Comp. Parasitol.
75(1), 2008, pp. 76–81

Hatching Behavior in *Heterodera glycines* in Response to Low Temperature*

EDWARD P. MASLER,[1,3] INGA A. ZASADA,[1] AND SANDRA SARDANELLI[2]

[1] Nematology Laboratory, Agricultural Research Service, U.S. Department of Agriculture, Beltsville MD 20705, U.S.A. and
[2] Plant Nematology Laboratory, Entomology Department, University of Maryland, College Park MD 20742, U.S.A.

ABSTRACT: *Heterodera glycines* eggs were exposed to low temperature (5°C) in the dark for various periods, and the effects of these treatments on hatching at 27°C were assessed. Low-temperature treatments caused a significant decrease in total percent egg hatch relative to untreated controls, but did not affect either the timing of egg hatch or the qualitative aspects of the hatch curve. Hatch curves comprised 3 distinct phases: hatch initiation (I), linear increase (II), and hatch rate decline (III). Hatch rates for all treatments were greatest during the first 12 d following hatch assay initiation, and rate decline occurred by day 14 regardless of treatment. Egg viability tests based upon vital staining demonstrated that refrigeration did not affect mortality, and monitoring progeny eggs obtained from plants inoculated with refrigerated eggs did not reveal any effect on hatching. Total percent hatch was directly dependent upon phase II linear increase rate. Depression of hatch by low temperature was not reversed when eggs were returned to 27°C. Results suggest that 1 or more developmental events were arrested, and that a diapause was induced.

KEY WORDS: developmental arrest, hatching, *Heterodera glycines*, low-temperature effect, plant parasite, soybean cyst nematode.

Plant-parasitic nematodes, including the soybean cyst nematode *Heterodera glycines*, are a serious threat to agriculture, accounting for more than $100 billion in annual losses globally (Chitwood, 2003). They present a continuing challenge for the development of new methods for control. Consequently, understanding fundamental biological events in the nematode life cycle is essential. Egg hatch in plant-parasitic cyst nematodes is a highly regulated event that ensures survival through heterogeneity of the egg population, thus preventing all eggs from hatching at once and reducing the risk of exposing newly hatched infective juveniles to conditions unfavorable for survival. Egg heterogeneity can vary with season and environmental conditions, and is the product of at least 2 types of eggs: those that hatch immediately with no specific stimulation, and those that express some form of dormancy, hatching only with passage of time and in the presence of specific environmental conditions (Zheng and Ferris, 1991; McSorley, 2003). Members of the dormant group respond to complex sets of physiological interactions among nematode, environment, and host plant (Rivoal, 1983; Hominick, 1986; Hill and Schmitt, 1989; Gaur et al., 2000). In examining survival strategies of plant-parasitic nematodes, extensive efforts have been directed toward dissecting the complexity of egg dormancy and hatching (Ishibashi et al., 1973; Hill and Schmitt, 1989; Perry, 1989; Zheng and Ferris, 1991; Yen et al., 1995; Perry, 2002), resulting in the assignment of eggs to various categories depending upon the type of hatching behavior demonstrated. Two major subcategories of dormancy are recognized: diapause and quiescence (Evans, 1987; Perry, 1989; Zheng and Ferris, 1991), each of which is characterized by a lowered metabolism and retarded development. Although recovery from quiescence usually follows a return to environmental conditions favorable to nematode survival and host infection, diapause is characterized by a more fundamental arrest of development, which can be released only after specific endogenous changes occur, even when favorable environmental conditions exist (Evans, 1987; Perry, 1989; Sommerville and Davey, 2002).

We are interested in determining how specific environmental factors affect development of *H. glycines* under conditions that minimize uncontrolled environmental cues. The purpose of the work reported here was to examine the qualitative and quantitative effects of extended exposure to low temperature (5°C) on hatching of *H. glycines* eggs.

[3] Corresponding author (email: Edward.masler@ars.usda.gov).

* Mention of trade names or commercial products in this publication is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the United States Department of Agriculture.

