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Diapause in the nematode *Globodera pallida*

ZANNA MUHAMMAD
Department of Biology, Imperial College at Silwood Park, Ascot. Berkshire SL5 7PY, England
Present address: Department of Biological Sciences, University of Maiduguri, P.M.B. 1069, Maiduguri, Borno State, Nigeria

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Abstract. Over a period of 12 months 'new' and 'old' cysts of *Globodera pallida* were hatched in potato root diffusate according to a novel 'nematode-response' hatching protocol. In this protocol, cysts were set to hatch at the beginning of autumn and then left to indicate when their hatching ability was exhausted (when less than 100 juveniles/replicate/week emerged) before another batch of cysts was set to hatch. At any time of the year for the 12 months this experiment was conducted there were cysts hatching. After 12 months of hatching, eight hatching curves were obtained. Based on the hatching curves of the 'new' and 'old' cysts, diapause was shown to be present in 'new' cysts in autumn, winter and early spring. However, diapause was absent in late spring and summer.

Infectivity assays to distinguish between juveniles obtained in the periods when cysts were in diapause and when cysts had overcome their diapause failed to show any significant difference in their infectivity. There was no significant difference in the number of eggs in 'new' and 'old' cysts. Based on this observation, it was suggested that high emergence in 'old' cysts may not be a result of few eggs in the cyst but rather due to absence of diapause. Also the presence of large numbers of eggs in 'old' cysts even after being stored for 12 months outdoors in the soil does not support the theories of spontaneous hatching, micro-organism induced hatching or persistence of hatching factors in the soil.

Introduction

Globodera rostochiensis cysts were found to contain viable eggs after eight years in soil [Franklin, 1937]. Various suggestions have been made to explain why some encysted eggs do not hatch, including the presence of inhibiting substances produced as a result of metabolic activities of the juveniles within the eggs during process of hatching [Ellenby, 1946], oxygen deficiency in the hatching medium [Wallace, 1959], seasonal variation [Calam et al., 1949] and genetic inheritance [El-Shatoury, 1978].

Shepherd and Cox [1967] and Oostenbrink [1967] considered that *G. rostochiensis* eggs which failed to hatch under optimum conditions were in diapause, and they compared the phenomenon with that found in insects. However, their evidence was confusing due to lack of fundamental information on (a) history of cysts, (b) species involved (prior to the work of Stone [1972] *G. pallida* was not distinguished as a separate species from

G. rostochiensis), (c) storage conditions of cysts, (d) hatching conditions of cysts, (e) hatching medium used, (f) how the hatching medium was produced and (g) host plants on which cysts were raised. Hominick et al. [1985] considered all these lapses in designing their experiments and concluded that one population of *G. rostochiensis* exhibited diapause.

In the work on *G. pallida* reported in this paper, the protocols of Hominick et al. [1985] are used, but with modifications in the hatching protocol. In this work, a 'nematode-response' hatching protocol was used as opposed to fixed calendar hatching times used by Hominick et al. [1985]. In the 'nematode-response' approach, cysts were set to hatch at the beginning of autumn (October, 1987) and then left to indicate when their hatching ability is exhausted (when less than 100 juveniles/replicate/week emerged), before another batch of cysts was set to hatch. The advantage of this approach is that, at any time of the year for the period this experiment was conducted (12 months) there are cysts hatching. Also 'new' and 'old' cysts were compared to elucidate diapause in *G. pallida*. The 'new' cysts are those extracted soon after maturity on host roots and 'old' cysts are those that were stored for one calendar year outdoors in a gravel plunge. The overall aim of the experiments is to examine whether or not there is a diapause in *G. pallida*.

Materials and methods

Setting up cultures

Cysts of *G. pallida* (Pa 2/3) were isolates that had been continuously grown outdoors on potato plants cv. Pentland crown for a number of years at the Scottish Crop Research Institute (SCRI) at Invergowrie, Dundee, UK. The cysts had been harvested from the 1985 season, extracted from soil and stored at SCRI at room temperature in the dark. On arrival at Silwood Park (IC), Ascot, UK on 29:2:87, these cysts were counted randomly into batches of 100, placed in glass vials and stored in the dark at 20 °C.

On 2:3:87 certified potato seed tubers cv. Pentland crown were set to sprout in greenhouse. Thirty 18 cm plastic pots with the drainage holes covered with fine muslin cloth were filled with steam sterilized loam: sand (2:1). A sprouting tuber of potato was planted into each pot and kept for 3 days in a greenhouse at 18–23 °C.

