



Diplogastrellus nematodes are sexually transmitted mutualists that alter the bacterial and fungal communities of their beetle host

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A recent accumulation of studies has demonstrated that nongenetic, maternally transmitted factors are often critical to the health and development of offspring and can therefore play a role in ecological and evolutionary processes. In particular, microorganisms such as bacteria have been championed as heritable, symbiotic partners capable of conferring fitness benefits to their hosts. At the same time, parents may also pass various nonmicrobial organisms to their offspring, yet the roles of such organisms in shaping the developmental environment of their hosts remain largely unexplored. Here, we show that the nematode *Diplogastrellus monhysteroides* is transgenerationally inherited and sexually transmitted by the dung beetle *Onthophagus taurus*. By manipulating artificial chambers in which beetle offspring develop, we demonstrate that the presence of *D. monhysteroides* nematodes enhances the growth of beetle offspring, empirically challenging the paradigm that nematodes are merely commensal or even detrimental to their insect hosts. Finally, our research presents a compelling mechanism whereby the nematodes influence the health of beetle larvae: *D. monhysteroides* nematodes engineer the bacterial and fungal communities that also inhabit the beetle developmental chambers, including specific taxa known to be involved in biomass degradation, possibly allowing larval beetles better access to their otherwise recalcitrant, plant-based diet. Thus, our findings illustrate that nongenetic inheritance can include intermediately sized organisms that live and proliferate in close association with, and in certain cases enhance, the development of their hosts' offspring.

fitness | microbiome | niche construction | symbiosis

Parents pass on more to their offspring than just genes. A wide breadth of nongenetic factors—including hormones, epigenetic marks, behavioral variants, and symbionts—can also be faithfully transmitted across generations, providing alternative and parallel mechanisms of inheritance and adaptation (1–3). Symbiosis in particular has emerged as a paradigm model for the inheritance of elements acquired in part from the environment but which are also transmitted across generations, often from host mother to her offspring (4, 5). The microbes associated with insects, for example, can provide them with important ecological functions, such as detoxification of defensive plant compounds (6), synthesis of essential nutrients (7), digestion of plant cell-wall components (8), protection from pathogenic microbes (9, 10), and resistance to heat (11) and desiccation (12). However, in addition to the microbiota of insects, multicellular organisms, such as nematodes (13–16), mites (17–19), and even other insects (20) commonly engage in close associations with insect parents and their offspring, and as such possess the potential to influence the health and fitness of their hosts, acting either in parallel to microbial interactants or potentially through them (21–24).

Nematodes in particular stand out as ubiquitous associates of many insects, yet the nature of their relationship is nearly universally considered detrimental (i.e., parasitic or entomopathogenic), commensal (i.e., phoretic), or on the spectrum between these poles (e.g., necromenic) (25–27). However, evaluating the relationships of nematodes to their invertebrate hosts in natural settings has

been challenging, and snapshots based on sampling efforts imply that many nuances of these relationships, especially from the view of the host, may go unnoticed (28). In addition to parasitic or simply phoretic associations, it is alternatively possible that nematodes can benefit their insect hosts, particularly in cases where the nutritional or defensive objectives of nematodes and insects align. Such cases have been described: for example, some pine-sawyer beetles (*Monochamus* spp.) and the plant-pathogenic nematodes (*Bursaphelenchus xylophilus*) they harbor both depend on the degradation of tree tissue for nutrition and the detoxification of defensive tree compounds, providing the conditions for an intricate, potentially mutualistic association between nematode and host (29, 30). In principle, cases like this should reveal mechanisms for how animal species, especially those assumed to be commensal or parasitic, may instead be symbiotic mutualists with their hosts.