MATERIALS AND METHODS

Nematode rearing

For all experiments, *H. glycines* were reared with the use of methods modified from Sardanelli and Kenworthy (1997). Nematode-susceptible *Glycine max* cv. Kent seeds were

sown in 250-ml plastic beakers containing 240 ml of a sand:tap water mix (4:1 v:v; 4 seeds/beaker), and grown at 27°C with constant moisture (Sardanelli and Kenworthy, 1997) under a 16-hr light:8-hr dark photoperiod (Gro-Lux wide spectrum lamps, Osram Sylvania, Danvers, MA). Shoots appeared 5 d after sowing, and 3,000 *H. glycines* eggs were inoculated near the roots of each plant. Plants were periodically pruned to a maximum height of 28 cm and were harvested 5 wk after inoculation by washing the roots free of sand. Nematode females were then rinsed from the roots and cleaned from remaining sand and debris with tap water. Eggs were released by rupturing the females with gentle pressure by sliding a rubber stopper over an 80-mesh sieve. Eggs were collected onto a 500-mesh sieve, rinsed with tap water, counted in 50–100-µl aliquots with the use of a dissecting microscope (×40) and a Sedgewick Rafter counting cell (ProSciTech, Thuringowa, Australia). Total numbers of eggs collected were estimated by counting at least 4 separate aliquots. Eggs collected from 1 group of 5-wk-old plants comprised a single collection replicate.

Hatching experiments

Hatching was monitored with the use of modified Baermann funnels containing tap water (pH = 7) covering eggs distributed across the surface of a nylon mesh support. Eggs per funnel varied from 5,300 to 50,000 depending upon number of eggs available, but were always held constant among all funnels of the same experiment.

To determine the influence of low temperature exposure on egg hatch, freshly collected eggs were stored in 50-ml polypropylene screw-cap tubes (10,800 to 16,800 eggs/ml of tap water) at 5°C for 1–8 wk. This was repeated with eggs collected from 3–4 separate *H. glycines* generations to provide independent treatment replicates. Two aliquots of the refrigerated eggs were removed at weekly intervals, placed on separate funnels at 27°C for hatching, and juveniles were collected at 1, 3, 5, 7, 10, 12, 14, 21, 28, and 35 d. Control eggs were placed immediately onto funnels with no exposure to low temperature and juveniles collected as above. The mean percent hatch at each collection day was based on at least 4 replicates (3 for 28-d refrigerated eggs) obtained from different generations. Daily percent hatch was calculated as [number of juveniles collected/number of eggs placed on funnel] × 100. Total cumulative percent hatch (TCPH) was calculated as the sum of daily percent hatches starting with day 1.

Egg viability was assessed with the use of methods modified from Shepherd (1962). Freshly collected (control) eggs and eggs after storage at 5°C for various periods were incubated in New Blue R stain dissolved at 0.4 mg/ml in distilled water. Eggs were dispensed into 25-mm-diameter wells of a 12-well polystyrene plate (Corning Plastics, Corning, NY) at 1,000–2,000 eggs per well in 700-µl New Blue R solution and incubated overnight at room temperature (25°C). Eggs were rinsed in tap water and then examined (×40). Normal, intact eggs took up no stain and remained translucent or opaque white. Eggs containing color (pink, blue, purple) were considered nonviable (dead embryo, compromised shell) and scored as dead. The percent dead eggs relative to total eggs sampled was calculated using eggs from at least 4 separate collection replicates. This was done for each temperature treatment and controls. Viability was also assessed by inoculating groups of 12 *G. max* with eggs refrigerated for 4–8 wk. Five weeks after inoculation, females from the 12 plants were harvested and pooled, eggs were collected, counted, and placed on Baermann funnels for hatching. Nonrefrigerated eggs were used as controls. After 28 d, both the mean total egg recovery and the TCPH were compared between refrigerated and control groups ($n = 3$–5).

Statistical analyses

Comparisons of means across all treatments were analyzed by 1-way analysis of variance, and comparisons of 2 means were determined with the use of *t* tests. Statistical analyses were done with the use of SigmaStat 3.1 (Systat Software, Point Richmond, CA) and KaleidaGraph (Synergy Software, Reading, PA). Data are presented as the mean ±1 standard error mean (SEM).

