Nematology, 2011, Vol. 13(7), 837-844

Effects of cyst components and low temperature exposure of *Heterodera glycines* eggs on juvenile hatching *in vitro*

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Received: 29 September 2010; revised: 14 December 2010
Accepted for publication: 15 December 2010; available online: 2 March 2011

Summary – The effects of low temperature treatment of *Heterodera glycines* eggs and the interaction of this treatment with egg condition and cyst influences were examined *in vitro*. Exposure of eggs to 5°C for 1 week followed by a return to normal culture temperature resulted in a 25-33% reduction in hatch after 2 weeks at 28°C but there was no effect on the timing of hatch. Hatch from encysted eggs was 40% lower than from free eggs at 2 weeks, and hatch from low temperature-encysted eggs was more than 60% lower during the same period. Encystment also altered the timing of hatch relative to free eggs from the same cohort. Hatch from free eggs in the presence of cyst contents was accelerated relative to free eggs without cyst contents, but the total cumulative percent hatch was not increased. Reduction in hatch as a result of low temperature treatment was significant only if the treatment was applied prior to the first juvenile stage (J1). J1 were not affected relative to the hatch of second-stage juveniles (J2). However, the effect of low temperature on earlier stages was not detected until development ceased at early J1 and later J1. Also, low temperature treatment affected the apparent locomotion of some newly hatched J2; 16-fold more J2 from treated eggs were retained on 30 μm pore sieves than those from control eggs. The depression of hatch by low temperature egg treatment was apparently the result of the residual effects on early embryo stages, leading to arrest of development prior to J2.

Keywords – cyst, development, embryo, endogenous factors, environmental factors, hatch depression, plant-parasitic nematodes, soybean.

Annual crop losses caused by plant-parasitic nematodes, including the soybean cyst nematode, *Heterodera glycines*, the most devastating pathogen of the soybean *Glycine max*, can be costed at billions of dollars worldwide (Chitwood, 2003). The importance of *G. max* to world agriculture and the challenges to the control of *H. glycines* (Niblack *et al*., 2006) require the continuing development of new control strategies, and this development must be supported by a fundamental understanding of the biology and behaviour of *H. glycines*. Such understanding is essential for the informed selection of vulnerable life cycle stages, such as hatching, that can provide targets for control.

Hatching in cyst nematodes is a highly regulated event (Perry, 2002) dependent upon a number of life cycle stages, and is designed to ensure survival by reducing the exposure of newly hatched second-stage juveniles (J2) to unfavourable conditions (Niblack *et al*., 2006). Hatching is a function of the complex integration of endogenous and environmental factors that influence the development and behaviour of the embryo and of the pre-hatch juvenile, and efforts have been made toward evaluating these factors in cyst nematodes (Okada, 1972a, 1974; Zheng & Ferris, 1991; Perry, 2002; Pridannikov *et al*., 2007).

We have integrated low temperature treatment of *H. glycines* eggs with various combinations of egg preparations to examine the interactions of external and internal factors on *H. glycines* hatch *in vitro*, and have examined embryo development over an extended period. Results indicate that factors suppressing hatch are additive, and demonstrate for the first time that low temperature treatment of early *H. glycines* embryos has a delayed effect on development.

Materials and methods

NEMATODES, REARING AND EGG COLLECTION

Heterodera glycines (NL1-RHp) was raised on soybean (*G. max*, cv. Kent) grown in sand-filled beakers us-

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Also available online - www.brill.nl/nemy
DOI:10.1163/138855410X552689

ing tap water in a constant moisture system (Sardanelli & Kenworthy, 1997), at 28°C and 16 h L : 8 h D photoperiod. Tap water was used throughout all assays to maintain consistency with rearing conditions. Cultures were inoculated with *ca* 2000 *H. glycines* eggs plant^{-1} and 5 weeks later plants were harvested, roots rinsed clean with water and cysts collected by gently stripping them from the roots. Cysts were rinsed with water and selected individually for uniformity under a dissecting microscope (20×) using jeweller's forceps. Depending upon the experiment, uniform batches of cream-coloured/light yellow lemon-shaped cysts and/or uniform batches of yellow lemon-shaped cysts were prepared. Eggs were obtained from individual cysts by crushing the cyst in water using a jeweller's forceps. Depending upon the experiment, eggs were either used directly or were first cleaned free of cyst materials by gentle rinsing with water using a micropipette. The selection and transfer of individual eggs were done using a Pasteur pipette drawn to a fine capillary over a low flame.

