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Biological Control - Parasitoids and Predators

Temperature-dependent Development, Survival, and Oviposition of *Laricobius osakensis* (Coleoptera: Derodontidae): A Specialist Predator of *Adelges tsugae* (Hemiptera: Adelgidae)

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Abstract

A predator, *Laricobius osakensis* Montgomery and Shiyake (Coleoptera: Derodontidae), is being mass-produced and released for the biological control of the invasive hemlock woolly adelgid (HWA), *Adelges tsugae* Annand (Hemiptera: Adelgidae). To better understand and predict the seasonality of this predator in North America, the development and reproduction of *L. osakensis* were evaluated at constant temperatures ranging from 5 to 22°C. The predicted seasonal biology was compared with data from field collections. *L. osakensis* did not complete development from egg to adult at the two lowest temperatures tested, 5 and 8°C, but did so at the highest temperature of 22°C. The minimum development thresholds were estimated for eggs (4.2°C), first (1.8°C), second (5.5°C), third (4.6°C), and fourth instar (4.1°C), prepupa (3.6°C), and pupa (7.5°C). Oviposition rates were significantly greater at 5 and 10°C than at 20 and 25°C. Head capsule width significantly increased for each of the four larval instars with a mean of 0.19, 0.26, 0.35, and 0.44 mm, respectively. Laboratory and field data were used to develop a phenology forecasting model to predict the occurrence of all developmental stages of *L. osakensis*. This model will allow land managers to more accurately predict the optimal timing for *L. osakensis* larval sampling throughout its established range.

Key words: temperature-dependent development, biological control, beneficial insect

Populations of both eastern hemlock, *Tsuga canadensis* L. (Pinales: Pinaceae), and Carolina hemlock, *Tsuga caroliniana* Engelmann (Pinales: Pinaceae) continue to decline 70 years after the first report of the accidental introduction (Souto 1996) of one of North America's most impactful forest pests, the hemlock woolly adelgid (HWA), *Adelges tsugae* Annand (Hemiptera: Adelgidae). These two hemlock species are important components of eastern North American forests. Eastern hemlock occurs in the Appalachian Mountains, north to New Brunswick, Canada, and west to the Great Lakes. Carolina hemlock has a much more limited distribution and grows in isolated locations in the southern Appalachians (Jetton et al. 2008). Eastern hemlock is a foundational species because it is associated with riparian ecosystems where it serves as food and refuge to a large component of fauna and flora (Dilling et al. 2007, Ingwell et al. 2012). The loss of these trees across the landscape, caused by HWA, is having lasting effects on the floral composition, shifting from hemlock dominant or codominant stands to hardwood dominant stands (Ellison et al. 2005, Eschtruth et al. 2006). Moreover, the loss of hemlock has caused adjacent aquatic and terrestrial habitats to increase in temperature and understory vegetative competition with invasive plants (Eschtruth et al. 2006, Siderhurst et al. 2010).

HWA was only considered an occasional ornamental pest of hemlocks for the first 30 years following its discovery in Virginia in 1951 (Souto 1996). However, in the 1980s, HWA populations underwent a dramatic range expansion causing observable decline of hemlocks on a landscape level (Ellison et al. 2005, Eschtruth et al. 2006, Siderhurst et al. 2010). With the exception of first instar crawlers and sexuparae, HWA is sessile. Primary dispersal of HWA occurs during the egg and crawler stage via wind or as phoronts on wildlife, including birds and arthropods (McClure 1990, Russo et al. 2016). HWA is currently established in at least 22 states in the United States and in a few eastern Canadian provinces (Virginia Tech 2021). As climate changes in the coming decades, HWA is projected to continue its northward dispersal into remaining un-infested areas of eastern hemlock (McAvoy et al. 2017, Kantola et al. 2019).

The HWA's bivoltine lifecycle comprises the sistens (overwintering) and the progrediens (spring) generations. The overwintering generation occurs from June to late March and the spring generation from late March to June, depending on latitude (McClure 1989, 1996; Gray and Salom 1996; Joseph et al. 2011). In Japan, HWA requires a primary host (tiger-tail spruce, *Picea torano* Voss. (Siebold ex K. Koch) (Pinales: Pinaceae)), and a secondary hemlock host (*Tsuga* spp.), to sustain alternating sexual and asexual generations (Havill et al. 2011). However, in HWA's new eastern North America range, tiger-tail spruce is absent, resulting in a strict anholocyclic life cycle, with both generations occurring on the secondary hosts, eastern and Carolina hemlocks (McClure 1989, Gray and Salom 1996).

Efforts to control HWA, using a classical biological control approach, began in the early 1990s. Of the eight insect agents evaluated and approved for release, *Laricobius* spp. have received the most attention. During quarantine studies, prior to a request for release, biological evaluations are conducted to determine an agent's host range. These studies are often accompanied with life history, phylogeny, morphometrics, and temperature response studies. The goals of these additional studies are to assemble enough evidence to identify and adequately understand the potential range once a biological control agent is released and to identify any potential negative factors associated with the agent. Morphometric (i.e., larval head capsule width) and temperature response studies supply researchers with important biological information such as the potential for species and larval stage identification and species-specific relationships between temperature and development, including thermal requirements and minimum temperature thresholds. Temperature-dependent studies also aid in optimizing rearing conditions, predicting establishment, potential range, rates of dispersal, and help build phenology models to optimize sampling and pest management efficiency (Frazer and McGregor 1992, Judd et al. 1993, 1994; Régnière 1996, Régnière et al. 2014).

Laricobius nigrinus Fender (Coleoptera: Derodontidae) and *Laricobius osakensis* Montgomery and Shiyake (Coleoptera: Derodontidae) have been mass produced by various governmental and academic agencies over the past 19 and 10 years, respectively (Foley et al. 2021). They are the only two *Laricobius* species released in eastern North America for the control of HWA (Mausel et al. 2010, Fischer et al. 2014). These two predator species have well documented biological traits that have made them among the most promising and impactful classical biological control agents against HWA. The larvae and adults of both species are host-specific and consume the eggs, nymphs, and adults of HWA (Vieira et al. 2011, Zilahi-Balogh et al. 2002). Additionally, *L. nigrinus* readily establishes in and outside of release sites and shows phenological synchrony with its host, HWA (Foley et al. 2019). In early fall *Laricobius* spp. adults emerge from the soil and migrate to the hemlock canopy to reassociate with HWA as they break aestivation (Mausel et al. 2008, Foley et al. 2022). Once in their arboreal habitat and towards the end of fall and the beginning of winter (end of December), *L. osakensis* begins oviposition which lasts until mid-spring (middle of May) (Vieira et al. 2013).

In controlled constant temperature studies, the relationship between insect fecundity, survival, and development rate in relation to temperature is usually nonlinear when examined over the full range of temperatures to which the species may be exposed and many models have been used to describe this (Chuine and Régnière, 2017). Extrapolations of temperature data from weather stations allow application of predictive models over large areas and provide valuable information for pest control professionals.

