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A bacterium from a novel *Caenorhabditis* niche induces opposing life history responses in *C. elegans* and *C. inopinata*

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A bacterium from a novel *Caenorhabditis* niche induces opposing life history responses in *C. elegans* and *C. inopinata*

A THESIS APPROVED FOR THE
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DEDICATION:

I dedicate this thesis to my grandmother, who taught me how to read and experience the joy of living, my grandfather, who taught me how to be empathetic towards experiences that are not my own and be dignified, and my mother, who taught me how to be selfless and care for others.

ABSTRACT:

Microbes are major drivers of host health, life history responses that influence reproduction and fitness, and evolutionary trajectories for all domains of life. The field of life history research has a rich history, while microbiome research is relatively recent. The evolution of microbe-dependent life history responses in divergent hosts is an avenue where these two fields intersect. *Caenorhabditis elegans* is a prominent model organism that has been utilized within many fields of biological research and has recently been used to study host-microbe interactions. Here, we use a morphologically and ecologically divergent *Caenorhabditis* species, *C. inopinata*, alongside its closest known relative, *C. elegans*, to investigate how microbe-dependent life history phenotypes evolve in hosts that occupy divergent ecological niches. To do this, we isolated bacteria from the ecological context of *C. inopinata*, reared both nematode species on these bacterial isolates, and measured life history responses and determined whether the responses have diverged or been conserved depending on what bacteria was consumed. We have identified a fig bacterial isolate, *Klebsiella sp.* WOUb2, that induces increased population growth in both nematode species in different ways. Individual reproductive output increases for *C. elegans* raised on *Klebsiella* sp. WOUb2, while developmental rate is more rapid for *C. inopinata*. The viability of *C. elegans* raised on *Klebsiella* sp. WOUb2 decreases, seemingly demonstrating a trade-off between increased reproductive output and survival. *Klebsiella sp.* WOUb2 also seems to modulate adult body length of *C. elegans*. Additionally, both foraging behavior and embryo-laying behaviors of *C. inopinata* and *C. elegans* were modulated by *Klebsiella sp.* WOUb2. We conclude that the evolution of a novel *Caenorhabditis* niche, the fig, has induced the evolution of microbe-dependent life history responses and behavior in *C. elegans* and *C. inopinata*.

INTRODUCTION:

Life exists within a microbe-rich world. Microorganisms are ubiquitous, and they interact with a wide range of taxa across all domains of life, significantly influencing their biology, physiology, and evolutionary trajectories (1). Organisms have co-evolved alongside microorganisms to form symbiotic, commensal, and antagonistic relationships, also called host-microbe interactions, and these relationships are ancient and have remained stable over evolutionary timescales (2). These interactions are numerous and have profound effects on host biology and health. For example, the Hawaiian bobtail squid (*Euprymna scolopes*) has a symbiotic relationship with the marine bacterium *Vibrio fischeri* where, through quorum sensing, *Vibrio fischeri* produces bioluminescence that benefits the squid while the squid provides shelter for the bacterium (3,4). In addition, through a complex set of interactions, *Rhizobia* form nitrogen-fixing nodules on legume roots that assist the growth and health of the plant (5). And, many species of leaf cutter ants tend to fungal gardens in their colonies, providing the fungi with nutrient sources and then later harvesting the fungus as a crop of sorts (6,7). Beyond well-established diseases caused by bacterial infections, complex disorders have also been connected to microbial drivers; diabetes and irritable bowel disease have been correlated with microbial dysbiosis in humans (8–10). Microbes are used by hosts to extract nutrients through digestion, but they can also synthesize micronutrients to their hosts that cannot be synthesized by the host itself (11).

The evolution and maintenance of these interactions largely depends on phylosymbiosis (12). Phylosymbiosis occurs when species engaged in a host-microbe relationship co-evolve simultaneously. To avoid extinction within new ecological contexts or a changing environment, all involved entities must be able to cope with those changes in one way or another (12). Both symbiotic and antagonistic host-microbe relationships evolve alongside each other in this manner. Additionally, because of the intimate ties between microbes and animals, many have proposed the idea of the "holobiont," which is the host and all of its associated microbes being considered as an individual unit as opposed to discrete, separate entities (13). The idea of the "hologenome" followed, which is the genomes of the host organism and all of its associated microorganisms being considered as a single unit that natural selection can act on (14).

Life history traits are individual components of a given organism's life history, meaning the total sum of the patterns that occur over development, reproduction, and survival. Historical life history traits include developmental rate, fecundity, viability, body size at different life stages, and reproductive effort (15,16). Organismal fitness is the sum of these components, being connected through physiology, development, and genetic mechanisms that enable an organism to interface with the external world, and this interlaced network of phenotypes results in correlations in these life history traits (16,17). Organisms have a finite amount of energy to invest into reproduction while maintaining internal homeostasis, and there is extraordinary diversity in the manner and amount of energy taxa across the tree of life put into each (15,16,18). This, along with differences in ecology and evolutionary history, result in one or more life history traits being diminished or boosted at the expense or benefit of other life history trait(s). It is apparent that microorganisms have extraordinary effects on the fitness of their hosts, so it is reasonable to conclude that microbes can be major drivers of life history phenotypes and their evolution. How do microbe-dependent life history responses evolve in divergent hosts? What are the molecular and genetic bases of host-microbe interactions and, consequently, their evolution? To explore these questions, we have opted to use *Caenorhabditis* nematodes.

Caenorhabditis nematodes, primarily *Caenorhabditis elegans*, have been used as a model system for the last fifty years within a variety of fields (19,20). There are a variety of traits that

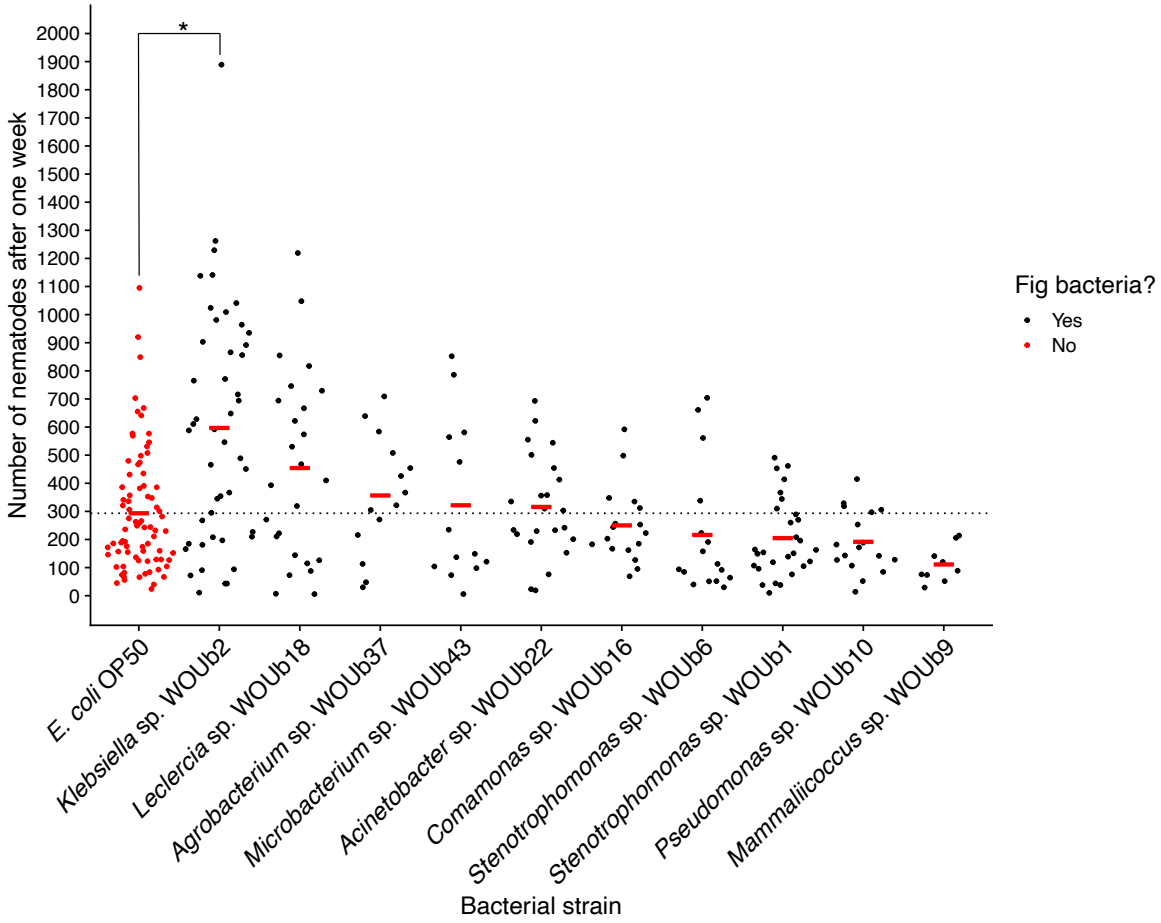

Figure 1: ***C. inopinata*** **harbors variable population growth when reared on bacteria from the fig environment.** Each datapoint represents the number of nematodes on a single plate at the end of the seven-day period for a given bacterial strain. Crossbars represent means. Sina plots are strip plots that take the contours of a violin plot. Failed and contaminated crosses were excluded (a figure including failed and contaminated crosses can be found in the supplemental materials; Supplemental Figure 1). *E. coli* OP50 was used as a control during each set of fig bacteria crosses; consequently, the *E. coli* OP50 data in this figure is an amalgamation of all the controls for each experiment (a figure showing each fig bacteria-control pairing can be found in the supplemental materials; Supplemental Figure 2). Additionally, for *Klebsiella* sp. WOUb2, the data in this figure is representative of two experiments. A second set of crosses was done for this bacterial isolate to confirm the significant results from the first set (Supplemental Figure 2). N crosses/plates = 80, *E. coli* OP50; N = 46, *Klebsiella sp. nov.*; N = 25, *Leclercia*; N = 14, *Agrobacterium*; N = 13, *Microbacterium*; N = 23, *Acinetobacter*; N = 17, *Comamonas*; N = 17, *Stenotrophomonas 2*; N = 28, *Stenotrophomonas 1*; N = 17, *Pseudomonas*; N = 0, *Mammaliicoccus*.

make these nematodes valuable as model organisms. They are bacterivores, meaning they are easy to culture and maintain on agar plates (19,20). Additionally, they have a rapid developmental rate, so many nematodes can be used in experiments at once in a high-throughput manner (19,20). And, they are highly genetically amenable (19,20). Traditionally, *C. elegans* has been reared on *E. coli* OP50 in laboratory settings (19); however, this is not what these nematodes eat in nature, and, until recently, the ecology of these nematodes was unexplored (21). Due of its status as a model

organism, determining the microbes *C. elegans* is associated with in nature while leveraging its power as a model organism has proved to be invaluable for studying host-microbe interactions (22,23). However, because *Caenorhabditis* nematodes generally have the same ecological niche, there has been no means to compare how *Caenorhabditis* microbial communities evolve across divergent ecological contexts until recently. We have opted to use the recently discovered *Caenorhabditis inopinata* alongside *C. elegans* (24).

C. inopinata is the closest known relative of *C. elegans*, and it is morphologically and ecologically divergent from *C. elegans* (24,25). *C. inopinata* is much larger than *C. elegans*. It also has distinct male and female sexes, whereas *C. elegans* is androdioecious (hermaphrodite/male). It develops more slowly than *C. elegans* but has a similar lifespan (26). The ecological niches of the two species also differ immensely: while *C. elegans* lives in rotting plant matter while having a cosmopolitan distribution (27–29), *C. inopinata* lives in fresh *Ficus septica* figs in only one part of the world, east Asia (24,25). Its life cycle is also associated with fig-pollinating wasps, using them as a dispersal mechanism, which is distinctly different than *C. elegans* (25). This divergence from its sister species makes this system ideal for studying how microbe-dependent life history responses evolve in divergent hosts.

Here, we have isolated bacteria from the natural ecological context of *C. inopinata* and systematically reared both *C. inopinata* and *C. elegans* on these bacterial isolates, measuring individual life history responses, behaviors, and how they are modulated by what bacterial isolate they were fed. We have identified one bacterial isolate, *Klebsiella* sp. WOUb2, that induced opposing life history responses compared to *E. coli* OP50 and altered nematode behavior.

RESULTS:

C. inopinata responds to microbes from its natural environment

After we isolated bacteria from the natural ecological context of *C. inopinata* and generated monocultures (see methods), we performed Sanger sequencing of the ITS region of the 16S rRNA gene for all forty-seven monocultures to ascertain genus-level identification for each isolate (Supplementary Table 1). From these identified genera, we selected representative strains from a select portion of them to rear, seed plates with, and perform population growth assays with *C. inopinata* nematodes (Figure 1).

Klebsiella sp. WOUb2 increases population growth of C. inopinata and C. elegans

To determine what effects different fig bacterial strains have on population growth of *C. inopinata*, we crossed L4 female nematodes with four males on agar plates seeded with a bacterial isolate, let the nematodes proliferate for a seven-day period, and counted the number of nematodes on the plates at the end of that period (Figure 1). For each set of fig bacterial crosses, crosses were also carried out on *E. coli* OP50 as a baseline control. Both failed and contaminated crosses were excluded from downstream analysis (a figure including failed and contaminated crosses can be found in the supplemental materials; Supplemental Figure 1).

