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The Relationship between Temperature and Development in *Globodera ellingtonae*

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Abstract: A new cyst nematode species, *Globodera ellingtonae*, was recently described from populations in Oregon and Idaho. This nematode has been shown to reproduce on potato. Because of this nematode's close relationship to the potato cyst nematodes, *G. rostochiensis* and *G. pallida*, an understanding of the risk of its potential spread, including prediction of potential geographical distribution, is required. To determine the development of *G. ellingtonae* under different temperatures, we conducted growth chamber experiments over a range of temperatures (10.0°C to 26.5°C) and tracked length of time to various developmental stages, including adult females bearing the next generation of eggs. Both the time to peak population densities of *G. ellingtonae* life stages and their duration in roots generally increased with decreasing temperature. Regression of growth rate to second-stage (J2) and third-stage (J3) juveniles on temperature yielded different base temperatures: 6.3°C and 4.4°C for J2 and J3, respectively. Setting a base temperature of 6°C allowed calculation of the degree-days (DD6) over which different life stages occurred. The largest population densities of J2 were found in roots between 50 and 200 DD6. Population densities of J3 peaked between 200 and 300 DD6. Adult males were detected in soil starting at 300 to 400 DD6 and remained detectable for approximately 500 DD6. By 784 to 884 DD6, half of the eggs in adult females contained vermiform juveniles. Given the similarity in temperature ranges for successful development between *G. ellingtonae* and *G. rostochiensis*, *G. ellingtonae* populations likely could survive in the same geographic range in which *G. rostochiensis* now occurs.

Key words: degree-day, development, *Globodera*, potato, soil temperature.

The potato cyst nematodes (PCN), *Globodera pallida* (Stone, 1973) Behrens, 1975 and *G. rostochiensis* (Wollenweber, 1923) Behrens, 1975, have been found on every continent except Antarctica and are quarantine pests in many countries (CABI, 2015). It has been estimated that in the United Kingdom, PCN cause a 9% loss of annual potato yield (Moxnes and Hausken, 2007). Although both species now have worldwide distributions, they both originate from South America (Evans et al., 1975). Transport patterns of these nematodes throughout the world are complex, with new populations often deriving from previously established non-South American populations (Subbotin et al., 2011; Boucher et al., 2013; Oro et al., 2014). Potato cyst nematodes can be transported to a new location by a variety of mechanisms including wind, water, animal movement (including on the shoes of humans), farm equipment, and transport of tubers (Turner and Evans, 1998). In many cases, PCN are considered to be invasive in their new environments (Singh et al., 2013).

In 2008, a *Globodera* type nematode was found in the United States in one Oregon and two Idaho fields with histories of potato production. This nematode was subsequently described as a new species in 2012: *G. ellingtonae* Handoo, Carter, Skantar, and Chitwood, 2012 (Handoo et al., 2012). Analysis of the internal transcribed spacer region of nuclear ribosomal DNA indicates that *G. ellingtonae* falls in a monophyletic group with both PCN and the tobacco cyst nematode *G. tabacum* (Lownsbery and Lownsbery, 1954) Behrens, 1975 (Skantar et al., 2011; Lax et al., 2014). Morphological and phylogenetic evidence indicate that *G. ellingtonae* also occurs in Chile and Argentina (Skantar et al., 2011; Lax et al., 2014). How the nematode arrived in Oregon and Idaho, how long it has been since first being transported from its presumable South American origins, and whether it was transported directly from South America or came via a secondary nonnative location is unknown. Because it has been shown to reproduce on potato, its status as a potato pathogen is of concern (Zasada et al., 2013; Lax et al., 2014).