On 28:4:87, thirty batches of 100 cysts were soaked in sterilized tap water (STW) for seven days in the dark at 20 °C. Then, on 5:5:87 each of the thirty pots containing a sprouting tuber of potato was inoculated with one batch of 100 pre-soaked cysts. Pots were maintained outdoors in a gravel plunge and watered when necessary throughout the growing period.

About three months later, as the potato foliage was senescing, the 30 pots were removed from the gravel plunge. The soil and the roots were

emptied into a large tray and dried slowly in a warm air chamber in the dark. After five days, the dry contents of 15 pots were extracted using a Fenwick can. The float containing the cysts was again dried in the dark in a warm air chamber. When the debris and the cysts were completely dry after three days, cysts were handpicked from the debris and randomly counted into batches of 50 and stored in glass vials at 20 °C in the dark. These cysts were referred to as 'new' cysts.

The soil from the remaining 15 pots was sieved to remove all traces of living potato roots or tubers, but retaining all cysts. Soil was then divided into sets of 18 cm plastic pots and returned to the gravel plunge out-doors (at the same time as the 'new' cysts began their storage at 20 °C in the dark). Here they were left for 365 days, after which the soil from each pot was extracted with a Fenwick can on 4:7:88. Cysts were dried, handpicked and counted into batches of 50 and stored in glass vials at 20 °C in the dark on 8:9:88. These cysts were referred to as 'old' cysts. In the absence of any living potato tissue to allow reproduction, the 'old' cysts are presumed to be identical to the 'new' cysts, the only difference being one of age.

Production of potato root diffusate (PRD)

On 5:5:87 potato root diffusate (PRD) from cv. Pentland crown was obtained and stored according to the protocols of Hominick et al. [1985].

Hatching system

Hatching was done according to a 'nematode-response' protocol (Fig. 1). 'New' and 'old' cysts were both stored in batches of 50 cysts in the dark (Fig. 1, S1, S2). Four replicates of 50 cysts each were hatched whenever required. During the experiments, each batch of cysts was kept on a small (7 mm diameter) 45 µm nylon hatching sieve, in 1ml of either STW or PRD in a well of 24 – well Linbro culture plates (Flow Laboratories, UK). The plates were individually wrapped in a black plastic sheet to limit exposure to light and while changing the medium care was taken to expose the cysts to minimal light.

The first batch of 'new' cysts were set to hatch in October 1987 by soaking in STW for two weeks (Fig. 1, H1), then hatched in PRD (Fig. 1, H2). Emerging juveniles were counted weekly until less than 100 juveniles/replicate/week emerged as adopted by Hominick et al. [1985]. Hatching was then concluded for these cysts which were rinsed in STW, dried and stored on their hatching sieves at 20 °C in the dark for a period of 12 months (Fig. 1, S3). Immediately another batch of 'new' cysts was set to hatch in a similar manner as the first batch. This protocol was used for all the remaining batches of 'new' cysts over a hatching time table of one year (Table 1).

