Stability of *Heterodera glycines* (Tylenchida: Heteroderidae) juvenile hatching from eggs obtained from different sources of soybean, *Glycine max*

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Summary – The hatching behaviours of *Heterodera glycines* second-stage juveniles (J2) from eggs obtained from laboratory and glasshouse cultures, and from the field, were examined *in vitro* using large scale (Baermann funnel) and small-scale (depression slide) assay designs. Hatching of J2 from cultured eggs was robust, whereas J2 from field eggs hatched very poorly *in vitro* ($<1\%$ total cumulative percentage hatch). Qualitative aspects of hatch were stable as hatching of J2 from eggs from all sources was linear from 2 through to 8-10 days. By contrast, quantitative aspects were more variable. Total cumulative percentage hatch typically ranged from 45 to 70% but approached 90% depending upon the source of the cultured eggs. Egg density (eggs/cm^2) affected hatch of J2 in the large scale *in vitro* system, with total cumulative percentage hatch significantly greater at 3100 eggs/cm^2 than at 420 eggs/cm^2. The poor hatch of J2 from field eggs was lost after two generations in culture and replaced by the typical hatch characteristics of J2 from eggs from an established culture. This included both qualitative and quantitative elements. Stability of *H. glycines* hatching behaviour and its reflection of dormancy and diapause of J2 are discussed.

Keywords – adaptation, behaviour, cyst nematode, diapause, dormancy, hatch assay, plant culture.

Hatching of second-stage juveniles (J2) from eggs of cyst nematodes is a result of complex interactions among nematode, host plant and environment. It is a portion of the nematode's life cycle that has been extensively researched and comprehensively reviewed (Jones *et al.*, 1998; Perry, 2000). Hatching behaviours of cyst nematode populations in the field are critical to nematode survival, and these behaviours are highly coordinated to exploit the availability of suitable plant hosts and to avoid exposure to unfavourable environmental conditions (Yen *et al.*, 1995; Sikora & Noel, 1996; Charlson & Tylka, 2003). Understanding hatching behaviours may lead to management tools that would reduce crop loss due to nematode damage.

A central strategy in this coordinated hatch is temporal. Not all J2 hatch at one time. In *Heterodera glycines*, eggs are distributed between an external gelatinous mass and

the cyst, with J2 from external mass eggs hatching first (Thompson & Tylka, 1997). In *H. glycines* and other cyst nematodes, response to environmental cues causes varying proportions of infective J2 within a cyst to hatch. The unhatched J2 remain dormant in the cyst until additional cues, which may change with conditions and season, occur (Ishibashi *et al.*, 1973; Zheng & Ferris, 1991; Gaur *et al.*, 1995; Thompson & Tylka, 1997). In addition to the influence of external factors, hatching is controlled by factors within the egg and the cyst (Okada, 1972a, b, 1974; Charlson & Tylka, 2003). To deal with this complex physiological system while investigating hatch regulation in *H. glycines*, we used rearing and hatching conditions that were designed to reduce influences from the outside environment. These approaches helped to reveal the stability of *H. glycines* hatch kinetics, illustrate changes in a *H.*

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glycines population transferred from field to culture, and detect egg influences on hatching.

Materials and methods

NEMATODES AND REARING

Heterodera glycines (population NL-RHp) had originally been collected from fields in the Eastern Shore region of Maryland and subsequently cultured in the laboratory for over 10 years, at approximately ten generations per year. Cultures were maintained on soybean, *Glycine max*, cv. Kent, at 27°C and a 16 h light : 8 h dark photoperiod. Cultures were grown in sand in 300 ml beakers, using a soil moisture control system (Sardanelli & Kenworthy, 1997). Standard rates of inoculation of *G. max* were 2000 *H. glycines* eggs per plant, four plants per beaker, done 5 days after *G. max* germination. New field population samples were prepared from *H. glycines* cysts collected from the Eastern Shore region of Maryland, near the original NL-RHp field collection site, during midsummer. The soybean field was not in commercial use and had characteristically loamy soil. The new *H. glycines* collection was termed Eastern Shore Field (ESF). Some of the collected ESF eggs were used to initiate a new laboratory population of *H. glycines*, cultured as described above, and referred to as Eastern Shore Culture (ESC). Other ESF eggs were used to set up a long-term (1 year) observation of *H. glycines* field eggs (9400 ESF eggs) maintained on a Baermann funnel (see below) in tap water. In Tennessee, *H. glycines* glasshouse cultures (TGC) were maintained on *G. max* cvs Bedford and DP415.

