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Impact of Thermal History on Tolerance of *Meloidogyne hapla* Second-stage Juveniles to External Freezing[1]

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Abstract: Low temperature induced physiological changes that increased the ability of second-stage juveniles of *Meloidogyne hapla* to survive external freezing. Second-stage juveniles in polyethylene glycol solution were exposed to −4, 0, 4, or 24 C, and then their survival was determined after ice-induced freezing of the suspensions at −4 C for 24 hours. Survival was greatest for juveniles exposed to 4 C before freezing. Some juveniles were killed by exposure to −4 C before freezing of the suspensions. The percentage of juveniles surviving freezing increased from about 30% to 80% within 12 hours of exposure to 4 C. This tolerance of external freezing was lost during subsequent exposure to 24 C. Longer exposures, of 1 to 15 days, to low temperature did not increase the percentage surviving external freezing, as compared to the 12-hour exposure, but reduced the tolerance of external freezing lost during subsequent exposure to 24 C for 48 hours.

Key words: acclimation, cold hardening, cryobiology, freezing tolerance, invertebrate, *Meloidogyne hapla*, nematode, overwintering, thermal history.

Laboratory studies indicate that tolerance of frozen conditions differs among *Meloidogyne* species with different geographic distributions (24). Eggs of *M. hapla* Chitwood, a species distributed widely throughout North America, survived exposure to −2 C for 12 days, whereas eggs of *M. javanica*, a species limited to areas where the soil does not freeze during winter, were killed when exposed to the same conditions (8). Survival of eggs and second-stage juveniles (J2) at 0 C was greater for *M. hapla* than for *M. incognita* (6,26), a species with cold tolerance intermediate to that of *M. hapla* and *M. javanica* (24).

Studies of many temperate invertebrates (1,4,7,13), including nematodes (20–22), have demonstrated that the ability to survive subzero temperatures changes seasonally. These studies indicate that tests of survival at low temperatures have little ecological relevance if environmental preconditioning is omitted from experimental protocols.

The hypothesis that environmental cues such as short photoperiods and low temperatures elicit physiological changes that increase the ability to survive subzero temperatures has been tested for numerous invertebrate species (1,4,7,13). We (11) and others (3,15) have demonstrated that nematodes also undergo cold hardening. In our previous study (11), survival of second-stage *M. hapla* in frozen polyethylene glycol solution was inversely related to prefreeze temperatures of 4 to 24 C. Exposure to 4 C for 12 hours resulted in a fourfold greater percentage of juveniles surviving freezing of the solution around them than exposure to 24 C. This cold hardening response also occurred in soil.

To understand better when and how thermal history affects the ability of second-stage *M. hapla* to survive external freezing, we examined the response of juveniles to a variety of temperature regimens and exposure times. Our objective was to determine the effects of exposure to temperatures below 4 C, age of juveniles, and fluctuating temperatures on the percentage of juveniles surviving external freezing.

Materials and Methods

Information on the population of *M. hapla* and general methods were published previously (11). The nematodes were reared on tomato (*Lycopersicon esculentum* cv. Rutgers) in a growth chamber at 24 C. Second-stage juveniles collected within 48 hours of hatching were cleaned, sus-

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pended in 5% polyethylene glycol (20,000 molecular weight, Sigma, St. Louis, MO), and adjusted to 125 individuals/ml. Samples, consisting of 1-ml aliquots of the suspension in 20-ml test tubes (16 mm diameter), were placed in refrigerated constant temperature baths (Neslab, Newington, NH) filled with an ethylene glycol:water solution (1:1 v:v) for exposure to the designated temperatures (±0.1 C). At various intervals, a subset of the samples was transferred to a bath at −4 C, and freezing of each sample was initiated by adding a 100-µl capillary tube containing 100 µl of ice. Samples were kept frozen for 24 hours, thawed at 4 C for 12 to 18 hours, and warmed to room temperature for 2 to 4 hours. The percentage of surviving J2 was determined for each sample on the basis of spontaneous movement and movement detected after prodding J2 with a fiberglass pick. Survival of J2 in nonfrozen samples was determined similarly. Each experiment was repeated and each replicate utilized J2 harvested from one tomato plant.

