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Effect of *Monochamus carolinensis* on *Bursaphelenchus xylophilus* Dispersal Stage Formation[1]

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Abstract: *Bursaphelenchus xylophilus* and its insect vector, *Monochamus carolinensis,* both develop within rapidly degrading xylem tissue of dying or recently cut trees of *Pinus* spp. The influence of *Monochamus* development on *B. xylophilus* dispersal stage formation was investigated. Nearly all nematodes extracted from wood surrounding beetle galleries were third-stage dispersal juveniles (J3). Formation of fourth-stage dispersal juveniles (J4) occurred almost exclusively in the presence of *M. carolinensis* late pupae and callow adults. This pattern was observed with live insects in naturally formed galleries, diet-reared insects in artificial galleries, and pulverized insects in artificial galleries. The molt from J3 to J4 appeared to be related to adult eclosion in *M. carolinensis.* We hypothesize that a genus-specific substance(s) associated with *Monochamus* adult eclosion ensures the *Monochamus-B. xylophilus* association.

Key words: *Bursaphelenchus xylophilus,* development, dispersal stage, *Monochamus carolinensis,* nematode, pine sawyer, pinewood nematode.

The pinewood nematode, *Bursaphelenchus xylophilus* (Steiner & Buhrer) Nickle, and its insect vectors in the genus *Monochamus* (Coleoptera: Cerambycidae) develop in close association within the wood of infested trees. *Bursaphelenchus xylophilus* colonizes and reproduces within new host trees or logs, reaching high population densities before switching from reproductive life stages to dispersal forms (Mamiya, 1984). The mechanisms that mediate the switch in *B. xylophilus* development from the reproductive forms to the third-stage dispersal juvenile (J3) are thought to be related to within-wood conditions and are independent of the presence or absence of insect vectors (Necibi, 1996; Warren and Linit, 1993).

Wood-boring beetles provide the only known means of transport for *B. xylophilus* from infested to uninfested host trees (Linit, 1988). Nematode J4 that surround beetle pupal chambers move into the chamber following adult eclosion and pack the tracheae of the beetle. The J4 are carried to new host trees during the feeding and oviposition activities of the beetle (Edwards and Linit, 1992; Kobayashi et al., 1984; Linit, 1988, Wingfield, 1983). The phoretic relationship between the nematode and beetle results in nematode transmission to new host trees and logs and is limited to the J4 stage of the nematode and the adult beetle (Kobayashi et al., 1984; Linit, 1988). Nematode and beetle development are synchronized within the dying host trees, suggesting that abiotic and biotic stimuli are critical for successful phoresy.

Warren and Linit (1993) collected *B. xylophilus* J3 and J4 from *M. carolinensis* (Olivier) larvae, pupae, and callow adults, although the numbers obtained from larvae were very low. The number of J4 was highest on newly formed, callow adults. Warren and Linit (1993) also reported that J4 did not develop in logs void of *Monochamus* activity.

The goal of this study was to investigate the influence of *M. carolinensis* life stages on formation of *B. xylophilus* dispersal stages adjacent to beetle galleries. The specific objectives were to determine: (i) the association of naturally developing *M. carolinensis* life stages with *B. xylophilus* dispersal stages, (ii) the effect of diet-reared *M. carolinensis* life stages on the development of *B. xylophilus* dispersal stages in artificial galleries, and (iii) the effect of pulverized *M. carolinensis* life stages on the development of *B. xylophilus* dispersal stages in artifical galleries.

MATERIALS AND METHODS

Naturally developing M. carolinensis: Logs used in this experiment were cut from *B.*