The goals of this study were to model the development and survival of immature life stages and to determine adult reproduction rates as functions of temperature for *L. osakensis*. Comparisons are then made with the development of *L. nigrinus* and *Laricobius kangdingensis* Zilahi- Balogh and Jelínek (Coleoptera: Derodontidae). Additionally, the head capsule width of each instar of *L. osakensis* was determined. The data from this study will provide valuable information to HWA managers interested in determining when to sample for specific life stages of *L. osakensis*.

Material and Methods

Effect of Temperature on Immature Development and Survival

In 2018 and 2019, the larvae used for this experiment were the F3 and F4 progeny of *L. osakensis* adults collected in Osaka, Japan in 2015. Prior to oviposition, adults were maintained as individual mating pairs in 50 × 9 mm polyester petri dishes (Fisherbrand, Waltham, MA) in an environmental chamber (I-35II, Percival Scientific Inc., Perry, IA), at a temperature reflecting the previous week's mean outdoor temperature at Blacksburg, VA. Once oviposition began, individual eggs (<24 h old) were removed with their respective ovisac, placed inside a petri dish containing moistened filter paper, and randomly assigned to a treatment temperature. The lid of the Petri dish was sealed with Parafilm to reduce moisture loss, and distilled water was added as a fine mist daily. Eggs within each petri dish were inspected daily for hatch, and each successive stadium. Because *L. osakensis* is active during the cooler months (October-to-April), six relatively low constant temperature treatments of 5, 8, 11, 15, 19, and 22°C were used, with a photoperiod of 12:12 (L:D) h. HWA used as food for the developing larvae were obtained from field sites near Blacksburg, VA. Each time food was brought in from the field, it was inspected for the presence of wild *Laricobius* spp. eggs and/or larvae. Food was replaced within each petri dish every 1–2 d. To ensure a continuous food source for the developing larvae through the duration of this experiment, HWA-infested eastern hemlocks branches were stored at 5°C to slow development (Salom et al. 2002).

A dissecting microscope was used to examine all life stages of *L. osakensis*. Egg hatch was indicated by presence of the empty chorion, and larval molts by exuviae. Once the developing larvae reached the final prepupal stage, ~2 mm of soil (Lamb et al. 2005), was added to each petri dish to simulate entry into the soil. The experiment ended when the adult emerged from the pupa. The number of days that each individual took to complete each life stage was recorded.

Effects of Temperature on Fecundity

This experiment was conducted in 2021 using the F1 progeny of adults collected in Osaka, Japan in 2019. A single mated adult was

placed into an individual 50 × 9 mm polyester petri dish (Fisherbrand) containing moistened filter paper. A hemlock twig with 15–20 HWA ovisacs with adults and eggs was then placed in the petri dish and wrapped in parafilm. The filter paper in the petri dishes was moistened every 3–4 d. The HWA-infested hemlock twig was replaced weekly and the number of *L. osakensis* eggs counted. The five temperature treatments used were; 5, 10, 15, 20, and 25°C at a photoperiod 12:12 (L:D) h, with sample sizes of 6, 9, 9, 12, and 17 adults, respectively. The experiment ended once no eggs were found in a petri dish for two consecutive weeks or when the adult died.

Field Observations

Field collections of *L. osakensis* were made at Hungry Mother State Park (HMSP) (36.8895 °N, –81.5243 °W, 693.5 m elevation), in Marion, VA from mid-December 2020 to late April 2021. During each sampling period, ~10 branches containing >150 HWA ovisacs were clipped, brought back to the laboratory, and examined under a dissection microscope, where the number of individuals of all life stages present for both HWA and *L. osakensis* were recorded.

Phenology Model

Constant temperature observations of development time, survival, longevity, and fecundity were used to calibrate the equations for each life stage using the maximum likelihood methods of Régnière et al. (2012). Because models of varying complexity were compared, we used the corrected Akaike Information Criterion (AICc) to compare likelihoods (Cavanaugh 1997) and choose the best models. The oviposition data collected at HMSP were used to fit a cumulative oviposition curve as a function of accumulated degree-days used as model input. An individual-based model was then created to be run within the BioSIM/11 software (Régnière et al. 2014). BioSIM provides weather-driven process models with location-specific temperature data based on nearby weather stations. Using local thermal gradients ('lapse rates'), it adjusts temperature for differences in latitude, longitude, and elevation between weather stations and simulation point. A georeferenced daily temperature database for all of northeastern North America was assembled from four sources: GHCN-D (https://www.ncei.noaa.gov/products/land-based-station/global-historical-climatology-network-daily, ISD-Lite (https://www.ncei.noaa.gov/products/land-based-station/integrated-surface-database), GSOD (https://www.ncei.noaa.gov/metadata/geoportal/rest/metadata/item/gov.noaa.ncdc%3AC00516/html#), and Environment Canada (http://climate.weather.gc.ca/index_e.html). Inverse distance-weighted averaging from the four weather stations nearest to the HMSP location was used to assemble a daily air temperature time series (Régnière et al. 2014). These stations were Mountain Empire Airport (36°53' N, 81°20' W, 780 m, distance 15.5 km, weight 0.471), Saltville 1N (36° 53' N, 81° 46' W, 528 m, distance 20 km, weight 0.209), Tazewell County Airport (37° 03' N, 81° 4' W, 808 m, distance 31 km, weight 0.164), and Richlands (37° 05' N, 81° 47' W, 582 m, distance 33 km, weight 0.156).

The optimal starting point for modeling *L. osakensis* phenology would be adult emergence from the soil in the fall. However, the timing of emergence, the pre-oviposition period, and the initiation of oviposition are not well understood and were not modeled. Consequently, 1 December was used for the initiation of oviposition and the starting date for degree-day accumulation to predict oviposition. The authors have not observed *L. osakensis* eggs in the field earlier than this date.

Head Capsule Measurements

L. osakensis larvae of a known life stage (L_1, L_2, L_3, or L_4) that died throughout the duration of the 2019 immature larval development study were preserved in 70% EtOH for head capsule measurements. The head capsule of specimens that survived to the adult stage was not measured. Head capsules were measured to the nearest 0.01 mm using a 3.5X-90X Stereo Zoom Microscope.

Data Analysis

Effect of Temperature on Immature Development

Provided here is a brief overview of the methods described in detail in Régnière et al. (2012), as implemented in the BioSIM/11's Curve Calibration component (Saint-Amant 2021a). This method posits that the duration (t, days) of a life stage for individual i is a function of temperature, $t_i = \frac{1}{R(T,\rho)}\varepsilon_i$, where $R(T, \boldsymbol{\rho})$ is development rate (1/duration), T is temperature (°C), and ε_i is an individual's relative development rate, a 'trait' belonging to a log-normal distribution with mean 1 and standard deviation σ_ε. Fast-developing individuals have $\varepsilon < 1$, slow individuals $\varepsilon > 1$. The values of parameter set $\boldsymbol{\rho}$ and the lognormal distribution parameter σ_ε are estimated by maximum likelihood using the development times of all individuals. Because the duration of *L. osakensis* larval stages can be very short (less than the observation period of one day at warm temperature), a temperature response was fit to the entire larval development period (instars 1–4), and a constant proportion of this total duration was attributed to each successive life stage based on our constant temperature observations.