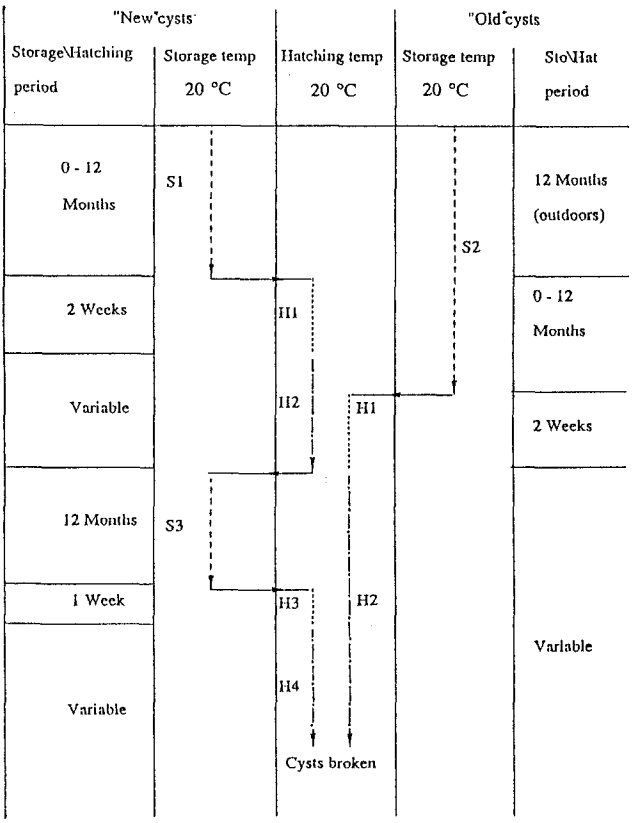

Fig. 1. 'Nematode-response' hatching protocols for 'new' and 'old' cysts of *G. pallida* incubated dry at 20 °C and hatched at 20 °C in PRD over a period of one and two calendar year(s).
Key:----- Storage period; ..... Soaking in STW; —·—·— Hatching in PRD.
S1 First storage period of 'New' cysts at 20 °C.
S2 First (and second) storage of 'old' cysts outdoor (and 20 °C).
S3 Second storage period (dry) of 'new' cysts for 12 months at 20 °C.
H1 First soaking ('new' and 'old' cysts) in STW.
H2 First hatching ('new' and 'old' cysts) in PRD until < 100 J2 emerged\week.
H3 Second soaking ('new'cysts) in STW.
H4 Second hatching ('new' cysts) in PRD after 12 months storage until < 10 J2 emerged\week.

'New' cysts were set to hatch for a second time after each replicate had had 12 months of dry storage at 20 °C. Cysts were first soaked in STW for one week (Fig. 1, H3) and then hatched in PRD (Fig. 1, H4). Emerging juveniles were counted weekly until less than 10 juveniles/replicate/week emerged; most juveniles emerged during first hatch. This protocol was applied to each replicate according to a strict time table (Table 1). Finally, after the completion of hatching, cysts were individually broken open under

Table 1. Time table of hatching of 'new' and 'old' cysts of *G. pallida*. 'New' cysts had second hatching after dry storage for one calendar year at 20 °C in the dark. All hatching in PRD at 20 °C in the dark

Hatching period	'New' cysts		'Old' cysts
	First hatch	Second hatch	First Hatch
October	5:10:87	5:10:88	5:10:88
November	16:11:87	16:11:88	16:11:88
December	21:12:87	21:12:88	21:12:88
February	15:2:88	15:2:89	15:2:89
April	4:4:88	4:4:89	4:4:89
May	9:5:88	9:5:89	9:5:89
July	4:7:88	4:7:89	4:7:89
September	12:9:88	12:9:89	12:9:89

a dissecting microscope using fine mounted needles and numbers of viable eggs (i.e. those eggs containing unhatched coiled second stage juveniles) estimated in each batch of cysts. The contents of the broken cysts were diluted in a fixed volume and thoroughly mixed to ensure even dispersion of eggs. Three aliquots were taken from the dilution of each batch and counted. Juveniles which were freed in the process of breaking the cysts were counted as viable unhatched eggs, while empty eggs were disregarded. Emergence was calculated as a percentage of the total hatched and unhatched viable eggs from each replicate.

The same protocol (Fig. 1) and strict time table (Table 1) was followed to hatch 'old' cysts, except that in this case cysts were first stored for 12 months outdoors and then for various periods at 20 °C in the dark (Fig. 1, S2). When emergence was less than 100 juveniles/replicate/week cysts were broken open and the number of viable unhatched eggs determined as previously mentioned. However, cysts were not stored and hatched the second time as in 'new' cysts due to lack of time.

Infectivity assay

Juveniles emerging during one week period from 'new' cysts were obtained and used for infectivity assay. Infectivity assay was done using three-week old tomato plants cv. Moneymaker planted into 9 cm plastic pots filled with loam: sand mixture (1:1). After three days, when plants were fully established in the potting medium, four replicates of the plants were each inoculated with approximately 1000 one week old juveniles. Pots were kept at 20 °C in a controlled temperature room with 16 h light and 8 h dark regime for two weeks; plants were watered lightly when necessary with 'phostrogen' solution. At the end of the two week period, plants were gently lifted, washed, stained in 0.05% acid fuchsin in lacto-glycerol

[Bridge et al., 1982] and the number of stained nematodes recorded and scored as percentage infectivity.

Eggs in 'new' and 'old' cysts

At the end of each hatching period, the total numbers of viable eggs in 'new' and 'old' cysts were determined and compared, to assess whether hatching had occurred in 'old' cysts during storage in the soil outdoors.