Exposure to subzero temperature: Half of 108 samples of J2 were initially exposed to 4 or 24 C for 24 hours, and 18 samples from each preliminary exposure of 4 or 24 C were then exposed to 4, 0, or −4 C. Any samples at −4 C that froze spontaneously were discarded. After 24, 48, and 96 hours, three samples from each temperature combination were frozen at −4 C, thawed, and examined for survival. The three remaining samples from each temperature combination served as nonfrozen controls. Five replicates were conducted, each with J2 from one plant.

Juvenile age: Ninety samples of J2 were placed in an incubator at 24 C. After 1, 3, 6, 9, and 12 days, 18 samples were transferred to a bath at 4 C. At the beginning of exposure to 4 C and after 3, 6, 12, and 24 hours, three samples from the group of 18 were frozen at −4 C and thawed, and survival was assessed as described. The three remaining samples served as nonfrozen controls. Three replicates were conducted, but due to low numbers of J2, the entire 12-day treatment was omitted from one replicate, and samples for the 24-hour, 4 C exposure were omitted from the 12-day treatment in another replicate.

Long-term exposure to 4 C and increase in temperature: In an incubator at 4 C were placed 126 samples. After 1, 3, 6, 9, 12, and 15 days, 21 samples were transferred to 24 C. At the beginning of exposure to 24 C and after 6, 12, 24, 36, and 48 hours of exposure, three samples were frozen at −4 C, thawed, and examined for survival. The three remaining samples served as controls. Three replications were conducted.

Fluctuating temperature: Sixty samples of J2 were assigned to three groups of 20, and each group was exposed to a different temperature regimen for 48 hours. The first group was exposed to two consecutive 24-hour cycles of 12 hours at 4 C followed by 12 hours at 24 C. The second group was kept at 24 C for 24 hours and then exposed to one cycle of 12 hours at 4 C followed by 12 hours at 24 C. The third group remained at 24 C during the entire 48-hour period. All samples were then exposed to another 24-hour cycle of 12 hours at 4 C and 12 hours at 24 C, during which two samples from each group were taken at nine times (3-hour intervals) and frozen. The two remaining samples from each group served as controls. Five replicates were conducted.

Long-term exposure to 4 C and survival at different freezing temperatures: In an incubator at 4 C were placed 75 samples of J2. At the beginning of the experiment and after 3, 6, 9, and 12 days, three of these samples were transferred to baths at 0, −2, −4, −6, and −8 C and frozen, and survival was determined. Three replicates were conducted.

Data analysis: For all experiments, the three samples (two for the fluctuating temperature experiment) assigned to each treatment combination within each replicate were treated as subsamples, and means of the subsamples were considered as single data points in the analysis. The replicates were treated as blocks.

In the first experiment, data for frozen

samples and nonfrozen controls were analyzed separately with a blocked three-factor split-split level analysis of variance (ANOVA) model (SAS Institute, Cary, NC). Preliminary exposure to 4 or 24 C was considered as the main factor, and the subsequent temperature and duration were considered as subfactors.

Data for all other experiments were analyzed with a blocked two-factor split-level ANOVA model. For the second experiment described, time at 24 C was the main factor and time at 4 C was the subfactor. For the third experiment, time at 4 C was the main factor and time at 24 C was the subfactor. For the fourth experiment, the number of cold–warm cycles was considered as the main factor and time was considered as the subfactor. For the fifth experiment, time at 4 C was the main factor and freezing temperature was the subfactor.

Mean comparisons were conducted with Fisher's protected least significant differences (LSD) calculated at $P = 0.05$. Different LSD were calculated for within-subfactor and across-factor mean comparisons (18).

