ISOLATING AND CHARACTERIZING THE *C.* sp. 34 DAUER LARVA

by

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Understanding the evolution of host seeking behavior is crucial to conceptualizing the evolutionary rise of parasitism and for the formulation of future treatments. Over a billion people are infected by parasitic nematodes, worms, globally. Amazingly, parasitic and non-parasitic nematodes have nearly identical pathways for developing the stress-resistant, dauer or infective, dispersal stages of their life cycles. One of the key divergent traits seen between infective and free-living is host specificity. The model organism *Caenorhabditis elegans* is promiscuous in it choice of transportation vector. *Caenorhabditis* species 34, a close relative of *C. elegans*, may represent a divergent phenotypic group of free-living worms with only one animal transportation vector. Using SDS to isolate dauers, *C. elegans* and *C. briggsae* were found to have higher dauer formation than *C.* sp. 34. When SDS was performed on 29 wild isolates of *C.* sp. 34, dauer frequency was again found to be lower than the other species, but variation in propensity to make dauers was seen in *C.* sp. 34. The *C.* sp. 34

dauer is significantly smaller than any other dauer measured and lacks the typical radial constriction found in dauers. However, it retains the classic dauer characteristic of pharyngeal constriction. Its unique dauer morphotype may be explained by the small size of its symbiotic partner, *Ceratosolen*. Additionally, conventional RNAi techniques using feeding to target dauer arrest genes failed to produce high amounts of dauers, but controls question the interoperability of the results. Fig plates were also generated as an alternative method to produce more dauers. Strangely, fig plates promoted nematode sterility and death. The unique dauer seen in *C.* sp. 34 may be evidence that the specificity of this species to its symbiont has played a large role in causing the divergent traits observed and open a promising door to possibly uncovering more about the evolution of host-specificity with future research.

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I would like to thank Patrick Phillips Ph.D., Gavin Woodruff Ph.D., and the entire Phillips Lab for their help and support in this endeavor. Prof. Phillips, thank you for giving me the opportunity to be a part of your lab and assisting me through the thesis process. Gavin, thank you for all the help, encouragement, and time. I am truly grateful for your patience and all the support you have given me. I would also like to thank my family and friends for their endless support in all my endeavors, especially my parents. And thank you Mr. Wagner for helping foster my passion for biology. I would also like to acknowledge the hundreds of thousands of worms that gave their lives for science.

Table of Contents

List of Figures

Introduction:

Parasitic Nematode Epidemiology

Parasitic nematodes pose one of the most daunting challenges to medical research. New-World and Old-World Hookworms and Whipworms have infected over one billion people (*Necator americanus* and *Ancylostoma duodenale,* and *Trichuris trichiura*). Just these three species alone are responsible for infecting 1/7 of the entire human population. (Hotez et al., 2007; de Silva et al., 2003; Else and Finkelman, 1998). People who are most affected by these diseases are the 2.7 billion who live on less than $2 a day (Hotez et al., 2005). Unfortunately, research in parasitic nematodes is greatly inhibited by the inability of many parasitic worms to be grown in laboratory cultures. Parasitic species of humans are obligate parasites, with one notable exception, and cannot be cultured or tested *in vitro* at specific life stages (Grant et al., 2006). *Strongyloides stercoralis* has both a free-living and parasitic life cycle, but this species alone is just one of a phylum filled with parasites. Nematode infections and transmitted diseases are known to cause defective growth, cognitive and learning impairment, anemia, and adverse maternal-fetal outcomes in pregnant women, all of which put a great burden on societies heavily affected by these parasites (Hotez et al., 2005).

Human Infection by Nematode Parasites

Infection can occur through fecal-oral transmission where the eggs and larvae of the parasite are deposited by a previous host's excretion. It is ingested either by contaminated food or water (Dubey, 2009). In a study investigating the prevalence of helminths in food vendors, 63.5% of vendors in Abeokuto, Nigeria were found to have

transmittable helminths even after being dewormed in the last four years (Iduwo and Rowland, 2006). There are also additional methods that parasites can use to infect humans. Transdermal infection occurs when worms excreted by a previous host penetrate the skin, generally feet, of a new host (Hotez et al., 2005). This is the strategy of the hookworm, *Ancylostoma duodenale*. Parasitic worms can also be transferred by vectors, like mosquitos or flies, and/or transferred from livestock to humans (Kambris et al., 2009). This strategy is seen in multiple species of worms that infect humans: *Wuchereria bancrofti* (elephantiasis), *Strongyloides stercoralis* (enterobiasis), and *Onchocerca volvulus* (river blindness). In all cases of infection, worms must develop into an infective stage in order to parasitize a host animal (Hotez et al., 2004). The infective stage in parasites facilitate their need to find new hosts by allowing them to be longer lived and resistant to stressors found outside the host's body. In the parasite life cycle this is the third larval stage (L3) (Viney and Crook, 2005). Understanding how the infective stage of parasitic species evolve is important, not only for adding to our general knowledge of ecological and evolutionary processes, but also for potentially finding ways to prevent infective stage parasites from invading humans in the future.

Parasitism Evolution

Parasitism has evolved from free-living ancestors multiple times independently in nematodes (Blaxter et al. 1998). The evolution of parasitism requires many pre-adaptations and independent changes for an organism to transition to parasitic from free-living (Dieterich and Sommer, 2009). This theory states that parasitism is a progression that goes from free-living (unassociated with symbionts), to phoretic (symbiotic transportation without parasitism), to necromenic (consuming the symbiotic

transport when it dies), to parasitic (taking nutrients from the living symbiont) that is facilitated by pre-adaptations (Dieterich and Sommer, 2009). Many of these pre-adaptations are present in free-living/phoretic nematodes. Their slender bodies and stress-resistant stages are conserved across the phylum despite tremendous genetic divergence (Kiontke and Fitch, 2005). Along with the conservation of this stress-resistant state, parasitism has evolved independently at least fifteen times in nematodes (Blaxter and Koutsovoulos, 2014). The frequency of the evolution of parasitism in nematodes indicates that species of nematode may easily transition from free-living to parasitism because of the conserved pre-adaptations. The high amount of conservation of these traits seen in both parasitic and free-living species provides us the opportunity to study free-living organisms and draw comparisons to their parasitic relatives. One of the most glaring instances of homology between parasites and free-living worms is the conservation of a stress-resistant L3 stage (Crook, 2014).

Infective Stage (L3) in Parasites

A parasitic worm must move from one host to another to complete its life cycle. The dispersal stage in a parasite's life cycle is called infective or filariform (for this thesis the stress-resistant L3 parasite stage will be referred to as infective). This stage is critical for host-seeking and surviving the harsh external environment (Rogers and Sommerville, 1963; Ellenby, 1968). Morphological changes common to parasitic infective L3 stage worms are a smaller buccal cavity and increases in the thickness of the cuticle or, like *Ancylostoma duodenale*, formation of a sheath in the infective stage using a previous molt's cuticle (Albarqi et al., 2016; Lee, 2002). These changes aid in surviving outside a host. In addition to those morphological changes, parasitic infective

stage worms slow down their metabolism to increase their lifespan and chances of finding a new host (Fernando, 1963). These infective worms of all types also have an extreme specificity to their host and especially to the microhabitat inside the host (Adamson and Caira, 1994). In order to understand the evolution of the infective L3 stage parasite comparison must be made between parasites and free-living species. Much of the previous work on the evolution of parasitism in nematodes has been done with the phoretic genus *Caenorhabditis*.

Dauer Stage (L3) in C. elegans

Genetic experimentation on *Caenorhabditis*, specifically *Caenorhabditis elegans,* has been conducted for over forty years (Frézal and Félix 2015). Like other model species, zebrafish (*Danio rerio*) or fruit flies (*Drosophila melanogaster*), *C. elegans* has a large brood size, relatively short lifespan, and is easily cultured in a laboratory setting (Corsi et al., 2015). Nematode research has facilitated Noble Prize-winning breakthroughs in understanding cell death, implementation of GFP, and the discovery of RNAi (Brenner 2003, Horvitz, 2003; Sulston 2003; Chalfie, 2009; Mello, 2007; Fire, 2007). Currently, nematode research is trying to uncover answers to many other evolutionarily-relevant, bio-medical, and gene mechanistic questions. Despite being the first multicellular organism to have its genome sequenced and forty years of genomic and molecular research, little is known about general *Caenorhabditis* ecology (Frézal and Félix, 2015; Fielenbach and Antebi, 2008). However, recent studies have uncovered more about its habitat and natural history. *C. elegans* is found on rotting plant material where they compete for food with other nematodes like *Pristionchus, Oscheius,* and *Caenorhabditis briggsae,* (Félix and Duveau, 2012). It exists in a boom

and bust life cycle where rapid proliferation on new plant material is met later by famine and a need for dispersal (Frézal and Félix, 2015). *C. elegans'* homologous life stage to the infective L3, the dauer L3, makes it an excellent candidate for research on the evolution of parasites.

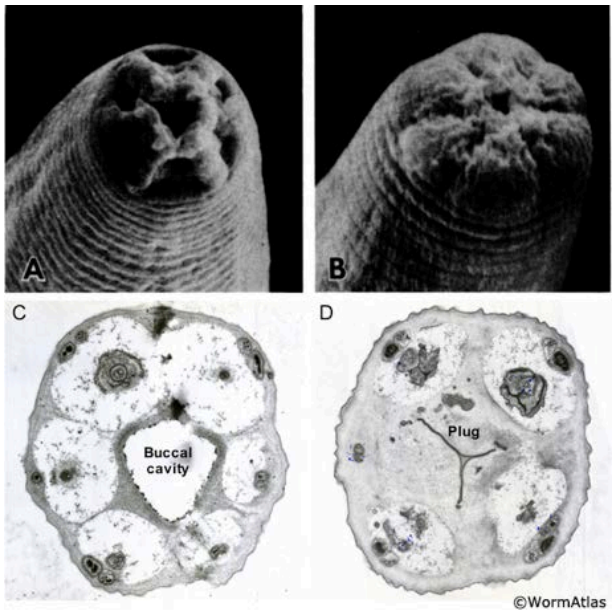


Figure 1: Reproductive L3 *C. elegans* and dauer larva L3 under electron scanning microscope. (Wormatlas.org, Originally: Riddle, 1998).

In this image, an electron scanning microscope captures the morphological differences between dauer and non-dauer *C. elegans*. The non-dauer (1A) does not have the buccal plug covering its mouth and thus is able to ingest bacteria. The dauer (1B), does have the buccal plug present and therefore is unable to ingest bacteria. 1C and 1D show a cross section of a non-dauer (1C) and dauer (1D). In image 1D, the mouth of the worm is obstructed by the plug.

In *C. elegans,* as the amount of food decreases and dauer pheromone increases, the population changes and a higher concentration of dauers is observed (Fielenbach and Antebi, 2008, Viney et al., 2003). This makes sense in the light of their boom and bust life cycle where the diapause-like state of dauer can be used in times of famine. For phoretic nematodes like *C. elegans* to find a transportation vector they must enter the

dauer L3 stage to disperse from the overpopulated and starved current location (Yoshiga et al., 2012). The dauer is a long lived, non-reproducing life stage that facilitates colonization and travel by making nematodes more stress resistant not unlike the parasitic infective stage (Hu, 2007). Dauers change from aerobic respiration to fermenting reserved fats to facilitate longer lifespans (Fielenbach and Antebi, 2008). Physiological changes in the dauer stage have been shown to create greater resistance to food, heat, and oxidative stressors (Larsen, 1993; Lithgow et al., 1995; Riddle and Albert, 1997).

C. elegans dauers are also morphologically different from their non-dauer counterparts (see Figure 2). They develop a buccal plug (see Figure 1) that covers the mouth opening to help prevent desiccation (Fielenbach and Antebi, 2008; Hu, 2007). The buccal plug also prevents the dauers from taking in any more nutrients, making this stage analogous to a semi-active hibernation. A semi-active hibernation refers to the worm's ability to lower its metabolic rate and no longer consume nutrients while still being able to move when provoked or sensing a transportation vector (Narbonne and Roy, 2009). In addition to the buccal plug, other morphological features like an increase in radial constriction (increase length to width ratio) are seen in dauers making them slimmer and elongated compared to normal non-dauer L3 (Riddle, 1988, Hu 2007). Additional morphological changes include: the elongation of the pharynx, the thickening of the cuticle, and a unique patterning of alae appear on the outside of the cuticle (visually they appear as racing stripes, but are a feature of the cuticle superstructure). These morphological changes found in dauers are detectable through

assays like general compound microscopy, electron microscopy, and fluorescence microscopy.

Free-living dauer larvae also perform a behavior called nictation. Nictation is defined as worms standing upright on their tails (Wolkow and Hall, 2011). As a part of this behavior it has also been shown that dauers respond to gravitational stimuli and

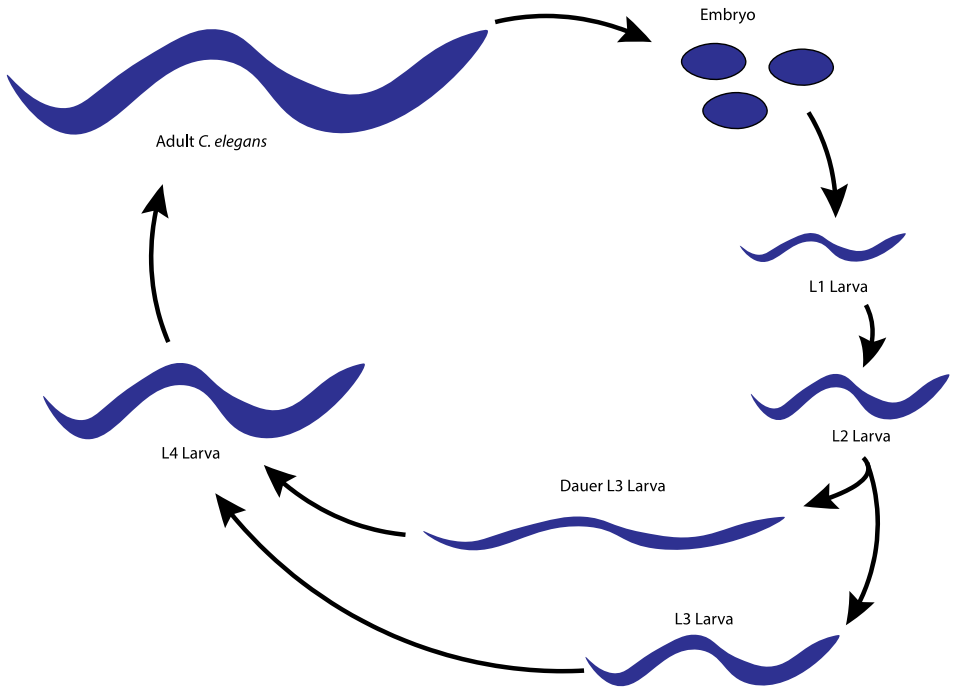


Figure 2: Life Cycle of *C. elegans* (Inspired by Wormatlas.org)

After L1 *C. elegans* either continues to live as larvae and grow into L4 and then adult or if factors like crowding (pheromones), starvation, or high temperature occur then *C. elegans* may switch to the long lived, stress resistant dauer phase.

even work together to form nictating masses (Félix and Duveau, 2012). Dauers of *C. japonica* have been shown to have a bias for traveling upward on vertical plates (Okumura et al., 2013). These behaviors are thought to facilitate attachment of phoretic

worms to invertebrate carriers to take them to new locations (Frézal and Félix, 2015). Both nictation and gravitational stimuli have been seen in parasitic species showing another level of homology between parasitic species and phoretic species of *Caenorhabditis* (Hart, 2006).