A compelling mechanism by which nematodes could benefit their insect host is as engineers of the microbial community closely associated with the host. The microbes that are carried with, or modified by, the presence of nematodes may have a crucial role in the health of the associated insect, nematode, or both. In the pine-sawyer beetle example, the insects likely benefit from bacterial species of *Serratia* and *Pseudomonas*, which are known to reduce the concentrations of defensive plant terpenes that would be harmful to insects (31, 32), and the growth of these bacteria is dependent on the presence of their nematode

Significance

Many organisms, from plants to humans, have long evolutionary histories with nematode worms. Historically these relationships have been assumed to be detrimental—as with intestinal or blood-borne parasites—or at best innocuous, to their hosts. However, this paradigm has been challenged recently, for example, with findings in mammals that worms can modulate their host's immune system and thereby thwart autoimmune disease. In our study, we describe a phenomenon wherein sexually transmitted and parentally provided worms benefit their insect hosts, possibly by "engineering" the microbiome present in the maternally constructed chamber where offspring develop. Given nematodes' association with many insects, particularly those with parental care, this phenomenon may be a more widespread feature of insect health.

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associates (22, 33, 34). Likewise, in some dung beetles, which commonly utilize herbivore dung as a food source during both larval and adult stages, it is known that bacteria are transmitted across generations from mother to offspring (35), enhancing larval growth (36). Because brood balls consist largely of nutritionally recalcitrant plant material, dung beetles likely benefit from the enzymatic capacities of microorganisms (37). At the same time, nematodes are well known to form close associations with dung beetles (15, 38, 39), yet whether and how nematodes interact with microbiota in ways that affect host health, development, or fitness is unknown. Here we assess the nature, consequences, and potential mechanisms of such a three-way interaction.

Specifically, we investigated potential interactions between insects, their nematodes, and local microorganisms using the dung beetle *Onthophagus taurus*. In this system, it is possible to manipulate nematode–insect interactions in a field-like setting, thereby allowing ecological tests of a likely widespread but often intractable type of interspecies interaction. In nature, adult *O. taurus* provide their offspring with larval nutrition in the form of a consumable “brood ball” constructed from mammalian herbivore (often cow) dung that supports beetle development from egg to adult. Furthermore, *O. taurus* mothers provide their offspring with a deposit of maternal fecal matter containing a fitness-enhancing microbiome (35, 36). Here we show that symbiotic nematodes may play a critical role in shaping the brood ball environment and resulting host benefits. Using both field animals and a controlled, artificial brood ball system, we (i) identify a species of nematode associated with both the genitalia of adult *O. taurus* and the developmental chambers of its offspring; (ii) show that the nematode is transmitted both vertically across generations and sexually during copulation between adult beetles; and (iii) show that its presence in the brood ball enhances fitness of host offspring. Finally, our results suggest (iv) a possible mechanism underlying these fitness benefits by showing that the presence of nematodes significantly and reliably alters abundance and composition of the bacterial and fungal communities in the offspring’s developmental environment.

Results and Discussion

Onthophagus Beetles Sexually and Vertically Transmit *Diplogastrellus* Nematodes. Field collections of *O. taurus* from Indiana and North Carolina revealed that both female and male beetles carried the morphospecies *Diplogastrellus monhysteroides*, specifically in their genitalia (Fig. 1 and *SI Appendix, Tables S1 and S2*). Using manipulative experiments, we demonstrated that these nematodes are vertically inherited; we transferred nematode-free beetle eggs to either nematode-free or *D. monhysteroides*-inoculated brood balls, bred the adults (within treatments) that emerged, and assessed their offspring for nematodes. Indeed, all beetle offspring derived from *D. monhysteroides*-treated parents ($n = 9$) inherited nematodes; in contrast, all beetle offspring derived from nematode-free parents ($n = 12$) lacked nematodes. Additionally, *D. monhysteroides* is sexually transmitted; when we performed another set of crosses to assess female to male transmission, all four previously nematode-free males mated to nematode-positive females acquired *D. monhysteroides*. Conversely, in the three crosses assessing male-to-female transmission, all previously nematode-free females acquired *D. monhysteroides* from their male *D. monhysteroides*-positive partners.

