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CO_2 drives the pine wood nematode off its insect vector

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Insects have developed special organs, spiracles and the trachea, for oxygen–carbon dioxide exchange to adapt to terrestrial life. The plant-parasitic nematode *Bursaphelenchus xylophilus*, also known as pine wood nematode (PWN), is vectored by pine sawyer beetles (*Monochamus* spp.) and causes destructive pine wilt disease, threatening the safety and stability of pine forest ecosystems. Unlike the free-living nematode model species *Caenorhabditis elegans*, PWN have two distinct life stages (dispersive and propagative), each requiring a unique host relationship ranging from symbiotic/commensal to parasitic. Its symbiotic vector beetle and the pine tree it ultimately infects represent dramatically different host environments within which it needs to successfully maneuver. In Asia, the symbiotic relationship between PWN and its host vector *M. alternatus* is very close (Figure S1A, see Supplemental Information). Previous studies have shown that third-stage juveniles (J_{III}) are attracted by specific terpenes produced by mature insect larvae and aggregate around pupal chambers in diseased trees [1] and fourth-stage juveniles (J_{IV}) are attracted to newly eclosed adults by ascarosides the beetles secrete [2]. These J_{IV}, sometimes up to 200,000 per beetle [3], then enter the tracheal system of the newly eclosed beetle, which is full of CO_2, for dispersal. Later, those nematodes depart from the spiracles to invade new healthy trees via the feeding wounds on pine branches made during beetles' feeding, thus starting a new cycle of infection, propagation and dispersal. The mechanism mediating the nematodes' departure remains unknown and remains an important unsolved focal point in the PWN life cycle. Our experimental evidence suggests acute CO_2 avoidance triggers this behavior.

CO_2 is not only an important product of animal respiration, but is also an important regulator for host detection, food location and mate finding in many animals, especially in insects [4]. Nematodes of different species or stages exhibit distinct behaviors upon sensing CO_2 [5]. In well-fed *C. elegans*, general avoidance occurs at 0.5% CO_2 [6] and acute avoidance occurs at ≥1% CO_2 concentration [7], while recent studies demonstrate there is a complex repertoire of experience-driven behaviors, as CO_2 can be attractive or repulsive, depending on life stage, recently experienced CO_2 levels and feeding state [8,9]. The potential effect that respiration of *M. alternatus* has on the dispersal of PWN, as it resides in the beetle's respiratory organs, is unknown.

We hypothesized that higher CO_2 concentrations resulting from the respiration cycles and bursts of CO_2 that occur during beetle maturation feeding triggers a CO_2 avoidance mechanism that serves as a signal to the nematodes to disembark from their vector. To test this, respiration rates of *M. alternatus* at various life stages (mature larva, pupa, adult) and treatments (food vs. no food, with nematodes vs. without nematodes, and over a range of different nematode loads) were measured, followed by single worm behavioral assays to demonstrate PWN's avoidance behavior.

At different stages of development, there were large differences in the insect's respiratory metabolic rate (Figure 1A). Respiration increases just before pupation, followed by a slow decrease and steady low respiration rates during the pupal stage, then a gradual increase near the end of this stage (see the distinctive 'U' shape, Figure 1A). As the insect becomes an adult, the rate of CO_2 release is higher than in the mature larva and pupa. In the adult, it shows a rhythmic fluctuation (Figure 1A) with higher metabolic rates and thus higher peak CO_2 releases in fed beetles (Figure S1B) and beetles carrying nematodes (Figure S1C).

Meanwhile, the respiration of 103 beetles (with nematodes, 4–8 d after emergence) was measured and mean CO_2 produced by each beetle was determined in relation to its nematode load (Figure 1B). These results suggest that the nematodes accumulated at intermediate carbon dioxide concentrations between 4.0×10^{-5} ml $\times$ s^{-1} and a threshold concentration of 1.7×10^{-4} ml $\times$ s^{-1} (see area enclosed by dashed lines, Figure 1B). When the mean CO_2 (ml $\times$ s^{-1}) reached this threshold, avoidance starts to become evident (see dashed line, Figure 1C), suggesting the nematodes were likely starting to show exit behavior or an upper limit to nematode loading.