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xylophilus-free trees in a 30-year-old stand of jack pine, *Pinus banksiana* Lamb., at the Thomas A. Baskett Wildlife Research and Education Center in Boone County, Missouri, between June and October 1995. The logs were 12- to 15-cm diam. × 45 cm long. The ends of each log were coated with paraffin to retard moisture loss. Blue stain fungus, *Ophiostoma minus* (Hedgc.) H. & P. Sydow, was inoculated into the logs 1 day after felling. Two fungal inoculation sites (1.27-cm diameter, 2 cm deep) were drilled through the bark and into the wood. The holes were on opposite sides and ends of each log, about 5-10 cm from each end. A 5-mm-diam. circular plug of agar containing *O. minus* was placed in each hole. The holes were plugged with styrofoam and sealed with petroleum jelly. Seven days later, two additional holes were drilled opposite the fungal inoculation sites for nematode inoculations. A 2-ml suspension, containing about 20,000 laboratory-reared *B. xylophilus* of varying life stages, was pipetted into each hole and the holes sealed as above. Nematodes were reared in the laboratory on *Botrytis cinerea* Pers. or *Monilinia fructicola* (Wint.) Honey grown on 9.3% malt agar. Nematodes were extracted with the Baermann funnel technique (Southey, 1986). Logs were held in the laboratory at room temperature, and the population of nematodes was allowed to increase within the logs.

Immediately after nematode inoculation, each log was placed in a 61 × 61 × 61-cm screen cage with about 20 *M. carolinensis* adult beetles and pine foliage. Each log remained in the cage for 48 to 72 hours until at least 25 beetle oviposition sites were found. The logs were placed in a growth chamber at 30 °C, LD 14:10 photoperiod, and 75% RH.

Sixty to 70 days after beetle oviposition the logs were cut horizontally into 10-cm-wide disks with a hand saw. Beetle galleries encountered were checked for the presence of a beetle life stage. Wood forming the walls of the gallery surrounding developing insects was removed by inserting a 0.79-cm-diam. drill bit into the gallery. Wood samples were irregular in volume due to the varying diameter and orientation of the galleries. *Monochamus carolinensis* mature larvae, pupae, and callow adults were removed from galleries. Twenty individuals of each life stage were collected for use in the study. Each wood sample was classified as adjacent to a mature larva, pupa, or callow adult and weighed immediately to determine wet weight. Nematodes were extracted with Baermann funnels. Wood sample dry weight was determined after nematode extraction by heating the wood chips to 125 °C for about 48 hours, and mositure content expressed as the percentage of dry weight. Twenty additional wood samples, taken at least 2 cm away from any beetle gallery, were used as nonadjacent control samples (Warren and Linit, 1993) and processed as above).

Bursaphelenchus xylophilus extracted from wood samples were classified as J3, J4, or non-dispersal life stages (combined) and expressed as the number of nematodes per gram of dry wood. J3 and J4 were distinguished from non-dispersal nematode life stages by the dark body coloration due to the presence of lipids (Mamiya, 1984). The J3 has a stylet, and the abdominal tip is rounded. The J4 has no stylet, and the abdominal tip is pointed.

Each *M. carolinensis* mature larva, pupa, and callow adult removed from a gallery was macerated and nematodes were collected with Baermann funnels. All *B. xylophilus* extracted were counted directly in a watch glass under a dissecting microscope. Nematodes were identified as J3, J4, or non-dispersal life stages (combined).

Analysis of variance (Proc ANOVA, SAS Institute, Cary, NC) was used to compare mean J3 and J4 densities among wood sample types. All data were square root-transformed to minimize variation in nematode densities. Similar analyses were used to compare mean densities of J3 and J4 among beetle life stages. Chi-square analysis was used to determine if the frequency of J3 or J4 association with *M. carolinensis* was independent of beetle life stage. Chi-square analysis was conducted using a BASIC program written in the Missouri Agricultural

Experiment Station (University of Missouri, Columbia, MO). Correlation analysis (SAS Institute) was used to determine if the number of J4 carried by adult beetles was related to wood moisture content or the density of J3 in the wood surrounding the gallery.

Live M. carolinensis in artificial galleries: Logs, 1 m in length, were cut from the bole of a jack pine. Three fungal inoculation sites (1.27-cm diam. × 2 cm deep) were drilled through the bark and into the wood. The holes were located at the center of the log and about 5 to 10 cm from each end of the log. The end holes were drilled on the opposing face of the center hole. The logs were inoculated with fungus and nematodes as described above. The logs were held at room temperature in the laboratory for 45 to 60 days after fungal inoculation, after which time J3 were present. The logs were sliced into 7-cm disks, and the two outer disks of each log were discarded to avoid excessive drying associated with the cut edges.