RESULTS

Heterodera glycines eggs hatched readily in tap water at 27°C and produced a characteristic hatching curve comprising 3 distinct phases (Fig. 1). Phase I occurred during day 1, with immediate egg hatch in all treatments. Phase II started at the end of day 1 and continued through day 12. During phase II, the linear hatch period through day 12 (LHP_{12}) and the total cumulative percent hatch (TCPH) increased linearly for all treatments as well as for the controls ($r^2 =$ 0.94–0.99). Phase III commenced from day 12 and was characterized by a marked decrease in hatch in all treatments (Fig. 1). Thus, although eggs exposed to low temperature exhibited a decrease in TCPH relative to controls after return to normal rearing temperature (27°C), they retained the multiphase hatch curve (Fig. 1).

After 12 d, total cumulative percent hatch in 1-wk refrigerated eggs was significantly lower ($P < 0.005$) than in controls, and TCPH was further reduced through 4-wk refrigeration (Table 1). In contrast, no significant difference was detected in TCPH among all egg groups during phase III ($P = 0.079$; days 12–35, Table 1). Although refrigeration affected TCPH, it did not affect hatch patterns over time. Regardless of treatment, more than 80% of TCPH measured on day 35 had already occurred by day 12, and less than 20% of TCPH occurred between days 12 and 35 (Table 1). The percent hatch during LHP_{12} clearly determined the quantitative hatch response of all eggs and, therefore, the relationship between TCPH at day 35 and total hatch during LHP_{12} was linear (Fig. 2; $r^2 = 0.98$).

Although LHP_{12} hatch rates for all treatments were linear, significant differences in TCPH between controls and refrigerated eggs appeared by day 5 (Fig. 1), when the mean TCPH for control eggs (30.11 ± 8.18) exceeded that for eggs refrigerated 5–8 wk (6.71 ± 1.29; $P = <0.008$). By day 7, all

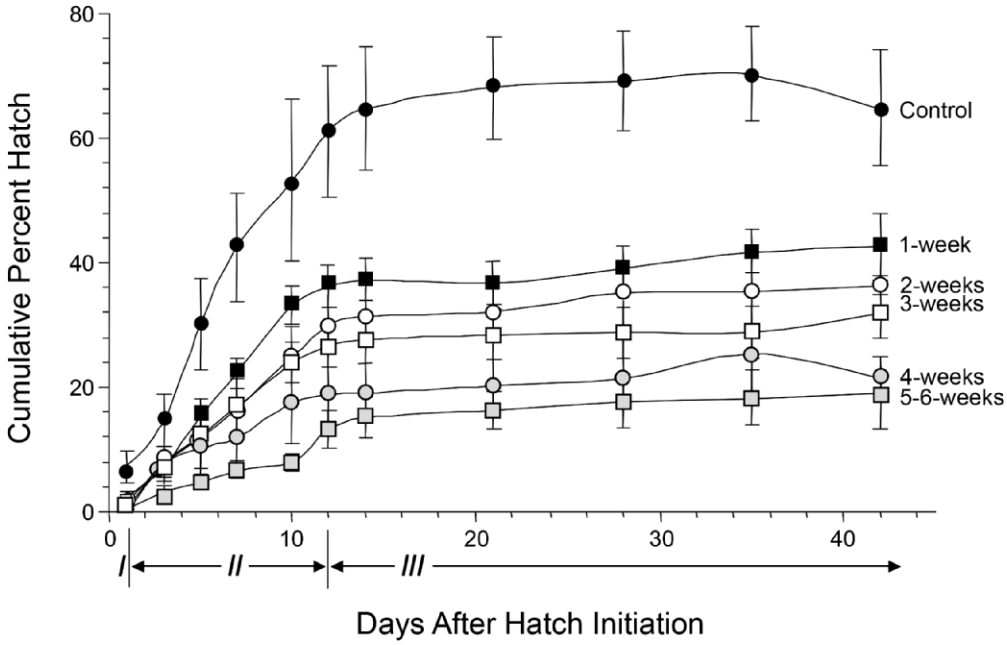

Figure 1. Effect of exposure to 5°C on the hatching of *Heterodera glycines* eggs. Data are expressed as mean ± SEM of total cumulative percent hatch on the indicated day. Means were derived from data collected from 4 separate *H. glycines* generations (except 4-wk refrigerated eggs, n = 3). Below the x axis, I, II, III indicate 3 phases of hatch curves as described in text.

