Effects of RNAi Feeding Experiments To Silence the *lgx-1* and F48E3.8 genes of *Caenorhabditis elegans*

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Abstract

Lymphatic filariasis affects a large area of the world and current preventative therapies are unable to control and eradicate it. Both ivermectin and DEC, the current drug treatments, are becoming less desirable because of an increased resistance by adult female worms and harmful side effects to human hosts, respectively (Babu *et al.*, 2006; Osei-Atweneboana *et al.*, 2011). The structural polymer, chitin, is found in the filarial nematodes that cause elephantiasis as well as in the free living nematode *C. elegans* (Harris *et al.*, 2000; Zhang, Foster, Nelson, Ma, & Carlow, 2005). By studying the effects of chitin modification in *C. elegans*, new preventative methods of lymphatic filariasis may emerge. This paper looked at the resulting changes to development and surviving progeny of silencing the *lgx-1* and F48E3.8 genes, both of which encode putative chitin deacetylase catalytic regions, in *C. elegans* through RNAi feeding experiments. Experiments silencing just the *lgx-1* gene showed a developmental delay while having no effect on brood size. Silencing both chitin deacetylase genes shows a combined effect of retarded development as well as a significant decrease in mean brood size. Silencing the *lgx-1* gene, expressed in the pharynx, may prevent complete elongation of the feeding organ resulting in a slower distribution of nutrients to the growing worm. The decrease in fertility seen by knocking down the F48E3.8 gene indicates that the conversion to chitosan is crucial in the control of pharyngeal elongation in developing embryos or ability to hatch from eggshells.

TABLE OF CONTENTS

I. Introduction

A. Lymphatic filariasis and *C. elegans*

Lymphatic filariasis, also known as elephantiasis, affects over 120 million individuals in 81 countries (WHO, 2011). This parasitic disease is caused by the roundworms *Wuchereria bancrofti*, *Brugia malayi*, and *Brugia timori* (WHO, 2011); all of which eventually reside in the lymphatic system of their human hosts. Acute symptoms such as swelling of lymph nodes and skin can leave victims in a state of painful disfigurement and disability. The disease is transmitted by parasite transfer through an insect vector, putting tropical countries at the highest risk (WHO, 2011).

Current antihelminthic therapies, such as ivermectin and Diethylcarbamazine (DEC), may need to be updated because of parasitic resistance and dangerous side effects in the hosts, respectively (Babu *et al.*, 2006; Mak, 2004; Osei-Atweneboana *et al.*, 2011). Ivermectin kills microfilariae while also preventing release of microfilariae from adult females by paralyzing the lower uterus (Osei-Atweneboana *et al.*, 2011). It does not, however, appear to have a strong antifilarial effect on the adult worms (Mak, 2004; Osei-Atweneboana *et al.*, 2011). More recently, studies have now shown that adult females are becoming resistant to anti-fecundity effects of the drug drastically reducing its overall effectiveness as a possible therapy (Osei-Atweneboana *et al.*, 2011).

DEC, one of the original treatments for the control of lymphatic filariasis, is still being used today in combination with newer drugs, such as ivermectin (Mak, 2004). DEC is also another microfilaricide, but unlike ivermectin, kills adult worms as well (Babu *et al.*, 2006; Mak, 2004). Though its ability to overcome parasitic infection is

strong, adverse reactions, from fever to uncontrollable itching, put the patient in a dangerous risk (Babu *et al.*, 2006).

A need for novel methods to protect the uninfected and treat the parasitized hosts has thus arisen. *Caenorhabditis elegans*, a free living nematode, may help provide insight for a new type of therapy. *C. elegans* shares many characteristics with filarial nematodes such as *B. malayi*; one to note is the use of chitin as a structural polymer in both somatic tissue and eggshells (Harris *et al.*, 2000; Zhang *et al.*, 2005). Chitin is not found in vertebrates; therefore it may be a suitable target for antihelminthics that should not carry adverse effects.

