Journal of Nematology 30(2):255–261. 1988.

Neutral Storage Lipid and Exit Behavior of *Bursaphelenchus xylophilus* Fourth-stage Dispersal Juveniles from their Beetle Vectors[1]

W. Terrell Stamps and Marc J. Linit[2]

Abstract: The J4 dispersal juvenile is a specialized life stage of the pinewood nematode, *Bursaphelenchus xylophilus* (Steiner & Buhrer) Nickle, that is transported by cerambycid beetles in the genus *Monochamus*. The mediation of J4 exit from beetle vectors is poorly understood. We hypothesized that decreasing neutral storage lipid in *B. xylophilus* J4 was related to behaviors leading to their exit from the beetle on which they are carried. J4 remaining within beetles and J4 exited from beetles were fixed, stained, and digitized for computerized image analysis of neutral storage lipid content. Nematodes remaining within beetles had significantly higher neutral lipid area than those that exited during the first 4 weeks of adult beetle life. Neutral storage lipid content of J4 from within beetles decreased dramatically at week 5 and remained low through week 10. Desiccation was thought to be the reason for this decrease. Neutral storage lipid was correlated with nematode exit and may contribute to *B. xylophilus* exit from vectors.

Key words: behavior, *Bursaphelenchus xylophilus,* lipid, nematode, pine wilt, pinewood nematode, staining.

Bursaphelenchus xylophilus (Steiner and Buhrer) Nickle is a secondary, and sometimes primary, pathogen of *Pinus* spp. The dispersal stage of the nematode is transported by cerambycid beetles in the genus *Monochamus* (Mamiya and Enda, 1972). The fourth-stage dispersal juvenile (J4) is a non-feeding, dauer-like juvenile stage that is adapted to unfavorable conditions (Mamiya, 1983). J4 enter the respiratory systems of newly eclosed adult beetles that have developed within pine and are carried to new host trees where the nematodes enter beetle oviposition or feeding wounds (Kobayashi et al. 1984; Linit, 1990; Wingfield and Blanchette, 1983).

The factors influencing J4 exit from beetles are not well understood. Volatile terpenes such as β-myrcene from pine have been suggested to stimulate exit from beetle vectors (Giblin-Davis, 1993; Hinode et al., 1987; Ishikawa et al., 1986), but the detection of these compounds by J4 cannot fully explain the pattern of nematode exit. Large numbers of J4 exit beetles in the apparent absence of pine volatiles (unpublished data). The temporal pattern of nematode exit does not coincide with the presence of the most likely pine volatile cues. Exit is miminal the first week after beetle emergence, when beetles are feeding on healthy pine and β-myrcene is present in high concentrations. Exit reaches a peak in 2 or 3 weeks, then gradually declines over the remaining weeks of the beetle's adult life (Kobayashi et al., 1984; Linit, 1989; Nakane, 1976).

The main food reserve for the non-feeding stages of numerous plant- and animal-parasitic nematodes is neutral lipid (Barrett, 1976; Storey, 1983, 1984; Van Gundy, 1965). Kondo and Ishibashi (1978) hypothesized that stored neutral lipid of J4 of *B. xylophilus* may be mobilized to glycogen for energy needs or may be used in the histogenesis of the intestine, median bulb, stylet, and reproductive organs in preparation for molting to the adult stage. Non-feeding, dauer juveniles of some species of nematodes utilize lipid at a linear rate of about 1% a day under normal conditions (Barrett, 1969; Wilson, 1965). *Bursaphelenchus xylophilus* J4 may use internal monitoring of utilization of neutral storage lipid (NS lipid) as a component in the initiation of exit behavior.

Factors intrinsic to the nematodes may mediate J4 exit from their beetle vectors. Changes in the concentrations of lipid, glycogen, or other compounds stored within

Received for publication 7 July 1997.

[1] Contribution from the Missouri Agricultural Experiment Station, Journal Series No. 12743.

[2] Department of Entomology, University of Missouri, Columbia, MO 65211

E-mail: linit@showme.missouri.edu

the nematode is one possible mechanism. We hypothesize that decreasing NS lipid in *B. xylophilus* J4 is related to behaviors leading to their exit from the beetle on which they are carried. The objectives of this study were to examine (i) the relationship of NS lipid content to exit of J4 by comparing NS lipid reserves in J4 within beetles with reserves in those having exited from beetles without extrinsic cues, and (ii) the temporal progression of NS lipid utilization in J4.

