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Temperature Affects Hatching Success of Cocoons in the Invasive Asian Earthworm *Amynthas agrestis* from the Southern Appalachians

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Abstract - Invasive Asian earthworms are increasingly common in the eastern USA where they are a major cause of terrestrial ecosystem disturbance. Among these, *Amynthas agrestis* (Crazy Worm, Alabama Jumper, and other common names) has been shown to alter above- and belowground food webs. Life-history traits of these earthworms are largely unknown, particularly in their invaded range. Here, we sought to answer questions about temperature effects on hatching success for cocoons of this species, using specimens collected from the southern Appalachian Mountains. We conducted 2 experiments investigating the effects of incubation temperature and the effect of varying the duration of cold temperature on hatching success. Of the temperatures tested, we found that cocoons hatched with greatest success at 10 °C, but our tests indicate a long duration at that temperature may be needed to result in an increase in hatching success. These results indicate that temperature and the duration of temperature exposure affect hatching success in this species. While our results contribute to the growing body of knowledge about the life-history traits of invasive Asian earthworms in the eastern US, more research is needed to provide a finer-resolution understanding of the optimum level and duration of temperatures for hatching success of *A. agrestis*.

Introduction

Invasive earthworms have been linked to biodiversity loss and major disruptions in ecosystem processes around the world (Hendrix et al. 2008). Although they are widespread and have serious impacts on both below- and aboveground aspects of ecosystem functioning (Burtelow et al. 1998; Hale et al. 2005, 2006), life-history traits of many species are unknown. However, such knowledge is critical in order to predict patterns of invasion and may be important for developing methods of control (Ikeda et al. 2015).

One such invasive earthworm, *Amynthas agrestis* (Goto and Hatai 1899) (Crazy Snake Worm, Alabama Jumper, Jumping Worm, and other common names), has spread and is a major cause of disturbance to terrestrial ecosystems in North America (e.g., Snyder et al. 2011). Although *A. agrestis* is native to Japan (Uchida 2004), this species has acquired a broad geographic distribution in its invaded

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range (CABI 2018, Callaham et al. 2003, Görres and Melnichuk 2012, Reynolds 1978), and continues to spread, often along with several related species (Chang et al. 2017a). *Amynthas agrestis* has been linked to alterations in soil chemistry, structure, and function (Qiu and Turner 2017), which have generated subsequent impacts on microbial (Chang et al. 2017b), invertebrate (Snyder et al. 2011), and vertebrate populations (Ziemba et al. 2016). Additionally, *A. agrestis* has been shown to alter established predator–prey interactions in soil food webs (Gao et al. 2017). Cascading effects of these invasive earthworms on native seedling recruitment and on plant–mycorrhizal interactions are only beginning to be explored in detail (Dobson and Blossey 2015, Paudel et al. 2016).

Formulating hypotheses regarding dispersal and control of the spread of these organisms has been somewhat difficult due to the lack of available information on their life-history traits (Callaham et al. 2006). *Amynthas agrestis* is known to have an annual life cycle in its native range (Uchida 2004), and is widely considered to demonstrate a yearly life cycle in North America, overwintering in the cocoon stage (Callaham et al. 2003, Reynolds 1978; but see Görres et al. 2018). Richardson et al. (2009) showed that the optimal temperature for the survival of *A. agrestis* from the southern Appalachians was 12 °C, although their soil- and litter-mixing activity was greatest at 25 °C and was heavily dependent on soil moisture. Questions remain about environmental cues for reproduction and cocoon maturation in this species, such as how seasonal fluctuations in temperature may act as indicators for initiation of hatching.

We sought to answer questions about cues for hatching of *A. agrestis* cocoons: (1) what is the optimal temperature for emergence from cocoons?, and (2) is duration of cold temperature important for hatching in this species? We hypothesized that the optimal temperature for cocoon hatching would be near 12 °C due to the field observations of Callaham et al. (2003). Reasoning that exposure to cold temperatures might be a cue to developing embryos, we further hypothesized that cocoon hatching would be dependent on duration of cold temperature and we predicted that hatching success would be highest when cocoons were incubated at temperatures near 12 °C for a short duration of time. Here, we performed laboratory incubations of cocoons of this species to address each question.

