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Seasonal Changes in the Dorsal Pharyngeal Gland Nucleolus of Unhatched Second-Stage Juveniles of *Globodera* spp. in Bolivia

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Abstract: Changes in the diameter of the nucleolus of the dorsal pharyngeal gland (DPGN) in unhatched second-stage juveniles (J2) of potato cyst nematodes, *Globodera* spp., were monitored for cysts recovered during two field experiments in the Bolivian Central Andes. In the first experiment, cysts were extracted from soil left fallow or supporting crops of potato, barley, lupin, or quinoa. The highest mean DPGN diameter for unhatched J2 occurred shortly after planting in January. The values were similar for individuals recovered from cysts associated with all cultivations. For cysts from potato plots, the lowest mean DPGN diameter of 2.26 ± 0.05 µm occurred in March, but the value increased again by May to 2.53 ± 0.05 µm. Similar seasonal changes were found for J2 under both nonhost crops and fallow with the smallest diameters recorded in May of 2.48 ± 0.02 µm and 2.34 ± 0.05 µm, respectively. Two possible factors might cause this significant seasonal change. First, some J2 may hatch early in the growing season, even in the absence of the host. This would enhance the proportion of dormant, unhatched J2 remaining in the cyst samples. Secondly, a seasonal change in the DPGN diameter may occur for most individuals with a transient fall value between January and March/May. A model defined by this study provides a good description of the observed effect, providing both factors are assumed to occur. The second experiment studied if changes in size of DPGN in response to a hatching stimulus are influenced by the cyst population age. The DPGN in unhatched J2 was measured for cysts recovered from soils that had supported potatoes that growing season or 2 or 4 years earlier. The unhatched J2 from the freshly cropped potato site showed the largest mean DPGN diameter of 3.66 ± 0.05 µm after 7 days in potato root diffusate, whereas those from the 4-year sample had the smallest value of 3.20 ± 0.05 µm. This significant difference may indicate a delayed response to the hatching stimulus with more prolonged J2 dormancy.

Key words: Bolivia, dorsal pharyngeal gland, *Globodera rostochiensis, Globodera pallida,* nematode, nucleolus, potato-cyst nematode, seasonal changes.

Potato is the fourth most important crop in terms of global yield and is grown in more countries than all food crops but maize (Horton and Anderson, 1992). It yields more calories per hectare than any other major food plant (Centro Internacional de la Papa, 1974) and so is often grown as a staple crop by subsistence farmers. For instance, there are 400,000 growers in Bolivia with an average cropping area of only 0.19 to 0.79 ha who depend on the potato (Programa de Investigación de la Papa, 1996). Poor people in this country spend up to 13% of their annual income on potatoes as their primary calorie source (J. Franco and G. Godoy, pers. comm.).

The potato has co-evolved in South America with the two species of potato cyst nematodes (PCN), *Globodera rostochiensis* and *G. pallida* (Evans et al., 1975). PCN are adapted to their seasonal host and can survive for many years in the absence of a potato plant. The unhatched second-stage juveniles (J2) depend on a host-mediated hatching stimulus to synchronize their life cycle with host availability (Hominick et al., 1985).

Several changes occur in unhatched PCN J2 in response to potato root diffusate (PRD), including an increase in oxygen consumption (Atkinson and Ballantyne, 1977a), changes in both the adenylate energy charge (Atkinson and Ballantyne, 1977b) and cAMP levels (Atkinson et al., 1987), and a change in eggshell permeability (Atkinson and Taylor, 1980). Only a few minutes' exposure to the root diffusate is required to trigger a response (Perry and Beane, 1982).

The three pharyngeal glands of PCN are swollen with secretions at eclosion (Doncaster, 1974). Secretory granules accumulate in the two subventral and the dorsal

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gland cells as a response to hydration, and all three gland cells are also affected by exposure to PRD (Perry et al., 1989; Smant et al., 1997). The diameter of the nucleolus in the nucleus of the dorsal pharyngeal gland (DPGN) in unhatched, unstimulated J2 of *G. rostochiensis* was 2.72 ± 0.10 μm (Atkinson et al., 1987). The DPGN diameter increased in response to PRD prior to hatching (Atkinson et al., 1987; Perry et al., 1989). This change in the DPGN is associated with the accumulation of secretory granules in the dorsal pharyngeal gland cell that are utilized during establishment of a cyst nematode in its host (Atkinson and Harris, 1989). Hatched J2 of *G. rostochiensis* had a DPGN diameter of 3.9–4.0 μm (Atkinson et al., 1987; Perry et al., 1989). This previous work established that the DPGN diameter provides an indirect indicator of readiness to hatch with values of ca. 3.0 μm for dormant individuals and ca. 4.0 μm for those about to hatch. Hatching is not known to occur before DPGN has increased in size.