BioSIM/11 offers the possibility of fitting one of 15 different thermal response equations with lower and upper thresholds. Because there were few observations near the lower and upper thermal limits, a regular-increment grid search method was used for those two parameters over a range of realistic values, estimating by maximum likelihood the remaining parameters of the set $\boldsymbol{\rho}$ (a variable number that depends on the equation being tested). The lower threshold was varied from 0 to 4°C, and the upper from 26 to 34°C, in increments of 1°C. The equation and set of estimates yielding the lowest AICc were chosen as the best model separately for eggs, larvae, prepupae, and pupae.

Linear regressions of development on temperature for each life stage (including larval stages separately) were fit with the model $R(T) = a + bT$, where $R(T)$ is average development rate, a and b are intercept and slope, respectively, and T is temperature (°C). The lower development threshold temperature is defined by $T_L = -a/b$, and the cumulative degree-day (C_{DD}) required to complete development above that threshold is given by $C_{DD} = 1/b$.

Effect of Temperature on Survival

Survival responses were also fitted according to the method of Régnière et al. (2012). Given the observed number of survivors N_s out of the initial number of individuals of a given stage N_i at the onset in a given treatment, the expected number of survivors is $\eta_s = N_i \times S(T, \boldsymbol{\rho})$ where $S(T, \boldsymbol{\rho}) = s(T, \boldsymbol{\rho})^t$ and $s(T, \boldsymbol{\rho})$ is daily survival, itself as a function of temperature T with parameter set $\boldsymbol{\rho}$, and t is the average time to complete the life stage. The set of parameters $\boldsymbol{\rho}$ was estimated by maximum likelihood, under the assumption that the number of survivors is Poisson-distributed with mean $N_i \times S(T, \boldsymbol{\rho})$. This function was chosen by minimizing the AICc among a set of 16 equations offered by BioSIM/11 (Saint-Amant 2021b) separately for each of the four life stages (egg, larva, prepupa, and pupa). Regression confidence bands (95%) around survival functions were obtained by the Monte Carlo method (Mazumdar 1995).

Effects of Temperature on Adult Longevity and Fecundity

The method used to analyze immature development was also used for adult aging (1/longevity), with the distinction that adult longevity

does not increase at high temperature and an upper 'aging threshold temperature' does not exist. Nineteen equations were tested with a lower threshold limit varying from 0 to 4°C in steps of 1°C, as was done for the other life stages.

To analyze and model fecundity, the approach of Régnière et al. (2012) was used, as implemented in BioSIM/11. This method assumes that a female oviposits a constant proportion of its remaining eggs each day. This proportion $O(T, \boldsymbol{\rho})$ varies as a function of temperature T and a set of parameters $\boldsymbol{\rho}$. The oviposition rate (F_t, number of eggs per female per day) was computed by dividing the total number of eggs per replicate by the number of days between sampling periods and used as a dependent variable. The total fecundity of individuals (F_i) follows a lognormal distribution with mean F_0 and standard deviation σ_F. The number of eggs laid each day by a female (F_t) at constant temperature was calculated using Equation 1:

$$F_t = F_i e^{-O(T,\rho)(t_t - t_0)} \quad (1)$$

where t_t is the number of days since emergence and t_0 is the preoviposition period (number of days between emergence from the pupa and first oviposition). The set of parameters values $\boldsymbol{\rho}$, F_0, and σ_F was estimated simultaneously by minimum likelihood, assuming the number of eggs laid in a given time interval by individual females is Poisson-distributed.

Phenology Model

Cumulative oviposition determined from the HMSP field egg and larval observations was fitted to a logistic equation, $CEC = [1/(1 + \exp(-(DD - \mu)/\sigma))]$, where μ and σ are parameters to be estimated by least-squared nonlinear regression, and DD is double sine degree-day accumulation (Allen 1976) after December first between lower (T_L) and upper (T_H) threshold values. The limited amount of data available (a single year in a single location) precluded accurate estimation of these thresholds. Instead, the values $T_L = 2.1$°C and $T_H = 20.1$°C, obtained in an earlier study of *L. nigrinus* (J. R. Foley, unpublished data) were used. This degree-day function provided the initial model input (new eggs entering the simulation) as a function of time. The full simulation model was written in C++. All code is open source, and can be found at: https://github.com/RNCan/WeatherBasedSimulationFramework/tree/master/wbsModels/LaricobiusOsakensis.

Head Capsule Width Measurements

Head capsule width data were checked for normality and then a linear model was used to determine differences in head capsule width between instars. Dyar's law (Dyar 1890) implies a geometric progression of head capsule width from one instar to the next. Dyar's ratio was calculated by dividing the head capsule width of one instar by the capsule width of the previous instar.

Results

Effect of Temperature on Immature Development and Survival

The development and survival observations reported in this study are summarized in Table 1. *L. osakensis* completed development from egg to adult at only four of the six constant temperature treatments (11, 15, 19, and 22°C), and average development rate increased significantly with temperature in all life stages (Fig. 1a; Table 2). Excluding the complete larval life stage (L1–4) and egg to adult development, the estimated lower-temperature threshold T_L ranged from 1.8 to 7.7°C, and the cumulative degree-day requirement ranged from $C_{DD} = 32.6$ to $C_{DD} = 147.5$ above T_L (Table 2).

The best nonlinear model to describe the developmental responses to temperature, based on the AICc, was that described by Régnière et al. (2012) (Fig. 1; Table 3):

$$R(T) = \psi \left\{ e^{k(T-T_b)} - \left[\frac{T_m - T}{T_m - T_b} e^{-k[(T-T_b)/\Delta T_b]} \right] - \left[\frac{T - T_b}{T_m - T_b} e^{k(T_m - T_b) - [(T_m - T)/\Delta T_m]} \right] \right\} \quad (2)$$

This development function provided a good description of each life stage's developmental response to temperature, although the behavior near the estimated upper thresholds (T_m) was not well supported by observations. The individual variability in development rates around the expected average value was well described by the lognormal distribution in the egg, larval, and prepupal stages (Fig. 1).

Although the proportion of total larval development time spent in each instar varied slightly with temperature, the average values 0.206, 0.203, 0.227, and 0.364 for instars 1–4, respectively, were used to divide larval development into instars. In the model, a larva's physiological age ranges from 0 at eclosion to 1 at pupation. Thus, when an individual larva's physiological age reached 0.206, it was considered to have molted to instar II, to instar III at age 0.409, and to instar IV at age 0.773.

A second-degree polynomial logistic regression model provided the best fit (based on the AICc) to survival response to temperature (Fig. 2; Table 4) for all life stages:

$$s = \frac{1}{1 + e^{-(k_0 + k_1 T + k_2 T^2)}}. \quad (3)$$

Eggs hatched at all temperatures tested. Daily and overall egg survival were high and did not vary significantly over the range of temperatures tested. Larvae did not develop at 5°C, and daily survival increased from 5% at 18°C to 83% at 22°C (Fig. 2). Overall larval survival was only 12% at 22°C (Fig. 2). Prepupae did not survive at 5°C. Daily survivorship was highest at 8°C (99%) and lowest at 18°C (96%). There was a slight increase in daily prepupal survival from 97 to 98% from 18 to 22°C. Pupal daily and overall survival responded similarly to temperature: there was no development at 5°C, and survival increased up to 18°C, and then decreased.