Materials and Methods

Monochamus carolinensis (Olivier) adults were examined for infestation of J4 upon emergence from logs of jack pine, *Pinus banksiana* Lamb, and maintained at 28 °C and 70% RH (Zhang et al., 1995). Infested beetles were marked with a binary labeling technique (Humphry and Linit, 1989) to document their date of emergence. Nematode-infested beetles of 11 ages (1, 7, 14, 21, 28, 35, 42, 49, 56, 63, and 70 days following adult beetle emergence) were placed in exit arenas on raised wire screen floors. J4 that exited the beetles in 24 hours were collected in distilled water in the bottom of the petri dishes. Beetles from which nematodes exited were processed in a Baermann funnel for 24 hours to collect resident J4 (those that remained within beetles) (Southey, 1986).

If no nematodes exited a beetle, the beetle was either returned to the laboratory colony or macerated for nematode extraction, as above. Beetles returned to the lab colony were reassayed weekly for up to 4 weeks. Additional beetles that were not assayed during the first 14 days of adulthood were added to the study to increase the number of time points, as younger beetles died or were processed for nematode recovery.

Nematode processing and lipid staining: The NS lipid reserves of the two groups of J4, those that exited beetles and resident J4, were quantified and compared. J4 from 1- to 70-day-old adult beetles were examined.

The low numbers of nematodes that typically exit beetles over a 24-hour period (a few hundred or less) required us to use a computerized digital quantification of stained NS lipid rather than the more traditional chemical quantification methods that require many thousands of nematodes (Fitters et al., 1997). J4 were fixed, and the droplets of unbound neutral lipid within the nematodes were then stained with oil red O following the procedure of Stamps and Linit (1995). Approximately 10 nematodes from a single beetle were then mounted on a microscope slide using the wax ring method (Southey, 1986).

NS lipid content of resident and exited J4: Individual stained J4 were recorded for 10 seconds with a high-resolution video camera mounted on a brightfield microscope, and images were videotaped. A frame grabber board in a desktop computer was used to caputure a single image of each of the J4 recorded.

Image analysis software (Mocha, SPSS, Chicago, IL) was used to determine total body area and NS lipid droplet area of each videotaped J4. A clearfield image was applied to each nematode to correct for uneven lighting across the image, thereby improving accuracy of the measurement process. A calibration was also performed on each image to convert raw pixels to millimeters.

Two "overlay planes" were created to measure the NS lipid area and the body area of each nematode. The planes were set to measure area (mm^2) and average light intensity (scale of 0–255; 0 = black, 255 = white) based on pixel intensity of the NS lipid and the unstained non-lipid area of the nematode body, respectively. Images of more than 1,500 nematodes from approximately 100 beetles were analyzed during the course of the study.

The percentage of body area occupied by droplets of NS lipid (percent lipid area) was measured on 5 to 10 nematodes from an individual beetle, and the mean value was used as the experimental unit in all subsequent data analyses. Light intensity values originally were intended to be used as an indicator of depth of NS lipid (i.e., overlapped droplets), but slight variations in

staining among wells of the tissue-culture plate outweighed subtle differences in NS lipid density among nematodes. Though area of NS lipid is a less accurate estimate than volume, area measurements usually are sufficient to indicate significant differences between treatments (Nwosu, 1978; Van Gundy et al., 1967).

Analysis of variance and least squares means tests were used to determine if percent NS lipid area differed significantly between resident and exited J4 from 1- to 70-day-old beetles. Least squares means tests were used to determine if NS lipid contents within each nematode group changed significantly over time. Pearson product moment correlations (r) were used to determine the relationship over beetle ages between percent beetles with exiting nematodes and percent NS lipid area of resident nematodes and percent resident nematodes with no stained lipid.

A second study was undertaken to determine the rate of NS lipid loss in J4 resulting from the energy expended during movement in the exit arena water. One or two *B. xylophilus*-infested beetles at each of five time points (1, 7, 14, 21, and 35 days) were processed in Baermann funnels to extract J4. The nematodes from each beetle were randomly assigned to one of two treatments: (i) immediate fixation in FA 4:1, or (ii) placement in distilled water in the bottom of exit arenas for 24 hours, followed by fixation. All nematodes were then stained and processed for image analysis, as above. Analysis of variance was used to compare percent NS lipid area of nematodes, pooled across beetle ages, between treatments.