treatments resulted in significantly lower hatch than the control (Fig. 1) ($P = <0.005$), and this trend was maintained through day 12 (Fig. 1; Table 1). Clearly, the slopes of the hatch curves during LHP_{12} varied among the treatments, and ranged from a maximum of 5% hatch/d for controls, to less than 1% hatch/d for eggs refrigerated 8 wk. The relationship between LHP_{12} slopes and length of refrigeration is shown in Figure 3. Length of refrigeration did not have a linear effect on hatch rate, as the regression ($r^2 = 0.93$) best fit a polynomial. Figure 3 demonstrates that the single largest effect on hatch occured within 1 wk of refrigeration, where the slope (3.2% hatch/d) had decreased 65% from the control. Also, the trend line suggests that refrigeration longer than 8 wk may have minimal additional effect on hatch rate.

Experiments to determine the consequences of low temperature on egg health found no effect on egg integrity or mortality (Table 2). Percent mortality remained between 15 and 20% regardless of treatment ($P = 0.21$), whereas TCPH at 12 d was significantly reduced ($P < 0.05$) in all treatments (Table 2). Because mortality was not affected, it cannot account for the decrease in hatch. Not surprisingly, refrigeration affected the number of eggs recovered 5 wk after *G. max* inoculation with treated eggs. The mean total number of eggs recovered from groups of 12 plants inoculated with control eggs ($4.9 \times 10^5 \pm 7.3 \times 10^4$) was more than 3.5-fold greater ($P = 0.007$) than the mean recovery from plants inoculated with eggs refrigerated 4–8 wk ($1.4 \times 10^5 \pm 1.810^4$). However, the hatching of progeny eggs derived from refrigerated eggs was not affected, because the mean TCPH at 28 d of all recovered eggs did not differ ($P = 0.369$) between the progeny of the control eggs ($TCPH_{28} = 65.28 \pm 8.54$) and progeny of refrigerated eggs ($TCPH_{28} = 55.00 \pm 5.54$).

DISCUSSION

Temperature is a regulator of metabolic rates in poikilotherms such as plant-parasitic nematodes and developmental rates are reduced when temperature declines (Van Gundy, 1965; Alston and Schmitt, 1988; Tzortzakakis and Trudgill, 2005). Depressing metabolism can retard development and prevent an animal from attaining what might be considered a critical developmental stage. Development beyond this critical stage cannot proceed without the action of

Table 1. Comparison of *Heterodera glycines* egg hatch through day 12 and between days 12 and 35 at 27°C following exposure to increasing lengths of time at 5°C. Eggs were collected, stored at 5°C for the times indicated, and hatched on Baermann funnels at 27°C over a period of 35 d.

	Hatch period			
	Days 0–12		Days 12–35	
Weeks at 5°C	Percent hatch*	(Proportion)†	Percent hatch*	(Proportion)†
0	60.9 ± 10.5 a	(86.8)	9.3 ± 3.7	(13.3)
1	36.1 ± 3.2 b	(91.2)	3.5 ± 0.9	(8.8)
2	29.9 ± 4.6 b,c	(90.3)	3.2 ± 0.9	(9.7)
3	26.6 ± 3.4 b,c	(91.7)	2.4 ± 0.9	(8.3)
4	18.7 ± 6.5 b,c	(83.1)	4.0 ± 2.2	(17.6)

* Total mean percent egg hatch ± SEM for the period indicated. Each mean represents 4–6 independent replications of *H. glycines* egg collections and were compared by 1-way ANOVA. Day 0–12 means followed by the same letter are not significantly different ($P < 0.005$). Day 12–35 means were not significantly different ($P = 0.079$).

† Proportion of total egg hatch (TCPH) over 35 d occurring during the period indicated.