Five holes (0.79-cm diam. × 2 cm deep) were drilled into the xylem at randomly selected locations on the disk to create an artifical gallery and simulate *M. carolinensis* pupal cells. The gallery, made with an electric drill and a bit, was large enough for insertion of a beetle life stage and to allow contact between the beetle and the gallery wall. Wood chips produced during gallery construction were weighed, and then nematodes were extracted and their numbers per gram of dry wood determined. After nematodes were extracted, each sample was heated and the moisture content determined as above. For each disk, the gallery that contained the greatest number of J3 was selected for insertion of an insect life stage. The remaining galleries were not used.

Monochamus carolinensis were reared on pulverized solid silkworm or painted lady butterfly culture medium, following the procedures of Necibi and Linit (1997). A single *M. carolinensis* was inserted into the selected artifical gallery on each disk. The exact ages of mature larvae were not known. The age of each individual of the remaining life stages was recorded as the number of days since pupation or adult eclosion. Twenty individuals of each life stage were used. Adult beetle antennae and legs were removed to facilitate insertion into and removal from the gallery. The gallery was sealed with a metallic plate held in place with push pins to prevent beetle escape. In addition, 20 styrofoam controls, the size of a beetle life stage, were inserted into galleries. The treatments, *M. carolinensis* beetle life stages and styrofoam controls, were removed from artifical galleries after 48 hours. Each individual was macerated and nematodes were collected. A drill bit was used to remove wood (1.27-cm diam × 3 cm deep) adjacent to the gallery wall after treatment removal. Moisture content and population densities of J3, J4, and non-dispersal life stages were determined for each wood sample.

Analysis of variance was used as in the previous section to compare the densities of J3 and J4 in wood surrounding artificial galleries of the different beetle life stages and within beetle life stage treatments. Chi-square analysis was used to test for independence of J3 and J4 association with *M. carolinensis* life stages.

Pulverized M. carolinensis in artificial galleries: The procedures followed in the previous experiment were duplicated with pulverized rather than live beetles. Individual beetles of appropriate life stages were pulverized with a mortar and pestel. About 0.5 ml of sterile distilled water was added to facilitate collection and transfer. The pulverized beetle life stage was immediately pipetted onto cotton pellets the size of the beetle life stage. Beetle larvae, early pupae, late pupae, callow adults, and sclerotized adults (n = 20 for each beetle group) were used in this experiment. Additionally, 20 cotton pellets with sterile distilled water only were used as controls. Each treated cotton pellet was inserted into a 0.79-cm diam × 2-cm deep artifical gallery that was sealed with a metallic plate attached with push pins. After 48 hours the cotton pellets were removed with forceps. Wood chips were collected from the gallery after cotton pellet removal, as described previously. Nematodes were collected from the wood chips and the cotton pellets. The number of nematodes was expressed per gram of

dry wood or per cotton pellet. Data collection and statistical analyses were the same as in the live insect experiment. Chi-square analysis was used to compare the frequency of J3 and J4 association among life stages of live and pulverized insects inserted into artificial galleries.

Results

Naturally developing M. carolinensis: Nearly all nematodes collected from wood surrounding beetle galleries were J3 (Table 1). J3 density was highest in the wood surrounding pupal galleries ($F = 14.22$, df = 3,76, $P = 0.0001$). A few J4 were collected from wood surrounding callow adults, but they were not found in other wood samples ($F = 2.79$; df = 3,76; $P = 0.0460$). Nearly all nematodes obtained from beetle life stages were J4 (Table 1). These were associated exclusively with callow adults ($F = 18.08$; df = 2,57; $P = 0.0001$). The number of J4 found on callow adults was not related to wood moisture content ($r = -0.070$, $P = 0.78$) or J3 density within the wood ($r = -0.015$, $P = 0.95$). Few J3 were collected from beetle life stages, but their density did not differ among the stages ($F = 2.02$; df = 2,57; $P = 0.1424$).

