Chapter 2

A LOCALIZATION STUDY OF ASCAROSIDE PRODUCTION

2.1 Abstract

According to earlier studies, loss-of-function *daf-22* mutants are unable to produce the short-chain ascarosides necessary for entry into dauer diapause or for behaviors such as male attraction. DAF-22, a thiolase responsible for the truncation step of peroxisomal β-oxidation of the ascaroside fatty-acid side chain and therefore necessary for the production of biologically active ascarosides, has been reported to be expressed in the intestine, hypodermis, and body wall muscle of the worm. In order to examine potential tissue-specific aspects of ascaroside production, we have selectively driven the expression of DAF-22 under tissue-specific promoters, conducted mass spectrometry-based analyses of the excretomes of each strain, and assessed several ascaroside-regulated worm behaviors. HPLC-MS analysis reveals that the intestine is the major site of ascaroside biosynthesis. However, several ascarosides are produced when a GFP::DAF-22 fusion is expressed in the body wall muscle and hypodermis of the worm. Furthermore, intestinal GFP::DAF-22 expression restored a large amount of dauer formation activity, while GFP::DAF-22 expression in all three individual tissues restored male attraction behavior to wild type-like levels.

2.2 Introduction

Despite its small size, *C. elegans* is comprised of multiple, well-defined tissues and organs that serve vital functions in the life of the worm. For example, the intestine is a large

organ that comprises approximately one third of the worm's entire body. Its main functions include the digestion of food following the grinding of bacteria in the pharynx, as well as the absorption and storage of nutrients.[1] The hypodermis forms the base of the nematode's body, stores nutrients, and secretes the worm's protective cuticle.[2-3] The body wall muscle, on the other hand, is responsible for the worm's sinusoidal movement. There are therefore many potential sites of ascaroside biosynthesis within the worm.

Although much of the biosynthetic pathway of ascarosides has yet to be elucidated, previous studies have shown that DAF-22, a thiolase responsible for the last step of peroxisomal β-oxidation of the lipid moiety attached to the 1'-position of the ascarylose sugar core, is necessary for the production of biologically active short-chain ascarosides (Figure 2.1).[4-5] Transgenic expression of an N-terminally tagged GFP DAF-22 fusion protein (GFP::DAF-22) under the control of the native *daf-22* gene promoter revealed that the protein is expressed in the intestine, the hypodermis, and the body wall muscle of the worm.[4, 6] Furthermore, when exclusively expressed in the intestine under the control of the *vha-6* promoter, GFP::DAF-22 rescue was able to restore some dauer inducing activity. It was therefore hypothesized that the intestine is the major site of ascaroside biosynthesis; however, the possible roles of DAF-22 expressed in the hypodermis and body wall muscle in the biosynthesis of ascarosides remained unexamined.[4] An additional hypothesis could be made that ascaroside production may occur at different locations within the worm and that the ascarosides produced may vary from tissue to tissue.

To investigate this issue, we created three strains of worms that drive the expression of GFP::DAF-22 specifically within the intestine, the hypodermis, or the body wall muscle

using tissue-specific gene promoters within a *daf-22(ok693)* mutant background. The ascaroside profile in each strain was examined by HPLC-MS analysis. Pheromone cues collected in each strain were then tested for activity in dauer formation, male attraction, and hermaphrodite repulsion.

2.3 Results and Discussion

2.3.1 Driving GFP::DAF-22 Expression within Individual Tissues

To selectively rescue DAF-22 function within a specific tissue, we integrated transgenes into the worm's genome that contained the sequence of a GFP::DAF-22 fusion protein under the direction of one of three different gene promoter sequences. For intestinal expression, we selected the promoter for the gene *vha-6*, which encodes an ortholog of subunit A of the membrane-bound domain of a vacuolar proton-translocating ATPase. This promoter was the same promoter sequence used previously by Butcher *et al*, and the *Pvha-6::gfp::daf-22* transgene in this study produced a similar expression pattern (Figure 2.2).[4]

To drive expression in the hypodermis, we used the short promoter sequence for the *dpy-7* gene, which encodes a collagen that is necessary for normal development and structure of the cuticle.[7] The *Pdpy-7::gfp::daf-22* transgene used in this study was expressed in a punctate manner within the hypodermis. This punctate pattern, which can also be seen in the expression pattern of the intestinal *Pvha-6::gfp::daf-22* transgene, may be due to the three amino acid serine-lysine-isoleucine peroxisomal tag at the C-terminus of the DAF-22 protein.

Expression was driven in the body wall muscle using the promoter sequence of *myo-3*, a gene which encodes the minor isoform of the myosin heavy chain. Expression of the *Pmyo-3::gfp::daf-22* transgene can be seen in the muscle running along the dorsal and ventral axes of the worm.

2.3.2 Ascarosides are Primarily Produced in the Intestine

In order to assess the ascaroside profile of each tissue-specific GFP::DAF-22 expressing strain, synchronized liquid cultures were grown from egg to young adult. Ascarosides excreted by the worms were extracted with ethanol from the lyophilized remnants of the culture supernatant, and identified from HPLC-MS chromatograms based on known fragmentation patterns, as previously described (Figure 2.3).[6, 8-9] As expected, the supernatant from *daf-22(ok693)* mutant cultures did not contain any short-chain ascarosides, as DAF-22 is required for the truncation of the lipid side chain. Worms expressing the GFP::DAF-22 fusion within the body wall muscle produced minimal amounts of ascr#3, typically one of the most abundantly produced ascarosides, as well as ascr#10, and ascr#18.

