1 **The effect of feeding behavior of *Monochamus alternatus* (Coleoptera:**
2 **Cerambycidae) on the departure of pine wood nematode, *Bursaphelenchus***
3 ***xylophilus* (Nematoda: Aphelenchoididae)**

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11

12 **Abstract**

13 In order to study the causes of pine wood nematode (PWN) departure from
14 *Monochamus alternatus*, the effects of the feeding behavior of *M. alternatus* on the
15 start date of the departure of PWN were studied. The start date of the departure of
16 PWN carried by the directly fed *M. alternatus* was 5–13 d after beetle emergence,
17 mainly concentrated within 6–10 d, with a mean ($\pm$ SD) of 8.02 ± 1.96 d. The start
18 date of the departure of PWN carried by the *M. alternatus* fed after starvation was
19 5–14 d after beetle emergence, mainly concentrated within 6–9 d, with a mean of 7.76
20 ± 2.28 d. The results show that there was no significant difference in the start
21 departure date of PWN between the two treatments. This shows that the feeding
22 behavior of *M. alternatus* is not the trigger for PWN departure. At the same time, it
23 was found that the motility of the PWN carried by *M. alternatus* at 8 d after
24 emergence was significantly greater than that of the PWN carried by the newly
25 emerged *M. alternatus*. And the PWN carried by *M. alternatus* at 8 d after emergence
26 was extracted more easily than the PWN carried by newly emerged beetles. These
27 results show that greater motility was associated with easier departure of PWN from
28 *M. alternatus*. In addition, transcriptome sequencing found that the level of oxidative
29 phosphorylation metabolism of PWN carried by beetles at 8 d after emergence was

significantly higher than that in the PWN carried by newly emerged beetle. High oxidative phosphorylation was associated with increased energy production and motility by the PWN and were the internal cause of the start of nematode departure.

Keywords: departure; feeding; lipid; *Monochamus alternatus*; motility; pine wood nematode

Introduction

Pine Wilt Disease (PWD) constitutes one of the most serious conifer diseases worldwide, affecting *Pinus* spp. from the Far East forestlands (Japan, China and Korea) (Cheng et al., 1983; Yi et al., 1989), and from Europe (Portugal and Spain) (Abelleira et al., 2011; Fonseca et al., 2012; Mota et al., 1999; Robertson et al., 2011). This disease causes significant economic and environmental damage to the countries affected, with large annual losses of timber (Mamiya et al., 2004), increased cost in management procedures, including disease and pest control (Mamiya et al., 2004; Yang et al., 2004). This disease is caused by the pine wood nematode (PWN), *Bursaphelenchus xylophilus* (Steiner & Buhrer) Nickle, and PWN transmission is dependent on vector insects, of which the main vector insect in East Asia is *Monochamus alternatus* Hope (Linit, 1989; Morimoto and Iwasaki, 1972). There are two developmental forms in the life cycle of the juvenile PWN, namely the propagative and the dispersal forms. Under favorable conditions, PWN molt into their propagative form, which then reproduce rapidly (Mamiya, 1984; Wingfield, 1983). However, under unfavorable conditions, e.g., high population density, nutritive deficiency, desiccation, and low temperature, the propagative second-stage juveniles will molt to produce dispersal third-stage juveniles, which will aggregate around the pupal chamber of the vector insects (Linit, 1988; Zhao et al., 2014). The dispersive third-stage juveniles molt to produce fourth-stage juveniles, which enter the tracheal system of the vector as the adult beetles emerge (Linit, 1988,1990; Necibi and Linit, 1998; Zhao et al., 2014); when the adult beetles feed on healthy pine trees, the fourth-stage dispersive juveniles to be transmitted to the healthy pine trees through wounds caused by the vector (Niu et al., 2012; Stamps and Linit,1988,2001; Zhao et

59 al., 2007).