To evaluate the risk of potential spread of this new nematode species, including prediction of potential geographical distribution, an understanding of the relationship between temperature and *G. ellingtonae* development is needed. As poikilothermic organisms, nematode developmental rates are closely tied to environmental temperatures, with optimal temperatures and upper and lower threshold temperatures varying between species (Trudgill, 1995). Potato cyst nematode populations are able to survive only where temperatures are great enough to allow completion of a life cycle by the end of a single growing season. Mature females develop into egg-filled cysts that overwinter to give rise to the next generation in the next growing season(s). Presumably, the different species of *Globodera* evolved optimal developmental temperatures corresponding with the temperature conditions during host growing seasons

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in their native regions (Grenier et al., 2010). However, boundaries of the native geographical ranges for the four *Globodera* species, both at recent and geological time scales, are uncertain (Oro et al., 2014). There is considerable overlap in current worldwide distributions of *G. rostochiensis* and *G. pallida*, with *G. rostochiensis* occurring in more countries than *G. pallida* (CABI, 2015). In South America, *G. ellingtonae* and *G. tabacum* have been reported in neighboring provinces of Argentina, but potential misclassifications of *Globodera* species, particularly before the description of *G. ellingtonae* as a new species, create uncertainty in records of species presence based on morphological evidence (Lax et al., 2014). In the United States, *G. pallida* is only known to occur in the state of Idaho, one of two states where *G. ellingtonae* has been detected, and *G. rostochiensis* only in the state of New York. Current known locations of *G. ellingtonae* provide little information on its optimal temperatures as it co-occurs regionally with *G. tabacum* (Lax et al., 2014), with the highest optimal temperature of the three *Globodera* in South America, and *G. pallida*, with the lowest optimal temperature, in Idaho.

Temperature optimums for each PCN have not only varied between studies but also within studies when different populations of the same species were included (Ellenby and Smith, 1975; Langeslag et al., 1982). There is general agreement that *G. rostochiensis* can continue development at higher temperatures than *G. pallida*, and *G. pallida* at lower temperatures than *G. rostochiensis*. Mugniery (1978) indicated that while developmental rates of PCN are fairly similar around 15°C, *G. pallida* develops more quickly than *G. rostochiensis* at temperatures less than 12°C and *G. rostochiensis* develops more quickly than *G. pallida* at temperatures greater than 18°C. Nonetheless, the developmental rates of both continued to increase as temperature increased up to 24°C (Mugniery, 1978). Similarly, reproductive values were higher for *G. pallida* than *G. rostochiensis* at temperatures less than 12°C and higher for *G. rostochiensis* at temperatures greater than 19°C (Foot, 1978). Hatching continued for *G. pallida* even when decreasing temperatures prohibited hatch of *G. rostochiensis* (Kaczmarek et al., 2014). *Globodera tabacum* has higher optimal temperatures than either PCN (Wang et al., 2001).

In this study, we conducted growth chamber experiments over a range of temperatures for which we tracked length of time to various *G. ellingtonae* developmental stages, including adult females bearing the next generation of eggs. Additionally, we propose a working base temperature with which accumulated degree-days can be calculated and used to predict developmental progress.

Materials and Methods

Growth chamber experiments: To establish the relationship between developmental rate and temperature, potato (*Solanum tuberosum*) was inoculated with *G. ellingtonae* eggs and grown at constant temperatures in two separate experiments. In 2013, plants were grown at 10°C, 15°C, and 20°C and in 2014, plants were grown at 17.2°C, 20°C, 23°C, and 26.5°C. Potato cv. Russet Burbank seed pieces treated with azoxystrobin and difenoconazole (Syngenta, Wilmington, DE) were started in trays of sterile soilless potting medium (Sun Gro Horticulture, Agawam, MA). When sprouts were approximately 6 cm tall individual stems were carefully separated and roots gently washed until most of the potting medium was removed. Plants were placed in a 10-cm diameter clay pot 1/3 filled with steam sterilized 1:1 sand:Willamette loam soil and inoculated with *G. ellingtonae* by pipetting 2 ml of a solution with ~1,250 eggs/ml water onto the roots. Each pot was then filled with the same soil for a total of approximately 500 g soil. To prepare the egg solution, cysts were freshly extracted from dried soil taken from field microplots at Powell Butte, OR, which had been planted with potato and inoculated with *G. ellingtonae* the previous growing season. The extracted cysts were crushed with a rubber stopper on nested 250- over 25-μm sieves, with eggs retained on the 25-μm sieve. Eggs were collected in water and repeatedly diluted and enumerated until the desired egg density was obtained.