However, it is key for this experiment to note that not all strains of *C. elegans* respond to the stimuli that produce dauers at the same rate. Tests have shown that between wild isolates and inbred lines of *C. elegans* there is a varying amount of response to the dauer pheromone and starvation (Vinney et al., 2003, Harvey et al., 2008). Some of these lines produce an expected amount of dauers while others have significantly lower or higher dauer production in response the same stimuli. These results indicated that natural history may play a significant role in the phenotypic plasticity of the dauer response. Quantitative trait loci (QTL) for dauer larva formation were detected on two *C. elegans* chromosomes (Harvey et al., 2008). The level of expression of genes associated with these loci may regulate the sensitivity of specific lines and species of *Caenorhabditis* to dauer triggering stimuli. The phenotypic plasticity seen in dauer initiation may also produce differences in species of *Caenorhabditis* that have different natural histories.

Evolution of Stress-Resistant L3

To test the question of if host-specific traits can be seen in culturable species along this phoretic-parasitic continuum, a comparison of the two most homologous life stages, L3, is most appropriate. Both parasitic worms and *C. elegans* use stress resistant stages to facilitate colonization of hosts or transportation vectors by increased longevity and resistance to stressors. In addition, dauers naturally occur during the third larval life

stage, seen in Figure 2, and go straight to L4 after leaving the dauer phase. It is in this L3 stage parasitic worms like *Strongyloides stercoralis* also choose between infective stage or normal L3 larva. This suggests that phoretic and parasitic stress-resistant L3 morphologies and behaviors are homologous.

Morphologically there are a lot of similarities between the parasitic infective and phoretic dauer life stages. Both have radial constriction that generates a slender and elongated body (Viney and Lok, 2007). However, parasites tend to be larger than *C. elegans* and other well studied *Caenorhabditis* dauers. Other parasitic species have unique morphological features including teeth-like structures (hookworms – *A. duodenale*) or spines and flanges (Blaxter and Koutsovoulos, 2014). Both types of worm also form a thicker cuticle, constricted pharynx, and generally a plug-like structure to help them withstand long exposure to environments outside of their niche. But the homology also extends further than just their morphologies; their genetics and behaviors also change to facilitate similar goals and indicate true homology between the infective and dauer stage.

Genetically there are also similarities seen in parasites more closely related to *Caenorhabditis*. In both *Strongyloides* and *C. elegans* an insulin signaling pathway is required for initiation of the stress-resistant phenotype (Massey et al., 2013; Hu 2007). Infective stage parasitic worms have shown to use isoforms of the insulin-like receptor DAF-2 for alternate and similar purposes. *daf*-2, a gene encoding the insulin-like receptor, regulates several pathways including arrest of the stress-resistant L3 stage (see Figure 3) in dauers of *C. elegans*. DAF-7 is a TGF- β ligand that activates the DAF-1/4

receptor complex to arrest the dauer stage (see Figure 4). In *C. elegans*, insulin-like receptors and TGF-β are utilized to regulate its endocrine system and go from one life

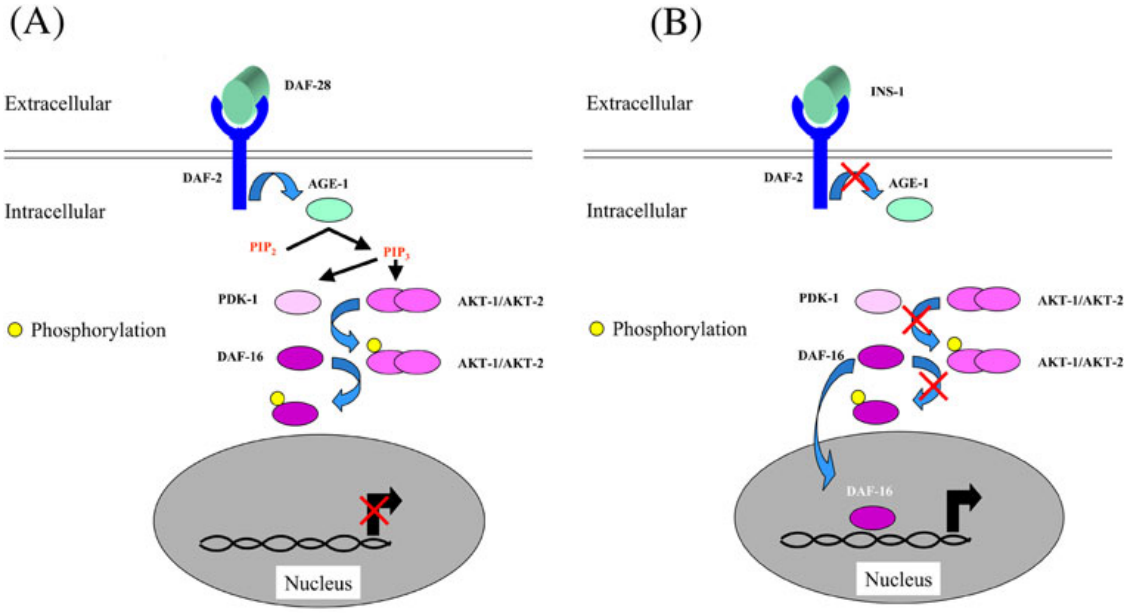


Figure 3: DAF-2 and AKT-1 and AKT-2 molecular pathway activated (A) and inhibited (B) (Ewbank, 2006)

Figure 3A shows the DAF-2 pathway; DAF-2 is stimulated by an insulin like growth factor which causes a intracellular signal cascade where PIP_3 binds to and activates AKT1/AKT-2 complex which in turn phosphorylates DAF-16 causing the transcription activator to be inactive and genes responsible for dauer to be repressed. In Figure 3B the same intracellular signal cascade is depicted, however both inhibition of the DAF-2 signal transduction and inhibition of the phosphorylation of DAF-16 (caused by inhibition of AKT-1/AKT-2) result in DAF-16 promoting dauer specific genes.

stage to the next including dauer L3 initiation or arrest (Ogawa et al., 2009). If the environmental conditions cue *C. elegans* to enter the dauer stage, DAF-2 is no longer able to inhibit the activation of dauer specific genes by DAF-16 through intracellular signaling cascades (McGehee et al., 2015). When these genes are inhibited by RNAi the

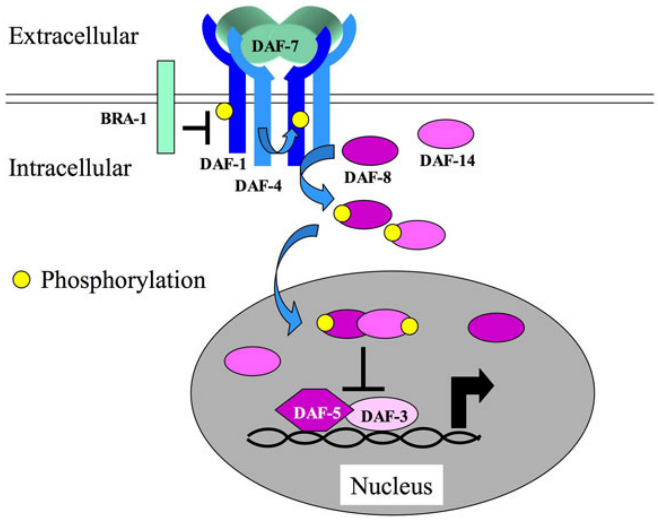


Figure 4: DAF-7 regulatory pathway (Savage-Dunn, 2015)

The DAF-7 TGF-β ligand binds to the DAF-1/DAF-4 complex which forms a tetramer to induce the intracellular signal cascade that leads to the inhibition of transcriptional activators that activate dauer specific genes. In RNAi mutants where DAF-7 is ablated the mRNA encoding the extracellular DAF-7 ligand are inhibited thus producing no DAF-7.

constitutive dauer phenotype is produced by a failure to exit the stress-resistant stage (Hu, 2007).

When investigating the parasite *S. stercoralis*, the DAF-2 protein homolog was found to be in two isoforms that had amino acid sequence changes that would be deleterious for *Caenorhabditis* (Massey et al., 2013). However, one of the isoforms was found to increase in concentration in the stages leading up to the infective stage (Massey et al., 2013). These isoforms may then serve similar purposes as the *Caenorhabditis* DAF-2. In addition, DAF-16 orthologues have also been identified in *A. caninum* and *S. stercoralis* (Geldmin et al., 2011; Massey et al., 2003). *A. caninum*'s *Ac*-DAF-16 protein has been indicated to be used similarly to DAF-16 in formation of the infective stage. When *Ac*-DAF-16 was inserted into *C. elegans daf-2*$^{-}$*/daf-16*$^{-}$ double

mutants, which are incapable of forming dauers, the dauer phenotype was rescued by inserting this parasitic gene into the free-living *C. elegans*. The insulin signaling proteins involved in regulating the stress-resistant stage are functionally conserved across these large evolutionary distances between free-living and parasitic worms.

However, there is one glaring divergent trait between dauer and infective stage worms. Both parasitic and free-living worms need to have a way to find their respective partner, but they have different requirements for specificity. Hallem et al. measured the response of BAG neurons, a neuron used in chemotaxis, of both *C. elegans* and parasitic species *S. carpocapsae* and *H. bacteriophora.* All relied on this type of neuron to detect and become attracted to CO_2 (Hallem et al., 2011). This indicates that *C. elegans* and the parasites have a general non-specific host seeking behavior. When the BAG neurons were ablated in *H. bacteriophora,* they failed to be attracted to specific host odors while non-ablated worms were attracted to them. In contrast, *C. elegans* was unresponsive to the pheromones of phoretic animal vectors as either adults or dauers when BAG neurons were ablated or left intact (Hallem et al., 2011). This shows that the BAG neuron function is conserved but divergent in parasites. Parasitic worms have evolved to become sensitive to specific host related cues while *C. elegans* specific phoretic partner associated molecules have yet to be discovered. Instead, much of the current research has found olfactory neurons of *C. elegans* to be associated with molecules pertaining to bacterial prey (Bargmann, 2006). It is unsurprising that parasitic infective worms need ways to recognize hosts at or almost at the species level because later obstacles in their life cycle like immune systems, host morphologies, and transmission methods, which are all dependent on the specific host of a single parasite

species. However, it would still be unsurprising if *C. elegans* does have many phoretic partner associated attractant behaviors as a way that *C. elegans* can get onto one of its many carriers as opposed to other organisms.

Current ecological research has found *C. elegans* to be extremely promiscuous in its choice of vector from one rotting fruit mass to another, choosing between isopods, univalves, and myriapods (Félix and Braendle, 2010). This lack of differentiation between phoretic vectors is common to many species of *Caenorhabditis* and forms a stark contrast between the well-known phoretic species and parasites (Jovelin et al., 2013). Being able to understand the infective stage's divergent traits from free-living dauers can help elucidate further breakthroughs with medical research and our general knowledge of evolutionary processes. The recently discovered *Caenorhabditis* species 34 is a species that fits all the criteria of culturability, phoretic-free-living, and, importantly, having a specific symbiotic partner.

Ecology of C. sp. 34

C. sp. 34 was recently discovered in Okinawa, Japan (see Figure 5) (Woodruff et al., 2016A). *C.* sp. 34 is a likely bacterivore that thrives in fig syconia of *Ficus septica*. Figs themselves have a well-established and researched symbiotic relationship with wasps. Inside the synconium are the fig's flowers which, like other flowers, require pollination by an animal pollinator. The fig depends on female pollinating wasps, *Ceratosolen*, to carry pollen from one fig to another. In return, the wasps use the syconium as a safe place for its offspring, and the developing progeny rely on the inflorescences for nutrients (Molbo et al., 2003).

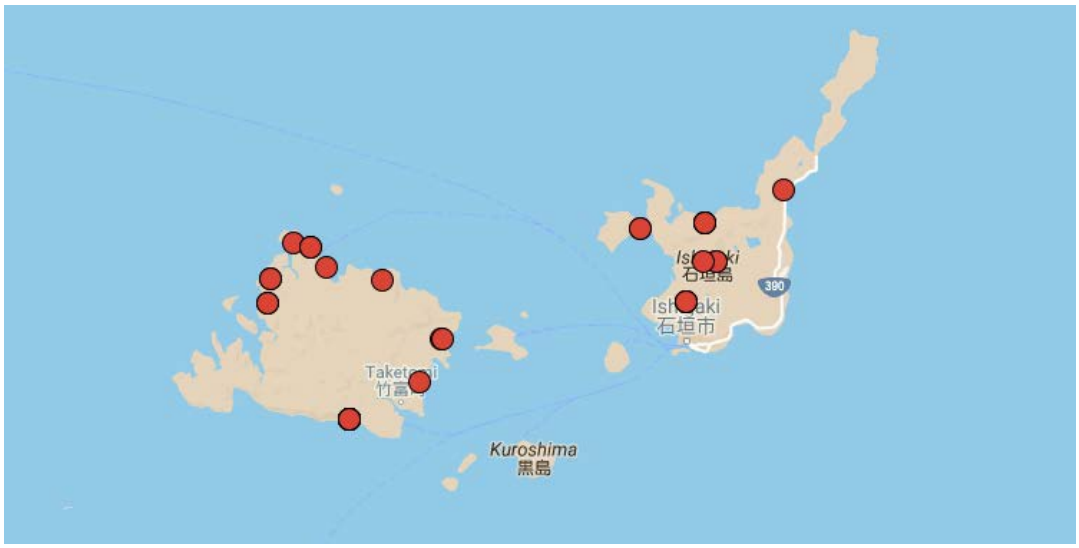
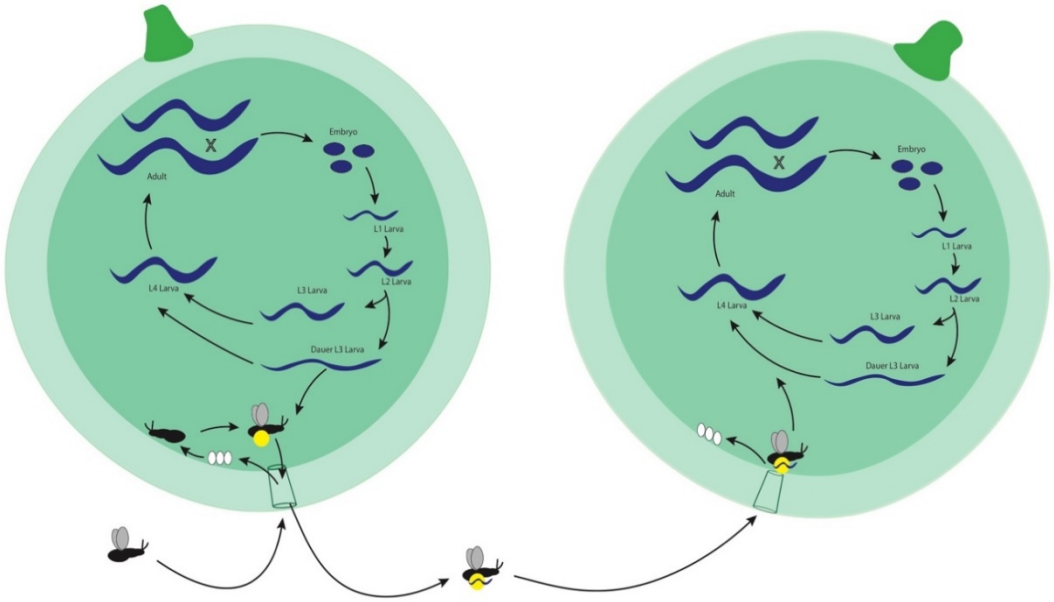


Figure 5: Islands of Okinawa were *C.* sp. 34 Wild Isolates Were Collected (Woodruff et al., 2016a)

The islands in the prefecture of Okinawa, Japan that were collected from include Yonaguni, Miyako, Iriomote, and Ishigaki. These islands are about 300 miles off of Taiwan and about 500 miles off of mainland China.