60 The PWN cannot be transmitted immediately after the emergence of *M.*
61 *alternatus*. A number of studies have investigated the time after *M. alternatus*
62 emergence when PWN transmission starts. Fourth-stage dispersive juveniles exit the
63 tracheal system of *M. alternatus* at 3–5 d (Hosoda and Kobayashi, 1977), at 5 d
64 (Jikumaru and Togashi, 2000), at 7 d (Aikawa, 2008), at 7–12 d (Wang et al. 2019), at
65 10 d (Togashi, 1985), or within 10 d after the emergence of the vector beetles (Enda,
66 1972).

67 What triggers the departure of PWN from its insect host? A number of studies
68 have investigated various factors: the degradation of neutral storage lipid was
69 correlated with nematode exit (Stamps and Linit, 1988); assumed β -myrcene to play
70 an important role in the transmigration of the PWN from the sawyer to the pine tree
71 (Ishkawa et al., 1986); PWN has a trait of spontaneous departure from *M. alternatus*,
72 and pine volatiles repress PWN departure from *M. alternatus*(Aikawa and Togashi,
73 1998); PWN departure behavior possibly endogenous natural factors (Aikawa and
74 Togashi, 1998; Stamps and Linit, 1988); when the CO₂ concentration in the trachea of
75 vector beetle reaches a certain critical value, PWN begins to escape from the beetle
76 (Wu et al., 2019). The feeding period is an important stage in the life cycle of the
77 vectors, as well as a key step in PWN transmission (Togashi and Shigesada, 2006;
78 Yoshimura et al., 1999), although whether the feeding behavior of vector is the factor
79 triggering PWN departure has not been studied. In order to further study the causes of
80 PWN departure from vector, we investigated the effect of the feeding behavior of *M.*
81 *alternatus* on PWN departure, the differences in motility and difficulty level of
82 extraction of the PWN carried by *M. alternatus* at 8 d after emergence and newly
83 emerged, and differences in the PWN transcriptome at these two time nodes.

84 **Materials and methods**

85 **Collection of infected wood and *M. alternatus***

86 In March 2019, dead specimens of *Pinus massoniana* trees, infested by *M.*
87 *alternatus* larvae and PWN, were collected at Bocun Forest Farm, Huangshan City,

88 Anhui Province in eastern China. The pine trees were cut into logs (1.0–1.2 m in
89 length) and maintained in outdoor insect cages (0.5 m × 0.5 m × 1.2 m), as shown in
90 Fig. 1 A. *M. alternatus* adults were collected daily (every 6 h) during the period of
91 adult beetle emergence from the logs.

92 The collected *M. alternatus* were divided into two groups and maintained in
93 culture flasks at 25°C. One group was fed (with fresh one-year-old pine twigs
94 collected from healthy *P. massoniana* trees, with the needles removed) immediately
95 after collection (“direct feeding”), whereas the second group was fed after a starvation
96 period of 4 d (in a preliminary experiment, no nematodes departure from beetles
97 within 4 days). In the present study, 41 *M. alternatus* were subjected to direct feeding
98 and 37 were fed after a starvation period; the feeding of the beetles is shown in Fig. 1
99 B.

Fig. 1 Collection and feeding of *Monochamus alternatus*

102 A: The dead pine trees were cut into logs and maintained in outdoor insect cages. *Monochamus*
103 *alternatus* adults were collected daily (every 2 h) during the period of adult beetles emergence. B: The
104 collected *M. alternatus* adults were divided into two groups and maintained in culture flasks at 25°C,
105 one of which was fed (with fresh one-year-old pine twigs with the needles removed) immediately after
106 collection, whereas the second group was fed after a starvation period of 4 d.

107 **Extraction, counting and identification of PWN**

108 The twigs of *P. massoniana* were replaced daily. The twigs had been fed by the
109 beetles were cut into pieces and the PWN were extracted using the Bellman funnel
110 method (Baermann, 1917). Twenty-four hours after extraction, the liquid at the
111 bottom of the funnel was collected in a 10-mL centrifuge tube. The number of PWN

112 was counted under a microscope (Zeiss Axio Lab. A1; Carl Zeiss, Göttingen,
113 Germsny) under 10×4 field.