Plants were grown in environmental chambers set to constant temperatures with 16 hr light/8 hr dark cycles. Incubation temperatures were recorded every 30 min throughout the experiment using WatchDog data loggers (Spectrum Technologies, Aurora, IL). For each chamber, all recorded temperatures were averaged to give the final "constant" temperature (thus the non-whole numbers for 17.2°C and 26.5°C). Individual temperature measurements varied no more than 1°C from the final average temperature. Plants were watered as needed and fertilized with a dilute N–P–K 20–20–20 (JR Peters, Allentown, PA) once a week. The experiments were initiated on 24 May 2013 and 12 February 2014. For each temperature, 68 pots were initiated to account for 17 sampling dates with 4 pots for each time point. In 2013, all temperatures were sampled on the same days after inoculation (DAI): 3, 5, 7, 10, 12, 14, 17, 20, 24, 27, 34, 41, 48, and 55. In 2014, sampling days differed by temperature based on predictions of peak developmental stages made from the 2013 data, with higher temperatures sampled more frequently initially to account for predicted faster transition between developmental stages. For 17.2°C, sampling days were 4, 7, 9, 13, 16, 19, 22, 26, 30, 33, 36, 40, 47, 54, 61, 68, and 77 DAI. For 20°C, sampling days were 3, 5, 7, 10, 12, 14, 16, 19, 22, 26, 29, 33, 40, 47, 54, 61, and 77 DAI. For 23°C, sampling days were 2, 3, 6, 8, 10, 12, 14, 16, 19, 22, 26, 29, 33, 40, 47, 54, and 77 DAI and for 26.5°C, sampling days were 2, 4, 5, 6, 7, 9, 10, 12, 14, 16, 19, 22, 26, 33, 40, 47, and 77 DAI.

Sampling methods: At each sampling day, four pots from each sampled temperature were destructively

harvested. Aboveground material was collected, dried, and weighed. The contents of pots were emptied into a container and roots were carefully removed from the soil so that disturbance to the surface of roots was minimized. To account for nematodes loosely attached to roots, the remaining soil clinging to the roots was rinsed with water into a 2-liter plastic beaker. To determine the number of adult males in the soil, the total weight of moist soil was determined and then a 250 g subsample was wet sieve extracted with males being collected on a 25-µm sieve, followed by sucrose gradient extraction (Byrd et al., 1966). The saved solution of soil washed from roots was also wet sieved by pouring the solution over nested 250- and 25-µm sieves. After sucrose centrifugation of 25-µm fraction, nematodes were again collected on a 25-µm sieve, washed into a 50-ml tube to a volume of 10 ml and duplicate 1 ml subsamples were counted using an inverted compound microscope (Leica, Buffalo Grove, IL). Combined total male counts in soil were calculated from both pot soil and root soil extractions. Once adult females were expected to have developed, the 250-µm sieve fraction from the root wash was saved and all females were collected and counted. Eggs from these females were enumerated by crushing females with a rubber stopper on a 125-µm sieve nested over a 25-µm sieve, washing the fraction collected on the 25-µm sieve with water into a 50-ml tube, and counting duplicate 1 ml samples. At the same time as enumerating eggs, the developmental state of the eggs were recorded, with the first 100 eggs counted from each sample categorized as either having a distinct vermiform shaped juvenile within the egg ("developed") or not.

The washed roots were collected, patted dry, weighed, stained with acid fuchsin, and stored in a 33% glycerol–33% lactic acid solution (Byrd et al., 1983). To extract stained nematodes from roots, roots were cut into ~3 mm pieces and processed with a blender in ~200 ml water for 20 sec on a high setting. The blended solution was poured over nested 250-, 125-, 25-µm sieves, washed well with water, and the contents of the sieves were washed separately into 50-ml tubes and adjusted to 10 to 20 ml volumes. Duplicate 1 ml samples from the 25- and 125-µm sieve fractions were counted using an inverted compound microscope. Adult females were enumerated from the entirety of the 250-µm sieve fraction under a dissecting scope. At the last sampling date, all soil from the pots was air-dried and cysts were extracted using a U.S. Department of Agriculture cyst extractor (Ayoub, 1980).

Base temperature calculation and statistics: Base temperatures were calculated by linear regression of the rate of growth on temperature and subsequent calculation of the x-axis intercept, i.e., the predicted temperature at which the rate of growth would equal zero (Trudgill, 1995). Rates of growth were calculated as the reciprocal of days required to reach the weighted mean peak population of infective second-stage (J2) and third-stage (J3) juveniles. The weighted mean peak was chosen as it decreases bias based on the choice of sampling days, since actual peaks may have occurred on a day that was not sampled. Tukey's honestly significant difference test was used to test for significant differences between temperatures in numbers of females and total number of eggs at the end of the 2014 experiment. All statistical analyses were performed and graphics made using the program R (R Core Team, 2014).