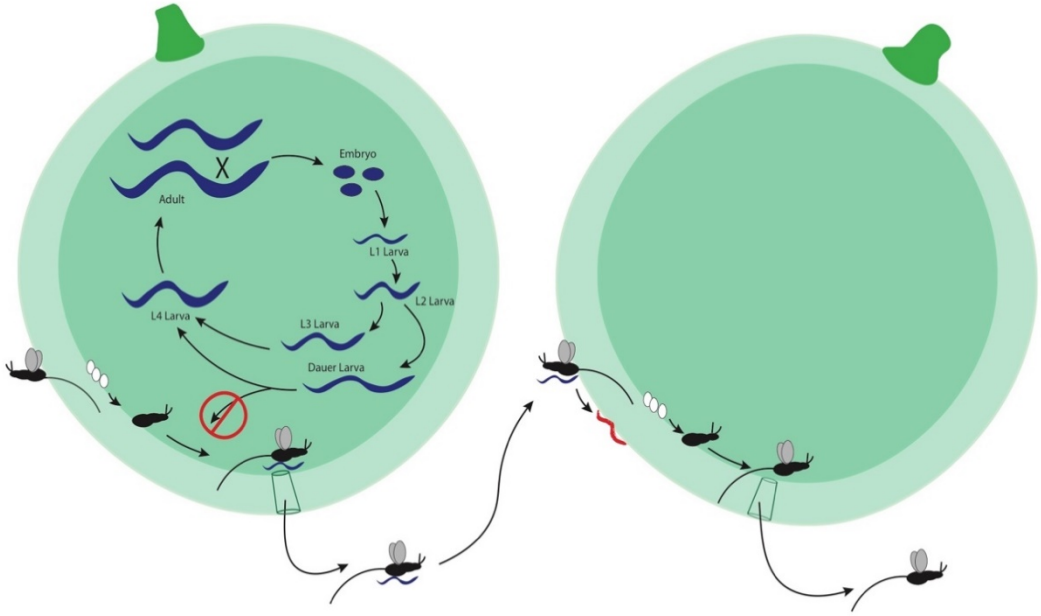


Figure 6: Life cycle of *C.* sp. 34

Above: The life cycle of *C.* sp 34 relies on the pollinating fig wasp to enter, lay eggs, and its offspring leave and return to another fig so allow the worms to complete their life cycle. Below: if a worm attaches to a parasitic fig wasp then it will no reenter a fig and thus die, producing an extremely negative selective pressure for choosing parasitic fig wasps.

As with most phoretic relationships, *C.* sp. 34 is thought to be a commensal partner of the pollinating fig wasp. It uses the tight interdependent relationship of the wasps and figs to its advantage to be transported from one fig to another with no confirmed positive or negative effects on the wasps or figs (Woodruff et al. 2016B). Once *C.* sp. 34 has located a host wasp that has matured inside the fig, it is theorized to attach itself for traveling. It then exits the stress resistant L3 stage to colonize the new fig habitat once it has arrived. This progression has been previously recorded in non-*Caenorhabditis* fig-associated nematodes (Giblin-Davis et al., 1995).

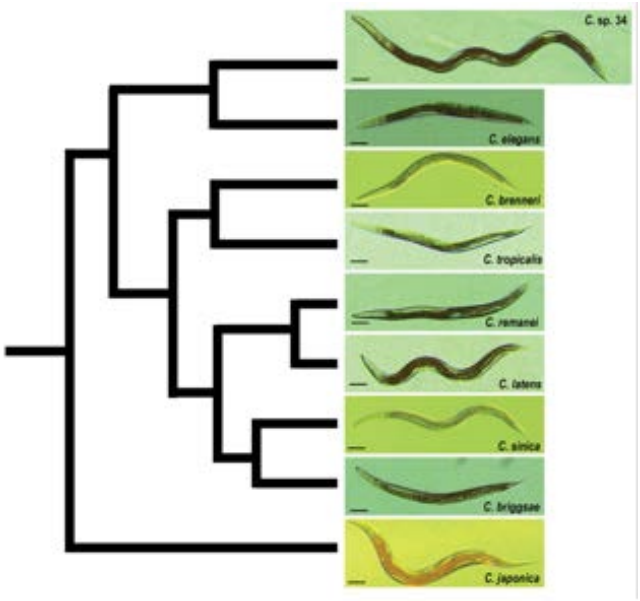


Figure 7: Phylogeny and Morphology Comparison of Caenorhabditis. (Woodruff et al., 2016a)

In this image *C.* sp. 34 has a clear morphological differences in size compared to other species of Caenorhabditis including its most related species, *C. elegans*.

There are also cheaters in this symbiotic fig and wasp system that *C.* sp. 34 must avoid. Non-pollinating (parasitic) wasps use an elongated ovipositor to deposit their offspring from the outside into figs without pollinating the internal flowers (Dunn et al., 2008). *C.* sp. 34 and these types of wasps are thought to have no relationship because the negative selective pressure of attaching oneself to the parasitic fig wasp is too great. If it did, the worm would completely erase its chances of reproducing because the parasitic fig wasp does not return to the inside of a synconium of another fig (see Figure 6). As parasitic fig wasps do not enter the fig, there is likely selection for host discrimination behavior in *C.* sp. 34 (see Figure 6). *C.* sp. 34 also must discriminate between pollinating fig wasp sexes. Only female pollinating fig wasps leave the fig and travel to a new fig to lay their eggs. The males stay at the fig where they developed and

inseminate the females before the females leave and the males die inside the original fig. The natural context of *C.* sp. 34 makes it an ideal candidate for studying the evolution of host-specificity.

Other species of *Caenorhabditis* have been observed to exhibit phoretic host-specificity, such as *C. japonica* (Okumura et al., 2012). *C. japonica* has shown to use specific host-seeking behaviors, but does not parasitize its symbiont, the shield bug, *P. japonensis*. Chemotaxis assays of *C. japonica* show a close association and choice to use *P. japonesis* as a phoretic vector over other species of shield bug (Okumura et al., 2012). These associations may also represent other members of this group of highly specific phoretic worms. However, *C. japonica* is not as closely related to the well-studied *C. elegans* as *C.* sp. 34 is (see Figure 7). Being within close phylogenic proximity to *C. elegans* allows for a more direct comparison between the two species and gives more power to the findings of this thesis. While the relationship between *P. japonesis* and *C. japonica* also represents a unique host-specific phoretic relationship, its phylogenic divergence lessens the impact of any differences seen in the stress-resistant stage. Because *C. japonica* and *C. elegans* are more distant a divergent trait is likely due to mutations over time than their separated natural histories. Instead, *C.* sp. 34. being very closely related to *C. elegans* lessens the chance of divergent traits being caused by random mutations.

Here, I intend to investigate and characterize the biology of the *C.* sp. 34 stress-resistant larvae dauer L3 to elucidate any divergent characteristics and to provide a potential model for studying the rise of host-specificity and the evolution of parasitism. By characterizing the dauer of *C.* sp. 34, the questions regarding the effect of natural

history on the stress-resistant stage can hopefully be investigated in this thesis and further using *C.* sp. 34. However, from observing *C.* sp. 34 starved plates it does not appear naturally to make L3 stress resistant dauer larvae in laboratory settings. To overcome this obstacle three methods, novel fig fruit infused media, RNAi, and natural variation (assayed by SDS) were used to induce or isolate the dauer phenotype. If one of these methods can generate dauers and allow us to characterize them, we can begin providing answers to questions regarding the evolution of host-specificity.

Methods:

Examining Strains of C. sp. 34 and Other Caenorhabditis for Dauers

C. sp. 34 has not been observed to generate dauer larvae in high numbers in laboratory conditions (Woodruff pers. comm.). To confirm this or indicate that other strains of *C.* sp. 34 make a disproportionate number dauers, sodium dodecyl sulfate (SDS) treatment was used to isolate dauers. SDS is a chemical which denatures proteins, breaks down lipid membranes, and is lethal to worms when ingested (Tukmachev et al., 1979). However, dauer worms have the buccal plug and thicker cuticles which prevent them from ingesting and being harmed by the SDS (Cassada and Russell, 1975). The SDS treatment was performed on *C.* sp. 34 (NKZ 2) as well as *C. briggsae* (JU1088), and *C. elegans* (N2). Worms were starved on NGM plates for 10 days before SDS treatment. They were then washed off in 1mL of M9 solution and half of the solution was transferred to a sterile Eppendorf tube labeled SDS and the other 500 mL was transferred to a sterile Eppendorf tube labeled control. The worms were then spun down at high speed for 30 seconds and washed with M9 three times. After incubating in SDS they were washed three times with M9 again and supernatant removed before plating the pellet on NGM plates. Observations were then made by enumerating the living and dead of both control and SDS exposed worms. For several strains, survivors of SDS treatment were picked and subjected to 30 more minutes of SDS treatment and observed again for survival. Statistical analysis of SDS treatments were done using Microsoft Excel to compile the data, R Studio for graphical

representations, Wilcoxon test rank sum test with continuity correction, and Welch's t-test were used to measure significance.

Fig Agar Plate Analysis

Using a procedure based on Choi et al., *C.* sp. 34 was plated on specially made fig agar plates. These plates were infused with the common fig, *F. carica*, to investigate cues for *C.* sp. 34 stress-resistant L3s. Fig plates were made using Anna and Sarah™ dried black mission figs. Figs (20) were blended in a standard blender until approximately 400 mL of fig slurry was generated in the blender. Distilled water was then added to the slurry until it became a lighter and smoother consistency. Typically, 400 mL of blended fig yields 1 L of fig agar solution. 250 mL of fig slurry was transferred into four 500 mL centrifuge bottle. Bottles were then centrifuged for 10 minutes at 8500 rpm. The supernanent was then transferred to a 1 L graduated cylinder and brought up to 800 mL with distilled water. To ensure a favorable pH for bacterial growth, pH was tested and generally yielded a highly acidic pH. pH was raised to between 6-7 using 40 drops of 4M NaOH. Fig solution was then added to a 4 L Erlenmeyer flask with 16 g of agar. Fig agar solution was then autoclaved using a long liquid cycle. After autoclaving, pro-microbial additives were added to the fig agar solution: 800 µL of 5 ng/mL of cholesterol, 800 µL of 1M CaCl, 800 µL of 1M $MgSO_4$, and 20 mL of 1M K_3O_4P. 10 mL of fig agar solution was plated into medium sized plates yielding approximately 80 total plates per pour. Using fig media plate, *C.* sp. 34 was plated on the fig plates and scored after 24 hours. Fecundity testing, numerating alive P0, and scoring dispersal around the fig plates was done by crossing 15 male L4

virgin to 5 L4 virgin female of the *C*. sp. 34 strain NKZ 2 and scoring the number of adults and progeny.

Inducing C. sp. 34 Stress Resistant Larvae via RNAi

To generate dsRNA for RNAi knockout of dauer constitutive genes, *daf*-2 and *akt-A*, and control genes, *dpy*-5, *dpy-2*, and *bli-1*, *C. elegans* coding sequences of interest were retrieved from Wormbase. These sequences were blasted to the *C*. sp. 34 genome to retrieve orthologues sequences (Gavin Woodruff, pers. comm.). Primers were designed with these *C*. sp. 34 sequences using the Primer3 software. For molecular cloning primers, the restriction sites of interest (SpeI (forward), ApaI (reverse)) were added to the 5' ends of the primers. After annealing, the DNA was stored in a -20°C freezer.

RNAi by feeding was used to inhibit dauer pathway genes. RNAi by feeding was done by generating a plasmid that encodes the dsRNA for the desired genes to inhibit in *E. coli.* PCR then amplified the DNA fragments. PCR product inserts and the L4440 vector plasmid were digested with the restriction enzymes ApaI and SpeI. Subsequent fragments were purified with Zymo DNA Clean and Concentrate columns and then ligated with T4 DNA ligase. Ligated DNA was then transformed into competent DH5-alpha *E. coli*. After confirming the presence of the insert via restriction digests, plasmids were re-isolated and transformed into competent HT115 (DE3) *E. coli*. HT115 has a defective RNAase and expresses the T7 RNA polymerase so is suitable for expressing large amounts of dsRNA. RNAi by feeding was performed using standard protocols by Asako Sugimoto (Ahringer, 2006). Plates were monitored continually after initial plating for any abnormal phenotypes and specifically for dumpy

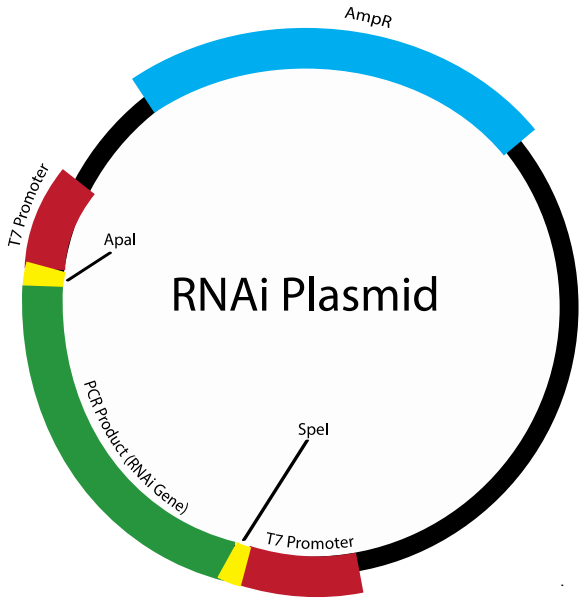


Figure 8: Plasmid for Transformation and RNAi

T7 promoters allowed for both forward and reverse DNA transcription of the insert. Incorporated into the plasmid is the AmpR gene for ampicillin resistance. Bacteria take up the plasmid in response to being seeded on LB + Amp plates and thus also incorporate the the sp. 34 coding sequence. SpeI and ApaI lyse the plasmid at the T7 promoter sites to allow for ligation or digestion of the PCR insert. DNA into the plasmids a restriction digest was performed using SpeI and ApaI restriction enzymes.

and blister phenotypes in *dpy-5* and *dpy-2* or *bli-1* bacteria. 24 hours after scoring *dpy* plates, dauer phenotype genes were tested for by Sodium Dodecyl Sulfide (SDS) (see "*Examining Strains of C. sp. 34 and Other Caenorhabditis for Dauers*" for SDS protocol).