Methods

Earthworm and cocoon collection

We collected adult *A. agrestis* earthworms from 3 locations: Great Smoky Mountains National Park, along the edge of a pond near US highway 129/Lake Chilhowee (35°31'57"N, 83°59'27"W); Nantahala National Forest, along the roadside of NC route 28 (35°26'31"N, 83°49'21"W); and Great Smoky Mountain Institute, Tremont, TN (35°38'23"N, 83°41'24"W). We dissected a fraction of the adult worms in order to confirm their identification as *Amynthas agrestis* (Reynolds 1978). Earthworms were collected from these locations during 17–19 June 2007, (see Table 1 for details) and stored in the laboratory in plastic culture containers at room temperature (22 °C) until adults had completed the life cycle (estimated to be

~100 days; Görres et al. 2016). We maintained soil in the cultures at water-holding capacity and periodically amended cultures with leaf material collected from the Great Smoky Mountains National Park. All material added to the cultures was air dried and crushed prior to use as feed in order to reduce the likelihood of introducing new cocoons into the cultures. In late October 2007, we collected cocoons from each culture by wet-sieving the soil and leaf material through a 1-mm sieve, transferring the material collected on the sieve into a plastic pan filled with water, and carefully sorting through this material with forceps and removing all encountered cocoons. We stored cocoons submerged in water for 7 days at room temperature (22 °C) before using them in the incubation trials. A total of 375 cocoons were collected from cultures for use in the incubations (Table 1).

Incubation at constant temperature

We placed cocoons originating from each population of earthworms into petri dishes and submerged them in tap water (20 mL) between 2 pieces of filter paper (Whatman #1 qualitative filter paper; 5.5 cm diameter) to prevent drying (Butt 1991; Lowe and Butt 2005, 2007). From each of the 3 populations, we placed 10 or 12 cocoons into separate petri dishes assigned to incubators at 6 different temperatures (5, 10, 15, 20, 25, and 30 °C) such that there was one dish of cocoons from each population at each temperature. Cocoons were then gradually adjusted to their assigned temperature by altering their temperature at 5 °C increments for 24 hours per increment until the assigned temperature was reached. We kept all incubators dark to simulate light conditions below the soil surface. We checked cocoons weekly to assess hatching and to add tap water as needed to keep them submerged. As hatchings occurred, we removed immature *A. agrestis* and their hatched cocoons from the petri dish. We recorded the number of hatched cocoons weekly for 31 weeks.

Cold-duration incubation

We placed cocoons produced from earthworms from the Lake Chilhowee location (Table 1) into petri dishes (10 cocoons per dish) which we assigned to 1 of 3 temperatures (5, 10, and 15 °C) and 1 of 3 durations (15, 30, or 60 days) such that there was 1 dish of 10 cocoons at each of the 9 possible temperature and duration combinations. Cocoons were gradually lowered to the assigned cold temperatures as described above and allowed to incubate at that temperature for the assigned duration. After the experimental incubation period, we transitioned the cocoons back to 20 °C in 5 °C increments for 24- hours per increment, a rate chosen to bring the cocoons back to

Table 1. Number of adult *Amynthas agrestis* collected from each of 3 locations and the number of cocoons produced from those individuals. All earthworms used for production of cocoons were collected 17–19 June 2007 from the locations indicated.

Collection location	Adults collected	Cocoons produced	Cocoons/ worm
Great Smoky Mountains National Park at Lake Chilhowee	13	210	16.2
Nantahala National Forest	3	89	29.7
Great Smoky Mountains Institute at Tremont	4	76	19.0

room temperature in a timely manner without being so rapid as to likely have a negative impact on hatching success (J.H. Görres, University of Vermont, Burlington, VT, pers. comm.). Cocoons then remained at 20 °C, and we assessed and recorded rates of hatching success (as described above) weekly for 31 weeks.