Results

Nematodes did not exit from 1-day-old adult beetles. Exited nematodes from older beetles were collected from an average of 30% of the beetles tested.

The percent NS lipid area of resident and exited J4 in beetles 28 days old or younger differed significantly between treatments ($F = 6.21$; df = 1,86; $P = 0.015$) and among beetle age ($F = 4.62$; df = 9,86; $P = 0.0001$). The interaction was not significant ($F = 0.49$; df = 8,86, $P = 0.857$). The percent NS lipid area of J4 exited from 7- to 28-day-old beetles was significantly lower than that of resident J4 (Fig. 1). Within-treatment NS lipid area did not decrease significantly over the first 28 days for both resident and exited JIV.

There was a significant, four-fold decrease in NS lipid area of resident J4 from beetles more than 28 days old, compared with the NS lipid areas of resident J4 from younger beetles (Fig. 1). Neutral stained lipid area did not decrease significantly in resident J4 from beetles at least 35 days or older. There was a decrease in the NS lipid area of exited J4 corresponding to the drop observed for residents, but the decrease was not significant. Also, the NS lipid areas of resident and exited J4 from older beetles did not differ within beetle age. The number of resident J4 containing few or no stained lipid droplets increased dramatically in beetles more than 28 days old. About half of the resident J4 from beetles beyond 28 days contained no stained lipid droplets. In comparison, only about 10% of resident J4 from younger beetles contained no stained lipid droplets (Table 1). This accounted for the significant decrease in NS lipid areas of resident J4 from the older beetles (Fig. 1). Nematodes collected from beetles only 1 day after emergence had large numbers of NS lipid droplets, and NS lipid areas had little variation compared to NS lipid areas of J4 from older beetles (Fig. 1). Fifty percent of 7- to 28-day-old beetles had exiting nematodes compared to 22% of 35-day-old and older beetles ($T = 3.012$, df = 8, $P = 0.017$). There was a significant positive correlation between the percentage of beetles with exiting nematodes and the percent NS lipid area of resident nematodes ($r = 0.665$, $P = 0.05$). There was a significant negative correlation between the percentage of beetles with exiting nematodes and the percentage of resident nematodes with no stained NS lipid ($r = -0.669$, $P = 0.049$). As the number of beetles with exiting nematodes decreased over time, the percent NS lipid in resident nematodes