C. elegans shares considerable sequence similarity with filarial nematodes and its ease of maintenance allows for low costs and large sample sizes. As a free living organism, it can live without a host and simply consume bacteria (either alive or dead). Furthermore, *C. elegans* reproductive nature is unique in that worms are either male (XO) or hermaphrodites (XX) with a ratio of 1:500 (Hodgkin, Horvitz, & Brenner, 1979). Therefore, most of the experimental worms will give birth to offspring. This paper focuses on chitin modification genes in *C. elegans* to test novel targets for filarial control.

B. Chitin and Chitosan

Chitin, a linear β-(1,4)-linked polymer of N-acetylglucosamine (GlcNAc), plays an integral role as a structural component in a variety of species and is believed to be the second most prevalent polysaccharide synthesized after cellulose (Merzendorfer, 2006). It is found in invertebrates and fungi, both as external and internal support structures strengthening cell walls and exoskeletons, and coating eggshells. (Arakane *et al.*, 2009; Luschnig, Batz, Armbruster, & Krasnow, 2006; Zhang *et al.*, 2005).

Its importance has been widely studied in numerous fungi and insect models by analyzing the specific genes that code for chitin synthases as well as chitinases. First purified in the 1980s in *Saccharomyces cerevisiae* (Cabib, Kang, & Au-Young, 1987), chitin synthases have since been extracted from higher eukaryotes, such as insects and nematodes (Cabib *et al.*, 1987; Tellam, Vuocolo, Johnson, Jarmey, & Pearson, 2000; Zhang *et al.*, 2005). Chitin synthase catalyzes glycosidic bond formation to add subunits of N- acetylglucosamine to increase the polymer length. The polymerization of UDP-N-acetylglucosamine produces insoluble chitin (Cabib *et al.*, 1987).

Many insects use chitin as a component of the exoskeleton, which is shed during molts. Therefore, chitin synthases are vital to the growth and development of the pre-adult and adult stages of the organism (Merzendorfer, 2006). This is clearly seen in the genus *Tribolium*, as well as other insects (Arakane *et al.*, 2009). *Tribolium* uses chitin in the extracellular matrix of the exoskeleton and alters the polymer to make the cuticle inflexible or bendable (Arakane *et al.*, 2009). Furthermore, chitin is found internally in several invertebrates. *Drosophila* synthesizes chitin in its embryonic tracheal lining (Luschnig *et al.*, 2006; Merzendorfer, 2006) while *Caenorhabditis* lines its pharynx with chitin (Zhang *et al.*, 2005). Another crucial role of chitin is in the structure of the eggshells in all nematodes (Zhang *et al.*, 2005). *C. elegans*, for example, contains two chitin synthases, one of which is located in the oocyte for eggshell development following fertilization (Zhang *et al.*, 2005).

It is possible because of chitin's rigidity that it must be altered in some way so that the organisms that employ it can grow. A unique characteristic of chitin is an acetyl group on the amine attached to the glucose ring (Figure 1). Following deacetylation of

this moiety, the free amine group renders the new molecule, chitosan, polar and more soluble in water (Merzendorfer, 2006). Chitosan is structurally similar to chitin except for a protonated amino group (Figure 1). Chitosan is much less rigid than its acetylated counterpart and a possible transitional polymer to allow for growth and development of the organism.

The N-deacetylation of chitin to chitosan has been the focus of research in a variety of insect and nematode models (Arakane *et al.*, 2009; Luschnig *et al.*, 2006). Gene sequences for nine separate chitin deacetylase (CDA)-like proteins have been characterized in *Tribolium castaneum* (Arakane *et al.*, 2009). *TcCDA1* and *TcCDA2* have been shown to affect all stages of life and their silencing prevented the beetle from shedding its old cuticle (Arakane *et al.*, 2009).