Effects of Temperature on Adult Longevity and Fecundity

A simple exponential model with lower threshold temperature (Logan et al. 1979) provided the lowest AICc for adult aging rate (the inverse of longevity) (Table 5):

$$R(T) = \psi e^{(k(T-T_b) - \exp[k((T-T_b)/\Delta T)])} \quad (4)$$

Adult longevity decreased with temperature, from an average of 80 d at 5°C to 15 d at 25°C (Fig. 3b). A Gaussian model (Taylor 1981)

$$O = \psi e^{-\frac{1}{2}\left(\frac{T - T_O}{\Delta T}\right)^2} \quad (5)$$

provided the best description of the relationship between temperature and the oviposition ratio, the proportion of remaining fecundity laid per day by individual females (Fig. 3d; Table 5). Total fecundity also varied with temperature, as a result of the interaction between daily oviposition rate and longevity (Fig. 3e). The model uses an average initial fecundity of $F_0 = 100.4$, assigned to each female as log-normally distributed random variable with a mean of 4.546 eggs/day, and standard deviation 0.355. The average daily oviposition rate varied with temperature, and was lowest at 25°C and highest at 10°C (Fig. 4).

Table 1. Percentage survivorship from the previous stage, mean and median duration (days = d), and the median rate (1/d) of development of *L. osakensis* for each respective life sage at six constant temperatures

Life stage	Temperature (°C)	*n*	Survival (%)	Duration (d)		Median rate
				Mean ± SD	Median	
Egg	5	38	79	57.1 ± 3.2	57	0.018
	8	58	86	26.1 ± 1.8	26	0.038
	11	60	87	13.7 ± 1.7	13.5	0.074
	15	58	100	10.3 ± 1.6	10	0.100
	19	62	89	7.1 ± 0.8	7	0.143
	22	75	95	5.2 ± 0.8	5	0.200
Larva 1	5	30	7	23.1 ± 2.3	23	0.043
	8	50	72	8.1 ± 2.2	8	0.125
	11	52	77	5.2 ± 1.3	5	0.200
	15	58	78	4.9 ± 1.4	5	0.200
	19	55	76	3.1 ± 0.9	3	0.333
	22	71	31	2.7 ± 0.8	3	0.333
Larva 2	5	2	100	21.5 ± 0.7	21.5	0.047
	8	36	78	9.2 ± 2.7	8.5	0.118
	11	40	88	6.3 ± 1.8	6	0.167
	15	45	82	4.4 ± 1.5	4	0.250
	19	42	93	3.5 ± 1.3	3	0.333
	22	22	50	2 ± 1.2	1.5	0.667
Larva 3	5	–	–	–	–	–
	8	28	71	14.1 ± 3.4	14	0.071
	11	35	86	6 ± 2.1	6	0.167
	15	37	92	4.5 ± 1.7	5	0.200
	19	39	69	3.6 ± 1.2	4	0.250
	22	11	73	2.1 ± 0.8	2	0.500
Larva 4	5	–	–	–	–	–
	8	20	65	17.2 ± 4.0	16.5	0.061
	11	30	73	11.5 ± 3.7	12.5	0.080
	15	34	76	7.8 ± 1.7	8	0.125
	19	27	70	5.1 ± 1.6	5	0.200
	22	8	100	4 ± 0.5	4	0.250
Larva 1–4	5	–	–	–	–	–
	8	20	52	48.6 ± 6.7	47	0.021
	11	30	51	29.0 ± 4.8	29.5	0.034
	15	34	58	21.7 ± 4.5	22	0.045
	19	27	44	15.2 ± 3.2	15	0.067
	22	8	11	10.7 ± 2.7	10.5	0.095
Prepupa	5	–	–	–	–	–
	8	13	–	38.1 ± 4.5	38	0.026
	11	22	55	19.9 ± 4.2	20	0.050
	15	26	85	13.1 ± 2.3	14	0.071
	19	19	84	10.3 ± 1.6	10	0.100
	22	8	75	8.3 ± 2.4	8.5	0.118
Pupa	5	–	–	–	–	–
	8	–	–	–	–	–
	11	12	55	29.3 ± 1.7	30	0.033
	15	22	84	21.3 ± 1.3	21.5	0.047
	19	16	84	14.9 ± 0.9	15	0.067
	22	6	75	7.8 ± 1.2	8	0.125
Egg to adult	5	–	–	–	–	–
	8	–	–	–	–	–
	11	12	20	91.8 ± 16.4	93	0.011
	15	22	38	66.4 ± 12	67.5	0.015
	19	16	26	47.5 ± 8.3	47	0.021
	22	6	8	32.0 ± 7.7	32	0.031

Field Study

L. osakensis eggs were present from the start of sampling on 29 December 2020 until 23 April 2021, with peak oviposition occurring in early March (Fig. 5A). The cumulative occurrence of *L. osakensis* eggs in the field was well described (R^2 = 0.98) by the logistic function