Examining Natural Variation in Dauer Formation in C. sp. 34

To test the phenotypic plasticity of *C.* sp. 34 dauer production, 29 wild isolates from different islands and inter-island locations were subjected to SDS. The SDS protocol was the same as previously stated in the methods for isolation of dauers in *C.*

elegans, *C. briggsae*, and *C.* sp. 34. Statistical analysis was also conducted Microsoft Excel and R studio.

Morphology Analysis

To confirm that the SDS resistant L3s were dauers, morphological characteristics were examined using several different methods. *C.* sp. 34 dauers and control *C. elegans, C. tropicalis,* and *C. briggsae* dauers, found by SDS were imaged under high-magnification DIC microscopes. The first characteristic examined was the length to width ratio (radial constriction). To ascertain the extent of radial constriction plates of N2 (*C. elegans*), and NKZ 2 (*C.* sp. 34) were grown to starvation (over 10 days) and were treated with SDS following the SDS assay protocol. Then agar slides were made by dropping 2% agarose solution on a slide and covering that slide with another slide perpendicular to it. Once the agar had cooled the slides were gently pulled apart, keeping the slide that the agar stuck to. Then 10 µL of NaN_3 was added onto the agar to paralyze the worms. Survivors of the SDS were plated into the NaN_3. Once approximately 20-100 survivors were picked onto slides and then moved to the DIC microscope. Images were taken using a Dino Eye Digital Eye Piece Camera. Analysis of the images of dauers was done using ImageJ software to compare morphological features of *C.* sp. 34 and *C. elegans* dauers (Abramoff, 2004). Using the segmented line tool, the length and width of worms was measured and then recorded into Microsoft Excel. Length to width ratios were then compared using R studio software. For comparison, I measured normal reproductive L3s lengths and widths by using a hatch off protocol and then imaging.

To synchronize the worm's life stages, plates of NKZ 2 and N2 were grown to adulthood (approximately 2 days at 25°C) and then washed off in M9. Worms were spun down and washed off two more times before being suspended in a bleach solution (33 mL diH_2O, 6 mL Bleach (Na^+ ClO^-), and 800 µL 10M KOH) and rotated for 7-9 minutes for N2 and 4-5 minutes for NKZ 2. Worms were then pelleted and washed off four times in M9 then left to rotate overnight. The next day L1s were plated on NGM plates and left to grow at 25°C for NKZ 2 until reaching L3 stage (approximately 2 days) and at 20°C for N2 until reaching L3 stage (approximately 2 days). These worms were then picked onto agar slides with 10 µL of NaN_3, photographed, and analyzed using the same techniques as L3 dauers.

Another characteristic measured was alae. These are difficult to see under normal DIC microscopy. The fluorescent dye Lipophilic Vital DiI was used to help identify them. Using a modified protocol from Schultz and Gumienny, 2012 *C.* sp. 34 alae were unable to be detected. Dauers were isolated using the SDS assay. After worms were isolated they were washed off 4X with M9 and then incubated for one hour on unseeded NGM plates to allow the cuticular lipids that DiI binds to, to be resynthesized. SDS is a harsh chemical that may destroy these lipids. Once the worms were incubated they were washed off the NGM plates with 0.5% Triton-100X in M9. Worms were spun down at 2000 rpm for 30 seconds. The supernatant was removed and the worms were washed off and spun down twice in M9. After removing the M9 supernatant, the worms were suspended in 400 µL of DiI working solution (30 µg/mL of DiI in M9) and left rotating at approximately 350 rpm for 3 hours. Eppendorf tubes containing DiI working solution and worms were covered with foil to prevent light exposure to the fluorescent

dye. After rotating the solution, it was spun down and the dye supernatant was removed. The pellet was re-suspended in 400 µL of M9 and plated on NGM plates seeded with 50 µL of OP50. Plates were then incubated for 30 minutes to allow the worms to crawl away from any residual fluorescent dye found in the M9. After 30 minutes the worms were picked onto agar slide plates and viewed under compound microscope with lasers to excite the dye. Unfortunately, it appears that the SDS may have destroyed lipids necessary for the dye to bind to the cuticle. However other observations were able to be made including ingestion of the dye.

Another documented feature of the dauer is pharyngeal constriction. Using the same protocol for measuring radial constriction, worms of *C.* sp. 34 and *C. elegans* were starved, subjected to SDS, and picked onto agar slides. These worms were then viewed under an inverted compound microscope. Pictures of the heads and pharynx of worms were taken using the same Dino Eye Digital Eye Piece Camera. The specific measurements taken for the pharynx was the width of the isthmus and the length of the entire pharynx from the back the terminal bulb to the tip of the opening. Statistical analysis was then done using Microsoft Excel and R studio.

Finally, a fourth test to confirm and characterize dauers was done using ESEM (Environmental Scanning Electron Microscope) to identify the buccal plug. The buccal plug structure is too small to be reliably identified using DIC microscopy, therefore requiring a greater magnification found in electron microscopy. ESEM allows one to image wet samples. This is done by allowing the chamber to not be a complete vacuum, but instead 1/76 of an atmosphere. Initially, when imaging, samples were placed on a heating/cooling stage so that the wet samples would not freeze or dehydrate, causing

distortion in the morphology of the samples when the first ESEM pictures were taken without the dehydration series. Humidity, temperature, and pressure where adjusted as needed to prevent freezing and dehydration while imaging the buccal cavity of *C. elegans* and *C*. sp. 34 L3s. Samples were incubated at -20°C in 100% ethanol until the next day of imaging.

To attempt avoiding the freezing/drying problem that may have distorted worms several additional steps were taken. Worms were fixed with glutaraldehyde overnight To remove water from the worms, a dehydration series followed with drying using CO_2 by means of the critical point method. After drying the worms were mounted and viewed.

Results:

C. sp. 34 Produce Few Dauers in the Lab

SDS treatment of the known *C.* sp. 34 strain, NKZ2, and strains of *C. elegans*

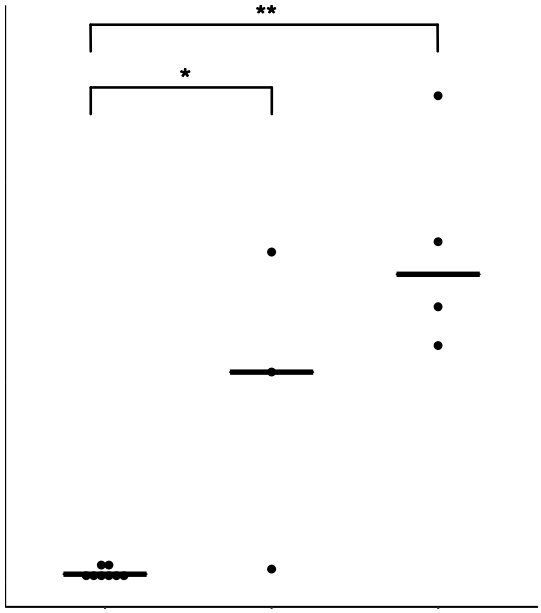


Figure 9: SDS Test: Percent Alive of Caenorhabditis Species

C. elegans produced the most survivors (33.5%), followed by *C. briggsae* (18.5%). *C.* sp. 34 had the least fraction alive (1.04% survival). *C. elegans* ($p = 0.004$) and *C. briggsea* ($p = 0.048$) did have percent of survivals that are significantly higher than *C.* sp. 34. Significance calculated using a Wilcoxon Rank Sum Test. *C. elegans* N = 4 treatments, 1825 worms. *C. briggsae* N = 3 treatments, 354 worms. *C.* sp. 34 N = 10 treatments, 4673 worms.

and *C. briggsae* provided support of the microscopic observations of starved plates that *C.* sp. 34 makes few dauers in normal lab conditions. Figure 9 shows a stark contrast in the amount of dauers with NKZ2 averaging 1.04% alive after SDS treatment. In comparison, strains of *C. elegans* and *C. briggsae* averaged 33.5% and 27.1% ($p = 0.004$ and $p = 0.04$, Wilcoxon Rank Sum Test). To further investigate the characteristics

of the dauer in *C.* sp. 34, increasing the rate of dauers formation was attempted using RNAi, fig-agar plates, and natural variation of strains in response to starvation.

RNAi of C. sp. 34

Using RNAi on *C.* sp. 34 proved to be more challenging than originally anticipated. The first attempts at feeding worms RNAi plasmid expressing *E. coli* failed to produce any significant noticeable response (see Figure 10). When dauer

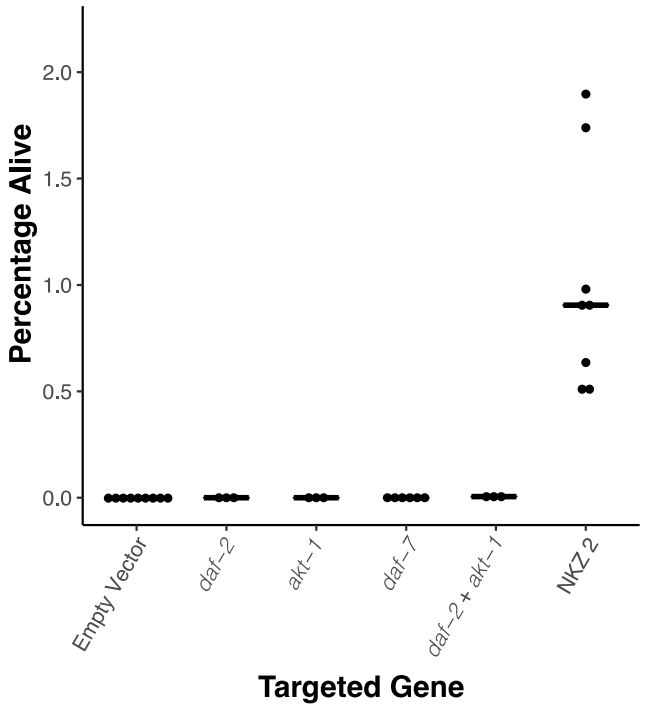


Figure 10: SDS of RNAi Targeted Genes Compared to Normal NKZ 2

None of the RNAi targeted genes elicited any significantly higher amounts of dauers than the normal SDS of *C.* sp. 34 grown with non-RNAi *E. coli* on NGM plates or the empty vector (L4440). Worms were treated with cutlures of RNAi feeding *E. coli*. N = 3+ treatments, 100 worms per RNAi test.

inhibiting genes *daf-2* and *akt-1* were targeted both separately and together no worms went into the dauers stage or were seen to be constitutively dauer. Worms subjected to RNAi targeting the *daf-2*, *daf-7*, *akt-1*, and *daf-2* and *akt-1* genes were placed in SDS to

isolate any dauers. None of the RNAi induced worms survived SDS (see Figure 10). This indicates that none of the worms exposed to RNAi produced the dauer constitutive phenotype seen in *C. elegans* when subjected to RNAi of *akt-1*, *daf-2*, and *daf-7*.

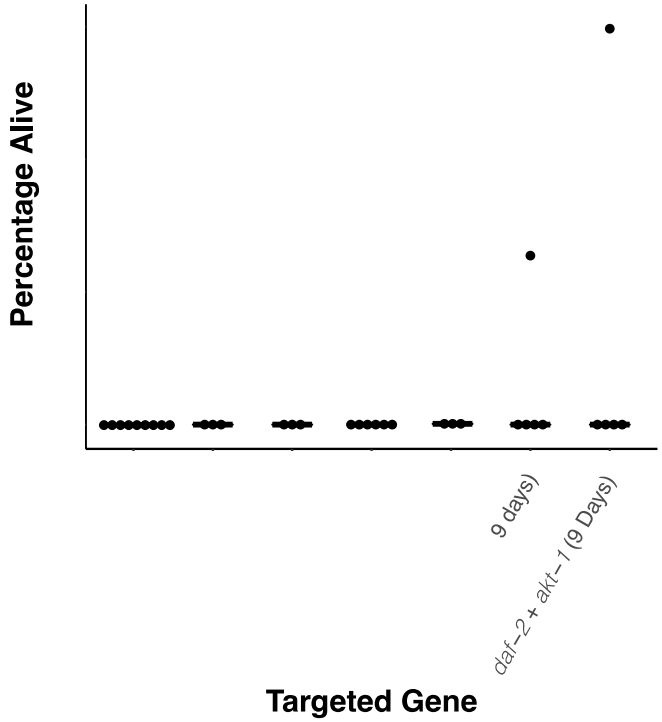


Figure 11: Comparing Extended Exposure to RNAi bacteria

To test if the time needed to expose the worms to transformed bacteria was needed, P1s could reproduce and F1 generation grow in the RNAi plates. These worms were then subjected to SDS after 9 days. This produced no significantly different result from the original 4 day experiment. N = 3+ treatments per RNAi gene targets, 100 worms per RNAi test.

One idea was that the RNAi needed more time to take effect, only resulting in phenotypes of the F1 generation. To test this, the time between plating embryos and treating with SDS was increased from 4 days to 9 days. This resulted in very little change in the survival of worms treated with *daf-2* or *akt-1* and *daf-2* and then subjected to SDS (see Figure 11). While there was some survival after SDS treatment in the 9 day

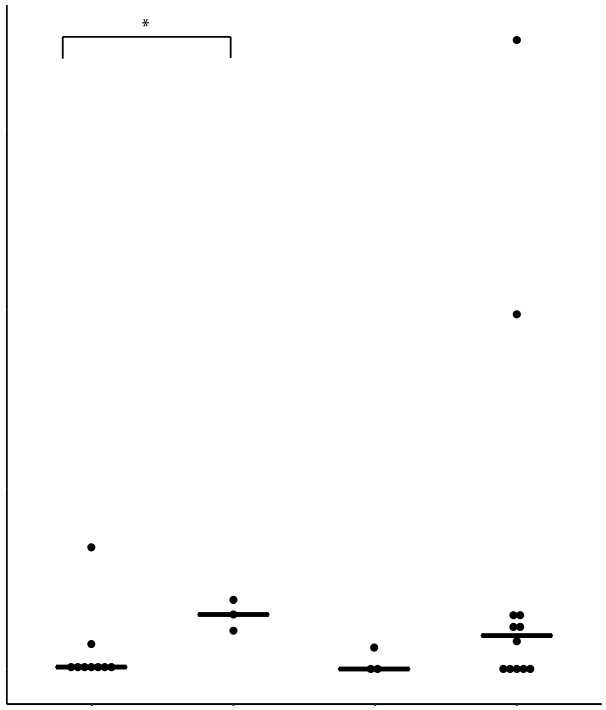


Figure 12: Empty Vector, *dpy-5*, *dpy-2*, and *bli-1* RNAi Fraction Dumpy

To measure the efficacy of RNAi, the control gene *dpy-5*, *dpy-2*, and *bli-1* was used to elicit a distinct phenotype to ensure that RNAi was successful. However, *dpy-5* and *bli-1* RNAi did not generate a significant ($p > 0.05$) number of mutant worms, but dpy-2 did generate a significantly higher number of mutants than the empty vector ($p = 0.03$). Statistical analysis was conducted using a Wilcoxon Rank Sum Test. N = 6, 665 worms (Empty Vector), N = 12, 894 worms (*dpy-5*), N = 3, ~144 worms (dpy-2), N = 3, 107 worms (*bli-1*).

daf-2 and *akt-1* + *daf-2* RNAi treated worms, the fraction of survival is exceedingly low and is most likely due to some of the F1 generation naturally reverting to dauer as the bacterial lawn is depleted.