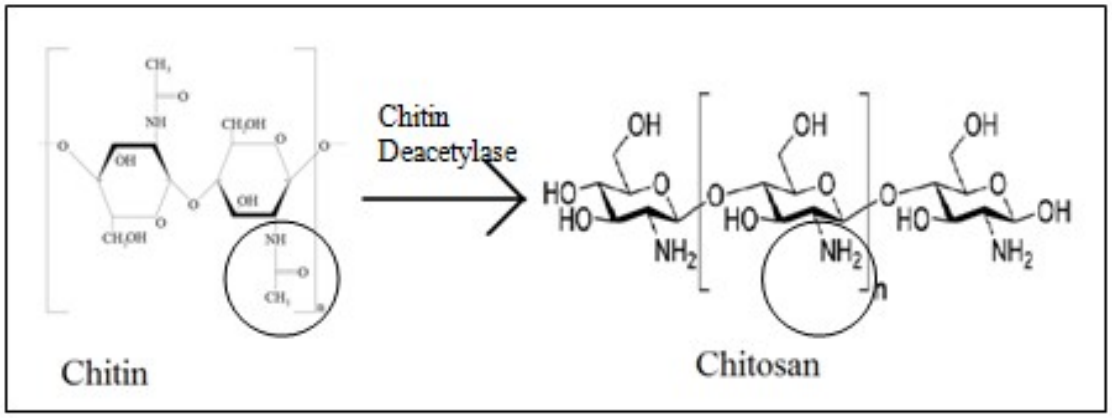


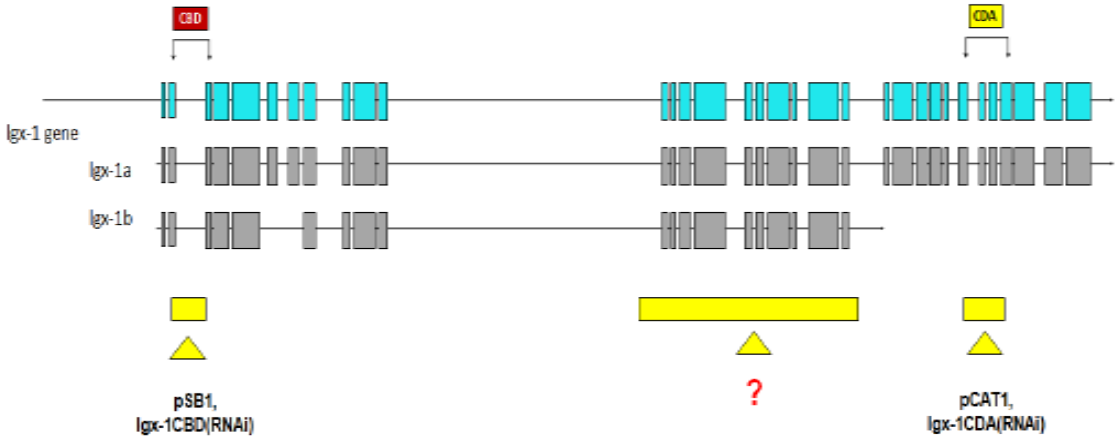
Figure 1: Conversion of chitin to chitosan by chitin deacetylase

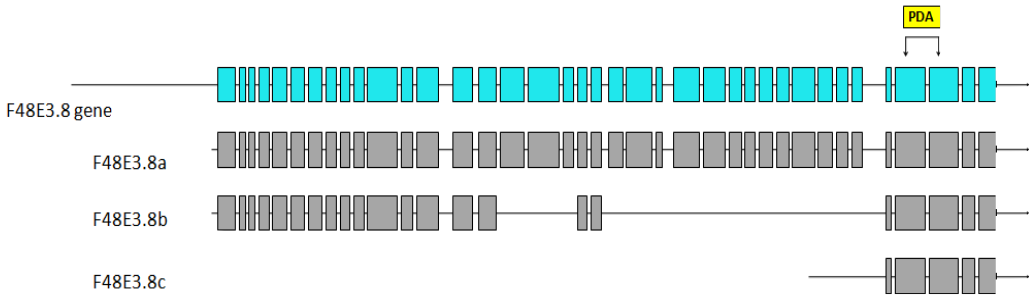
Similar growth defects are seen in *Drosophila* when *serpentine* and *vermiform* genes were silenced (Luschnig *et al.*, 2006). These genes encode matrix proteins for chitin modification, including the deacetylation of chitin. Gene silencing resulted in disproportionate tracheal elongation and death of the insect embryo (Luschnig *et al.*, 2006). If the conversion of chitin to chitosan could not occur, an inability to molt and/or control its length may present itself in organisms that use the polysaccharide as part of its exoskeleton or tracheal lining (Arakane *et al.*, 2009; Luschnig *et al.*, 2006).