***Diplogastrellus* Nematodes Increase the Fitness of Their Beetle Hosts.** The inclusion of *D. monhysteroides* in artificial brood balls significantly enhanced *O. taurus* beetle larval growth ($\chi^2 = 4.09$, $df = 1$, $P = 0.04$) by enhancing growth rate ($\chi^2 = 4.34$, $df = 1$, $P = 0.04$). In other words, the beetles were larger at pupation not because they took longer to develop but because they grew more during a given amount of time (Fig. 2). Additionally, we found that this fitness advantage is likely to be conditional in nature, as field-collected beetles varied for the presence of *D. monhysteroides* (*SI Appendix, Supplementary Material and Methods*), possibly because the benefits of possessing these nematodes is context-dependent in ways not captured by this study. Taken together, these findings show that *D. monhysteroides* are conditional mutualists of

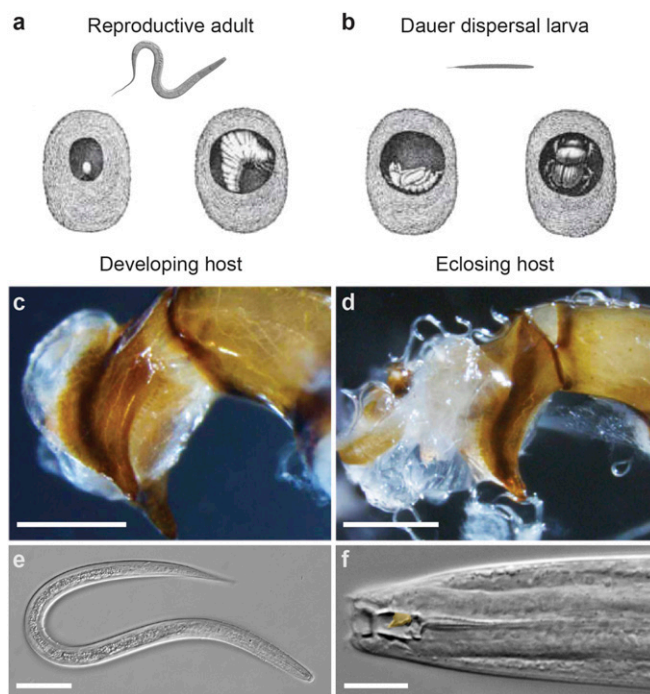


Fig. 1. *D. monhysteroides* is closely associated with reproduction of the dung beetle *O. taurus*. Nematodes are known associates of brood balls, developmental chambers made by beetles from dung to protect and nourish their young. (A) When a beetle deposits an egg in a brood ball, nematodes exit their dauer (J3D) stage and ultimately achieve high population densities in the brood ball as the beetle offspring develops (15). (B) As the beetle larva nears pupation, nematodes again arrest as dauer larvae, thereafter leaving the brood ball with their beetle host. This single species of brood ball-associated nematode was found at high densities on *O. taurus* genitalia: compare nematode absence and presence in C and D, respectively. (Scale bars for C and D, 0.5 mm.) (E) Dauer of *D. monhysteroides*, a facultative larval type with a closed mouth and covered in wax that confers physiological resilience in aerial (above-ground) habitats and thus long-distance dispersal with hosts. (Scale bar, 20 μm .) (F) An adult female *D. monhysteroides*, showing a dorsal tooth (false-colored orange) hypothesized to allow feeding on fungi as well as bacteria (40). (Scale bar, 5 μm .)

