RNAi knockdown of a putative chitin deacetylase in *Caenorhabditis elegans*

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Abstract

Filarial parasites pose a major problem to the developing world, yet treatments for infections by parasitic nematodes are limited, and not always effective. Recent studies have shown that drug resistance is emerging, which emphasizes the gap that needs to be filled by a new treatment option. Chitin metabolism in the nematode is an appealing target for new drugs, as this process is not shared by mammalian hosts.

Chitin deacetylase (CDA), which converts chitin into chitosan, is present in the pharynx of the free-living nematode *Caenorhabditis elegans*, a model organism for parasitic worms. Here, we examine the RNAi knockdown of one putative chitin deacetylase gene, F48E3.8. RNAi experiments show that this knockdown is associated with reductions in brood size, and may also cause developmental delays. We also attempted to visualize the morphology associated with CDA knockdown using eosin Y, a specific stain for chitosan. Though further knockdown experiments and imaging remain to be completed, F48E3.8 may represent an important step in the development of *C. elegans*, and its homologue in parasites may be a potential drug target as well.

Introduction

Background

Lymphatic filariasis (LF) is considered a neglected tropical disease, affecting 120 million people in 80 developing countries (WHO 2002). While the mortality rate for LF is relatively low compared to the "Big 3" diseases (HIV, malaria, and tuberculosis), the economic impact for affected people and communities is profound. Disability adjusted

life years (DALY) measures this impact and provides a means to compare the neglected diseases with those that have a more obvious impact. The DALY for LF is 5 million, and of the neglected tropical diseases, the impact of LF is second to only the soil transmitted helminths (Hotez 2008). This represents a huge impact for societies that are affected by LF, in addition to the disability and disfigurement that individuals face.

US drug companies have very little motivation to put R&D money into drugs that would need to be donated or sold at a reduced price to the developing world; consequently, current treatments for LF are limited to ivermectin, albendazole, and diethylcarbamazine (which cannot be used in areas endemic with onchocerciasis). However, studies are showing emerging parasite resistance to ivermectin in sub-Saharan Africa (Schwab 2005). Thus, there is an important need for new drugs targeting LF as current infections become more resistant to available treatments.

We are using *Caenorhabditis elegans* as a model organism for the parasites that cause LF: *Wuchereria bancrofti, Brugia malayi,* and *B. timori*. In the laboratory setting, *C. elegans* is useful for a number of reasons: first, it does not require an animal host to complete its life cycle, as the parasites do. Additionally, *C. elegans* was the first multicellular organism to be sequenced (*C. elegans* Sequencing Consortium 1998). RNA interference (RNAi), which is arguably the most useful new laboratory technique in recent years, was first characterized in *C. elegans*. As a species, *C. elegans* undergoes many developmental processes similar to other species in the phylum Nematoda, which includes the parasites that cause LF. The process of chitin metabolism in nematodes is of particular interest to the lab. Humans do not possess chitin, so a drug interrupting chitin metabolism in a parasite may be less likely to cause harmful side effects in the patient.

Overview of Chitin in Nematodes

Chitin is a polymer consisting of repeating units of β1-4 linked N-acetylglucosamine. It is a rigid and insoluble molecule, and most commonly known as a significant portion of insect exoskeletons; crustaceans and fungi also use chitin in a structural capacity. Some insects, including the fly *Drosophila melanogaster*, contain chitin in their epithelial tubes. Many pathogenic yeasts, including *Cryptococcus neoformans*, have chitin-containing cell walls.

Many species have chitin deacetylase (CDA) enzymes, which removes the acetyl group from the chitin monomer, converting it to chitosan. Deacetylation leaves chitosan with a free amine, which affects the physical properties of chitosan: it is more flexible and slightly more soluble than chitin.

A number of hypotheses have been proposed for why an organism would possess a chitin deacetylase. The first is to aid in digestion: a CDA could be active in an organism's gut to assist in polysaccharide breakdown. Secondly, CDA could act on endogenous chitin—in *Tribolium castaneum*, CDA activity is required for molting (Arakane 2009). In *D. melanogaster*, CDA knockdown in developing embryos led to unnaturally elongated epithelial tubes, which adopted a bent morphology to fit into the limited space of the epithelium. In this organism, chitin deacetylase action is required for termination of these tubes (Luschnig 2006, Wang 2006), and the bent tubes could result in decreased ability to move molecules through the embryo.

The yeast *C. neoformans* requires CDA activity for normal development. Knockdown of this enzyme results in daughter cells that do not fully separate from the parental cell during budding (Banks 2005, Baker 2007). Additionally, cells with the

CDA knockdown have difficulty keeping melanin, a virulence factor, within their cell walls (Baker 2007). CDA activity also contributes to virulence in the fungus *Collectotrichum lindemuthianum* (Blair 2006), which infects plants, and a polysaccharide deacetylase (PDA) is a virulence factor in the bacteria *Streptococcus pmeumoniae* (Blair 2005). Deacetylation in both of these species likely aids virulence by making it more difficult for chitinases and other digestive enzymes to recognize the pathogens.

C. elegans, like all nematodes, contains chitin in its eggshell and pharynx (Veronico 2001, Zhang 2005), and chitin synthases have been identified in numerous nematode species, including parasitic nematodes (Harris 2000, Veronico 2001, Harris 2002, Fanelli 2005). A bioinformatic search performed by Luschnig revealed one gene with sequence similarity to other chitin deacetylases (2006). A second gene was later discovered bioinformatically in our lab, and further, and our lab demonstrated the somatic expression of one or both of these putative CDAs during *C. elegans* growth (Ronald Heustis, personal communication).

C. elegans may deacetylate pharyngeal chitin for a variety of reasons. As in the fruit fly, CDA may be necessary for proper development of the *C. elegans* pharynx. Thus, CDA action on pharyngeal chitin could be crucial to the ability of the worms to feed. A CDA could also be active in the pharyngeal lining to assist in breakdown of ingested bacteria. Alternatively, CDA activity could protect the nematode from attack by microbes that contain chitinases to consume chitin as a food source (Mercer 1992). Chitinases digest chitin, but cannot bind to chitosan, so microbes that enter the nematode digestive system would not be able to easily break down the worm's pharyngeal lining. Lastly, though *C. elegans* is not an infective species, it is possible that chitin deacetylase

could contribute to virulence in the filarial parasites. Polysaccharide deacetylation helps pathogenic yeasts and bacteria remain undetected by digestive enzymes like chitinases, so CDA activity could play the same role during the parasite infective stage.

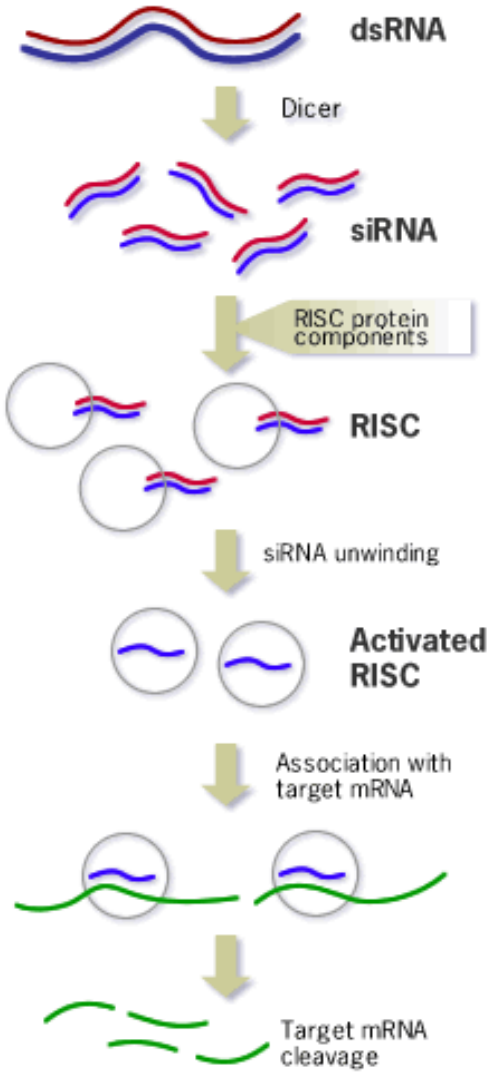


Figure 1: Mechanism of RNA interference. courtesy of Ambion http://www.ambion.com/techlib/append/RNAi_mechanism.html

RNA interference in C. elegans

Andrew Fire and Craig Mello won the Nobel Prize in Physiology or Medicine for their discovery of RNAi in 1998. The mechanism of this process was later elucidated (Figure 1): double-stranded RNA (dsRNA) is cleaved into ~21-23 bp long fragments (small interfering RNA, or siRNA) by an enzyme, Dicer (Bernstein 2001). The siRNAs are then captured by a protein complex known as the RNA-induced silencing complex (RISC), which includes the Argonaute protein. Argonaute selects the strand with the greater AT/GC ratio on the 5' end and loads it into the RISC complex (Rand 2005). The now single-stranded "guide strand" seeks out complementary mRNA messages, and the RISC complex degrades the mRNA, thus lowering protein expression in a cell (Hammond 2000).

RNAi Laboratory Methods

RNAi knockdown can be achieved in *C. elegans* by one of three ways: soaking (Tabara 1998), feeding (Timmons 1998), or microinjection (Fire 1998). Soaking involves incubating worms in a medium containing dsRNA of the gene of interest, while in the feeding protocol, worms are fed with bacteria expressing the dsRNA. Microinjection involves directly injecting the dsRNA into the nematode. Generally, microinjection produces the most effective knockdown, followed by soaking and feeding; however, it is also more expensive and difficult to perform.