A one-way ANOVA was carried out followed by a Tukey post-hoc test to make pairwise comparisons of all groups. Most fig bacteria did not increase or decrease population growth (Tukey post-hoc; p adj. > 0.05). However, the fig bacterial isolate *Klebsiella sp.* WOUb2 increased the population growth of *C. inopinata* (Tukey post-hoc; p adj. < 0.001). There was an average difference of 303.48 nematodes, which is a 103% mean increase in population growth. This result was confirmed across two experimental trials (Supplemental Figure 2). Each plate was seeded with approximately the same bacterial biomass (see methods), and after inoculation on the plates, the

density of bacteria on the newly formed lawns appeared to be similar. This seems to be in accordance with the growth rates observed when growing the bacteria in liquid culture; however, we have not quantified nor obtained metrics of exactly how much bacteria each plate had on it nor determined doubling times for each bacterial isolate. It is possible that there could have been differences in absolute biomass on each plate, and this could, in turn, have downstream effects regarding nematode physiology which would have implications for these findings. However, the relative amounts of bacteria on each plate were similar for *E. coli* OP50 and *Klebsiella* sp. WOUb2, at least from anecdotal observation.

After noticing that our *Klebsiella sp.* WOUb2 isolate raised the mean population growth of *C. inopinata* by one hundred three percent (Figure 1), we performed a BLAST to compare our *Klebsiella* sp. WOUb2 16S Sanger sequence against sequences in the NCBI GenBank database. We found that our sequence shares high identity with a cultured bacterial isolate (Sequence ID: LC269173.1) from Kanzaki et al., the group that published the *C. inopinata* genome paper (24). This cultured isolate was shown to increase population growth of *C. inopinata* in a statistically

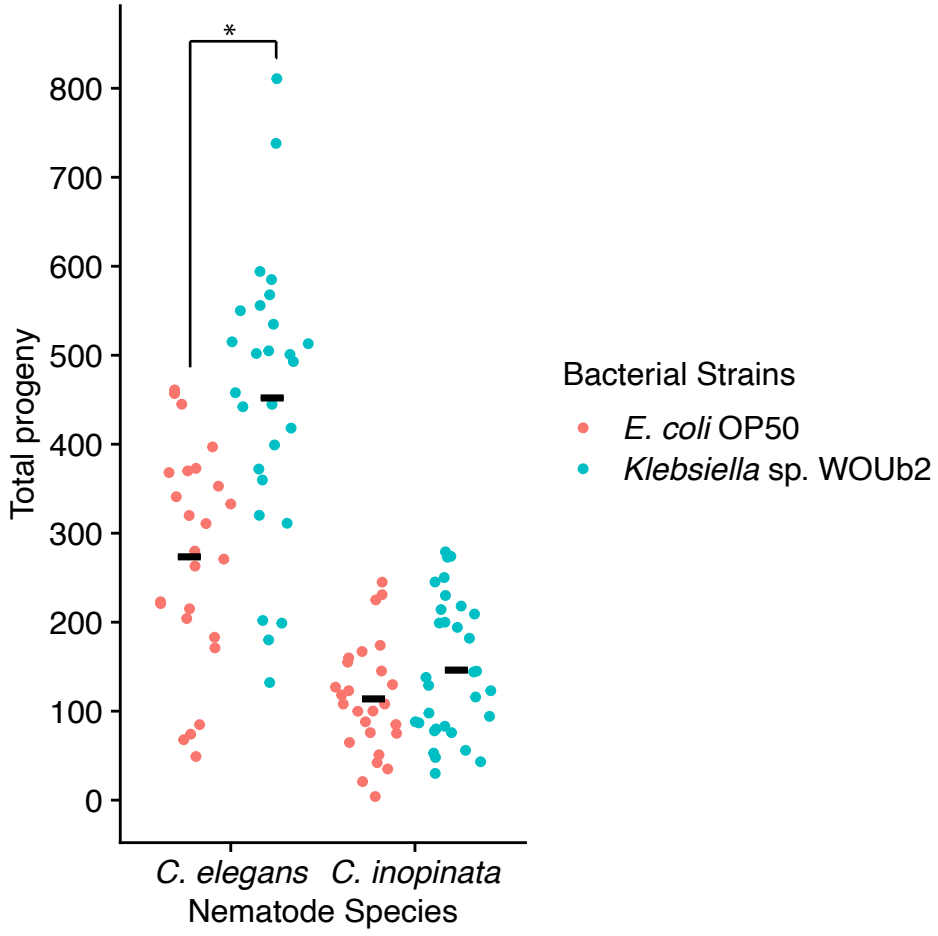

Fig. 2: ***Klebsiella sp.* WOUb2 increases individual fecundity for *C. elegans*, but not *C. inopinata***. Each datapoint represents the number of embryos produced by an individual nematode from a given treatment. Crossbars represent means. Sina plots are strip plots that take the contours of a violin plot. N individual females = 26, *C. inopinata* – *E. coli* OP50; N = 32, *C. inopinata* – *Klebsiella sp. nov.*; N = 25, *C. elegans* – *E. coli* OP50; N = 27, *C. elegans* – *Klebsiella sp.* WOUb2.

significant manner in that paper, and our work here has reproduced those results. This isolate seems to also be tied to work done with another fig from the genus *Ficus*, *Ficus hispida*, which is also associated with *Ceratosolen* pollinating wasps and *Caenorhabditis* nematodes, for the other top *Klebsiella* sp. WOUb2 16S Sanger sequence BLAST hit mentions these organisms (30). The BLAST output table from this result can be found in the supplementary materials (Supplementary Table 2)

To determine whether *Klebsiella sp.* WOUb2 also increased the population growth of *C. elegans* strains *fog-2 (q71)* and *PD1074*, crosses and data collection were carried out in the same manner as the *C. inopinata* population growth experiments using only *Klebsiella sp.* WOUb2 and *E. coli* OP50 (Supplemental Figure 3). *Klebsiella sp.* WOUb2 also increased the population growth rate of *C. elegans* PD1074 (Cohen's *d* effect size; *d* = 1.011517) and *C. elegans fog-2(q71)* (Cohen's *d* effect size; *d* = 1.004257).

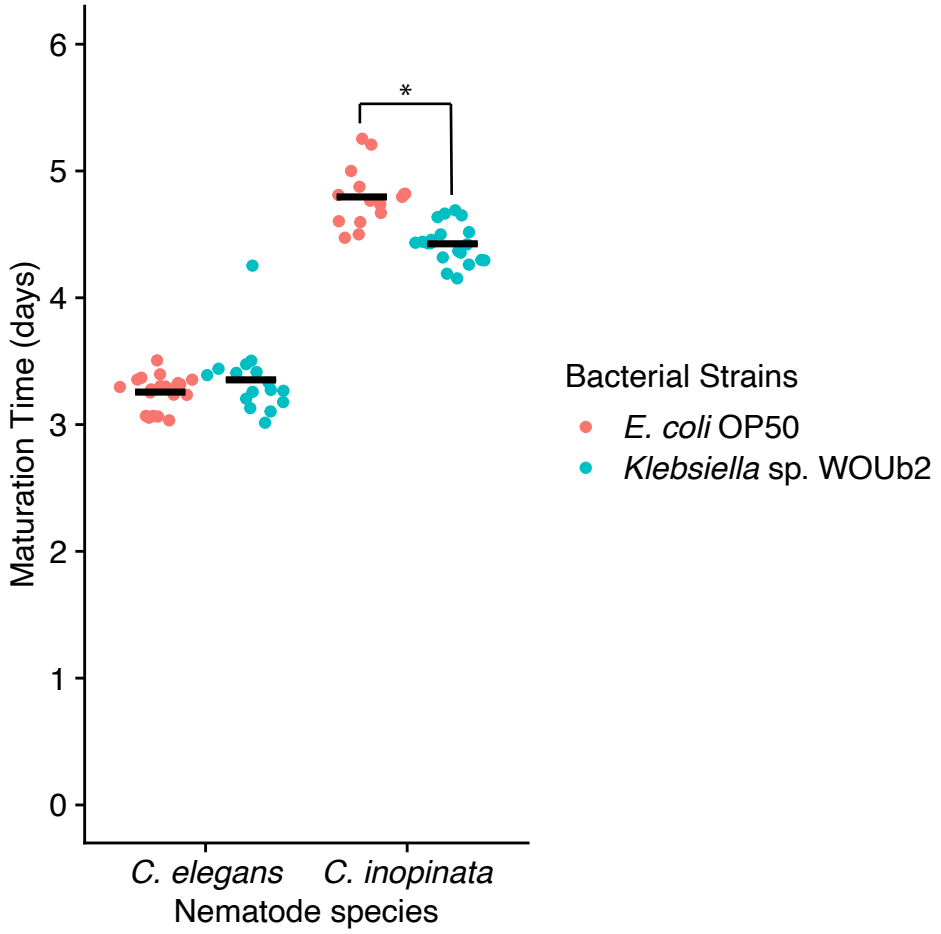

Fig. 3: ***Klebsiella sp.* WOUb2 increases the developmental rate of *C. inopinata*, but not *C. elegans*.** Each datapoint in this figure represents the day approximately half of the nematodes on a given plate reached the adult milestone. Crossbars represent means. Sina plots are strip plots that take the contours of a violin plot. N plates = 20, *C. elegans* – *E. coli* OP50; N = 16, *C. elegans* – *Klebsiella sp.* WOUb2.; N = 15, *C. inopinata* – *E. coli* OP50; N = 20, *C. inopinata* – *Klebsiella sp.* WOUb2.

Individual fecundity increases for C. elegans reared on Klebsiella sp. WOUb2 but not C. inopinata

To determine the effects *Klebsiella sp.* WOUb2 has on individual female nematode fecundity, *C. elegans* (*fog-2 (q71)*) and *C. inopinata* L4 females were placed into continuous mating conditions on plates seeded either with *Klebsiella sp.* WOUb2 or *E. coli* OP50, and the total reproductive output of individual females was measured (Figure 2). There was no significant difference between the *C. inopinata* treatments (Tukey post-hoc; p adj. > 0.05); however, there was a significant difference in individual female reproductive output between *C. elegans* (*fog-2 (q71)*) reared on *Klebsiella sp.* WOUb2 and *E. coli* OP50 (Tukey post-hoc; p adj. < 0.001).

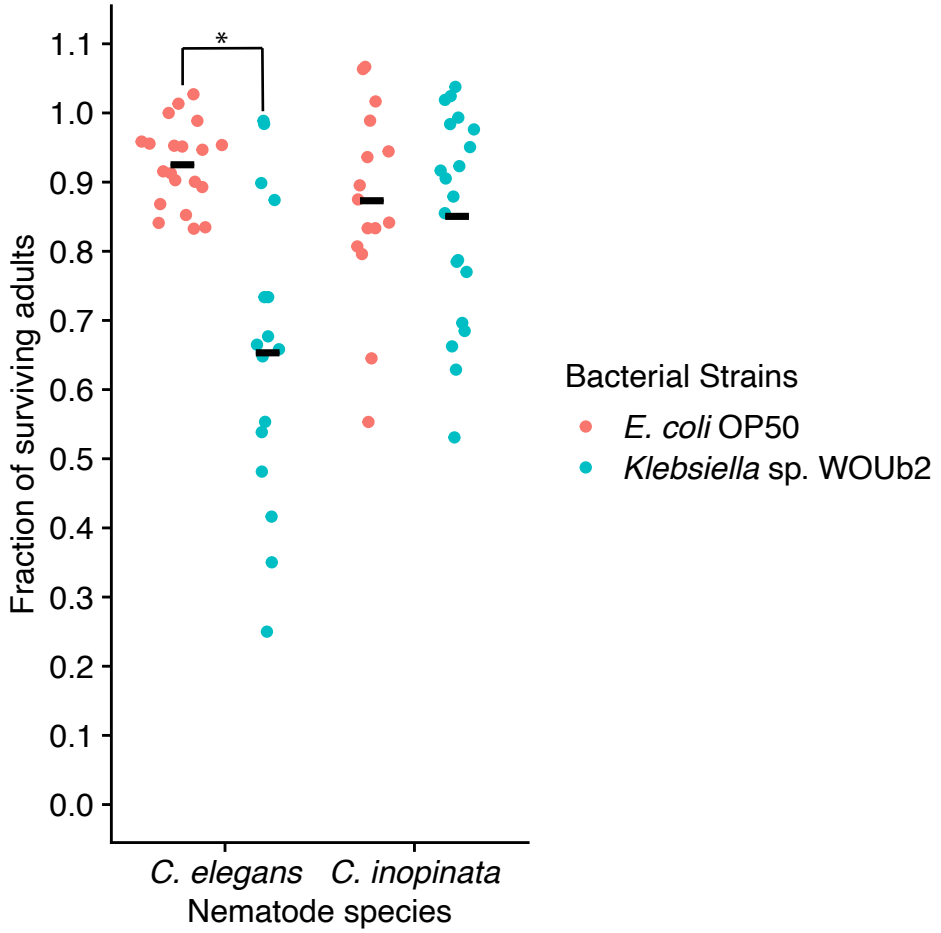

Fig. 4: ***C. elegans* shows decreased viability when reared on *Klebsiella sp.* WOUb2.** From our developmental timing experiment, we ascertained embryo-to-adult viability for nematodes reared on a given plate. Each datapoint represents the fraction of embryos on a given plate that survived to adulthood. *C. elegans* was reared at 20°C and *C. inopinata* was reared at 25°C. Crossbars represent means. Sina plots are strip plots that take the contours of a violin plot. N plates = 20, *C. elegans – E. coli* OP50; N = 16, *C. elegans – Klebsiella sp.* WOUb2.; N = 15, *C. inopinata – E. coli* OP50; N = 20, *C. inopinata – Klebsiella sp.* WOUb2.

Reproductive maturation occurs sooner for C. inopinata reared on Klebsiella sp. WOUb2 while viability decreases for C. elegans

To determine the effects *Klebsiella sp.* WOUb2 has on nematode developmental timing, *C. elegans* (*fog-2 (q71)*) and *C. inopinata* were reared from embryos to adulthood on plates seeded with either *Klebsiella sp.* WOUb2 and *E. coli* OP50. Each treatment's developmental rate was tracked by tabulating the number of nematodes at each developmental milestone (larval, L4, adult, gravid adult) each day. Tabulation of developmental timing ceased when the number of adults on a given plate stabilized for three consecutive days or when it became difficult to distinguish the next generation from the nematodes being tracked. The median timing for reproductive maturity was calculated and plotted for each plate in all treatment groups (Figure 3).