Results

The time to peak population densities of J2 in roots generally increased with decreasing temperature. At 26.5°C the peak infective J2 population occurred at 6 DAI, while at 10°C an initial peak was seen at 27 DAI and a second peak at 48 DAI (Fig. 1A). Concomitantly, the duration of the J2 population in roots generally increased with decreasing temperature, with sizeable presence (densities greater than 3% of maximum levels) extending over 48 days at 10°C to only 19 days at 26.5°C. There was evidence of multiple waves of infection of roots by J2, particularly at 10°C, 15°C, and 20°C, with the pattern sometimes extending into the J3 life stage. The trend of increasing duration of a life stage with decreasing temperature persisted for J3. The timing of peak occurrence for this life stage, however, showed less variation with temperature, with J3 at 20°C, 23°C, and 26.5°C all peaking at 14 DAI, 17.2°C at 22 DAI, 15°C at 24 DAI, and 10°C at 55 DAI, which was the last sampling day for that experiment (Fig. 1B). To adjust for similar peak population times but different overall duration of population presence at different temperatures, weighted mean times to maximum population densities were calculated and used to determine temperature-dependent growth rates. Peak population densities for fourth-stage (J4) juvenile males were 21, 24, 24 to 28, 35, and 41 DAI at 26.5°C, 23°C, 20°C, 17.2°C, and 15°C, respectively (data not shown).

Adult males began appearing in the soil at 19 to 20 DAI at 20°C, 23°C, and 26.5°C; 30 DAI at 17.2°C; and 41 DAI at 15°C and remained in the soil for greater than 3 wk at all temperatures (Fig. 2). No adult males were found at 10°C during the 55 days of the experiment. Presence of adult females was not sampled for in the first set of experiments until 48 DAI. At this time, a mean of 50 (±12 standard error) adult females were recovered at 15°C and 170 (±11) at 20°C. At 55 DAI, a mean of 93 (±26) adult females were recovered at 15°C and 100 (±27) at 20°C. No adult females were found at 10°C at either 48 or 55 DAI. In the second experiment, detection of adult females corresponded with the appearance of males in soil (data now shown).

Egg development occurred earlier as temperature increased from 17.2°C to 23°C (Fig. 3A). Vermiform juveniles were detected in 10% of eggs at 38, 47, and 57 DAI and in 50% of eggs at 52, 60, and 70 DAI at temperatures 23°C, 20°C, and 17.2°C, respectively. Egg development data obtained at 26.5°C were excluded from