Statistical analyses

We analyzed the data for hatching from the incubations at constant temperatures using a repeated measures analysis of variance test (ANOVA) with population as a block and incubation temperature as the explanatory variable. We applied an arcsine transformation to proportion data of hatching success in order to meet assumptions of ANOVA. We used Tukey's honest significant difference (HSD) to determine differences among treatments.

We performed a logistic regression with the data from the cold-duration incubation to detect differences in the probability of hatching over the assessment period of the treatment combinations of incubation temperature and cold duration. Model selection was performed by using Akaike information criterion (AIC) values (Johnson and Omland 2004). We performed all analyses for both experiments using Rstudio version 3.3.1 (RStudio Team 2016).

Results

Incubation at constant temperature

Temperature was found to be a significant factor in determining hatching success ($F_{5, 12} = 8.40$, $P < 0.001$). The highest mean hatching (64.4%) occurred at 10 °C followed by 25 °C (16.7%) and 15 °C (8.9%) (Fig. 1A). Across all treatments, 15.5% of cocoons hatched. The results of Tukey's HSD performed on the cumulative proportion of hatched cocoons showed that although hatching success at 10 °C differed significantly from 5 °C, 20 °C, and 30 °C, it did not differ from 15 °C and 25 °C. We used population origin as a blocking factor in our initial analysis and found that this factor was insignificant with respect to hatching success. We therefore dropped this factor in subsequent analyses. Additionally, week of hatching varied among the temperature treatments with cocoons held at 10 °C hatching during weeks 17–20, while cocoons held at other temperatures hatched during the first 10 weeks, if at all ($F_{1,12} = 11.87$, $P = 0.002$; Fig.1B).

Cold-duration incubation

Comparison of AIC values revealed that the model where temperature and cold duration were allowed to interact together, but not with week of hatching, was the best-fitting model (Table 2). We did not detect any treatment effects of temperature

Table 2. AIC values used for model selection in the cold-duration incubation.

Model	df	AIC	ΔAIC	Weight
(1) Cold duration * Temperature + Hatching week	11	982.73	0.00	0.960
(2) Cold duration * Temperature * Hatching week	19	989.16	6.43	0.038
(3) Cold duration + Temperature + Hatching week	7	996.14	13.41	0.001

or cold duration (Table 3). Week of hatching was a significant variable in the model regardless of the applied treatment, i.e., cocoons hatched at the same rate regardless of the temperature x duration treatment applied. Across all treatments, 14.4% of cocoons hatched, and all of these hatched during weeks 8–21 of the assessment period.