EGG COLLECTION AND JUVENILE HATCHING

Heterodera glycines cysts were harvested from laboratory plant culture by rinsing roots of infected plants under a gentle stream of tap water while massaging roots by hand to release cysts. Cysts were collected on a 150 μm pore sieve, then transferred to a beaker of tap water and rinsed five times by gentle agitation and decanting to separate cysts from sand and other debris. Cysts were collected from field soil samples by agitation in tap water and decanting as above. Individual cysts were collected by using a pair of jeweller's forceps, cleaned from debris by rinsing in tap water, and collected on standard metal sieves (250 or 150 μm pore). Eggs were released from rinsed cysts using gentle pressure from a rubber stopper ground against a 180 μm pore sieve in

tap water. Released eggs were collected on a 35 μm pore sieve. Egg totals were calculated from representative aliquots counted on a Sedgewick-Rafter cell (Probing and Structure, Thuringowa, Queensland, Australia) under 60× magnification. Hatching was monitored using Baermann funnels with 6000-88 000 eggs per funnel containing 120 ml tap water. Each funnel was fitted with a nylon mesh (3 mm gaps) disc covered with Scotties® tissue (Kimberly-Clark, Philadelphia, PA, USA). Eggs were distributed on top of the tissue located 3-5 mm below the water surface, and incubated at 27°C. Hatched J2 were removed and counted daily, using the same counting method as for eggs.

IN VITRO CULTURE

Heterodera glycines (NL-RHp) were also maintained in sterile root culture consisting of *G. max* cv. Kent root explants grown on 1.5% nutrient agar with Gamborg's medium (Huettel, 1990). Females were harvested by hand, and the external gelatinous mass was separated from the cyst using jeweller's forceps. Encysted eggs were collected by the rupturing of individual cysts using forceps and then extruding the eggs into water. Eggs from cysts or external masses containing eggs were transferred separately into wells of glass depression slides, 4-125 eggs/ 0.5 ml distilled water per well, and incubated at 27°C. J2 were counted daily to monitor hatching.

Data are expressed as means ± standard error, and means are compared using *t*-values and one-way ANOVA.