ASSAYS

Control and experimental treatments

For each assay, control groups (eggs or cysts) were incubated at 28°C immediately upon setup of the assay (day 0). Observations and counts were initiated on day 0 and continued through to day 14. Treatment groups of eggs or cysts were set up in assay plates in parallel with controls but the plates were immediately placed at 5°C. After incubation for 7 days at 5°C, the treatment plates were transferred to 28°C; the day of transfer was considered day 0 for the treatment groups. Observations and counts were initiated on day 0 and continued through to day 14.

Cyst integrity

Individual, uniform cysts (yellow, lemon-shaped) were either left intact or crushed individually in water to release eggs. The released eggs were rinsed 2-3× with water to remove the cyst wall and contents. Single, intact cysts or eggs prepared from single cysts (100-200 eggs), were transferred to wells of 24-well polystyrene assay plates (Falcon, Beckton Dickinson, Franklin Lakes, NJ, USA) containing 500 μl of water. For each experiment, two identical plates (control and treatment) were prepared, each with 12 replicate cysts and 12 replicate egg preparations. In egg wells, all eggs and J2 were counted on day 0, and J2 were counted daily thereafter. In the cyst wells, J2 were counted daily. On day 14, cysts were crushed and all eggs and non-emerged J2 were counted. The experiment was repeated once ($n = 24$ for each of four treatments). Percentage hatch from free eggs was calculated as (((J2 on day$_x$ − J2 on day$_0$)/eggs on day$_0$) × 100). Percentage hatch from encysted eggs was calculated as ((J2 on day$_x$)/(J2 on day$_x$ + (J2 + eggs in cyst day$_{14}$)) × 100).

Direct and indirect cyst contents hatch assays

Cysts were collected, sorted for uniformity as above, and transferred to 200 μl PCR tubes (Fisherbrand, Fisher Scientific, Pittsburgh, PA, USA) containing 50 μl water (1 cyst per tube). Each cyst was crushed with a jeweller's forceps to release eggs, and the entire cyst wall-cyst content-egg (typically 100-200 eggs) preparation was transferred to an individual well of a 12-well polystyrene assay plate (Falcon) containing 2 ml of water. This procedure was used for direct hatch measurement assays.

For indirect hatch measurement assays, cyst wall-cyst content-egg preparations were obtained as above, and transferred onto 12 mm diam. sieves fitted into the plate wells containing 2 ml of water. Sieves were prepared with Netwell plate inserts (Corning Costar, Corning, Corning, NY, USA) consisting of a *ca* 11 mm diam. plastic collar supporting a 500 μm pore polyester mesh. A circle of 30 μm pore nylon mesh (Spectrum Laboratories, Rancho Dominguez, CA, USA) was placed on top of the 500 μm mesh and secured in place with a tight-fitting ring cut from a polypropylene tube. The entire assembly was placed in the plate well, suspended just above the well bottom. This method served to keep hatched J2, which moved through the mesh into the plate well, physically separated from the egg and cyst pieces that were retained on the sieve.

Each plate comprised 12 replicates and plates were prepared as control and treatment pairs. For the direct assay format, eggs and J2 were counted on day 0, and J2 were counted daily thereafter. For the indirect assay format, only J2 were counted daily from day 0 through to day 14. On day 14, all eggs and J2 retained on the sieve were counted. The experiment was repeated once ($n = 24$ for each of four treatments). Direct percent hatch was calculated as (((J2 on day$_x$ − J2 on day$_0$)/eggs on day$_0$) × 100), and indirect percent hatch was calculated as ((J2 on day$_x$)/(J2 on day$_x$ + (J2 + eggs on sieve day$_{14}$)) × 100).

Individual egg assay

Cysts were selected for uniformity as above, and cream-coloured/light yellow and yellow cysts were used. Eggs were released in water by crushing the cyst with a jeweller's forceps, and then rinsed 2-3× with water

to remove the cyst wall and contents. Using the Pasteur capillary, individual eggs were transferred to wells of a 96-well microtitre plate (Stripwells; Corning Costar) with 100 μl water in each well. For each experiment, a set of 15-46 eggs was picked from 3-8 different cysts, with approximately half of each set of eggs used for the control group and half for the treatment group. Plates were covered with perforated microtitre plate sealing tape (X-Pierce, Excel Scientific, Victorville, CA, USA) and kept in a humid chamber to prevent evaporation.