The frequency of association of J3 with beetles was independent of beetle life stage ($\chi^2 = 2.73$, df = 2, $P = 0.155$), while the association of J4 was highly dependent upon beetle life stage ($\chi^2 = 40.00$, df = 2, $P < 0.001$) (Table 1). Very few non-dispersal stages were recovered from wood samples or beetle life stages.

Live M. carolinensis in artificial galleries: Beetle life stages used for insertion into artificial galleries were categorized as mature larvae, early and late pupae, non-sclerotized and sclerotized adults, and formed discrete age groups. The mean ages in days (± SD) since pupation were 4.2 (± 2.47) for early pupae and 11.40 (± 1.19) for late pupae, while the numbers of days after eclosion were 1.60 (± 1.10) for callow adults and 10.15 (± 2.18) for sclerotized adults. Mature larvae were selected by appearance, based on personal experience, that indicated they were close to pupation.

J3 within-wood density increased dramatically between wood samples taken during gallery construction and those collected 48 hours later, after insect removal (Table 2). The life stage-specific increases ranged from 85% to more than 1,200% and were independent of initial density and insect life stage. The greatest increase was noted in wood from galleries in the control treatment. Nearly all nematodes collected from

Table 1. Numbers of third-stage (J3) and fourth-stage (J4) dispersal juveniles of *Bursaphelenchus xylophilus* per gram of dry wood and from *Monochamus carolinensis* activity within natural galleries (n = 20 for each wood sample type).

Nematode source	Dispersal stage	Beetle life stage	Number of nematodes		Beetles with nematodes
			Mean[a]	SD	
Wood	J3	Larva	23.20 b	29.21	
		Pupa	218.40 a	355.11	
		Callow adult	17.20 b	24.93	
		Nonadjacent	1.25 b	2.77	
	J4	Larva	0.00 b	0.00	
		Pupa	0.00 b	0.00	
		Callow adult	1.30 a	3.84	
		Nonadjacent	0.00 b	0.00	
Insect	J3	Larva	0.20	0.69	2
		Pupa	2.65	7.79	7
		Callow adult	0.85	1.89	6
	J4	Larva	0.00 b	0.00	0
		Pupa	0.00 b	0.00	0
		Callow adult	5,265.50 a	8,726.42	15

[a] Means within a nematode life stage grouping followed with the same letter do not differ significantly according to a test of least significant differences ($P \leq 0.05$). Statistical analyses were conducted on square-root transformed data (sqrt [x + 0.5]); means of non-transformed data are reported.

TABLE 2. Numbers of *Bursaphelenchus xylophilus* collected from wood during artificial gallery construction and after removal of *Monochamus carolinensis* (n = 20 for each beetle life stage).

Nematode life stage	Beetle treatment	Gallery construction		Insect removal	
		Mean	SD	Mean[a]	SD
Third-stage dispersal juveniles	Larva	3.80	3.22	32.90	25.33
	Early pupa	10.65	20.94	48.50	62.10
	Late pupa	7.50	8.62	51.75	42.35
	Callow adult	36.55	79.79	67.85	74.98
	Adult	5.35	7.68	51.75	60.81
	Control	3.50	2.35	48.55	39.69
Fourth-stage dispersal juveniles	Larva	0.00	0.00	0.00	0.00
	Early pupa	0.00	0.00	0.00	0.00
	Late pupa	0.00	0.00	1.60	3.65
	Callow adult	0.00	0.00	2.65	1.05
	Adult	0.00	0.00	0.40	0.91
	Control	0.00	0.00	0.00	0.00

[a] For each nematode life stage, means within the same column do not differ significantly according to analysis of variance ($P \leq 0.05$). Statistical analyses were conducted on square-root transformed data (sqrt [x + 0.5]); means of non-transformed data are reported.

wood surrounding beetle galleries upon removal of live *M. carolinensis* life stages were J3 (Table 2). J3 density did not differ among the wood samples surrounding the different beetle life stages ($F = 1.11$; df = 5,114; $P = 0.3581$). A few J4 were collected from wood surrounding live late pupae, callow adults, and sclerotized adults, but J4 were not obtained from other wood sample types ($F = 1.96$; df = 5,114; $P = 0.0896$).