In the CO_2 avoidance bioassays, where avoidance is defined as a single forward-moving nematode rapidly shrinking after sensing the gas and immediately reversing direction (see Video S1), the nematodes clearly display strong avoidance response when CO_2 reaches a certain concentration (see two arrows, Figure 1C). Furthermore, acute avoidance behavior at this CO_2 concentration of 3.3×10^{-4} ml $\times$ s^{-1} (1% CO_2) was most prominent in fourth-stage juveniles (J_{IV}), in stark contrast to other stages and also *C. elegans* adults (Figure 1D). There were no differences in CO_2 avoidance between the two concentrations tested in adults of either nematode species, but on the contrary, the higher CO_2 treatment had significantly lower avoidance in PWN third-stage juveniles (see J_{III}, Figure 1D) compared with the lower CO_2 treatment because J_{III} are still aggregating in the pupal chamber. This suggests higher CO_2 concentrations from developing pupae also mediate further accumulation of nematodes in addition to those J_{III} attracted by specific ratios of terpenes produced by mature insect larvae [1], together with J_{IV} attraction to ascaroside-producing adults [2], helps to explain how up to more than 200,000 J_{IV} nematodes can load into a single beetle [3].

Given the lower number of nematodes in adult beetles at high CO_2 concentrations (Figure 1B) and the responses of nematodes to CO_2 at 1% concentration (Figure 1C,D), these results suggest that acute CO_2 avoidance is the likely mechanism triggering the exit behavior of fourth-stage juvenile (J_{IV}) nematodes. Knowledge of the signals of interspecific communication in this symbiosis sheds light on a long overdue puzzle regarding PWN exiting from its vector beetle and could inspire new avenues to control transmission of pine wilt disease.

SUPPLEMENTAL INFORMATION

Supplemental information includes one figure, one video, experimental procedures, and supplemental references, and can be found at https://doi.org/10.1016/j.cub.2019.05.033.