While *C. elegans* does not reach reproductive maturity at different times depending on their bacterial rearing condition (Tukey post-hoc; p adj. > 0.05), *C. inopinata* reached reproductive maturity 0.369 days faster when reared on *Klebsiella sp.* WOUb2 compared to *E. coli* OP50 (Tukey post-hoc; p adj. < 0.001). Additionally, because the numbers of initial embryos and surviving adults per plate were known, the fraction of surviving adults was calculated for each plate and

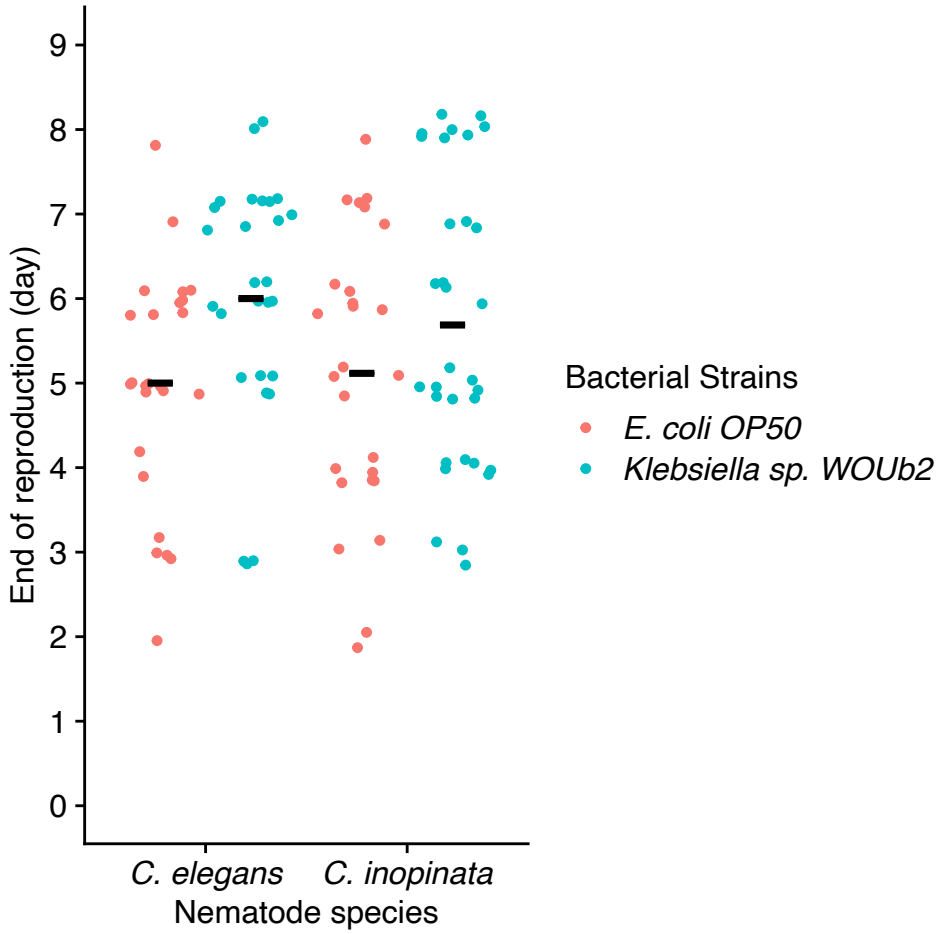

Fig. 5: ***Klebsiella* sp. WOUb2 nor *E. coli* OP50 impacts the length of the reproductive period.** Each datapoint represents the day reproduction ended for an individual female nematode. Crossbars represent means. Sina plots are strip plots that take the contours of a violin plot. N individual females = 26, *C. inopinata* – *E. coli* OP50; N = 32, *C. inopinata* – *Klebsiella sp.* WOUb2; N = 25, *C. elegans* – *E. coli* OP50; N = 27, *C. elegans* – *Klebsiella sp.* WOUb2.

plotted for each experimental treatment (Figure 4). *C. elegans* nematodes reared on *Klebsiella sp.* WOUb2 shows a dramatic drop-off in viability when compared to *C. elegans* reared on *E. coli* OP50 and both *C. inopinata* treatments (Cohen's *d* effect size; *d* = -1.800643), and this drop-off is significantly different from each of these groups (Tukey post-hoc; *p* adj. < 0.01).

Reproductive duration changes slightly for nematodes reared on Klebsiella sp. WOUb2

Because the total reproductive output of individual females was tracked for each treatment group over time, comparisons of reproductive duration between each group were possible. Reproductive output was tracked for individual nematodes until a given individual did not produce progeny for three consecutive days. The first day out of these three consecutive days was deemed the time reproduction ended for a given individual, and these times were noted and plotted (Figure 5). Irrespective of nematode species, there is a modest and significant increase in reproductive duration due to *Klebsiella sp.* WOUb2 (Tukey post-hoc; *p* adj. < 0.05). However, at the species level, there is no significant impact of bacterial strain on reproductive duration.

Klebsiella sp. WOUb2 affects the body size of C. elegans and not C. inopinata

After synchronizing *C. inopinata* and *C. elegans* according to their developmental timing reared on both *Klebsiella sp.* WOUb2 and *E. coli* OP50 (Figure 3) so that the early adult life stage would be reached at the same time for all experimental groups, the body size of each group was measured. For both species, male nematodes were smaller than their female counterparts (Tukey post-hoc; *p* adj. < 0.001). Neither *Klebsiella sp.* WOUb2 nor *E. coli* OP50 altered the length of adult *C. inopinata* females or males (Tukey post-hoc; *p* adj. > 0.05); however, both male and female *C. elegans* nematodes saw an increase in adult length when reared on *Klebsiella sp.* WOUb2 compared to *E. coli* OP50 (Tukey post-hoc; *p* adj. < 0.001; Figure 6). As for width, *Klebsiella sp.* WOUb2 increased the width of adult *C. elegans* males and females compared to *E. coli* OP50 (Tukey post-hoc; *p* adj. < 0.001; Supplementary Figure 4), and it also increased the width of adult *C. inopinata* males (Tukey post-hoc; *p* adj. < 0.01; Supplementary Figure 4).

Both *C. inopinata* and *C. elegans* females are larger than their male counterparts (Figure 6). The effect size of the difference between *C. inopinata* males and females reared on either *Klebsiella sp.* WOUb2 (Cohen's *d* effect size; *d* = -3.532645 (large)) or *E. coli* OP50 (Cohen's *d* effect size; *d* = -3.944066 (large)) was similar, and we know that there was no significant difference in body length for *C. inopinata* reared on *Klebsiella sp.* WOUb2 or *E. coli* OP50 (Figure 6); thus, the slope between male and female *C. inopinata* nematodes is very similar irrespective of bacterial rearing differences (Supplemental Figure 5). However, both male and female *C. elegans* nematodes reared on *Klebsiella sp.* WOUb2 were much larger than their counterparts reared on *E. coli* OP50 (Figure 6), and the effect sizes of the difference between *C. elegans* males and females reared on either *Klebsiella sp.* WOUb2 (Cohen's *d* effect size; *d* = -7.673693 (large)) or *E. coli* OP50 (Cohen's *d* effect size; *d* = -4.148126 (large)) were very different from each other. The increased size of *C. elegans* reared on *Klebsiella sp.* WOUb2 makes the slope between the two sexes more similar to C. *inopinata* also reared on *Klebsiella sp.* WOUb2 (Supplemental Figure 5), and, thus, the difference of body size between sexes is more consistent for these nematode species reared on this bacterium. However, because the slopes of the two species reared on *E. coli* OP50 are more different than those for *Klebsiella sp.* WOUb2, there is variation in the difference in adult body length between the sexes for each species reared on *E. coli* OP50.

C. inopinata and C. elegans larvae have different bacterial preferences

To determine whether *C. elegans* and *C. inopinata* prefer different bacteria and, thus, display varying foraging behavior, choices between *Klebsiella sp.* WOUb2, *E. coli* OP50, and *Stenotrophomonas sp.* WOUb1 were offered in a pairwise manner upon the hatching life stage (Figure 7). Larval bacterial choice was measured by seeding two of these bacterial species on opposite sides of a plate with nematode embryos being plated in the center. After ten hours, the number of nematodes present within each bacterial lawn and the number of nematodes not on either lawn were measured for each plate. This was done for all possible bacterial combinations

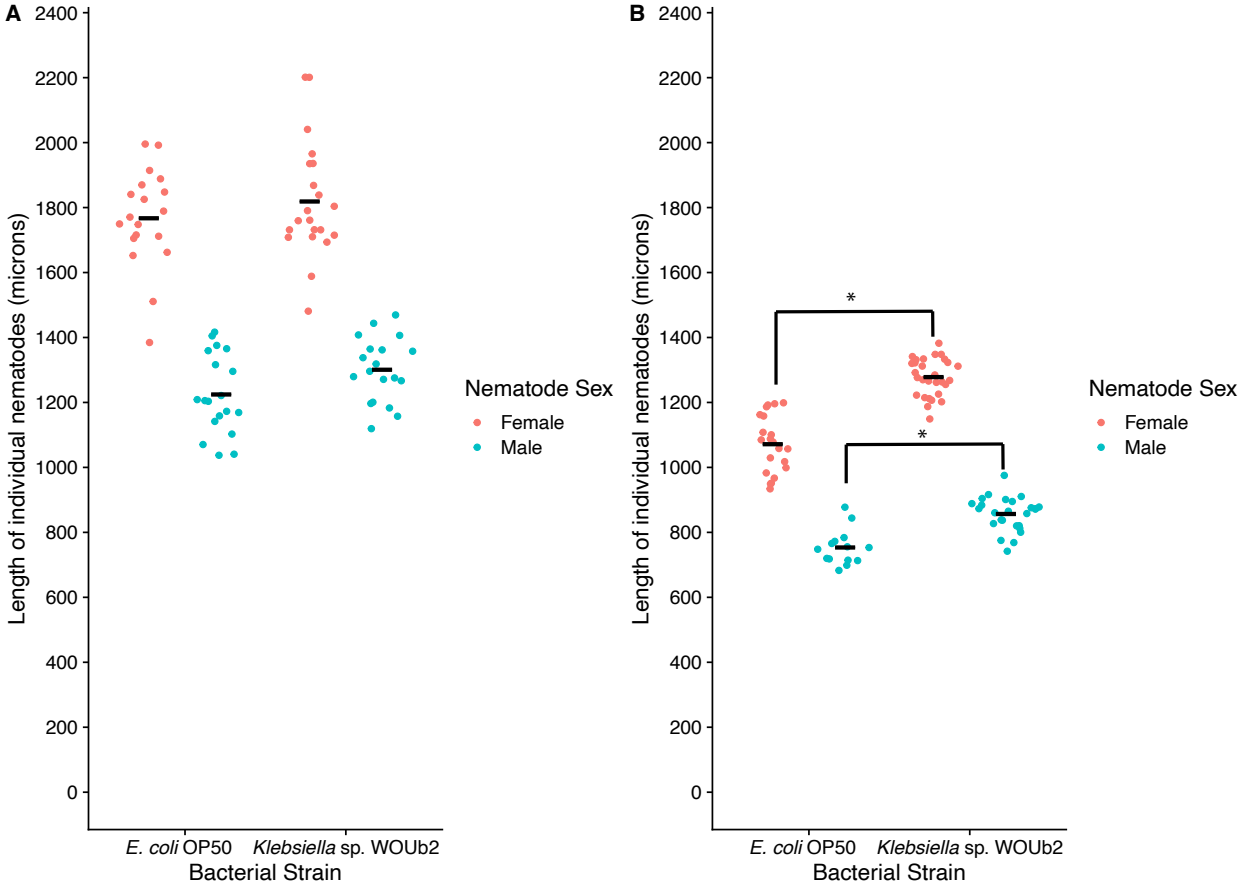

Fig. 6: ***Klebsiella* sp. WOUb2 impacts the length *C. elegans* compared to *E. coli* OP50.** Each datapoint represents the length of an individual nematode in a given treatment that was mounted on an agarose slide and measured in Fiji. A) Comparing the length of *C. inopinata* reared on *E. coli* OP50 and *Klebsiella sp.* WOUb2. B) Comparing the length of *C. elegans* reared on *E. coli* OP50 and *Klebsiella sp.* WOUb2. Crossbars represent means. Sina plots are strip plots that take the contours of a violin plot.

(*Klebsiella sp.* WOUb2, *E. coli* OP50, and *Stenotrophomonas sp.* WOUb1). Between nematode species for the same pairwise bacterial choice combinations, the fraction of total larvae not on any bacterial lawn was significantly different (Tukey post-hoc; p adj. < 0.001). Within each species, the fraction of total larvae not on any lawn was consistent across bacterial choice combinations (Tukey post-hoc; p adj. > 0.05). *C. inopinata* only showed one strong preference: when presented with a choice between *E. coli* OP50 and *Klebsiella sp.* WOUb2, *C. inopinata* larvae preferred *Klebsiella sp.* WOUb2 (Tukey post-hoc; p adj. < 0.001) while the fraction of larvae on *E. coli* OP50 or no lawn at all did not differ (Tukey post-hoc; p adj. > 0.05). For the other two choice

combinations, *C. inopinata* larvae were found on bacteria more than not being on any lawn (Tukey post-hoc; p adj. < 0.05), however, there were no strong preferences for either bacterial choice (Tukey post-hoc; p adj. > 0.05). *C. elegans* displayed stronger, less ambiguous preferences. *C. elegans* larvae preferred both *Klebsiella sp.* WOUb2 and *Stenotrophomonas sp.* WOUb1 over *E. coli* OP50 (Tukey post-hoc; p adj. < 0.001) while preferring *Stenotrophomonas sp.* WOUb1 over *Klebsiella sp.* WOUb2 (Tukey post-hoc; p adj. < 0.05).