$$\mathrm{CEC} = \frac{1}{1 + e^{-(D-\mu)/\sigma}},$$

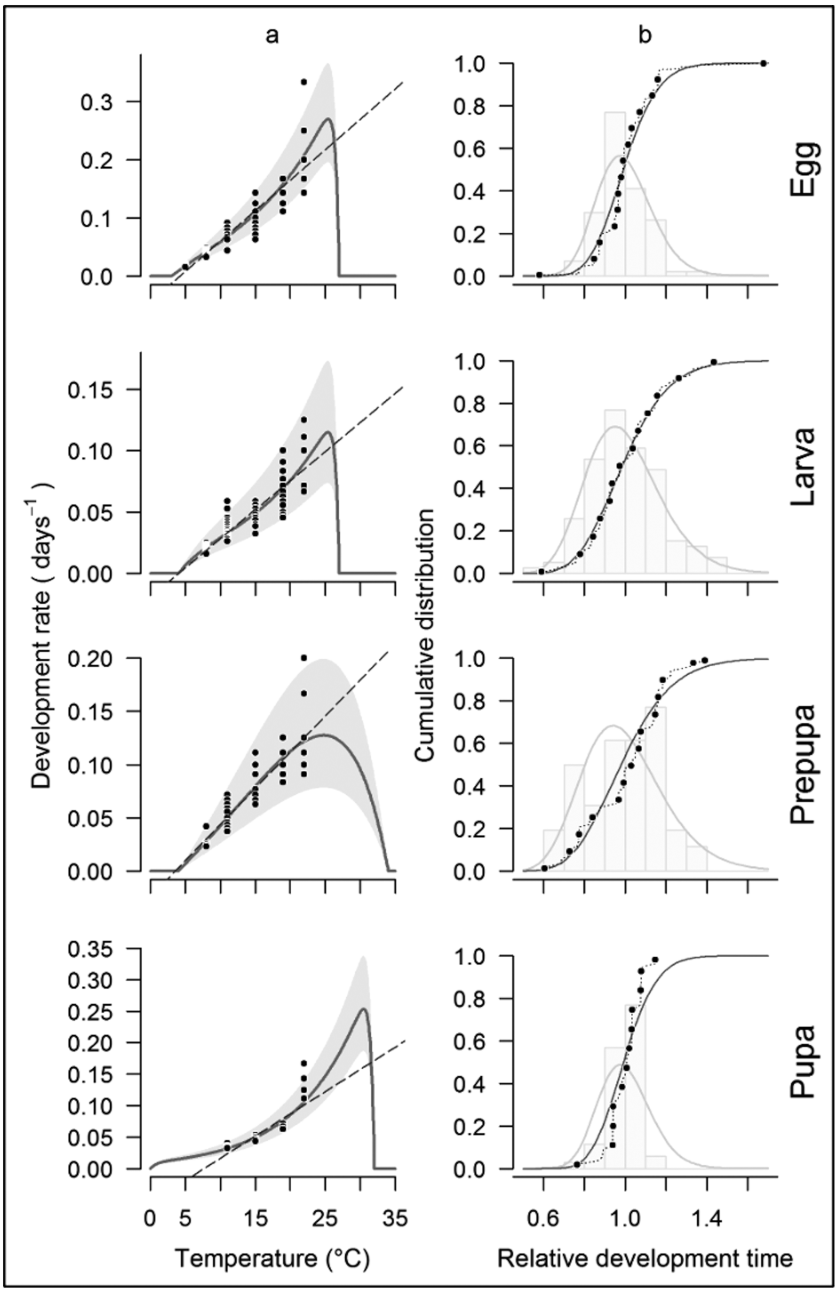

Fig. 1. Development of *Laricobius osakensis* at different constant temperatures in each life stage. Row 1: eggs; row 2: larvae (instars 1–4); row 3: prepupae; row 4: pupae. Column a: development rate as a function of temperature; gray areas: percentiles [1–99] of individual development rates. Column b: frequency distribution (grey) and cumulative distribution (black) of development times relative to mean (lines: log-normal distribution).

where D is the accumulation of degree-days from December first between the thresholds of 2.1 and 20.1°C, with estimates of μ = 105.1 and σ = 88.6 (Fig. 5B). In late March, occurrence *L. osakensis* larvae coincided with a dramatic decrease in the number of HWA eggs present (Fig. 5A). The simulation model closely reflected the observed larval abundance data (Fig. 5). The optimal time to sample *L. osakensis* larvae is 10 d before *L. osakensis* larvae have reached 50% of their density. This time period coincides with when *L. osakensis* adults have finished laying eggs (late March) and before larvae begin dropping from hemlock trees to the ground (late April). At this time, the maximum density of *L. osakensis* larvae per HWA occurs, and an accurate density ratio can be determined from sampling at this time. A 10-day sampling period based on the model was used to allow sufficient time to sample larvae and accommodate for any microclimatic variability (Fig. 5). An example of the model's full output of HWA phenology is illustrated in Fig. 6.

Head Capsule Measurement

Frequency distributions of head capsule measurements separated out four nonoverlapping peaks, corresponding to four larval instars (Table 6). We found a significant increase in larvae head capsule width for each successive larval instar of ($F_{3,88}$ = 653.4, P < 0.001, Table 6). The head capsule width of insects follows a constant geometric progression as they develop from one instar to the next (Dyar 1890). Dyar's ratios were relatively constant and ranged from 1.34

Table 2. Parameters of linear regression ($r = a + bT$), minimum temperature threshold ($TL = -a/b$), and cumulative degree-days ($CDD = 1/b$) to complete each stage based on development at constant temperatures (°C) of *Laricobius osakensis*

	Parameters						
Life stage	*a*	*b*	df	r^2	*P*	T_L (°C)	C_{DD}
Egg	−0.044	0.0104	349	0.90	0.0x	4.2	95.9
Larva 1	−0.035	0.0196	314	0.65	0.0x	1.8	50.9
Larva 2	−0.161	0.0307	186	0.46	0.0x	5.2	32.6
Larva 3	−0.114	0.0249	148	0.12	0.0x	4.6	40.1
Larva 4	−0.058	0.0140	118	0.61	0.0x	4.1	71.6
Larva 1–4	−0.019	0.0047	118	0.76	0.0x	4.0	211.6
Prepupa	−0.024	0.0068	84	0.74	0.0x	3.5	147.5
Pupa	−0.054	0.0071	52	0.72	0.0x	7.7	141.7
Egg to adult	−0.010	0.0018	52	0.83	0.0x	5.5	556.7

Table 3. Parameters of Eq. (2) describing the developmental responses of immature stages of *Laricobius osakensis* to temperature (°C), including the lognormal variance term σ_ε

Life stage	*n*	Ψ	*K*	T_b	T_m	ΔT_b	ΔT_m	σ_ε	*AICc*
Egg	351	0.03962	0.08769	3	27	0.9287	0.5	0.135	1352.5
Larva 1–4	119	0.01959	0.08478	4	27	0.3596	0.5	0.184	697.7
Prepupa	88	0.07032	0.08388	4	34	50.00	10.64	0.201	445.7
Pupa	56	0.00959	0.10923	0	32	0.1000	0.5	0.128	260.1

to 1.36 mm for all larval stages with the exception of the fourth instar, which showed a slight decrease compared to the other three instars (Table 6).

Discussion

This is the first paper that quantifies the effect of temperature on *L. osakensis* development. Temperature-dependent developmental studies have been conducted for *L. nigrinus* (Zilahi-Balogh et at. 2003) and *L. kangdingensis* (Gatton et al. 2009). *L. nigrinus* was approved for release in early 2000 (Zilahi-Balogh et al. 2003); in contrast, a petition for the release of *L. kangdingensis* was never submitted nor was the species released. *L. osakensis* and *L. kangdingensis* share a sister clade and thus, are more closely related to one another than they are to *L. nigrinus* (Montgomery et al. 2011). *L. osakensis* did not complete development from egg to adult at two of the lower temperatures tested (5 and 8°C) (Table 1). Gatton et al. (2009) reported that *L. kangdingensis* failed to develop from egg to adult at 6 and 9°C. Zilahi-Balogh et al. (2003) also reported that *L. nigrinus* completed development at 6 but not at 9°C. The lower minimum development threshold from egg to adult for *L. osakensis* (5.5°C) was slightly warmer but still similar to both *L. nigrinus* (4°C) and *L. kangdingensis*, but also similar to that of the spring generation of HWA (3.9°C) (Salom et al. 2002). The developmental parameters (i.e., thermal requirements and thresholds) of HWA's overwintering generation (sistens) are currently unknown. Moreover, the reciprocating impact of the sistens developmental parameters on the phenology of *L. osakensis* is difficult to gauge. If *L. osakensis* adults do not begin oviposition until after the sistens begin oviposition (as these data suggest), the development of *L. osakensis* larvae would largely be unaffected by the development of the sisten nymphs. However, the initiation of oviposition by *L. osakensis* could strongly depend on the developmental rate of the sistens by virtue of when sistens eggs first appear.