O. taurus, and that their benefit is conferred during the beetles’ postembryonic development.

Nematodes Dynamically Shift the Relative Abundance of Fungi to Bacteria in the Host Developmental Environment. Because *Diplogastrellus* nematodes likely feed on bacteria, fungi, or both in nature (40), we hypothesized that increased beetle fitness correlated with nematode-induced changes to brood ball microbiota. To assess microbial abundances and communities in the host larval environment, we manipulated nematode presence across two types of dung: dung frozen before the experiments, which eliminates other species of free-living nematodes, and dung collected fresh from a pasture (for late time points only). This two-pronged approach allowed us to assess the influence of *D. monhysteroides* in the absence of other nematodes, while also allowing us to capture potential *D. monhysteroides*-mediated differences in more complex microbial communities that might be missed due to the freezing procedure. When we performed these manipulations, we found that inoculation with *D. monhysteroides* influenced the relative abundances of fungi and bacteria in brood balls, and this effect varied based on the time at which the samples were taken and the type of dung. Specifically, the inclusion of *D. monhysteroides* resulted in a significantly lower fungi-to-bacteria ratio in the early time-point samples: that is, brood balls sampled 7 d after inoculation with worms ($\chi^2 = 4.99$, $df = 1$, $P = 0.03$) (Fig. 3A). In contrast, there was a significant interaction effect between the

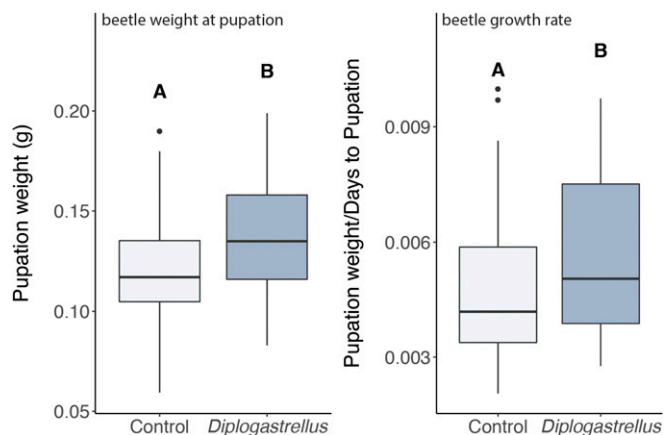


Fig. 2. The presence of *Diplogastrellus* nematodes enhances overall growth and growth rate of *O. taurus* larvae. Weight was measured from beetle larvae within 24 h of pupation and growth weight was determined by dividing the pupation weight by the number of days an individual spent as a larva. Lower and Upper box hinges include first and third quartiles; whiskers, $1.5 \times$ the interquartile range. Letters indicate groups with significantly ($P < 0.05$) different means. Dots represent outliers (data greater than $UQ + 1.5 \times IQD$ or less than $LQ - 1.5 \times IQD$, where UQ = upper quartile, LQ = lower quartile, and IQD = interquartile distance).

inclusion of *D. monhyseroides* and dung type on the fungi-to-bacteria ratio in late time-point samples (i.e., 21 d after inoculation; $\chi^2 = 6.93$, $df = 1$, $P = 0.01$) and a post hoc test revealed that the significant effect was obtained in brood balls constructed from dung collected fresh from a pasture, such that the inclusion of *D. monhyseroides* resulted in a significantly higher fungi-to-bacteria ratio ($t = 3.95$, $df = 14$, $P = 0.01$) (Fig. 3B). Taken together, our results suggest that the relative abundances of fungi and bacteria in the brood ball can be modified by *D. monhyseroides*, but that the direction of these effects differs through beetle larval ontogeny.

Nematodes Change Bacterial and Fungal Communities in the Host Developmental Environment. Using the samples derived from dung frozen before the start of the experiment (see previous section), we sought to determine whether the presence of *D. monhyseroides* influences the bacterial community structure of brood balls constructed in the absence of other nematodes. Significant differences were observed for both early and late time points [$F_{1, 10} = 2.65$, $P = 0.02$ and $F_{1, 9} = 3.16$, $P < 0.001$] when unweighted, but not when weighted UniFrac was used, indicating that the presence of *D. monhyseroides* influences community membership (what taxa are present) rather than community composition (how much of each taxon is present) (Fig. 4A). The corresponding fungal communities of the same brood balls were similarly significantly affected by the presence of *D. monhyseroides*, and again the effect was evident at both early and late time points [$F_{1, 10} = 4.38$, $P < 0.001$ and $F_{1, 10} = 5.05$, $P = 0.01$] (Fig. 4B). Thus, our results suggest that both bacterial and fungal communities of brood balls are influenced by the presence of *D. monhyseroides* nematodes throughout beetle larval ontogeny.

