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Seasonal Biochemical Changes in Eggs of *Heterodera glycines* in Missouri[1]

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Abstract: Changes in the carbohydrate (glucose, trehalose, and glycogen) and total protein contents of eggs retained within *Heterodera glycines* cysts were monitored monthly in a field microplot experiment conducted from March 1993 to March 1995. Treatments included two near-isogenic lines of soybean cv. Clark differing for date of maturity, and one corn hybrid. The soybean lines were planted in microplots infested with *H. glycines* at a high average initial population density (Pi) (23,810 eggs/100 cm^3 soil), and the corn was planted in microplots infested at high (24,640) and low (5,485) Pi. Soil temperatures at 15 cm depth and rainfall were monitored. Carbohydrate contents varied in the same pattern, with the highest levels measured before planting (May) and after harvest (October) in both years. Neither Pi nor soybean isoline had an effect on any measured response, but the carbohydrate contents of eggs from corn and soybean microplots differed during the overwinter (October–May) periods ($P < 0.0001$). Trehalose accumulation was negatively correlated with soil temperature ($r = -0.78$ and $r = -0.84$, $P = 0.0001$, July through November 1993 and 1994, respectively), which reflects its role as a cryoprotectant. In contrast to the pattern for carbohydrates, total protein was lowest before planting and after harvest, and highest (>20 μg/1,000 eggs) June through October. Protein content was unaffected by plant cultivar or species. Protein and carbohydrate levels in *H. glycines* eggs showed seasonal changes that appeared to be primarily temperature-dependent.

Key words: biochemistry, dormancy, *Glycine max,* hatching, *Heterodera glycines,* soybean cyst nematode, trehalose.

Egg dormancy is important for overwinter survival of the soybean cyst nematode *Heterodera glycines* Ichinohe in North America during cold winter periods (37). Dormancy in other species may be affected by several factors, including temperature, moisture, oxygen content, and salt content in soil (13,15,20,31). Dormancy of *H. glycines* eggs may be affected by soil temperature (1,19) and host phenology (19); however, we found that dormancy of *H. glycines* eggs retained within the cyst was not affected by host phenology and was independent of soil temperature (37).

The mechanism of dormancy in *H. glycines* is unknown, but biochemical changes associated with dormancy of some parasitic nematodes have been described (2,6,32). Nematode eggs are composed primarily of carbohydrates, proteins, and lipids (9). Carbohydrates and lipids provide the energy for either the development or reproduction of nematodes and also serve as storage materials for overwinter survival (9,26). In *Nematodirus battus,* an animal-parasitic nematode, the lipid content of dormant eggs decreased from 30% to 15% of the dry weight during an 8-week chilling period at 5 °C. In contrast, total carbohydrate, glycogen, and trehalose contents of *Nematodirus battus* eggs increased during chilling (4,5). The lipid content of unhatched dormant *Globodera pallida* and *G. rostochiensis* eggs declined during overwinter survival in the soil (32).

Trehalose functions as a cryoprotectant that is involved in diapause of insects and some nematodes (3,10). Trehalose is a non-reducing disaccharide formed by two glucose molecules joined through the 1-carbon and may account for a large percentage of a nematode's dry weight (9). Eggs of *G. rostochiensis* may contain 6.7% trehalose by dry weight (12), and the highest levels of trehalose (7.9% of the dry weight) have been found in *Ascaris lumbricoides* eggs (18). Trehalose exists in the perivitelline fluid of the egg and plays a

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role in the hatching of eggs (27–29). In *N. battus,* chilling the eggs results in increased trehalose concentration and lowered supercooling point (5,6).

Further characterization of dormancy in *H. glycines* would be facilitated by the availability of a biochemical or genetic marker. In addition, a marker would save time and money in applied research programs, in which experimental results are affected by dormancy in the inoculum. The objective of this study was to determine the seasonal changes in *H. glycines* egg chemical composition in order to identify a biochemical marker for dormancy.