This work was done to i) determine if DPGN diameter is a reliable indicator for detecting seasonal changes in unhatched J2 under field conditions; and ii) investigate if speed of response of unhatched J2 to potato root exudate is influenced by the duration of their previous dormancy in the field.

Materials and Methods

Field experiments in Toralapa: Toralapa Field Station is situated in the Department of Cochabamba, Bolivia, at an altitude of about 3,300 m a.s.l. and latitude 18°S. Rainfall at Toralapa Field Station is normally above 50 mm/month from November to March (the summer period), but it is much lower for the rest of the year. During the potato-growing season, the average air temperature is relatively stable (ca. 6–10 °C), but the minimum daily temperature drops from April (ca. 2 °C) to about August (ca. –4 °C). Soil temperature at a 10-cm depth, measured at 08:00 drops from April (11 °C) to at least July (6 °C) (Programa de Investigación de la Papa, 1996). Rainfall, moisture levels, and soil and air temperatures during the 1995–96 potato growing season were similar to the 10-year average (Programa de Investigación de la Papa, 1996). Planting time for potato in the Bolivian Andes is not only defined by the calendar; it also depends upon the start of the rainy season, which varies between October and December. Harvest is about 6 months after planting (Programa de Investigación de la Papa, 1997).

Changes in the diameter of the DPGN of PCN J2 during the potato growing season in Bolivia: Several crops were planted or plots were left fallow at the field site in a randomized complete block design (Little and Hills, 1978) with three replicates. Crops used were potato, *Solanum tuberosum andigena* cv. Waycha (susceptible to both PCN species); quinoa, *Chenopodium quinoa;* lupin, *Lupinus mutabilis;* and barley, *Hordeum vulgare.* Each sub-plot was 7 m^2 with four rows of crop plants in a field naturally infested with PCN (30–50 viable eggs/g soil). Tests before the experiments using both PCR techniques and stylet measurements indicated that the population within the field trial area was >90% *G. rostochiensis. Globodera pallida* was not positively identified at the site and so was not present at a density that could influence the effects recorded. The last potato crop at the site was 1 year before the trial. Planting was delayed until December when spring rainfall occurred, and harvest took place in early July 1996. The land was then prepared for subsequent use before the first likely spring rainfall in September.

PCN samples were taken in January, February, March, May, and August 1996. Cysts were extracted from wet soil with a Fenwick can (Southey, 1986) and immediately crushed to release eggs and J2, fixed in TAF (formalin 7% v/v; triethanolamine 2% v/v; distilled water 91% v/v) (Southey, 1986), and sent air-mail to the University of Leeds, United Kingdom. In Leeds, the fixed, crushed cysts were stored at 4 °C until further use. For analyses, the crushed cysts and released J2 stored in TAF were mounted on microscope slides; glass cover slips were sealed with nail varnish. DPGN diameters were measured only in J2 released from eggshells. Preliminary experiments established that TAF fixation and subsequent procedures did not result in a change in DPGN diameter.

Changes in the diameter of the DPGN of PCN J2 from cysts up to 4 years old: After harvest in August 1995, PCN cysts were collected from several fields with a known crop history at Toralapa Field Station. They were extracted from wet soil, dried, and sent air-mail to the University of Leeds, United Kingdom. On arrival, cysts were put into PRD for 7 days at 18 °C in the dark. Afterward, cysts were crushed and J2 released from eggshells were mounted on a microscope slide. PRD was obtained by the method of Fenwick (1949).

Image analysis: An Olympus BHT system microscope was used with differential interference contrast to measure the diameter of the DPGN of J2. The image was visualized with a ×40 objective lens, a ×6.7 ocular, and a monochrome camera. Measurements were made using the Quantimet 500 image analysis program (Leica, Cambridge, UK). The diameter of each nucleolus was measured four times, and the mean was calculated and recorded.