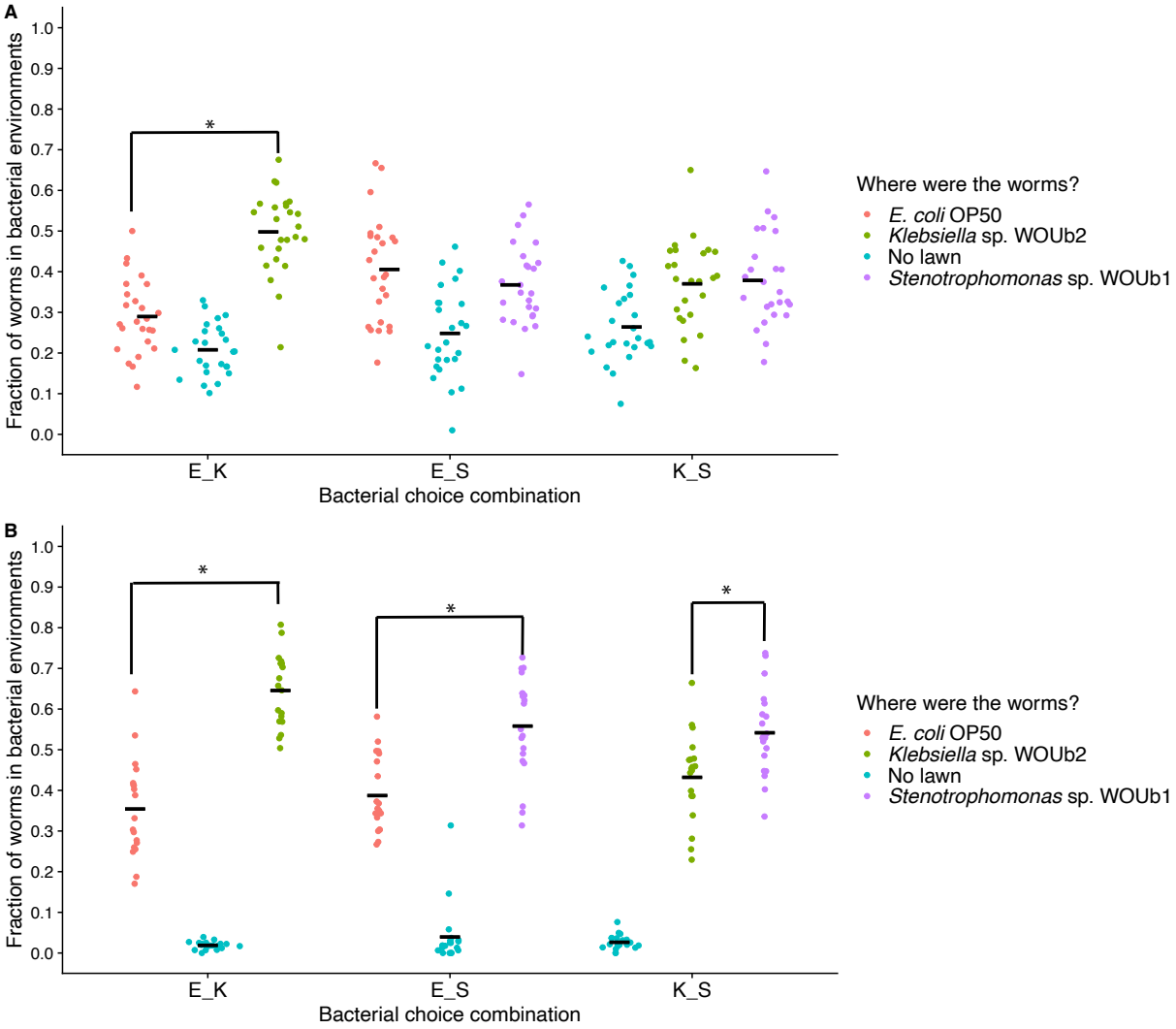

Fig. 7: ***C. elegans* and *C. inopinata* larvae respond differently to bacteria choice assays.** Each datapoint represents the fraction of nematodes in a given bacterial lawn (or no lawn) for a single plate in a given bacteria-choice treatment. A) Choice experiment for *C. inopinata*. B) Choice experiment for *C. elegans*. Note: *E_K* represents a choice between *E. coli* OP50 and *Klebsiella sp.* WOUb2; *E_S* represents a choice between *E. coli* OP50 and *Stenotrophomonas sp.* WOUb1, and *K_S* represents a choice between *Klebsiella sp.* WOUb2 and *Stenotrophomonas sp.* WOUb1. post-hoc; p adj. < 0.05; Supplemental Figure 6).

Gravid C. inopinata females prefer to lay their embryos in Klebsiella sp. WOUb2

To determine whether embryo-laying behavior differed depending on immediate bacterial environment differences, we placed single gravid females onto plates seeded with either *E. coli*

OP50 or *Klebsiella sp.* WOUb2. Although there was no difference in the fraction of total embryos individual *C. elegans* females laid on *E. coli* OP50 lawns or *Klebsiella sp.* WOUb2 lawns (Tukey post-hoc; p adj. > 0.05), *C. inopinata* females laid higher fractions of total embryos on *Klebsiella sp.* WOUb2 than *E. coli* OP50 (Tukey post-hoc; p adj. < 0.05; Figure 8). However, these results correspond to data where ten or less total embryos being laid by a given individual female were filtered out. When taking into consideration the unfiltered data, *C. elegans* females laid higher fractions of total embryos on *Klebsiella sp.* WOUb2 than *E. coli* OP50 (Tukey post-hoc; p adj. < 0.05; Supplemental Figure 6) and, although the means shifted downwards, *C. inopinata* females still laid higher fractions of total embryos on *Klebsiella sp.* WOUb2 than *E. coli* OP50 (Tukey

DISCUSSION:

It has been demonstrated that bacterial isolates from the ecological context of *C. elegans* have profound effects on its biology and life history phenotypes (22,23,31,32). Due to the power of the *C. elegans* system, the field of host-microbe interactions using *Caenorhabditis* nematodes has been blooming as of late. In *Caenorhabditis* nematodes, the effects microbes exert effects on host life history phenotypes is dependent on both genotype and environmental context, and microbial community composition is conversely correlated with host strain or genotype (33,34). It is plausible that microbial communities in these nematodes (and eukaryotes more generally) play a key role in evolutionary trajectories of said hosts; microbes can change community composition and evolve divergent physiological responses due to environmental alteration in a more rapid manner than their hosts, for hosts have vastly longer generation times and miniscule population sizes compared to their microbial counterparts (35). Consequently, because microbes significantly impact host fitness, their community composition can be altered by host genotype or ecological niche, and host genotype may correspond to alternate host-microbe responses, evolution and divergence of either the host or the microbe into novel ecological contexts could alter the components of the entire network on a fundamental level. Using *C. elegans* and its divergent relative *C. inopinata* alongside microbes from a novel *Caenorhabditis* niche, the fig, makes it possible to disentangle how host-microbe interactions (and the molecular and genetic bases of these interactions) evolve in divergent species through laboratory experimentation, especially in the context of life history.

To investigate how host-microbe interactions may have diverged between *C. elegans* and *C. inopinata*, we first isolated forty-seven bacterial isolates from the natural environment of *C. inopinata* and systematically reared nematodes on ten of these isolates to determine how natural microbial communities affect various components of nematode life history and behavior. We measured how these ecologically relevant bacterial strains affected general population growth of *C. inopinata* compared to the standard *E. coli* OP50 control strain OP50 (19) starting from a single cross (Figure 1). Then, we identified one of our bacterial isolates, *Klebsiella* sp. WOUb2, not only increases the population growth of *C. inopinata* after one week compared to other bacterial isolates, but it also induces opposing life history responses between *C. inopinata* and *C. elegans*. Developmental timing and individual fecundity contribute differentially to increased population growth in these two species when reared on our *Klebsiella* sp. WOUb2 isolate. Additionally, foraging behavior was different for nematodes of the same species being offered different bacterial choices, and foraging behavior also varied between species. And, our *Klebsiella sp.* WOUb2 isolate also influenced embryo-laying behaviors of both *C. inopinata* and *C. elegans*.

***C. inopinata* shows either no response or drastic response when reared on fig bacteria compared to *E. coli* OP50**

Most fig bacteria did not influence the population growth of *C. inopinata* compared to *E. coli* OP50 in statistically significant manners, but our *Klebsiella* sp. WOUb2 isolate did (Tukey post-hoc; p adj. < 0.001; Figure 1). Isolates belonging to the same genera as *Klebsiella* sp. WOUb2, *Klebsiella* (JUb54 and JUb38), have been shown to increase population growth and attract *C. elegans* even after being trained on other isolates (23,36). The lack of responses to other bacterial isolates, however, is surprising, for several genera that were used in our population growth experiment have been demonstrated to alter the population growth of *C. elegans* in extraordinary manners (22). While mean population growth of *C. inopinata* reared on our *Microbacterium* strain was approximately equivalent to *E. coli* OP50 (Figure 1), two *Microbacterium* strains (MYb45 and MYb50) resulted in population growth around one hundred times less than that of *E. coli* OP50 in *C. elegans* (22). Our *Pseudomonas*, *Stenotrophomonas*, and *Acinetobacter* strains did not influence population growth in *C. inopinata* significantly (Figure 1), but corresponding genera have altered *C. elegans* population growth in variable manners (22,23). There may be species differences between similar genera used in this study and others, however, so each nematode species would need to be reared on the exact same bacterial isolates to determine whether there has been a true divergence of host-microbe response in this instance. Yet, the lack of sensitivity of *C. inopinata* to a majority of our bacterial isolates raises the question of what facets of *C. inopinata* biology have evolved to produce this result.

C. elegans has a cosmopolitan distribution but is especially prevalent in in temperate environments (27–29). It seems to have a stable, species-specific microbiome at higher bacterial taxonomic levels that differs from surrounding substrates across vast geographic distances (22,37), yet, because of its vast spatial distribution, it encounters a wide range of biotic and abiotic stimuli. *C. inopinata*, however, is found in only one region of the world, lives in an extraordinarily specific ecological niche, and is associated with a single invertebrate carrier as its dispersal mechanism (24,25); consequently, it is reasonable to call this species a specialist relative to its sister species, *C. elegans*. Previous studies have suggested that specialization can impede a species' ability to respond to environmental change as efficiently as a generalist species, imposing a constraint that may result in extinction as resource availability, resource type, or numerous other biotic and abiotic factors change (38–40). Has *C. inopinata*'s ability to respond to wide sets of bacteria taxa been altered as it evolved to live in the fig compared to its widespread relative, *C. elegans*? One way to test this would be rearing *C. inopinata* on non-fig bacterial isolates associated with *C. elegans* and other nematodes and measuring both life history and transcriptomic responses. It is also possible that we simply did not use enough bacterial isolates in our population growth experiment to detect the true range of the nematode's response to bacteria from its natural environment. And, it is important to note that *C. inopinata* is, compared to other *Caenorhabditis* species, less fecund, less viable, and develops much more slowly (26). Consequently, it may be unsurprising that this nematode does not respond to different food sources the same way *C. elegans* does.

Klebsiella* sp. *WOUb2 induces opposing life history phenotypes in closely related nematodes

Both *C. inopinata* and *C. elegans* show increased population growth responses when reared on our *Klebsiella* sp. WOUb2 isolate (Figure 1; Supplementary figures 1 and 2). Our data suggests that there has been divergence in the manner in which each species arrives at this shared overall response. Individual *C. inopinata* nematodes reared on either *E. coli* OP50 or *Klebsiella* sp. WOUb2 produced the same number of embryos on average (Tukey post-hoc; p adj. > 0.05), but

individual *C. elegans* nematodes reared on *Klebsiella* sp. WOUb2 produce more embryos on average than their counterparts reared on *E. coli* OP50 (Tukey post-hoc; p adj. < 0.05; Figure 2). However, *C. elegans* nematodes reared on either *E. coli* OP50 or *Klebsiella* sp. WOUb2 do not differ in the age at which reproductive maturity is reached, yet *C. inopinata* nematodes reared on *Klebsiella* sp. WOUb2 reach reproductive maturity approximately a third of a day faster than their counterparts reared on *E. coli* OP50 (Tukey post-hoc; p adj. < 0.05; Figure 3). Although each species shows increased population growth when reared on *Klebsiella* sp. WOUb2, *C. inopinata* achieves it through a faster developmental timing, and *C. elegans* achieves it through increased individual reproductive output.