Materials and Methods

Experiments were conducted in field microplots at the University of Missouri Agronomy Research Farm near Columbia, Missouri, from March 1993 to March 1995. A description of the experimental site has been published (37). The 1-m^2 microplots were surrounded by fiberglass barriers that extended 0.53 m below the soil surface. Initial population (Pi) densities of *H. glycines* race 3 in the microplots in February 1993 were either high (23,810 ± 950 eggs/100 cm^3 soil) for soybean and high (24,640 ± 1,030 eggs/100 cm^3 soil) or low (5,485 ± 230 eggs/100 cm^3 soil) for corn.

Microplots were planted to two near-isogenic soybean lines ("isolines") provided by R. S. Nelson (USDA soybean Germplasm Collection, Urbana, Illinois) differing from Clark (Maturity Group III) for maturity (e_2, 10 days earlier, and E_1, 20 days later) and one corn hybrid (Pioneer 3563). Clark is susceptible to *H. glycines;* corn is a non-host and was included as a check for host effects. Forty-five soybean seeds or 24 corn seeds were planted through the center of each microplot and thinned by hand to 24 soybean seedlings or 10 corn seedlings 7 days after emergence. Soybean or corn was planted to extend the microplot row beyond the fiberglass boundary, and rows were planted 0.76 m on either side of the microplot row so that the row within the microplot was subject to conditions similar to those within a field canopy.

The experimental design was an incomplete block (two soybean isolines at one Pi and one corn isoline at two Pi, for four treatments) replicated four times. Plant treatments were assigned arbitrarily within Pi classes. The planting dates were 3 June 1993 and 15 June 1994, and harvest dates were in October for both years. Soil temperature was monitored with a datalogger (Li-Cor, Lincoln, NE) at a depth of 15 cm.

Soil samples were collected at monthly intervals. In each microplot, six 2.5-cm-diam, cores were taken 20 cm deep from within 20 cm of the row. Each sample was mixed thoroughly and a 100-cm^3 subsample (measured by water displacement) was processed with a semi-automatic soil elutriator (11) in which the cysts were collected on a 75-μm-pore sieve. Eggs were freed from the cysts by a mechanical method (25) and collected on a 25-μm-pore sieve. Egg suspensions were concentrated by centrifugation in a 2.5-M sucrose solution (7). For comparison with eggs from the microplots, and as a control for the effects of laboratory and greenhouse conditions during the hatching tests and biochemical analysis described below, each set included eggs from a greenhouse isolate that does not exhibit seasonal reduction in hatching (hereafter referred to as the check isolate). Eggs from the check isolate were extracted at the same time and in the same manner as eggs from the microplots.

Biochemical analyses for trehalose, glycogen, total carbohydrate, and total protein also were conducted on eggs from each microplot plus the check isolate. Each 1,000-egg allotment for biochemical analysis was transferred to a 1.5-ml microcentrifuge tube and subjected to centrifugation for 10 minutes at 3,000*g*. The supernatant liquid was discarded, 0.5 ml sterile water was added, and the centrifugation was repeated twice to remove all traces of sugar solution. Finally, 0.5 ml sterile water was added to each microcentrifuge tube in an ice bath. A micro-ultrasonic cell dis-

rupter (Kontes, Vineland, NJ) was used to disrupt the eggs. The disrupter was used in three 45-second bursts, with 1-minute cool-down periods between bursts.

Trehalose analysis: Tubes containing disrupted eggs were centrifuged at 3,000*g*. The supernatant liquid was retained, and 0.5 ml sterile water was added. The centrifugation was repeated twice, resulting in a 1.5-ml sample. A 1.5-ml aliquot of 95% (v/v) ethanol was added to each sample in a 10-ml glass tube and the tubes placed on ice for 1 hour and then centrifuged at 3,000*g* to remove insoluble materials. Solvent was removed, under vacuum, at 50°C on a rotary evaporator. Remaining traces of solvent were removed by placing the samples in a vacuum desiccator containing phosphorus pentoxide.

Trehalose was converted to the trimethylsilyl-(TMS)-derivative (34) and quantitatively measured after gas chromatography with a flame ionization detector. Results are reported relative to a standard curve prepared with authentic D-(+)-trehalose (Sigma, St. Louis, MO).