As hypothesized, the intestine does appear to be the major source of ascaroside biosynthesis, with *Pvha-6::gfp::daf-22* transgenic worms producing 50-100% of each ascaroside compared to levels generated by wild type N2 worms, including both simple ascarosides and ascarosides modified at the 4'-position of the ascarylose sugar. As digestion and absorption of nutrients occurs in the intestine, as well as nutrient storage, it makes sense that ascaroside production occurs where the building blocks are most likely to be found.

Worms expressing the GFP::DAF-22 fusion protein within the hypodermis also produce a significant amount of ascarosides, albeit generally less than the quantities

displayed by intestinal expression of GFP::DAF-22. Hypodermal GFP::DAF-22 expression resulted in ascr#3 levels similar to intestinal rescue, but significantly less quantities of ascr#5, and 2'-glucosylated ascr#4. Although previous studies did not predict the hypodermis as potential location for ascaroside biosynthesis, the tissue is known to play a role in nutrient storage, and therefore could harbor the building blocks necessary for ascaroside production. Interestingly, hypodermal GFP::DAF-22 rescue demonstrated wild type levels of ascr#10 and its 4'-modified derivatives, icas#10 and osas#10. Research has shown that lysosome-like organelles within the intestine, called the gut granules, are required for the 4'-modification of ascarosides. Therefore, the question arises as to whether the hypodermis contains the machinery necessary to modify 4'-modified ascarosides, whether simple ascarosides are synthesized in the hypodermis and then transported to the intestine for 4'-modification, or whether there is leaky expression of the *Pdpy-7::gfp::daf-22* transgene within the intestine.

2.3.3 Intestinal Rescue of GFP::DAF-22 Restores Dauer Formation

Originally, ascarosides were identified as the major components of dauer pheromone, a chemical signal that directs entry into and exit out of dauer diapause.[10] Previous research demonstrated that a *daf-22* loss-of-function mutant is unable to produce the pheromone necessary to induce entry into dauer diapause; however, partial dauer formation activity can be restored through transgenic expression of GFP::DAF-22 within the intestine.[4, 11]

In order to probe whether driving DAF-22 expression in the body wall muscle and hypodermis, the two other tissues in which DAF-22 is naturally expressed within the worm,

can restore dauer formation activity, integrated strains containing either a *Pmyo-3::gfp::daf-22* or *Pdpy-7::gfp::daf-22* transgene were grown in liquid culture. Dauer pheromone was then collected via ethanol extraction of the boiled supernatant. Exogenous pheromone was then added to plate agar on which eggs were allowed to hatch and develop, and dauer formation activity was assessed (Figure 2.4).

As expected, exogenous pheromone collected from *daf-22(ok693)* loss-of-function mutant worms induced no dauer formation in N2 worms, whereas wild type dauer pheromone elicited dauer formation in 70 ± 5% (± SD) of larva. Pheromone generated by intestinal expression of GFP::DAF-22 restored approximately half of the dauer formation activity compared to the N2 control (35 ± 4%). GFP::DAF-22 rescue within the body wall muscle and hypodermis produced dauer pheromone that induced much lower levels of dauer formation at 6 ± 2% and 4 ± 2%, respectively. This is consistent with the initial hypothesis that ascaroside biosynthesis primarily occurs within the intestine. However, although very modest, GFP::DAF-22 expression in both the body wall muscle and hypodermis does restore dauer formation compared to the *daf-22(ok693)* mutant. This correlates with the HPLC-MS analysis that reveals ascaroside biosynthesis occurs within the hypodermis, and to a lesser extent, within the body wall muscle.

The main components of dauer pheromone are ascr#1, ascr#2, ascr#3, ascr#5, ascr#8, and icas#9. Although the low resolution HPLC-MS data could not resolve ascr#1 from other metabolites, it was revealed that ascr#3 is produced in all three rescue strains, though minimally in the body wall muscle. Furthermore, ascr#5 was found in the supernatant of intestinal and hypodermal GFP::DAF-22 rescue cultures, as well as icas#9. The intestine

produces greater quantities of all the aforementioned dauer-inducing ascarosides compared to the hypodermis, which may explain why dauer pheromone derived from *Pvha-6::gfp::daf-22* worms rescues higher levels of dauer formation than pheromone derived from worms expressing the *Pdpy-7::gfp::daf-22* transgene. It is important to note that the conditions in which cultures were grown for HPLC-MS analysis and dauer pheromone collection were vastly different. As ascaroside production is regulated by the nutritional status of the worm, this may explain why dauer-inducing pheromones ascr#2 and ascr#8 were not detected in the well-fed cultures from the HPLC-MS analysis.[12]

2.3.4 GFP::DAF-22 Rescue Restores Male Attraction Behavior

Hermaphrodite worms are known to produce a chemical cue that attracts males.[13-14] Guided fractionation studies revealed ascr#2 and ascr#3 as the major components of the male attracting signal. Although incapable of attracting *C. elegans* males alone, ascr#4 was shown to synergize with ascr#2 and ascr#3 to enhance male attraction.[5] We therefore sought to examine whether or not tissue-specific GFP::DAF-22 expression could rescue male-attracting activity in pheromone-deficient worms.

Pheromone cues were collected from *Pvha-6::gfp::daf-22*, *Pmyo-3::gfp::daf-22*, and *Pdpy-7::gfp::daf-22* expressing worms by soaking thoroughly washed synchronized adult hermaphrodites in buffer. The biological activity of each cue was tested by measuring the number of male worms in regions containing cue compared to a control buffer region and calculating a chemotaxis index (Figure 2.5). Media conditioned with *C. elegans* hermaphrodites from each tissue-specific GFP::DAF-22 strain demonstrated the ability to attract male worms to a similar degree as media conditioned with wild type N2 worms

(Figure 2.6). The male attraction chemotaxis indexes of cues collected from *Pvha-6::gfp::daf-22*, *Pmyo-3::gfp::daf-22*, and *Pdpy-7::gfp::daf-22* expressing hermaphrodite worms were 0.72 ± 0.16, 0.71 ± 0.14, and 0.71 ± 0.06, respectively. In comparison, pheromone collected from wild type N2 worms produced a male attraction chemotaxis index of 0.82 ± 0.10. As expected, all cues were significantly more attractive than the buffer control (chemotaxis index of 0.03 ± 0.28) and pheromone collected from the *daf-22* loss-of-function mutant (chemotaxis index of 0.28 ± 0.31).