In the laboratory, RNAi in *C. elegans* has been enhanced by the discovery of RNAi hypersensitive strains. Such strains have deleterious mutations in genes that would normally inhibit RNAi: for example, *eri-1* mutant worms have a nonfunctional siRNA-specific exonuclease (Kennedy 2004). We use the strain NL2099 (Simmer 2002), which is mutated in the RNA-dependent RNA polymerase *rrf-3*. The exact reason for this RNAi hypersensitive phenotype has not been identified, though Simmer hypothesized that wild-type RRF-3 may compete with the RNA polymerases RRF-1 and EGO-1, which are required for RNAi.

Previous Research

Previously, I established the presence of chitosan in the *C. elegans* pharynx by staining with eosin Y. Eosin is commonly used as a stain in histology, but recently it was suggested to bind specifically to chitosan *in vitro* (Chatterjee 2005). *In vivo*, Baker used eosin Y to stain the chitosan-containing cell walls of the yeast *C. neoformans* (2007).

Last year, I concluded that eosin Y bound to structures in the *C. elegans* pharynx by comparing eosin Y-stained worms (Figure 2) with worms incubated in 4-methylumbelliferone, which is not specific for any structures in the nematode (Meredith Rogers, Bio 193, Fall 2009). The non-specific stain was necessary to ensure that the worms were not simply ingesting the molecule. Though a definitive conclusion cannot yet be made about the specificity of eosin Y for chitosan, my results support the hypothesis that chitosan exists in the *C. elegans* pharynx.

I also looked at how chitosan content changes during nematode development by imaging different stages of wild-type worms stained with eosin Y, and concluded that chitosan content in the pharynx appears to increase in proportion with the increased size of the pharynx as the nematode grows (Meredith Rogers, Bio 194, Spring 2010).

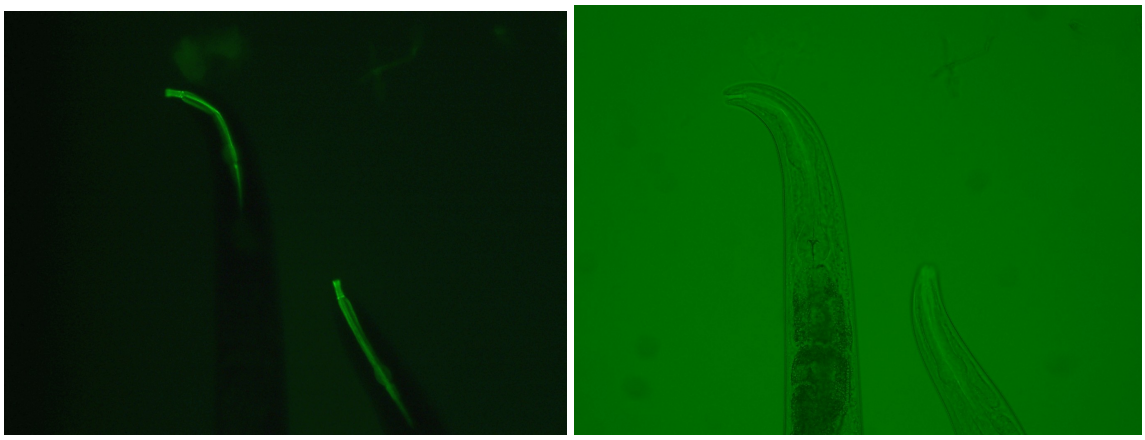


Figure 2. Eosin Y specifically stains the *C. elegans* pharynx.

Currently, the laboratory is investigating both putative CDAs that had been identified: *lgx-1* and F48E3.8. Knockdown of *lgx-1* appeared to cause delays in nematode development, but F48E3.8 had not been previously studied using RNAi. Last year, a portion of the F48E3.8 cDNA, yk1130a03, was cloned into an expression vector

for this purpose.

In this study, I attempted to knock down the gene by two different RNAi methods: soaking and feeding. Soaking requires a substantial amount of dsRNA, which is obtained using *in vitro* transcription. Despite numerous attempts to troubleshoot the *in vitro* transcription reaction, there was very little dsRNA yield. Instead, worms were fed with *E. coli* expressing the F48E3.8 dsRNA. The worms were observed at 48 and 72 hours for potential knockdown effects on time to development and progeny numbers. I also imaged eosin y-stained knockdown and control worms to see whether there were visible differences in pharynx morphology.

Here, I report the results of F48E3.8 knockdown in an RNAi hypersensitive strain of *C. elegans*. This knockdown appears to cause decreased brood size, although further repeats of these feeding experiments are recommended to support this conclusion. Initial eosin y staining revealed no obvious differences in the pharynges of knockdown and control worms, but this should be explored further.

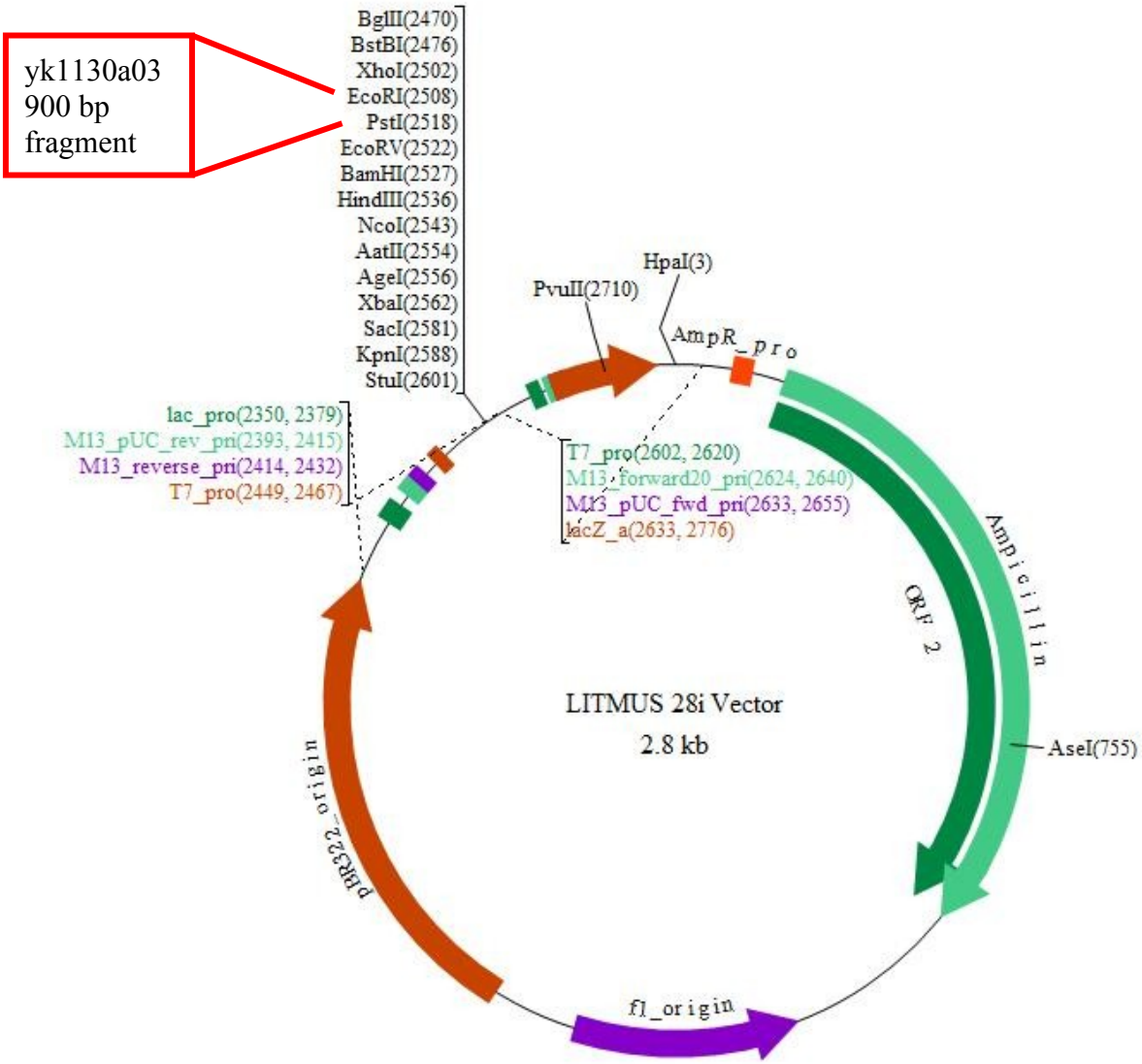
Methods

Plasmid

A fragment of F48E3.8, yk1130a03 (courtesy of Y. Kohara, National Institute of Genetics, Japan), was previously ligated into a Litmus v28i (New England Biolabs) expression vector (Sam Cho, Bio 94, Spring 2010) (Figure 3). The plasmid was transformed into *E coli* strain HT115 (DE3), which can be induced by IPTG to express recombinant DNA inserted into the plasmid between two T7 promoters.

In-Vitro Transcription

In vitro transcription reactions were performed according to the Ambion MAXIscript T7 Kit protocol (Ambion), except that the chloroform extraction was not performed and DNase treatment was carried out only once. The plasmids were linearized using EcoR1 and HindIII restriction enzymes in separate reactions. This created two linearized plasmids: one with a T7 promter flanking one strand of the F48E3.8 gene fragement, and another with a promoter flanking the opposite strand of the fragment. pAL41 β-Actin, supplied with the kit, was used as control DNA. 1 μg linearized plasmid DNA was combined with 2mM dNTPs (final concentration), 10x reaction buffer, and 30 Units T7 RNA polymerase, and incubated for 1 h at 37ºC. The samples were then visualized by gel electrophoresis on RNAse-free agarose gels (1%).



http://www.biovisualtech.com/bvplasmid/LITMUS_28i_Vector.jpg
Figure 3. Map of Litmus 28i vector with 900 bp portion of yK1130a03 insert ligated into the EcoR1 site.