Total carbohydrate analysis: Disrupted egg suspensions were transferred from 1.5-ml microcentrifuge tubes to 10-ml glass tubes. One ml of 0.2% (w/v) anthrone in concentrated sulfuric acid was added to each glass tube. The solution developed a blue color after being shaken for a few minutes at room temperature (17). Absorbance was determined at 620 nm, and results are reported relative to a standard curve prepared with D-(+)-glucose (Sigma, St. Louis, MO).

Glycogen analysis: Disrupted egg suspensions were centrifuged at 3,000*g*. The supernatant liquid was decanted into a 2-ml serum bottle placed in an ice bath, and the precipitate was homogenized in 0.45 ml sterile water and 0.05 ml 10% trichloroacetic acid with an ultrasonic cell disrupter (14). The supernatant liquid was collected and the procedure repeated once. The supernatant liquids were mixed with 0.5 ml of cold 95% (v/v) ethanol and allowed to stand 30 minutes and then centrifuged 5 minutes at 3,000*g*. The supernatant was discarded and the precipitate was dis-

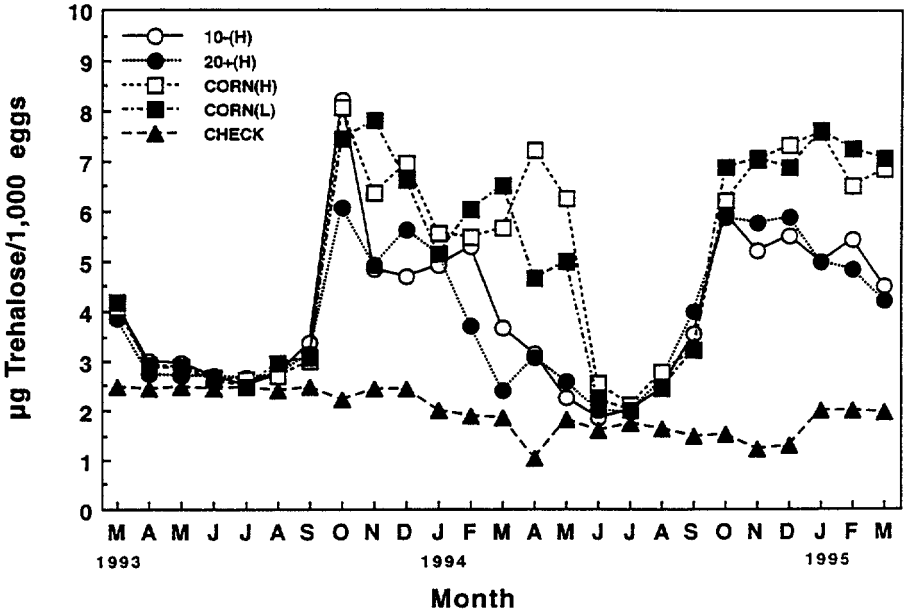

FIG. 1. Changes in the trehalose content of eggs of *Heterodera glycines* extracted from cysts collected from field microplots planted with near-isogenic soybean lines that differed from Clark soybean for date of maturity (10− = 10 days earlier and 20+ = 20 days later) and corn. Microplots were infested with high (H) or low (L) initial population of *H. glycines*. Check refers to a greenhouse isolate of *H. glycines* produced on Essex soybean. Data are means of four replications.

solved in 0.5 ml sterile water and then reprecipitated by adding 1 ml cold 95% ethanol. The precipitate was collected after centrifugation at 3,000*g* and dried in the oven at 60 °C. Dried precipitate was dissolved in 0.5 ml sterile water and enzymatically hydrolysed with amyloglycosidase (from *Aspergillus niger*) (Sigma, St. Louis, MO) at 50 °C for 1 hour. Pure glycogen (type III, from rabbit liver) (Sigma, St. Louis, MO) was used as a standard for this chemical analysis. The glycogen content was determined by the anthrone-sulfuric acid method (17).

Total protein analysis: The disrupted egg suspension was transferred from a 1.5 ml microcentrifuge tube to a 10-ml glass tube. Total protein was determined by the Markwell method (24) and reported relative to a standard curve prepared with bovine serum albumin (BSA) (Sigma, St. Louis, MO).