Although ascr#2 was not detected in any culture including N2, ascr#3 was detected in cultures of all three tissue-specific rescue strains. Furthermore, ascr#4 was produced in worms expressing the *Pvha-6::gfp::daf-22* transgene at levels similar to wild type, and to a lesser extent in *Pdpy-7::gfp::daf-22* expressing worms. Although ascr#3 levels produced by rescue of GFP::DAF-22 in the body wall muscle were very low compared to wild type and the other rescue strains, the concentrations of ascr#3 required to induce male attraction lies within the pico- to nanomolar range. Therefore, only very small quantities of ascr#3, such as those displayed by the HPLC-MS data for GFP::DAF-22 rescue in the body wall muscle, are required to attract male *C. elegans* worms, which could explain the near wild type levels of male attraction achieved by pheromone cues collected from *Pmyo-3::gfp::daf-22* rescue worms.

2.3.5 Hermaphrodite Conditioned Media Does Not Induce Repulsive Behavior Amongst Hermaphrodites

Ascarosides have also been shown to regulate social behaviors such as aggregation and repulsion. Dauer pheromone components ascr#2, ascr#3, and ascr#5 were all shown to be repulsive to *C. elegans* hermaphrodite worms.[15] On the other hand, icas#3 and icas#9 were shown to be effective hermaphrodite aggregation signals.[16]

In order to test whether tissue-specific rescue of GFP::DAF-22 causes differences in social behavior, we collected pheromone cues from each transgenic strain by soaking synchronized hermaphrodite worms in buffer for either 6 or 24 hours, and compared how hermaphrodite worms react to each cue compared to a buffer control in a four-quadrant assay (Figure 2.7). A chemotaxis index of 1 indicates a high level of attraction, whereas a chemotaxis index of -1 indicates a high level of repulsion.

Calculated chemotaxis indexes from the repulsion assay were all close to a value of zero, and therefore revealed worms were neither repelled nor attracted to the 6 and 24 hour cues (Figure 2.8). In fact, cues derived from all three tissue-specific GFP::DAF-22 rescue strains, wild type N2 worms, and *daf-22(ok693)* loss-of-function mutant worms produced chemotaxis indexes similar to the buffer control. While the *daf-22(ok693)* cue may not have attracted or repelled hermaphrodite worms due to a complete lack of ascarosides, it may be possible that the cues generated by wild type worms and each tissue-specific strain contained conflicting signals. While all three tissue-specific GFP::DAF-22 rescue strains produce ascr#3, a repulsive ascaroside, all three tissues also produce icas#3, an aggregation pheromone. It is therefore possible that the two signals cancel each other out.

2.4 Conclusions

In summary, we have found that driving the expression of GFP::DAF-22 within the intestine, hypodermis, or body wall muscle within a *daf-22* loss-of-function background is able to rescue varying levels of ascaroside biosynthesis. HPLC-MS analysis of liquid culture supernatants reveals that the intestine is the main site of ascaroside biosynthesis with hypodermal GFP::DAF-22 rescue also producing significant levels of biologically active ascarosides.

The expression of the *Pdpy-7::gfp::daf-22* transgene did not diffuse through the entire hypodermis, but rather appeared in a punctate pattern. While this may be due to the three amino acid serine-lysine-isoleucine peroxisomal tag, whether GFP::DAF-22 expression is localized to the peroxisomes should be confirmed. This may be accomplished by constructing and injecting a *Pdpy-7::mcherrySKL* peroxisomal marker into the gonads of *Pdpy-7::gfp::daf-22* worms. Fluorescence microscopy may then be used to assess potential colocalization of the mCherry marker and GFP::DAF-22 within the peroxisomes.

Our results also indicated that hypodermal rescue of GFP::DAF-22 results in the production of 4'-modified derivatives of ascr#10. Previous research has indicated that 4'-modification of ascarosides requires the gut granules, lysosome-like organelles within the intestine. To assess possible leaky expression of GFP:DAF-22 within the gut of the hypodermal rescue worms, one could cross the *Pdpy-7::gfp::daf-22* expressing worms with *glo-1(zu39)* or *pgp-2(kx48)* strains, which contain mutations that cause a complete lack or have a reduced number of gut granules, respectively. The presence of 4'-modified

ascarosides in the excretome of such an animal would indicate that the hypodermis does contain the biological machinery required for 4'-modification.

Furthermore, the quantities of ascr#10 derivatives produced by hypodermal rescue of GFP::DAF-22 surpass the levels displayed by intestinal rescue. While higher resolution HPLC-MS studies would be needed to confirm this result, it may indicate that the 4'-modified ascarosides observed may not be due to leaky expression of the transgene. Other genes necessary for the formation of gut granules such as *pgp-2*, a member of the ABC transporter family, and *apb-3*, an adaptin, are also expressed in the hypodermis. Therefore, combined with the punctate expression pattern of *Pdpy-7::gfp::daf-22*, these results could suggest the presence of gut granule-like structures in the hypodermis. To probe this issue, worms expressing the *Pdpy-7::gfp::daf-22* transgene could be stained with LysoTracker Red, a dye that labels lysosome-related gut granules. Fluorescence microscopy could then be used to test for colocalization of GFP::DAF-22 with possible gut granule-like structures within the hypodermis.