C. The Putative Chitin Deacetylase Genes *lgx-1* and F48E3.8

In *C. elegans*, two putative chitin deacetylase (CDA) catalytic regions have been identified on the *lgx-1* and F48E3.8 genes. At least two transcripts of the *lgx-1* gene are known; of the two, *lgx-1a* contains both CBD (chitin binding domain) and CDA regions while *lgx-1b* contains a truncated sequence, ending with exon 21. Both transcripts contain a CBD in exons 2 and 3 while exons 27-30 contain the CDA found in *lgx-1a* (Figure 2). An unusually large intron also separates the 11th and 12th exon.

lgx-1 (13,315 bp)
RNAi constructs inc. Yuji Kohara insert





Through *in situ* experiments, *lgx-1* transcripts have been located in the pharynx of *C. elegans* (http://nematode.lab.nig.ac.jp/). Because the pharynx is lined with chitin (Zhang *et al.*, 2005), the conversion to chitosan may be used for elongation during molts. Knock out mutations or silencing of the *lgx-1* gene may therefore cause morphological problems which could lead to worm death or delayed growth similar to *vermiform* and *serpentine* silencing in *Drosophila* (Luschnig *et al.*, 2006).

The F48E3.8 gene codes for another putative chitin deacetylase protein (Figure 3). It was first identified through bioinformatics by Ronald Jason Heustis, Tufts (personal communication). Very little is known about this gene and its supposed catalytic properties in deacetylating chitin. Based on previous research of *lgx-1* silencing, the F48E3.8 gene underwent RNA interference experiments to determine its impact, if any, on the growth and development of *C. elegans* (personal communication).

D. RNA Interference (RNAi)

To study the purpose of chitin and chitosan in *C. elegans*, RNAi feeding techniques were used and the resulting phenotypes were analyzed. RNA interference (RNAi) is a method of gene silencing in which small fragments of double stranded RNA (dsRNA) cause the degradation of entire mRNA strands with complementary sequences.

This natural ability evolved as a defense against viruses that contain their genomic code as dsRNA. Once detected, the dsRNA is cut by an RNase III family protein called dicer into 21-23- nucleotide RNA sequences called small interfering RNAs (siRNA) (Elbashir, Lendeckel, & Tuschl, 2001). The siRNA are then bound to the argonaute protein forming an RNA Induced Silencing Complex (RISC) (Hammond, Bernstein,

Beach, & Hannon, 2000). This enzyme catalyzes the degradation of complementary mRNA sequences. Without a full mRNA sequence, no protein is synthesized and the gene is said to be silenced. By inserting a fragment of dsRNA of the *lgx-1* and *F48E3.8* genes into *C. elegans*, we can study the change in phenotype when chitin deacetylation is blocked.

We chose RNAi feeding experiments to silence the putative chitin deacetylase genes in *C. elegans*. This method was preferred because of its low cost and synthesis of dsRNA *in vivo* (Kamath, Martinez-Campos, Zipperlen, Fraser, & Ahringer, 2001). Selective media is used to stimulate RNA synthesis in bacteria which form dsRNA and are subsequently fed to L3 stage worms.