Statistical analysis: Two statistical programs were used, Excel 5 and SPSS 6.1, to analyze the data sets. An ANOVA single-factor analysis was conducted, and significance was tested at the 5% confidence level.

Results

Changes in the diameter of the DPGN of PCN J2 during the potato growing season in Bolivia: The diameter of the DPGN was measured for 1,723 J2 gathered from cysts collected from potato, nonhost crops, and fallow plots. The mean diameter ± standard error of the mean for DPGN for hatched individuals of this population was 4.04 ± 0.04 µm. Values for unhatched J2 in each sampling month are shown in Table 1 for all five sets of treatment plus the combined nonhost data (quinoa, lupin, barley). The highest value for DPGN diameter occurred in January for all treatments, with a range in mean values of 2.85 to 3.01 µm. For cysts from potato plots, the mean value fell to the lowest value of 2.26 ± 0.05 µm in March before an increase in both May and August. Each mean differed significantly from that preceding it ($P < 0.05$) except that the values for May and August were similar. Data for all nonhost crops were combined for comparison with the samples from the potato and fallow plots. Similar trends (U-shaped curves) in DPGN diameters also were found in J2 from the nonhost and fallow plots. The lowest means were 2.48 ± 0.02 and 2.34 ± 0.05 µm, respectively, in May, with a significant increase in size by August to 2.81 ± 0.02 or 2.69 ± 0.06 µm (Table 1). For nonhost crops, each mean differed significantly from that preceding it ($P < 0.05$) except when March and May were compared. The values for cysts from fallow changed between all samples except February and March. The data sets for both quinoa and lupin plots also showed similar U-shaped curves (Table 1).

The proportions of animals with different

TABLE 1. Seasonal changes in the mean diameter (µm) ± standard error of the nucleolus in the nucleus of the dorsal pharyngeal gland in unhatched *Globodera* spp. J2 during the growing season. Cysts were collected from a field experiment at Toralapa, Cochabamba, Bolivia.

Crop	January	February	March	May	August
Potato	2.86 ± 0.06 a	2.53 ± 0.05 b	2.26 ± 0.05 c	2.53 ± 0.05 b	2.54 ± 0.03 b
Fallow	3.01 ± 0.06 a	2.56 ± 0.05 b	2.52 ± 0.06 b	2.34 ± 0.05 c	2.69 ± 0.06 d
Quinoa	2.96 ± 0.04 a	2.70 ± 0.04 b	2.48 ± 0.05 c	2.46 ± 0.03 c	2.85 ± 0.04 d
Lupin	2.85 ± 0.05 a	2.66 ± 0.05 b	2.55 ± 0.08 bc	2.51 ± 0.06 c	2.83 ± 0.05 a
Barley	2.85 ± 0.05 a	2.57 ± 0.05 b	2.52 ± 0.08 b	2.43 ± 0.06 b	2.58 ± 0.05 b
Nonhost Crops	2.89 ± 0.03 a	2.66 ± 0.03 b	2.52 ± 0.03 c	2.48 ± 0.02 c	2.81 ± 0.02 d

Nonhost crops are the combined data of quinoa, lupin, and barley. Means within the same row followed by the same letter are not significantly different ($P > 0.05$; t-test).

nucleolus sizes are shown for January, March, and August for potato, nonhost, and fallow data, respectively (Figs. 1–3). Individual values observed for these unhatched J2 ranged from 1.8 to 3.8 μm. For potato, the majority of J2 in the January sample had a nucleolus diameter above 2.8 μm, whereas in March nearly all J2 had a size below 2.8 μm; by August most individuals had a value of 2.6 to 2.8 μm (Fig. 1). Similar, but not such marked, changes were observed in the nonhost data (Fig. 2). The January results showed that many more J2 had a nucleolus diameter greater than 2.8 μm than occurred for the March data, with the August results intermediate (Fig. 2). In January, most J2 from fallow plots had a DPGN diameter of >2.6 μm, whereas in March few diameters were >2.6 μm; most of the August values from fallow plots were equally spread over a range from 2.4 to 3.0 μm (Fig. 3).