Analysis of results

The statistical package 'Statistix' (NH analytical software, St. Paul MN SS117 USA) was used to perform the chi-squared test to assess whether the hatching curves in 'new' and 'old' cysts differed significantly from one another in any hatching period; using the hatching curve of 'old' cysts as 'expected'. Also the total number of eggs in 'new' and 'old' cysts were tested with one way analysis of variance (ANOVA) to assess whether there were significant difference between their contents during any hatching period.

Results

The results of hatching 'new' and 'old' of *G. pallida* produced eight hatching curves (Fig. 2) according to the 'nematode-response' hatching protocol. Hatches of 'new' cysts in October, November, December, February and April produced less than 50% emergence; while hatches in May, July and September produced about 80% emergence. In all hatches, peak emergence was in the third or fourth week of hatching, except for those cysts hatched in May where there were two peaks, the first peak starting in the second week and the second in the fifth week. The shortest emergence period was eight weeks shown by cysts hatched in October, February and April; while the longest was 12 weeks in July. Dry storage of all 'new' cysts for 12 months after first hatch and subsequent second hatching in PRD produced no further emergence at any periods (Fig. 2).

However, 'old' cysts showed significant differences ($P < 0.05$) in their emergence compared to 'new' cysts. Cysts hatched in October had the lowest emergence of less than 20%, those hatched in April, May and July had 50–60% emergence, while cysts hatched in November, December, February and September had the highest emergence of about 80%. All peaks of emergence in 'old' cysts occurred in the second and third weeks except for those cysts hatched in November which had various peaks. The longest emergence period of 10 weeks was observed in cysts hatched in November, while all other hatches had an emergence period of four to five weeks.