Although dauer formation activity restored by GFP::DAF-22 rescue in each tissue roughly correlates with the quantities of dauer-inducing ascarosides observed in the HPLC-MS analysis, it is likely that the ascaroside profile of the dauer pheromone used in the dauer formation assays differs from that observed in the cultures used in our HPLC-MS studies. Ascaroside production varies upon multiple environmental factors, including nutritional state. Therefore, in future, it may be necessary to run an additional HPLC-MS analysis on the dauer pheromone, which was collected from starved worm cultures, in order to get a more complete ascaroside profile of actual dauer pheromone produced by each tissue-specific GFP::DAF-22 rescue strain.

Overall, this study demonstrates that ascaroside biosynthesis is a complex process that involves multiple tissues and organelles. Furthermore, ascarosides can have both distinct and redundant functions in regulating worm behavior, and thus further studies in ascaroside perception are also important.

2.5 Figures

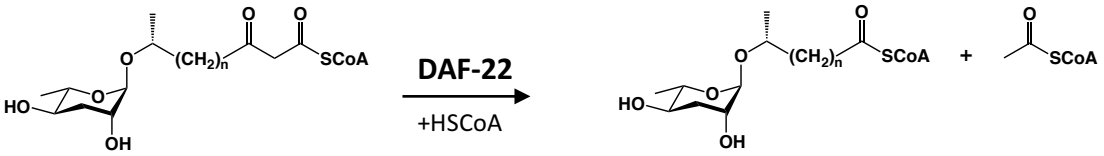

Figure 2.1: The Role of DAF-22 in Short-Chain Ascaroside Biosynthesis. DAF-22 comprises the fourth and last step of peroxisomal β-oxidation of the ascaroside lipid side chain in which the carbon chain is truncated by two carbons, which are released in the form of acetyl-CoA.

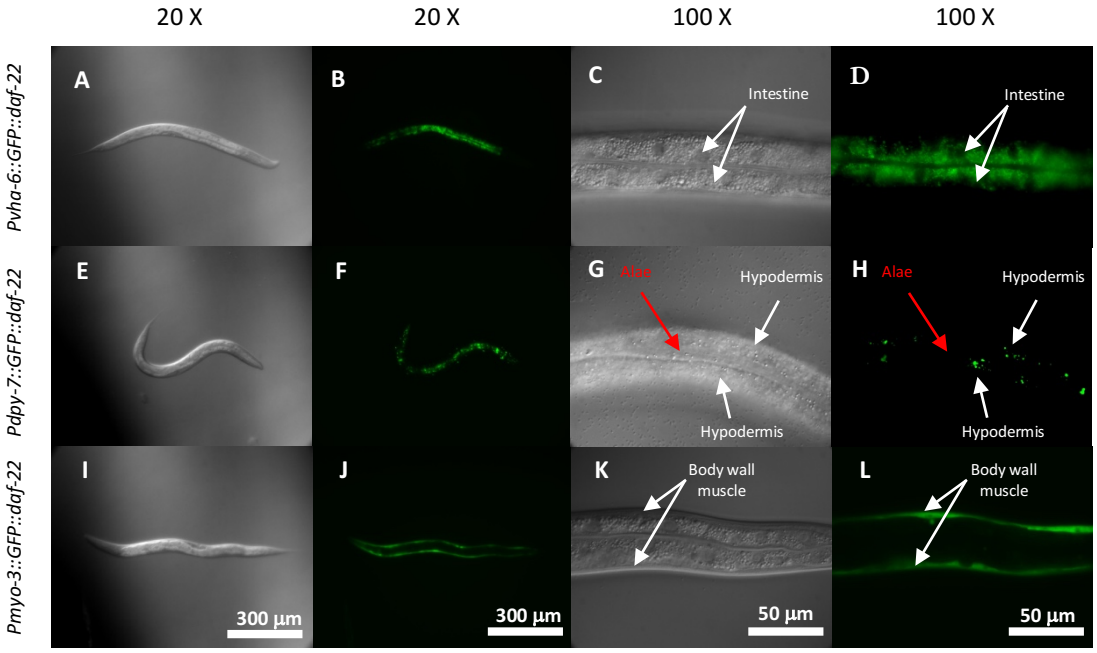

Figure 2.2: Tissue-Specific GFP::DAF-22 Expression. DIC and GFP fluorescence microscopy images of L4 larva expressing a GFP::DAF-22 transgene at 20X and 100X magnification. (A-D) *Pvha-6::gfp::daf-22* expresses in the intestine (white arrows). (E-H) *Pdpy-7::gfp::daf-22 expresses* in a punctate manner within the hypodermis (white arrows). The top focal plane is in focus, displaying the alae (red arrow). (I-L) *Pmyo-3::gfp::daf-22* expresses within the body wall muscle (white arrows).