Changes in the diameter of the DPGN of PCN J2 from cysts up to 4 years old: The 272 J2 measured in this experiment were unhatched but stimulated by PRD for 7 days. The diameter of the DPGN ranged from 2.4 to 4.6 μm. The DPGN of J2 from the youngest cysts showed the largest mean diameter (3.66 ± 0.05 μm), and J2 from the oldest cysts had the smallest (3.20 ± 0.05 μm) (Table 2). Differences between the three treatments were significant ($P < 0.05$). The youngest J2 demonstrated a marked peak in DPGN diameter at around 4.0 μm (Fig. 4). The 2-year-old J2 showed a bimodal distribution with peaks at 3.0 μm and 4.0 μm, respectively. A distinct peak at 3.0 μm was observed for the 4-year-old J2. Both the skewness and the kurtosis of the data increased over time (Table 2; Fig. 4).

Discussion

The mean diameters of the DPGN for J2 from nonhost plots in this study were 2.48 to 2.89 μm. These values are similar to those of J2 under laboratory conditions before exposure to PRD (Atkinson et al., 1987; Perry et al., 1989). The highest frequency of DPGN diameter measurements >3.0 μm occurred in January. By March few J2 had a nucleolus diameter >3.0 μm, whereas by August the values from many individuals exceeded 3.0 μm. The fall in DPGN diameter in spring in the field in the absence of potatoes may be a consequence of the hatch of some of the J2 that had a relatively large DPGN diameter. The cumulative annual hatch at this field site was ca. 36% (Holz, unpublished) in all

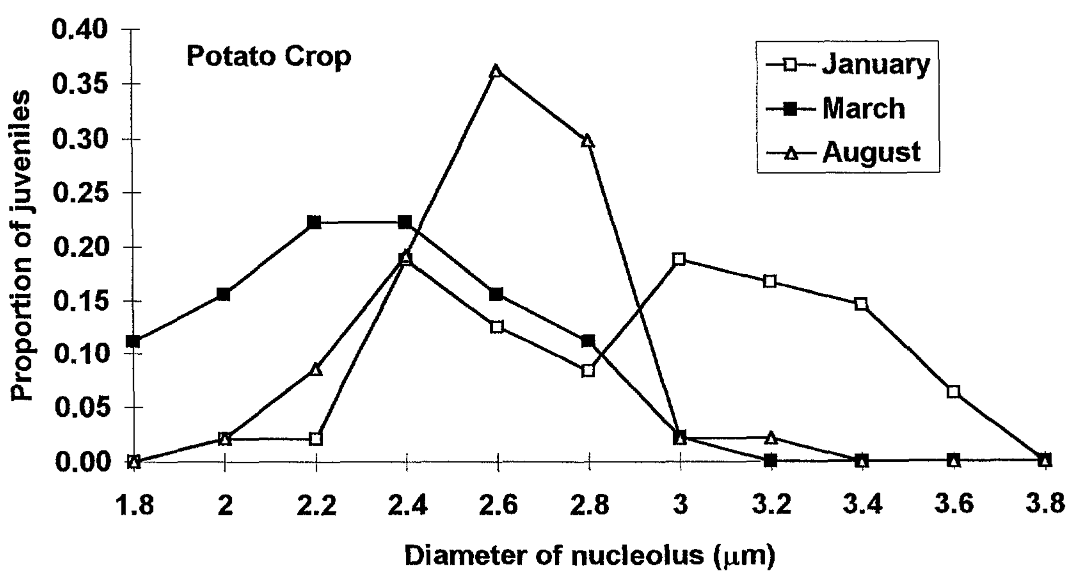

Fig. 1. Season changes in the proportion of individuals with different diameters of the nucleolus in the nucleus of the dorsal pharyngeal gland in unhatched *Globodera* spp. Second-stage juveniles during the potato growing season. Cysts were collected from potato plots at Toralapa, Cochabamba, Bolivia.