Candidate Microbial Taxa for Mediating Nematode-Dependent Fitness Effects on Hosts. Differential-abundance analyses performed between groups with and without nematodes revealed several bacterial and fungal taxa whose abundance may be selectively changed or regulated by *D. monhyseroides* ($P_{adj} < 0.05$) (See *SI Appendix*, Figs. S3–S5 and Datasets S2–S7). This effect was seen at both early and late time points, as well as in artificial brood balls constructed from either fresh or previously frozen dung. First, we identified candidate bacteria that may contribute to the nutritional needs of *O. taurus*, particularly microbes that metabolize otherwise inaccessible compounds in a plant-based diet; for example, *D. monhyseroides* enhanced the abundances of *Dysgonomonas* and

Sphingobacterium (Bacteroidetes: Chitinophagaceae), which as gut flora of wood-feeding beetles (41–46) and termites (47) are known to digest lignocellulose—also a major component of dung and brood balls—for their hosts; likewise, *D. monhyseroides* modulated the abundances of *Nocardioidea* (Actinobacteria), which include known lignocellulose decomposers (48) associated with termite hosts (49). Furthermore, *D. monhyseroides* consistently enhanced abundances of bacterial taxa known both to degrade plant biomass and to associate with other insects. Such microbes included members of Firmicutes and Planctomycetes, which contain plant-decomposing species associated with longhorn beetles (45, 50), and *Comamonas* and *Acinetobacter* (Proteobacteria), xylanase-expressing bacteria associated with the Colorado potato beetle (46), red turpentine beetles (51), and the cabbage white butterfly (52). In addition to cultivating bacteria that may provide nutritional benefits, *D. monhyseroides* also mediated levels of potentially harmful bacteria: for example, *D. monhyseroides* reduced levels of a *Desulfovibrio* sp., which is a known associate of *O. taurus* (35) and may provide nutrients to dung beetle larvae, but which also produces toxic hydrogen sulfide as a byproduct (53). Finally, *D. monhyseroides* consistently regulated abundances of several fungal taxa, which based on the spore-feeding habit of diplogastriid nematodes may have included the early suppression of potentially harmful fungi, although the relatively limited molecular classification of fungi makes their potential effects harder to predict.

Inherited “Macrobiotics” Are Engineers of Microbial Communities and Benefit Their Developing Hosts. Insects are ecologically diverse, and their symbiotic partners—including bacteria, archaea, protists, and fungi (8, 54–56)—are thought to be critical factors underlying their successful colonization of novel habitats (57, 58). In this study, we show that the nematode *D. monhyseroides* lives and reproduces in close association with the dung beetle *O. taurus*, is transmitted both sexually during adult copulation and vertically