The majority of nematodes collected from beetle life stages were J4 (Table 3). J4 were obtained in greatest number from *M. carolinensis* late pupae and callow adults, and to a lesser degree from sclerotized adults ($F = 5.40$; df = 5,114; $P = 0.0002$). Very few J4 were associated with early pupae, and none were collected from larvae or the controls. The number of J4 on callow adults was not related to wood moisture content ($r = -0.301$, $P = 0.20$) or J3 density within the wood ($r = -0.132$, $P = 0.58$). J3 were found in greatest numbers on early pupae, late pupae, and callow adults ($F = 6.35$; df = 5,114; $P = 0.001$). The frequency of association of J3 and J4 with beetles was dependent upon beetle life stage ($\chi^2 = 51.14$, df = 5, $P < 0.001$; and $\chi^2 = 54.50$; df = 5; $P < 0.001$, respectively).

TABLE 3. Numbers of *Bursaphelenchus xylophilus* collected from live *Monochamus carolinensis* within artificial galleries (n = 20 for each beetle life stage).

Nematode life stage	Beetle treatment	Mean[a]	SD	Beetles with nematodes
Third-stage dispersal juveniles	Larva	0.00 b	0.00	0
	Early pupa	4.40 a	6.31	14
	Late pupa	5.10 a	12.67	12
	Callow adult	9.80 a	18.67	11
	Adult	0.30 b	1.34	1
	Control	0.00 b	0.00	0
Fourth-stage dispersal juveniles	Larva	0.00 c	0.00	0
	Early pupa	0.05 c	0.22	1
	Late pupa	14.25 a	32.33	16
	Callow adult	12.05 ab	36.60	11
	Adult	5.55 bc	12.84	6
	Control	0.00 c	0.00	0

[a] For each nematode life stage, means followed with the same letter within the same column did not differ significantly according to a test of least significant differences ($P \leq 0.05$). Statistical analyses were conducted on square-root transformed data (sqrt [x + 0.5]); means of non-transformed data are reported.

Pulverized M. carolinensis in artificial galleries: A similar increase in J3 within-wood density between wood samples was noted for artificial galleries that received pulverized beetle life stages (Table 4). Although initial densities were lower than in the wood removed during artificial gallery construction for the live insects, the percentage increases between the initial and subsequent wood samples were similar. Increases in J3 densities were noted in wood surrounding all life stages, including the control. Nearly all nematodes obtained from wood surrounding beetle galleries after removal of cotton pellets containing pulverized insects were J3 (Table 4). The highest J3 density was found in the wood surrounding adult galleries, while the lowest was associated with the wood surrounding the control galleries (F = 3.92; df = 5,114; P = 0.0026). A few J4 were obtained from wood surrounding late pupae, but J4 were not collected from other wood samples (F = 1.00; df = 5,114; P = 0.4211).

The density of J3 from cotton pellets was highest for pulverized adults and lowest on the controls (F = 6.55; df = 5,114; P = 0.0001) (Table 5). J4 were collected only from cotton pellets with pulverized late pupae and callow adults (F = 7.78; df = 5,114; P = 0.0001). No J4 were associated with larvae, early pupae, sclerotized adults, or controls. The number of J4 found on cotton pellets containing pulverized callow adults was not related to wood moisture content (r = −0.024, P = 0.92) or J3 density within the wood at the time of insect removal (r = 0.317, P = 0.89). The frequency of J3 association was independent of beetle life stage (χ^2 = 5.89, df = 5, P = 0.137), while the association of J4 was dependent on life stage (χ^2 = 36.29, df = 5, P = 0.001).

The frequency of J4 association among beetle life stages was independent of insect treatment, i.e., live insects vs. pulverized insects (χ^2 = 4.20, df = 3, P = 0.2401). Larvae were excluded from the analysis because there were no J4 associated with these life stages in either treatment. Controls were excluded because they were made of different materials in the two procedures. A similar trend was found for J3 association (χ^2 = 20.33, df = 4, P = 0.2998). Controls were excluded, as above. Thus, the mean number of dispersal-stage *B. xylophilus* associated with *M. carolinensis* life stages inserted into artificial galleries was generally higher for live insects than pulverized insects, but the pattern of association did not differ.