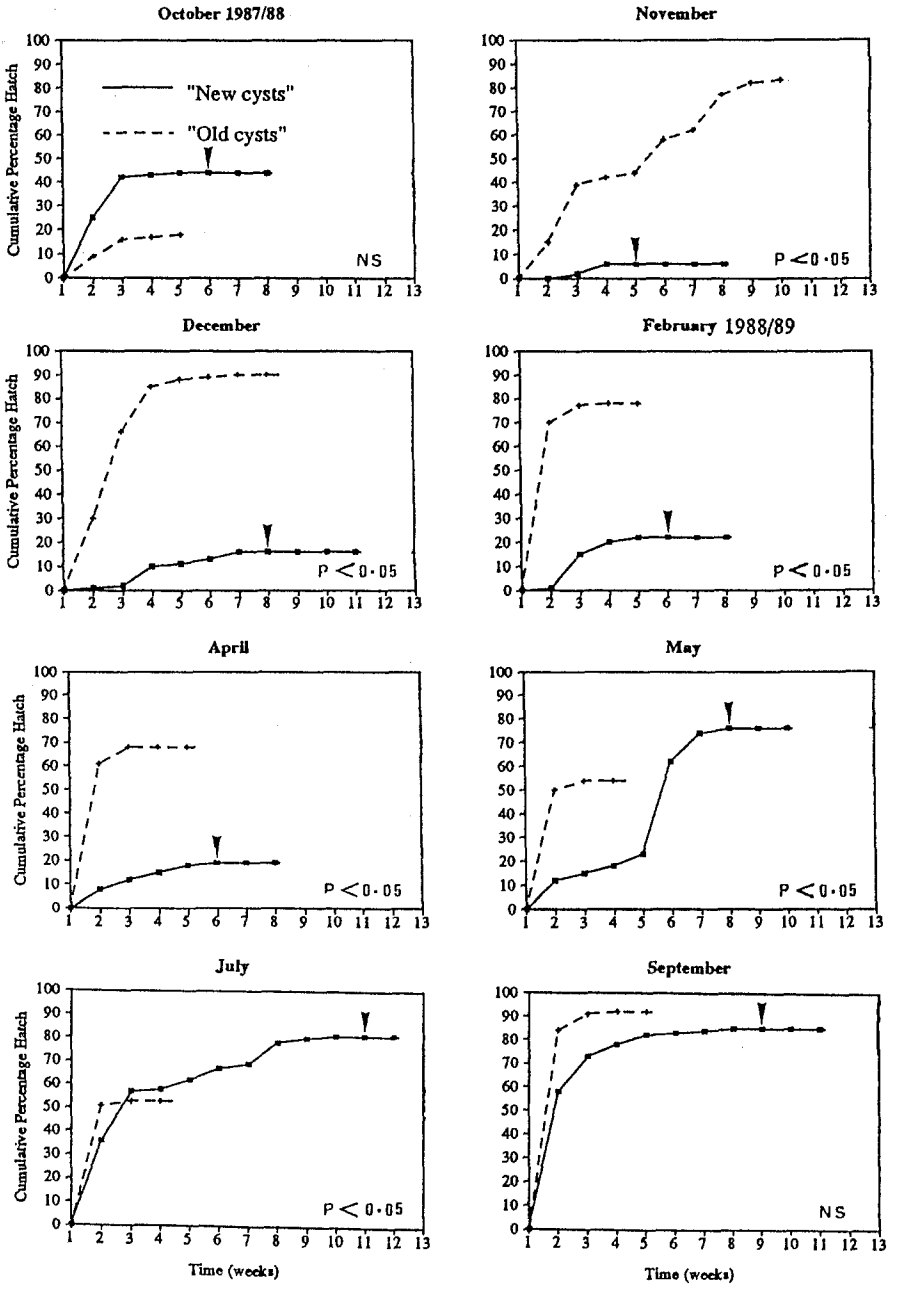

Fig. 2. Cumulative percentage hatch of 'new' and 'old' cysts of *G. pallida* stored dry at 20 °C for various periods before hatching in PRD at 20 °C over a hatching cycle of one and two calendar year(s). Key: ↓ End of first hatching period in 'new' cysts (H1 and H2). Continuing line indicates resumption of hatch (H3 and H4) after storage period S3.

Hatching curves in 'new' and 'old' cysts were significantly different ($P < 0.05$) in November, December, February, April, May and July hatches, suggesting the presence of diapause in these periods, since emergence in 'old' cysts were considered to be the 'expected' values. However, hatching curves in October and September were not significantly different, suggesting the absence of diapause in these periods (Fig. 2).

The results of infectivity assay of juveniles following emergence from 'new' cysts failed to distinguish juveniles emerging during diapause periods and non-diapause periods (Fig. 3). There were eight infectivity assays none of which is significantly different from the other.

The numbers of eggs in 'new' and 'old' cysts were significantly different ($P < 0.05$) only in cysts hatched in November (Table 2). The near equality of number of eggs in 'new' and 'old' cysts (obtained during other months) shows the absence of spontaneous hatching in 'old' cysts. This result also confirms the absence of hatching induced by micro-organisms or as a result of persistence of hatching factors in the soil, even when 'old' cysts were stored outdoor in soil for 12 months. Therefore, maximum emergence observed in the hatching behaviour of the 'old' cysts cannot be attributed to hatching of few eggs in the cysts, but rather due to absence of diapause. Also the converse may be true, those cysts that exhibited low emergence may be because diapause has not been overcome regardless of the 12 months outdoor storage.

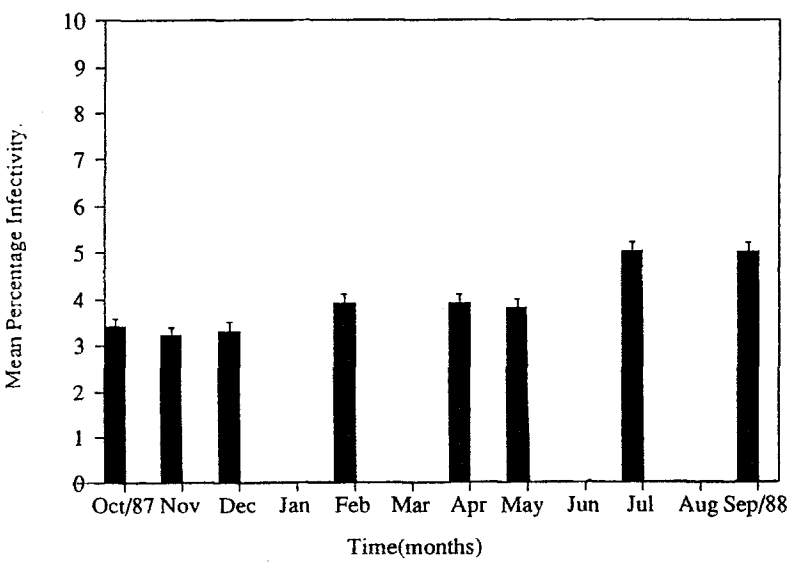

Fig. 3. Infectivity of *G. pallida* on tomato plant cv. Money maker over a hatching cycle of 12 months. Juveniles were from 'new' cysts and inoculation was done with about 1000 one week old juveniles/plant with four replicates. Lines above bars indicate standard error of the mean.