Growing Conditions

Strain NL2099, *rrf-3* (RNAi hypersensitive) *C. elegans* worms were plated onto NGM (nematode growth medium) plates (Stiernagle 2006) and fed with OP-50 *E. coli*. Worms were grown at 16°C until they were used in the feeding experiments.

RNAi

RNAi knockdown was performed by feeding *C. elegans* with bacteria expressing dsRNA corresponding to the F48E3.8 gene. HT115 (DE3) *E. coli* containing the F48E3.8 insert in Litmus 28i vector (named v28i.F48E3.8) or a control (vector without insert) were grown overnight at 37°C with shaking in 3 ml LB + 100μg/ml ampicillin and 15μg/ml tetracycline. Bacteria (0.1 ml) were plated onto NGM plates containing 25 μg/ml carbenicillin and 1 mM IPTG, as described by Wood (Wood 1988), and grown overnight at 37°C. One L3 worm was placed onto each plate and grown at 20°C. At 48 h, the original worm was removed from the plate. At 48 and 72 h, the number of progeny and their developmental stages were recorded.

Eosin Y Staining

After 5 days, knockdown and control worms were washed from plates with M9 buffer (22 mM KH_2PO_4, 22 mM Na_2HPO_4, 85 mM NaCl, 1 mM $MgSO_4$) and spun at 200xg at 4°C for 5 minutes. Supernatant was removed, the pellet was washed with 5 ml M9 buffer, and spun down again. The supernatant was removed, and the pellet was resuspended in 5 ml McIlvaine's buffer (0.1 M citric acid, 0.2 M Na_2HPO_4, pH 6.0) and spun down at 600xg at 4°C for 5 minutes. The supernatant was removed, leaving 0.5 ml of buffer + worms. The staining was performed as described by Baker (2007). 30 μl eosin Y (5mg/ml in 70% ethanol) was added to the tube for a final concentration of 0.3 mg/ml. The samples were incubated in the dark, on ice, for 10 minutes. 3 ml McIlvaine's buffer was added to the tube to wash, and the worms were spun down again at 600xg. Supernatant was removed, and the wash was repeated 2 times. After the last

wash, the supernatant was removed, leaving 0.5 ml of buffer + worms.

Microscopy

Knockdown and control worms, both with unstained controls, were imaged on an Olympus BX40 fluorescent microscope with a Chroma 31000 FITC filter.

Statistical Analysis

Mean numbers of progeny for knockdown and control worms were compared using a two-tailed Student's T test via SPSS.

Results

RNAi

We were unable to use soaking methods for RNAi because we did not obtain sufficient dsRNA from the *in vitro* transcription reaction, despite a number of attempts to troubleshoot the protocol (Figure 4). Other members of the lab had been successful with RNAi by feeding, so the soaking protocol was put aside.

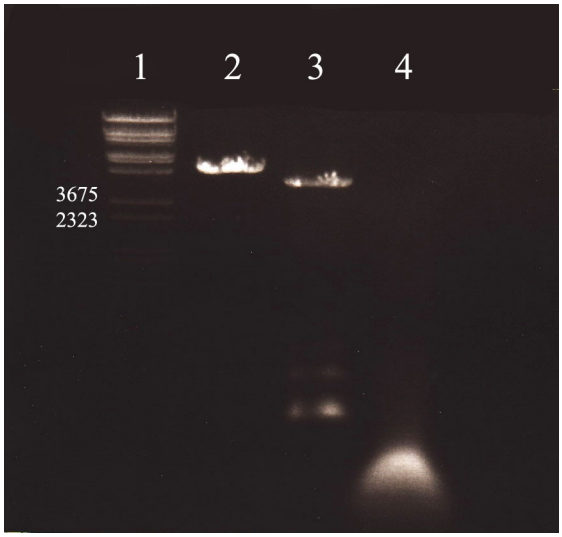


Figure 4. *In vitro* transcription of F48E3.8 fragment. Successful *in vitro* transcription would have yielded a 900 bp RNA fragment of the F48E3.8 gene, while the streak seen on the gel was less than 700 bp. Lane 1: λBst E molecular weight marker. Lane 2: Linearized v28i.F48E3.8 plasmid DNA. Lane 3: Control pAL41 β-Actin RNA. Lane 4: Annealed F48E3.8 fragment RNA.

The first feeding knockdown was performed as an unblinded experiment. After feeding L3 worms (strain NL2099 RNAi hypersensitive) with bacteria expressing dsRNA against F48E3.8 (experimental) or bacteria containing the expression plasmid without the F48E3.8 insert (control), knockdown worms had a significant reduction in brood size at 48 h (two-tailed t test, $p < 0.001$, $df = 11$, $t = -10.89$) and 72 h ($p < 0.001$, $df = 11$, $t = -6.02$) hours compared to control worms (Table 1, Figure 5). At 48 h, the experimental plates had one-third the number of progeny as the control plates. At 72 h, the experimental plates contained less than one half the progeny of the control plates. However, two of the experimental plates yielded zero progeny, which makes the knockdown appear to have a greater effect. A significant reduction in hatched progeny was also seen at 48 h ($p < 0.001$, $df = 11$, $t = -5.34$) and 72 h ($p < 0.001$, $df = 11$, $t =$

-5.12), as well as in the late-stage progeny at 72 h ($p = 0.001$, $df = 11$, $t = -4.43$).

To account for the potential effect of experimenter bias, the next feeding experiments were performed blinded: plates were randomly assigned numbers by another member of the laboratory, so that bias could not affect the size of the L3 worm assigned to each plate or the progeny counts post-treatment.

Table 1. Unblinded feeding experiment data.

	48 h		72 h	
	Experimental	Control	Experimental	Control
Total Progeny	74	223	177	390
Mean Progeny	6.17*	18.58	14.75*	32.5
Progeny (excluding eggs)	11	34	150	317
Mean Hatched Progeny	.92*	2.83	12.5*	26.4
Total L3, L4, and Adults	0	0	83	181
Mean Late Stage Progeny	N/A	N/A	6.92*	15.1

* represents statistically significant difference in mean ($p<0.05$) using two-sided T test

The blinded knockdown of F48E3.1 led to a less dramatic difference in the numbers of progeny in the experimental and control groups compared to the unblinded experiment. The first blind trial (Table 2, Figure 6) yielded an overall reduction in brood size of the experimental group compared to the control; however, this reduction was only quarter-fold. Two plates in the experimental group yielded zero progeny, while one plate in the control group yielded zero progeny. One control plate contained 57 progeny at 48 h, 10 more than the next highest number (Appendix A). The mean hatched experimental progeny was significantly less than the control ($p < 0.001$, $df = 11$, $t = -14.24$). At 48 h,

the experimental plate L1 stage progeny was only 7% of the L1 controls. At 72 h, the difference in progeny counts was most apparent at the L1, L4 and adult stage worms: the L4 experimental group was ½ that of the control group, while the L1 and adult stages were each ¼ that of the control.

Table 2. Data for trial 1 of unblinded feeding experiment.

	48 h		72 h	
	Experimental	Control	Experimental	Control
Total Progeny	131	223	140	192
Mean Progeny	10.92	18.58	11.66	16
Progeny (excluding eggs)	4	61	123	167
Mean Hatched Progeny	0.33*	5.08	10.25	13.9
Total L3, L4, and Adults	0	0	70	92
Mean Late Stage Progeny	N/A	N/A	5.83	7.67

* represents statistically significant difference in mean ($p<0.05$) using two-sided T test

The second blinded feeding experiment again yielded an overall reduction in brood size, but this reduction was not significant (Table 3, Figure 7). At 48 h, both experimental and control groups contained L3 and L4 stage larvae, which indicates that some parental worms were already at L4 or adult stage at the beginning of the feeding experiment. However, the number of L3 and L4 worms in the experimental group at 48 h was significantly less than those in the control group ($p < 0.001$, $df = 11$, $t = -12.09$).

Table 3. Trial 2 of blinded feeding experiment.

	48 h		72 h	
	Experimental	Control	Experimental	Control
Total Progeny	468	486	500	545
Mean Progeny	39.0	40.5	41.67	45.42
Progeny (excluding eggs)	100	136	445	461
Mean Hatched Progeny	8.33	11.3	37.08	38.42
Total L3, L4, and Adults	5	33	284	299
Mean Late Stage Progeny	.42*	2.75	23.67	24.92

* represents statistically significant difference in mean ($p<0.05$) using two-sided T test

The third unblinded trial had a very slight, and insignificant, reduction in knockdown brood size compared to the control (Table 4, Figure 8). Instead, a delay in development was observed in the experimental group (Figure 7, Panel B). In accordance with this delay at 72 h, there were significantly fewer L3, L4, and adult worms in the experimental group than in the control ($p = 0.026$, $df = 11$, $t = -2.57$).

Table 4. Blinded feeding experiment, trial 3.

	48 h		72 h	
	Experimental	Control	Experimental	Control
Total Progeny	135	139	145	154
Mean Progeny	11.25	11.58	12.08	12.83
Progeny (excluding eggs)	4	0	127	141
Mean Hatched Progeny	0.33	N/A	10.58	11.75
Total L3, L4, and Adults	0	0	8	19
Mean Late Stage Progeny	N/A	N/A	0.67*	1.58

* represents statistically significant difference in mean ($p<0.05$) using two-sided T test

The last blinded trial yielded more worms for both the experimental and control groups than any of the other trials (Table 5, Figure 9). At 72 h, there were fewer total progeny in the experimental group than in the control, but at 48 h, there were significantly more hatched experimental group progeny than hatched control group progeny ($p = 0.026$, $df = 11$, $t = -2.57$). No shift was seen in the developmental curve for this trial.

Table 5. Trial 4 of unblinded feeding experiment.