114 The molecular identification of nematodes was carried out to confirm that the
115 extracted nematodes were PWN. 40 µL nematode liquid was transferred to a 1.5-mL
116 Eppendorf tube with 40 µL of nematode lysis solution (Zhang et al., 2017) and 5 µL
117 of protease K (Takara, Dalian, China). The mixture was then subjected to
118 centrifugation at 2,000 ×g for 1 min before heating for 45 min at 65 °C in a constant
119 temperature water bath, with the temperature then being raised to 95 °C for 10 min.
120 Samples were then centrifuged at 12,000 ×g for 3 min, before collecting the
121 supernatant and storing it at 4°C (Yang et al., 2020).

122 The 10-µL detection mixture consisted of 3.5 µL of the supernatant, 5 µL of
123 Premix Ex Taq (Takara, Dalian, China), and 1.5 µL of a mixture of probe (5'-TGCAC
124 GTTGT GACAG TCGT-3') and primers (F: 5'-GAGCA GAAAC GCCGA CTT-3', R:
125 5'-CGTAA AACAG ATGGT GCCTA-3') in a PCR tube which was centrifuged at
126 2,000 ×g for 30 s. The detection mixture was analyzed by quantitative real-time PCR
127 (qPCR) with the LineGene K Plus Real-Time PCR Detection System (FQD-48A;
128 Hangzhou Bioer Technology Co. Ltd. Hangzhou, China) for the presence of PWN
129 (constant temperature: 95 °C for 20 s; 40 cycles at 95 °C for 15 s, and 60 °C 129 for
130 20 s) (Yang et al., 2020).

131 **PWN motility and extraction percentage**

132 PWN were extracted for a 4-h period from newly emerged *M. alternatus* and
133 from beetles at 8 d after emergence, using the Bellman funnel method (Baermann,
134 1917). The PWN were washed with sterile water and treated in the dark for 10
135 minutes. Then, the motility of PWN was observed under a light microscope (Zeiss
136 Axio Imager. M2; Carl Zeiss, Göttingen, Germsny) and videos were taken (Zeiss
137 AxioCam HRc). The motility of the PWN carried by the beetles is shown in
138 supplementary video A (the motility of the PWN carried by the newly emerged *M.*
139 *alternatus*) and video B (the motility of the PWN carried by the beetles at 8 d after
140 emergence).

141 PWN were extracted from ten newly emerged *M. alternatus* and from ten beetles

142 at 8 d after emergence, with PWN being collected and counted every 6 h. The
143 percentage of PWN for the total extraction was determined over different extraction
144 periods (0–12 h, 12–24 h, 24–36 h).

145 **Transcriptome sequencing (Biomarker Technologies, Beijing, China)**

146 *RNA quantification and qualification*

147 The total RNAs (three biological replicates of each of the two groups) of PWN
148 were extracted, using the TRIzol Plus RNA Purification kit (Invitrogen, Waltham,
149 MA, USA). RNA concentration was measured using NanoDrop™ 2000
150 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). RNA integrity
151 was assessed using the RNA Nano 6000 Assay Kit of the Agilent Bioanalyzer 2100
152 system (Agilent Technologies, CA, USA).

153 A total amount of 1 µg RNA per sample was used as input material for the RNA
154 sample preparations. Sequencing libraries were generated using NEBNext®Ultra™
155 RNA Library Prep Kit for Illumina® (NEB, Ipswich, MA, USA) following
156 manufacturer's recommendations and index codes were added to attribute sequences
157 to each sample.

158 *Clustering and sequencing*

159 The clustering of the index-coded samples was performed on a cBot Cluster
160 Generation System using TruSeq PE Cluster Kit v4-cBot-HS (Illumina) according to
161 the manufacturer's instructions. After cluster generation, the library preparations were
162 sequenced on an Illumina platform and paired-end reads were generated.

163 *Quality control*

164 Raw data (raw reads) of fastq format were firstly processed through in-house perl
165 scripts. In this step, clean data (clean reads) were obtained by removing reads
166 containing adapter, reads containing ploy-N and low quality reads from raw data. At
167 the same time, Q20 (%), Q30 (%), GC-content (%) and sequence duplication level of
168 the clean data were calculated. All the downstream analyses were based on clean data
169 with high quality.