Table 2. Total number of eggs in batches of 50 'new' and 'old' cysts (means and standard deviation (SD) of four replicates) of *G. pallida*

Hatching period	Total number of eggs in batches of 50 cysts (means and SD)	
	'New' cysts	'Old' cysts
October	20998 ± 892	18987 ± 1699
November	29497 ± 2413	18875 ± 1590*
December	24034 ± 2672	23672 ± 3483
February	25940 ± 852	28902 ± 3171
April	24128 ± 1661	22443 ± 1925
May	27818 ± 2542	23645 ± 2981
July	24307 ± 1177	23244 ± 2667
September	26334 ± 1468	31919 ± 4174

Discussion

Based on the 'nematode-response' hatching protocol, the pattern of the hatching curves in 'new' and 'old' cysts showed that hatchings in November, December, February, April, May and July are significantly different ($P < 0.05$), indicating the presence of diapause in 'new' cysts during these periods. However, hatchings in October and September were not significantly different, indicating absence of diapause in 'new' cysts during these periods. These results suggest the presence of diapause in 'new' cysts in autumn, winter and early spring, while in late spring and summer diapause was absent (Fig. 2).

These results further suggest synchronization between the hatching pattern of 'new' cysts of *G. pallida* and the development of its potato host plants. Diapause sets in during autumn and winter, corresponding to the post-harvest period; while it is overcome in spring and summer corresponding to periods when potato plants start growing and are liable to infection by potato cyst nematodes (PCN). This specialization of *G. pallida* differs from the erratic behaviour of its close relatives *G. rostochiensis*, where Hominick et al. [1985]. reported diapause to be present during autumn and winter, while Muhammad [1990] reported diapause to be present during autumn, winter, spring and mid-summer.

Problems of assessing diapause in PCN are compounded when studying *G. pallida*, because no direct studies had been conducted before on this species. Also, it was only in 1972 that Stone [1972] described *H. rostochiensis* as different from *H. pallida*, while Behrens [1975] separated PCN from *Heterodera* to form a new genus *Globodera*. Therefore, it is often difficult to determine the species used for studies before 1972.

Storage of cysts of *G. rostochiensis* outdoors for a period of 12 months overcomes diapause [Hominick et al., 1985; Muhammad, 1990]. However

in *G. pallida* even when cysts were stored outdoor in soil for 12 months ('old' cysts), some eggs still exhibit diapause (Fig. 2). The hatching patterns show high emergence in autumn and winter, then a decreased emergence in spring and summer and an almost complete suppression of emergence in mid-summer. This trend may suggest that, because diapause lasts for a long period in the 'new' cysts, it may require a much longer storage period before the diapause can be broken. Alternatively, *G. pallida* may have multiple diapause as suggested previously for *G. rostochiensis* [Oostenbrink, 1967; Shepherd and Cox, 1967].

A second hatching of 'new' cysts in PRD (Fig. 2) did not increase emergence in any of the hatching periods. In some of the hatching periods, the first hatch in PRD (May, July and September) result in about 80% emergence. As a result there were few juveniles left in the cysts for further emergence. However, in those cysts where emergence was less than 50% (October, November, December, February and April) it was expected that restimulation with PRD after one year of dry storage would elicit further emergence. This did not happen (Fig. 2), probably because such a treatment (period of first hatching in PRD followed by 12 months dry storage) might have removed or altered the protection afforded by the eggshell and trehalose to the unhatched juvenile, and as a result the juveniles become susceptible to desiccation [Perry, 1989]. In this work, diapause has been demonstrated to occur in *G. pallida*, but the mechanism for both induction and termination still remain poorly understood except, for some inferences that may be derived from work done with *G. rostochiensis* [Hominick, 1979; Hominick et al., 1985; Hominick, 1986; Muhammad, 1990].

Infectivity assessment (Fig. 3) to determine differences due to period of emergence showed no differences between juveniles of *G. pallida* obtained during periods when cysts were in diapause and periods when diapause was absent. The results show the near perfect synchronization of the hatching of *G. pallida* to the growing phase of the potato plants in the field. These results and observations agree with those of Storey [1984] who reported a lack of difference in infectivity between cysts of PCN which were dormant for one, four or seven years.

In practical terms, these results may have wider implications for our present day crop protection management for PCN. Therefore, a recognition of diapause in all our management strategies will improve our ability in evolving a robust control measure in the future.

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