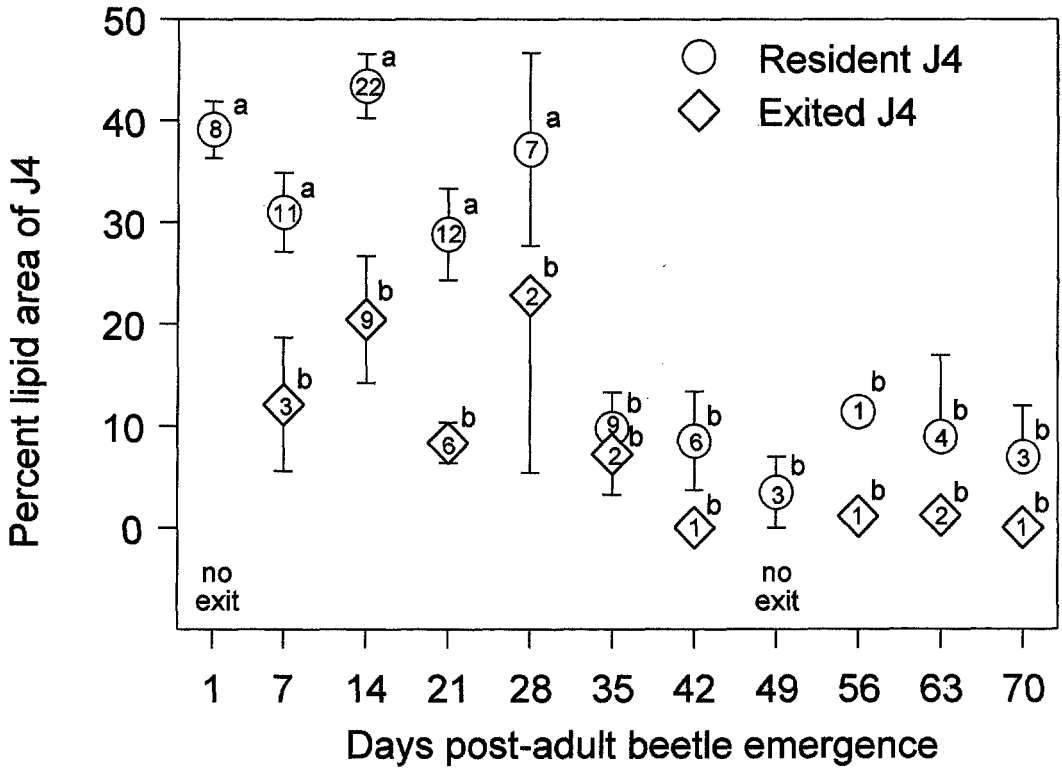

FIG. 1. Percentage of neutral storage (NS) lipid contents of resident J4 dispersal juveniles of *Bursaphelenchus xylophilus* (○) and exited J4 (◇) over 10 weeks post-adult beetle emergence (beetle age 1, 7, 14 . . . 70 days). Data points are means of the NS lipid percentage of 5 to 10 resident or exited J4 per beetle, the numbers within each data point are the number of beetles examined, and the error bars are standard errors. "No exit" indicates exited J4 were not collected for that time point. Mean values followed by the same letter did not differ significantly within treatments (percentage NS lipid area) across beetle age and between treatments within beetle age at $P = 0.05$, according to least squares means tests.

decreased and the number of resident J4 with no stained lipid increased. Most nematodes exited from beetles 2 to 4 weeks old, with the number decreasing during the remainder of the beetle's life.

TABLE I. Percentage of total number of individual resident J4 dispersal juveniles of *B. xylophilus* without stained neutral storage (NS) lipid. Means followed by the same letter do not differ significantly from one another at $P = 0.05$.

Adult beetle age (days)	No. of J4 examined	Percentage of total J4 without stained NS lipid
1	52	6 a
7	94	1 a
14	215	11 a
21	115	17 a
28	56	14 a
35	75	57 b
42	51	63 b
49	20	80 b
56	10	60 b
63	28	68 b
70	20	65 b

The NS lipid of J4 removed from beetles and placed in distilled water in exit arenas for 24 hours did not differ significantly ($F = 0.10$; df = 1,12; $P = 0.735$) from resident J4 that were fixed immediately upon removal.

DISCUSSION

The temporal pattern of *B. xylophilus* exit from its insect vector has been described by several researchers (Kobayashi et al., 1984; Linit, 1989; Nakane, 1976; Togashi, 1985) and appears to be similar for all *Monochamus* species. J4 exit is generally very low during the first 5 to 10 days following adult beetle emergence. Exit is highest during the next few weeks and then declines as adult beetles reach an age of 5 weeks or more. The

mechanisms that underlie this pattern are poorly understood.

A distinct bimodal pattern of J4 exit from beetle vectors was observed in this study. Most nematodes exited beetles within the first 4 weeks following adult beetle emergence, and a greater percentage of beetles tested during the first 4 weeks of adult life carried nematodes that exited. The rate of nematode exit from adult beetles greater than 4 weeks of age was dramatically lower.

No J4 exit was observed from 1-day-old beetles. The J4 exiting from 7- to 28-day-old beetles had significantly lower NS lipid area than resident J4 from beetles of the same age range. This suggests that J4 behavior changes are associated with changes in NS lipid area or that a significant amount of lipid was utilized during the exit process. Work conducted with other nematode species and the results of the present study do not support the latter conjecture.

A variety of nematode species use lipid at low rates under normal conditions (Barrett, 1969; Wilson, 1965). Nwosu (1978) determined that excessive activity resulting from artificial and continuous stimulation by neostigmine bromide for 8 hours was required for a loss of 10% lipid in *Ancylostoma tubaeforme*. In the present study, there was a threefold difference in estimated NS lipid between resident and exited J4 from 7- to 28-day-old beetles. In contrast, the NS lipid of J4 removed from beetles and placed in distilled water in exit arenas for 24 hours did not differ significantly from resident J4 that were fixed immediately upon removal from beetles. Thus, it is unlikely that the difference observed in NS lipid area between resident and exited J4 was due to energy consumption during the exit process.