Results

Exposure to subzero temperature: The percentage of J2 surviving external freezing was always least ($P \leq 0.05$) for J2 exposed to −4 C before freezing and greatest ($P \leq 0.05$) for J2 exposed to 4 C before freezing (Fig. 1). The analysis of variance indicated a significant ($P \leq 0.01$) two-way interaction between temperature and duration of exposure; for each preliminary exposure, the percentage of J2 surviving external freezing increased with the duration of exposure to 4 C and decreased with the duration of exposure to −4 C. Duration of exposure to 0 C had no consistent effect on the percentage surviving external freezing. There was also a significant ($P \leq 0.01$) two-way interaction between preliminary exposure and temperature. Of J2 exposed to 0 or −4 C, survival after freezing of the suspensions was greater for those receiving the preliminary 4 C exposure.

The tubes were inspected several times per day during the prefreeze incubations, and ice was never observed in the suspensions at −4 C. Nevertheless, some J2 died during the prefreeze incubation at −4 C

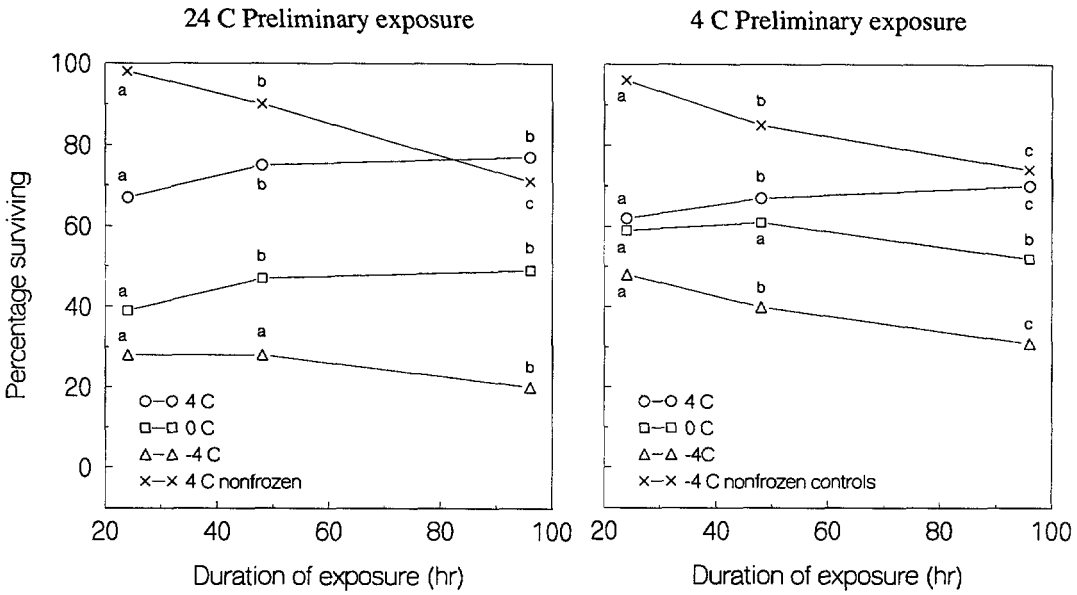

FIG. 1. Effects of exposure to 4, 0, and −4 C (nonfrozen) on the percentage of second-stage juveniles of *Meloidogyne hapla* surviving external freezing. The juveniles received preliminary 24-hour exposures to 24 C (left) or 4 C (right). Each point represents the mean of five replicates. Points within the same temperature treatment labelled with different letters are significantly different ($P \leq 0.05$).

(Fig. 1). Survival of the J2 at −4 C decreased through time, from 97% after 24 hours to 72% after 96 hours. Preliminary exposure to 4 or 24 C did not affect survival at −4 C. No J2 died at 0 or 4 C.

Juvenile age: For all periods of time after hatch, the percentage of J2 surviving external freezing increased ($P \leq 0.01$) during the subsequent 24-hour exposure to 4 C (Fig. 2). The level of tolerance of external freezing attained during the 4 C exposure declined ($P \leq 0.01$) with an increase in length of incubation at 24 C. Survival after exposure to 4 C for 24 hours and freezing of the suspensions was greater for J2 held for 1, 3, or 6 days after hatching than for J2 held for 9 or 12 days ($P \leq 0.05$). There was 24% mortality in nonfrozen controls held for 12 days before the low-temperature exposure, and there was no mortality of J2 held for shorter durations. Intestines of J2 were noticeably translucent after 6 days and completely clear after 9 days.