Discussion

Bursaphelenchus xylophilus and *M. carolinensis* develop in a close symbiotic relationship within host trees. We believe that the devel-

TABLE 4. Numbers of *Bursaphelenchus xylophilus* collected from wood during artificial gallery construction and after removal of pulverized *Monochamus carolinensis* (n = 20 for each beetle life stage).

		Gallery construction		Insect removal	
Nematode life stage	Beetle treatment	Mean	SD	Mean[a]	SD
Third-stage dispersal juveniles	Larva	7.35	14.58	12.80 bc	11.11
	Early pupa	4.75	11.34	42.05 a	41.68
	Late pupa	3.80	3.12	31.40 ab	28.80
	Callow adult	2.50	2.04	29.00 abc	30.07
	Adult	5.15	6.72	53.60 a	73.13
	Control	1.35	1.98	12.20 c	12.81
Fourth-stage dispersal juveniles	Larva	0.00	0.00	0.00	0.00
	Early pupa	0.00	0.00	0.00	0.00
	Late pupa	0.00	0.00	0.30	1.34
	Callow adult	0.00	0.00	0.00	0.00
	Adult	0.00	0.00	0.00	0.00
	Control	0.00	0.00	0.00	0.00

[a] For each nematode life stage, means followed with the same letter within the same column do not differ significantly according to a test of least significant differences ($P \leq 0.05$). Statistical analyses were conducted on square-root transformed data (sqrt [x + 0.5]); means of non-transformed data are reported.

TABLE 5. Number of *Bursaphelenchus xylophilus* collected from pulverized *Monochamus carolinenis* within artifical galleries (n = 20 for each beetle life stage).

Nematode life stage	Beetle treatment	Mean[a]	SD	Beetles with nematodes
Third-stage dispersal juveniles	Larva	81.90 b	119.69	20
	Early pupa	120.10 b	181.85	18
	Late pupa	141.60 b	152.80	17
	Callow adult	95.40 b	27.38	18
	Adult	232.40 a	195.12	19
	Control	13.45 c	13.65	20
Fourth-stage dispersal juveniles	Larva	0.00 b	0.00	0
	Early pupa	0.00 b	0.00	0
	Late pupa	12.05 a	36.59	8
	Callow adult	1.60 a	2.70	8
	Adult	0.00 b	0.00	0
	Control	0.00 b	0.00	0

[a] For each nematode life stage, means followed with the same letter within the same column do not differ significantly according to a test of least significant differences ($P \leq 0.05$). Statistical analyses were conducted on square-root transformed data (sqrt [x + 0.5]); means of non-transformed data are reported.

opment of *B. xylophilus* J4 is dependent, at least in part, on that of *M. carolinensis*. Warren and Linit (1993) reported that *B. xylophilus* J4 did not develop in pine logs devoid of insects. In the present study, the formation of J4 in pine logs occurred almost exclusively in the presence of *M. carolinensis* late pupae and callow adults, whether live or pulverized, in natural or artificial galleries. Thus, the molt of *B. xylophilus* from the J3 to the J4 dispersal stage was temporally synchronized with *M. carolinensis* adult eclosion. Occurrence of the nematode molt in the presence of pulverized late pupae or callow adults suggests that a chemical substance or suite of substances associated with *M. carolinensis* adult eclosion is involved in J4 formation.

J3 were not frequently found on any of the live beetle life stages. They were, however, collected from cotton pellets containing each of the beetle life stages. This may have been a result of J3 accumulation in the water contained in or leaking from the cotton pellet into the artificial gallery.

J3 density was greater in wood surrounding pupae in natural galleries than in wood surrounding artificial galleries containing any life stages of live or pulverized insects. J3 density in the wood surrounding artificial galleries increased in the 48 hours following gallery construction, even in the absence of *M. carolinensis* life stages. This may have been in response to changes in wood chemistry as the xylem was exposed to O_2 and other gases or changes in the quantity or species composition of fungi on the walls lining the gallery. J3 density in the wood surrounding natural pupal galleries was greater than that in wood surrounding the artificial galleries, perhaps in response to an attractive, insect-produced, substance. If released or volatilized slowly, this substance would have been present in the natural galleries of this study but not necessarily in the artificial galleries due to the short residence time of the beetle life stages. Thus, no life stage-specific aggregation would be expected.