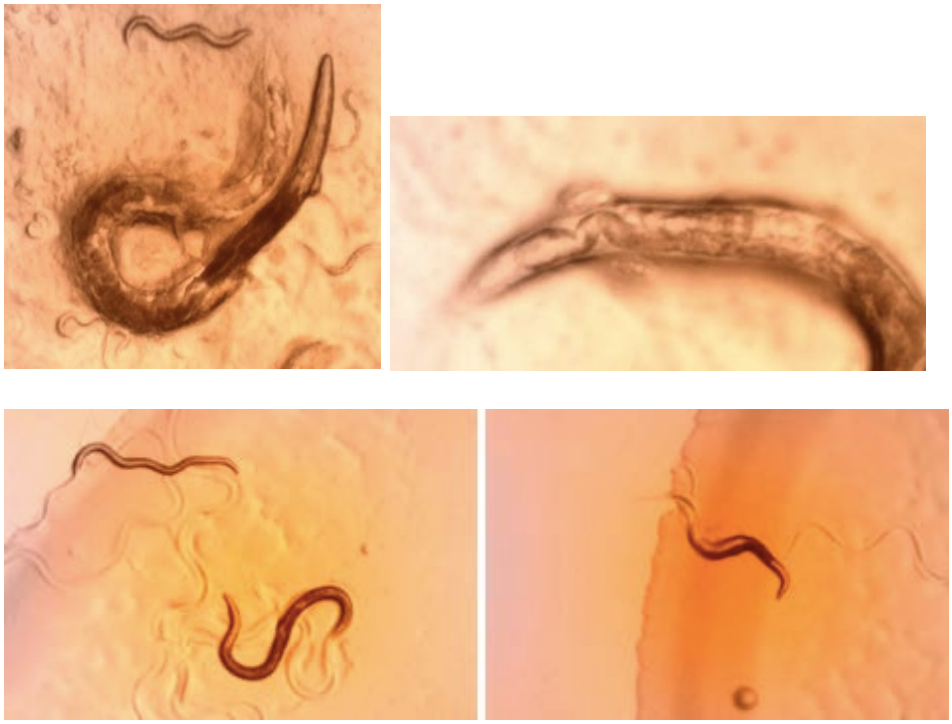


Figure 13: RNAi Generated Mutant Phenotypes

Figure 13: Top: Two worms treated with *bli-1* RNAi, the tail region (arrow) had a distinct internal structure that was common most of the older adult worms. 16 Bottom-left: Shows wild-type *C.* sp. 34. These worms were given low amounts of dsRNA resulting in a failure to express the dumpy phenotype. Figure 13 bottom-right shows a phenotypically dumpy *C.* sp. 34. The worm contains the anatomical features of an adult but in a noticeable shorter body. These worms were given a higher amount of *dpy-5* dsRNA during RNAi by soaking (photo credit: Gavin Woodruff).

The results of the RNAi of the dauer arrest genes was put into question by the results of the controls. As expected, the empty vector generated no phenotypic abnormality, but the control genes *dpy-5, dpy-2,* and *bli-1* failed to produce a high amount of dumpy or blistering phenotype worms. While the potency of the control phenotypes was not high, *dpy-2* still had significantly more dumpy phenotype expressing worms on average than the empty vector ($p = 0.03$, Wilcoxon Rank Sum Test) (see Figure 12). It is noted that the *bli-1* worms exhibited an abnormal phenotype

that has not been previously recorded from worms treated with *bli-1* RNAi. These worms appeared to have detachments of the internal structure from the cuticle at the tail and midsection. The internal structure could be seen bunching up and zig-zagging on many of the worms treated with *bli-1* (see Figure 13). The normal phenotype of *bli-1* worms is cuticle detachment around the head forming a very recognizable blister-like structure. As the positive controls were largely ineffective, it is clear that our RNAi experiments are not inhibiting gene activity to the degree seen in typical *C. elegans* experiments.

Fecundity Assay of Fig Media Plates

To see if a fig-like environment would help *C.* sp. 34 go through its natural life cycle or if there was something in the fig that could trigger dauer larva formation, novel fig media was made to replicate a fig. After optimizing the media to meet the requirements of *E. coli*, OP50, fecundity tests were done to test *C.* sp. 34 receptiveness to the fig media. Surprisingly, the worms did not respond well to the novel fig media (see Figure 14). In some tests, the agar appeared to kill them, but those that lived did not lay any embryos while *C.* sp. 34 plated on NGM at the same stage averaged 38 embryos per test. Additionally, the location of the worms on the agar plates indicates that those worms platted on fig plates were not concerned with feeding or reproducing, but were trying to leave. Most of the worms alive on fig plates were either outside the lawn (generally far outside it) and even on the glass walls of the agar plate (see Figure 15). Worms plated on NGM were generally found in the bacterial lawn. Because the worms would not lay any embryos and would die on the fig media the experiment was not continued further.

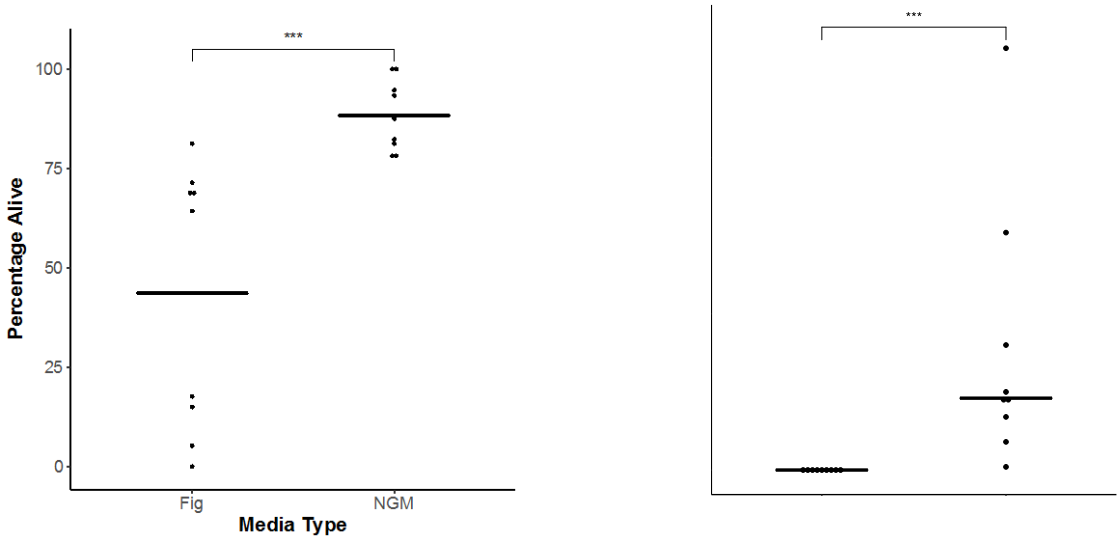


Figure 14: Left: Number of Alive, Right: Number of Embryos Laid per Fecundity Test

Left: The percentage of alive worms after fecundity test proved to be significantly different (p = 0.0009) between the two media (fig and NGM). However, there appears to be a large difference between two sets of worms on the fig plates. These was done in two tests, however both tests had split results. The fig plates were all poured from the same solution. Right: The number of embryos laid is greatly different between *C*. sp. 34 platted on fig media and NGM (p = 0.0001). Those plated on fig media had no embryos laid, while NGM averaged 38 embryos per fecundity test. Statistical analysis was conducted using a Wilcoxon Rank Sum Test. N = 9, 15 female and 5 male per plate (total = 180).

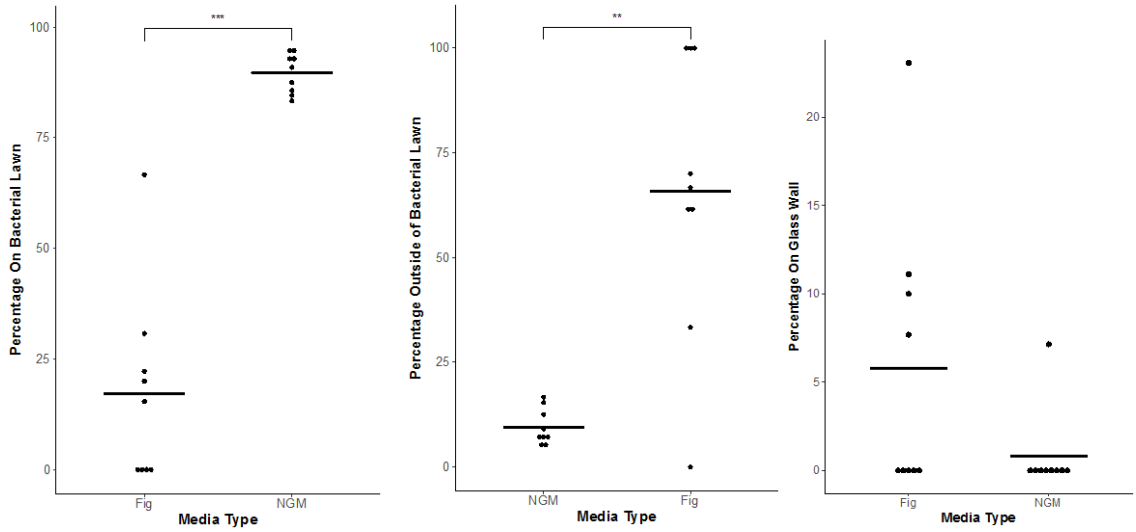


Figure 15: Fig and NGM Media Worm Dispersal

Left: *C.* sp. 34 plated on NGM plates had significantly higher amount actively feeding in the bacterial lawn (average = 13.7) than fig media (average = 1.7) ($p = 0.0003$). Middle: More worms were found outside of the bacterial lawn on fig plates (average = 4.3) than the NGM plates (average = 1.4) ($p = 0.005$). Right: There was no statistical difference between number of worms found on the glass walls of fig (average = 0.6) or NGM plates (average = 0.1) ($p = 0.09$). Statistical analysis was conducted using a Wilcoxon Rank Sum Test. Fig: N = 9, 180; worms NGM: N = 9, 180 worms

Natural Variation in Dauer Formation

When SDS treatment was applied to the 29 wild isolates from Okinawa the percentage alive was as low or lower than the original *C.* sp. 34 strain NKZ2, except NKZ88 (see Figure 16). Both NKZ2 and NKZ88 had significantly higher amounts of dauers compared to other wild isolates ($p = 2.24 \times 10^{-7}$, $p = 3.64 \times 10^{-9}$, Wilcoxon Rank Sum Test) while NKZ27 and NKZ75 are only slightly significantly higher than the other isolates in comparison to NKZ2 and NKZ88 ($p = 0.04$, $p = 0.04$ Wilcoxon Rank Sum Test). The island of origin, Iriomote and Ishigaki, do not appear to be a factor in the rate of dauer formation. There was no significant difference between isolates from

one island compared to another (p = 0.69,Wilcoxon Rank Sum Test). NKZ88's high number of survivors is similar to the number of survivors to that of NKZ2 (p = 0.59, Wilcoxon Rank Sum Test) (see Figure 17). In total, none of the wild-isolates indicated to have a huge increase in the variation in generating dauers. However, the rates of dauers seen in NKZ2 and NKZ88 were still higher than RNAi dauer arrest targeted worms. To ensure that stress-resistant larva seen surviving the SDS assay were not due to a phenomenon in *Caenorhabditis* called "bagging" where larvae are encapsulated in the carcass of an adult female worm and thus could be spared from the SDS, SDS was re-administered to survivors of a first round of SDS for two strains of *C.* sp. 34 (Figure 18). While the percentage of survivors is not high there were several worms that survived independent full immersion in SDS (p = 0.04, Wilcoxon Rank Sum Test). This indicates that these worms were not L1 larva that managed to avoid SDS by being encased in a gravid female carcass. It is also key to note that when observations were continued of worms that survived SDS they proceeded through their life cycle and reproduced.

and Location:
Iriomote
Ishigaki

Figure 16: Natural variation in Dauer Formation of *C.* sp. 34 Wild Isolates

SDS testing of the 29 wild isolates collect from the field yielded no significantly higher results in fraction alive than NKZ 2. Fraction alive of NKZ 2 and NKZ 88 were found to be significantly higher than other isolates ($p = 2.24 \times 10^{-7}$, $p = 3.64 \times 10^{-9}$) and NKZ 27 and 75 were found to slightly significantly higher ($p = 0.04$, $p = 0.04$). Island of origin, Iriomote (red) and Ishigaki (blue) was not found to be a factor in survivorship from SDS testing ($p = 0.69$). Isolates with zero survivors, except NKZ 43, were not included in Figure 13. 12 out of 29 strains had zero survivors. Statistical analysis was conducted using a Wilcoxon Rank Sum Test. N = 5 treatments per strain (average), 477.3 worms (average).

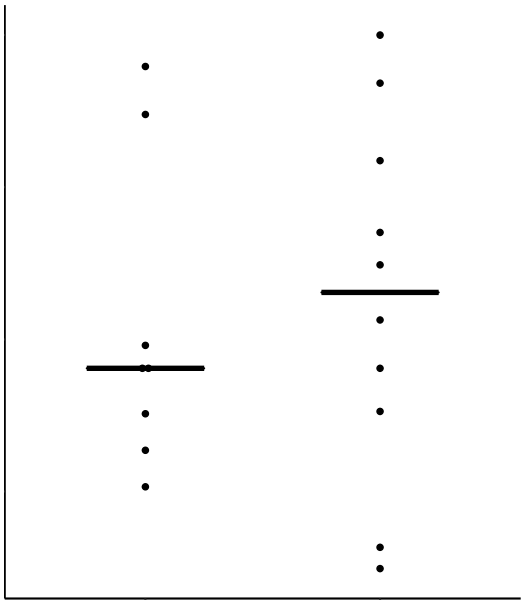


Figure 17: Comparison of NKZ 88 and NKZ 2 Survivors

NKZ 2 and NKZ 88 produced similar amounts of survivors to the SDS treatment. NKZ 88 was the only strain to produce a statistically similar number of survivors ($p = 0.59$). Statistical analysis was conducted using a Wilcoxon Rank Sum Test. N = 6 treatments, 4426 worms (NKZ 2), 10 treatments, 3974 worms (NKZ 88)

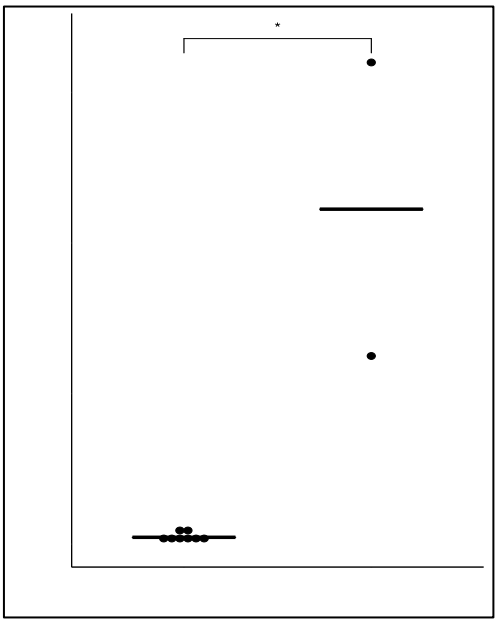


Figure 18: *C.* sp. 34 Extended Exposure to SDS

When worms that survived were subjected to 30 minutes in SDS (following the typical SDS assay protocol detailed in Methods) they were picked from plates they were platted on and placed back into a solution of 1% SDS. These worms were then washed off and re-platted and observed for survivorship. There is a significant ($p = 0.04$) increase in survival of worms exposed again. Statistical analysis was conducted using a Wilcoxon Rank Sum Test. *C.* sp. 34 1st Exposure: N = 10 treatments, 4673 worms, *C.* sp. 34 2nd Exposure: N = 2, 19 worms.