Data were subjected to analysis with PC-SAS (SAS Circle, Cary, NC) procedures: general linear models (GLM) for analysis of variance and single-degree-of-freedom comparisons, and CORR for product-moment correlations.

Results

Trehalose analysis: Eggs of the check isolate of *H. glycines* contained between 1 μg and 2.5 μg trehalose/1,000 eggs during the entire experiment from March 1993 to March 1995 and did not follow a seasonal pattern (Fig. 1). The trehalose content of eggs from the microplots declined from March to April 1993 and then remained below 3 μg/1,000 eggs until July 1993. Trehalose content increased to more than 6.2 μg/1,000 eggs in October 1993. Moreover, the trehalose content of eggs from the corn treatments was higher than that of eggs from the soybean treatments from November 1993 to May 1994 (P = 0.0001), but there were no differences between the two soybean isolines. From February to May 1994, the trehalose contents of eggs produced on either soybean isoline decreased as from March to May 1993 and did not differ, but the trehalose content of eggs from the corn treatments remained >5 μg/1,000 eggs. After May 1994, the trehalose contents of eggs from soybean and corn treatments declined, and no treatment effects were detected. Trehalose

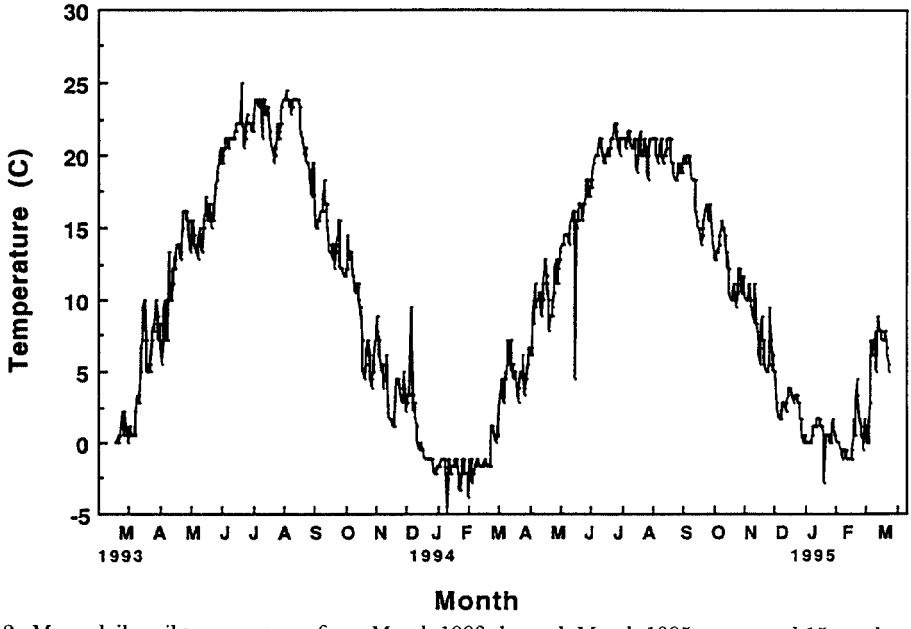

FIG. 2. Mean daily soil temperatures from March 1993 through March 1995, measured 15 cm deep, in field microplots at the University of Missouri Agronomy Research Farm near Columbia, Missouri.

contents of all treatments increased after July 1994 in a pattern similar to that for 1993, i.e., differences were detected in trehalose contents of eggs from the corn and soybean treatments ($P = 0.001$) but not between soybean isolines or between Pi levels on corn.

The mean daily soil temperature at 15 cm soil depth was recorded from March 1993 to March 1995 (Fig. 2). The temperature increased from March to July 1993 and remained between 20 °C and 25 °C through September 1993. The temperature declined gradually to below 0 °C from December 1993 to March 1994. The temperature curve of 1994 was similar to that of 1993. Trehalose content was inversely correlated with soil temperature from July to November 1993 ($r = -0.78$, $P = 0.0001$) and July to November 1994 ($r = -0.84$, $P = 0.0001$).