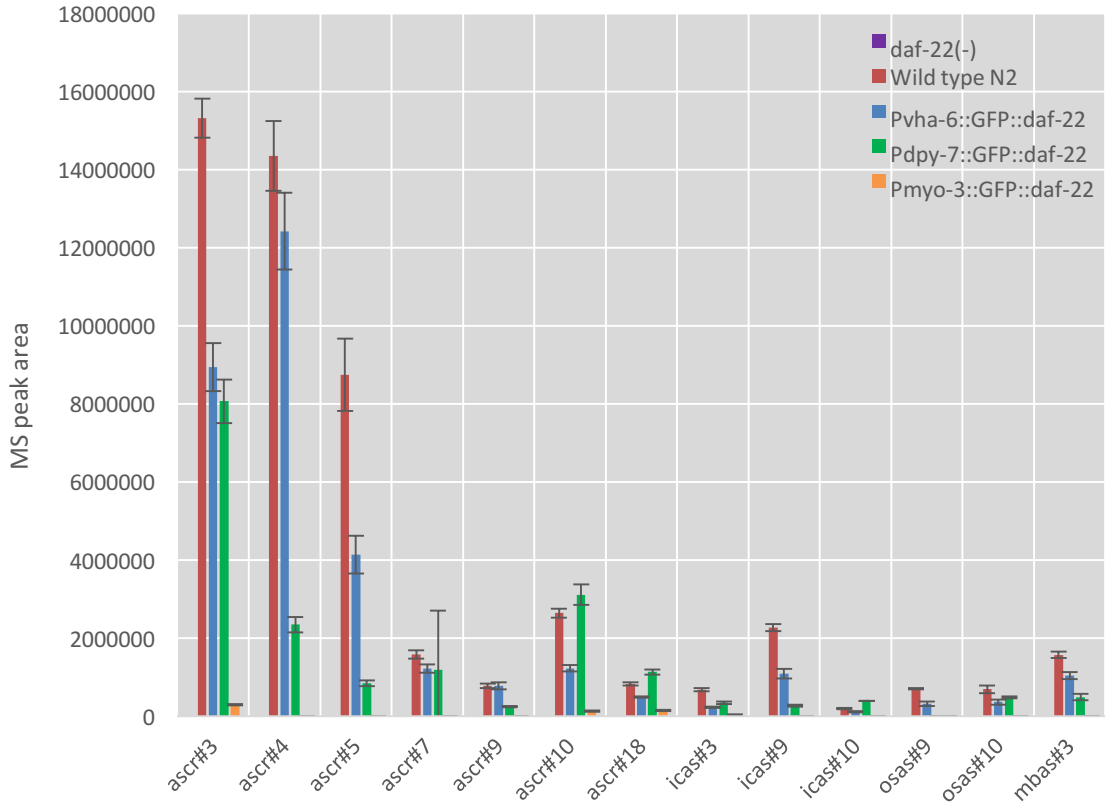

Figure 2.3: Tissue-Specific Ascaroside Profiles. HPLC-MS analysis of liquid culture supernatant reveals ascaroside profiles of worms expressing GFP::DAF-22 in the intestine (*Pvha-6::gfp::daf-22*), the hypodermis (*Pdpy-7::gfp::daf-22*), and the body wall muscle (*Pmyo-3::gfp::daf-22*). Averages and standard error of the mean were calculated from three biological replicates. Daumone, or ascr#1, could not be resolved from other metabolites and therefore could not be assessed.

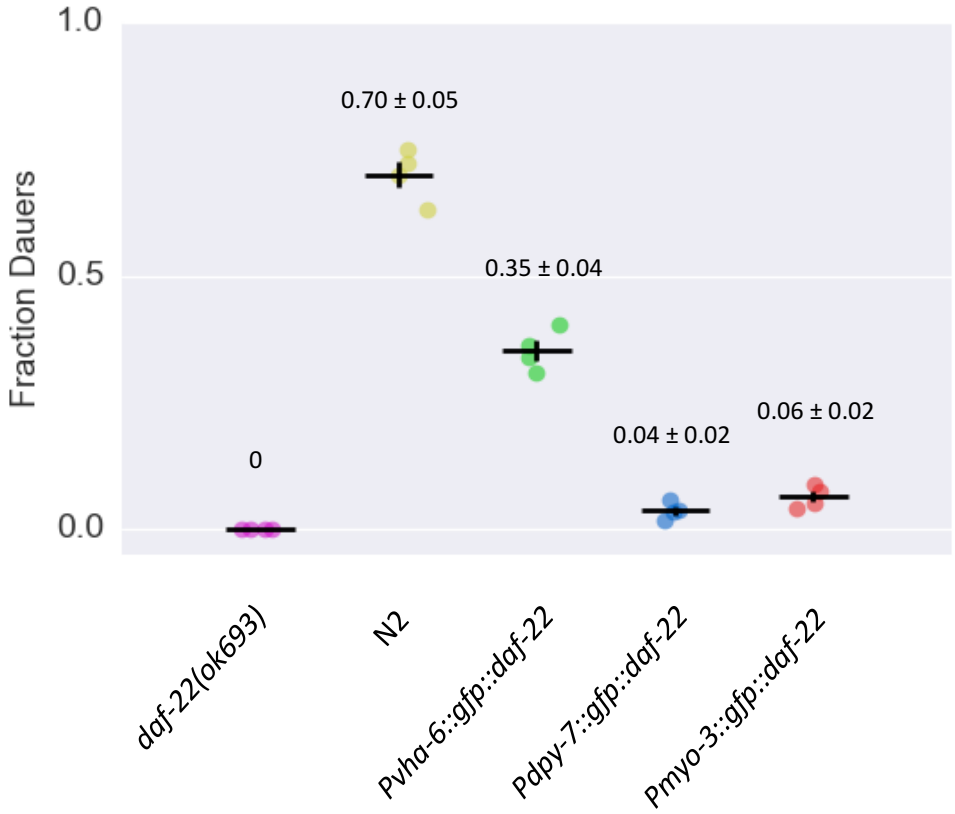

Figure 2.4: Dauer Formation Activity is Restored by GFP::DAF-22 Rescue. Dauer formation activity, based on worm morphology, was measured in N2 worms exposed to exogenous dauer pheromone collected from *daf-22(ok693)* loss-of-function worms, wild type N2 worms, and worms expressing GFP::DAF-22 in the intestine (*Pvha-6::gfp::daf-22*), hypodermis (*Pdpy-7::gfp::daf-22*), or body wall muscle (*Pmyo-3::gfp::daf-22*). Dauer pheromone collected from all three tissue-specific transgenic GFP::DAF-22 strains demonstrated partially restored dauer formation ability compared to pheromone derived from loss-of-function *daf-22(ok693)* worms. However, intestinal GFP::DAF-22 expression restored the most activity. Averages and standard error of the mean were calculated from a minimum of four trials.