After transfer to the plate well, each egg was examined with an inverted microscope, at 40× and 100× magnification as needed to observe selected features, and assigned to one of four development stages. Assignments were based upon an arbitrary four-step visual classification scale with an associated numerical score. Eggs that were opaque or dark with distinct uniform spherical structures were considered as early embryos and classified as EE (score = 0.5). Eggs that showed signs of change, including non-uniform shading, irregular interior structures, or both, were termed early first-stage juvenile (J1e; score = 1). Eggs containing an obvious vermiform structure were classified as J1 (score = 1.5). The final category was hatched J2 (score = 2). Classification was done by four different people to minimise personal bias.

For each of six experiments, cysts from a different harvest were used to obtain eggs. Within each experiment, all eggs were scored every day for 14 days, and the percent of eggs in each stage was calculated as (number of individuals in stage$_x$/total number of individuals in EE + J1e + J1 + J2)×100. Data are expressed as the mean percent of each stage on each day across all experiments (n = 6). With additional experiments, the numerical scores for all individual eggs on day 14 were used to compute the mean numerical scores for six groups of eggs based upon stage on day 0 (EE, J1e, J1) and treatment (control, low temperature). Each group included 49-208 individual eggs (N).

Data analysis

Individual means were compared using Student's t-test with Mann-Whitney unequal sample size post-test or paired observations as appropriate. Multiple means were compared using one-way ANOVA with Tukey's Multiple Comparison Test. P is indicated for each comparison. Data exploration and statistical analyses were done using JMP (SAS Institute, Cary, NC, USA) and GraphPad Prism (GraphPad Software, La Jolla, CA, USA) computer software programs.

Results

Encysted *vs* free eggs

Hatching from encysted and free eggs generated distinctly different hatch curves (Fig. 1). The entire 14-day curves for both of the free egg groups (control and treated) could be fitted non-linearly (4th order polynomials; r^2 = 0.99), whereas the 14-day curves for the encysted egg groups were linear (r^2 = 0.98). However, all curves were linear ($r^2 \geqslant 0.98$) through to at least day 8. The daily hatch rates from free eggs were highest through to day 8, at 12.2% and 8.1% for the control and treated groups, respectively. Rates declined sharply between days 8 and 14 to 1.8% (control) and 2.0% (treated). By contrast, the daily percentage hatch from encysted eggs remained constant through to day 14 for both the control (4.4%) and treated (2.8%) groups. Also, the means for cumulative percentage hatch from free eggs differed signif-

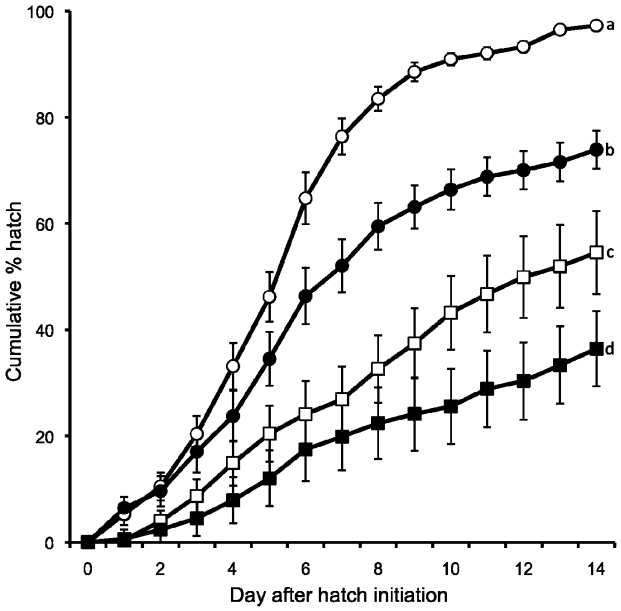

Fig. 1. *Comparison of hatching of second-stage juveniles (J2) of* Heterodera glycines *from control and 5°C treated eggs obtained from individual cysts or from eggs contained within control and 5°C treated cysts. Percentage hatch from free eggs was calculated as ((J2 on day$_x$ − J2 on day$_0$)/eggs on day$_0$) × 100. Percentage hatch from encysted eggs was calculated as ((J2 on day$_x$)/(J2 on day$_x$ + (J2 + eggs in cyst day$_{14}$)) × 100). Means at 14 days were compared by Student's* t*-test and those followed by different letters are significantly different (*P < *0.05, encysted control* vs *encysted treated;* P < *0.005 all others). Free control:* ○*; free treated:* ●*; encysted control:* □*; encysted treated:* ■.

icantly by day 7 (control *vs* treated; $P < 0.05$), whereas the means for cumulative percentage hatch from encysted eggs did not differ significantly ($P < 0.05$) until day 10.