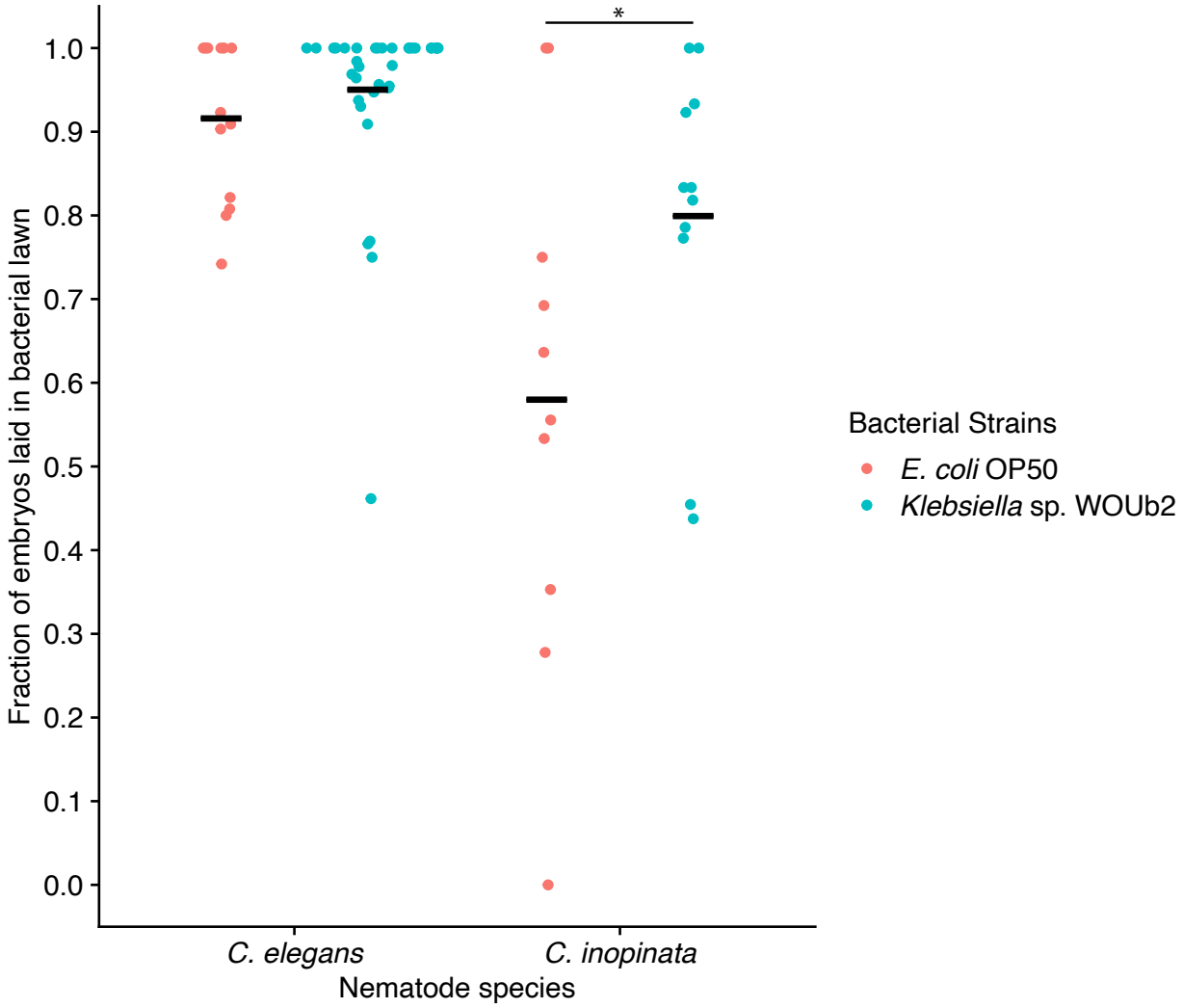

Fig. 8: **Gravid *C. inopinata* nematodes preferentially lay their embryos on *Klebsiella sp.* WOUb2 compared to *E. coli* OP50.** Each datapoint represents the fraction of total embryos laid in a given bacterial environment for a given nematode species by a single gravid female. Only data where worms laid greater than or equal to ten embryos was included here (a figure including all data can be found in the supplemental materials; Supplemental Figure 6).

The viability of *C. inopinata* does not differ between nematodes reared on *E. coli* OP50 and *Klebsiella* sp. WOUb2, yet *C. elegans* shows decreased viability when reared on *Klebsiella*

sp. WOUb2 (Figure 4). This seemingly indicates a trade-off occurring between viability and maternal fecundity. This trade-off is a well-documented phenomenon (15,16). For example, *Teleogryllus oceanicus* crickets, demonstrate a trade-off between high fecundity and low viability (41). There are also examples of seasonal variation for this trade-off in *Drosophila melanogaster*, for organisms experience a wide range of environments, resource types, and resource availability (42,43). In *Callirhytis quercusbatatoides* wasps that induce galls on the stem of its host plant, *Quercus virginiana*, gall size is positively correlated with offspring number; however, because increased gall size results in increased predation from avian predators, there is negative correlation between offspring number and offspring survival (44). Because organisms only have so much energy to invest into individual survival and reproduction, it makes sense that increased numbers of offspring might result in either less energy allocated to each individual offspring or less energy to invest in maternal survival, resulting in decreased viability overall (15,16,18). Furthermore, the ecological niche of a majority of *Caenorhabditis* species is rotting plant material where bacterial food is thought to be sparse due rapid turnover of this niche (27), and it has been speculated that this has resulted in the evolution of a more rapid developmental rate and increased fecundity within those *Caenorhabditis* species compared to *C. inopinata* (26). We speculate that the evolution of these responses also stems from species-specific host-microbe interactions when encountering beneficial microbes such as members of the bacterial phyla *Proteobacteria* (which our *Klebsiella* sp. WOUb2 isolate is a member of), which have been found to dominate the ecological niche of *C. elegans* (22,23). If few members within a given nematode population encounter food, increasing individual reproductive output when encountering food that can better sustain the population may be key to reaching a fitness optimum for avoiding extinction of the population alongside an increased developmental rate. However, in our experiments, we provided adequate food to the nematodes, so it is very surprising that the viability of *C. elegans* decreased when reared on *Klebsiella sp.* WOUb2. We speculate that there may be stage-specific effects of *Klebsiella sp.* WOUb2 relating to pathogenicity, where larval or embryonic stage nematodes are more susceptible to infection or host-pathogen interactions with *Klebsiella sp.* WOUb2 while adult nematodes may receive the benefits conferred by this bacterium. However, pathogenic traits of *Klebsiella sp.* WOUb2 have yet to be observed, and this is merely speculation based on the pathogenicity of other notable *Klebsiella* species (45,46). Another surprising aspect of our viability data is that the viability of *C. inopinata* is moderately higher compared to what other studies have observed (26). The ranges of the proportion of surviving *C. inopinata* nematodes reared on either *Klebsiella* sp. WOUb2 or *E. coli* OP50 both span fifty percent to one hundred percent, with a majority of the data being at or above eighty percent survival. We expected there to not only be a difference between the viability of *C. elegans* and *C. inopinata* generally due to prior observation, but also for there to be a difference in nematode viability between bacteria.

We also looked at reproductive duration. Both the onset and cessation of reproduction have been considered as fundamental life history traits, for earlier onset of reproduction is negatively correlated with organismal lifespan and reproductive duration (15,16,47). For example, previous studies have demonstrated a genetic trade-off between early reproduction and lifespan in *Bactrocera cucurbitae* melon flies (48), and others have observed shortened lifespans of Columbian ground squirrels that successfully reproduced early compared to those that reproduced later (49). From invertebrates to mammals, the principles of this trade-off (and others) remain consistent. Although *C. inopinata* reared on *Klebsiella* sp. WOUb2 reaches reproductive maturity a third of a day earlier than its counterparts raised on *E. coli* OP50, it is unknown whether this difference is large enough to drive a and shortened lifespan phenotype, for we did not measure this

life history response. Neither *C. inopinata* nor *C. elegans* reared on *E. coli* OP50 or *Klebsiella* sp. WOUb2 differed in their total reproductive span (Figure 5; measured in days). This is unsurprising, for previous studies have observed that reproductive duration does not differ between *C. inopinata* and *C. elegans* (26).

The last life history trait we assayed was adult body size depending on bacterial rearing conditions. *C. inopinata* is larger than *C. elegans* by a considerable amount (24,26). Although body size has been measured before in *C. inopinata*, we were interested in seeing whether body size can be modulated by fig bacteria in this *Caenorhabditis* species, as differing *E. coli* strains have been shown to alter the body size of *C. elegans* (50). We found that both *C. inopinata* males and females did not have different adult body lengths when reared on *Klebsiella sp.* WOUb2 or *E. coli* OP50 (Tukey post-hoc; p adj. > 0.05; Figure 6). Larger animals tend to take more time to develop, for larger animals have more body mass to synthesize. Body size and developmental timing have been correlated across a wide variety of taxa (51,52). We saw an increase in developmental rate for *C. inopinata* reared on *Klebsiella sp.* WOUb2, yet body size was not different; this either suggests that this difference in maturation rate is not enough to drive a difference in body size or the genetic mechanisms behind these life history traits are not coupled in this instance. This is consistent with the body of *C. elegans* literature. Most mutant genes in the *C. elegans* genome corresponding to an alteration of body length do not affect developmental rate (26). It is reasonable to suspect that these responses may not be intertwined in this context. We also observed that both adult *C. elegans* males and females were larger when reared on *Klebsiella sp.* WOUb2 compared to *E. coli* OP50 (Figure 6), *C. elegans* reared on *Klebsiella sp.* WOUb2 also increased individual reproductive output compared to *E. coli* OP50 (Figure 2), and developmental timing of *C. elegans* does not differ irrespective of whether it is reared on *Klebsiella sp.* WOUb2 and *E. coli* OP50. The developmental rate aspect is, yet again, consistent with the literature (26), and because nematode body size is usually positively correlated with fecundity (53), the fact that we saw an increase in individual reproductive output was also expected. Additionally, there seems to be variable levels of sexual dimorphism between *C. elegans* and *C. inopinata* as it relates to adult body length depending on what bacteria the nematodes were reared on. In *C. elegans*, *Klebsiella sp.* WOUb2 increases the extent of body size sexual dimorphism; however, in *C. Inopinata*, there is no environment-dependent impact on sexually dimorphic body size (Supplementary Figure 5).

Although we have observed *Klebsiella sp*. WOUb2 influencing several nematode life history traits, the extent to which this isolate influences *C. inopinata* living in figs remains unclear. Previous studies have demonstrated that developmental stages of *Ceratosolen* fig wasps and *C. inopinata* are correlated (25); this makes sense due to the intertwined nature of the *C. inopinata* life cycle and these fig wasps, for *C. inopinata* uses the wasps as a dispersal mechanism (25). To understand how bacteria within the fig modulate life history traits of *C. inopinata* in nature, the abundances of microbes across fig, nematode, and wasp developmental stages could be measured and compared against each other. A given microbe being more abundant within the fig lumen may increase the likelihood of it being consumed by nematodes. Measuring the distribution of microbe abundances in the fig across time as well as their effects on nematode life history in the lab could reveal further insights into how host-microbe interactions evolved in this context. After all, these nematodes are not consuming bacteria lawns consisting of only one bacterium inside the fig, they are interacting with an entire microbial community with individual species presumably being present in varying abundances at once. Furthermore, measuring how individual members of the fig microbial community interact with each other and their net metabolic output through

metabolomic and metatranscriptomic approaches would be useful in determining the genetic and molecular bases of what compounds modulate *C. inopinata* life history phenotypes.

Foraging behavior and embryo-laying behavior are modulated by bacteria

It has been demonstrated that *C. elegans* has preferences for what bacteria it eats (54–56), and these preferences are often mediated by chemosensory cues that nematodes use to discriminate between different bacterial species (29). *Caenorhabditis* nematodes can sense chemicals and metabolites that are released by bacteria in the environment, and these are what cause *C. elegans* and other *Caenorhabditis* species to be attracted to a given bacteria (29); depending on the evolutionary context of a given *Caenorhabditis* species, the cues that result in attraction may differ for that species since bacterial community composition and, consequently, their metabolic output will differ on an environmental basis as well as the genetic background of divergent *Caenorhabditis* species. The genome of *C. elegans* encodes a family of over one thousand chemoreceptor genes (57).

To study the bacterial preferences of *C. elegans* in an ecologically relevant manner, many groups have used the CeMbio resource (37). It has been found that *C. elegans* shows strong preferences for the odors emitted by three out of the twelve CeMbio strains (CEent1: *Enterobacter hormaechei*, JUb66: *Lelliotia amnigena*, and BIGb3093: *Pantoea nemavictus*) over *E. coli* OP50 (58). In the same study, gas chromatography paired with mass spectrophotometry was used to identify that all three of these isolates released isoamyl alcohol (58), which has been previously identified in the literature as a strong attractant for *C. elegans* (59–61). These three bacterial isolates also increased the developmental speed of the nematode compared to when it was reared on *E. coli* OP50 (58); six additional isolates from the CeMbio resource also increased developmental speed compared to *E. coli* OP50, however, *C. elegans* did not show a strong preference for these six strains over *E. coli* OP50 (58). This information reveals a key point, however: the bacterial food of *Caenorhabditis* nematodes can affect life history traits.

Here, we have used bacteria (*Klebsiella sp.* WOUb2 and *Stenotrophomonas sp.* WOUb1) isolated from *C. inopinata*'s ecological niche, the fig, alongside *E. coli* OP50 to investigate whether foraging and embryo-laying behaviors have diverged between *C. inopinata* and *C. elegans*. We found that *C. inopinata* larvae only showed strong preferences when presented with a choice between *Klebsiella sp.* WOUb2 and *E. coli* OP50 (Tukey post-hoc; p adj. < 0.001), while *C. elegans* larvae had strong preferences in every choice combination used here with *Stenotrophomonas sp.* WOUb1 being most preferred, *Klebsiella sp.* WOUb2 being the next-most preferred, and *E. coli* OP50 being least preferred (Figures 8 and 9). Additionally, while around a quarter of *C. inopinata* larvae were not on any bacterial lawn at the time of observation (consistent across choice combinations), *C. elegans* larvae were hardly found off of a bacterial lawn regardless of bacterial choice combination (Figure 8).