Long-term exposure to 4 C and increase in temperature: For all durations of exposure to 4 C, the percentage of J2 surviving external freezing decreased ($P \leq 0.05$) during the first 12 hours of exposure to 24 C (Fig. 3). The decline in tolerance of external freezing after transfer to 24 C was reduced ($P \leq 0.01$) by a longer duration of prior exposure to 4 C. For J2 exposed to 4 C for one day, survival after external freezing dropped 69% in the first 12 hours at 24 C and remained unchanged thereafter ($P \leq 0.05$). For J2 exposed to 4 C for 12 days, survival dropped 15% in the first 12 hours at 24 C and remained unchanged thereafter ($P \leq 0.05$).

Fluctuating temperatures: The percentage of J2 surviving external freezing increased during the 12 hours at 4 C and decreased during the 12 hours at 24 C (Fig. 4). The magnitude of change in survival after external freezing was inversely related ($P \leq 0.05$) to the number of prior cold–warm cycles. At the beginning of the cold phase of the cycle, survival after external freezing was greater ($P < 0.05$) for J2 exposed to two prior cold–warm cycles than for J2 exposed to one prior cycle or constant 24 C. After 12 hours of cold exposure, there

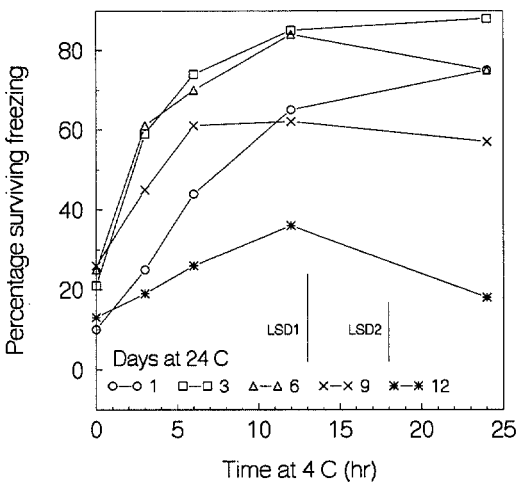

FIG. 2. Effects of duration of exposure to 4 C on the percentage of second-stage juveniles of *Meloidogyne hapla* surviving external freezing. The juveniles were initially kept at 24 C for 1, 3, 6, 9, or 12 days after hatching. For 1-, 3-, 6-, and 9-day data, each point represents the mean of three replicates. The 12-day data are means of two replicates except for the 24-hour 4 C exposure, which represents one sampling. LSD1 and LSD2 are for comparing means ($P \leq 0.05$) across different age groups or within the same age group, respectively.

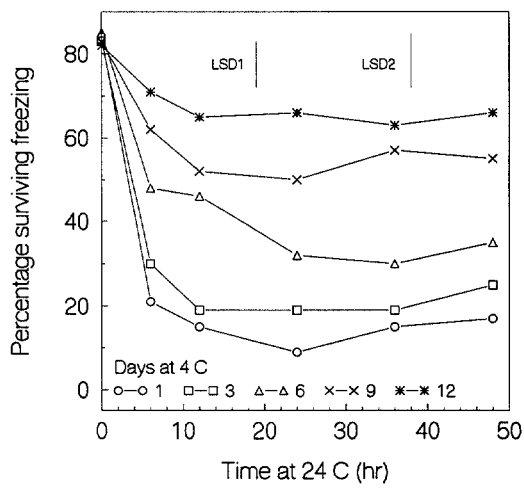

FIG. 3. Effects of exposure to 24 C, after exposure to 4 C for 1 to 12 days, on the reduction in percentage of second-stage juveniles of *Meloidogyne hapla* surviving external freezing. LSD1 and LSD2 are for comparing means ($P \leq 0.05$) within the same 4 C preconditioning regimen or across different preconditioning regimens, respectively.