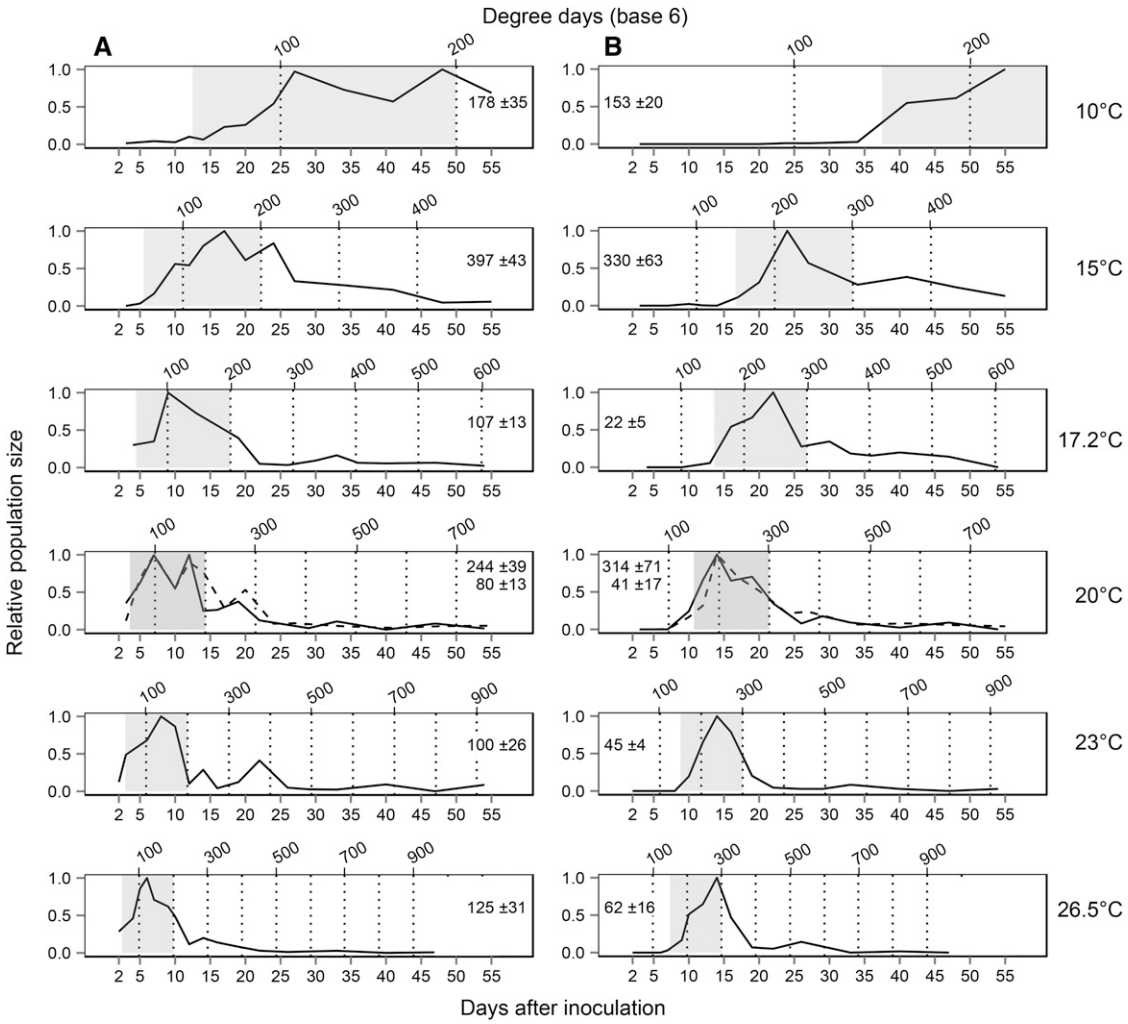

Fig. 1. Presence of *Globodera ellingtonae* (A) second-stage juveniles (J2) and (B) third-stage juveniles (J3) in roots at different temperatures across time. The y-axis is scaled in proportion to the largest mean population size, which is given (±standard error) on the right side of each box for J2 and the left side for J3, reached for each temperature. Days after inoculation are along the bottom x-axis, and accumulated degree-days above 6°C (DD6) values are along the top x-axis for each temperature graph. Vertical dotted lines delineate 100 DD6 intervals. The gray boxes highlight 50 to 200 DD6 for J2 and 150 to 300 DD6 for J3. The dotted line in the 20°C graph indicates 2013 data and the solid line indicates 2014 data.

analysis as our sampling schedule did not extend into the period of egg development at this temperature. At the final sampling, 77 DAI, there was a trend of increasing total cyst numbers as temperature increased from 17.2°C to 26.5°C (Fig. 3B), with significantly more females at 23°C and 26.5°C than at 17.2°C ($P < 0.05$). Total egg numbers (Fig. 3C), however, increased significantly between 17.2°C and 23°C, but significantly decreased between 23°C and 26.5°C ($P < 0.05$).

Base temperature calculations: Regression of growth rates, i.e., the reciprocal of days to weighted mean peak population densities, yielded different base temperatures: 6.3°C ($R^2 = 0.94$) and 4.4 °C ($R^2 = 0.97$) for J2 and J3, respectively (Fig. 4). Setting a base temperature of 6°C allowed determination of the accumulated degree-days above 6°C (DD6) at which different life stages occurred and subsequent comparison to studies with *G. rostochiensis*. The largest population densities of J2 were found in roots between ~50 and 200 DD6, although smaller numbers of J2 extended to 400 DD6 (Fig. 1A). The largest population densities of J3 were found in roots between ~150 and 400 DD6, generally peaking between 200 and 300 DD6 (Fig. 1B). Population densities of adult males started to increase in soil at about 300 DD6 between 15°C and 20°C but after 400 DD6 at 23°C and 26.5°C (Fig. 2). Once present in the soil, adult males remained detectable for an additional ~500 DD6. The DD6 when egg development was evident, i.e., ~10% of eggs had vermiform juveniles, were very similar at 17.2°C, 20°C, and 23°C: 640 to 660 DD6. The DD6 when 50% of eggs contained vermiform juveniles were more variable, with 884, 840, and 784 DD6 at 23°C, 20°C, and 17.2°C, respectively.