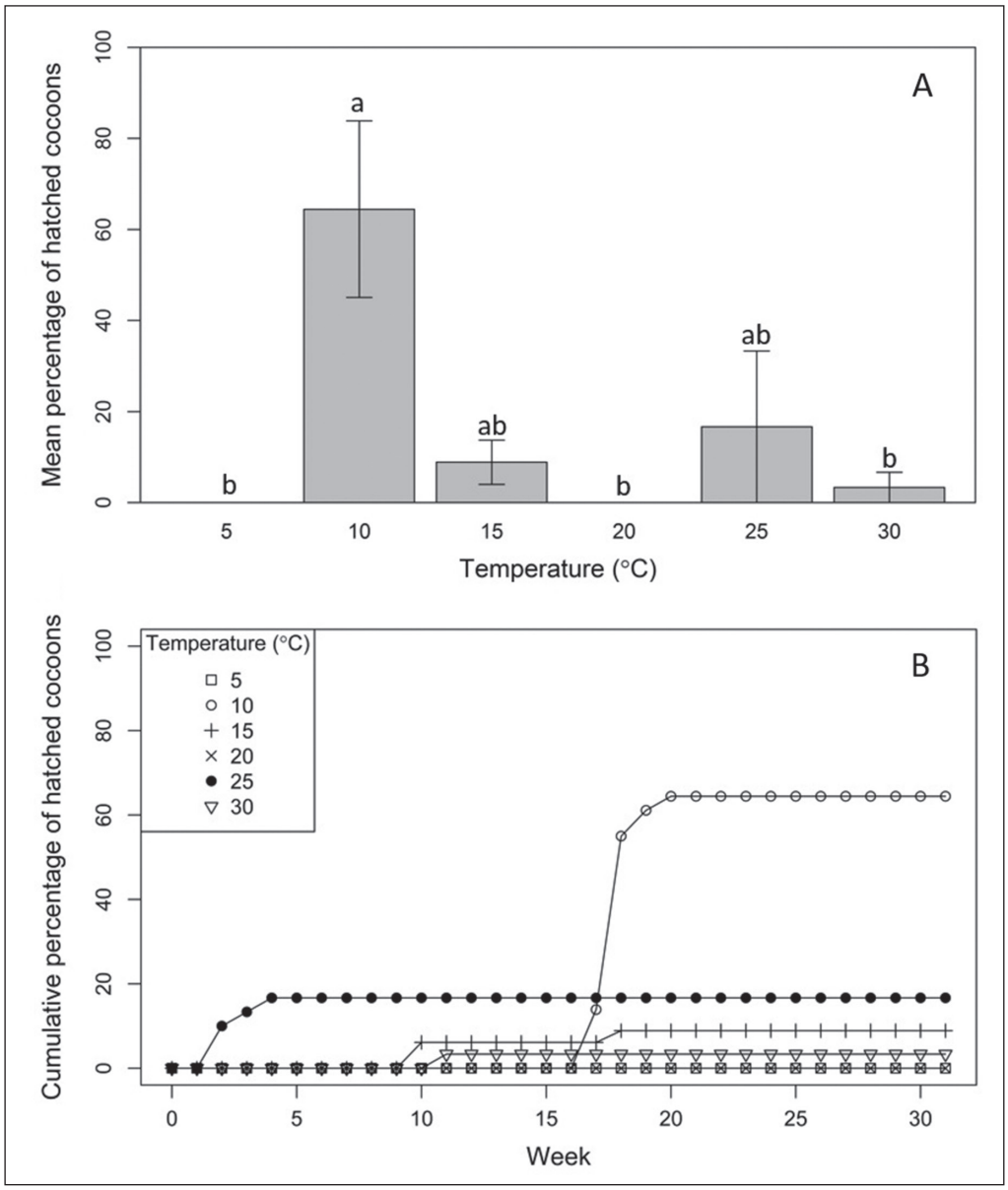

Figure 1. Hatching success of *Amynthas agrestis* earthworm cocoons in the incubation at constant temperature experiment. Cocoons were held at each of 6 temperatures and monitored for 31 weeks for hatching. (A) Mean (± SE) percentage of hatched cocoons at each temperature at the end of the constant temperature incubation. Letters indicate significant difference derived from Tukey HSD test. (B) Cumulative percentages of hatched cocoons over the 31-week assessment period at the 6 different temperature treatments.

Discussion

Our study provides evidence that temperature is an important abiotic factor affecting the hatching of *A. agrestis* cocoons. In the constant-temperature experiment, we found that the highest mean percentage of hatching (64.4%) occurred at 10 °C (Fig. 1a). This is consistent with our hypothesis that hatching would be highest near 12 °C as reported from field observations (Görres et al. 2016) of *A. agrestis* hatching in Vermont forests. Contrary to our hypothesis in the cold-duration incubation, we found no differences in hatching success in the cold-temperature duration treatments (Table 3). However, the conclusions to be drawn from that second experiment are limited due to the relatively short duration of the longest exposure treatment in light of the results from our first experiment.