Radial Constriction in C. sp. 34 and Other Caenorhabditis

Using SDS is the classic approach to isolating dauers of *C. elegans*, but due to the different natural history and morphology in adult stages, *C.* sp. 34 dauers could easily have differing morphological characteristics in the dauer stage. Therefore, morphological features that have been well documented in *C. elegans* like length to width ratio are good points of comparison of other *Caenorhabditis* species to *C.* sp. 34. Specific common characteristics of dauers can help us understand if the *C.* sp. 34 dauer

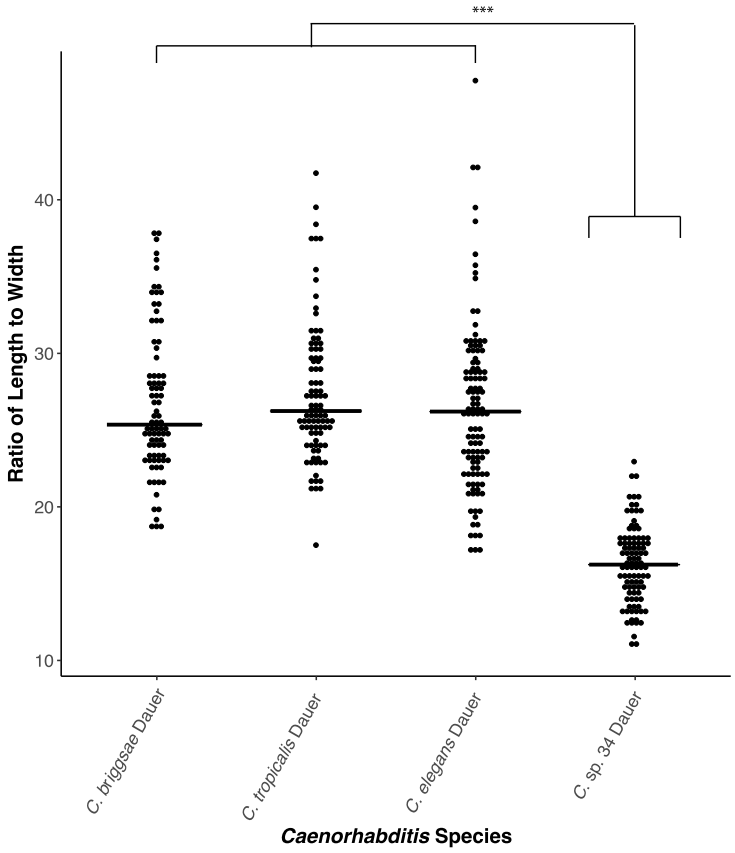


Figure 19: Length to Width Ratio of Dauer Species

C. sp. 34 averaged 16:1 length to width ratio while *C. elegans* averaged 26:1 ($p < 2.2 \times 10^{-16}$), *C. briggsae* averaged 26:1 ($p < 2.2 \times 10^{-16}$), *C. tropicalis* averaged 27:1 ($p < 2.2 \times 10^{-16}$). There was no significant difference between non-*C.* sp. 34 Caenorhabditis length to width ratio ($p > 0.05$) Statistical analysis was conducted using a Welch's Two Sample T-test. N = 3, 97 total dauers for *C.* sp. 34, N = 1, 113 total dauers for *C. elegans*, N = 1, 85 total dauers for *C. briggsae*, N = 1, 93 total dauers for *C. tropicalis*.

has changed due to its unique natural history. The first analysis was of length, width, and the ratio of length to width of the stress-resistant L3s in *C.* sp. 34 and other *Ceanorhabditis*. Dauers of *C. elegans* have a typical length to width ratio of 30:1 (Riddle, 1988). *C.* sp. 34 has a far lower ratio of length to width (16:1) as opposed to *C. elegans* which was measured in this experiment to have a width to length ratio of 26:1.

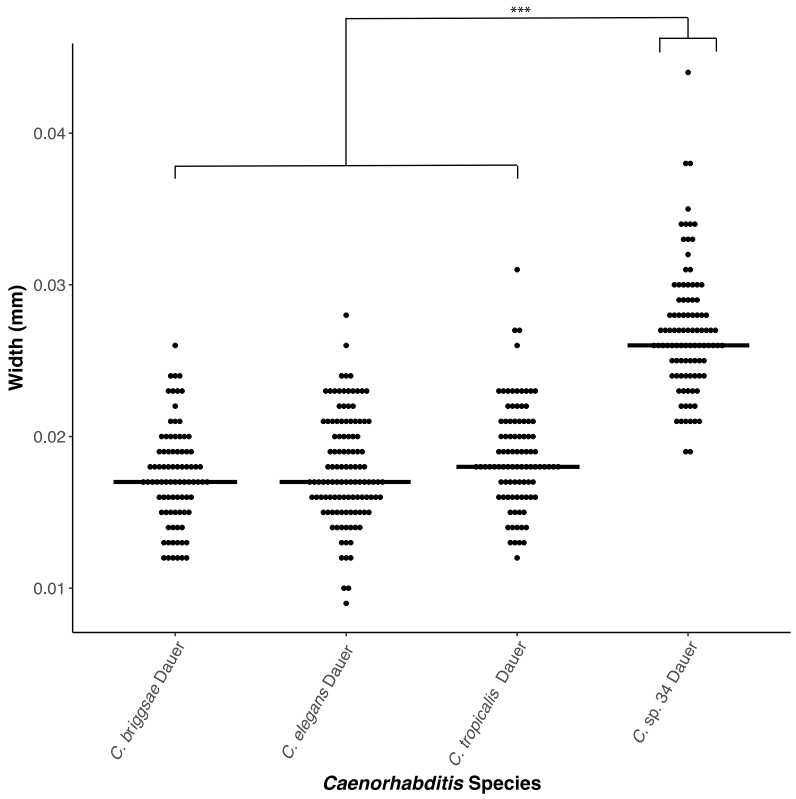


Figure 20: Width of Dauer Species

C. sp. 34 averaged 0.026 mm in width while *C. elegans* averaged 0.018 mm ($p < 2.2 \times 10^{-16}$), *C. briggsae* averaged 0.017 mm ($p < 2.2 \times 10^{-16}$), and *C. tropicalis* averaged 0.018 mm ($p < 2.2 \times 10^{-16}$). Statistical analysis was conducted using a Welch's Two Sample T-test. N = 3, 97 total dauers for *C.* sp. 34, N = 1, 113 total dauers for *C. elegans*, N = 1, 85 total dauers for *C. briggsae*, N = 1, 93 total dauers for *C. tropicalis*.

C. briggsae also had 26:1 ratio and *C. tropicalis* had a ratio of 27:1 (see Figure 19). Strangely, *C*. sp. 34 is significantly ($p < 2.2 \times 10^{-16}$, Welch's Two Sample T-test) smaller in length to width than *C. elegans*, *C. briggsae*, and *C. tropicalis* indicating the opposite size differentiation found in the adult form of these worm species. All three of the non- *C*. sp. 34 dauers had no significant difference between them ($p > 0.05$, Welch's Two Sample T-test). When looking at the specific parameters (length and width) *C*. sp.

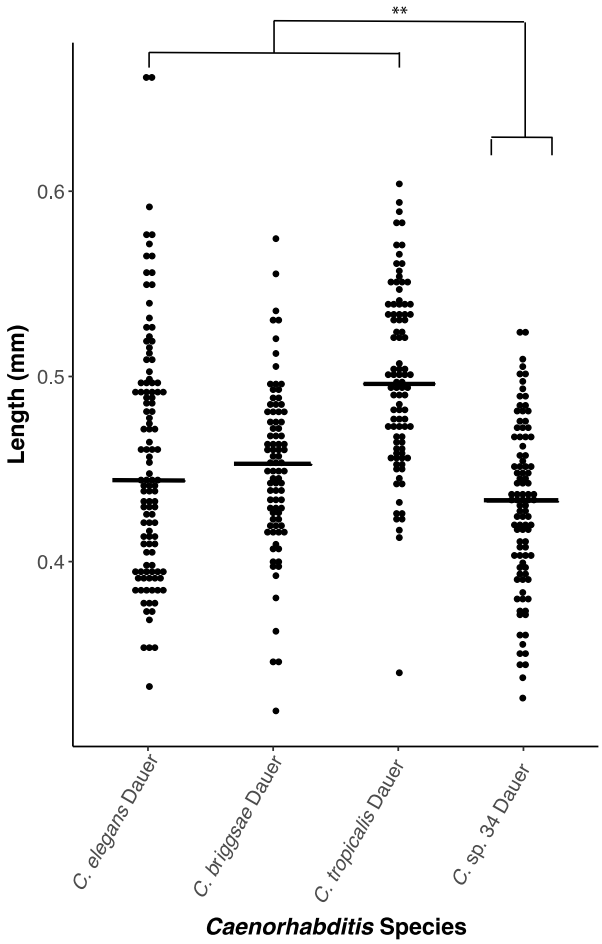


Figure 21: Length of Dauer Species

Below: *C.* sp. 34 averaged 0.43 mm in length and *C. elegans* averaged 0.46 mm (p = 0.0006), *C. briggsae* averaged 0.45 mm (p = 0.001), *C. tropicalis* averaed 0.49 mm ($p < 2.2 \times 10^{-16}$). Statistical analysis was conducted using a Welch's Two Sample T-test. N = 3, 97 total dauers for *C.* sp. 34, N = 1, 113 total dauers for *C. elegans*, N = 1, 85 total dauers for *C. briggsae*, N = 1, 93 total dauers for *C. tropicalis*.

34 is both significantly wider ($p < 2.2 \times 10^{-16}$, Welch's Two Sample T-test) and shorter ($p < 0.01$, Welch's Two Sample T-test) than the other species of phoretic *Caenorhabditis* (see Figure 20 and 21). However, width is the most noticeable difference between the species of dauers tested. These significant changes in the size

parameters indicate that the *C.* sp. 34 dauer morphotype is divergent from other well studied *Caenorhabditis*.

To confirm that there is a difference between the L3 stress resistant dauer stage and L3 non-dauer stage the length to width ratio of non-dauer L3s were measured using

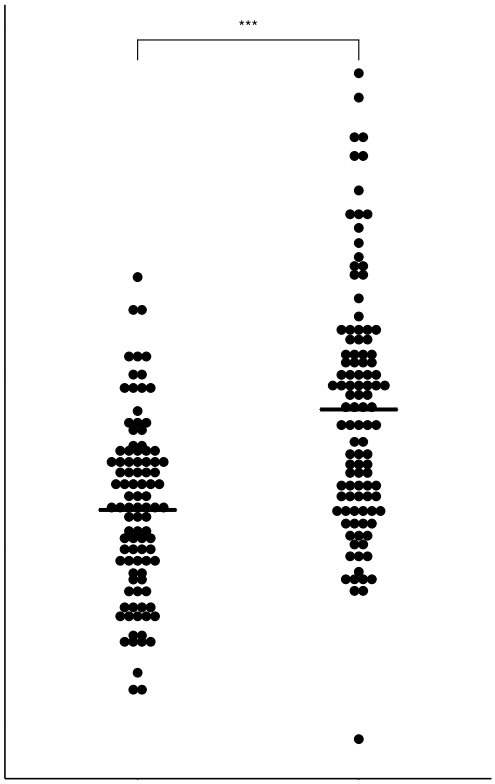


Figure 22: *C.* sp. 34 Dauer L3 vs non-Dauer L3 Length to Width Ratio

When comparing the width to length ratios of both non-dauer L3s (L3) and dauer L3s there is significant difference between the two groups ($p = 2.2 \times 10^{-9}$). Statistical analysis was conducted using a Welch's Two Sample T-test. N = 3, 97 total L3 dauers, N = 1, 107 L3. *C.* sp. 34: N = 3, 97 total L3 dauers, N = 1, 107 L3

synchronized plates and Image J. When *C.* sp. 34 dauers were compared to non-dauer L3s there was a significant difference between the two life stage sizes ($p = 2.2 \times 10^{-9}$, Welch's Two Sample T-test) (see Figure 22). When *C. elegans* non-dauer L3s were

compared to *C. elegans* dauers the difference between the two were unsurprisingly large ($p < 2.2 \times 10^{-16}$, Welch's Two Sample T-test) (see Figure 23). *C.* sp. 34 L3s average 18.9:1 in length to width ratio as compared to the dauer 16:1. The morphological changes between L3 and dauer appear to have reversed in *C.* sp. 34. These worms no longer indicate the classic radial constriction that is common throughout not only

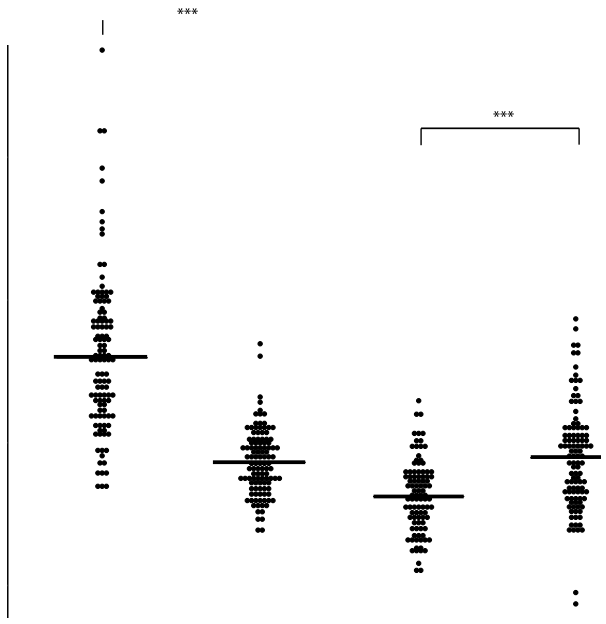


Figure 23: *C.* sp. 34 and *C. elegans* Dauer and L3 Length to Width Ratios

C. sp. 34 has less, but still significant, difference between the L3 and dauer L3s ($p = 2.2 \times 10^{-9}$) when compared to *C. elegans*. The difference that is seen is that the dauer is smaller than the L3. There is a sharp contrast with *C. elegans* who's dauers are significantly larger than the L3 ($p < 2.2 \times 10^{-16}$). Interestingly, the ratio of length to width of L3s between *C.* sp. 34 and *C. elegans* are not different ($p > 0.05$). Statistical analysis was conducted using a Welch's Two Sample T-test. *C.* sp. 34: N = 3, 97 total L3 dauers, N = 1, 107 L3. *C elegans*: N = 1, 113 total L3 dauers, N = 1, 109 total L3.