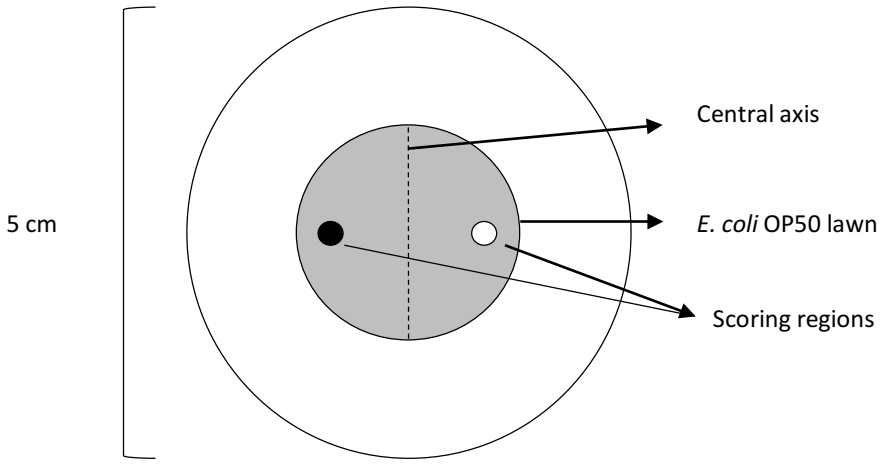

Figure 2.5: Male Retention Bioassay. A representation of the bioassay used to measure male retention by a hermaphrodite generated cue. Media conditioned with adult hermaphrodites was spotted on one side of the plate with wash buffer placed on the other side as a control. Male worms were placed along the central axis and behavior was recorded for 20 minutes, from which a chemotaxis index was computed.

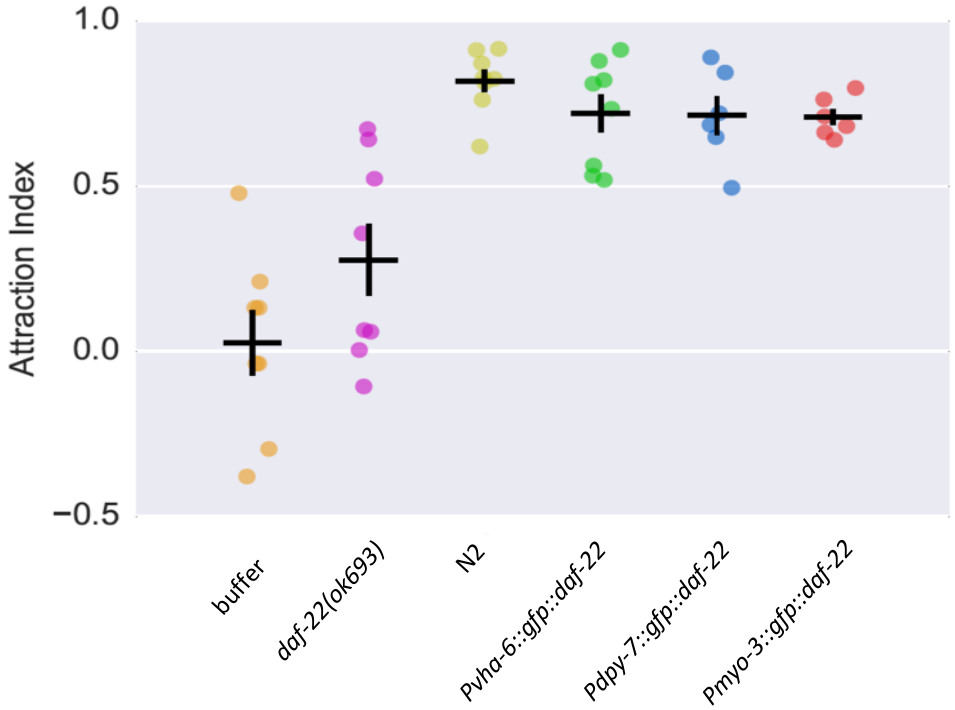

Figure 2.6: Male Attraction is Restored by GFP::DAF-22 Rescue. Male worm response (attraction index) to cues generated by hermaphrodites expressing GFP::DAF-22 in the intestine (*Pvha-6::gfp::daf-22*), hypodermis (*Pdpy-7::gfp::daf-22*), and body wall muscle (*Pmyo-3::gfp::daf-22*) are compared to cues generated by wild type N2 or *daf-22(ok693)* worms and a buffer control. Pheromone cues collected over a period of four hours from each tissue-specific GFP::DAF-22 transgenic strain were sufficient to restore male attractive behavior similar to wild type levels. Averages were calculated from a minimum of 6 trials. Error bars represent the standard error of the mean between trials.