NL2099 is a strain of *C. elegans* that has the *rrf-3* gene knocked out. The mutation of this gene causes a hypersensitivity to RNAi because of the absent RNA directed RNA polymerase (RdRP) (Maine, 2010; Simmer *et al.*, 2002; Simmer *et al.*, 2003). Normally, both the RRF-3 and the RRF-1 (another RdRP) proteins compete for reactions with siRNAs. The RRF-3 inhibits somatic RNAi where as RRF-1 stimulates it. The *rrf-3* gene knockout eliminates this competition and the RNAi signal is amplified, silencing the gene more efficiently (Maine, 2010).

The amplified signal must then be transmitted throughout the organism. The silencing effects of ingested dsRNA move throughout *C. elegans* because of the SID-1 protein (Jose, Smith, & Hunter, 2009). The SID-1 dsRNA channel protein is used to extend the silencing effects of RNAi to cells sensitive to the message (Winston, Molodowitch, & Hunter, 2002). It has been shown that SID-1 is only vital in the import of dsRNA, while the export is independently controlled (Jose *et al.*, 2009).

The *E. coli* strain, HT115(DE3), fed to the worms is useful in RNAi experiments because of the absence of RNase III (Timmons, Court, & Fire, 2001). Without this enzyme, dsRNA sequences are not degraded. This allows multiple worm generations to feed on this strain and continuously induce gene silencing through RNA interference (Timmons *et al.*, 2001).

In this paper, RNAi feeding experiments were used to silence the chitin deacetylase genes *lgx-1* and F48E3.8, preventing chitin modification. The subsequent effect on phenotypes in development and brood size were then used to understand the role of chitosan in the organism *C. elegans.*

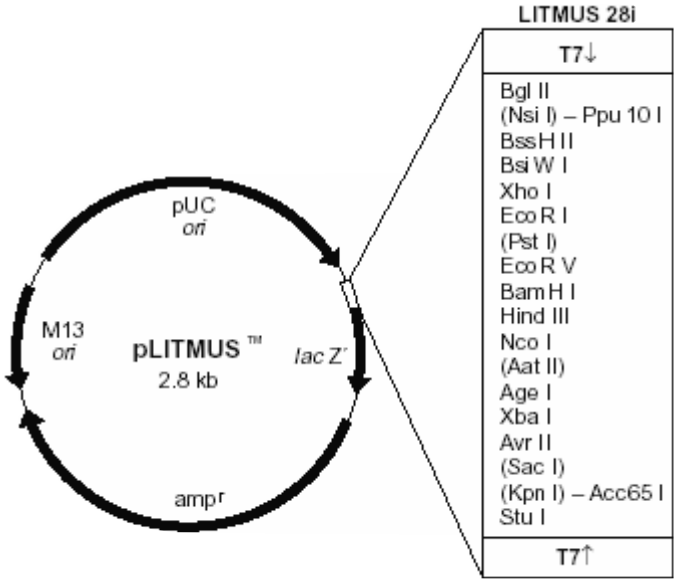
II. Methods

To study the role of chitin deacetylase, RNAi feeding experiments were utilized to silence the *lgx-1* and *F48E3.8* genes in *C. elegans*. The *rrf-3* deficient strain of *C. elegans*, NL2099, was used because of its hypersensitivity to RNAi (Simmer *et al.*, 2003). This hypersensitivity is increased at warmer temperatures, while the brood size is significantly decreased (Simmer *et al.*, 2002). To strike a balance between brood size and RNAi hypersensitivity, the feeding experiments were conducted at 20°C, with the exception of Trial 5, which used N2 wild type worms at 25°C.

Two plasmids containing sequences from the *lgx-1* and F48E3.8 genes were transformed into HT115(DE3) cells. Fragments from these genes were inserted into the Litmus 28i vector flanked by two T7 RNA polymerase promoters in inverted orientation (Figure 3) (Kamath *et al.*, 2001).