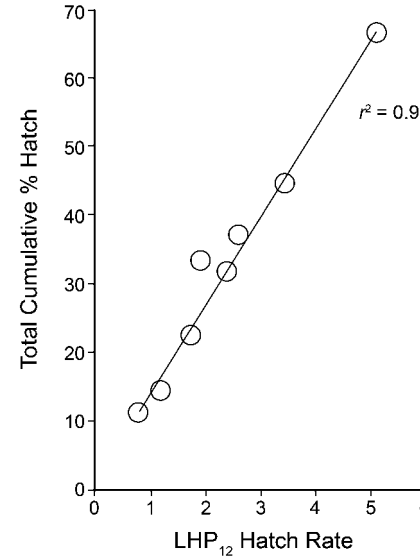

Figure 2. Relationship between total cumulative (%) hatch of *Heterodera glycines* eggs at 35 d and the hatch rate (slope) during the linear hatching period of 12 d (LHP_{12}). Best-fit curve by linear regression.

internal molecular signals. Issuance of these signals requires more than a simple return to normal conditions, and may depend upon several factors, including environmental state, host plant signal, and time. Hatching of *H. glycines* eggs was reduced by exposure to low temperature, a treatment chosen to mimic an environmental factor to which eggs may be exposed in the field. Treatments were conducted on freed eggs to minimize confounding influences from the cyst, which contains hatch-inhibiting and -stimulating factors (Okada, 1972a, b; Ishibashi et al., 1973; Okada, 1974; Charlson and Tylka, 2003), to reduce interactions among eggs that may occur at high egg densities within the cyst, and to minimize residual influence from the host plant by rinsing freed eggs in water. Under these conditions, the egg and developing embryo respond primarily to the treatment, reduced temperature, whereas other environmental influences are minimized. The effect of this response was a decrease in total egg hatch, but qualitative aspects of the *H. glycines* egg-hatching curve were clearly independent of treatment. All curves retained the characteristic 3-phase form, and a near-immediate hatch of a small percentage of eggs in phase I was always observed. Such rapid hatch has been reported in studies on egg hatch from *Heterodera schachtii* cysts (Zheng and Ferris, 1991), and indicates a class of eggs requiring no further development to hatch; they may be considered fully hatch developed. The percent of these fully hatch developed eggs decreased as time of exposure to low temperature increased, a result that also fits with the observation of reduced nematode reproduction on *G. max* inoculated with refrigerated eggs. However, even eggs refrigerated for up to 8 wk hatched at a low level in phase *I*. While low temperature should not have any long-term effect on fully hatch-developed eggs, it may alter those eggs in less advanced stages of development. It is notable that Alston and Schmitt (1988) estimated the basal temperature threshold for development of *H. glycines* to be 5°C. This suggests that low-temperature treatments can depress metabolism and prevent development to a critical stage. Such conditions should cause dormancy in some eggs that are not fully hatch developed. It would be of interest to explore the effects of additional low temperatures on hatch and hatch recovery.

Dormancy is also indicated by the linear phase II of the hatch curve during which only the hatch rate and not its linearity was affected by treatment. During this period, only eggs that were hatch committed proceeded to hatch. They were not prepared for immediate hatch and required various degrees of further development. Those eggs that eventually hatched must have attained a critical developmental stage by the time of first exposure to low temperature, such that their return to 27°C restored their normal metabolic, developmental, and linear hatch rates. This

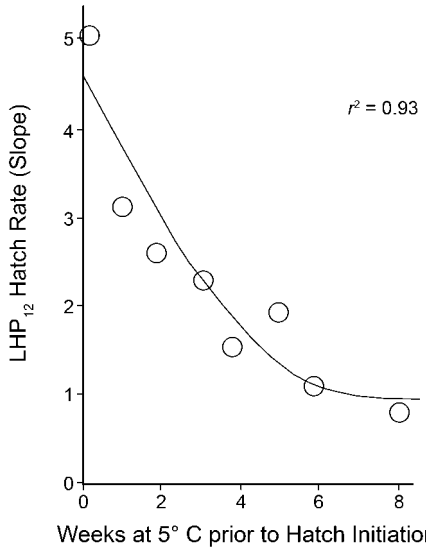

Figure 3. Relationship between length of refrigeration of *Heterodera glycines* eggs and hatch rate (slope) during the linear hatching period of 12 d (LHP_{12}). Best-fit curve by nonlinear (polynomial) regression.

linear hatching must be a basic and robust feature of cyst nematode egg hatch activity, as it has also been demonstrated with cyst nematodes under quite different experimental conditions (Zheng and Ferris, 1991; Sikora and Noel, 1996).