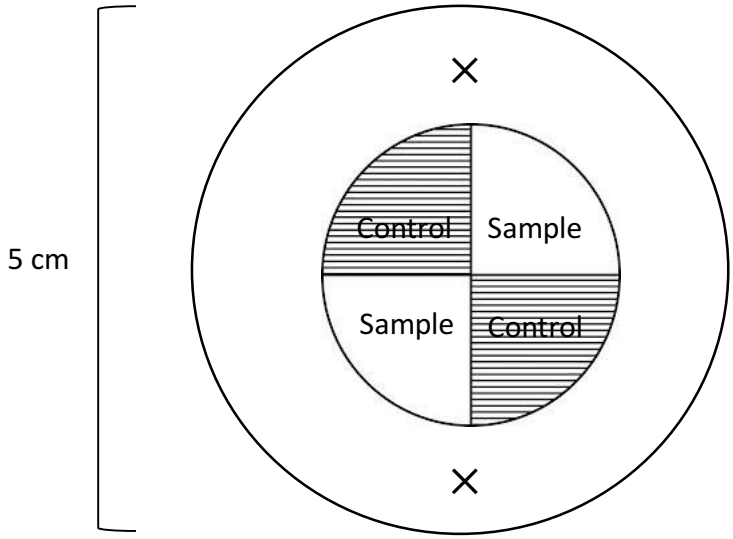

Figure 2.7: Hermaphrodite Repulsion Assay. A representation of the bioassay used to measure repulsion behavior. Buffer control was applied to the shaded quadrants and pheromone cue collected from hermaphrodite worms was applied to the white quadrants. The two X's denote where washed hermaphrodite worms were placed on the plate. Behavior was recorded for 10 minutes, from which a chemotaxis index was calculated.

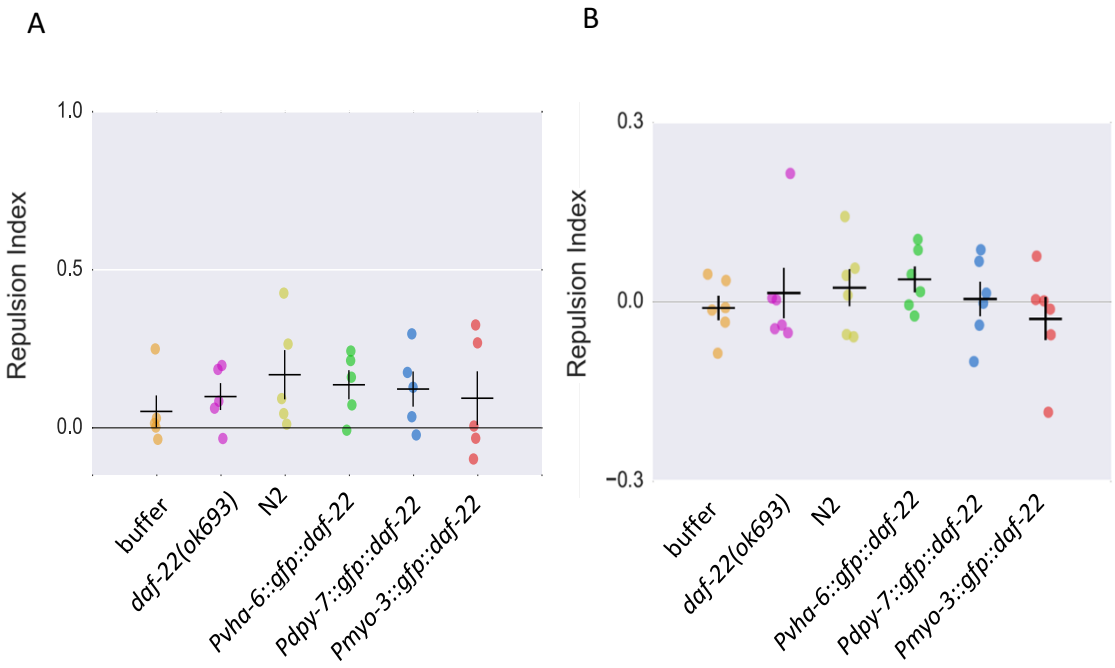

Figure 2.8: Hermaphrodites Neither Attract nor Repel Each Other. Adult hermaphrodite worm response (repulsion index) to cues generated by hermaphrodites expressing GFP::DAF-22 in the intestine (*Pvha-6::gfp::daf-22*), hypodermis (*Pdpy-7::gfp::daf-22*), and body wall muscle (*Pmyo-3::gfp::daf-22*) compared to cues generated by wild type N2 or *daf-22(ok693)* worms and a buffer control. Cues were collected either over (A) 6 hours, or (B) 24 hours. Averages were calculated from a minimum of 5 trials. Error bars represent the standard error of the mean between trials.

2.6 Materials and Methods

2.6.1 C. elegans Strains and General Culture Methods

All strains were maintained at room temperature on standard Nematode Growth Medium (NGM) agar plates, which were made with Bacto agar (BD Biosciences) and seeded with *Escherichia coli* OP50 bacteria grown overnight as food.[17] Wild type reference Bristol strain N2, CB1490 *him-5(e1490)*, and RB859 *daf-22(ok693)* mutant strains were obtained from the Caenorhabditis Genetics Center (Minneapolis, MN).

2.6.2 Construction and Expression of GFP::DAF-22

To construct plasmid pAEA1 (*Pvha-6::gfp::daf-22*), the *gfp::daf-22* cDNA fragment and *vha-6* promoter sequence were amplified by PCR from plasmid HYM433, generously provided by H.Y. Mak using primers oAA017, oAA018, oAA019, and oAA020.[4] The 3'UTR region of the *unc-54* gene was PCR amplified from plasmid pPH93, a gift from the Bargmann Lab, using primers oAA015 and oAA016. These three segments were inserted into backbone vector pBluescript SK(+) between KpnI and ClaI restriction sites using the Gibson assembly system (New England BioLabs, USA).[18]

To construct plasmids pAEA3 (*Pdpy-7::gfp::daf-22*) and pAEA4 (*Pmyo-3::gfp::daf-22*) and, the *dpy-7* promoter was amplified by PCR from genomic DNA using 5' primer oAA013 and 3' primer oAA014 and the *myo-3* promoter sequence was PCR amplified from plasmid jc10ssfo es1 using 5' primer oAA011 and 3' primer oAA012. Each promoter was subsequently inserted between KpnI and AscI restriction sites of a pAEA1 vector backbone. Transgenic lines were generated by microinjecting a mixture containing

either pAEA1, pAEA3, or pAEA4 (10 ng/μL), a *Pmyo-2::dsRed* co-injection marker (5 ng/μL), and 1 kb DNA ladder (New England BioLabs, Massachusetts, 85 ng/μL) into the germ line of adult hermaphrodite *daf-22(ok693)* worms using standard techniques.[19] Extrachromosomal arrays were integrated into the background genome via X-ray irradiation. All integrated strains were outcrossed ten times with *daf-22(ok693)*, which had previously been outcrossed ten times with wild type N2 worms.