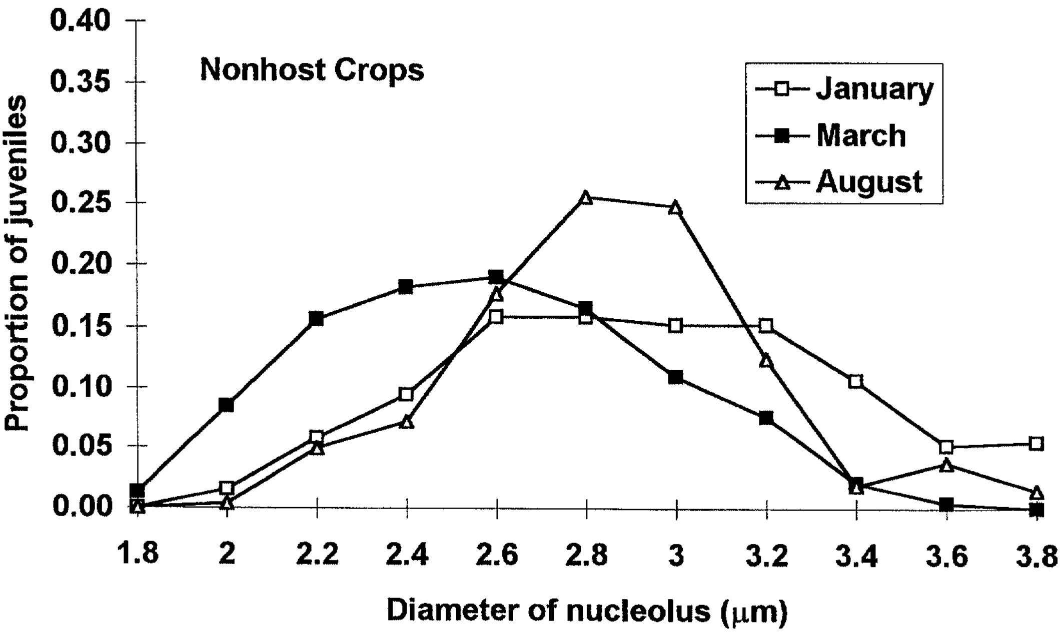

FIG. 2. Seasonal changes in the proportion of individuals with different diameters of the nucleolus in the nucleus of the dorsal pharyngeal gland in unhatched *Globodera* spp. Second-stage juveniles during the potato growing season. Cysts were collected from nonhost crop plots at Toralapa, Cochabamba, Bolivia.

plots, with a higher proportion of hatch when potatoes were grown. However, the hatching of the 36% of J2 with the greatest values for DPGN diameters in January would not alone result in the reduced mean value obtained for individuals in March. A second possibility is that a seasonal reduction in the DPGN diameter occurred for those J2 that remained in their eggs between January and March to May. We have generated a model

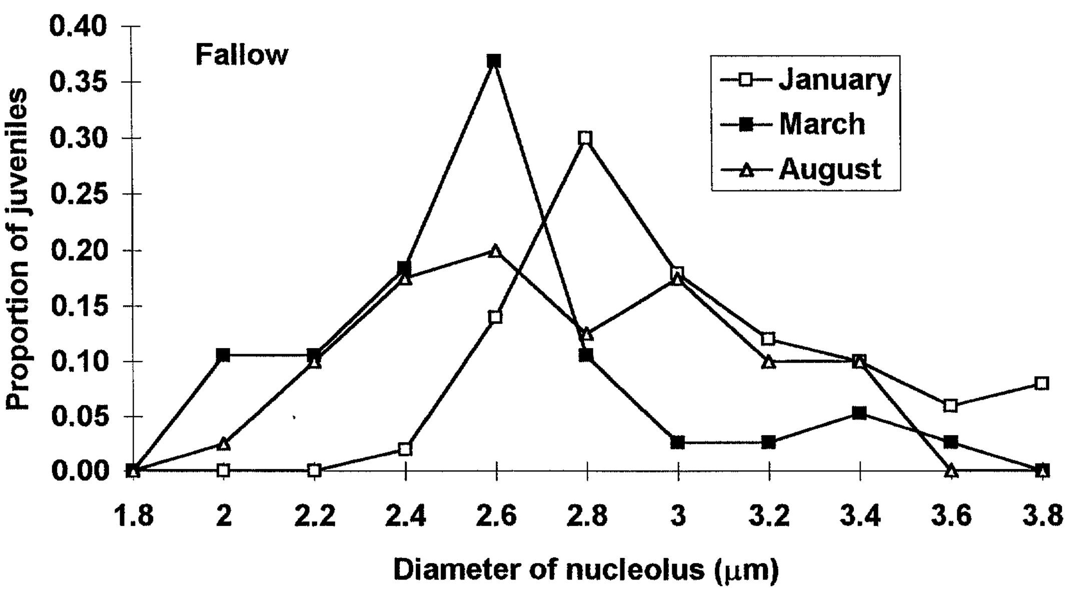

FIG. 3. Seasonal changes in the proportion of individuals with different diameters of the nucleolus in the nucleus of the dorsal pharyngeal gland in unhatched *Globodera* spp. Second-stage juveniles during the potato growing season. Cysts were collected from fallow plots at Toralapa, Cochabamba, Bolivia.