Worms were plated on nematode growth medium (NGM) with 25µg/mL carbenicillin and 1.0mM of IPTG (Wood, 1988). This medium is a healthy living

environment for *C. elegans* as well as a stable growing platform for the *E. coli* strain HT115(DE3). The carbenicillin added to the NGM was used to select for HT115(DE3) cells that were successfully transformed with the Litmus 28i vector, which carries an ampicillin resistance gene (Figure 3). To stimulate dsRNA production, the bacteria were allowed to grow on plates containing IPTG, an allolactose analog. The T7 RNA polymerase gene is controlled by a *lac* promoter in the HT115(DE3) strain.



To initiate RNAi, L3 stage worms were placed on NGM plates (Day 0) that had previously been seeded with bacteria according to the following protocol:

On Day -2, the *E. coli* strain HT115(DE3), carrying a Litmus vector, was inoculated into LB + 100µg/mL ampicillin (amp) + 12.5µg/mL tetracycline (tet) plates at 37ºC for 18-24 hours. Two groups of bacteria were used, an experimental and control. The experimental group of bacteria contained either the *lgx-1* fragment yk1621.B03 or the F48E3.8 gene fragment yk1130.A03. Both fragments were inserted into the Litmus

28i vector and transformed separately into the HT115(DE3) cells. The control group contained transformed cells with an empty Litmus 28i vector.

On Day -1, two to three drops of the bacterial suspension were plated on the $NGM_{carb+IPTG}$ plates without spreading. These were left to grow at room temperature overnight. An L3 stage worm was then placed on the center of the plate on the drop of bacteria (Day 0). The plates were then maintained at 20ºC for the remainder of the experiment.

The initial worm was removed on Day 2 (48 hours) and the progeny were counted and staged (egg, L1, L2, L3, L4, adult). On Day 3 (72 hours), progeny were again counted and staged (Table 1).

Two-tailed statistical tests using SPSS were run on mean number of hatched worms (L1-adult stage).

Table 1: Feeding Protocol Timeline

Day	Action
-2	Bacterial Inoculation
-1	Bacterial Plating
0	L3 Worm Placement
2	Original Worm Removal; Progeny Counted and Staged
3	Progeny Counted and Staged

III. Results

The *lgx-1* and F48E3.8 genes were chosen to be knocked down because of putative chitin deacetylase regions as indicated from bioinformatics. Silencing these genes allowed for a better understanding of chitosan's role in the development of *C. elegans*. Trials 1, 2, and 3 used RNAi feeding experiments to silence only the *lgx-1* gene from the NL2099 strain of *C. elegans*. Trials 4, 6, 7, and 8 silenced both the *lgx-1* and

F48E3.8 genes using the same RNAi experiments in Trials 1, 2, and 3 on the NL2099 strain worms. Trial 5 also silenced both genes; however, it was conducted on the N2 wild type strain of *C. elegans* at 25°C (Table 5).

During Trial 1, a significant difference was found between the mean number of worms on the experimental and control plates at the 48-hour time point (df=11; t= -4.683; p=0.001) (Table 2B). By 72 hours though, the total number of worms found on both plates was relatively consistent (Table 2A). Interestingly, however, a majority of the *lgx-1* knockouts were in the L2 stage while the control group had an equal number of worms in the L3 and L4 stage (Figure 4). This effect was most apparent in the 72-hour time point.

A similar effect was seen during Trial 3. Once again, at the 72-hour mark, a developmental delay was observed such that a larger proportion of the experimentally fed worms were identified as L2 stage while a majority of the control worms were in the L3 and L4 stage (Figure 6).

Trial 2 showed different results. Although the experimental and control groups did not differ significantly in mean progeny at either time point (Table 2B), fewer worms survived by the second time point. Both the LGX-1 deficient and control worms were identified as L3 stage showing equal growth rates (Figure 5).