	48 h		72 h	
	Experimental	Control	Experimental	Control
Total Progeny	589	546	767	833
Mean Progeny	49.08	45.5	63.92	69.4
Progeny (excluding eggs)	212	161	693	745
Mean Hatched Progeny	17.67*	13.42	57.75	62.1
Total L3, L4, and Adults	0	0	259	324
Mean Late Stage Progeny	N/A	N/A	21.58	27

* represents statistically significant difference in mean ($p<0.05$) using two-sided T test

Eosin Y Staining and Imaging

Eosin Y staining was performed on F48E3.8 knockdown worms to visualize any morophology associated with the RNAi knockdown. There may have been a slight decrease in fluorescence intensity in knockdown worms compared to control (Figure 10), but the movement of live worms made it difficult to definitively determine whether staining intensity was affected. Attempts to paralyze the worms using tricaine/tetramisole resulted in nonspecific fluorescence (data not shown).

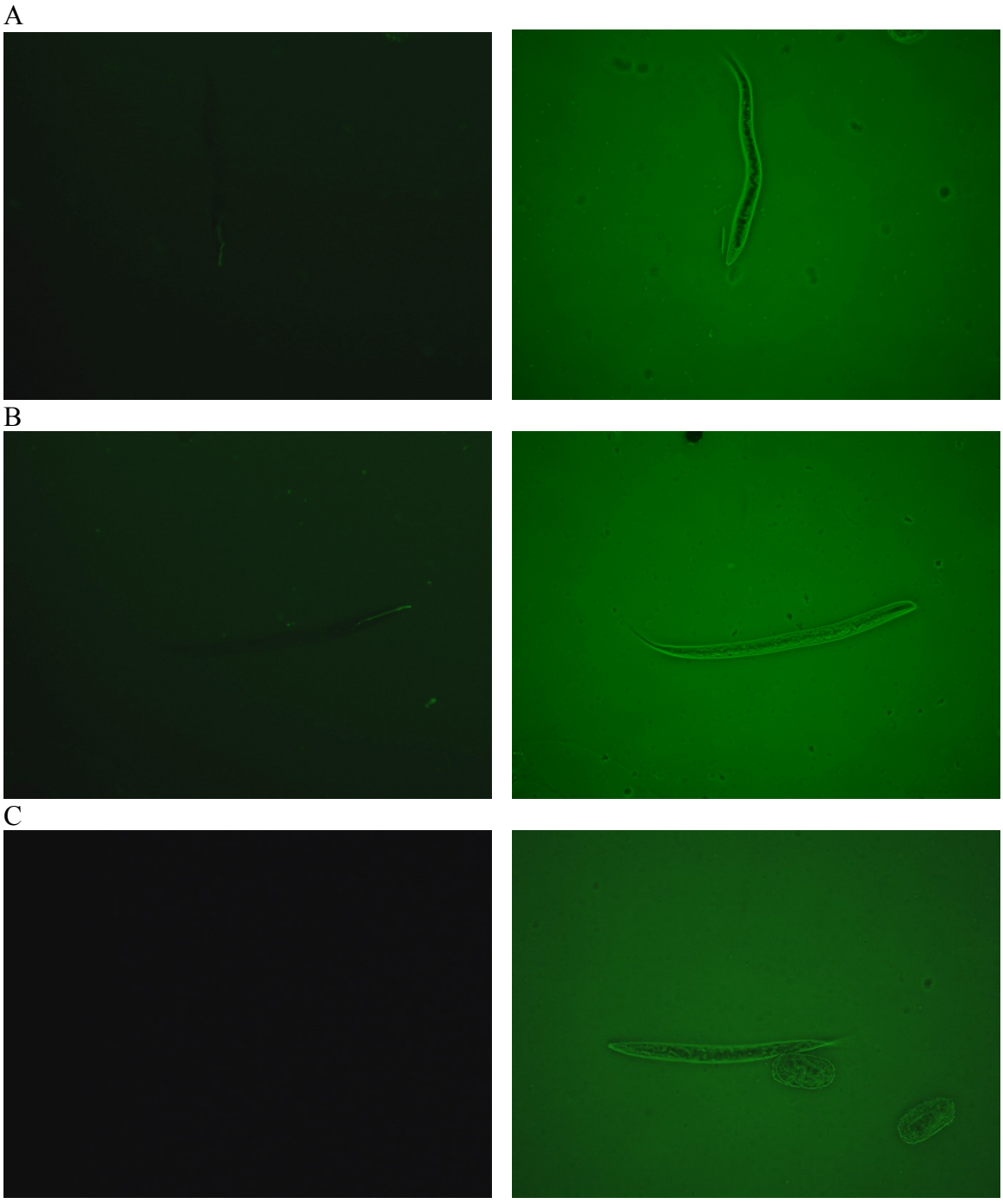


Figure 10. F48E3.8 knockdown may cause decreased fluorescence intensity with eosin Y. Knockdown and control worms were incubated on ice in 0.3 mg/ml eosin Y in McIlvaine's buffer for 10 minutes. After washing, worms were imaged. Fluorescent images were taken with an exposure time of 500 msec. Panel A: F48E3.8 knockdown eosin Y staining, fluorescence and bright field. Panel B: RNAi control eosin Y staining, fluorescence and bright field. Panel C: unstained control, fluorescence and bright field. Contrast and brightness of fluorescent images were adjusted equally for better visualization on print.

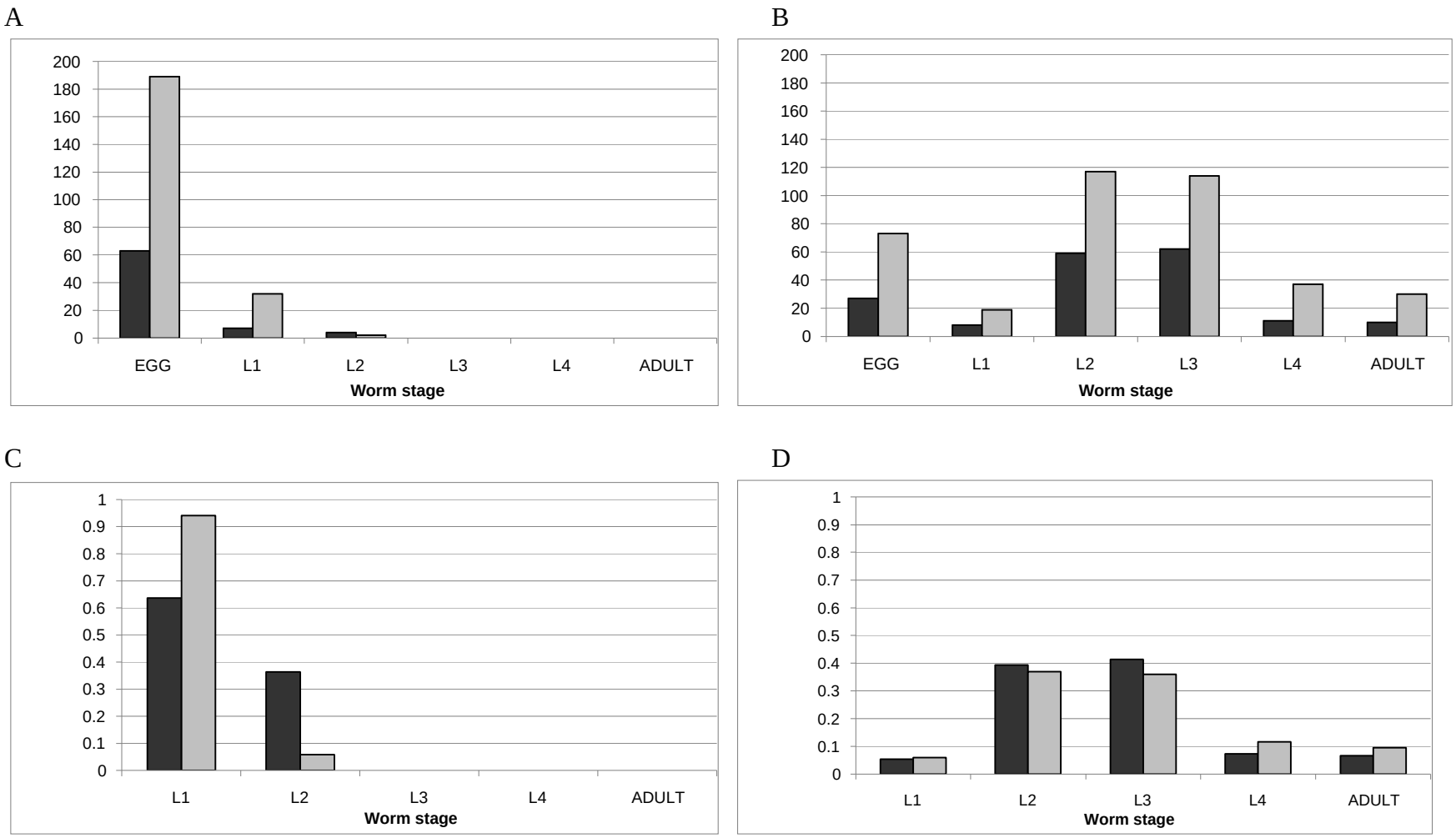


Figure 5. RNAi knockdown of F48E3.8. In an unblinded RNAi experiment, NL2099 *C. elegans* fed on bacteria expressing double-stranded RNA against F48E3.8 had half the number of progeny as worms fed on control bacteria. Dark bars: experimental; light bars: control. Total progeny at each stage at each stage of experimental and control worms were compared at 48 hours (A) and 72 hours (B), and the fraction of total progeny at each stage at each stage, not including eggs, were compared at 48 (C) and 72 (D) hours.