Results

Hatch of *H. glycines* was remarkably robust and consistent, as illustrated by the hatching patterns of J2 from eggs obtained from *G. max* plant culture and *G. max* sterile root culture (Fig. 1A) and from within the cyst or within the external mass (Fig. 1B). Hatching kinetics in the large-scale Baermann system (plant culture eggs) and the small-scale depression slide system (sterile root culture eggs) were nearly identical. In each system, hatching increased linearly ($r^2 = 0.98$-0.99) through 10 days, then slowed markedly. The total cumulative percentage hatch was also similar in each system (63.9 ± 4.8%, n = 24, funnels; 67.5 ± 4.8%, n = 71, depression slides). The data in Figure 1A for the sterile root culture eggs represent the combined hatch of J2 expressed from cysts and eggs present in the external mass. When data from these
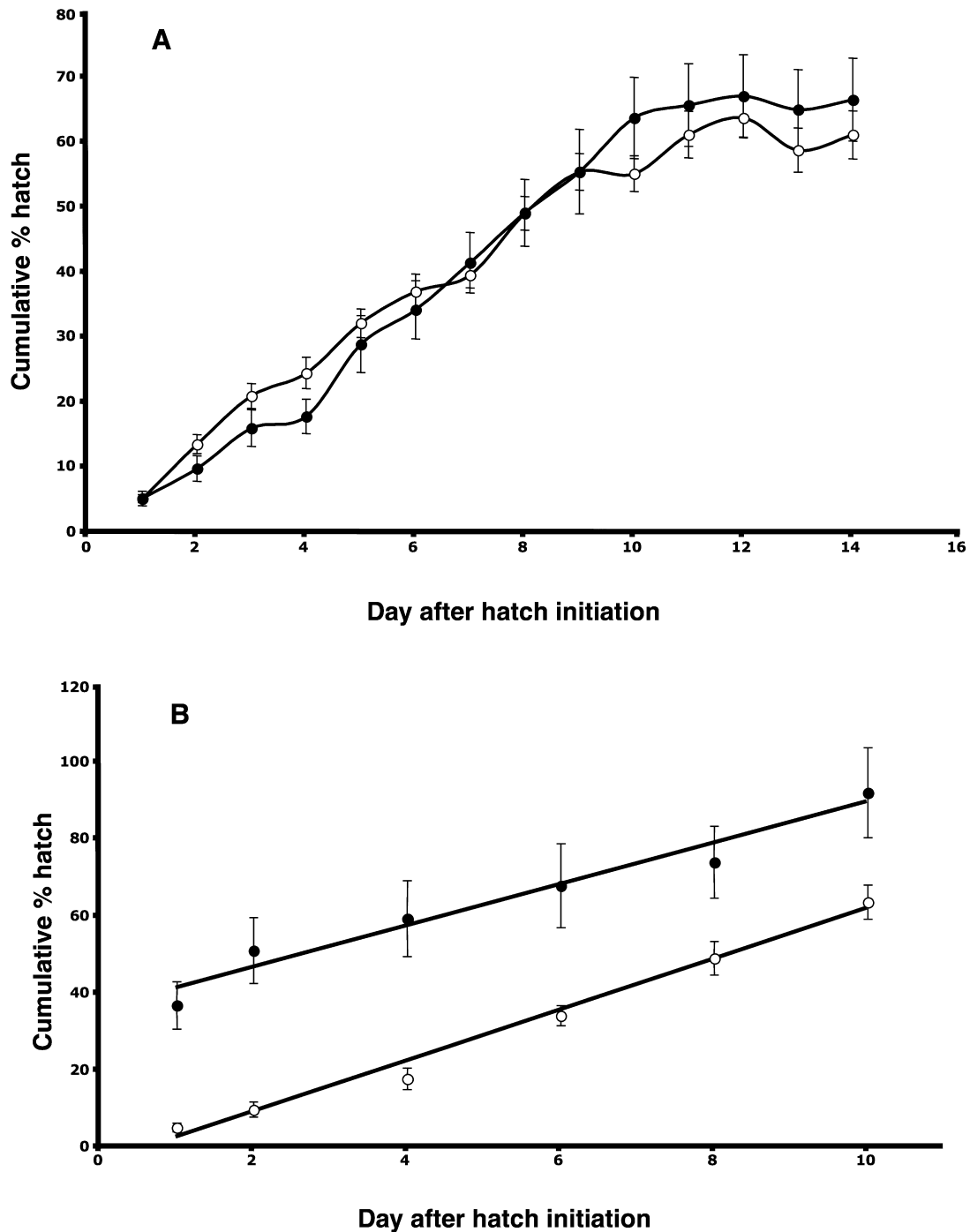