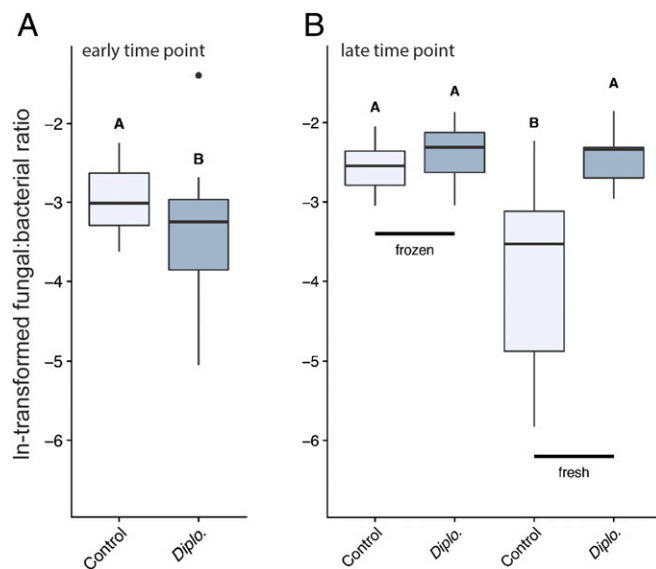


Fig. 3. Relative fungal and bacterial abundances are influenced by *Diplogastrellus* nematodes across time points and dung types. The abundance of fungal and bacterial biomass was estimated by RT-qPCR. Lower and Upper box hinges include first and third quartiles; whiskers, $1.5 \times$ the interquartile range. Letters indicate groups with significantly ($P < 0.05$) different means. Dot represents an outlier (data greater than $UQ + 1.5 \times IQD$ or less than $LQ - 1.5 \times IQD$, where UQ = upper quartile, LQ = lower quartile, and IQD = interquartile distance). (A) Seven days after inoculation with nematodes, brood balls exhibit a lower abundance of fungal to bacterial biomass relative to controls ($P = 0.03$). (B) Twenty-one days after inoculation with nematodes, brood balls made from fresh dung have a higher abundance of fungal to bacterial biomass relative to controls ($P = 0.01$). *Diplo.*, *D. monhyseroides*.

Diplogastrellus nematodes for inoculation were collected in the following manner: after cleaning adult male *O. taurus* with sterile water, their aedeagi were dissected and placed in M9 buffer + 0.1% Tween 20 + ampicillin. Tween 20 and ampicillin were included to mechanically and chemically eliminate bacteria from the beetle that might otherwise be transferred with the nematodes to the artificial brood balls, and the effectiveness of these treatments was confirmed by plating treated worms on lysogeny broth plates, which did not yield any bacterial colonies after 24 h of incubation. Because we expected *D. monhyteroides* dauers to float on the buffer surface, given the presence of wax found on their body surface, dauers were readily collected from the surface of the buffer: after “floating” for 30 min, ~20 *D. monhyteroides* dauers were picked onto artificial brood balls.

Because the presence of a developing beetle may influence the behaviors and ecological functions of the nematodes within a brood ball, we generated nematode-free *O. taurus* eggs for all experimental treatments. We randomly selected 24 colony-raised female beetles and placed them in a cylindrical, light-impermeable ovipositing container filled to a height of 21 cm with sterilized soil (70). After adding ~200 g of homogenized cow dung, we covered these containers with window screen and perforated black plastic foil. After 5 d, brood balls produced by females were collected and dismantled to harvest eggs. Eggs were rinsed with 0.1% Tween 20 and then sterile water and then transferred to a 2% agar plate for visual inspection to ensure that eggs were completely free of nematodes. Cleaned eggs were then transferred to 12-well plates containing artificial brood balls. Additionally, inspection of brood balls at the end of the experiments confirmed the absence of nematodes throughout development in the nonmanipulated, control brood balls.

Measurement of Beetle Fitness. Additional experiments were performed with the artificial brood ball system to test whether the inclusion of *D. monhyteroides* nematodes influenced the growth of *O. taurus* beetle larvae. Artificial brood balls were constructed from previously frozen dung and nematode inoculations were performed as described above. Each plate received three to six eggs (among separate wells), and each plate became a treatment replicate with all eggs within a plate receiving the same treatment. Each open (lidless) plate was placed in a larger plastic, tight-lidded container, such that nematodes were strongly prevented from traversing wells and completely prevented from contaminating adjacent replicates. These replicate containers were randomized with respect to treatment in a climate-controlled room at 26 °C and a 16L:8D cycle. Four rounds of the experiment were conducted to collect sufficient replication. In total, 72 and 86 beetles from control and *D. monhyteroides* treated brood balls, respectively, were measured. Control brood balls were checked periodically for nematode contamination (none was detected). Measurements recorded were pupal weight, sex, and days to pupation. To analyze functional data, mixed models were used on natural log-transformed response variables (weight at pupation and growth rate; growth rate was determined by developmental time, i.e., the days between hatching and pupation) using the R package nlme (71). Fixed factors were: (i) nematode treatment; (ii) beetle sex; the interaction of (i) and (ii); and (iii) the experimental round from which the data were collected. The random variable was replicate container. Significance of fixed factors was determined by comparing nested models with likelihood ratio tests.