170 *Comparative analysis*

171 The adaptor sequences and low-quality sequence reads were removed from the

172 data sets. Raw sequences were transformed into clean reads after data processing.
173 These clean reads were then mapped to the reference genome sequence
174 (<https://www.ncbi.nlm.nih.gov/genome/?term=Bursaphelenchus+xylophilus>). Only
175 reads with a perfect match or one mismatch were further analyzed and annotated
176 based on the reference genome. HISAT2 tools soft were used to map with reference
177 genome.

178 *Gene functional annotation*

179 Gene function was annotated based on the following databases: Nr (NCBI
180 non-redundant protein sequences); Nt (NCBI non-redundant nucleotide sequences);
181 Pfam (Protein family); KOG/COG (Clusters of Orthologous Groups of proteins);
182 Swiss-Prot (A manually annotated and reviewed protein sequence database); KO
183 (KEGG Ortholog database); GO (Gene Ontology).

184 *Differential expression analysis*

185 Differential expression analysis of two conditions/groups was performed using
186 the DEseq software. DEseq provide statistical routines for determining differential
187 expression in digital gene expression data, using a model based on the negative
188 binomial distribution. The resulting P values were adjusted using the Benjamini and
189 Hochberg's approach for controlling the false discovery rate (FDR). Genes with an
190 adjusted *P*-value < 0.01 found by DEseq were assigned as differentially expressed.

191 *KEGG pathway enrichment analysis*

192 KEGG (<http://www.genome.jp/kegg/>) was used to identify biological pathways that
193 were enriched in a gene list more than would be expected by chance. KOBAS (Mao et
194 al., 2005) software was used to test the statistical enrichment of differentially
195 expressed genes (DEGs) in KEGG pathways.

196 **Statistical analysis**

197 Statistical analysis was performed using SPASS 19.0 (IBM, Armonk, NY, USA).
198 One-way analysis of variance (ANOVA) was used to analyze the differences of the
199 start departure time of PWN from *M. alternatus* feeding after starvation and direct
200 feeding. Mann-Whitney test was performed to analysis the differences of difficulty
201 level of extracted PWN from *M. alternatus* at 8 d after emergence and from newly

emerged beetles.

Results

Effect of *M. alternatus* feeding behavior on PWN departure from beetle

After molecular identification, it was confirmed that the nematodes extracted in this study were all pine wood nematodes.

Data from the study into the time after *M. alternatus* emergence required for PWN start departure from the beetles are shown in Table 1. It can be seen that the mean ($\pm$ standard deviation, SD) start time for PWN departure from directly fed *M. alternatus* was 8.02 ± 1.96 d after beetle emergence, and there was no significant difference between male and female beetles fed directly ($P=0.47$). The mean start time for PWN departure from *M. alternatus* fed after starvation was 7.76 ± 2.28 d after beetle emergence, with no significant difference between male and female beetles ($P=0.19$). There was also no significant difference in the time that PWN started to departure from the beetles under the two feeding regimes ($P=0.34$), with no significant differences in the start time of PWN departure from female ($P=0.58$) or male ($P=0.12$) beetles under the two feeding regimes.

Table 1. The start date of pine wood nematode (PWN) departure from *Monochamus alternatus*

Treatment	Direct feeding ^a		Feeding after starvation ^b	
Sex of beetles	♀	♂	♀	♂
Days after emergence of <i>Monochamus alternatus</i>	5	6	5	5
	6	6	6	6
	6	6	6	6
	6	6	6	6
	7	6	6	6
	7	6	6	6
	7	7	6	6
	7	7	7	6
	7	7	7	7
	7	8	7	7

	7	9	7	7
	7	9	8	8
	7	9	8	9
	8	10	9	9
	8	10	9	9
	9	10	9	12
	9	11	10	
	9	12	11	
	10	12	12	
	10		13	
	10		14	
	13			
Average (mean $\pm$ SD)	7.82 $\pm$ 1.82	8.26 $\pm$ 2.13	8.19 $\pm$ 2.54	7.19 $\pm$ 1.80
	8.02 $\pm$ 1.96		7.76 $\pm$ 2.28	