Our results show that in the constant-temperature experiment, cocoons held at different temperatures took varying amounts of time to hatch (Fig. 1b), with cocoons held at 10 °C hatching during the time period of 17–20 weeks (119–140 days), and cocoons held at other temperatures hatching during the first 10 weeks, if at all (no cocoons held in the 5 °C or 20 °C treatments hatched). The sudden and short-lived hatching period of those cocoons held at 10 °C suggest that *A. agrestis* cocoons near 10 °C require a specific amount of time (in this case, at least 119 days) in order for embryo development to occur, and that once that time has elapsed, mass emergence from cocoons may be expected. That possibility was reinforced by the findings of our second experiment, where shorter periods of expsoure to 10 °C (15, 30, and 60 days) were not long enough to induce hatching in most cocoons. Future studies investigating longer durations of temperatures near 10 °C could further clarify this matter. Additionally, questions remain about diurnal and seasonal temperature fluctuations, which can vary greatly across the invaded range of *A. agrestis*, and which might influence embryonic development and hatching dynamics. Studies that expose coccoons to temperature levels, durations, and rate of change that more closely approximate those they are likely to experience seasonally in natural settings may provide important insight into

Table 3. Model outputs for the logistic regression of the cold-duration incubation data (model 1 from Table 2). NS indicates nonsignificant differences, and SE indicates standard error.

Coefficients	Estimates (± SE)	*z*-value	*P*-value
Intercept	-2.28 (950.9)	-0.024	NS
Duration 30 days	5.67×10^{-10} (1.34×10^{3})	<0.001	NS
Duration 60 days	0.017 (950.9)	0.018	NS
Temp. 10 ºC	0.197 (950.9)	0.021	NS
Temp. 15 ºC	3.63×10^{-10} (1.34×10^{3})	<0.001	NS
Hatching week	0.116 (0.013)	10.997	<0.001
Duration 30 * Temp. 10 ºC	-0.470 (1.34×10^{3})	<0.001	NS
Duration 60 * Temp. 10 ºC	-17.26 (950.9)	-0.018	NS
Duration 30 * Temp. 15 ºC	-5.67×10^{-10} (1.34×10^{3})	<0.001	NS
Duration 60 * Temp. 15 ºC	-17.18 (1.65×10^{3})	-0.010	NS

potential climactic limits to invasion and degree of successful establishment in habitats with different climate regimes.

A closer examination of the hatching patterns we observed provides several further topics for discussion. Firstly, when hatching occurred at temperatures higher than 10 °C, it occurred earlier than when hatching occurred at 10 °C. This finding is consistent with observations made by Lowe and Butt (2005) of lumbricid earthworm species. However, in contrast to their observations, in our study, hatching success declined more than 40% at temperatures warmer than 10 °C. These results could indicate that extensive periods of time at temperatures higher than 10 °C are detrimental to developing *A. agrestis* embryos. This is notable as temperatures in the region are projected to warm (Kunkel et al. 2013), and could potentially alter the phenology and/or the successful hatching of this aggressive invader (Görres et al. 2018). Secondly, we found that hatching success at 10 °C differed significantly from 5, 20, and 30 °C, but not 15 or 25 °C. We suspect this may be due to high variability in hatching success, and low statistical strength due to low replication in our experimental design, and not necessarily a result of the experimental treatments. Finally, it is noteworthy that our experiment was designed to determine the optimum temperature for hatching in *A. agrestis*, and to do this we used 5 °C increments, thus leaving a ± 5 °C margin of error for our conclusion that 10 °C represents the optimal temperature. Clearly, finer resolution estimates for temperature optima for this species will require further study, employing smaller increments between tested temperatures and a greater number of replications to more clearly discern statistically significant differences.

In the assessment period of the cold-duration experiment, cocoons hatched at the same rate (weeks 7–21) regardless of the applied temperature or cold-duration treatments. Although these results were contrary to our hypothesis, they could suggest some type of cue for hatching that is not related to temperature or cold duration, but rather may be related to time since last cold exposure. However, results from our incubation at constant temperature experiment may contradict this idea because the highest rate of hatching was in the 10 °C treatment, in which cocoons were held until they hatched or until the end of the assessment period. However, it may be inappropriate to compare the timing of hatchings of the 2 experiments because cocoons held in the incubation at constant temperature trials were held at experimental temperatures until they hatched, or until the end of the assessment period, whereas the cocoons in the cold-duration incubation trials were at room temperature during the assessment period.