The upper development threshold for the spring generation of HWA, the prey of *L. osakensis*, was predicted to be between 22 and 27°C (Salom et al. 2002). Gatton et al. (2009) and Zilahi-Balogh et al. (2003) reported failure to develop from egg to adult in *L. kangdingensis* and *L. nigrinus* at temperatures of 18 and 21°C, respectively. *L. osakensis* completed development from egg to adult at 22°C, the highest temperature tested in this study (Tables 1 and 5). This predator can develop and survive at higher constant temperatures than *L. nigrinus* and *L. kangdingensis*. Our results suggest that *L. osakensis* may be a better fit as a biological agent than *L. nigrinus* in the southern portion of HWA's distribution.

Additional research is needed to more precisely estimate the lower and upper thermal limits of *L. osakensis* development and survival, perhaps using temperature transfers as suggested by Régnière et al. (2012) and Wardlaw et al. (2022).

The mean development time from egg to adult for *L. osakensis* from 11 to 19°C was on average 4.2% slower than for *L. nigrinus* at comparable temperatures (12–18°C) (Zilahi-Balogh et al. 2003) (Table 1). *L. kangdingensis* only completed development at 12 and 15°C, and comparing these temperatures to those tested in this study (11 and 15°C), *L. osakensis* completed egg to adult development 23% slower on average (Gatton et al. 2009). There was no statistical difference in oviposition rates between 5, 10, and 15°C, and 15 and 20°C (Fig. 4). Based on percent survivorship data, however, 10°C was optimal for oviposition for this species. For larval growth, 15°C was the optimal, whereas, 15–18°C was the optimal for the prepupal and pupal life stages, as measured by survivorship (Fig. 3; Table 1). *L. osakensis* is more heat tolerant than *L. nigrinus* or *L. kangdingensis* as evidenced by their survivorship, fecundity, and rate of development (Figs. 1–3; Tables 1 and 2).

Over the past 10 years, temperature requirements used to rear *L. osakensis* at Virginia Tech have been largely based on those of *L. nigrinus* (Zilahi-Balogh et al. 2003, Salom et al. 2012, Foley et al. 2021). Salom et al. (2012) suggested that the techniques used to rear *L. nigrinus* are also applicable to rearing other *Laricobius* species. The apparent success in rearing >70,000 *L. osakensis* since 2011 (Foley et al. 2021), and similar optimal thermal thresholds presented here for *L. osakensis*, support this recommendation.

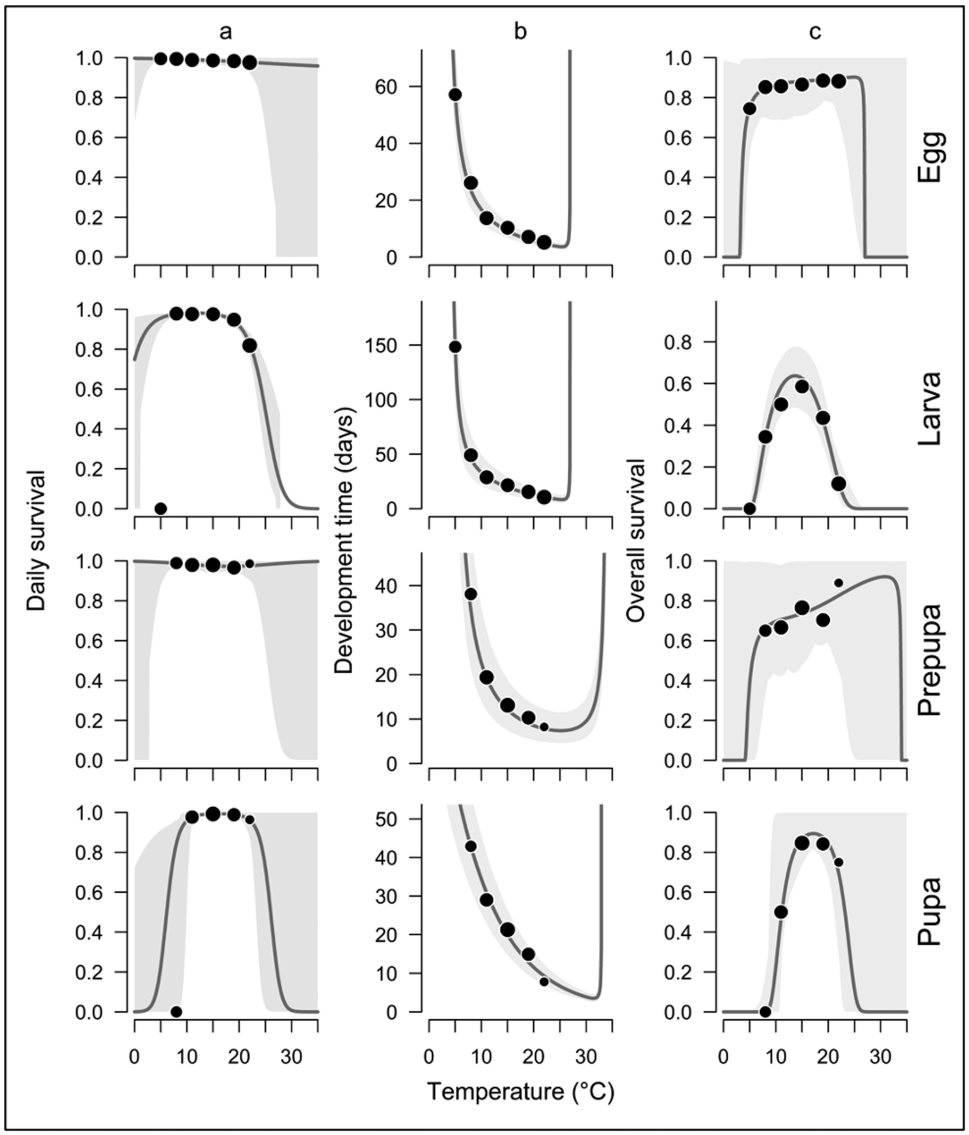

Fig. 2. Survival of *L. osakensis* as a function of temperature in four life stages. Row 1: eggs; row 2: larvae; row 3: prepupae; row 4: pupae. Column (a) daily survival. Column (b) stage duration. Column (c) overall survival daily survival and stage duration. Shaded areas: 95% confidence interval on regression lines in (b) Eq. (1), in (c) Eq. (2). Widening at extremes caused by a lack of data.

Table 4. Parameters of Eq. (3) describing the survival responses of immature stages of *Laricobius osakensis* to temperature (°C)

		Parameters				
Life stage	*n*	k_0	k_1	k_2	Maximum log-likelihood	AICc
Egg	411	−5.906	0.1299	−0.001460	−17.71	53.42
Larva 1–4	351	−1.087	−0.5072	0.02209	−13.15	44.29
Prepupa	120	−6.276	0.2963	−0.008149	−11.7	53.41
Pupa	86	8.133	−1.635	0.05088	−8.7	47.41

High rates of larval mortality were observed throughout the duration of this study. However, high rates of larval mortality have consistently been noted for both *L. nigrinus* and *L. osakensis* (Foley et al. 2021) and have been attributed (but not limited) to moisture and handling time (Fig. 2; Table 1) (Zilahi-Balogh et al. 2003, J.R.F., personal observations). While each experimental unit was moistened regularly, saturation levels were not recorded. Efforts to minimize the amount of handling during daily inspection were made but likely played a role in total survivorship. Therefore, the degree of mortality for each respective life stage and temperature should be interpreted carefully.