Despite these differences in hatching behaviour, hatch from both free and encysted eggs was suppressed by low temperature treatment. Mean cumulative percent hatch on day 14 from free eggs was 97.2 ± 0.69 for controls *vs* 73.9 ± 3.53 for the treated group ($P < 0.005$). The mean cumulative percentage hatch from encysted eggs on day 14 was also greater ($P < 0.05$) in the control group (54.5 ± 7.85) than in the treated group (36.4 ± 7.09). Comparisons between the two egg environments showed that total cumulative percentage hatch from encysted eggs was lower than that from free eggs when either the control ($P < 0.005$) or treated ($P < 0.05$) groups were analysed. Finally, the number of J2 remaining in cysts opened on day 14 represented the same mean percentage of all J2 in both the control and treated groups (14.1 ± 5.73 and $13.4 \pm 3.48\%$, respectively; $P > 0.5$).

Direct and indirect hatch assay

Hatching dynamics were similar whether J2 hatch was observed directly or indirectly using sieves to separate eggs from hatched J2. The shapes of the hatch curves, as well as the timing of hatch rate changes, were the same for all four groups (Fig. 2). All curves were linear through to day 8 ($r^2 \geqslant 0.98$), with daily hatch of 7.8% and 5.6% for direct control and direct treated groups and 6.6% and 6.3% for indirect control and treated groups, respectively. Beginning on day 9, hatch rates began to decline in all of the groups, and between days 9 and 14, the percentage hatch per day was between 1.7% (direct controls) and 0.9% (indirect treated). Based upon the daily percentage hatch values, hatching was approximately 80% complete by day 8 for all groups.

Differences between the cumulative percentage hatch means of the direct control ($46.1 \pm 3.13\%$) and direct treated groups ($34.7 \pm 3.66\%$; $P < 0.05$) appeared on day 5, whereas significant differences between the cumulative percent hatch means of the indirect control (64.1 ± 4.15) and indirect treated (54.1 ± 3.23) groups ($P < 0.05$) were not apparent until day 9. On day 9, the cumulative percent hatch for each control group was significantly higher ($P < 0.05$) than that for the corresponding treated group (direct treated, $48.3 \pm 2.33\%$).

No significant differences were observed between total cumulative percentage hatch means of the control groups or between total cumulative percentage hatch means of the treated groups at any time during the assay period. Notably, however, the number of J2 remaining on the sieves at day 14 differed significantly ($P < 0.001, n = 24$) between the control and treated groups. Based upon the percent of all the J2 that had hatched, only $0.4 \pm 0.62\%$ remained from the control group but 16-fold more ($6.8 \pm 0.64\%$) remained from the treated group.

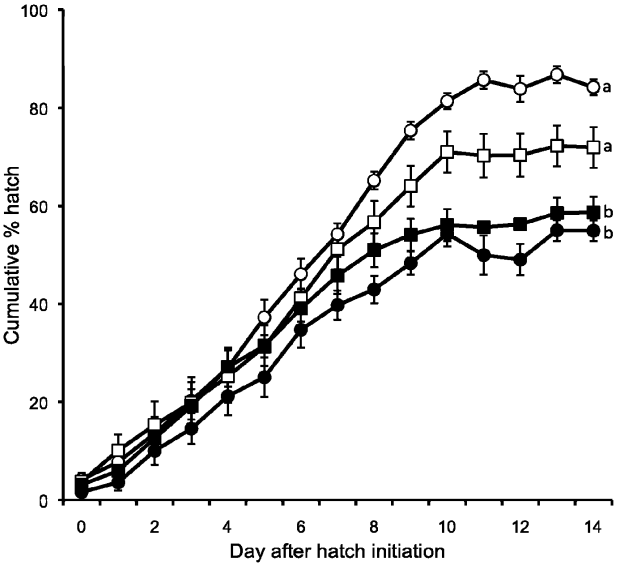

Fig. 2. *Comparison of hatching of second-stage juveniles (J2) of* Heterodera glycines *from control and 5°C treated eggs obtained from individual cysts with direct and indirect J2 counting. Direct percentage hatch was calculated as ((J2 on day$_x$ − J2 on day$_0$)/eggs on day$_0$) × 100. For indirect percentage hatch calculations, all eggs and juveniles remaining on the sieves at 14 days were counted and percentage hatch determined as ((J2 on day$_x$)/(J2 on day$_x$ + (J2 + eggs on sieve day$_{14}$)) × 100). Means at 14 days were compared by Student's* t*-test and those followed by different letters are significantly different (*P < 0.005). *Direct control: ○; direct treated: ●; indirect control: □; indirect treated: ■.*