219 a: The date of PWN start departure from *Monochamus alternatus* which was fed directly after
220 emergence, with 41 beetles. b: The date of PWN start departure from *Monochamus alternatus* was
221 which fed after a 40-d starvation period, with 36 beetles. There was no significant difference in the
222 start date of PWN departure from beetles between the two treatments ($P=0.34$), with no significant
223 differences of PWN departure from female ($P=0.58$) or male ($P=0.12$) beetles.

224 The start date of PWN departure from *M. alternatus* fed directly was mainly
225 concentrated in the period 6–10 d after emergence, with the 36 *M. alternatus* in this
226 category accounting for 87.8% of the total. The start date of departure of PWN from
227 *M. alternatus* fed after the 4-d starvation treatment was mainly concentrated in the
228 period 6–9 d after emergence, with the 29 *M. alternatus* in this category accounting
229 for 78.4% of the total. There was no significant difference between the two treatments
230 in peak period of PWN start departure from beetles ($P = 0.078$).

231 These results indicated that the feeding behavior of *M. alternatus* had no
232 significant effect on the departure of PWN. It also suggest that the consequent
233 metabolic changes caused by different feeding treatments had no effect on the

234 departure of PWN, and volatiles from pine twigs also had no effect on the departure
235 of PWN (when the beetles were exposed to starvation, they were not stimulated by
236 volatiles.).

237 Comparisons of PWN motility

238 After dark treatment for 10 minutes (to simulate conditions in the body of the
239 beetle), the PWN carried by the newly emerged beetles were less motile than the
240 PWN which carried by the beetles at 8 d after emergence (supplementary videos A
241 and B). At the same time, it was found that the PWN carried by *M. alternatus* at 8 d
242 after emergence were easier to extract than the PWN carried by the newly emerged
243 beetles (Table 2). A higher proportion of PWN being collected within 12 h from the
244 beetles at 8 d after emergence than from the newly emerged beetles ($P < 0.01$). These
245 findings suggest that the higher motility PWN were easier extracted from *M.*
246 *alternatus* was associated with their departure from the vectors.

247 Table 2 The percentage of pine wood nematodes (PWN) collected in different extraction periods

	NO.	The time of PWN collected		
		0-12 h	12-24 h	24-36 h
Newly emerged beetles	1	29%	50%	21%
	2	35%	53%	12%
	3	26%	49%	25%
	4	38%	49%	13%
	5	25%	61%	14%
	6	29%	46%	25%
	7	41%	40%	19%
	8	27%	51%	22%
	9	33%	51%	16%
	10	31%	55%	14%
Beetles at 8 day after	11	80%	15%	5%
	12	84%	11%	5%
	13	91%	6%	3%

	14	81%	13%	6%
	15	77%	16%	7%
	16	75%	17%	9%
	17	72%	20%	8%
	18	81%	15%	4%
	19	83%	12%	5%
	20	70%	23%	7%
Mean $\pm$ SD	a	31% $\pm$ 5.2%	51% $\pm$ 6.0%	18% $\pm$ 4.9%
	b	79% $\pm$ 6.2%	15% $\pm$ 4.8%	6% $\pm$ 1.8%

248 The mean $\pm$ SD percentage of PWN collected during different extraction periods relative to
249 the total extraction number (a: extracted from newly emerged *M. alternatus*. b: extracted from *M.*
250 *alternatus* at 8 d after emergence.). within 0-12 hours, the proportion of PWN extracted from
251 beetles at 8 d after emergence was significant higher than that from newly emerged beetles ($P <$
252 0.01).