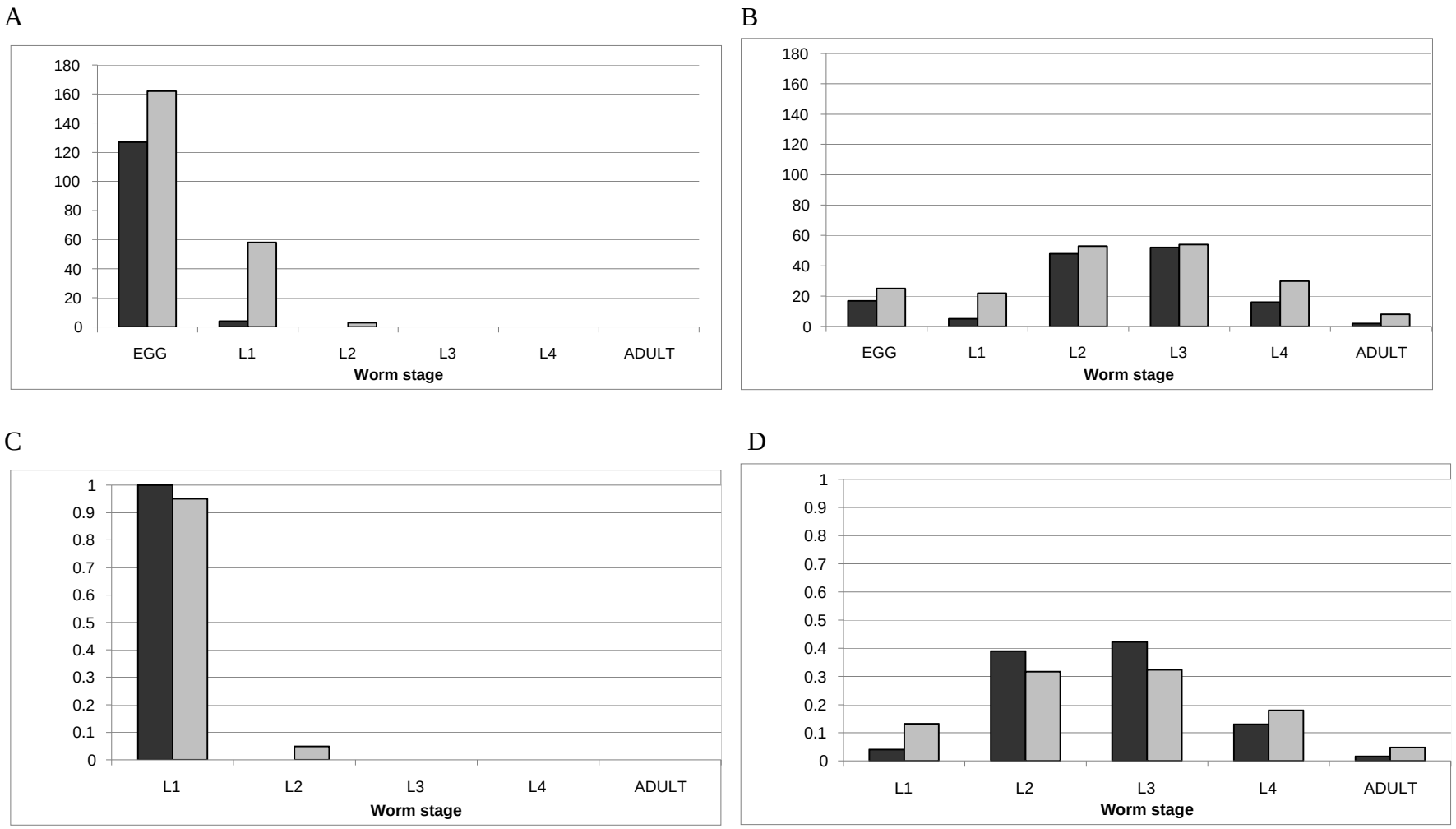


Figure 6. Trial 1 of a blinded RNAi knockdown of F48E3.8. After feeding NL2099 *C. elegans* on bacteria expressing dsRNA against F48E3.8, knockdown worms produced less progeny than controls. Dark bars: experimental; light bars: control. Absolute number of progeny of experimental and control worms were compared at 48 hours (A) and 72 hours (B), and the fractions of total hatched progeny were compared at 48 (C) and 72 (D) hours.

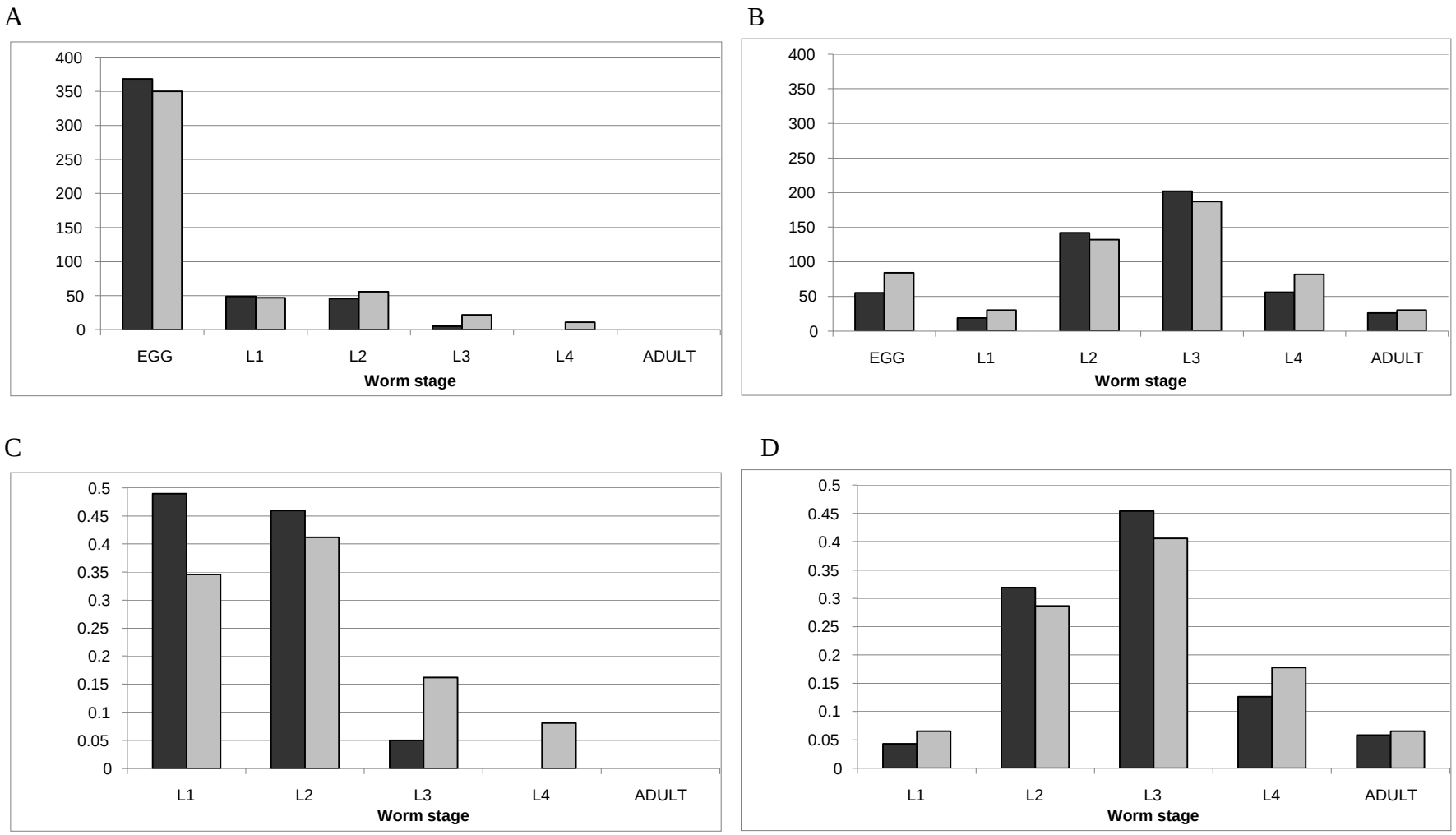


Figure 7. RNAi knockdown of F48E3.8, Trial 2. In a repeat of the blinded RNAi experiment, NL2099 *C. elegans* fed on bacteria expressing dsRNA against F48E3.8 appeared to have slight developmental delays. Dark bars: experimental; light bars: control. Total progeny at each stage of experimental and control worms were compared at 48 hours (A) and 72 hours (B), and the fraction of total progeny at each stage, not including eggs, were compared at 48 (C) and 72 (D) hours.