TABLE 2. Diameters of the nucleolus in the nucleus of the dorsal pharyngeal gland (DPGN) in unhatched but potato root diffusate-stimulated *Globodera* spp. J2. All cysts were collected at the end of the 1995 growing season at Toralapa, Cochabamba, Bolivia, from fields with varying lengths of time since the last potato crop.

Years since last potato crop	DPGN diameter ± SE (μm)	Skewness	Kurtosis
0	3.66 ± 0.05 a	−0.15	−1.18
2	3.39 ± 0.04 b	0.20	−1.07
4	3.20 ± 0.05 c	0.86	0.09

Means for DPGN diameter followed by different letters are significantly different ($P < 0.05$, t-test) (SE = standard error).

showing that a combination of both factors provides a good description of the observed effect. The model generates a loss of 36% of J2 using an exponential decline curve. It assumes all animals with a DPGN diameter of 4.0 μm in January do hatch by March, with the probability of hatch declining to 10% for individuals with a DPGN diameter of only 1.6 μm in January. This loss of individuals plus a reduction in DPGN diameter of 0.4 μm of all unhatched J2 between January and March provides a good fit to the observed data (Fig. 5). It is not possible to generate such a good fit using only values for either one or the other of the two effects that are combined in the model.

Potato plants and PCN co-evolved in Central and South America. Therefore, PCN are well adapted to the seasonal nature of this crop in the Andes. A J2 hatching in March, for example, would not complete its life cycle before the potato harvest. By August, the DPGN diameter indicates that some J2 may be prepared to hatch even before the host is present. The actual planting date varies between years depending on the onset of spring rainfall. Such readiness to hatch may favor completion of the life cycle by minimizing the delay in responding to host presence. This would favor PCN in the short growing season that prevails at high altitude in Bolivia. The observed change between January and March occurred under all crops, suggesting that the unhatched J2 respond to other factors than host presence, such as seasonal changes in temperature or soil moisture levels. Previous work has shown seasonal biochemical changes in unhatched *Heterodera glycines* J2 in the field that are not related to plant cover or host phe-