Glycogen and total carbohydrate analysis: Glycogen and total carbohydrate contents changed synchronously. The highest levels were measured before planting (June) and after harvest (October) in both years (Figs. 3,4). The glycogen content of eggs was more than μg/1,000 eggs from March to June 1993 and then decreased in July 1993 (Fig. 3). The glycogen content increased gradually from July to November 1993 and also was negatively correlated with soil temperature during this period ($r = -0.91$ $P = 0.0001$). From November 1993 to May 1994, glycogen content of eggs from two soybean isolines did not differ ($P = 0.64$), whereas the glycogen contents of eggs from soybean treatments were lower than those of eggs from microplots planted to corn ($P = 0.0001$). After May 1994, glycogen contents of eggs from all treatments declined until July 1994 and increased again through July 1994 to December 1994.

The total carbohydrate content remained higher than 18 μg/1,000 eggs during March and April 1993 (Fig. 4) but declined in May and June 1993 and was maintained at a low level of >6 μg/1,000 eggs until October 1993. Total carbohydrate content increased after October 1993 and remained at about 16 μg/1,000

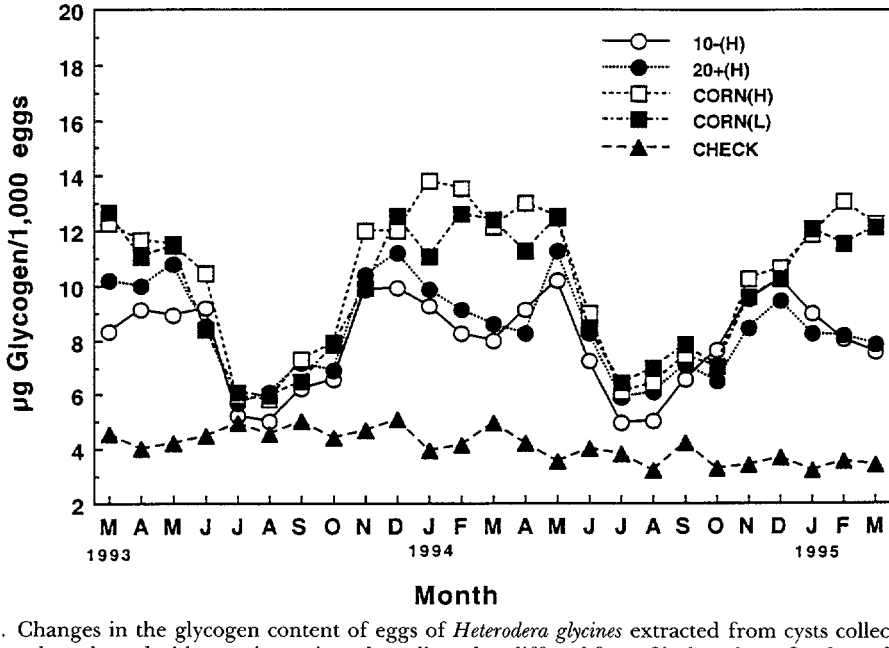

FIG. 3. Changes in the glycogen content of eggs of *Heterodera glycines* extracted from cysts collected from field microplots planted with near-isogenic soybean lines that differed from Clark soybean for date of maturity (10− = 10 days earlier and 20+ = 20 days later) and corn. Microplots were infested with high (H) or low (L) initial population of *H. glycines*. Check refers to a greenhouse isolate of *H. glycines* produced on Essex soybean. Data are means of four replications.

eggs for both soybean isolines and corn, with no significant differences between them. The pattern between June, 1994 and March 1995 was similar.

Total protein analysis: The pattern in change of total protein was the converse of total carbohydrate and glycogen, with the lowest level occurring before planting and after harvest, and highest (>20 μg BSA/1,000 eggs) levels measured from June through October 1993 and 1994 (Fig. 5). (The total protein content of the check isolate of *H. glycines* was below 10 μg BSA/1,000 eggs throughout the study. No differences were detected between eggs from microplots planted to corn and eggs from microplots planted to soybean.