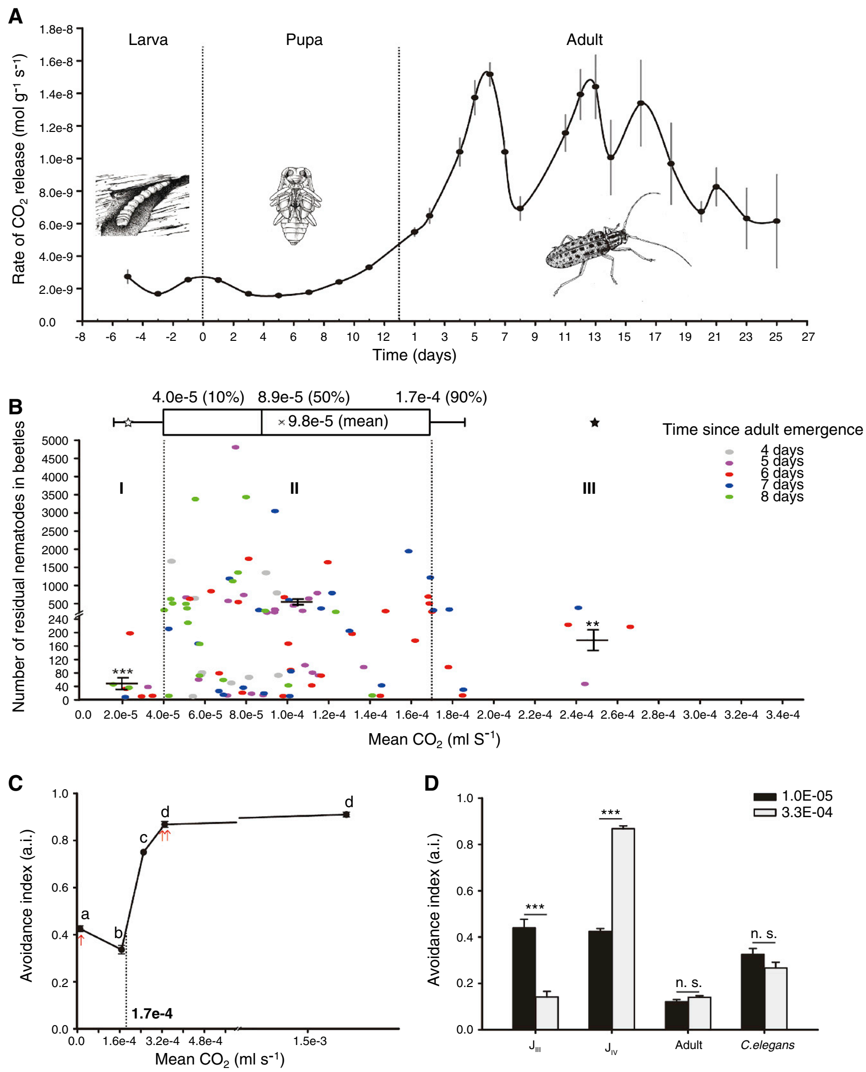

Figure 1. CO_2 respiration rates of beetles and nematode CO_2 avoidance index.
(A) Changes in the rate of CO_2 release in different stages of *Monochamus alternatus*. The dotted line denotes divisions between each life stage, data points are mean ± SE at specific time points. $n_{larva} = 20$, $n_{pupa} = 72$, $n_{adult} = 231$. (B) The relationship between mean CO_2 produced by the beetle and the vector's capacity to carry nematodes. The horizontal axis represents the mean CO_2 exhaled by each *M. alternatus* adult, and the vertical axis represents the number of *B. xylophilus* residing in the corresponding beetles. Nematodes tended to accumulate at intermediate CO_2 concentration ranges as shown by the dashed lines according to the box-and-whisker plot (whiskers depict 1.5 × IQR with extreme tails of the distribution depicted by unfilled star = 1%, filled star = 99%, respectively). Kruskal-Wallis test showed differences in the mean numbers of nematodes in beetles among groups I (lower CO_2 range), II (intermediate CO_2 range) and III (upper CO_2 range) ($X_2^2 = 12.157$, $p = 0.002$, $n = 103$) and Dunnett's T3 post-test showed in comparison to intermediate CO_2 concentrations (group II), significantly lower mean numbers of nematodes accumulated at low and high CO_2 concentration (groups I and III), **, $P < 0.01$; ***, $P < 0.001$, horizontal lines and bars within each group represent mean ± SE. (C) The response of J_{IV} to different concentrations of CO_2. Error bars represent mean ± SE, one arrow = air (0.03% CO_2, 2 ml min⁻¹), two arrows = 1% CO_2 (2 ml min⁻¹), different letters show significant differences in avoidance index, $P < 0.01$, one-way ANOVA with Tukey's multiple comparison test, n = 19–21 trials per point. CO_2 avoidance starts to become evident at 1.7×10^{-4} ml × s⁻¹ (see vertical dashed line, which also corresponds to right dashed line in Figure 1B) and acute avoidance occurs at 3.3×10^{-4} ml × s⁻¹ (see two arrows = 1% CO_2 concentration, and Video S1) and higher concentrations. (D) The acute avoidance response of different nematodes. J_{III}, J_{IV} and adult were *B. xylophilus* and *C. elegans* were adults. Each bar represents the mean ± SE. The statistical differences between two concentrations in each group were calculated with independent t test. ***, $P < 0.001$, n. s. = no significance.

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AUTHOR CONTRIBUTIONS

Y.W. and J.S. designed the study; Y.W. performed the experiments; Y.W., L.Z., J.D.W. and J.S. wrote the manuscript.

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