253 **Analysis of the PWN transcriptome from *M. alternatus* of different ages**

254 Analysis of transcriptome sequencing results found that expression of the
255 oxidative phosphorylation pathway genes of PWN, which extracted from their vector
256 beetles at 8 d after emergence, was greatly up-regulated, compared with PWN which
257 extracted newly emerged beetles (Fig. 2). A total of 36 differentially expressed genes
258 were enriched in this pathway, 35 of which were up-regulated; this pathway was
259 enriched with the most abundant up-regulated genes (Details: Supplementary material
260 S1). This indicated that PWN carried by the beetles at 8 d after emergence were in an
261 oxygen-rich environment. At the same time, the expression of genes associated with
262 fatty acid degradation (fatty acid β -oxidation) in PWN extracted from the beetles at 8
263 d after emergence was also significantly up-regulated (**Fig. 3**) (Details: Supplementary
264 material S2). The increase in fatty acid degradation and oxidative phosphorylation
265 would generate a considerable amount of energy, which promote the enhancement of
266 PWN motility.

Fig. 3 Fatty acid degradation pathway and gene expression profiles in pine wood nematodes, showing genes up-regulated (red) or down-regulated (blue) in PWN extracted from 8-d beetles relative to newly emerged beetle.

The important genes (encoding long-chain acyl-CoA synthetase [EC:6.2.1.3], acetyl-CoA C-acetyltransferase [EC:2.3.1.9] and 3-hydroxyacyl-CoA dehydrogenase [EC:1.1.1.35]) for fatty acid β -oxidation were all up-regulated. Expression of the gene encoding acyl-CoA oxidase [EC:1.3.3.6], which inhibits fatty acid β -oxidation, was down-regulated.

Discussion

A number of studies have investigated the factors which affect PWN departure from vectors, although differences in results have been reported. Stamps and Linit (1988) reported that neutral storage (NS) lipid was correlated with PWN exit and may contribute to PWN departure from vectors. A rolling fulcrum model of the integration of intrinsic (NS lipid) and extrinsic (volatiles) cues is proposed to explain the behavioral ontogeny of fourth-stage dispersive juveniles in relation to the beetle vector (Stamps and Linit, 2001). In the rolling fulcrum model, NS lipid content is proposed as a modifier of PWN response to beetle and tree produced volatiles, with

295 juveniles with the lower NS lipid content being attracted to β -myrcene, a pine volatile,
 296 whereas PWN with the higher NS lipid content were attracted to toluene, a beetle
 297 cuticular hydrocarbon (Stamps and Linit, 1998, 2001). Ishkawa et al. (1986)
 298 examined the attractant effect of volatile components in *Pinus densiflora*, to the PWN
 299 (dispersive PWN that extracted from *M. alternatus*), and assumed that β -myrcene
 300 played an important role in the transmigration of the PWN from the vector to the pine
 301 tree and for the movement of the PWN inside the pine wood. However, in the present
 302 study, we believe that volatiles were not the cause of PWN departure from *M.*
 303 *alternatus*.

304 Provided autoclaved (without natural volatiles) and fresh pine twigs to *M.*
 305 *alternatus* respectively, the results suggested that PWN had a trait of spontaneous
 306 departure from *M. alternatus* and the volatiles from fresh *P. densiflora* twigs
 307 repressed PWN departure from beetles (Aikawa and Togashi, 1998). Stamps and Linit
 308 (1988) examined the effects of various pine volatiles on PWN departure, suggested
 309 that PWN departure behavior possibly controlled by endogenous nature factors.
 310 Volatiles, previously thought to be attractants, may actually act more as aggregation
 311 stimulants than as attractants, with PWN moving randomly until the desired chemical
 312 is reached, whereupon movement decreases or stops (Futai, 1980; Stamps and Linit,
 313 1998). Volatiles may only play a role in fourth-stage dispersal juveniles movement
 314 from beetles into pine wood (Stamps and Linit, 1988, 1998).