Caenorhabditis, but also throughout more distantly related species (Castelletto et al., 2009; Hu 2007).

When comparing the size of the *C.* sp. 34 dauer to its other life stages, L1-Adult, the length of dauers are statistically the same length as L2s ($p = 0.57$, Welch's Two Sample T-test) (see Figure 24). When comparing the widths there is a significant

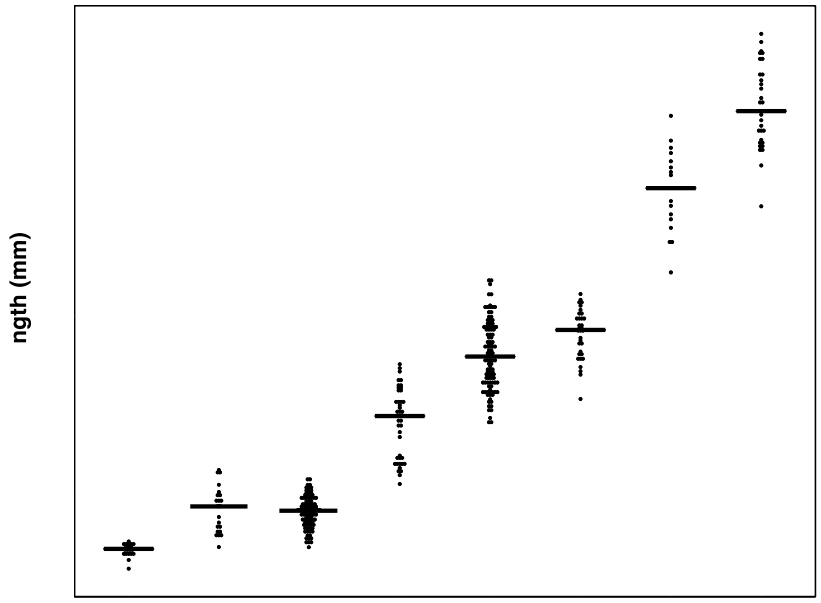


Figure 24: Length of All *C.* sp. 34 Life Stages

Data collected by Gavin Woodruff Ph.D. was compared to data collected for *C.* sp. 34. Surprisingly, length of dauers correlates with L2 ($p = 0.57$) and not L3 or is significantly higher than L3 as seen in *C. elegans*. L3-1 is data for L3 collected by Gavin Woodruff Ph.D. L3-2 was collected in this experiment. Statistical analysis was conducted using a Welch's Two Sample T-test.

divergence from any life stage as it is in between the width of L2 and L3 worms ($p = 0.00023$, $p < 2.2 \times 10^{-16}$, Welch's Two Sample T-test) (see Figure 25). When these parameters are combined, *C.* sp. 34 does appear to have a distinct length to width ratio

that is far lower than any life stage that is near it (see Figure 26). This result bolsters the idea that the stress-resistant form isolated by SDS is indeed a distinct morphotype from the other normal life stages of *C*. sp. 34 (see Figure 27).

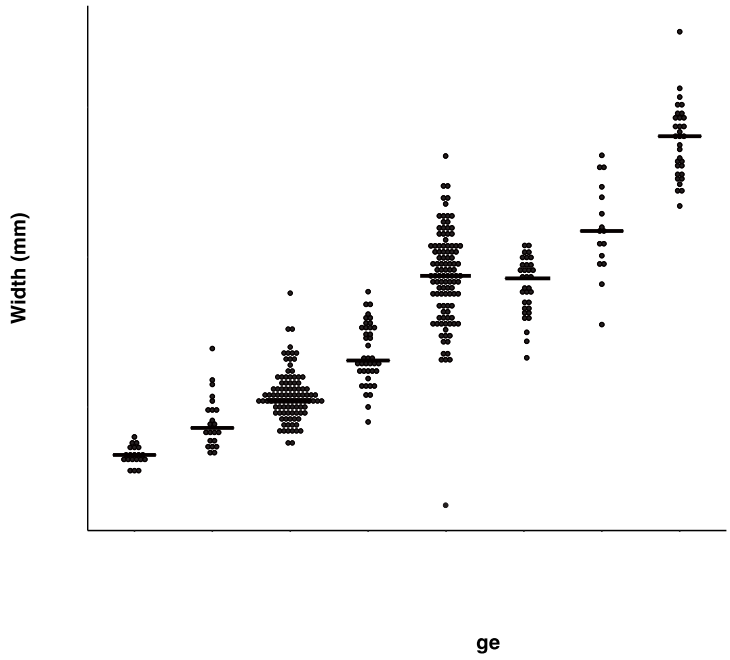


Figure 25: Width of All *C*. sp. 34 Life Stages

Data of *C*. sp. 34 life stage width collected by Gavin Woodruff Ph.D. was compared to the data for dauers. Dauers were found to have a distinct width compared to other life stages. L3-1 is data for L3 collected by Gavin Woodruff Ph.D. L3-2 was collected in this experiment. Statistical analysis was conducted using a Welch's Two Sample T-test.

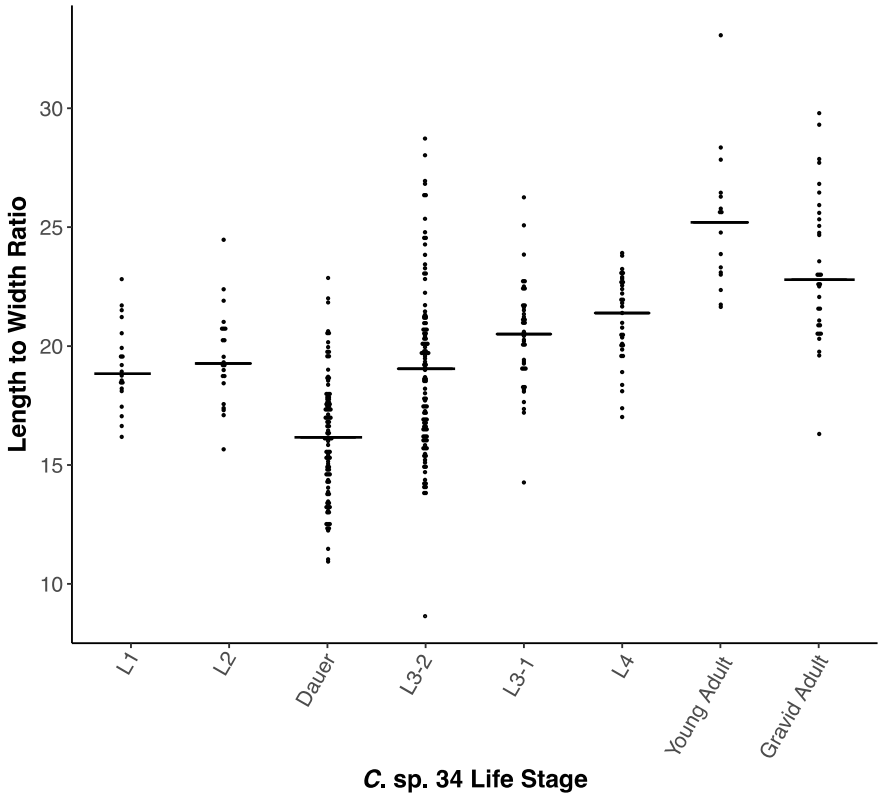


Figure 26: Length to Width Ratio of All Life Stages of *C.* sp. 34

The dauer of *C.* sp. 34 has a distinctly lower length to width ratio than any other life stage. Even L1, L2, and L3 have close length to width ratios while the dauer has a lower length to width ratio then all of them ($p = 4.98 \times 10^{-8}$ (L2), $p = 4.47 \times 10^{-10}$ (L3)). This indicates that there is a distinct morphotype of the *C.* sp. 34 dauer. Statistical analysis was conducted using a Welch's Two Sample T-test.

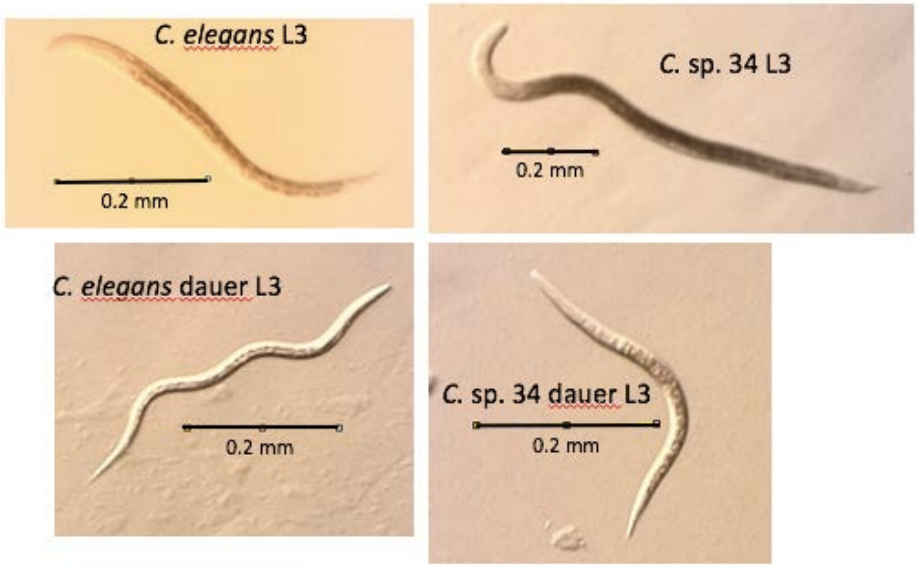


Figure 27: Morphotypes of *C. elegans* and *C.* sp. 34 L3s and Dauers

The shapes of the dauer of both species are distinctly different. C. elegans dauers have the long radial constriction while C. sp. 34 dauers appear to smaller and wider than non-dauer L3s.

Pharyngeal Constriction

In addition to radial constriction, another characteristic of the dauer is the elongation and narrowing of the pharyngeal structure. An image of the pharynx of both L3 and dauer of *C. elegans* can be seen in Figure 28. When measuring the length of the entire pharynx versus the width of the isthmus (central tube connecting the terminal blub and metacorpus) *C. elegans* was unsurprisingly found to have an immense amount of constriction and elongation between L3s and dauers. *C.* sp. 34 did also have a significant amount of constriction in the dauer stage compared to the normal L3 (see Figure 29). The commonality between the *C. elegans* and *C.* sp. 34 dauer does not wholly justify this being a true dauer form, but does indicate that this stress-resistant form may have retained some dauer characteristics. While there is constriction of

Figure 28: Pharyngeal Constriction in *C. elegans* Dauers

The pharynx structure seen in this Figure is the central structure slightly outlines in black. It starts at the buccal opening and ends at the back of the terminal bulb. *C. elegans* dauers have a distinct constriction of the pharynx. As can be seen by the difference in the two images, left: *C. elegans* L3, right *C. elegans* dauer.

C. sp. 34 pharynx, it is important to note that the level of constriction is not as extreme as seen in *C. elegans* dauers and non-dauer L3s.

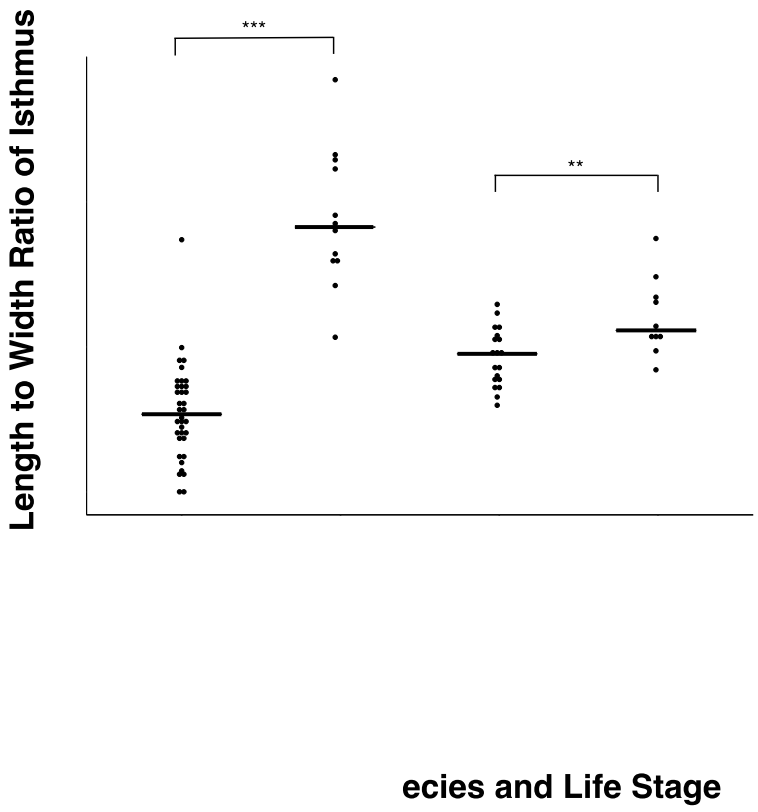


Figure 29: Pharyngeal Constriction of *C. elegans* and *C.* sp. 34 Dauers and L3s

C. elegans has a significant amount of pharyngeal constriction between L3 and dauer ($p < 0.001$). While *C.* sp. 34 has a lot less constriction than *C. elegans* there is still a significant amount of pharyngeal constriction seen between L3 and dauer ($p < 0.01$). This indicates that the dauer of *C.* sp. 34 retains some of the morphologies seen in typical *Caenorhabditis*. Statistical analysis was conducted using a Welch's Two Sample T-test. *C.* sp. 34: N = 1, 10 total dauers, N = 1, 19 L3. *C elegans*: N = 1, 12 total dauers, N = 1, 36 total L3

Lipophilic Vital DiI Cuticle Staining

Another key feature of *C. elegans* dauers is the presence of alae on the cuticle. To detect if these structures were also present in *C.* sp. 34 stress-resistant L3s, lipophilic vital DiI was used to make the cuticular structures of the worms fluoresce. However, the cuticle proved to be more difficult to stain than previously anticipated. SDS used to isolate dauers appeared to also destroy the lipids that DiI uses to bind with. When

performing DiI on mixed stage populations, avoiding SDS, neuronal, pharyngeal, and generally rectal and volval structures were seen, but the alae and annuli (cuticle structures generally seen using DiI) were not commonly visible. This would point to the SDS not being a factor in the binding of dye to the cuticle. Other factors like rotations per minute of shaking may have a larger role in producing better results and will be tested in further experimentation.

ESEM Buccal Cavity Imaging

To further investigate if the buccal plug is present in the *C.* sp. 34 stress resistant L3s ESEM was used to obtain finer images of the buccal cavity of both reproductive and stress-resistant L3s. Unfortunately, optimization of the protocol for *C.* sp. 34 preparation for ESEM imaging has prevented us from gathering images of the buccal plug for this thesis. Continued optimization should produce better images and allow for a clear picture of the buccal cavity to find any dauer-like structures.