Individual egg observations

Treatment and observation of individual eggs revealed hatching results similar to those using pooled eggs, but revealed additional features of hatch dynamics as well. Individual observations clearly defined a three-part hatching curve for both the control and treated groups (Fig. 3A). Between days 0 and 5, the daily hatch rates were 1.1% for controls and 0.3% in the treated group, but significant differences between the cumulative percentage hatch means of the two groups first appeared on day 7 ($P < 0.05$). Between days 6 and 9, the daily hatch

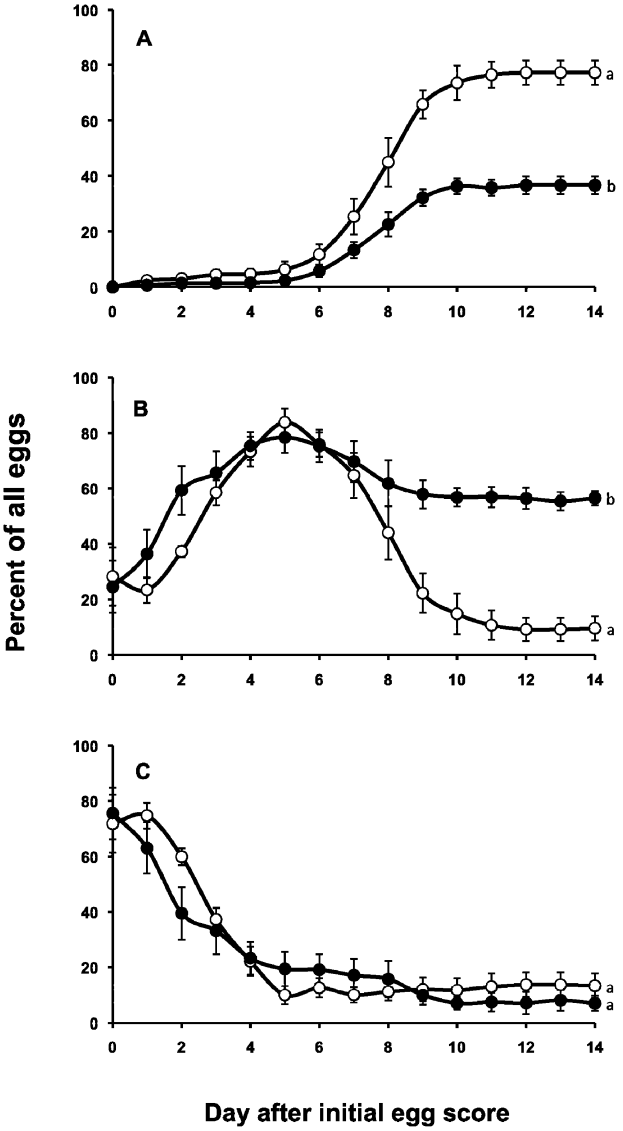

Fig. 3. Heterodera glycines *development in response to 5°C treatment of eggs followed by recovery at 28°C assessed by observation of individual eggs. Individual* H. glycines *eggs were collected, and incubated either immediately at 28°C (control) or stored at 5°C for 7 days (treated) and then incubated at 28°C. All eggs were scored for developmental stage daily for 14 days as described. Four stages (EE, J1e, J1, J2; see text) were assigned and the percent of each stage was calculated as: (number individuals in stage$_x$/total individuals in EE + J1e + J1 + J2) × 100. This was done for a minimum of six experiments, and each data point represents the mean ± standard error percentage across all experiments (*n = 5-6*). Means on day 14 were compared using Student's* t*-test, and means followed by different letters are significantly different (*P < 0.01*). Control data are indicated by open circles (○); treated data by solid circles (●).*

rates increased to 18.2% and 7.9% in the control and treated groups, respectively. By day 10, daily percentage hatch rates declined to below 1% for groups. The final (day 14) total cumulative percentage hatch mean for controls ($77.2 \pm 4.37\%$) was more than twice that for the treated group ($36.6 \pm 3.15\%$; $P < 0.01$).