Given the robust nature of *H. glycines* egg hatch, those eggs that had not reached the critical developmental stage necessary for hatching, and were thus unable to resume development following a return to 27°C, must have been fundamentally changed. A consequence of this change was a developmental arrest that may have been induced by endogenous signals responding to altered metabolism and/or to low temperature *per se*. The nonlinear relationship between phase II linear hatch rate and length of time of refrigeration can be explained if a range of developmental stages was present, with some stages more sensitive to low temperature induction of arrest than others. Eggs that resumed development after a return to normal conditions were likely not fundamentally affected by the treatment, because the same multiphasic and qualitative aspects of hatch patterns that characterize control egg hatch were observed in all of the treated eggs. The developmentally arrested eggs that did not hatch may require additional cues, beyond simple restoration of normal conditions, to resume development, and are probably in diapause. Whatever the physiological effects of refrigeration on hatching, they do not carry through to the progeny,

Table 2. Effects on hatch and mortality of *Heterodera glycines* eggs stored for increasing lengths of time at 5°C. Eggs were collected, stored at 5°C for the times indicated, and either hatched on Baermann funnels at 27°C or incubated in New Blue R at 25°C as described. Each mean represents 4–6 independent replications of *H. glycines* egg collections and were compared by 1-way ANOVA.

Weeks at 5°C	Cumulative percent hatch*	Percent mortality†
0	61.0 ± 10.4 a	15.5 ± 1.1
1	36.0 ± 2.8 b	15.3 ± 1.6
4	18.6 ± 5.6 b	15.0 ± 2.0
6	15.2 ± 3.0 b	19.7 ± 1.6

* Cumulative percent hatch values are the means ± SEM measured 12 d after initiation of hatch test. Means followed by the same letter are not significantly different ($P < 0.05$).

† Percent mortality values are the means ± SEM for eggs examined from each of the corresponding treatments. Means were not different ($P = 0.214$).

because TCPH was the same regardless of refrigeration treatment of parent eggs.

The third feature of the hatch curves was a dramatic decline in the hatch rate after day 12, the timing of which was not affected by treatment. The low hatch rate during phase III is present for all treatments including the control, and may represent a separate category of normally slow-developing eggs, what Zheng and Ferris (1991) call "delayed development."

Because all qualitative features of *H. glycines* hatch curves were shared by treatments and the control, the major effect of the treatments was quantitative. If low temperature simply slowed metabolism, one would expect that eggs returned to normal conditions would regain normal metabolic rates and approach the total percent hatch of the control. For example, ongoing work in our laboratory on nematode egg hatch in soil (Zasada and Tenuta, 2005) indicates that significant suppression of *H. glycines* egg hatch can occur at pH > 10. In preliminary in vitro experiments, we found that elevated pH suppressed egg hatch but that this suppression was rapidly reversed by a return to tap water. No hatching differences were observed between these recovered eggs and controls. Such a reversal was not the case with low temperature, suggesting that the mechanisms of hatch suppression by these 2 treatments were different, and not *de facto* linked to developmental arrest. The argument is made, then, for the induction of diapause by low temperature, in which development was arrested and was not restored even after a return to normal conditions (Perry, 1989; Sommerville and Davey, 2002).

Two different types of diapause have been described (Evans, 1987; Zheng and Ferris, 1991). Facultative diapause is induced by environmental conditions, and is overcome only with time and the presence of amenable conditions. Obligate diapause is required for normal development, is regulated by endogenous messengers, but is overcome with time and specific internal and external conditions (e.g., temperature, soil conditions, influence of host plant). Although it was not possible with our present experiments to determine the type of diapause expressed by *H. glycines* eggs, the additional diapause induced by low temperature was likely of the same type exhibited by the subset of control eggs that did not hatch, because only quantitative differences were observed. Further, it would be interesting to identify the molecular components involved in dormancy and diapause of hatch-developed and arrested nematode eggs.

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