A	Trial 1	Trial 2	Trial 3	
48 hrs	Experimental	41	12	100
	Control	96	24	152
72 hrs	Experimental	143	65	336
	Control	130	59	313

B	Trial 1	Trial 2	Trial 3	
48 hrs	Experimental	3.42*	1	8.333
	Control	8.00	2	12.67
72 hrs	Experimental	11.92	5.42	28.00
	Control	10.83	4.92	26.08

Both the *lgx-1* and F48E3.8 genes were silenced in Trial 4 and a combined response was seen. Not only were a majority of the experimental worms identified as L2 by the 72-hour time mark (Figure 7), but there was a very significant decrease in mean brood size at both time points (48 hour: df=11; t= -8.843;. $p<0.0001$; 72 hour: df=11; t= -11.023; $p<0.0001$) (Table 3B).

The chitin deacetylase deficient worms in Trial 6 also showed a developmental delay and a significant decrease in brood size at both time points (48 hour: df=11; t= -20.875; $p<0.0001$; 72 hour: df=11; t= -4.546, $p=0.001$) (Table 3A; Table 3B). Unlike previous trials, most of the experimental group was in the L3 stage while the control group was still split even between the L3 and L4 stages (Figure 9).

No delay effect was apparent in Trial 7 or Trial 8. The majority of worms in both groups were in the same life stage at both time points (Figure 10; Figure 11).

Furthermore, not only was there no brood size reduction in Trial 7, the experimental group had about a 25% increase in total hatched worms at 72-hours (Table 3A). Trial 8, however, had a significant difference in brood size at 48-hours (df=11, t= -3.360; p=0.006).

Both groups in Trial 5 – which used the N2 strain – grew with the same rate with a bulk of the progeny in the L3 stage by 72-hours (Figure 8). The mean brood size of the control group, however, was significantly larger than the experimental group at both time points (48: df=11; t= -5.945; p<0.0001; 72: df=11; t= -2.222; p=0.048) (Table 4B).

A	Trial 4	Trial 6	Trial 7	Trial 8	
48 hrs	Experimental	26	65	149	281
	Control	67	345	162	388
72 hrs	Experimental	63	435	500	571
	Control	271	818	398	650

B	Trial 4	Trial 6	Trial 7	Trial 8	
48 hrs	Experimental	2.17**	5.42**	12.42	23.42*
	Control	5.58	28.75	13.5	32.33
72 hrs	Experimental	5.25**	36.25*	41.667	47.58
	Control	22.58	68.17	33.17	54.17

A	Trial 5	
48 hrs	Experimental	93
	Control	315
72 hrs	Experimental	250
	Control	427

B	Trial 5	
48 hrs	Experimental	7.75**
	Control	26.25
72 hrs	Experimental	20.83*
	Control	35.58

Trial	Gene(s) Silenced
1	*lgx-1*
2[x]	*lgx-1*
3[x]	*lgx-1*
4	*lgx-1* and F48E3.8
5[x]	*lgx-1* and F48E3.8
6	*lgx-1* and F48E3.8
7[x]	*lgx-1* and F48E3.8
8[x]	*lgx-1* and F48E3.8

IV. Discussion

Blocking the conversion of chitin to chitosan causes developmental changes in phenotype in *C. elegans*. Trials 1, 2, and 3 only silenced the LGX-1 chitin deacetylase. Other than the mean brood size difference at the first time point in Trial 1, no difference was seen in total progeny between the control and LGX-1 deficient worms across these trials. The effect, instead, presented itself as a developmental delay in the first and third trials (Figure 4 and 6).

The lack of effect in Trials 2, 7, and 8 can be attributed to inconsistencies in methods as well as contamination. Experimental and control plates used in both Trial 2 and Trial 7 were found to have mold spores growing in the media of some plates while other plates had dried out, forcing the nematodes to congregate in corners of the plate with no bacteria. This could explain the low total progeny in both groups and the lack of a developmental delay in Trial 2 (Table 2A), and the lack of any effect in Trial 7 (Table 3A). Though Trial 8 had a significant decrease in mean brood size at 48-hours (Table 3B), the effect was gone at 72-hours and no developmental delay was apparent in the experimental group.