Assessment of Vertical and Sexual Transmission of *D. monhyteroides*. After all experimental animals were collected, males and females were paired within treatments and transferred to individual, shallow containers, such that 10 families each of control and nematode-treated beetles were generated. Pairs were kept and fed in these containers for 1.5 wk, at which point they were transferred as pairs to deep containers that facilitate brood ball creation and burial. Soil in both types of containers had previously been autoclaved at 150 PSI for 30 min to ensure no extraneous nematodes were transferred to the breeding pair. Four families from each of the treatments produced brood balls. After 5 d, brood balls were collected from these deep containers and transferred to 12-well plates without further manipulation; a subset of these individuals were then used for an experiment assessing sexual transmission, and one to four individuals from each family were raised to eclosion to assess vertical transmission. Upon eclosion, beetle genitalia were dissected to determine whether nematodes had been transmitted between generations.

Because all adult beetles born during the vertical transmission experiments always had *D. monhyteroides* if they were treated with *D. monhyteroides* during development, we assumed that the subset of individuals from the same treatment groups used for the sexual transmission experiment would show the same phenotypes (as *D. monhyteroides* presence cannot be assessed nondestructively). We thus created two types of crosses for assessing sexual transmission: *D. monhyteroides*-possessing females to control males ($n = 4$) and *D. monhyteroides* possessing males to control females ($n = 3$). After allowing individuals to mate for 1 wk, they were collected and their genitalia dissected and scored for nematode presence.

DNA Extraction for Comparisons of Bacterial and Fungal Communities. Artificial brood balls with beetle eggs were generated for RT-qPCR and microbial profiling as described above. Ultimately, three replicate plates per treatment and two brood balls per plate were used for extractions for a total of $n = 6$ per treatment. Extractions were performed immediately after samples were taken: 7 and 21 d after nematode inoculation for early and late time-point samples, respectively. Early time-point samples were only taken from frozen dung. Six individuals among the fresh dung samples died during development, so these samples were removed from the analysis (three in each of the control and *D. monhyteroides* treated brood balls, leaving $n = 3$ for samples constructed from fresh dung). Samples were taken from brood balls in ~200-mg amounts and were processed with the Qiagen PowerSoil kit. Extracted DNA was stored at -80 °C until further processing for both RT-qPCR and microbial profiling.

Quantification of Relative Bacterial and Fungal Abundances. The relative abundance of bacterial to fungal biomass can be estimated from environmental samples using taxon-specific, RT-qPCR (72, 73). Further details on bacterial and fungal quantification, including taxon-specific standards (SI Appendix, Table S3) and assessment of absolute values, are provided in SI Appendix, Supplementary Materials and Methods.

Comparative Microbial Profiling of Brood Balls. Bacterial and fungal taxa were sequenced and statistical analyses of 16 S and ITS rRNA sequences were performed as described in SI Appendix, Supplementary Materials and Methods.

Quantification of Host-Associated Community Identity and Composition. We calculated unweighted pairwise Unifrac distances (74) among bacterial communities. We used Bray–Curtis dissimilarity to determine whether fungal communities differed between treatments. For statistical analyses, data were placed into three subsets to compare conditions that were most biologically relevant, namely with control vs. *D. monhyteroides*-treated samples taken at (i) an early time point from frozen dung; (ii) a late time point from frozen dung; and (iii) a late time point from fresh dung. Statistical analyses were performed as described in SI Appendix, Supplementary Materials and Methods.

Identification of Candidate Taxa Regulated by *D. monhyteroides* in Brood Balls. To determine which taxa were differentially abundant among samples, sequence data were placed into three subsets to contrast conditions that were determined to be biologically relevant, as described above. Each subset was formatted for the DESeq2 (75) package in R, and DESeq2 was executed using the phyloseq package, as this method for identifying differentially abundant taxa is considered to be more conservative (i.e., in avoiding false positives) than comparing proportions of or rarefying microbial data (76). Lists of bacterial and fungal taxa were trimmed to include only those taxa identified to family level.

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