Uniformly high amounts of NS lipid in J4 from 1-day-old beetles were correlated with the absence of exiting J4 during the first week of an adult beetle's life (in the absence of plant or fungal clues). J4 resident in older beetles showed greater variation in NS lipid area, with a significant portion of the population sampled having few or no NS lipid droplets. The change in variation of NS lipid area from low to high over the first 4 weeks in resident J4 corresponded to the onset of exit behavior. Changes in NS lipid content in resident nematodes were correlated with the percentage of beetles with exiting nematodes.

Decreased NS lipid area in resident nematodes from older beetles (35 days or more) indicated a physiological change. Resident J4 in beetles at least 5 weeks old became brown and dried in appearance and were unable to move when placed on a glass slide. These desiccated J4 could be rehydrated in water in the laboratory, but this process is unlikely to occur within a beetle's trachea under natural conditions. Thus, the majority of resident J4 in beetles older than 5 weeks may be unable to exit because of desiccation or depleted energy reserves. The lack of NS lipid may reflect the energy cost of converting neutral lipid to other compounds for surviving desiccation. In *Aphelenchus avenae,* lipid content dropped to 60% of its original value in the first 72 hours of slow drying, with a concomitant increase in trehalose and glycerol, both of which were implicated in survival under drying conditions (Evans and Womersley, 1980). Lipid also decreases upon rehydration. These processes may explain the decrease in the NS lipid of resident J4 from older beetles. J4 may use NS lipid upon entering a desiccated state and use still more NS lipid when rehydrated during extraction in a Baermann funnel. Dessication as a method of survival under adverse conditions is a well-documented component of the life histories of many nematode species (Evans and Womersley, 1980; Van Gundy, 1965; Wharton et al., 1985).

Though evidence from other species suggests lipid can be a minor component of the energy required for host invasion (Van Gundy et al., 1967), mobility of *B. xylophilus* resident J4 may be affected when NS lipid is depleted. NS lipid area of resident J4 from beetles at least 4 weeks old decreased significantly compared to J4 from younger beetles. This decrease correlated with decreases in the numbers of exiting nematodes and a decrease in percent beetles with exiting nematodes, as reported in this study and by others

(Kobayashi et al., 1984; Linit, 1989; Nakane, 1976; Togashi, 1985). Consequently, J4 mobility may have been compromised and these older J4 may have been unable to exit because of dessication or lack of energy reserves in the form of NS lipids. Robinson et al. (1985) suggested that lipid depletion below some minimum in *G. rostochiensis* compromised its ability to reach, invade, and move to feeding sites in roots.

Nematodes continued to exit at later time points, though at much lower rates, and these nematodes may have been from deeper tracheae within the beetles. A dry, brown ball of nematodes often can be observed within the atrium of the first abdominal spiracle of older beetles. Upon removal of this ball, lighter colored, less desiccated J4 can be seen within the tracheal system. Consequently, a large number of J4 remaining within older beetles may be desiccated, unable to exit, and contain very little NS lipid, while a small number of J4 within the beetle's tracheae, having avoided desiccation, may still be able to exit the beetles.

Many nematode species rely on environmental cues for exiting the dauer state or for host location and penetration (Clarke and Hennessy, 1984; Dropkin, 1989; Golden and Riddle, 1984a,b; Prot, 1980). In contrast, the exit behavior of *B. xylophilus* apparently follows a fixed temporal pattern, with exit maximized during the second to fourth weeks of an adult beetle's life (Enda, 1972; Hosoda and Kobayashi, 1977, 1978; Linit, 1989; Nakane, 1976). In the present study, J4 exit occurred over distilled water in the absence of pine volatiles and rate of exit was correlated with beetle age. This suggests that processes within the nematode or beetle are related to exit behavior. J4 occupy beetle trachea, a space external to the beetle hemocoel. Thus, it seems unlikely that nematode exit would be linked to changes in the physiology of the vector. We believe that exit rate is related to lipid content of the J4, which is a function of J4 age and correlated with adult beetle age. However, we recognize that lipid may be simply a co-correlate to another intrinsic factor that involves nematode exit. J4 exit based on lipid depletion may be a "fail-safe" default mechanism that operates if external cues fail to initiate exit. If operative, this mechanism suggests that all J4 would eventually exit; however, exit would be inhibited if desiccation occurred prior to exit. Neutral lipids are not the only energy-rich compounds in dispersal juveniles of *B. xylophilus*. Other storage compounds such as glycogen should be examined in relation to the exit behavior of this nematode.