Mortality during the subterranean portion of the *Laricobius* spp. life cycle is in excess of 60% (Foley et al. 2021, 2022). This period encompasses pupation, aestivation, and adult emergence. High levels of mortality were observed in our study. None of the prepupae (n = 13) tested at 8°C and only 55% of those tested at

Table 5. Parameters of functions describing the longevity [Eq. (4)] and fecundity [Eq. (5)] responses of *Laricobius osakensis* to temperature

	Parameters					*n*	AICc
Adult longevity	Ψ	k	T_b	ΔT	σ_ε		
	0.0288	0.1066	4.0	99.98	0.401	71	542.6
Oviposition	Ψ	T_o	ΔT	F_o	σ_F		
	0.015178	10.9	6.5351	100.4	0.355	252	1690.5

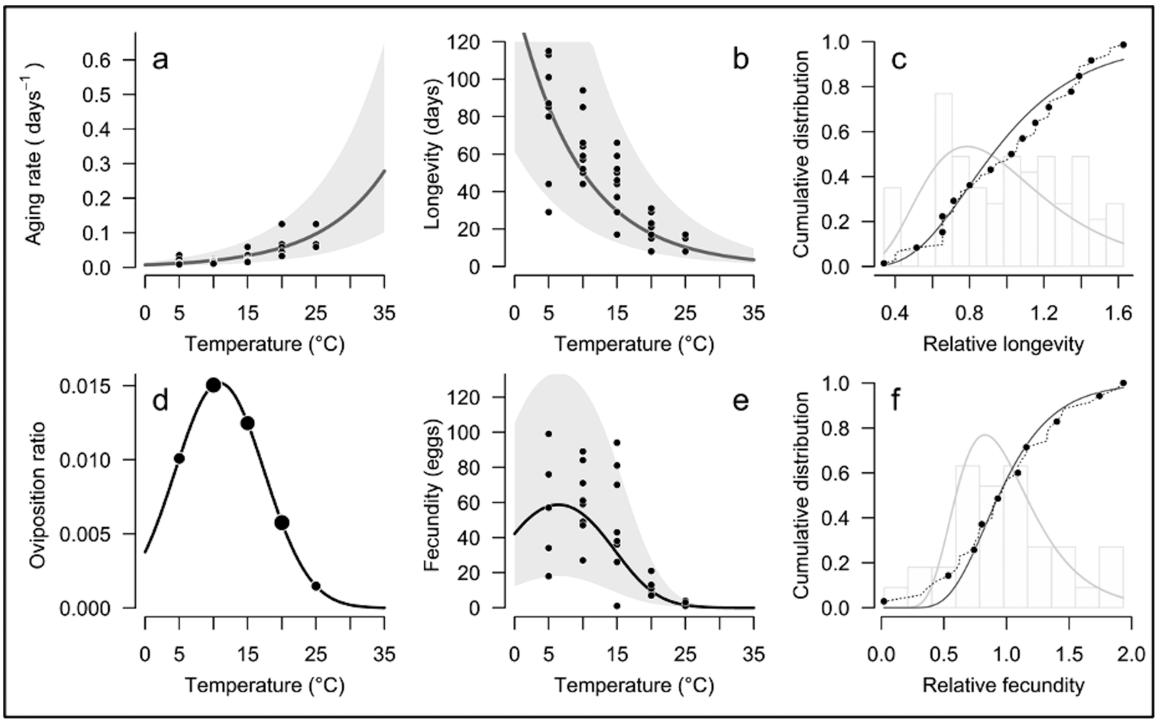

Fig. 3. (a) Adult aging rate and (b) longevity as a function of temperature (lines: Eq. (1)), (c) density distribution of relative longevity (line: lognormal). (d) Oviposition ratio of remaining eggs as a function of temperature. Dot sizes are proportional to the number of eggs laid (line: Eq. (4)). (e) Fecundity as a function of temperature (line: Eq. (3)). (f) Density distribution of relative fecundity (line: lognormal). Shaded areas in (a), (b), (d), and (e): [1–99] percentiles.

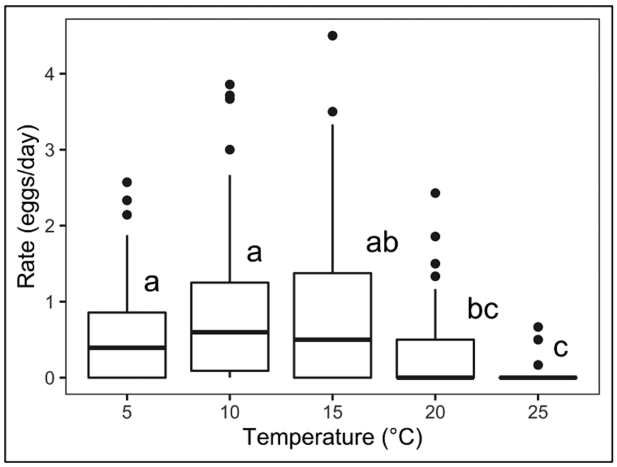

Fig. 4. Oviposition rate for *Laricobius osakensis* at five constant temperatures. Treatments with the same letter are not significantly different at the 5% level (Tukey's HSD test). ANOVA ($P < 0.01$; $F = 12.5$; treatment df = 4; error df = 329).

11°C (n = 12) completed development (Table 1). However, at the highest temperatures tested in this study (15–22°C), subterranean survivorship was highest, which is consistent with the natural conditions of this system in western Virginia. From 1980 to 2016, the average daily high temperatures for western Virginia from mid-to-late April when most of these insects are dropping from the trees to their subterranean habitat, are between 16 and 21°C (Rienecker et al. 2011).

L. osakensis, as with other species of *Laricobius*, has four larval instars (Clark and Brown 1958, Franz 1958, Clark and Brown 1960, Zilahi-Balogh et al. 2003, Gatton et al. 2009). This is the first documentation of larval head capsule widths for *L. osakensis*. The mean head capsule widths for each *L. osakensis* larval instar were smaller than that of head capsule reported for *L. nigrinus* and *L. kangdingensis* (Zilahi-Balogh et al. 2003, Gatton et al. 2009). However, the range of head capsule widths for each respective instar of *L. nigrinus*, *L. kangdingensis*, and *L. osakensis* overlap. The ability to distinguish various *Laricobius* spp. based on larval head capsule width is not possible (Montgomery et al. 2011).