Discussion

Globodera ellingtonae showed developmental progress at all temperatures tested, from 10°C to 26.5°C.

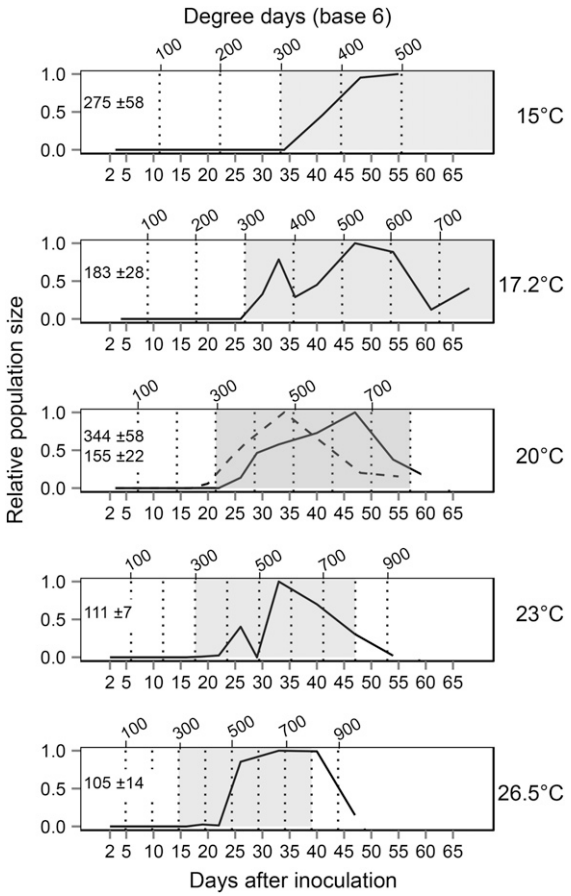

FIG. 2. Presence of *Globodera ellingtonae* males in soil at different temperatures across time. The y-axis is scaled in proportion to the largest mean population size, which is given (±standard error) on the left side of each box, reached for each temperature. Days after inoculation are along the bottom x-axis, and accumulated degree-days above 6°C (DD6) values are along the top x-axis for each temperature graph. Vertical dotted lines delineate 100 DD6 intervals. The gray boxes highlight 300 to 800 DD6. The dotted line in the 20°C graph indicates 2013 data and the solid line indicates 2014 data.