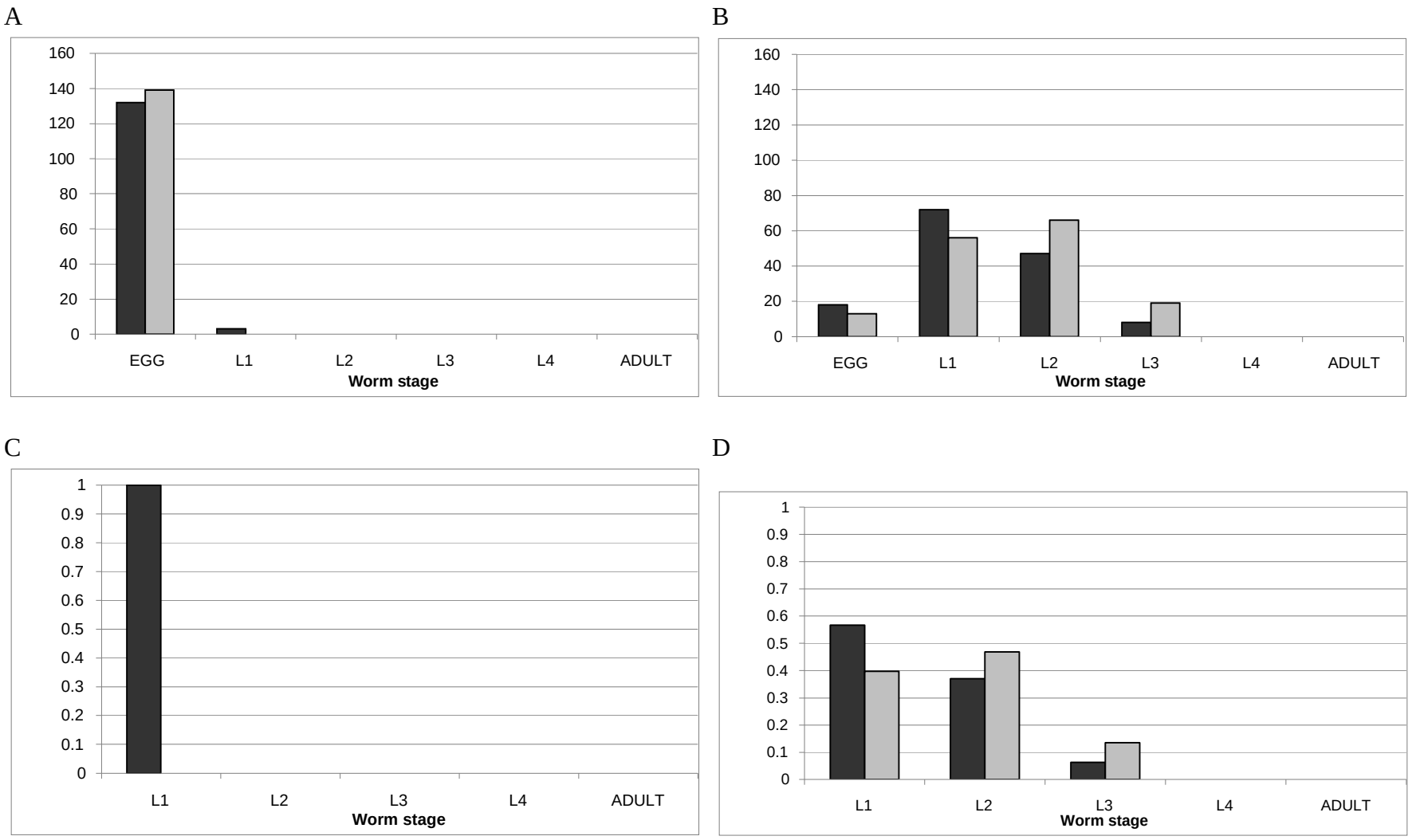


Figure 8. Trial 3 of blinded RNAi knockdown of F48E3.8. There appeared to be a small delay in the growth of NL2099 *C. elegans* fed on bacteria expressing double-stranded RNA against the putative CDA F48E3.8, compared to control worms. Dark bars: experimental; light bars: control. Total progeny at each stage of experimental and control worms were compared at 48 hours (A) and 72 hours (B), and the proportion of hatched progeny were compared at 48 (C) and 72 (D) hours.

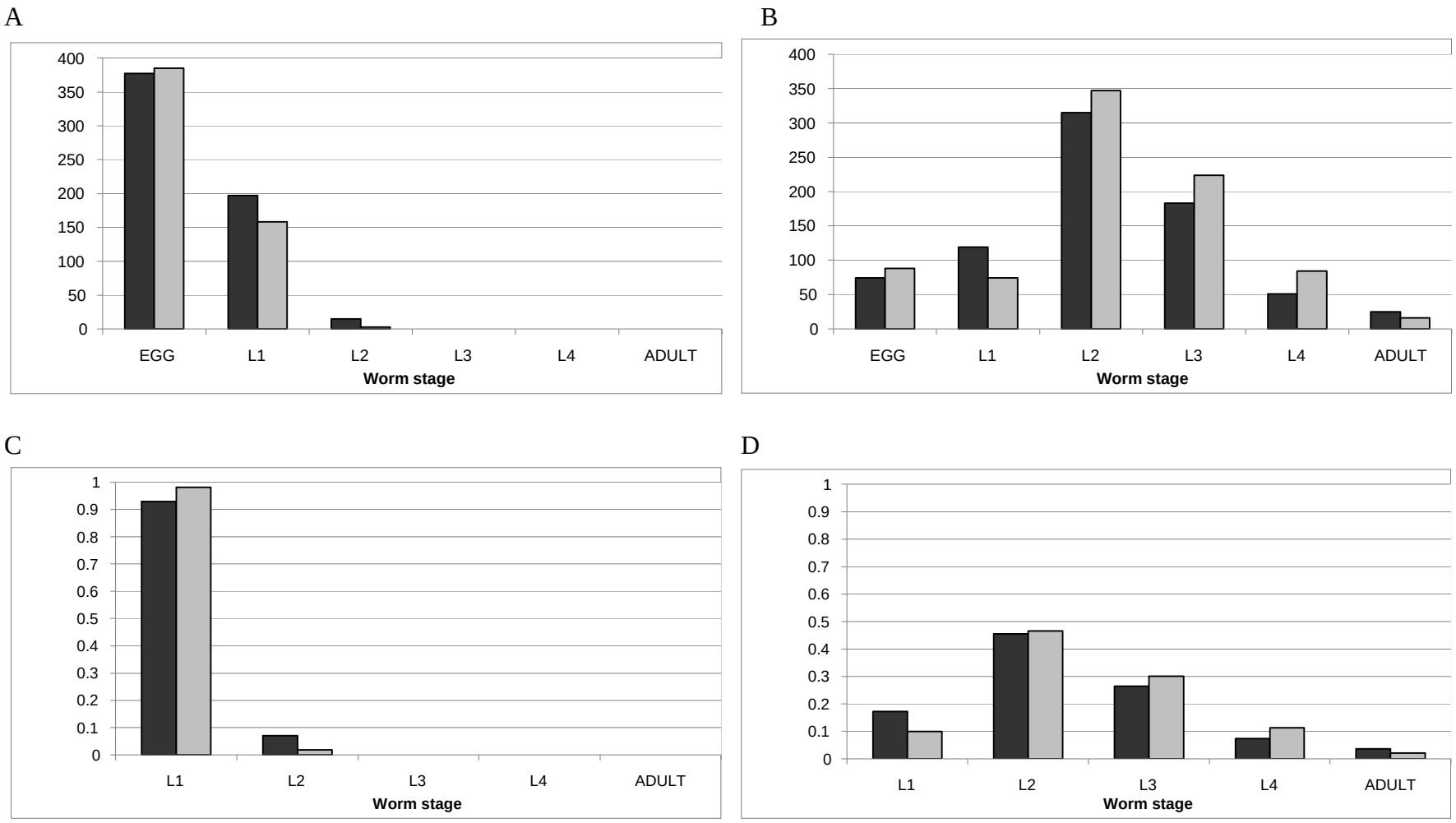


Figure 9. RNAi knockdown of F48E3.8. In Trial 4 of a blinded RNAi experiment, NL2099 *C. elegans* fed on bacteria expressing double-stranded RNA against F48E3.8 had a small decrease in the number of progeny compared to controls. Dark bars: experimental; light bars: control. Total progeny at each stage of experimental and control worms were compared at 48 hours (A) and 72 hours (B), and the fraction of total progeny at each stage, not including eggs, were compared at 48 (C) and 72 (D) hours.

Discussion

Here, a change in phenotype was observed following the knockdown of the *C. elegans* putative chitin deacetylase, F48E3.8, using RNAi by feeding. While overall the knockdown appeared to lead to a decrease in brood size, there was considerable variation among individual experiments, and within experiments. Within experiments, progeny counts could range between 0 and 87 (Appendix A, Unblinded Trial 2). The most likely reason for 0 progeny counts is that moving the worm from a stock plate to the experimental plate stresses or kills it. Additionally, the majority of worms in wild-type populations are hermaphroditic, but mutations in the *rrf-3* gene, which causes RNAi sensitivity, create a 7-10-fold higher incidence of males (Simmer 2002). Therefore, worms that produced 0 progeny could have been males, since they do not produce eggs. Abnormally high progeny counts are best explained by accidentally picking an L4 or adult worm, rather than an L3 worm, on day 0 of the experiment.

In an unblinded experiment, experimenter bias could result in either occurrence: unequal handling of L3 larvae, leading to death or infertility in the knockdown experimental group, or picking larger worms for the control group. However, a blinded experiment should eliminate these sources of bias. In analyzing the data, we decided not to omit plates that had no progeny for this reason.

In the unblinded experiment, statistically significant differences in mean worm counts were seen at both 48 and 72 h, for all worm stages compared (total progeny, hatched progeny, and L3/L4/adult progeny). Considering that each blinded trial had only one statistically significant difference in any mean, this further emphasizes the profound

effect that experimenter bias can have on results. In this trial, 3 experimental group plates contained 0 progeny. The experimental group was plated first, which may suggest a relatively simple explanation for the difference in means between the two groups: inexperience with handling worms would likely be more evident in the group that was plated first.

Interestingly, in each unblinded trial, the statistically different means were detected in the latest stage progeny available at 48 hours, or for trial 4, the latest stage progeny at 72 hours. For example, Trial 3 showed a statistically significant reduction in the mean number of L3, L4, and adult progeny compared with the control at 48 hours. In trial 2, on the other hand, no worms in either the experimental or control group reached the L3 stage at 48 hours. In this trial, there were instead significantly fewer hatched progeny in the experimental group at 48 hours compared to the control. Trial 4 can be explained by the fact that the control group had not hatched at 48 hours—the worms picked in this trial were likely L2s, rather than L3s. These results indicate a brief delay in development for the F48E3.8 knockdown group. At the later time point, the experimental group catches up to the control.