315 In the present study, feeding behavior of the beetles had no significant effect on
 316 the time of PWN departure from *M. alternatus*. The results also indicated that
 317 volatiles (starvation-treated beetles were not stimulated by volatiles) of pine and the
 318 metabolic changes of *M. alternatus* caused by feeding behavior had no effect on the
 319 departure date of PWN. The two main behaviors of *M. alternatus* following
 320 emergence are feeding and breathing. Have study reported that, after emergence, the
 321 respiratory rates of *M. alternatus* increased, which led to a rise in CO₂ production rate
 322 (Wu et al., 2019). When the CO₂ concentration in trachea reached a certain critical
 323 level, PWN began to escape from the beetle (Wu et al., 2019), with to avoid CO₂
 324 (Bretscher et al., 2008). In the present study, we observed that the PWN carried by the

325 newly emerged beetles exhibited less motility than the PWN carried by the beetles at
 326 8 d after emergence, with the PWN carried by *M. alternatus* at 8 d after emergence
 327 were easier to extract than the PWN carried by newly emerged beetles, suggest that
 328 the date of departure of PWN from vector might be related to their motility. PWN
 329 movement requires energy, lipid degradation provides the energy needed for exercise
 330 (Stamps and Linit, 1988,1998), with lipid fully degradation to produce large amounts
 331 of energy (β -oxidation) requiring sufficient O₂. Respiratory behavior leads to a critical
 332 level of CO₂ in the trachea of *M. alternatus* (Wu et al., 2019), with the O₂
 333 concentration in the beetle trachea also reaching a critical level when breathing (the
 334 concentration of O₂ will increase when inhaling).

335 Tian et al. (2017) carried out analysis of metabolism-related genes and showed that
 336 the metabolic level of fourth-stage dispersive juveniles (carried by newly emerged
 337 beetles) was much lower than that in third-stage dispersive juveniles (in dead pine).
 338 This was due to the decreases in enzymatic activity in those pathways involving
 339 metabolism of glycolysis, oxidative phosphorylation, tricarboxylic acid cycle,
 340 gluconeogenesis, etc. (O'Riordan and Burnell, 1989). The expression of the sorbitol
 341 dehydrogenase gene in the fourth-stage dispersal juveniles (carried by newly emerged
 342 beetles) was up-regulated (Tian et al., 2017), this enzyme may be involved in ethanol
 343 fermentation under hypoxic conditions (Mcelwee et al., 2006), providing basic energy
 344 for PWN. These findings indicated that the PWN were in a hypoxic environment in
 345 the early stage after entering the trachea of *M. alternatus* (Tian et al., 2017). In the
 346 present study, the low motility of PWN carried by newly emerged beetles also
 347 supported that PWN are in a state of low oxygen and low energy.

348 In the present study, the greater motility of PWN (carried by *M. alternatus* at 8 d
 349 after emergence) were easier departure from *M. alternatus*. The greater motility
 350 showed that, under these circumstances, PWN had a strong metabolism and abundant
 351 energy. A large amount of lipid degradation in PWN, after a period of emergence of
 352 the beetle (Stamps and Linit, 1988), and oxidative phosphorylation requires sufficient
 353 oxygen sustained stimulation. In the present study, transcriptome sequencing found
 354 that expression of the genes associated with oxidative phosphorylation level of PWN

carried by the beetles at 8 d after emergence was significantly higher than that in PWN carried by newly emerged beetles. The thermodynamic power of the oxidative phosphorylation electron transfer chain comes from the strong affinity of oxygen for electrons (Wang et al., 2002), indicating that PWN in the trachea of beetles (at 8 d after emergence) obtained sufficient oxygen, with the oxygen obtained by PWN can only come from the respiration of the beetles.

In summary, our view is that the increased of respiration after adult vector emergence (Wu et al., 2019) causes an increase in O₂ concentration in the trachea, which causes the increase of oxidative phosphorylation level in PWN, which, in turn, then causes the degradation of lipid (oxidative phosphorylation and β -oxidation are coupled (Wang et al., 2002)). This increase in oxidative phosphorylation in PWN releases energy, which increases the motility of PWN, and is the direct internal cause of their departure from the vector beetle, with the increased motility being the factor necessary for PWN departure from the vector.

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Conflicts of interest

The authors declare no conflict of interest. The sponsors had no role in the design, execution, interpretation, or writing of the study.

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