In the native range of *L. osakensis* and HWA (Japan), eggs of both species were found in December and January at two of the three field sites used to quantify their phenology, (Vieira et al. 2013). In this study, *L. osakensis* eggs were present on the first sampling date (29 December) whereas HWA's eggs were not present until two months later (17 February). While *L. osakensis* eggs were present two months prior to the appearance of HWA eggs, these eggs did

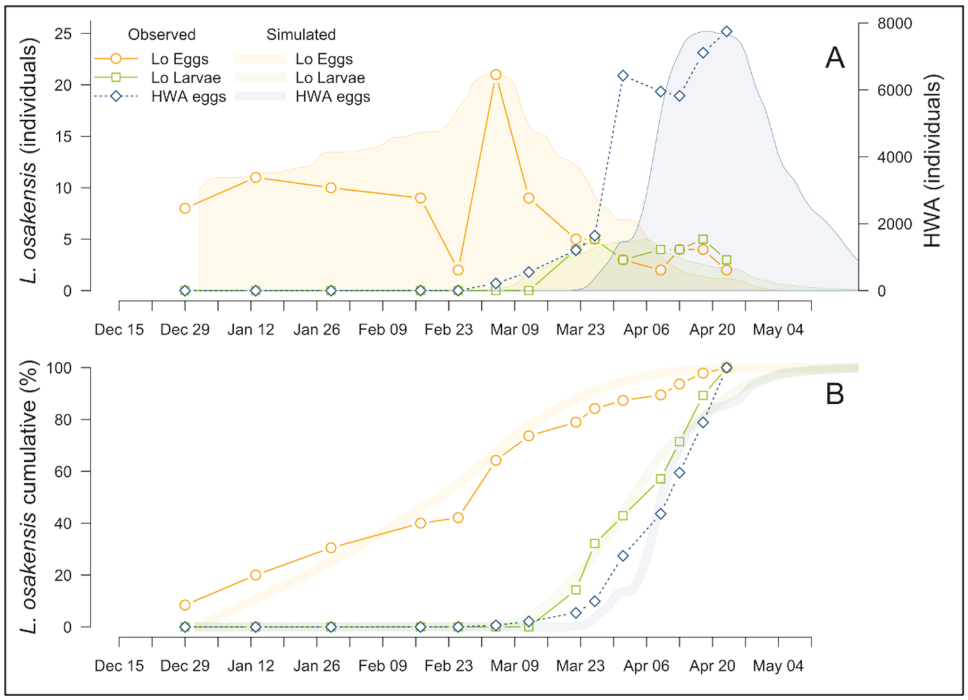

Fig. 5. Observed and simulated abundance of eggs and larvae of *L. osakensis* under field conditions at Hungry Mother State Park, Marion, VA from 2020 to 2021. (A) number of individuals. (B) cumulative.

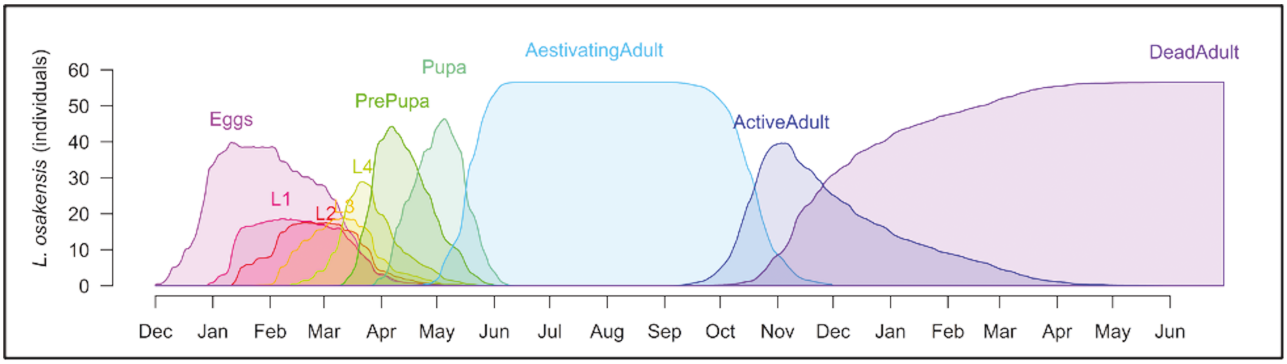

Fig. 6. Number of individuals in each successive *Laricobius osakensis* life stage over time, as predicted by the model for Hungry Mother State Park, Marion, VA for 2020–2021.

Table 6. Mean (±SD) head capsule width and Dyar's ratio (head capsule width/previous head capsule width) measurements of *Laricobius osakensis* larvae

		Head capsule width (mm)		
Instar	*N*	Mean	Range	Dyar's ratio
I	40	0.19 ± 0.01	0.16–0.20	
II	15	0.26 ± 0.01	0.24–0.28	1.36
III	20	0.35 ± 0.04	0.20–0.40	1.36
IV	17	0.44 ± 0.02	0.40–0.48	1.34

not hatch during this period indicated by the absence of larvae. *L. osakensis* eggs not hatching until HWA eggs were present suggests good synchrony between the predator and prey. (Figs. 5 and 6). During the fifth sampling period (25 February 2021) there was a dramatic decrease in the total number of eggs from the previous and following sampling periods. It is unclear if this was an artifact of the sampling procedure or if this decrease was associated with egg mortality or recently eclosed larvae leaving the HWA ovisac due to a lack of available eggs to consume. We observed the phenology of this predator and prey complex in only one location, HMSP. While *L. osakensis* can be found at many of their original release sites (Mooneyham et al. 2016, Toland et al. 2018), there is still a limited number of available field sites that are thought not be colonized by other *Laricobius* species. Colonization of a site by other *Laricobius* spp. occurs through the unintentional release of *Laricobius rubidus* LeConte (Coleoptera: Derodontidae) or *L. nigrinus* inadvertently reared for release (Toland et al. 2018, Foley et al. 2021), through dispersal of *L. nigrinus* from nearby release sites (Foley et al. 2019, Jubb et al. 2021), or through the dispersal of *L. rubidus* from adjacent sites where eastern white pine (*Pinus strobus* L.) are infested with pine bark adelgid (*Pineus strobi* Hartig) (Hemiptera: Adelgidae).

The model that predicted peak larval density was similar to the field data collected from HMSP and appeared to track peak larval

density well (Fig. 3). However, sampling did not start prior to the beginning of *L. osakensis* oviposition. Furthermore, sampling could not be continued past 23 April 2021, when larvae were still present in the trees. However, if field observations were continued it would likely have continued to follow the model predictions. The most effective and least time-consuming method to confirm *Laricobius* spp. adult presence at any particular site is through beat sheet sampling (Mausel et al. 2010). However, this method does not work as well for *Laricobius* spp. larvae and can often result in false negatives (Mausel et al. 2010, Toland et al. 2018). Aside from yet-to-be-developed eDNA techniques, the highest likelihood of confirming the presence and therefore establishment of *Laricobius* spp. at any site is to sample for larvae through branch clippings (Mausel et al. 2010, Toland et al. 2018, Jubb et al. 2021). The density or ratio of *Laricobius* spp. to HWA can also be determined by clipping branches when sampling for larvae. Details for this method can be found at Virginia Tech (2021). The development of this phenological forecast model will allow HWA land managers to more confidently and precisely predict when to sample for *L. osakensis* larvae.

Acknowledgments

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