The reductions in brood size that were seen in the knockdown of F48E3.8 are consistent with double knockdown of both putative chitin deacetylases, F48E3.8 and *lgx-1* (Kenneth Brand, Senior Honors Thesis, 2010-2011). *lgx-1* knockdown resulted in delayed development, with no reduction in brood size. The double knockdown appeared to combine the effects of the single knockdowns: both delayed knockdown and reduced brood size were observed. The different effects of these knockdowns could be due to temporal or spatial differences in protein expression.

Nematodes may deacetylate chitin for a number of reasons. The worm's own chitin could be deacetylated, or chitin from an external source could be a target of CDA. Intrinsically, CDA activity may be necessary for pharynx assembly or for protection from bacteria that consume chitininous structures. In parasitic worms, CDA could act as a virulence factor, assisting the nematode in evading detection by a host. Extrinsically, CDA may assist in digestion of ingested bacteria that contain chitin. The virulence factor can be ruled out for *C. elegans*, as this is a free-living nematode. A chitin deacetylase could aid in digesting bacteria, but not in this experiment, as *E. coli* does not produce chitin. Additionally, in the laboratory setting, the worms are probably not being infected by microbes.

The most likely model for F48E3.8 is that it is involved in proper assembly of the pharyngeal lining. Perhaps worms deficient in F48E3.8 have difficulty feeding, and do not allocate as many resources to reproduction, in favor of keeping themselves alive. Alternatively, F48E3.8 knockdown during embryogenesis could have negative organizational effects on reproductive development. During development, the embryo must feed for growth. If F48E3.8 allows normal growth by encoding for a pharyngeal chitin lining, then knockdown of F48E3.8 may cause an abnormal pharynx, as seen in *D. melanogaster* (Luschnig 2006, Wang 2006), with which developing worms would have difficulty feeding. In chitosan, the free amine group released by deacetylation may help anchor the polysaccharide to proteins in epithelial cells. Alternatively, the free amine itself may interact with epithelial cells as a signal to turn off the chitin synthase gene, or it could interact with the chitin synthase protein. Chitin synthase is an integral membrane protein, so chitosan could directly inhibit this protein, perhaps by preventing newly

synthesized chitin from exiting the active site of the enzyme, or by occupying the enzyme active site and preventing new substrate binding. In F48E3.8 knockdown, perhaps a malnourished state caused by abnormal pharynx development inhibits transcription of genes that encode normal reproductive development, since this would be considered inessential compared to the survival of the individual worm.

F48E3.8 expression in the eggshell or reproductive tract cannot be completely ruled out, though my earlier eosin Y staining resulted in no egg fluorescence (Meredith Rogers, Bio 193, Fall 2010). The *C. elegans* eggshell contains chitin, so F48E3.8 could be necessary for proper egg development, which would explain the reductions in brood size that were seen in this experiment. The CDA might be necessary for egg formation, or it could be essential for hatching.

Initially, we had planned to perform RNAi knockdown by soaking worms in a medium containing dsRNA of the F48E3.8 gene, as the soaking method is said to produce a greater knockdown (Tabara 1998). After the first *in vitro* transcription reaction yielded no RNA for either F48E3.8 or the control included with the Ambion Maxiscript kit, the protocol was repeated with careful mixing of the stock solutions. The control yielded RNA, but again, no F48E3.8 RNA was detected. Ethanol precipitation of the original v28i.F48E3.8 plasmid before the *in vitro* reaction and DNase treatment before running the gel yielded only a streak on the agarose gel that was much smaller than the expected RNA product. Other members of the lab had previously experienced difficulty obtaining RNA from the *in vitro* transcription reaction (Lawrence Yen, Senior Honors Thesis, 2009-2010; Sam Cho, Bio 94, Spring 2010).

Though the soaking protocol was abandoned in favor of the feeding method, which has been successfully performed a number of times in the lab, the soaking method is still worth pursuing. A more potent knockdown of F48E3.8 could reveal new or enhanced effects on worm development and brood size, and could help determine whether parasitic CDAs should be examined as potential drug targets.

Eosin Y was reported to be a specific stain for chitosan (Chatterjee 2005), and this is consistent with my earlier imaging experiments, where eosin Y specifically bound to the *C. elegans* pharynx. Additionally, when *C. elegans* proteins were run on a substrate gel containing chitin, eosin Y specifically bound to bands corresponding to CDA after an incubation period that allowed the CDA to deacetylate the gel chitin to chitosan (Juliet Fuhrman, personal communication). Imaging after eosin Y staining may be a useful technique for visualizing how the CDA knockdown contributes to the phenotypic effect that was observed in the feeding experiments.

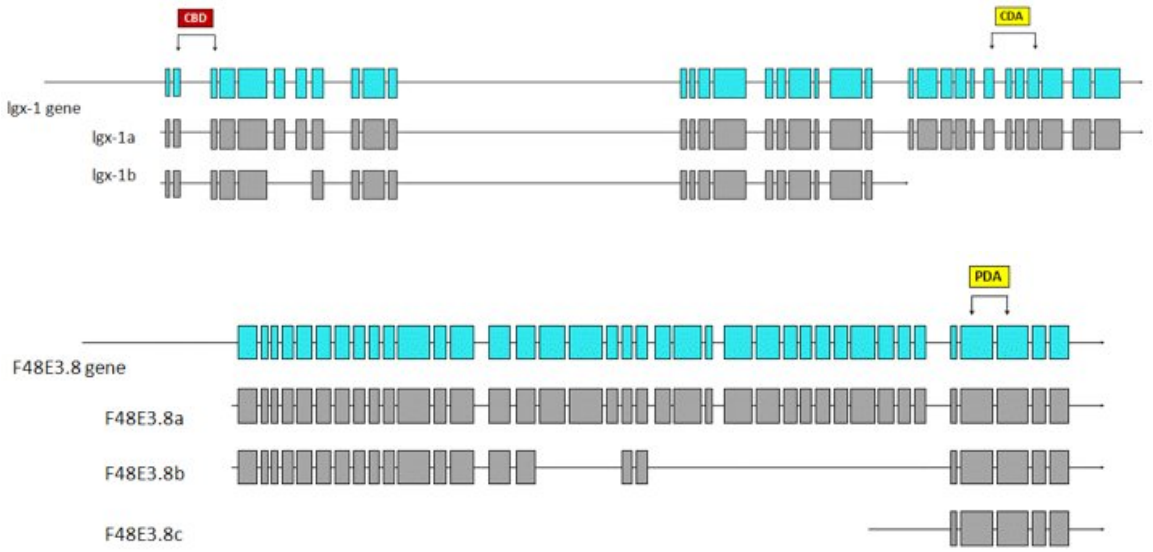
Eosin Y staining of knockdown worms was not explored as extensively as originally intended. It is very difficult to image live worms: capturing fluorescence signals requires exposure times of 500msec or more, and in this time period, live worms can move, blurring the picture. One attempt was made to paralyze the worms with tricaine and tetramisole as I previously described (Meredith Rogers, Bio 194, Spring 2010); however, the paralytic led to nonspecific fluorescence throughout the worm. This effect had previously been seen with other paralytics, but not as extensively with tricaine/tetramisole. An older stock of the paralytic was used for this imaging, so contamination of this stock could be the reason for nonspecific staining.

If CDA knockdown causes a morphological disruption in the worm pharynx, similar to the *serp* and *verm* CDA knockdown in *D. melanogaster* (Luschnig 2006, Wang 2006), then eosin Y staining could reveal this changed morphology. Additionally, assuming eosin Y is specific for chitosan over chitin, then the intensity of the stain should be decreased in knockdown worms compared to control worms. Morphological differences between experimental and control groups were not seen in the live imaging. Differences in intensity are more difficult to determine, because the pharynx is a 3D structure, and our fluorescence microscope captures only a 2D image at a single plane of focus. Simply focusing the microscope objective at a different depth can lead to an apparent difference in intensity that may not actually exist. Thus, the need to paralyze the worms before imaging is even more apparent—the worm motion can move the brightest part of the pharynx in and out of focus of the microscope. Once the worms are successfully paralyzed, a z-step microscope could be used to obtain a 3D image of the entire worm pharynx, allowing us to more easily determine the greatest fluorescence intensity.

Eosin Y staining and imaging of knockdown worms needs to be repeated successfully to allow us a complete understanding of the phenotypic effects of CDA knockdown. The staining could also be done for *lgx-1* and double knockdown worms.

Earlier eosin Y staining provided strong evidence that the *C. elegans* pharynx contains chitosan. Protein substrate gels run in the lab support protein expression of at least one CDA in the worm soma to catalyze this chitin modification (Hong Ng, Fall 2010). Two proteins of 207 and 145 kDa had CDA activity, and comparison with the

expected protein sizes from gene summaries suggests that these CDAs are *lgx-1* and F48E3.8, respectively (Figure 11).



(Ronald Heustis, Personal Communication)

Figure 11. Gene summary reveals two potential transcripts for *lgx-1*, one with a chitin deacetylase domain, and three potential transcripts with CDA domains for F48E3.8. Predicted protein sizes of *lgx-1* are 135 (without CDA domain) and 207 kDa; predicted protein sizes of F48E3.8 are 41, 145, and 269 kDa (Wormbase).

Conclusions and Future Directions

Aside from answering interesting biological questions about the role of chitin deacetylation in nematode development, CDA knockdown presents an exciting step towards understanding potential drug targets for infection by filarial parasites. Parasites pose a challenge because, as eukaryotic organisms, they are much more similar to humans than simpler bacteria. Because humans and parasites have similar structures and developmental processes, a drug that kills a parasite could have harmful side effects in

the host. Mammals do not possess chitin, and therefore, chitin metabolism could be an excellent target for new antiparasitic therapies.