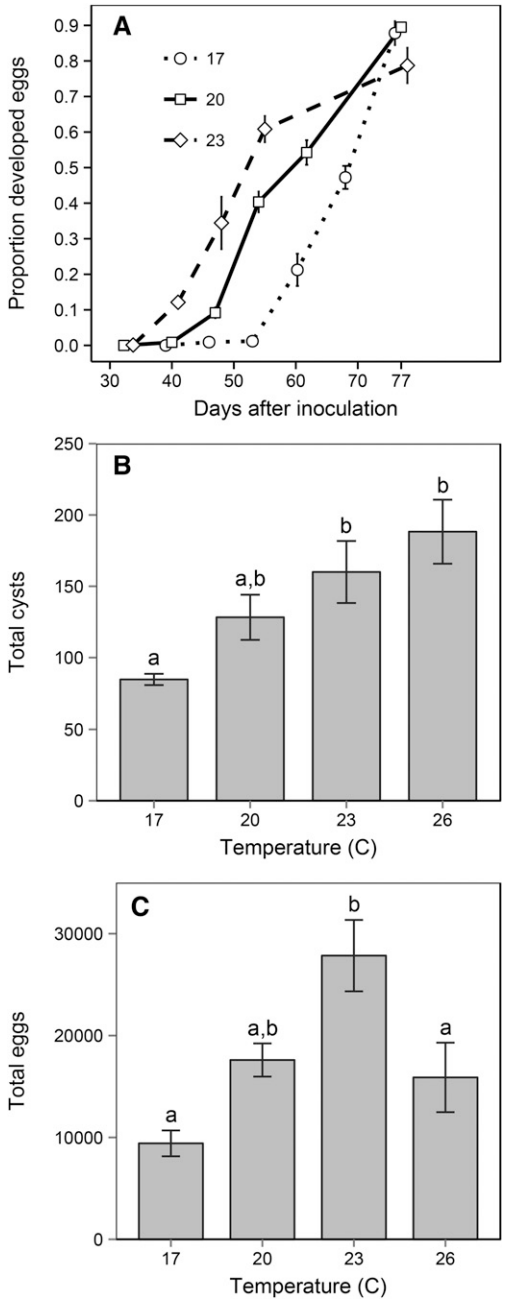

FIG. 3. (A) Proportion of developed eggs (containing vermiform juveniles) in adult females across time at temperatures 17.2°C, 20°C, and 23°C. (B) Mean cyst counts for the final sampling of the 2014 experiment at 77 days after inoculation (DAI) for temperatures 17.2°C, 20°C, 23°C, and 26.5°C. (C) Mean total egg counts from all cysts for the final sampling of the 2014 experiment at 77 DAI for temperatures 17.2°C, 20°C, 23°C, and 26.5°C. Each data point is the mean of four replicates. Error bars designate standard errors of means. Means with common letters do not differ significantly (Tukey's honestly significant difference; $P < 0.05$)

Multiple waves of infection were seen at 20°C and less. This is consistent with the observation of a slight increase in hatch rate at day 10, following the most pronounced hatch seen at day 3 after exposure to potato root diffusate (Zasada et al., 2013). Although reproductive stages were not reached within the 55-day observation time frame at 10°C, development from the J2 to J3 stages during that time indicates that 10°C is not a lower limit for development. There was no delay in J2 or J3 development at the highest tested temperature evaluated, 26.5°C. However, males appeared in soil at a later DD6 for temperatures 23°C and 26.5°C than the lower temperatures. It is possible that the transition from J3 to male is effected differently by temperature than other developmental transitions. Interestingly, although cysts at 77 DAI were more numerous at 26.5°C than at the other temperatures, they contained fewer eggs than the 23°C treatment, suggesting a potential upper limit of optimal egg development somewhere between those two temperatures. In this case a potentially confounding factor is temperature-based physiological changes in the host potato plant that result in indirect effects of temperature on nematode development. However, there was no evidence that potato plant growth was reduced at 26.5°C as compared to 23°C, at least up to the final biomass measurement of the 26.5°C

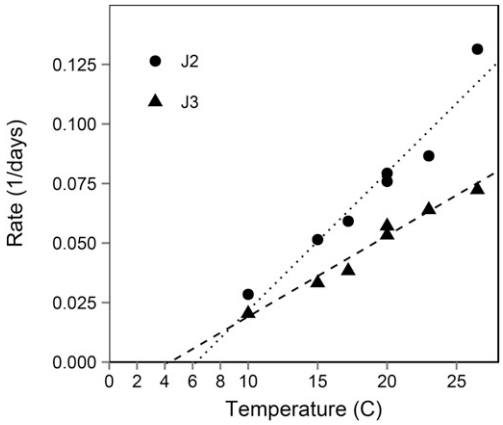

FIG. 4. Linear regression of rate of growth to reach second-stage (J2) and third-stage (J3) juveniles of *Globodera ellingtonae* at various temperatures. The y-axis is the reciprocal of weighted mean days post inoculation for nematodes to reach the associated developmental stage. Regression analysis based on length of time to J2 yielded a base temperature of 6.3°C (R^2 = 0.94) and time to J3 yielded a base temperature of 4.4°C (R^2 = 0.97).

treatment at 47 DAI (data not shown). More experiments at a range of higher temperatures will be required to determine at what temperatures developmental rate begins to decline and stop. However, when testing hatch rates of *G. ellingtonae* at different temperatures, we observed no hatch at 30°C during a 4-wk incubation period, but when those cysts where placed at 20°C they hatched at normal rates (unpubl. data).