Across all treatments in both experiments, only 15% of cocoons hatched, suggesting that although temperature is an important factor for hatching in *A. agrestis*, other factors are likely involved in hatching success or failure. Moisture has been found to be a strong determinant in the spatial distribution of *A. agrestis* in the southern Appalachians (Snyder et al. 2011), and soil moisture and temperature are known to interact to affect growth and reproduction in other earthworm species (Presley et al. 1996). While cocoons used in this experiment were submerged in water, it is possible that *A. agrestis* cocoons have a narrow

range of suitable moisture levels that limited their hatching success in this study. In fact, subsequent studies (Ikeda et al. 2015) have used different approaches to determine cocoon viability, performing dissections of incubated cocoons to evaluate whether a live embryo is present, rather than waiting for cocoons to hatch. They did this because it is not clear that filter paper in a petri dish provides conditions ideal for emergence from cocoons (a process that may require abrasion or friction provided by mineral particles in the soil environment). Indeed, when Ikeda et al. (2015) performed such dissections on cocoons incubated for 16 weeks in petri dishes and filter paper, they found cocoon viability rates of up to 60% in a control group of cocoons. It should be noted, however, that dissected embryo viability is not considered the same metric as successful hatching, which was used here. Another possible consideration with respect to submersion of cocoons in our study is the relationship between dissolved oxygen (DO) concentrations in freshwater relative to temperature (cooler water has more DO than warmer water; Horne and Goldman 1994). It is possible that DO concentrations were low enough at the higher temperatures used in our incubations, that there may have been an effect on cocoon viability and/or hatching. However, the diffusion distances between atmosphere and cocoons were extremely small, and we do not expect that DO was limiting to embryo development. Further, this method for incubating cocoons has been reported to be successful and have no negative consequences for hatching (Butt 1991, Lowe and Butt 2005). A final factor that may have influenced our results is that adult worms were collected and held in the laboratory until their life cycle was complete. Therefore, cocoons were produced at some point between June and November, and we were not able to track cocoon age as it might relate to hatching success. However, because we randomly selected from the pool of all produced cocoons, it is likely that cocoons used in this study represented the entire age distribution. Future studies may investigate if age of cocoon is a useful factor in determining hatching success.

Invasive earthworms continue to spread in North America, and it is clear that they frequently become well established once introduced into deciduous forests (Szlavecz et al. 2018). *Amynthas* invasion has been described as far north as Vermont (Görres and Melnichuk 2012), Michigan (Greiner et al. 2012), and Wisconsin (Laushman et al. 2018), and it is suggested this invasion may continue into even colder climates. At least 1 record of occurrence exists for Canada, and it is expected that they will continue to colonize farther north (Moore et al. 2017). Further, winter hatching has been described in the northeastern US, though it is unclear how this might affect *Amynthas* populations over time (Görres et al. 2018). Further, the findings of Görres et al. (2018) emphasizes the observations of Chang et al. (2017a), that *A. agrestis* is frequently found in conjunction with 2 other morphologically similar species: *A. tokioensis* (Beddard) and *Metaphire hilgendorfi* (Michaelsen). Although we performed dissections on several individuals collected for these experiments to ensure they were, in fact, *A. agrestis*, we cannot entirely rule out the possibility that some of the adult worms used may have been representatives of these other species.

Exotic earthworms contribute to the general pattern of invasive species altering ecosystems in North America. An understanding of the life-history traits (e.g., time of hatching, time of maturity) of such organisms will be a critical first step to the development of management plans for ecosystems susceptible to invasion. We strongly encourage future studies that identify life-history traits of these organisms.

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