The sensitivity of *Caenorhabditis elegans* to environmental cues changes with nematode age (Golden and Riddle, 1982, 1984a). In *B. xylophilus*, changes in NS lipid or other intrinsic factors may alter the nematode's sensitivity to plant- or insect-derived volatiles. This interaction may result in J4 exit behavior that is synchronized with beetle activity and host plant condition to ensure nematode transmission to new host trees. The role of environmental cues, particularly as they interact with internal states such as lipid reserves, needs to be investigated.

Literature Cited

Barrett, J. 1969. The effect of aging on the metabolism of infective larvae of *Strongyloides ratti*, Sandground. Parasitology 59:3–17.

Barrett, J. 1976. Energy metabolism in nematodes. Pp. 11–70 *in* N. A. Croll, ed. The organization of nematodes. London: Academic Press.

Clarke, A. J., and J. Hennessy. 1984. Movement of *Globodera rostochiensis* (Wollenweber) juveniles stimulated by potato root exudate. Nematologica 30:206–212.

Dropkin, V. H. 1989. Introduction to plant nematology. New York: John Wiley and Sons.

Enda, N. 1972. Removing dauerlarvae of *Bursaphelenchus lignicolus* from the body of *Monochamus alternatus*. Transactions of the Annual Meeting of the Kanto Branch, Japanese Forestry Society 24:32. (In Japanese).

Evans, A. A. F., and C. Womersley. 1980. Longevity and survival in nematodes: Models and mechanisms. Pp. 193–212 *in* B. M. Zuckerman, ed. Nematodes as biological models, volume 2. New York: Academic Press.

Fitters, P. F. L., E. M. J. Meijer, D. J. Wright, and C. T. Griffin. 1997. Estimation of lipid reserves in unstained living and dead nematodes by image analysis. Journal of Nematology 29:160–167.

Giblin-Davis, R. M. 1993. Interaction of nematodes with insects. Pp. 302–344 *in* M. W. Khan, ed. Nematode interactions. London: Chapman and Hall.

Golden, J. W., and D. J. Riddle. 1982. A pheromone influences larval development in the nematode *Caenorhabditis elegans*. Science 218:578–580.

Golden, J. W., and D. J. Riddle. 1984a. The *Cae-*

norhabditis elegans dauer larva: Developmental effects of pheromone, food, and temperature. Developmental Biology 102:368–378.

Golden, J. W., and D. J. Riddle. 1984b. *Caenorhabditis elegans* dauer-inducing pheromone and an antagonistic component of the food supply. Journal of Chemical Ecology 10:1265–1280.

Hinode, Y., Y. Shuto, and H. Watanabe. 1987. Stimulating effects of β-myrcene on molting and multiplication of the pinewood nematode *Bursaphelenchus xylophilus*. Agricultural and Biological Chemistry 51:1393–1396.

Hosoda, R., and K. Kobayashi. 1977. Drop-off procedures of the pinewood nematode from the pine sawyer. Transactions of the Annual Meeting of the Kansai Branch, Japanese Forestry Society 28:255–258 (in Japanese).

Hosoda, R., and K. Kobayashi. 1978. Drop-off procedures of the pinewood nematode from the pine sawyer (II). Transactions of the Annual Meeting of the Kansai Branch, Japanese Forestry Society 29:131–132 (in Japanese).

Humphry, S. J., and M. J. Linit. 1989. Effect of pinewood nematode density on tethered flight of *Monochamus carolinensis* (Coleoptera: Cerambycidae). Environmental Entomology 18:670–673.

Ishikawa, M. Y., Shuto, and H. Watanabe. 1986. β-Myrcene, a potent attractant component of pine wood for the pinewood nematode *Bursaphelenchus xylophilus*. Agricultural and Biological Chemistry 50:1863–1866.