Fig. 1. *A: Comparison of patterns of* Heterodera glycines *juvenile hatch from eggs either mass collected from* Glycine max *plant culture and assayed in a large scale Baermann funnel system (open circles) or collected from females hand picked from* G. max *sterile root culture and assayed using a micro scale depression well system (filled circles); B: Hatch curves illustrating linearity of hatch from root culture eggs either expressed from cysts (open circles; $r^2 = 0.96$) or present in the external gelatinous mass (filled circles; $r^2 = 0.99$).*
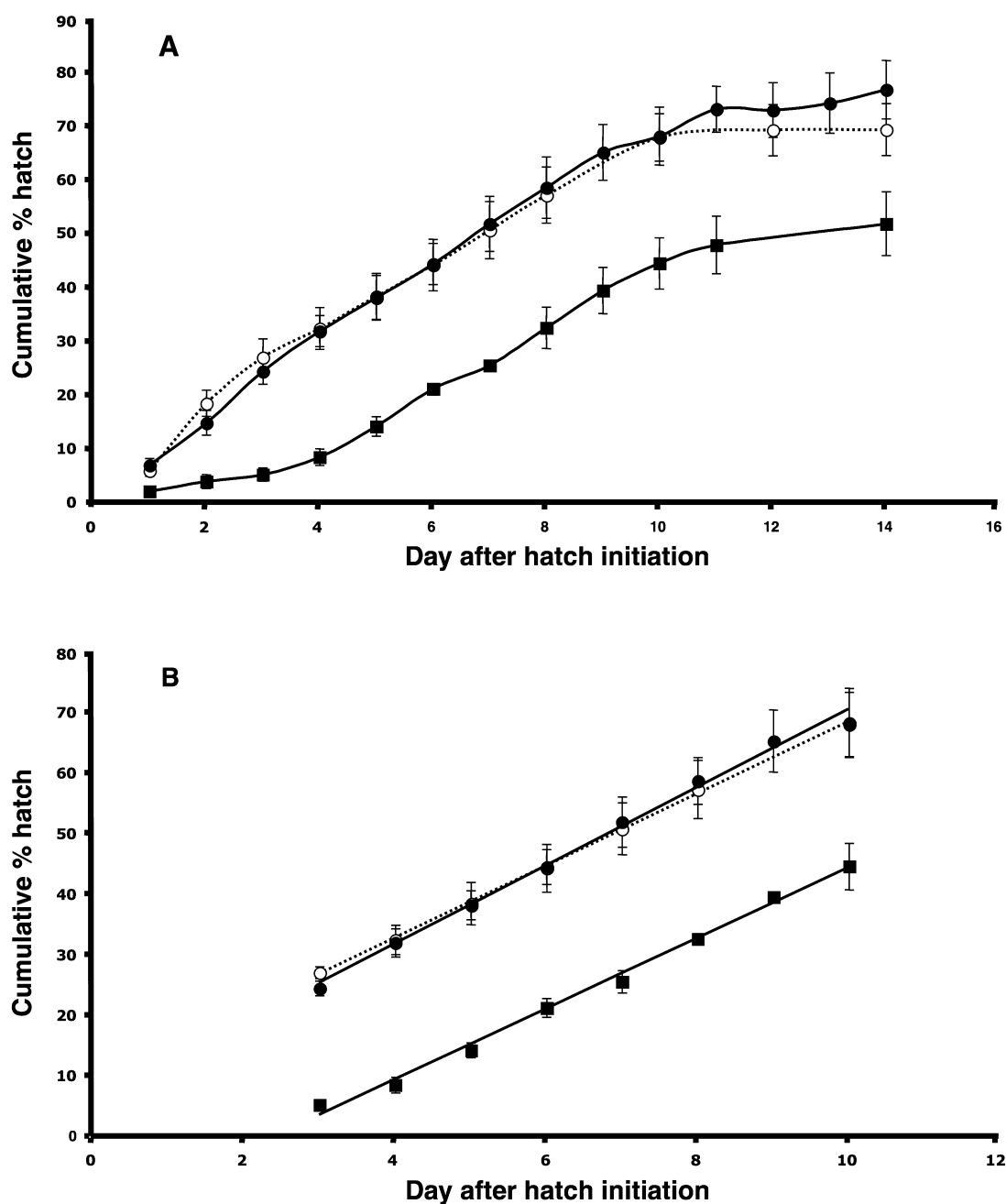
Fig. 2. *A: Hatch patterns of* Heterodera glycines *from eggs maintained in long-term laboratory culture (NL-RHp, filled circles), short-term laboratory culture (ESC, open circles) and glasshouse culture (TGC, filled squares); B: Linear portion of hatch curves from NL-RHp (filled circles), ESC (open circles), and TGC (filled squares).* $r^2 = 0.99$ *for each.*