2.6.3 Preparation of Nematode Culture and Metabolite Extracts

Gravid hermaphrodite worms were collected and treated with a 2:1:7 mixture of 5% NaClO, 5M NaOH, and double distilled water for 2 minutes. Eggs were washed three times with M9 buffer and resuspended at a concentration of 2 eggs/μL in 100 mL of S-complete medium supplemented with *E. coli* HB101. Cultures were grown at 22°C on a rotary shaker until worms reached the young adult stage. Metabolite extractions were performed as previously reported.[9] Liquid cultures were centrifuged, and the supernatant and worm pellets were frozen separately. After lyophilization, the dried supernatant was extracted with 35 mL of 95% ethanol at room temperature for 12 hours. Extracts were then dried *in vacuo*, resuspended in methanol and analyzed by LC-MS. All cultures were grown in three biological replicates.

2.6.4 HPLC-MS Analysis

As performed previously, low resolution HPLC-MS was performed using the Agilent 1100 Series HPLC system equipped with an Agilent Eclipse XDB-C18 column (250 mm x

9.4 mm, particle size 5 μm), connected to a Quattro II or Quattro Ultima mass spectrometer.[6, 9] A 0.1% acetic acid-acetonitrile solvent gradient was used starting with an acetonitrile content of 5% for 5 minutes after injection which was then increased to 100% over a period of 40 minutes. Metabolic extracts were analyzed by HPLC-ESI-MS in the negative ionization mode using a capillary voltage of 3.5 kV and a cone voltage of -40 V. Ascarosides were detected as $[M-H]^-$ ions and confirmed through comparisons with the retention times of synthetic standards.

2.6.5 Dauer Formation Assay

The preparation of crude dauer pheromone and dauer formation assay were performed as previously described with modification.[20] Crude pheromone was extracted with 95% ethanol from exhausted liquid culture medium and resuspended with double distilled water. Assay plates were prepared the day before each experiment by adding crude pheromone to NGM agar containing no peptone and dried overnight at room temperature. Heat-killed *E. coli* OP50 was used as a limited food source and was prepared by resuspending overnight OP50 cultures in S-basal at a concentration of 8 g/mL and heating at 100°C for 5 minutes. On the day of the experiment, 2 μL of heat-killed *E. coli* OP50 was spotted onto each pheromone plate. Ten young adult worms were picked onto each plate and allowed to lay 50-60 eggs before being removed. Another 18 μL of heat-killed OP50 was added to the plates as a food source for the unhatched larvae. Plates were incubated for 48 hours at 25°C, after which dauers and non-dauer worms were counted based on morphology.

2.6.6 Male Retention Assay

To collect pheromone cures, worms were selected as L4 larva and allowed to grow overnight into adult worms. Synchronized adults were washed four times in M9 buffer, then allowed to soak in M9 buffer in the cap of an inverted 1.5 mL Eppendorf tube at a concentration of 1 worm/μL. After 4 hours, and centrifugation, the supernatant was collected and stored at -20°C. As described by Choe *et al*, a 16 mm lawn of *E. coli* OP50 was grown overnight on 5 cm NGM agar plates at 20°C the day prior to the experiment.[21] Two 0.6 μL spots, one a control consisting of the last M9 wash cycle and the other the pheromone cue, were placed on opposite sides of the bacterial lawn using a transparent template. The liquid was allowed to settle into the agar. 10 male worms, isolated as L4 larva the night before the experiment, were placed along the central axis of the bacterial lawn and video recording began immediately upon worm placement. Trials were recorded for 20 minutes at a frame rate of 1 frame per second using the behavior chamber and camera set up described by Chai *et al*.[22] The results were averaged from at least six different trials. To compare worm occupancy in each scoring region, automated software was used to calculate the Chemotaxis Index, as outlined by Bargmann *et al*.[23]

2.6.7 Hermaphrodite Repulsion Assay

Pheromone cues were generated similarly to those used for the male retention assay, but were collected over a period of 6 or 24 hours. Repulsion behavior was assessed using the protocol outlined previously.[22] On day 1, 10 well-fed gravid hermaphrodite worms were picked onto NGM agar plates seeded with *E. coli* OP50 the night before, and allowed to lay

eggs for six hours. The worms were then removed from the plates and progeny were allowed to grow at 20°C for three days into adults. On day 3, 35 mm chemotaxis agar plates were prepared and allowed to dry overnight at room temperature. On day 4, synchronized adult worms from two NGM plates were washed three times in chemotaxis buffer. During the second wash, 1 μL of M9 buffer (quadrants 1 and 4) or cue (quadrants 2 and 3) were added to the 35 mm assay plate using a transparent template. After complete washing, worms were transferred to the assay plate above and below the circular treated region and a folded tissue was used to absorb excess wash buffer. Using video capture software, behavior was recorded for 10 minutes at a frame rate of 1 frame per second. The experiment was repeated at least 5 times for each pheromone cue.

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