Kobayashi F., A. Yamane, and T. Ikeda. 1984. The Japanese pine sawyer beetle as a vector of pine wilt disease. Annual Review of Entomology 29:115–135.

Kondo, E., and N. Ishibashi. 1978. Ultrastructural differences between the propagative and dispersal forms in pinewood nematode. *Bursaphelenchus lignicolus*, with reference to the survival. Applied Entomology and Zoology 13:1–11.

Linit, M. J. 1989. Temporal pattern of pinewood nematode exit from the insect vector *Monochamus carolinensis*. Journal of Nematology 21:105–107.

Linit, M. J. 1990. Transmission of pinewood nematode through feeding wounds of *Monochamus carolinensis* (Coleoptera: Cerambycidae). Journa of Nematology 22:231–236.

Mamiya, Y. 1983. Pathology of the pine wilt disease caused by *Bursaphelenchus xylophilus*. Annual Review of Phytopathology 21:201–220.

Mamiya, Y., and N. Enda. 1972. Transmission of *Bursaphelenchus lignicolus* (Nematoda: Aphelenchoididae) by *Monochamus alternatus* (Coleoptera: Cerambycidae). Nematologica 18:159–162.

Nakane, I. 1976. Drop-off of the dauerlarvae of the pinewood nematode from the pine sawyer. Transactions of the Annual Meeting of the Kansai Branch, Japanese Forestry Society 27:252–254. (in Japanese).

Nwosu, A. B. C. 1978. Energy cost of locomotory activity in larval hookworms. International Journal of Parasitology 8:85–88.

Prot, J. C. 1980. Migration of plant-parasitic nematodes towards plant roots. Revue de Nématologie 3: 305–318.

Robinson, M. P., H. J. Atkinson, and R. N. Perry. 1985. The effect of delayed emergence on infectivity of juveniles of the potato-cyst nematode *Globodera rostochiensis*. Nematologica 31:171–178.

Southey, J. F., ed. 1986. Laboratory methods for work with plant and soil nematodes. Ministry of Agriculture, Fisheries, and Food Ref. Book. 402. London: Her Majesty's Stationery Office.

Stamps, W. T., and M. J. Linit. 1995. A rapid and simple method for staining lipid in fixed nematodes. Journal of Nematology 27:244–247.

Storey, R. M. J. 1983. The initial neutral lipid reserves of juveniles of *Globodera* spp. Nematologica 29:144–150.

Storey, R. M. J. 1984. The relationship between neutral lipid reserves and infectivity for hatched and dormant juveniles of *Globodera* spp. Annals of Applied Biology 104:511–520.

Togashi, K. 1985. Transmission curves of *Bursaphelenchus xylophilus* (Nematoda: Aphelenchoididae) from its vector *Monochamus alternatus* (Coleoptera: Cerambycidae) to pine trees with reference to population performance. Applied Entomology and Zoology 20:246–251.

Van Gundy, S. D. 1965. Factors in the survival of nematodes. Annual Review of Phytopathology 3:43–68.

Van Gundy, S. D., A. F. Bird, and H. R. Wallace. 1967. Aging and starvation in larvae of *Meloidogyne javanica* and *Tylenchulus semipenetrans*. Phytopathology 57:559–571.

Wharton, D. A., J. Barrett, and R. N. Perry. 1985. Water uptake and morphological changes during recovery from anabiosis in the plant-parasitic nematode *Ditylenchus dipsaci*. Journal of Zoology 206:391–402.

Wilson, P. A. G. 1965. Changes in lipid and nitrogen content of *Nippostrongylus brasiliensis* infective larvae aged at constant temperature. Experimental Parasitology 16:190–194.

Wingfield, M. J. and R. A. Blanchette. 1983. The pinewood nematode, *Bursaphelenchus xylophilus*, in Minnesota and Wisconsin: Insect associates and transmission studies. Canadian Journal of Forest Research 13:1068–1076.

Zhang, X., W. T. Stamps, and M. J. Linit. 1995. A nondestructive method of determining *Bursaphelenchus xylophilus* infestation of *Monochamus* spp. vectors. Journal of Nematology 27:36–41.