two sources are plotted separately (Fig. 1B), differences are observed in total percentage hatch each day through to day 10 ($P < 0.10$), with J2 from eggs from the external mass (filled circles, Fig. 1B) hatching at a higher percentage than from eggs freed from the cyst (open circles). However, hatch rates were not statistically different ($P > 0.10$; slope $= 5.98 \pm 0.58$, freed eggs; 4.18 ± 1.00, external mass eggs).

Hatching behaviours of J2 obtained from different geographic sources and with different culture histories were examined on the funnel system and some qualitative features were compared (Fig. 2A). The Maryland cultures of *H. glycines* maintained on *G. max* for over 100 generations (NL-RHp, solid circles) or maintained on *G. max* for seven generations (ESC, open circles) had nearly identical hatching patterns. Both NL-RHp and ESC J2 hatched linearly from 3-10 days, and hatch rates of each began to decline at the same time (*ca* 11 days). J2 from eggs obtained from the Tennessee glasshouse cultures (TGC, black squares) exhibited a very similar hatch pattern, linear between 3-10 days with a decline in hatch rate after 10 days. Quantitative comparisons (Fig. 2B) reveal that between 3-10 days, the linear percentage hatch rate (curve slope) for NL-NHp J2 (filled circles, $6.64 \pm 0.45\%$) was marginally higher ($P < 0.05$) than that for ESC J2 (open circles, $5.39 \pm 0.38\%$), but there were no statistical differences ($P > 0.3$) between the hatch rates of NL-RHp and TGC (filled squares, $5.79 \pm 0.46\%$) or between those of ESC and TGC. While the total cumulative percentage hatch at 10 days was the same ($P > 0.7$) for NL-RHp ($69.9 \pm 5.1\%$) and ESC ($68.3 \pm 4.0\%$), percentage hatch for each was greater ($P < 0.005$) than that for TGC J2 ($44.8 \pm 4.4\%$).

In contrast to *H. glycines* eggs obtained from culture, there was a very low hatch of J2 from eggs obtained directly from the field. At 10 days, J2 from ESF eggs had a total cumulative percentage hatch of <1. However, hatching of ESF J2 was apparently stimulated by exposure to *G. max*, since infection of *G. max* occurred when cultures were inoculated with ESF eggs (Table 1). Initial infection rates were low, since after one generation fewer eggs were recovered than were inoculated (recovery rate $= 0.46$). However, after two generations, the new cultures (ESC) obtained from ESF eggs produced three times the number of eggs inoculated (Table 1), and between two and ten generations the egg recovery rate for ESC eggs was the same ($P = 0.73$) as the rate for the established NL-RHp culture during the same period.

Using NL-RHp eggs in the funnel system, we demonstrated an apparent positive effect on J2 hatch by the presence of neighbouring eggs (Table 2). The mean total percentage hatch of J2 was higher ($P < 0.05$) in funnels loaded at high egg population density (3100 eggs/cm^2) than in funnels loaded at low egg population density (420 eggs/cm^2) on each day from day 2 to day 10. In addition, hatch rates were linear for each treatment ($r^2 = 0.99$) but were significantly different ($P < 0.01$) with daily per-

Table 1. *Change in hatch of second-stage juveniles of* Heterodera glycines *following transfer from field to laboratory culture as estimated by recovery of eggs from infected* Glycine max.

Egg source	Generations in culture	Egg recovery ratio[a]
ESF[b]	1	0.46
ESC[b]	2	3.06
ESC[b]	9	4.97 ± 0.93[c]
NL-RHp	>100	5.53 ± 1.09[c]

[a]Eggs recovered/eggs inoculated.
[b]Eggs collected from the field (ESF) were used for the initial inoculation of *G. max* in establishing a new source of laboratory-cultured eggs (ESC).
[c]Mean egg recovery ratio from generations 2 through 10 (9 generations of ESC) was not different ($P = 0.73$) from that for established culture generations produced over the same 14-month period.

Table 2. *Egg density* vs *hatch level. Mean total cumulative percentage hatch from* Heterodera glycines *eggs incubated at low and high egg population densities.*

	Egg population density[a]	
Hatch day	Low	High
2	8.39 ± 1.33	26.40 ± 3.13
4	21.53 ± 4.60	45.82 ± 3.83
6	30.61 ± 5.80	58.94 ± 4.30
8	42.78 ± 7.34	65.35 ± 4.89
10	53.08 ± 7.79	74.26 ± 6.15

[a]Baermann funnels were loaded at low density (420 eggs/cm^2) or high density (3100 eggs/cm^2) with eggs from an established laboratory culture (NL-RHp). Second-stage juveniles were collected every 2 days for 10 days and total cumulative percentage hatch was calculated after each collection. Mean total cumulative hatch for low density (n $= 4$) and high density (n $= 6$) preparations on all days was compared by one-way ANOVA. Means at each day were significantly different ($P < 0.05$) between the two treatments.

centage hatch rates of $8.30 \pm 0.44\%$ for the high-density funnels and $2.30 \pm 0.35\%$ for the low-density funnels.