Discussion

A concurrent study showed that dormancy induction of *H. glycines* eggs within cysts, as indicated by decreasing hatching rates during the soybean growing season, was unrelated to soil temperature (37). Furthermore, soil temperature increases in the spring were accompanied by only small increases in hatching rates, demonstrating that dormancy-breaking was also independent of temperature as the primary determinant. Conversely, this study found that trehalose accumulation was highly correlated with decreasing soil temperature, and when soil temperatures increased in the spring, trehalose content decreased. Thus, although dormant eggs may have high trehalose levels, trehalose accumulation and dormancy induction are independent events. Similar seasonal relationships were observed for glucose, glycogen, and protein contents.

The analyses we performed on carbohydrate and protein levels, in addition to trehalose, allow us to make some observations on the biochemistry of *H. glycines* relative to other nematodes. The main energy source of many plant-parasitic nematodes and free-living nematodes is lipid (8), which we were unfortunately not able to measure efficiently; however, in some cases, as in the microbivorous *Panagrellus redivivus* and *Turbatrix aceti* (36), it is glycogen. In the presence of oxygen, *P. redivivus*

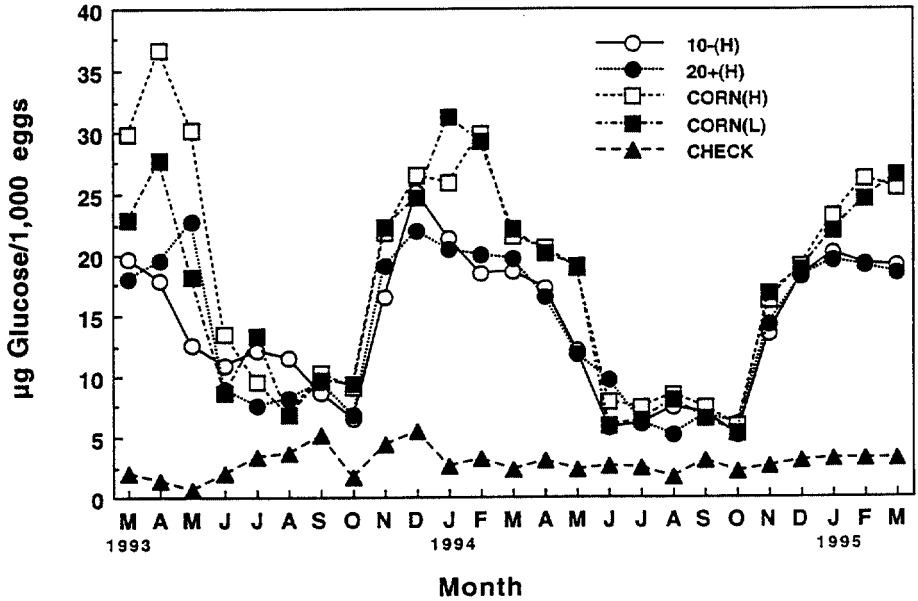

FIG. 4. Changes in the total carbohydrate content of eggs of *Heterodera glycines* extracted from cysts collected from field microplots planted with near-isogenic soybean lines that differed from Clark soybean for date of maturity (10− = 10 days earlier and 20+ = 20 days later) and corn. Microplots were infested with high (H) or low (L) initial population of *H. glycines*. Check refers to a greenhouse isolate of *H. glycines* produced on Essex soybean. Data are means of four replications.