Changes in percentage hatch (*i.e.*, appearance of J2) were accompanied by corresponding changes in the percentages of eggs containing embryos in the J1e/J1 stages (Fig. 3B). In each group, the category percentage peaked at day 5 (means were not different) then declined by day 9 to $57.9 \pm 5.12\%$ for controls and $22.2 \pm 7.13\%$ for the treated group ($P < 0.05$). The percentage for the control group declined further, to a low of $9.6 \pm 4.41\%$ on day 14, whereas the percentage in the controls remained steady ($54.5 \pm 2.56\%$ on day 14).

Most remarkable was the fate of the EE ("early embryo") stage eggs (Fig. 3C). While they represented between 70 and 80% of all individuals in both the control and treatment groups on day 0, the EE stage accounted for only $13.2 \pm 4.53\%$ (controls) and $6.87 \pm 2.73\%$ (treated) on day 14, and these means were not different ($P > 0.5$). Analysis of numerical scores of more than 700 individual eggs (Table 1) revealed that by day 14, the level of development, including J2 hatch, was the same for all control eggs regardless of the embryo stage on day 0. The J1 stage was not affected by low temperature treatment since the mean day 14 numerical score was the same as those for all of the controls. By contrast, exposure of embryos at stages EE or J1e on day 0 to low temperature resulted in significantly reduced ($P < 0.05$) day 14 numerical scores.

Discussion

Metabolism and development in poikilotherms such as plant-parasitic nematodes are processes dependent upon environmental temperatures and the rates of these processes can decline at reduced temperatures. Hatching in plant-parasitic nematodes is the result of highly regulated metabolic and developmental events that are responsive to both endogenous and environmental factors, including temperature. Such biological mechanisms are necessary to ensure population survival in the field. They are also features that provide useful experimental approaches with which to examine plant-parasitic nematode

A: Comparison of daily percentage of J2; B: Comparison of daily percent of J1e + J1 (combined). C: Comparison of daily percent of EE.

Table 1. *Developmental scores of control and 5°C-treated* Heterodera glycines *eggs on day 14 of incubation at 28°C as a function of egg score at day 0.*

Initial stage	Group	Day 14 numerical score
EE (208)	Control	1.8 ± 0.03^{a}
J1e (49)	Control	1.9 ± 0.05^{a}
J1 (121)	Control	1.9 ± 0.02^{a}
EE (191)	Treated	1.4 ± 0.04^{b}
J1e (53)	Treated	1.5 ± 0.06^{b}
J1 (112)	Treated	$1.7 \pm 0.04^{a,c}$

Individual *H. glycines* eggs were collected, incubated and scored as described in Materials and methods. Day 14 numerical scores for all eggs, including those that had produced J2, were sorted into six groups according to the initial (day 0) stage (EE, J1e, J1) and control or treated status. Group mean numerical scores were compared by one-way ANOVA with Tukey's Multiple Comparison Test. Means followed by different letters are significantly different ($P < 0.05$). Numbers in parentheses are the number of eggs scored (n) in each group. EE, early embryos; J1e, early first-stage juvenile; J1, first-stage juvenile.

behaviour and development *in vitro* and *in vivo* (Charlson & Tylka, 2003; Masler *et al*., 2008).

One such experimental approach involves the exposure of eggs to low temperature, followed by a period of recovery, and the evaluation of subsequent residual effects on development and hatching (Masler *et al*., 2008). We have expanded the examination of these responses, and incorporated egg condition with temperature treatment to generate a detailed view of *H. glycines* hatch dynamics and to reveal some fundamental hatch behaviours.

Hatch of J2 from encysted eggs was significantly lower than from free eggs, in concert with reports comparing hatch from *H. glycines* egg mass eggs and encysted eggs (Thompson & Tylka, 1997) and the evidence for hatch depressive compounds in cyst contents (Okada, 1972a; Pridannikov *et al*., 2007). Since the effects of low temperature and encystment on hatch suppression were additive, different biochemical and molecular mechanisms were probably affected by the two conditions. This is not surprising if low temperature affected a general depression of metabolic activity and encystment exerted more specific effects. In addition to possible endogenous hatch suppressors *per se*, confining eggs in tight contact with each other might facilitate chemical communication that represses hatch.