It has been previously found that the *lgx-1* gene is expressed in the pharynx of *C. elegans* (http://nematode.lab.nig.ac.jp). Based on silencing studies on the chitin deacetylases in insect models, worm death of *C. elegans* was expected (Arakane *et al.*, 2009; Luschnig *et al.*, 2006). The failure to modify of chitin in *Drosophila* caused excessive elongation of the tracheal tubing and the normal function of the trachea was lost (Luschnig *et al.*, 2006). If the pharynx in *C. elegans* was not deacetylated, improper feeding may result causing the death of worms. Since no brood size reduction was seen,

the loss of function of the *lgx-1* gene is not a lethal mutation. Even without the chitin deacetylase, the worm can still use its pharynx for feeding, perhaps preventing elongation and forming a smaller organ. A less developed pharynx, however, might indicate a reduced rate in breaking down food and supplying the body with nutrients. This decrease in rate could account for a developmental delay, but not a reduction in hatched progeny. It can be speculated, therefore, that the role of LGX-1 may be to convert the rigid polymer chitin to the more pliable molecule chitosan. Once chitin is deacetylated, the pharynx can lengthen and then another enzyme can add the acetyl group back.

Bioinformatics show another putative chitin deacetylase region on the F48E3.8 gene in *C. elegans.* Trials 4-8 attempt to demonstrate the resulting effects of losing both chitin deacetylases. In both Trials 4 and 6, a developmental delay and significant decrease in mean brood size between experimental and control worms was seen (Figure 7; Figure 9; Table 3B). Trial 5 did not have a delay in development, but still showed a significant reduction in brood size (Table 3B).

Because another chitin deacetylase is present, it is possible to assume that the F48E3.8 translated enzyme compensates for the loss of LGX-1. The F48E3.8 protein could catalyze chitin to chitosan in the pharynx at the same locations as LGX-1, allowing for some elongation and function of the organ. The results, however, do not support this conclusion. When only the F48E3.8 gene is silenced through RNAi feeding experiments, reduction in brood size is seen (personal communication with Ronald Jason Heustis). The data in this paper shows a combined effect when both genes are silenced; not only are the experimental worms growing more slowly, less of them survive. If the hypothesis

that the F48E3.8 gene simply compensates for the absence of LGX-1, then only a reduction in brood size would be seen.

Since a loss of the F48E3.8 gene produces a lethal phenotype, one of two tissues can potentially be the site of action for the enzyme. This gene might act similarly to the *verm* and *serpentine* genes in *Drosophila* and cause excessive elongation when silenced resulting in embryonic death (Luschnig *et al.*, 2006). The worms that are able to overcome this phenotype are still affected by the developmental delay effects of the absent LGX-1. This is supported by the effect seen in Trial 5 on the N2 wild-type worms. The silenced F48E3.8 gene effect was seen on the experimental plates while the *lgx-1* delay effect disappeared. If there is substantially less expression of the *lgx-1* gene than F48E3.8, the resulting phenotype from knocking down the F48E3.8 gene may mask the delay effect. Additional trials using N2 worms and using qPCR to quantify expression levels of both F48E3.8 and *lgx-1* genes will lead to more meaningful conclusions.

Chitin in the eggshells of *C. elegans* (Zhang *et al.*, 2005), may be modified by the F48E3.8 enzyme. It could be expressed by either the parent or the unhatched offspring in order to weaken the shell and allow for hatching. Worms that have the F48E3.8 gene silenced will lay progeny that may be unable to emerge from the egg which would be observed as a reduction in brood size. Worms that do hatch are, once again, subjected to the delay effects of the silenced *lgx-1* gene.

In situ experiments can be conducted to test the areas in which the F48E3.8 gene is expressed and more meaningful conclusions can then be made. It is clear that chitin modification to chitosan is vital to the development of the nematode *C. elegans.*

Silencing of either one or both *lgx-1* and F48E3.8 genes result in some sort of phenotype changes in the nematode.

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