As previously discussed, *C. inopinata* is a specialist in terms of its ecology compared to *C. elegans*, and nearly the entirety of *C. inopinata*'s life is contained within the fig. Bacteria may not be as sparsely dispersed throughout this environment as it would be for *C. elegans*, for the turnover of the *C. elegans* niche is high while the figs *C. inopinata* lives in persist for longer durations (24,25,27). Additionally, *C. elegans* has a cosmopolitan distribution, encountering a wide variety of environments (27–29). Taking this into consideration, *C. inopinata* may have evolved to not have as discerning when it comes to finding food as *C. elegans* would in its natural context, for

bacteria are present all around it in the fig suspension and, thus, would require less searching to find. Additionally, because *C. elegans* is associated with a variety of environments (and, thus, bacteria) and *C. inopinata* is only associated with bacteria found in *Ficus septica* figs, *C. inopinata* may have lost sensory capabilities or odorant receptors for a wide range of bacterial taxa (and their excreted metabolites) while *C. elegans* may have retained these capabilities due to its extensive geographic distribution, enabling it to be more discerning than its specialist counterpart. Interestingly, *C. inopinata* has lost approximately seventy-five percent of its G protein-coupled receptor genes compared to *C. elegans* (24), and these receptors are implicated in various forms of stimuli response such as odorants, hormones, and neurotransmitters (62). This could also explain why *C. elegans* larvae were hardly found anywhere besides a bacterial lawn in our choice experiment while a significantly larger portion of *C. inopinata* larvae were not on a bacterial lawn at all. This is largely speculative and would need to be investigated independently, however.

We also measured embryo-laying responses of gravid *C. elegans* and *C. inopinata* females on plates seeded with either *Klebsiella sp.* WOUb2 and *E. coli* OP50 (Figure 8 and Supplemental Figure 6). We found that *C. inopinata* females, on average, laid a higher fraction of their total embryos within *Klebsiella sp.* WOUb2 lawns compared to *E. coli* OP50 (Tukey post-hoc; p adj. < 0.05; Figure 8). For *C. elegans*, however, there was no difference in the fraction of total embryos laid in *Klebsiella sp.* WOUb2 lawns compared to *E. coli* OP50 (Tukey post-hoc; p adj. > 0.05; Figure 8). These results only consider nematodes that laid ten or more embryos. When considering nematodes that laid less than ten embryos, *C. elegans* laid a greater total fraction of embryos in *Klebsiella sp.* WOUb2 lawns compared to *E. coli* OP50 (Tukey post-hoc; p adj. < 0.05; Supplemental Figure 6). The mean for the fraction of total embryos laid on *Klebsiella sp.* WOUb2 hardly changed, yet the mean for the fraction of total embryos laid on *E. coli* OP50 dropped considerably. We know from our choice experiment that *C. elegans* is certainly capable of finding bacteria, so it is surprising that, when laying less embryos, *C. elegans* still chooses to lay them within *Klebsiella sp. WOUb2* yet does so to a significantly lesser degree for *E. coli* OP50. Seeing as *Klebsiella sp.* WOUb2 raises population growth of *C. elegans* through the increase of individual reproductive output (Figure 2 and Supplemental Figure 3) compared to *E. coli* OP50, is it possible that, to maximize fitness when producing low numbers of progeny, the nematode chooses to lay embryos according to where its offspring will be most fit? One way to address this would be to do another choice experiment but doing it with gravid females instead of larvae.

Limitations and caveats

There are limitations and caveats to the work presented here. Although we tested how several *Ficus septica* bacterial isolates affected population growth and several other life history responses for nematodes reared on *Klebsiella* sp. WOUb2, as mentioned above, it is unclear which microbes *C. inopinata* actually eats in its natural context and whether we captured the range of its nutrition in this study. It is also unknown how abundant each of these nutritional sources is within the fig; consequently, although we know that it is possible for microbes to affect nematode fitness, the degree to which our isolates modulate the phenotype of *C. inopinata* in the fig remains to be seen. Longitudinal studies of the microbial communities within *Ficus septica* across fig stage must be done to begin disentangling this remaining question. A laboratory setting does not faithfully recreate all features of these animals' natural environment, so even though these isolates are more ecologically relevant than *E. coli* OP50, there are still aspects of the study that do not completely imitate how these nematodes live in the fig. Aspects of nematode behavior, and the behavioral differences between *C. inopinata* and *C. elegans* due to ecological niche divergence, may also

impact what we have measured here. Future studies focusing on behavioral responses of each nematode in response to bacteria within and outside of its ecological context could illuminate further divergence of these sibling species. Furthermore, just as work has been done to quantify the microbiome of *C. elegans* (37), quantifying the microbiome of *C. inopinata* could not only enable studies using the microbes genuinely associated with the nematode as opposed to microbial community members that may not be as relevant, but by comparing the microbiome of *C. inopinata* and *C. elegans*, the evolution of host-microbe associations due to the divergence of both the nematodes and microbial communities can be quantified through evolutionary genetic means.

For our life history experiments, there are four major limitations. The first one is that we used a mutant strain, *fog-2 (q71)*, for all of our experiments. It could be argued that our experiments do not truly represent *C. elegans* as it would be found in nature. However, this was done to control for reproductive mode differences between *C. inopinata* and *C. elegans*, and although it is possible that the mutation may have had an impact on our results, it is not predicted to do so in a significant manner (63). The second limitation is the differences in rearing temperature between *C. elegans* and *C. inopinata* during our developmental timing experiment (and consequently, our viability results also). *C. elegans* develops much faster than *C. inopinata* (26), and this is especially apparent when reared at higher temperatures. When reared at temperatures exceeding the ambient temperature it experiences in nature (27–29), *C. elegans* shows increased stress responses (64,65). The lifespan of *C. elegans* also decreases considerably with increased temperature; at 16°C, the life span of *C. elegans* is twenty-three days, but at 25°C this decreases to nine days (65). However, *C. inopinata* is typically grown at 25°C in the lab, for this more closely resembles the temperature of its natural environment and represents an optimal temperature (24,26). We reared *C. elegans* at 20°C and *C. inopinata* at 25°C. We reared both strains in their respective optimal, non-stressful environmental conditions and, thus, limited the effects of stress on developmental timing and viability. The same experiment should be run again but with all temperature-species-bacteria combinations to ensure that our results are not merely due to a global effect from temperature. The third limitation is a caveat(s) within our viability results. Besides the rearing temperature differences between *C. elegans* and *C. inopinata* outlined above, there were some plates where the viability seems to exceed one hundred percent. This is most likely due to undercounting embryos after the initial egg lay immediately preceding our developmental timing experiment. Additionally, it is possible for nematodes to crawl off of agar plates, potentially driving viability downwards. The fourth limitation is that, for the *C. elegans* treatments in the developmental timing experiment, measurements were only taken once per day. Although we observed no differences in developmental timing for *C. elegans* nematodes reared on *E. coli* OP50 or *Klebsiella* sp. WOUb2, it is possible that there are in fact differences that we could not detect. Because these nematodes develop on a more rapid scale than *C. inopinata*, the number of measurements required may need to be scaled to this difference in developmental timing. This also has potential implications for our body size data. If we did not accurately capture differences between developmental rate, then one experimental condition could be farther ahead or behind in development compared to another, and, thus, our measurements of *C. elegans* size may or not be comparable and *Klebsiella sp.* WOUb2 may not actually increase length or width.

Conclusion

Host-microbe interactions impact all domains of life and drive not only evolutionary trajectories, but also life history evolution. Here, we reared *C. inopinata* and *C. elegans* on bacteria isolated from the natural environment of *C. inopinata*, *Ficus septica* figs, and measured nematode

life history responses. One of our isolates, *Klebsiella* sp. WOUb2, has revealed divergent means between the species to achieve increased population growth compared to nematodes reared on *E. coli* OP50. Notably, *C. inopinata* achieves this through increased developmental rate while *C. elegans* has increased fecundity. The increased production of progeny by *C. elegans* reared on *Klebsiella* sp. WOUb2 comes at the cost of viability, revealing the evolution of a trade-off between the two life history traits. Body size was found to increase for *C. elegans* reared on *Klebsiella sp.* WOUb2 but not for *C. inopinata*, and it is unknown whether this is due to genetic or nutritional differences. Studies measuring temporal microbial community member abundance in the fig and determining the core microbiome of *C. inopinata* is needed to further disentangle the evolution of both microbes and *Caenorhabditis* nematodes living in divergent ecological niches. Additionally, further studies of nematode behavior using more bacteria from various environments could further elucidate why we saw differences in foraging and embryo-laying behavior.

METHODS:

Strains and maintenance

To isolate bacteria from the natural environment of *C. inopinata*, *Ficus septica* figs were sampled in Taipei, Taiwan in 2019. Figs were dissected in 4 ml of sterile M9 buffer (Brenner 1974) in a sterile petri dish. 200 µl of fig suspension was transferred to a sterile LB plate and left at room temperature. Subsequently, monocultures were generated on LB media reared at room temperature. Stocks were preserved by adding 1.5 mL of a saturated liquid culture in LB to 0.5 ml of 60% glycerol; these were then frozen in liquid nitrogen and stored at -80°C. Genus identity was determined via PCR and Sanger sequencing of the ITS region of the 16S rRNA gene (list of all bacterial strains used in this study alongside their 16S sequences can be found in Supplemental Table 1).

All nematodes were reared on peptone-free Nematode growth medium (NGM) plates. Peptone-free plates were used to control for the amount of available bacterial biomass on the NGM plates (22,37,66). All bacterial strains were reared in LB broth at 37°C to an optical density of 0.6 at 600nm (67). This, in conjunction with the peptone-free plates, was done to ensure that approximately the same amount of cellular material would be placed onto each experimental plate. For each experimental plate, 150ul of this bacterial solution was used for plate seeding. The *C. inopinata* strain NKZ35 (24) and *C. elegans* strain *fog-2(q71)* JK574 (63) were used for all experiments in this work. Wild-type *C. elegans* strains are androdioecious (hermaphrodite/male), but *C. inopinata* is gonochoristic (female/male). Therefore, to account for differences in reproductive mode between these two species when measuring life history trait responses, the *C. elegans fog-2(q71)* mutant line was also used. This mutant line prevents spermatogenesis only in *C. elegans* hermaphrodites while it is maintained in males (63), making the stocks functionally gonochoristic.

Population growth

Synchronized *C. inopinata* NKZ35 and *C. elegans fog-2 (q71)* populations were generated through bleaching (68), and embryos acquired in this process were placed onto plates seeded with the bacterial strain that experimental crosses would be made on. These plates were then incubated at 25°C until nematodes reached the L4 stage. For each cross, four male nematodes and one female nematode were placed onto a single plate seeded with a given bacterial strain. Nematodes were then allowed to proliferate for seven days, and then the number of nematodes on each plate was counted (Figure 1). All estimates of population growth on a given fig bacterial strain were paired

with controls where animals were reared on *E. coli* OP50. Failed crosses were those where either the L4 female died prior to reproduction or the number of nematodes at the end of one week did not exceed the initial amount (five nematodes: four males and one female). Contaminated crosses were those where bacteria with different morphology than the one the agar plate was initially seeded with was observed, and thus was excluded.

Individual fecundity and reproductive duration

Synchronized *C. inopinata* NKZ35 and *C. elegans fog-2 (q71)* embryos were generated through bleaching (68) and were raised on *Klebsiella sp.* WOUb2. or *E. coli* OP50 at 25°C. Due to differences in developmental timing between *C. inopinata* and *C. elegans* (24,26), the *C. elegans fog-2 (q71)* populations were generated two days after the *C. inopinata* NKZ35 treatments. This allowed us to conduct the following experiment for all treatments at the same time (as the nematodes reached the L4 stage the same time despite their differences in growth rate). When animals reached the L4 stage, crosses were performed to determine individual female fecundity (with one female and four males per plate). Every day, parents were transferred to a new plate, and the number of embryos and larvae generated on the previous plate were counted (Figure 2). For animals moved to a new plate, fresh males were also added (if needed) to maintain a consistent male:female ratio of 4:1. Parents were moved (and progeny counted) until no progeny were generated for three consecutive days (or the female died). The span from when reproduction began to when reproduction ended for a given individual female was recorded as the reproductive duration (Figure 5).

Developmental timing and viability

Nematode populations (reared on either *Klebsiella sp.* WOUb2. or *E. coli* OP50) were synchronized by allowing gravid *C. inopinata* NKZ35 and *C. elegans fog-2 (q71)* females to lay embryos for six hours on the same respective bacterial strain. Females were then removed, and plates were reared at 20°C for *C. elegans fog-2 (q71)* and 25°C for *C. inopinata* NKZ35. Then, every day, the number of animals at each of four developmental stages (embryo, L1-L3 larva, L4 larva, and adult) were counted. Plates were counted until the number of adults did not increase for three consecutive days. The median time to reach every developmental milestone (L1-L3 larva, L4 stage, and adult stage) was inferred as in (26). Briefly, animals were coded as having reached or not reached a given milestone (for every day a given plate was measured). For each plate, a logistic model fit was generated (with the formula (milestone status ~ time)), and the time at which half of the nematodes were predicted to reach the milestone was noted (the median time to adulthood is reported in Figure 3). In addition to this, because the number of starting embryos and final number of adults was known for each plate, viability for each experimental treatment could be determined. Viability was measured as the fraction of embryos surviving to adulthood (Figure 4).

Body size

Using our developmental timing data (Figure 3), we synchronized populations of *C. elegans fog-2 (q71)* and *C. inopinata* NKZ35 nematodes reared on *Klebsiella sp.* WOUb2. or *E. coli* OP50 so that they would reach the young adult life stage at approximately the same time. Then, using 3% agarose in DI water as a mounting agent, we constructed microscope slides to take photographs of adult male and female nematodes from all experimental groups. Next, we pipetted 20 microliters of 0.35 mM Levamisole and placed it onto the 3% agarose mount; this is a paralytic

agent. And, we placed nematodes from each sex-bacteria-nematode species treatment group onto distinct slides in the freshly pipetted Levamisole for each group. Using an Axioscope5 (Zeiss), we took photographs of a control slide with defined lengths using various optical lenses using Zen software (69), and then we took photographs of nematodes from each experimental group. Finally, using Fiji (70), we measured the lengths and widths of all nematodes in each experimental group after defining how many pixels corresponded to a given amount of microns. Lengths and widths for each treatment group were plotted (Figure 6 and Supplementary Figure 4).