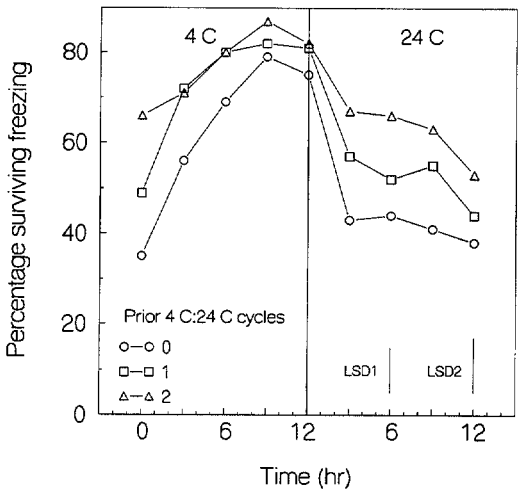

FIG. 4. Effects of exposure to a 24-hour cycle of 12 hours at 4 C and 12 hours at 24 C on the percentage of second-stage juveniles of *Meloidogyne hapla* surviving external freezing. The juveniles were previously exposed to none, one, or two prior cycles. Each point represents the mean of five replicates. LSD1 and LSD2 are for comparing means ($P \leq 0.05$) within the same preconditioning regimen or across different preconditioning regimens, respectively.

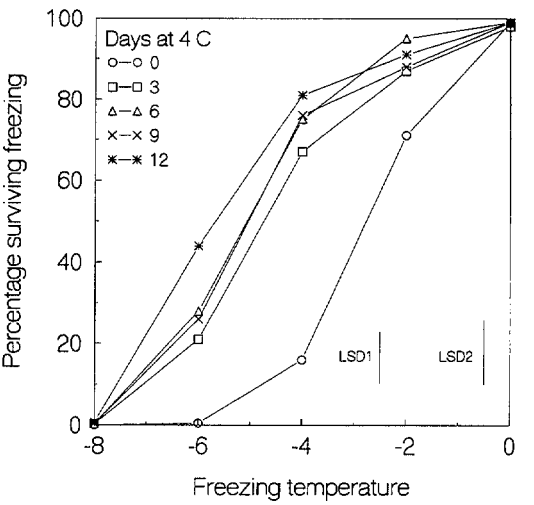

FIG. 5. Effect of exposure to 4 C for 0, 3, 6, 9, or 12 days on the percentage of second-stage juveniles of *Meloidogyne hapla* surviving external freezing at −2, −4, −6, and −8 C. Each point represents the mean of three replicates. LSD1 and LSD2 are for comparing means ($P \leq 0.05$) within the same preconditioning regimen or across different preconditioning regimens, respectively.

were no differences in survival after external freezing for J2 with different thermal histories. After 12 hours at 24 C, the percentage of J2 surviving external freezing was again greater ($P \leq 0.05$) for J2 exposed to two prior cold–warm cycles than for J2 exposed to one prior cycle or constant 24 C.

Long-term exposure to 4 C and survival at different freezing temperatures: Exposure to 4 C increased the percentage of J2 surviving external freezing at −6, −4, and −2 C. Survival after external freezing at −6 C was greater ($P \leq 0.05$) for J2 exposed to 4 C for 12 days than for those exposed to 4 C for 9, 6, or 3 days (Fig. 5). For other freezing temperatures, survival was not significantly affected by the duration of exposure to 4 C.

DISCUSSION

In this and previous studies (11,12), the survival of second-stage *M. hapla* exposed to frozen conditions was affected by thermal history. Tolerance of external freezing increased rapidly during exposure to low temperature and decreased during subsequent warming. Furthermore, the decrease in tolerance of external freezing during warming was reduced by long-term exposure to low temperature.

The dynamic nature of *M. hapla* J2 tolerance of external freezing illustrates the importance of examining thermal history when conducting laboratory studies to assess the low temperature limits of survival for populations. Failure to consider thermal history could result in estimates of survival at subzero temperatures that may not reflect survival of nematodes in the field. The duration of exposure to low temperature needed for increasing the tolerance of external freezing of *M. hapla* J2 in this study indicates that previously used preconditioning regimens for *M. hapla* (11,12, 23,26) were sufficient to ensure high tolerance.