Regulations of PCN precluded our directly comparing PCN developmental rates in the same experimental environment as *G. ellingtonae*. However, calculating a base temperature not only allows for prediction of developmental rates in new field situations, but also allows for comparison to studies in which the degree-days to different developmental stages of known PCN have been calculated. There is no single prescription for base temperature calculations (Trudgill, 1995). Generally, temperature is plotted against the inverse of time required to advance from a particular developmental stage to another chosen developmental stage, i.e., developmental rate (Trudgill, 1995). In many non-PCN systems, the time required for a complete life cycle, e.g., J2 to second generation J2, is used. However, second generation J2 may not immediately hatch from PCN eggs due to an entry into a varied duration of diapause after their maturation (Perry and Gaur, 1996), making the use of that time interval untenable. Another consideration in measuring time between stages is what marker during a developmental phase will be used as start and end points. This could be the first observation of an individual at that stage or when a certain proportion of individuals have reached that stage (Trudgill, 1995). A limitation of both, but particularly the former, is that the day of initial observation or peak is limited to the days on which sampling occurs. Due to that issue, we chose to use a weighted average time to maximum population density of each stage to calculate growth rates.

Use of varying developmental transitions to calculate base temperatures for other *Globodera* have yielded varying results. Mugniery (1978) used the time interval between combinations of stages ranging from J2 to J4 and calculated base temperatures ranging from 1.1°C to 5.5°C for *G. pallida* and 5.5°C to 6.9°C for *G. rostochiensis*. Using embryogenesis and two to three populations of both PCN species, Langeslag et al. (1982) calculated base temperatures in the range of 4.5°C to 6.8°C for *G. pallida* and 5.9°C to 6.3°C for *G. rostochiensis*, depending upon the population. Others have chosen a base temperature not by regression of developmental rates on different temperatures but by observation of the lowest temperature at which nematodes are detected in soil, observed to hatch or sustain developmental processes. A base temperature of 11°C has been proposed for *G. tabacum* guided by the observation that development ceased at 10°C but occurred at 12°C (Ambrogioni et al., 2000). Our calculations using times to different developmental points also resulted in slightly different base temperatures, 6.3°C based on time to J2 and 4.4°C based on time to J3, but were in the same range as those calculated by Mugniery (1978) and Langeslag et al. (1982) for the other PCN. We chose to use a base temperature of 6.0°C because we observed no hatch at temperatures <5°C (unpubl. data), and this temperature facilitates comparisons with development of *G. rostochiensis*, for which a DD6 is sometimes used.

The time between developmental stages of *G. ellingtonae* determined in this study was similar to those found for both PCN (Mugniery, 1978). Ebrahimi et al. (2014) tracked development of both *G. pallida* and *G. rostochiensis* concurrently in field microplots in Belgium. Because they used a base temperature of 6°C to calculate degree-days to developmental stages of *G. rostochiensis*, we can compare our results to theirs. The first *G. rostochiensis* J2 in roots, J3, J4, male, female, and cysts were observed at 145, 145, 216, 291, 291, and 401 DD6, respectively. Excepting the time to J2, these values are very similar to what we observed for *G. ellingtonae*. Grown concurrently at the same environmental temperatures, *G. pallida* developed more quickly than *G. rostochiensis* (Ebrahimi et al., 2014). Therefore, on the basis of the comparison with that study, the temperature–development relationship for *G. ellingtonae* is more similar to that of *G. rostochiensis* than of *G. pallida*.

Given the similarity in temperature ranges for successful development between *G. ellingtonae* and *G. rostochiensis*, *G. ellingtonae* populations likely could survive in the same geographic range in which *G. rostochiensis* now occurs. Although *G. rostochiensis* currently occurs in more countries worldwide than *G. pallida*, it is unclear whether environmental temperatures or historical distribution patterns (e.g., transport of nematodes

between countries, particularly on potato propagules) have played a larger role in determining their ranges. Clearly, transport of *G. ellingtonae* to new locations is undesirable, especially given the wide range of temperatures at which it will develop. Because development–temperature relationships can vary between populations of the same PCN species, the South American populations of *G. ellingtonae* may have different relationships to temperature than the Oregon population and will require separate testing.

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