Bacterial foraging choice

Peptone-free NGM plates were seeded with three different bacterial isolates (*Klebsiella sp.* WOUb2, *E. coli* OP50, and *Stenotrophomonas sp.* WOUb1; two bacterial strains per plate in three possible combinations) placed equidistant from the center of the plate on opposite sides. Each lawn was seeded with 30 microliters of bacteria grown to an optical density of 0.6 at 600nm. Seeding locations were marked using a 3D-printed template that was placed on the underside of the plate, and the center of the plates were also marked using the same template to give an indication of where to place the embryos used in the experiment. Nematodes were reared on *Microbacterium sp.* WOUb43 to remove choice bias, and then we ascertained embryos after bleaching populations of *C. elegans fog-2 (q71)* and *C. inopinata* NKZ35 nematodes; these embryos were placed onto the previously marked centers of choice plates. After ten hours, the number of nematodes in each lawn (and not in a lawn at all) were counted, and the fraction of nematodes in each bacterial environment for each treatment combination was plotted (Figure 7).

Embryo-laying

Peptone-free NGM plates were seeded with 50 microliters of either *Klebsiella sp.* WOUb2 or *E. coli* OP50. Each seeding location was marked using a 3D-printed template that was placed on the underside of the plate, and the center of the plates were also marked using the same template to give an indication of where to place the gravid nematodes used in the experiment. We synchronized populations of *C. elegans fog-2 (q71)* and *C. inopinata* NKZ35 nematodes, and both prior-to and after synchronization, we reared them on the bacterium they would eventually be placed on. Once gravid females were observed for both species of nematode reared on either *Klebsiella sp.* WOUb2 and *E. coli* OP50, we took individual gravid females and placed them onto plates seeded with the same bacteria they were reared on. After six hours, we removed the female nematodes and counted how many embryos were laid both on and off the bacteria lawn. The fraction of total embryos laid on the lawn was plotted for each species-bacteria combination (Figure 8 and Supplementary Figure 6). Because some nematodes inevitably laid low numbers of embryos, we filtered out data where female nematodes laid less than ten embryos (Figure 8; a figure showing all data can be found in the supplemental materials; Supplemental Figure 6).

Statistics and R Packages

All analyses performed and plots created were done using R Statistical Software (v4.2.1; R Core Team 2022). The R package tidyverse (71) is an umbrella package that contains *ggplot2* (72), *tidyr* (73), and *dplyr* (74); these packages were used to work with and plot data obtained in the life history experiments outlined above. Additionally, *cowplot* (75) was used for the theme of all the figures, and *ggforce* (76) was used to create sina plots within *ggplot2* using the argument *geom_sina()*. The package *reshape2* (77) was used to convert data between wide and long formats using the *melt()* function. The base R function *aov()* was used to perform analysis of variance

analyses (ANOVA). The base R function *TukeyHSD()* was used to perform post-hoc analyses on the ANOVA results by measuring all pairwise differences among sample means for significance while controlling for the probability of Type I errors. Effect sizes were calculated using the *cohensD()* function from the package lsr (78).The package lmtest (79) was used to perform a liklelihood ratio test for our linear models constructed using our body size data with the function *lrtest()*. All data and code have been deposited on Github (https://github.com/austincolelink/Austin-Link-Thesis).

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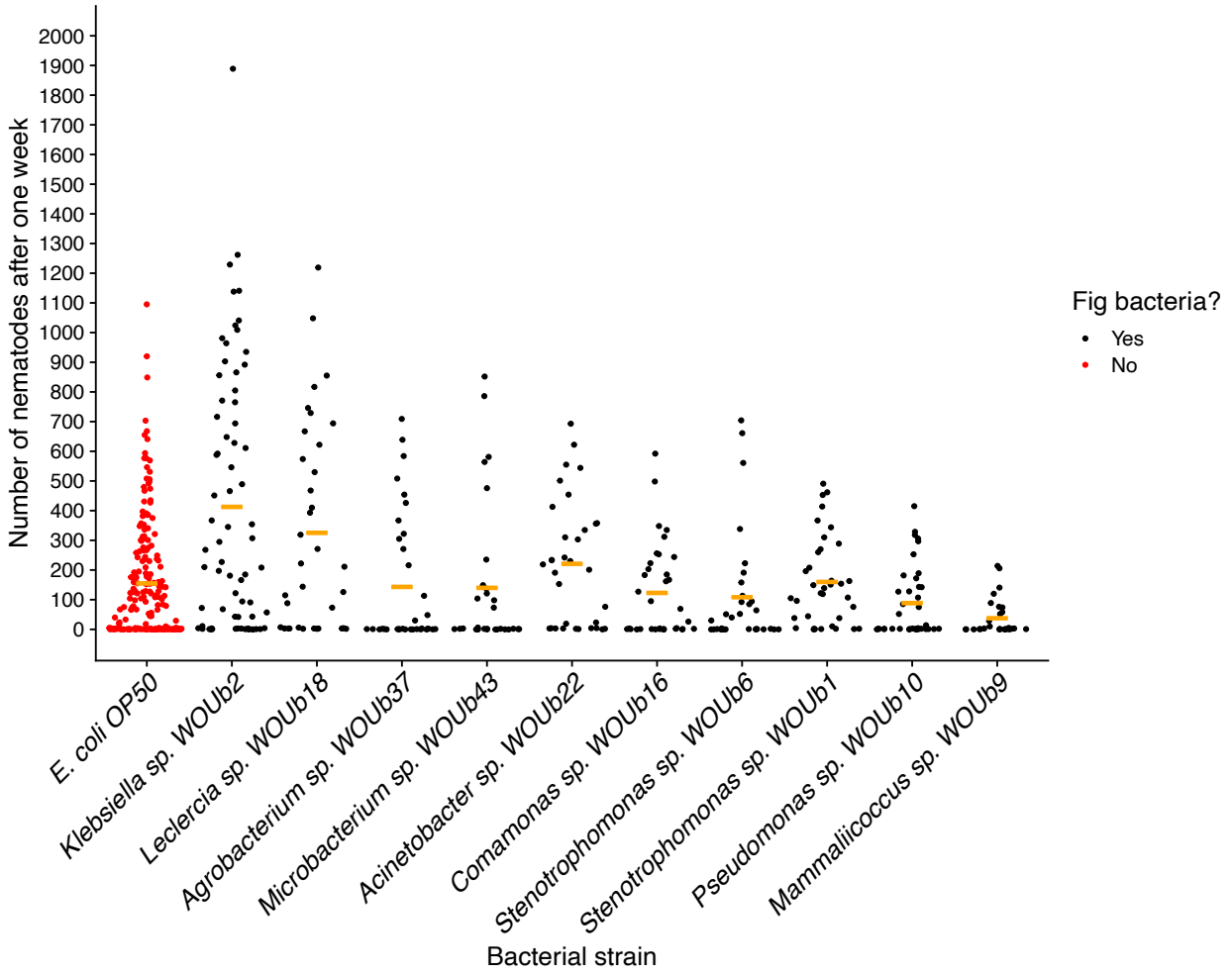
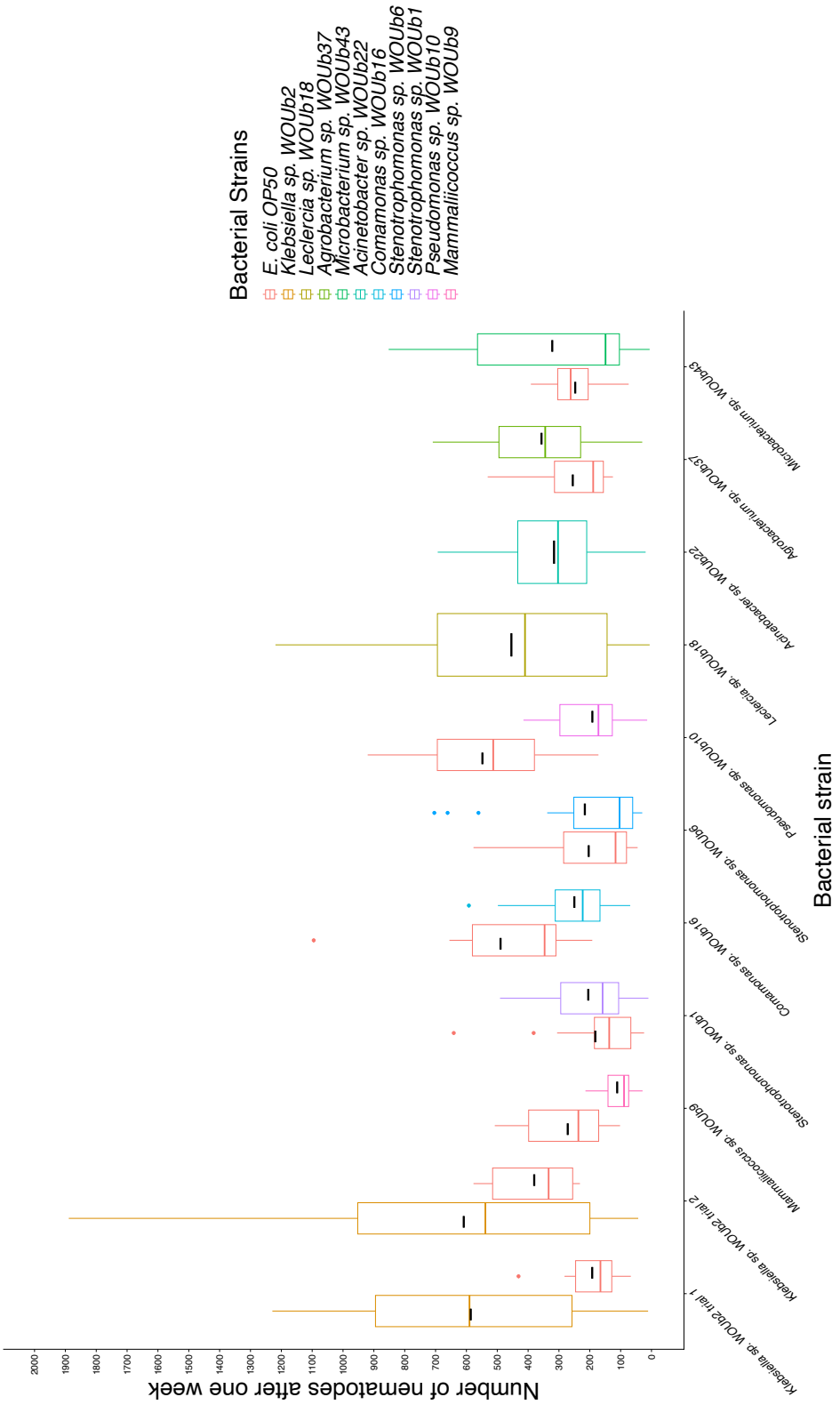
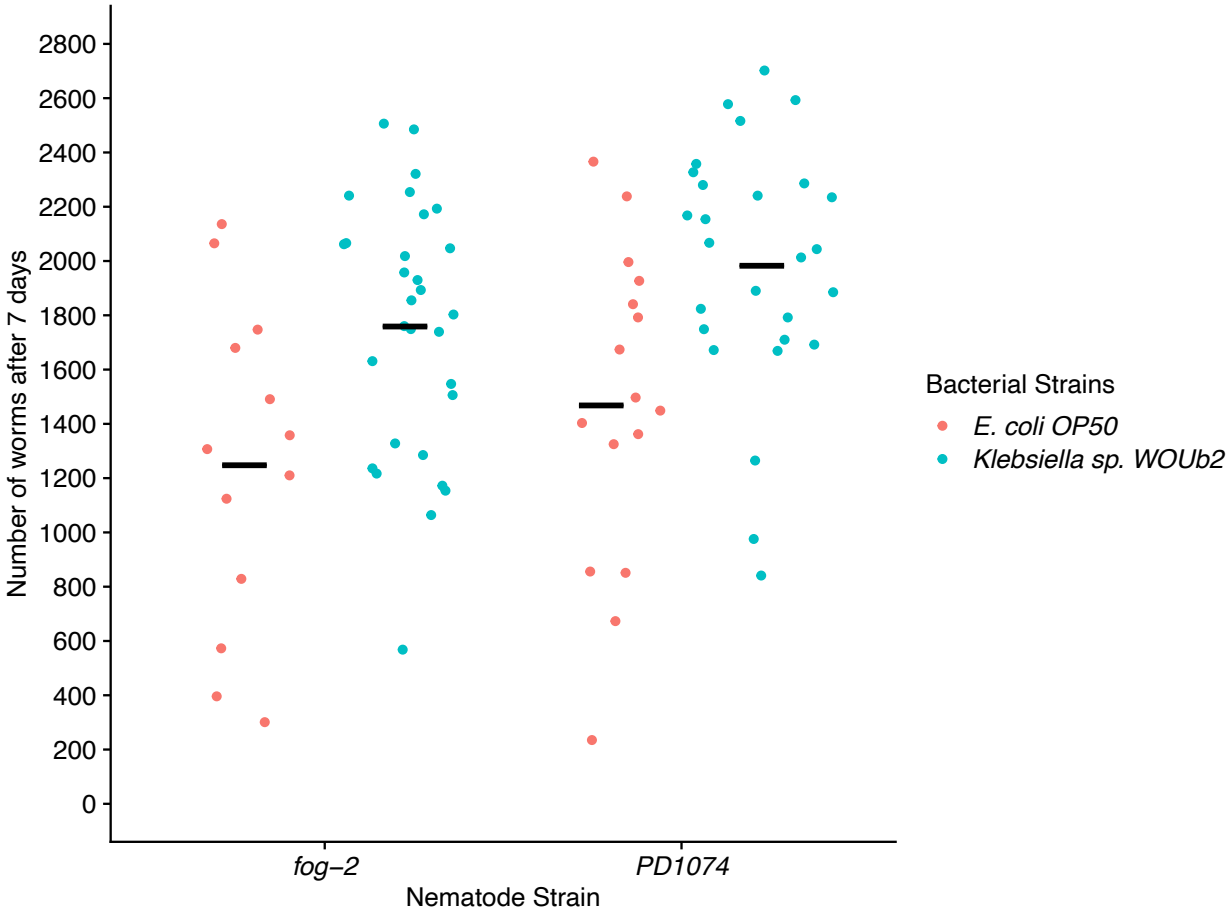
Supplemental Figures:



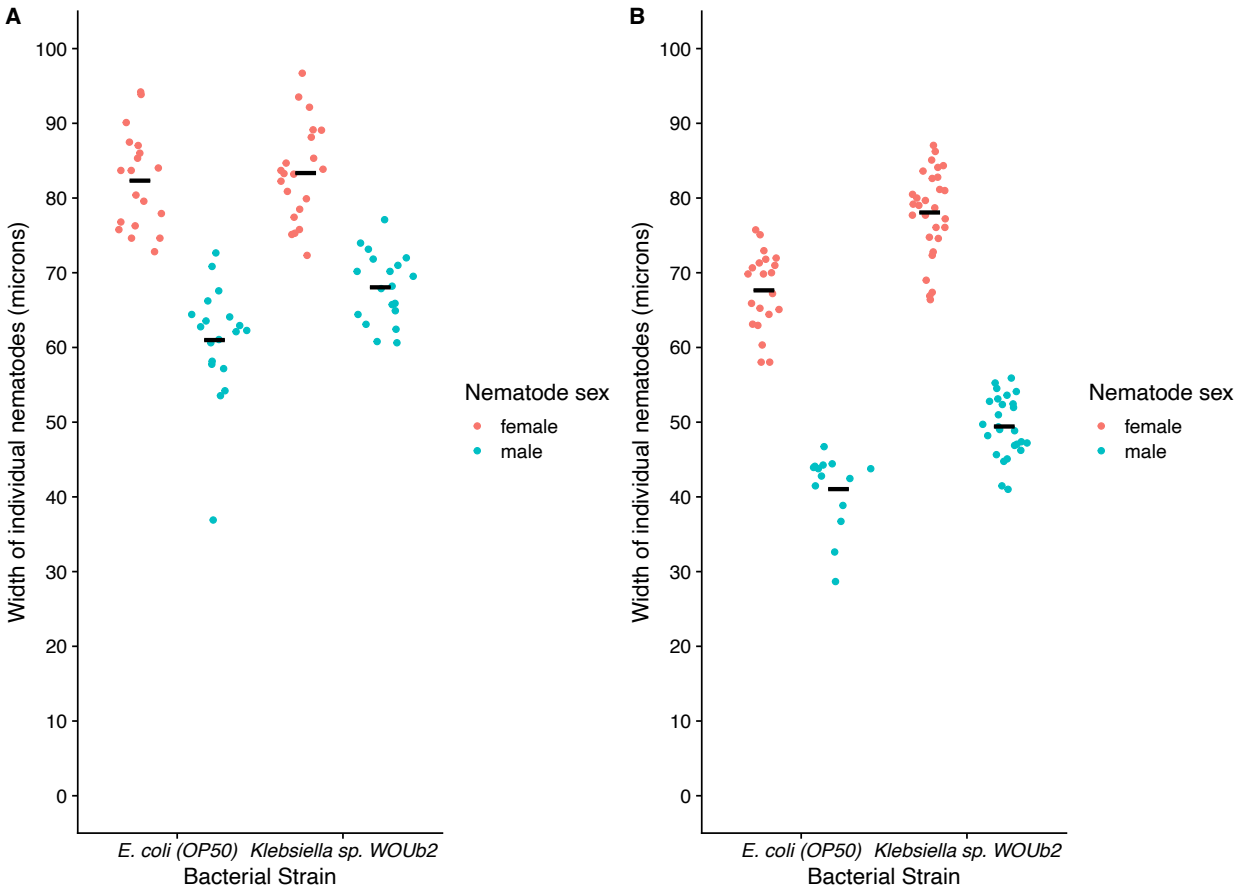
Supplementary Fig. 1: ***C. inopinata*** **harbors variable population growth when reared on bacteria from the fig environment (failed and contaminated crosses included).** Each datapoint represents the number of nematodes on a single plate at the end of the seven-day period for a given bacterial strain. Crossbars represent means. Sina plots are strip plots that take the contours of a violin plot. *E. coli* OP50 was used as a control during each set of fig bacteria crosses; consequently, the *E. coli* OP50 data in this figure is an amalgamation of all the controls for each experiment (a figure showing each fig bacteria-control pairing can be found in the supplemental materials; Supplemental Figure 2). Additionally, for *Klebsiella* sp. WOUb2, the data in this figure is representative of two experiments. A second set of crosses was done for this bacterial isolate to confirm the significant results from the first set (Supplemental Figure 2).



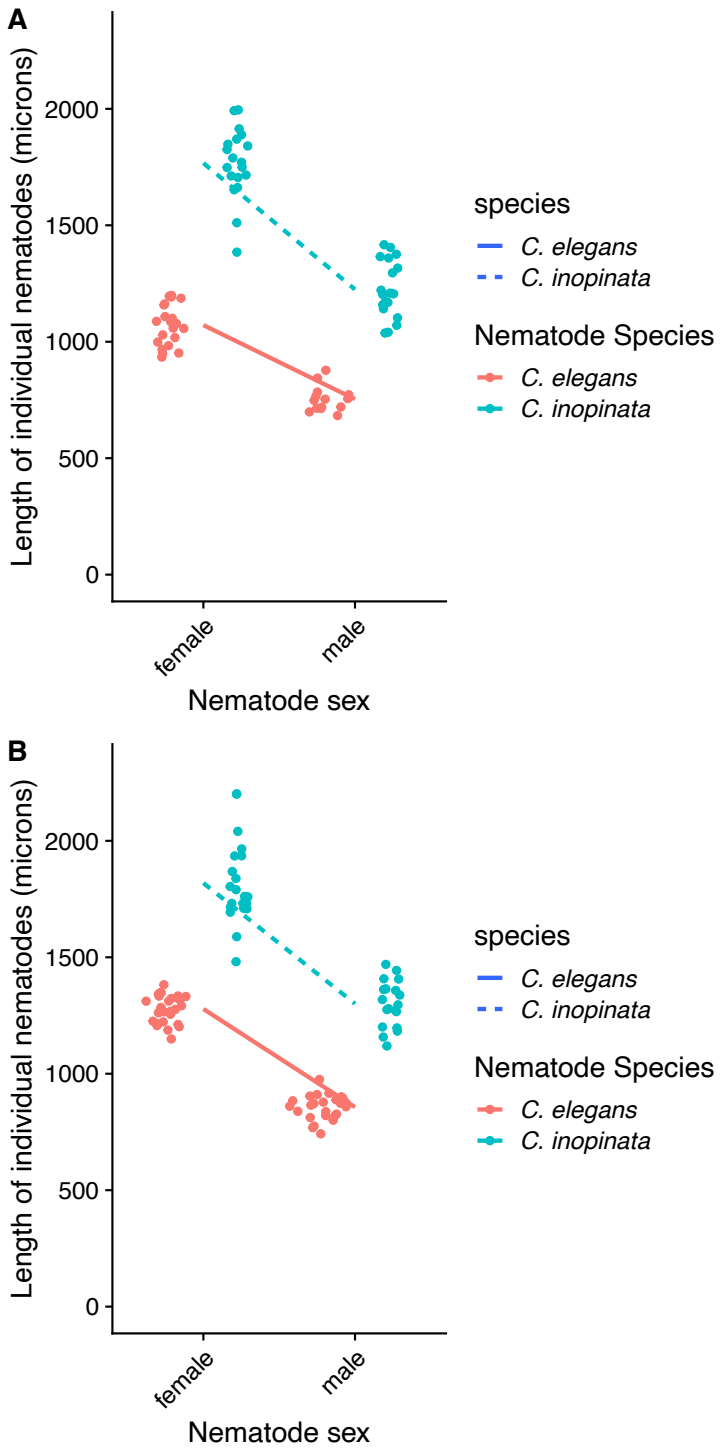
Supplementary Fig. 2: ***E. coli* OP50 – fig bacteria pairings from our population growth experiment (Figure 1).** Failed and contaminated crosses were excluded (a figure including failed and contaminated crosses can be found in the supplemental materials; Supplemental Figure 1).



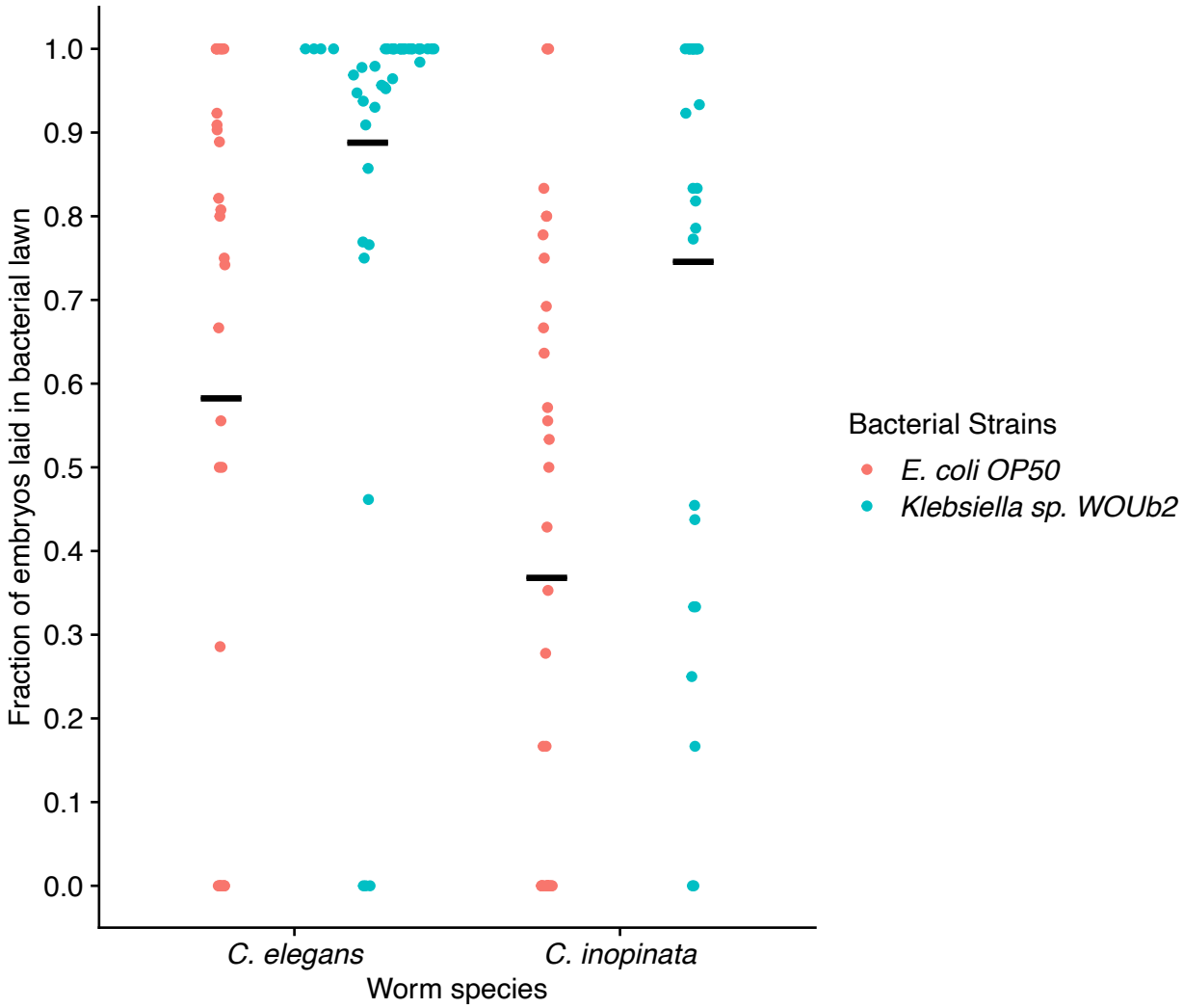
Supplementary Fig.3: ***C.* elegans, like *C. inopinata*, shows increased population growth reared on *Klebsiella sp.* WOUb2 compared to *E. coli* OP50.** Each datapoint represents the number of nematodes on a single plate at the end of the seven-day period for a given bacterial strain. Crossbars represent means. Sina plots are strip plots that take the contours of a violin plot.



Supplementary Fig. 4: ***Klebsiella* sp. WOUb2 impacts the width of *C. elegans* compared to *E. coli* OP50.** Each datapoint represents the width of an individual nematode in a given treatment that was mounted on an agarose slide and measured in Fiji. A) Comparing the width of *C. inopinata* reared on *E. coli* OP50 and *Klebsiella sp.* WOUb2. B) Comparing the width of *C. elegans* reared on *E. coli* OP50 and *Klebsiella sp.* WOUb2. Crossbars represent means. Sina plots are strip plots that take the contours of a violin plot.



Supplementary Fig. 5: **Comparing the slopes of female to male nematode lengths in *Klebsiella sp.* WOUb2 and *E. coli* OP50.** Each datapoint represents the length of an individual nematode of a given sex. A) *E. coli* OP50 comparison. B) *Klebsiella sp.* WOUb2 comparison.



Supplementary Fig. 6: **Both nematode species preferentially lay their embryos on *Klebsiella sp.* WOUb2 compared to *E. coli* OP50 considering low amounts of embryos laid.** Each datapoint represents the fraction of total embryos laid in a given bacterial environment for a given nematode species by a single gravid female.