Discussion

Hatching patterns of *H. glycines* J2 from eggs maintained in *G. max* laboratory culture, *G. max* sterile root culture, or on *G. max* in the glasshouse, were remarkably similar. Although total cumulative percentage hatch varied, the pattern of hatching, including a distinctive linear phase of approximately 7 days in all cases, was quite

robust regardless of egg source or hatch conditions. The rapid and linear hatch is markedly higher than reported previously (Tefft *et al.*, 1982; Thompson & Tylka, 1997; Charlson & Tylka, 2003). One contributing factor may be the method of egg collection and processing used in the current study, which involves less aggressive egg rinsing and fewer processing steps than typically reported (Thompson & Tylka, 1997; Charlson & Tylka, 2003). Differences in the degree of egg washing may result in different amounts of hatch effectors removed from the eggs. Materials from the surface of *Globodera rostochiensis* eggs have been shown to stimulate hatching (Pridannikov *et al.*, 2007) and our data suggest a hatch stimulator may be associated with *H. glycines* eggs.

To evaluate the stability of hatching of *H. glycines* J2, we examined both egg source and hatching conditions. The established NL-RHp culture had originated from material collected from fields on the Eastern Shore region of Maryland and was cultured subsequently for decades at Beltsville. A new collection (ESF) of *H. glycines* was from fields in the same Eastern Shore region and was used to initiate a new laboratory culture (Eastern Shore Culture; ESC). A third source of eggs was provided from glasshouse cultures from Jackson, Tennessee (TGC). Hatch characteristics of *H. glycines* from the Jackson glasshouse TGC culture were similar to that from the Beltsville NL-RHp culture, having an average maximum percentage hatch higher than previously published reports. The ESF population of *H. glycines* adapted quickly to plant culture conditions and exhibited the same hatching kinetics as those observed with NL-RHp. All populations examined (root culture, long maintained plant culture, newly established plant culture, glasshouse culture) produced hatches that were linear from days 3-10, had similar hatch rates (slopes), and resulted in high total percentage hatch. It is important to mention that, although the total percentage hatch of J2 from eggs from either cysts or the external egg mass observed in the present study was higher than that reported in *H. glycines* (Thompson & Tylka, 1997), in each study the total percentage hatch of J2 from the egg mass eggs was greater than that of J2 from encysted eggs. The endogenous mechanisms of *H. glycines* hatch are clearly robust enough to override geographic origin, culture conditions and method of hatch assay.

Just as noteworthy as hatch stability is the maintenance of heterogeneity. Developmental heterogeneity as it relates to hatching has been described previously in a number of cyst nematode species (Zheng & Ferris, 1991; Yen

et al., 1995; Perry, 2000), with types of dormancy and hatch suppression discussed. We observed that J2 from *H. glycines* eggs collected from the field exhibited varied hatching behaviours and detected sub-populations of J2 defined by hatch characteristics. Some J2 hatched in water, some responded to the presence of a host plant, but most did not hatch. Even after selection under constant laboratory culture conditions, a process that favours rapid hatching and infection, the *H. glycines* population does not exhibit homogeneous hatching behaviour. Although proportions change (*e.g.*, a majority of J2 hatch in water), sub-populations of J2 persist. The 30-40% of J2 in eggs obtained from the controlled culture environment that did not hatch under favourable conditions may have been in some state of dormancy. Thus, even within populations sheltered from environmental cues and exposed to continuous selection pressure, some of the underlying heterogeneity of the founding field eggs, including hatch suppression, is retained.

J2 in eggs collected from *H. glycines* field populations hatched at very low rates even after extended periods, suggesting that factors repressing J2 hatch are strong and persistent. Earlier work showed that hatching from eggs of *H. glycines* obtained from culture (Ishibashi *et al.*, 1973; Wong *et al.*, 1993) is markedly higher than those from eggs obtained from field or non-cultured populations (Okada, 1971, 1972a, b; Thompson & Tylka, 1997; Charlson & Tylka, 2003). We have shown that changes resulting in increased hatching in egg populations transferred from field to culture occurred rapidly. This hatch increase may account for the increased production of females on plants after only two generations in culture, although other factors such as J2 motility and infectivity need to be examined. However, given the persistent nature of hatch suppression, the rapid change in the physiological state of the egg population from field to culture is remarkable.