J4, the phoretic stage, was collected almost exclusively from live or pulverized insects and only rarely from the wood surrounding natural or artificial galleries. *Monochamus carolinensis* callow adults removed from natural galleries carried a greater number of J4 than live callow adults inserted into artificial galleries. This may have resulted from (i) insufficient time for nematodes to respond to the attractive substance, (ii) a minimum response time needed for nematode conversion from J3 to J4 due to physiological changes involved in this molt, or (iii) the absence of the correct concentration or proper mixture of more than one substance in a single beetle life

stage that would have precluded completion of the process. Differences in the construction and architecture of natural vs. artificial galleries also may have contributed to the observed differences in J4 density on callow adults.

J4 were collected in equal numbers from live late pupae and callow adults in the artificial galleries, but only from the pulverized late pupae on cotton. Very few J4 were obtained from the pulverized callow adults, although the frequency of association was similar between the beetle treatments. Volatile chemical components of the pulverized adults may have been lost during processing and handling, resulting in reduced J4 formation. *Monochamus carolinensis* callow adults were more successful than older, sclerotized adults in inducing J4 formation, suggesting that J4 molt-inducing substances are gone within a few days of beetle eclosion. This phenomenon has been observed in a similar beetle-nematode system, *M. galloprovincialis* Olivier and *B. mucronatus* Mamiya and Enda (Tomminen, 1992). Tomminen (1992) speculated that the production of hormones, as suggested by Ishibashi and Kondo (1977), was more prolific in callow adults than in the sclerotized adults and was responsible for differential J4 association with the two adult beetle forms.

Numerous species of insects develop within *B. xylophilus*-infested xylem under field conditions, but only *Monochamus* spp. carry significant numbers of J4 upon emergence from host trees (Linit et al., 1983; Mamiya, 1984). Twelve species of Cerambycidae other than *Monochamus* are known to carry J4 upon emergence from nematode-infested trees (Linit, 1988). The non-*Monochamus* cerambycids, however, carried very low numbers of J4 compared to *Monochamus* spp. CO_2 is believed to play a role in *B. xylophilus* attraction to insect vectors (Miyazaki et al., 1978). However, only *Monochamus* late pupae and adults carried J4, although larvae and early pupae also produced CO_2 during respiration. In contrast, pulverized late pupae did not respire, but J4 were collected from the cotton containing this life stage. Shuto and Watanabe (1987) suggested that vector-produced substances, such as toluene and o-xylene, might be important in the attraction of the nematode to *Monochamus* beetles because high concentrations of nematodes are not found around the chambers of other insect species inhabiting the wood. We believe that one or more genus-specific substances associated with *Monochamus* adult eclosion trigger the J3-J4 molt and act as an isolating mechanism, ensuring the adult *Monochamus*-J4 association. We further hypothesize that the absence of such substances inhibits this association with other wood-inhabiting species of insects and with immature stages of *Monochamus*.

Mechanisms controlling *B. xylophilus* dispersal-stage formation and movement onto *Monochamus* beetles remain poorly understood. The interaction between *B. xylophilus* and its insect vector involves a series of specific cues. It is likely that these cues are multiple and complex, including insect activity, chemical substances produced during insect molts, and volatile substances produced by the vector. J4 development in the presence of pulverized callow adults suggests that J4 formation was associated with the chemistry of the beetle eclosion process and not with the behavior or activity of the beetle alone. Once formed in the presence of a *Monochamus* adult, *B. xylophilus* J4 may follow a CO_2 gradient into the metathoracic spiracles of its vector. Further research is necessary to isolate, assay, and identify substances associated with *Monochamus* adult eclosion that will help to explain the symbiotic relationship of these organisms and the nature of the isolating mechanism that excludes other wood-boring beetles as vectors.

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