Hatching from *H. glycines* eggs exposed to low temperature for 1 week never fully recovered to control levels (Masler *et al*., 2008), suggesting a fundamental developmental arrest. Since the present experiments extended for 14 days, the usual limit to our analyses of free eggs, we cannot say whether the final cumulative percentage hatch from encysted eggs would have approached that of free eggs, but the shapes of the hatching curves suggest that this might only be a remote possibility.

However, the hatching curves strongly indicate that total hatch from encysted eggs exposed to low temperature would never approach that of control encysted eggs. Thus, whatever developmental suppression encystment caused, it must have been enhanced by low temperature and was confined to J2 hatch, since the low temperature component did not affect the ability of hatched J2 to leave the cyst. What these suppression factors are is an important issue. Does the cyst respond chemically to low temperature with an increase in natural hatch suppressors? Does low temperature induce developmental arrest in some encysted eggs? The results of the cyst-low temperature experiments – generation of four similar but highly distinct hatching profiles – indicate that combinations of multiple suppression strategies can be a powerful approach to the discovery of hatch control mechanisms.

Low temperature suppression of hatch was equally measured by direct and indirect assays, as neither the control nor treated hatch curves differed between assay types. However, the subtle but significant difference between the retention of J2 on sieves requires more examination. The greater number of J2 from treated eggs that were retained was not enough to account for the difference in total cumulative percent hatch between control and treated groups. Although the retained J2 may simply indicate slow residual hatching following the drop in hatch rate, they may have been less capable of locomotion than their cohorts, and less likely to move through the sieve. This suggests a small but important residual effect of the low temperature treatment on J2 viability. Each of these possibilities is now being examined for biochemical or behavioural differences between retained and other J2.

Hatch curves for the free eggs in the direct-indirect experiments and the encystment experiments had different profiles. In one case, hatching was essentially over by day 10, and in the other it continued at least through to day 14. An important distinction between the two experimental methods was in the preparation of the eggs. In the encystment experiments, freed eggs were rinsed and removed from cyst contents. In the direct-indirect comparison experiments, freed eggs were always in contact with cyst components and the eggs were not

rinsed. These preparation differences, and the differences in the timing of hatch rate changes, suggest that the cyst contents accelerated hatching but did not increase total percentage hatch. Increased hatch in the presence of cyst and egg components has been reported for *H. glycines* (Okada, 1972b, 1974; Charlson & Tylka, 2003), and *Globodera rostochiensis* (Pridannikov *et al*., 2007) but total percent hatch was much lower than in the present work. Cyst contents, then, may affect both the timing and level of hatching.

The effects of low temperature on *H. glycines* eggs were confined to developmental stages prior to the appearance of distinct J1. Alston and Schmitt (1988) demonstrated that development from early stage *H. glycines* embryos was linearly dependent upon temperature, and presented preliminary data suggesting that development could continue at a basal level at 5°C, allowing for some development to J2 after increasing the temperature to 24°C; however, these observations were based upon a small sample. We have conclusively demonstrated, with large numbers of eggs, that early-stage *H. glycines* embryos indeed recover from long-term low temperature exposure and proceed with development. In addition, our data show that development from early stage embryos proceeded at the same rate in both the control and treated groups, and that on day 14 there was no difference in early embryo numbers between the groups. Thus, the decreased percent hatch observed in the treated group cannot be attributed to an arrest of development in early stages. In addition, the J1 stage was not affected by low temperature treatment relative to J2 development.

We thus conclude that low temperature treatment of *H. glycines* eggs results in a residual effect on development that causes an apparent arrest at the J1 stage, and that this residual effect is induced sometime during the EE or J1e stages, before the J1 is formed. Understanding developmental arrest at any life-cycle stage is important for developing nematode control strategies, and discovering the mechanisms involved in the delayed arrest observed here will be an important next step. Various possibilities, ranging from modifications in the eggshell that could preclude hatch to metabolic changes that might result in a type of diapause or dormancy, all need to be examined. Nevertheless, a fundamental and persistent change in *H. glycines* development was induced by the low temperature exposure.

Acknowledgements

We thank our student interns, Ms Alyssa Metzler and Ms Asmita Nagarkar, for expert technical assistance in the preparation and scoring of individual egg assays, and Dr David Chitwood for his critical reading of the manuscript. 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 US Department of Agriculture.

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