Even for the *M. hapla* J2 preconditioned at 4 C, survival dropped sharply between freezing temperatures of −4 and −6 C. In soil, moisture (12,26) and perhaps other factors affect nematode survival at subzero temperatures. Such factors could reduce

the slope of the effect of freezing temperature on survival in soil. Bélair (5) reported that field populations of *M. hapla* in Quebec survived winter soil temperatures as low as −16 C.

Our results contrast with some previous studies on *M. hapla* and other nematodes. Prior exposure to low temperature does not affect survival of *M. incognita* J2 in soil at 0 C (6). This species is unable to survive for extended periods in frozen soil (26) and may not possess the physiological adaptations necessary for surviving subzero temperatures. Studies monitoring changes in nematode population densities (10,17, 23) also failed to establish a relationship between thermal history and mortality in soil at subzero temperatures, perhaps due to the confounding effects of temperature on reproduction and natality. For example, Sayre (23) found that emergence of second-stage *M. hapla* from egg masses was reduced by exposure to low temperature prior to freezing. It is likely that the eggs matured more rapidly at warmer temperatures and that the data reflect differences in initial rate of eclosion rather than survival.

Of the temperature regimens we tested, the optimal temperature for developing tolerance of external freezing was near 4 C. Survival after external freezing for J2 exposed to 0 or −4 C in this study, or to 8–24 C in a previous study (11), never exceeded values attained at 4 C. It is possible that tolerance of external freezing increases more slowly at temperatures below 4 C and was not evident within the exposure times that we used.

In previous research (11) we found that some *M. hapla* J2 were killed by exposure to 0 C. The mortality of nonfrozen controls at −4 C in this study confirms that some second-stage *M. hapla* are killed by low temperatures without external freezing. Mortality caused by a rapid reduction in temperature without freezing, referred to as cold shock or direct chilling injury, occurs in bacteria, protozoans, algae, higher plants, and insects (4,13,14,19). Indirect chilling injury results from sustained exposure to low temperature rather than rapid cooling rate (14,19). Because mortality of the *M. hapla* J2 occurred despite prior exposure to 4 C for 24 hours and the rate of mortality was relatively slow, the J2 were more likely killed by indirect chilling injury.

Some nematodes accumulate low molecular weight carbohydrates and polyols during exposure to low temperature (2) and desiccation (9,16,27). Our research (12) indicates that *M. hapla* J2 may be desiccated by external freezing. The physiological changes associated with tolerance of external freezing may resemble those associated with desiccation tolerance in other species.

If carbohydrate or polyol cryoprotectants are synthesized from lipid reserves in *M. hapla* J2 during exposure to low temperatures, then starvation could reduce tolerance of external freezing by reducing lipid reserves and the amount of cryoprotectant produced from them. The intestines of *M. hapla* J2 were noticeably translucent at 6 days and completely clear at 9 days after hatching, indicating a decrease in lipid reserves (25). Nonetheless, even after 12 days, which resulted in 24% mortality in nonfrozen controls, exposure to low temperature induced an increase in percentage of J2 surviving external freezing. Identifying the cryoprotectants accumulated during exposure to low temperatures and quantifying the relationships among cryoprotectant concentration, tolerance of frozen conditions, and lipid reserves would provide stronger evidence for the relationship between the ability to survive frozen conditions and starvation.

Although the percentage of J2 surviving external freezing reached maximal levels within ca. 12 hours, the relationship between duration of exposure to 4 C and decline in tolerance of external freezing during subsequent exposure to 24 C indicates that additional physiological changes also occur. Seasonal changes in insect freezing tolerance and supercooling ability often involve the accumulation of several different cryoprotectants at different times (4). The

rapid induction of tolerance of external freezing in second-stage *M. hapla* may be the result of initial cryoprotectant accumulation. The more slowly accumulating and stable form may result from the production of different cryoprotectants or other physiological changes, such as increased membrane fluidity or permeability or the synthesis of new enzymes.

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