Discussion:

Natural Variation of Dauer Formation in C. sp. 34

The goal of this experiment was to investigate if the *C.* sp. 34 stress-resistant dauer larvae has any divergent characteristics because of its specific phoretic relationship and to provide a potential model for studying the rise of host-specificity. Host-specificity is thought to be one of the key pre-adaptations necessary for a species to evolve from free-living to parasitic. But before we could characterize the dauer of *C.* sp. 34 we first needed to identify and isolate it. Three methods were attempted, RNAi, fig-media, and natural variation. Of all three of the attempted approaches to produce a workable amount of the dauer of *C.* sp. 34, natural variation established itself as the most successful and reliable method.

C. sp. 34 being from several different islands in Okinawa makes it an ideal candidate to investigate differences in sensitivity to dauer pheromones and decreases in food concentration between strains. Previous research had shown that *C. elegans* varies in its propensity to make dauers by strains from different origins (Viney et al., 2003). The SDS treatment of the known strain of *C.* sp. 34, NKZ2, and the other *Caenorhabditis* species helped solidify the observed trait that *C.* sp. 34 generates extremely few dauers (see Figure 9). This was also supported by the SDS treatment of the 29 wild isolates. Except for NKZ88, all the wild isolates performed worse than NKZ2 having between zero and two percent alive per treatment (see Figure 16). NKZ88 did not have a significantly higher number of survivors, but had nearly the exact same number of survivors as NKZ2 (see Figure 17). The uniformity of results indicates that

this trait of low dauer generation in laboratory conditions is not unique to NKZ2 but is consistent in the species and found in strains that are separated by miles of ocean on different island locations.

However, within the strains, NKZ2, 88, 27, and 75 all had significantly higher amounts of survivors than the other strains. These higher producers of dauers have no connection between island location and dauer formation, but this shows a variation within the species. This poses the question, what about those strains make them more prone to produce dauers? This question was not explored in this experiment, but would be an interesting enquiry to answer in future experimentation. A possible area for investigation is the variation in density of growth of each species. If one species is able to grow to higher densities than the others it can produce higher amounts of dauer pheromone and reduce the concentration of food more quickly. Thus, these actions should generate more dauers in comparison to the other less-dense strains.

To further ensure that this natural variation was not due to random gravid female bagging, worms that survived one round of 30 minute SDS exposure were re-exposed to SDS for 30 additional minutes and then observed. Close to 50% were found to survive being independently introduced to SDS indicating that these worms were resistant to SDS due to means other than bagging.

Characterizing the C. sp. 34 Dauer

To further characterize the dauers of *C.* sp. 34, they were imaged with DIC microscopy after SDS treatment. Using the one of the most prolific survivors of SDS treatment, NKZ2 (*C.* sp. 34), and N2 (*C. elegans*), the images showed an interesting change in morphology. *C.* sp. 34 showed a smaller length to width ratio than that of *C.*

elegans. When observing *C. elegans* and *C.* sp. 34 survivors of SDS treatment there were also clear morphotypes as one would expect of a life stage like dauer (see Figure 29). While *C.* sp. 34 survivors did not have the radial constriction seen in *C. elegans* and other *Caenorhabditis* species it did have uniformity to it shape. The body was significantly smaller than the L3 while retaining its width. The body increased in width from the tail up until pharynx where it then shrunk back down to the buccal cavity. The presence of this morphotype commonly seen within survivors of SDS indicates that these are a dauer-like stage. In addition, the length to width ratio of the dauer does not correlate with any other life stage. However, the length of the dauer has significant resemblance to the L2 life stage of *C.* sp. 34. It should be noted that in very distantly related species of nematode there are some that do not undergo a stress-resistant L3 stage, but instead have a stress-resistant L2 (Lee, 2002). If *C.* sp. 34 had a stress-resistant L2 or L2-like morphology, this would be very unique. However, the symbiotic partner of *C.* sp. 34, the pollinating fig wasp, has a length of approximately 1-2 mm (Frank, 1984). It would then make sense that *C.* sp. 34 would produce a smaller dauer to stay on the small transportation vector. These comparisons do not prove that it is a stress-resistant L2. Further testing including SDS of starved specific life stages, not mixed populations, could help identify if this is an L2 or very small L3.

When comparing the length to width ratio of *C.* sp. 34 SDS resistant L3s with other phoretic *Caenorhabditis, C. briggsae, C. tropicalis, C. elegans, C.* sp. 34 is significantly smaller than all three. All of these species of dauer had a length to width ratio of 26:1 (*C. briggsae*), 27:1 (*C. tropicalis*), 26:1 (*C. elegans*) indicating that non-specific phoretic species of *Caenorhabditis* have larger L3 stress resistant stages. Again,

the lack of radial constriction in the dauer of *C.* sp. 34 may indicate a characteristic change in *C.* sp. 34 that could be a result of its unique natural history. The findings of these measurements of the stress-resistant stage also appear to be at odds with its adult morphology. *C.* sp. 34 is renowned for its large size that makes it so divergent from other *Caenorhabditis* at the adult stage. So far, the only explanation for this differentiation between species is that *C.* sp. 34's specific phoretic vector and life cycle has resulted in these characteristic changes.

However, when pharyngeal constriction was measured *C.* sp. 34 retained constriction of the pharynx. While it was not as much as *C. elegans,* it was significant. The presence of pharyngeal constriction gives weight to the claim that this is a very distinct dauer morphotype. To further define the morphology of this unique dauer we investigated if it presented the buccal plug feature. Using environmental scanning electron microscopy (ESEM) we attempted to take images of the dauer and non-dauer L3 buccal cavity. Unfortunately, preparation of the dauer caused morphological damage. In research after this thesis we hope to obtain images of the buccal plug once we optimize the protocol for ESEM preparation. The buccal plug is one of the most key features that separates this life stage form any other. To get better images, staining the worms with osmium tetroxide after isolation and sputter coating them with gold-palladium after the dehydration series are the next additional steps to the protocol. Once images of it can be taken then a truly definitive answer to what stage this dauer is may be possible.

RNAi and Fig Media

To investigate the genomic nature of the dauer and to attempt a rate of dauer formation closer to other *Caenorhabditis* species, RNAi was applied to *C.* sp. 34 embryos and gravid females to specifically block dauer repressive genes. RNAi inhibits the activity of targeted transcripts via complementary base pairing of small-interfering RNA (siRNA) and protein-transcript (mRNA to ribosome) interactions (inability to translate RNA) (Fire et al., 1998). The genes *daf-2* and *akt-1* are well known dauer genes in *C. elegans* and due to *C.* sp. 34's close phylogeny to *C. elegans*, homologs were easily found. In initial testing RNAi proved to be unsuccessful in eliciting a response in both the dauer genes and with the control *dpy-5* gene, but after optimization of the protocol, *dpy-2* and *dpy-5* elicited dumpy phenotypes and *bli-1* had an unusual phenotype that had not been previously recorded for *bli-1* RNAi treated worms. *dpy-5* and *dpy-2* genes encode a collagen and a collagen hydrolase, respectively (De Lucas et al., 2015; Torpe and Pocock, 2014). These genes create shorter and stout looking worms compared to normal adult phenotypes (Riddle, 1997). *bli-1*, encodes a collagen that is involved in cuticle strut assembly. When this protein is missing, the cuticle can detach from the body and form what appears as blisters around the worm, typically around the head (Riddle, 1997). These genes were chosen as controls for their success rate in RNAi in *C. elegans*, the clarity of the phenotype in detection, and because they have been observed to be homologous between *Caenorhabditis* species (Wei et al., 2013). It is very unlikely that the dauer arrest and control genes which have been so highly conserved throughout the phylum would change within the *Caenorhabditis* genus.

Recent research using *C. elegans*, *Strongyloides stercoralis,* and *Ancylostoma caninum* has shown that orthologues of DAF-2 and DAF-16 are used in the infective stage and can induce the dauer state when orthologous *daf-16* gene from the parasite is inserted into *daf-16* defective *C. elegans* (Gelmedin et al., 2011). It is also important to note that the potency of RNAi on neurons (which is where the dauer pathway is activated) is lower than other areas that can be targeted. In addition, the rate of false negatives in RNAi is around 30% in *C. elegans* (Ahringer, 2006). The high number of false negatives is produced by a lack of protein degradation in the time between platting on RNAi (thus inhibiting that synthesis of that protein) and scoring. To account for this the dauer arrest genes were continued with longer exposures to RNAi, but this had very little effect. Possibly, *C.* sp. 34 is hypersensitive to dauer arrest proteins and would need even longer exposure on RNAi plates to generate the desired dauer constitutive phenotype. Another method to increase the dauer constitutive phenotype in *C.* sp. 34 is injection of the dsRNA directly into the gonads of males and scoring the progeny. In addition, methods like CRISPR could be used to generate desired mutant phenotypes in *C.* sp. 34.

The other method used to attempt to generate more dauers, fig agar plates, unfortunately was very unsuccessful in eliciting any type of positive response from *C.* sp. 34 L4s. Many worms that were plated on fig-media plates either died within 24 hours or were appearing to flee the bacterial lawn and agar plate. In addition, none of them laid any embryos. It was unexpected that the *C.* sp. 34 would be so abhorrent to the fig-media because their natural ecological niche is in figs. However, two problems with the fig media may have contributed to the results. The first is that the fig-media

was not made from the figs found in Okinawa and secondly, the figs in the media were from dried fruit that had presumably been picked long before the package arrived at the lab and culture could be made. Both factors may play a large role in explaining why the worms did not like the media. *C.* sp. 34 is unique for spending most of its life, if not all, in one type of fresh fig. Most *Caenorhabditis* discovered have been found on different types of rotting plant material and not specific fresh plant material. The results seen could be due to some unexpected overpowering substance like sugar or salt content in the dried figs that was unaccounted for. Continued research into refining the specific substances of complex novel media can hopefully produce better results. In addition, collecting figs and fig wasps from Ishigaki and Iriomote may be a more appropriate test in the future. Still other tests that could be done to help establish the stress-resistant form of *C.* sp. 34 would be RT-PCR of specific genes like *Hsp90* which are transcriptionally active in this otherwise quiescent state (Hoogewijs et al., 2008). In addition, RNA-seq for mRNA transcriptional profiles that match *C. elegans* dauers should provide similar insights as RT-PCR (Dalley and Golob, 1992). This would offer another piece of hard evidence that the stage observed in this experiment is the dauer stage found in nematodes.

Limitations and Alternative Hypothesis

There is the possibility that this stage is not a homolog to the dauer of other *Caenorhabditis* species. While these stress-resistant dauers of *C.* sp. 34 seen in this experiment have a distinct morphology and some similar characteristics of *C. elegans* dauers there may be other explanations for their stress-resistant state. One is that a specific molt of *C.* sp. 34 may provide enough cuticle protection and decrease in

pharyngeal pumping to avoid death by SDS. An analysis of pharyngeal pump rates of SDS survivors could help delineate if the morphotype is a unique dauer or a uniquely stress-resistant molting event. It seems unlikely that both pharyngeal pumping and a form of false sheathing would happen at the same time so often in *C*. sp. 34. In addition, if this is the result of decreases in pumping and a false sheath then why do other molting stages other than L2 or L3 fail to survive SDS when transitioning?

During the RNAi testing of *C*. sp. 34 there was a substantial amount of contamination. Sterile technique and other precautions were used to avoid contamination, but towards the end of the RNAi experimentation there appeared to be a yellow film that quickly developed over where the *E. coli* was platted. The worms then appeared to favor these different bacteria that quickly outcompeted the RNAi bacteria. Glass containers of the liquid media, which was used to grow *E. coli,* as well as other steps in the RNAi method were tested for contamination and found to have none. One explanation is that the bacteria was on the wild *C*. sp. 34 themselves and couldn't be easily separated. Attempting RNAi again with a better protocol for cleaning the worms before platting them on the RNAi plates may result in increased potency of both dauer arrest and control genes.

It is also important to note that throughout this experiment typical dauer behavior like nictation were not noticed on starved, SDS isolated, or mix stage population plates. These observations were not tested in this experiment, but *C*. sp. 34 may rely less on nictation and more on their olfactory neurons to locate the pollinating fig wasp. Classic chemotaxis assay with pheromones from male and female pollinating fig wasp and parasitic fig wasp could help establish if the *C*. sp. 34 dauers have

diverged from other *Caenorhabditis* in their dauer's behavior. Now that *C*. sp. 34 dauers have been isolated in this experiment testing can be done to attempt to establish the role of host-specificity on behavioral characteristic changes in *C*. sp. 34. Alternatively, nictation in *C*. sp. 34 may require the presence of the pollinating fig wasp female. These tests could provide convincing data that the *C*. sp. 34 dauer has evolved key host-specific sensing that other *Caenorhabditis* do not need nor have. From here the olfactory neurons and other features of *C*. sp. 34 can be investigated for divergent traits from *C. elegans*. Performing assays like Hallem et al., 2011 and ablating neurons could provide a door for an entire area of genetic research. *C*. sp. 34 could be used as a model for studying inter-cellular pathways and genes relating to host-specificity. From there comparisons can be drawn to free-living and known parasitic pathways.

Significance of Research

The experiments conducted in this thesis show that *C*. sp. 34 does has a divergent morphotype of the stress-resistant dauer. The short and wide stature of the *C*. sp. 34 dauer is likely an adaptation specific to the small size of its phoretic partner the pollinating fig wasp. *C*. sp. 34's general morphology is just one part of many different lines of research that can be conducted to understand how host-specificity arises in nematodes. More experiments can be done with *C*. sp. 34 to understand specific behavioral changes. The implications of this are great. A better understanding of the evolution of host-specificity may give us previously unknown insights into how to treat or prevent infectious parasites in the future. Researching host-specificity in nematodes parasitism can aid in preventing the rise of nematode parasitism not only in humans, but also in human associated organisms like livestock and plants for agriculture.

Conclusion:

The assays conducted in this thesis have shown that *C.* sp. 34 has a divergent dauer life stage that has yet to be seen in other species of *Caenorhabditis*. Because *C.* sp. 34 is such a close relative of *C. elegans* this provides us with a unique opportunity to see how two very phylogenically related species with radically different natural histories have changed. *C.* sp. 34's stress resistant L3 appears at a very low rate in response to classic dauer stimuli like crowding and starvation. There is a variation in the rate of formation of dauers within wild isolates, but none of the isolates have a rate higher than 3%. The dauer of *C.* sp. 34 also has a distinct morphotype that is highly divergent from closely related *Caenorhabditis*. Its short and wide appearance may be a result of its specific natural history with a small phoretic vector like the pollinating fig wasp. However, *C.* sp. 34 dauer retains some classic dauer characteristics like pharyngeal constriction. While additional testing is required to give more power to this claim, these divergent traits may be the result of the unique natural history of *C.* sp. 34 and additional testing may allow us to better understand the evolution of host-specificity using *C.* sp. 34. Testing the behavior, strategies, and pathways of this partner-specific *Caenorhabditis* can then give us insights into how parasitic species evolve host-specificity. From there, we can use the knowledge gained from *C.* sp. 34 and test parasitic species to understand how the free-living to parasitic theory unfolds in nematodes.

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