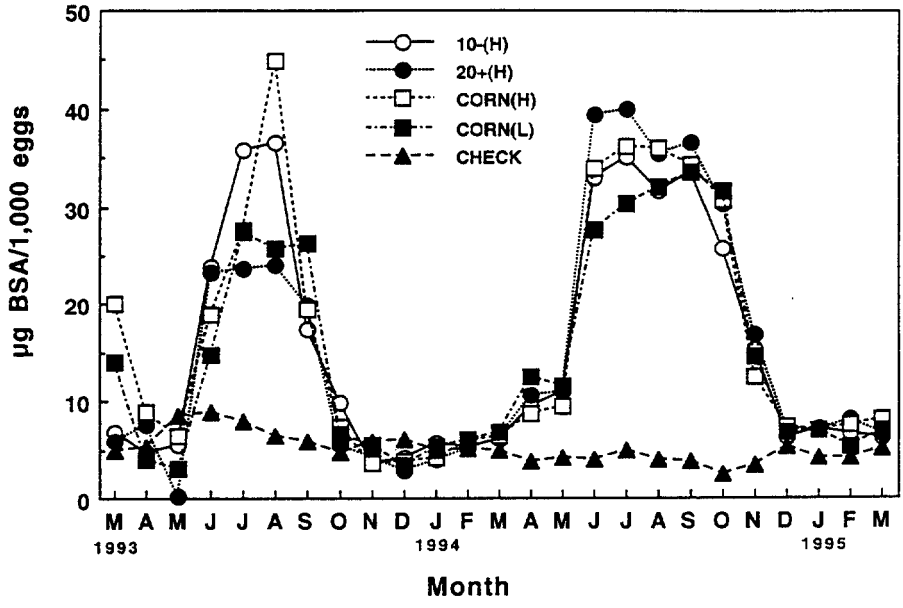

FIG. 5. Changes in the total protein content of eggs of *Heterodera glycines* extracted from cysts collected from field microplots planted with near-isogenic soybean lines that differed from Clark soybean for date of maturity (10− = 10 days earlier and 20+ = 20 days later) and corn. Microplots were infested with high (H) or low (L) initial population of *H. glycines*. Check refers to a greenhouse isolate of *H. glycines* produced on Essex soybean. Data are means of four replications. The total portein content is relative to a standard curve prepared with bovine serum albumin (BSA).

and *T. aceti* can accumulate and use lipid as their major source of energy, whereas, in the absence of oxygen, these nematodes can accumulate and use glycogen as their major energy source. Madin and Crowe (22) and Madin et al. (23) reported that *Aphelenchus avenae* can use lipid and glycogen during anhydrobiosis and convert them into trehalose and glycerol. Lipid and glycogen contents both declined rapidly during anhydrobiosis, whereas trehalose and glycerol contents both increased rapidly (23). In addition, a positive correlation exists between nematode survival and synthesis of trehalose and glycerol (22). During recovery from anhydrobiosis, trehalose and glycerol levels decreased and glycogen content increased (16). Womersley and Smith (35) also reported that the highest trehalose contents were found in the desiccated plant-parasitic nematodes *Anguina tritici* and *Ditylenchus dipsaci,* but glycerol content was low. In contrast, two microbivorous nematodes, *P. redivivus* and *T. aceti,* had high concentrations of glycerol (35). Krusberg (21) observed that trehalose from desiccated *D. dipsaci* and *A. tritici* is synthesized from glycogen and not lipid, and that the lipid content remains unchanged; however, utilization of lipid during desiccation also has been reported in *Meloidogyne naasi* (28), *Globodera* spp. (32), *Pratylenchus thornei* (33), and *Heterodera oryzae* (30). This study's results were obtained from analyses performed on eggs from cysts within soil, and presumably in a hydrated state. Under these conditions, the glycogen and trehalose contents of eggs of *H. glycines* increased at the same time; therefore, lipid rather than glycogen may be the resource for trehalose synthesis in *H. glycines,* whereas glycogen serves as the main carbohydrate storage material. In this study, both trehalose and glycogen contents of *H. glycines* eggs increased as soil temperatures decreased; therefore, both glycogen and trehalose may act as energy-storage materials for overwinter survival.

The biochemical changes we monitored

in eggs were apparently unrelated to plant species or host phenology. We observed differences only in the overwinter period in carbohydrate contents of eggs extracted from microplots planted with corn and soybean, which suggested that the differences were due to the ages of the eggs, i.e., those from corn microplots were at least 1 year older than those from the soybean microplots. As with dormancy induction (37), we found no differences associated with the different maturity dates of the soybean isolines. The inverse relationship between total protein and total carbohydrate content, lack of effects due to plant species, and lack of change in protein content of the check isolate suggests that most of the proteins synthesized are involved in activities not directly related to either dormancy or parasitism. Differential protein analysis would provide more insight into the implications of the protein content changes than measurement of total protein.

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