoresced nuclei. It is likely that this egg was either from *L. rubidus* brought in on infested hemlock branches used for food, or a fertilised egg that occurred as a result of mating inside of the soil containers. These data provide further evidence that the eggs found in the Ln × Lo dishes were sterile and not fertilised.

4.3. *Mate choice and mate change*

The results of the mate choice test suggest that there is no fitness cost in the form of a lower production of progeny when *L. osakensis* and *L. nigrinus* occur together. However, there were a number of complications with this experiment specific to this study system.

For this experiment, it was necessary to determine which beetles were male and female prior to placement in the sleeve cages due to the low probability of choosing two males and females correctly. Because the sex of the adults could not be determined *a priori* without causing harm to their reproductive organs, the beetles were mated before the experiment and separated into individual dishes and then checked for eggs to determine which were males and females. This probably could have been done without pre-mating the beetles, since *Laricobius* spp. females produce sterile eggs, but this information was not confirmed at the time. Since females can most

likely store sperm for two months, three weeks was probably not long enough for females to expend all of sperm in the spermatheca from previous intraspecific mating. Therefore, the progeny from interspecific crosses could have come from intraspecific mating that took place prior to the experiment. However, given the fact that the two *Laricobius* spp. do not appear to exhibit mate choice preference, it is likely that both intra- and interspecific copulations took place in the sleeve cages. For this reason, this experiment suggests that no fitness costs are likely from interspecific copulation due to some form of sperm replacement (e.g. sperm from a different *Laricobius* spp. replacing sperm from a prior intraspecific encounter) or through damage to the reproductive organs. Conversely, the mate change experiment showed that the mean number of eggs produced by the inter-intraspecific Ln × Ln cross was relatively low compared with the intra-intraspecific Ln × Ln cross (87.1: 12.9). This may suggest a fitness cost to *L. nigrinus* following copulation with *L. osakensis*. The sample size for the mate change experiment was much lower than that of the mate choice experiment; therefore, it is likely that the results from the mate choice experiment are more representative of potential fitness costs due to interspecific mating between these two species. Regardless, the inconsistency in the results of the two experiments suggests that fitness costs between these two species may require further study.

In summary, we found no evidence that *L. osakensis* and *L. nigrinus* can hybridise. This is consistent with genetic evidence suggesting that *L. osakensis* is in a divergent clade with other Asian *Laricobius* spp. and is not closely related to either *L. nigrinus* or *L. rubidus* (Montgomery et al., 2011). Whether there are fitness costs when *L. osakensis* and *L. nigrinus* mate interspecifically is not completely clear and may need to be investigated further. Given that *L. osakensis* was found to produce eggs earlier in the season and at a higher rate than *L. nigrinus*, its success as a biological control agent may be superior to that of *L. nigrinus*.

Acknowledgements

The authors would like to thank Gina Davis, Peter Thomasson, Jacquelyne Brown, Ben Aigner, Christine Cho, Carrie Jubb, Meredith Schrader, McKinzie Mann, Linda Manden, Nick Brehm, Natalie Morris, Kara Tourje, Chandler Barton, Ryan Mays, and Tom McAvoy for their help at the Virginia Tech Insectary and Quarantine Lab, and Joanne Klein, Brenna Traver, Grace Mulholland, Jacquelyn Martin and Stephanie Vece for their help in the Molecular Lab.

Disclosure statement

No potential conflict of interest was reported by the authors.

Funding

This work was supported by USDA Forest Service Cooperative Agreements 07-CA-11420004–161 and 11-CA-11420004–133.

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