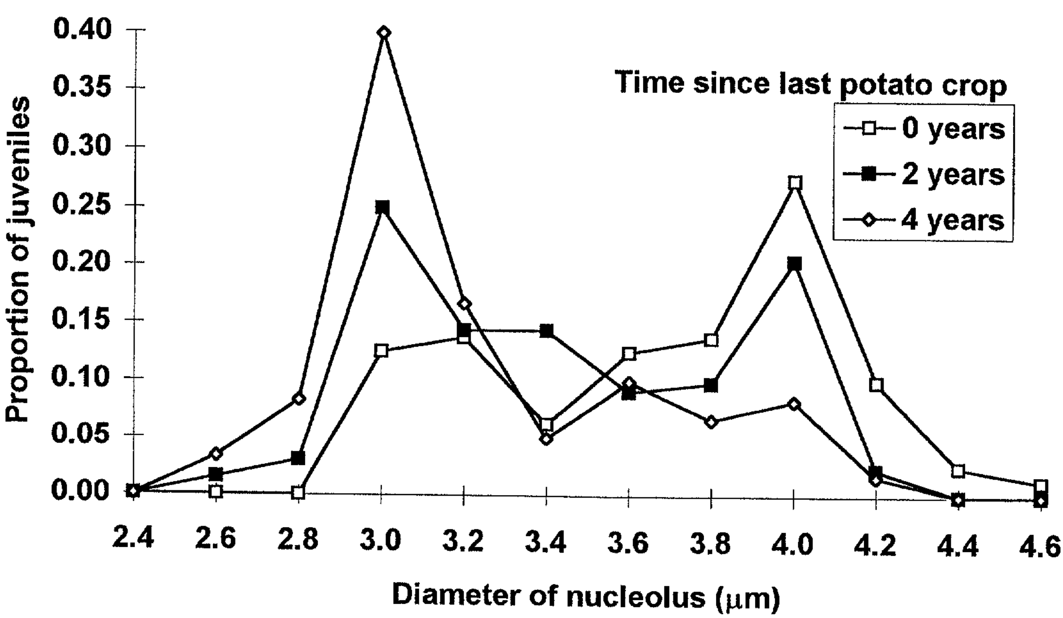

FIG. 4. Changes in the proportion of unhatched *Globodera* spp. Second-stage juveniles with different diameters of the nucleolus in the nucleus of the dorsal pharyngeal gland after stimulation with potato root diffusate for 7 days. All cysts were collected in August 1995 at the end of the growing season at Toralapa, Cochabamba, Bolivia. The cysts came from fields at 0, 2, and 4 years after the last potato crop.

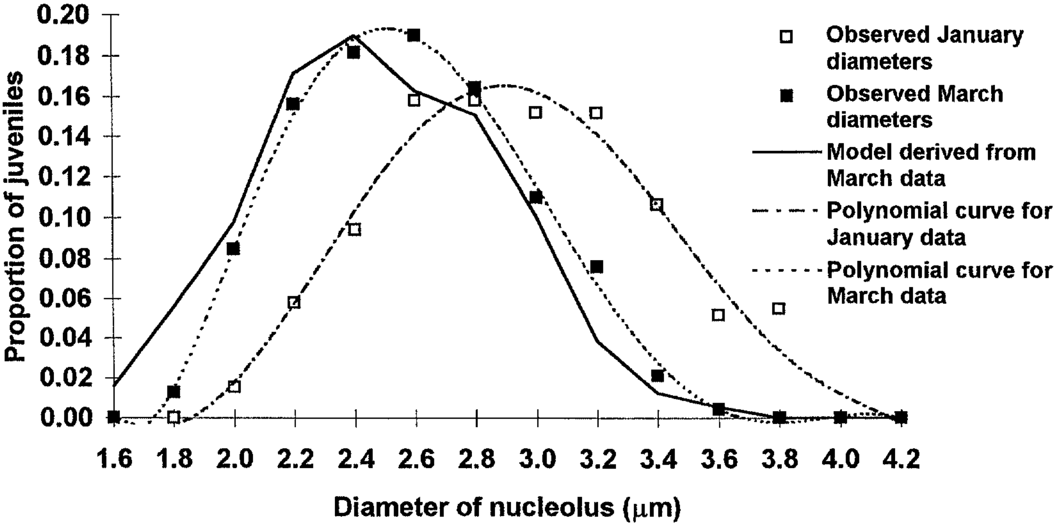

FIG. 5. A comparison between observed data in January and March for the diameter of the nucleolus in the nucleus of the dorsal pharyngeal gland (DPGN) of unhatched second-stage juveniles from *Globodera* spp. cysts collected from nonhost crop plots at Toralapa, Cochabamba, Bolivia. Polynomial curves are fitted to the two data sets. In addition, a curve generated by a model is provided for the March data. The model is based on assuming a hatch of 36% of the individuals recorded in January by March, and a mean decline in value of 0.4 μm for DPGN diameter for the remaining individuals.

nology (Yen et al., 1996). Further work may be of value to clarify the distinct possibility that J2 show an annual rhythm in their readiness to hatch in the field.

The data from potato plots are complicated by the recruitment of new cysts to the population by May, resulting in a mix of older and newly recruited cysts in May and August. Second-stage juveniles from new cysts are likely to enter diapause (Hominick et al., 1985), and this may explain the relatively low mean value recorded in August in comparison to values of cysts without new recruitment in fallow plots.

The graphs in Figure 4 show a bimodal distribution of values for J2 exposed to PRD for 7 days. Previous work suggests that nematodes with a nucleolus diameter of >3.8 μm are highly likely to hatch within a few days (Atkinson et al., 1987; Perry et al., 1989). In contrast, juveniles with a nucleolus diameter of around 3.0 μm have little if any response to the hatching stimulus. Clearly, the proportion of animals indicated as ready to hatch after 7 days of stimulation declined with the duration of their previous dormancy. This may indicate that prolonged dormancy is correlated with a longer period of preparation to hatch in response to PRD. It may also reflect the accumulation of less responsive individuals in cysts as their more responsive siblings hatch. Further work is required to determine if the effects reported in this work occur widely in potato fields.

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