J2 that did not hatch in any of our experimental treatments may have been in diapause. Two characteristics that define diapause are the induction of developmental arrest by environmental cues, such as seasonal changes, and a temporary irreversibility. Thus, even under favourable conditions, development does not resume unless a minimal intrinsic developmental level has been reached (Sommerville & Davey, 2002). Field eggs were subject to environmental cues and, even when maintained under conditions clearly favourable for hatching, the majority of J2 did not hatch. Thus, J2 in these eggs satisfied the above definition of diapause. The laboratory cultures were to some

extent insulated from an uncontrolled and variable environment with its associated cues. One would expect that physiological and biochemical changes, induced by such cues, would be absent from laboratory cultures. Yen *et al.* (1996) demonstrated the occurrence of dramatic seasonal fluctuations in carbohydrate and total protein levels in *H. glycines* harvested from microplots. In the same study, they reported that similar fluctuations were absent from *H. glycines* maintained as glasshouse cultures. Interestingly, Meyer *et al.* (1997) observed a seasonal dormancy or diapause with regard to *H. glycines* female production in sterile root culture.

Indeed, whether the J2 in plant-cultured eggs that did not hatch under our experimental conditions were in diapause, or in some other form of dormancy and were not exposed to a proper hatching stimulant, is not clear. However, since only two generations in culture were required for most *H. glycines* J2 to hatch readily in water whereas over 95% of J2 in field eggs did not hatch, the combination of molecular controls that regulate *H. glycines* hatch clearly must undergo rapid selection. This molecular selection is undoubtedly limited since a large proportion of J2 in cultured eggs failed to hatch in water and, remarkably, this proportion remained constant in all subsequent generations. This indicates that the molecular controls that suppress hatching are more strongly preserved than those that facilitate it.

Identification of specific molecules is needed to understand the endogenous regulation of hatching. In *H. glycines*, both promoters and inhibitors of hatching are present in the cyst (Okada, 1972a, b, 1974; Charlson & Tylka, 2003). Materials that stimulate hatching in cyst nematodes have been extracted from the eggs of *H. glycines* (Okada, 1972b; Charlson & Tylka, 2003) and from eggs and cysts of *G. rostochiensis* (Pridannikov *et al.*, 2007). A positive correlation between cyst population density and J2 hatch in *H. glycines* has been reported (Schroeder & MacGuidwin, 2004), similar to our observations with *H. glycines* eggs.

In *G. rostochiensis* and *H. glycines*, the age of the cyst also contributes to biochemical changes that can affect hatching (Kaul, 1962, cited by Perry, 2000; Okada 1972a; Charlson & Tylka, 2003). One such change in *G. rostochiensis* cysts is the elevation of catechin levels during the autumn and winter, when hatch should be suppressed (Kaul, 1962). Catechin and its derivatives are known to inhibit matrix metalloproteases (Demeule *et al.*, 2000; Oneda *et al.*, 2003). A gene encoding a matrix metalloprotease has been characterised from *H.*

glycines (Kovaleva *et al.*, 2004), and its expression in the egg increased and decreased in time with hatching. Knowledge of the regulation of this and other related genes in *H. glycines* field *vs* cultured eggs, and in other cyst nematodes with different hatching requirements (*e.g.*, *G. rostochiensis*; Perry, 1997) should provide clues to the identities of endogenous molecular controls.

Various rearing and assay procedures were used to demonstrate a high level of stability in the hatch of J2 of *H. glycines*. Hatch repression apparently induced by environmental cues rapidly declined after transfer of eggs to culture, and eggs themselves appeared to influence hatching. However, a significant proportion of J2 always remained unhatched under assay conditions. It is thus clear that fundamental mechanisms used by *H. glycines* for hatch regulation (*e.g.*, diapause) persist in populations selected for rapid hatch and reared for generations in the laboratory insulated from most environmental cues. Questions surrounding the molecular components, which participate in this regulation, are both fundamentally and practically important as we seek to understand cyst nematode development and management.

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