RNAi knockdown of F48E3.8 did not cause developmental arrest or death; however, this does not rule out CDA as an important enzyme in nematode development. The RNAi feeding method may be less potent than other RNAi methods for CDA knockdown. It would be interesting to know the effects of F48E3.8 knockdown by soaking or microinjection.

Additionally, RNAi knockdown was recently performed *in vivo* in the parasite *B. malayi* (Song 2010). This would be an intriguing method to test whether parasitic CDA presents a realistic target for new drug treatments.

F48E3.8 knockdown should be repeated, but it appears that worms deficient in this protein product produce fewer offspring. Eosin Y staining and imaging may provide visual evidence of this phenotypic effect. Regardless of the implications in parasitic nematodes, it appears evident that chitin deacetylation represents an important step in *C. elegans* development, consistent with prior studies in other invertebrates.

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Appendix: Raw data of feeding experiments.

Table 1. Unblinded feeding experiment raw data.
48 H
EXPT

	EGG	L1	L2	L3	L4	ADULT	Total Progeny	Total Progeny (no eggs)	Total L3,L4, Adult
1	6	0	0	0	0	0	6	0	0
2	6	2	0	0	0	0	8	2	0
3	10	0	0	0	0	0	10	0	0
4	5	0	0	0	0	0	5	0	0
5	0	0	0	0	0	0	0	0	0
6	4	0	0	0	0	0	4	0	0
7	0	0	0	0	0	0	0	0	0
8	10	2	1	0	0	0	13	3	0
9	6	1	1	0	0	0	8	2	0
10	5	1	2	0	0	0	8	3	0
11	2	1	0	0	0	0	3	1	0
12	9	0	0	0	0	0	9	0	0
Total	63	7	4	0	0	0	74	11	0

48 H
CONT

	EGG	L1	L2	L3	L4	ADULT	Total Progeny	Total Progeny (no eggs)	Total L3,L4, Adult
1	7	0	0	0	0	0	7	0	0
2	21	2	0	0	0	0	23	2	0
3	7	5	2	0	0	0	14	7	0
4	7	4	0	0	0	0	11	4	0
5	24	5	0	0	0	0	29	5	0
6	19	0	0	0	0	0	19	0	0
7	6	3	0	0	0	0	9	3	0
8	13	0	0	0	0	0	13	0	0
9	25	3	0	0	0	0	28	3	0
10	16	4	0	0	0	0	20	4	0
11	20	1	0	0	0	0	21	1	0
12	24	5	0	0	0	0	29	5	0
Total	189	32	2	0	0	0	223	34	0

72 H
EXPT

	EGG	L1	L2	L3	L4	ADULT	Total Progeny	Total Progeny (no eggs)	Total L3,L4, Adult
1	1	0	2	5	0	0	8	7	5
2	0	0	12	9	0	0	21	21	9
3	7	0	5	3	0	0	15	8	3
4	3	1	6	1	0	0	11	8	1
5	0	0	0	0	0	0	0	0	0
6	1	2	5	12	2	3	25	24	17
7	0	0	0	0	0	0	0	0	0
8	5	1	7	11	2	2	28	23	15
9	4	0	4	9	1	2	20	16	12
10	2	0	2	6	6	3	19	17	15
11	1	1	0	1	0	0	3	2	1
12	3	3	16	5	0	0	27	24	5
Total	27	8	59	62	11	10	177	150	83

72 H
CONT

	EGG	L1	L2	L3	L4	ADULT	Total Progeny	Total Progeny (no eggs)	Total L3,L4, Adult
1	4	0	3	4	0	0	11	7	4
2	7	0	4	6	7	5	29	22	18
3	4	0	1	5	4	6	20	16	15
4	6	0	6	4	6	5	27	21	15
5	6	0	32	26	7	8	79	73	41
6	1	0	17	13	2	0	33	32	15
7	5	6	14	11	5	0	41	36	16
8	6	1	4	6	0	2	19	13	8
9	8	8	16	8	0	0	40	32	8
10	8	0	5	15	3	4	35	27	22
11	12	2	10	7	1	0	32	20	8
12	6	2	5	9	2	0	24	18	11
Total	73	19	117	114	37	30	390	317	181

Table 2. Trial 1 blinded.
48 H
EXPT

	EGG	L1	L2	L3	L4	ADULT	Total Progeny	Total Progeny (no eggs)	Total L3,L4, Adult
1	2	0	0	0	0	0	2	0	0
2	0	0	0	0	0	0	0	0	0
3	10	0	0	0	0	0	10	0	0
4	0	0	0	0	0	0	0	0	0
5	5	0	0	0	0	0	5	0	0
6	31	0	0	0	0	0	31	0	0
7	19	0	0	0	0	0	19	0	0
8	3	0	0	0	0	0	3	0	0
9	2	0	0	0	0	0	2	0	0
10	4	0	0	0	0	0	4	0	0
11	12	0	0	0	0	0	12	0	0
12	39	4	0	0	0	0	43	4	0
Total	127	4	0	0	0	0	131	4	0

48 H
CONT

	EGG	L1	L2	L3	L4	ADULT	Total Progeny	Total Progeny (no eggs)	Total L3,L4, Adult
1	5	0	0	0	0	0	5	0	0
2	3	0	0	0	0	0	3	0	0
3	20	0	0	0	0	0	20	0	0
4	0	0	0	0	0	0	0	0	0
5	2	0	0	0	0	0	2	0	0
6	6	0	0	0	0	0	6	0	0
7	9	0	0	0	0	0	9	0	0
8	38	1	0	0	0	0	39	1	0
9	21	22	3	0	0	0	46	25	0
10	12	8	0	0	0	0	20	8	0
11	38	19	0	0	0	0	57	19	0
12	8	8	0	0	0	0	16	8	0
Total	162	58	3	0	0	0	223	61	0

72 H
EXPT

	EGG	L1	L2	L3	L4	ADULT	Total Progeny	Total Progeny (no eggs)	Total L3,L4, Adult
1	0	2	0	0	0	0	2	2	0
2	0	0	0	0	0	0	0	0	0
3	0	0	6	4	0	0	10	10	4
4	0	0	0	0	0	0	0	0	0
5	0	1	6	0	0	0	7	7	0
6	12	0	6	12	4	0	34	22	16
7	1	1	11	5	0	0	18	17	5
8	0	1	2	0	0	0	3	3	0
9	1	0	1	0	0	0	2	1	0
10	0	0	1	4	0	0	5	5	4
11	0	0	5	8	0	0	13	13	8
12	3	0	10	19	12	2	46	43	33
Total	17	5	48	52	16	2	140	123	70

72 H
CONT

	EGG	L1	L2	L3	L4	ADULT	Total Progeny	Total Progeny (no eggs)	Total L3,L4, Adult
1	0	0	2	2	0	0	4	4	2
2	0	0	2	1	0	0	3	3	1
3	1	0	11	8	3	0	23	22	11
4	0	0	0	0	0	0	0	0	0
5	0	0	2	0	0	0	2	2	0
6	0	0	5	1	0	0	6	6	1
7	5	0	6	5	8	2	26	21	15
8	8	22	15	5	0	0	50	42	5
9	2	0	1	19	17	6	45	43	42
10	1	0	4	2	0	0	7	6	2
11	6	0	3	7	2	0	18	12	9
12	2	0	2	4	0	0	8	6	4
Total	25	22	53	54	30	8	192	167	92

Trial 3. Trial 2 blinded.
48 H
EXPT

	EGG	L1	L2	L3	L4	ADULT	Total Progeny	Total Progeny (no eggs)	Total L3,L4, Adult
1	16	6	7	1	0	0	30	14	1
2	31	4	6	1	0	0	42	11	1
3	14	2	2	0	0	0	18	4	0
4	31	2	0	0	0	0	33	2	0
5	32	0	0	0	0	0	32	0	0
6	34	8	7	0	0	0	49	15	0
7	8	10	14	2	0	0	34	26	2
8	23	3	5	1	0	0	32	9	1
9	45	3	3	0	0	0	51	6	0
10	49	7	1	0	0	0	57	8	0
11	43	3	1	0	0	0	47	4	0
12	42	1	0	0	0	0	43	1	0
Total	368	49	46	5	0	0	468	100	5

48 H
CONT

	EGG	L1	L2	L3	L4	ADULT	Total Progeny	Total Progeny (no eggs)	Total L3,L4, Adult
1	20	6	9	0	0	0	35	15	0
2	32	6	3	0	0	0	41	9	0
3	4	0	0	0	0	0	4	0	0
4	33	0	0	0	0	0	33	0	0
5	40	0	2	0	0	0	42	2	0
6	50	4	3	0	0	0	57	7	0
7	59	2	5	0	0	0	66	7	0
8	33	7	4	1	0	0	45	12	1
9	21	5	19	5	0	0	50	29	5
10	12	11	11	16	11	0	61	49	27
11	38	4	0	0	0	0	42	4	0
12	8	2	0	0	0	0	10	2	0
Total	350	47	56	22	11	0	486	136	33

72 H
EXPT

	EGG	L1	L2	L3	L4	ADULT	Total Progeny	Total Progeny (no eggs)	Total L3,L4, Adult
1	3	2	17	11	1	0	34	31	12
2	6	0	6	24	15	3	54	48	42
3	2	0	10	21	3	1	37	35	25
4	3	0	12	7	0	0	22	19	7
5	0	0	6	21	4	0	31	31	25
6	11	2	14	15	7	4	53	42	26
7	3	0	3	22	18	12	58	55	52
8	12	0	1	9	7	6	35	23	22
9	6	3	14	27	1	0	51	45	28
10	1	2	33	21	0	0	57	56	21
11	2	9	14	8	0	0	33	31	8
12	6	1	12	16	0	0	35	29	16
Total	55	19	142	202	56	26	500	445	284

72 H
CONT

	EGG	L1	L2	L3	L4	ADULT	Total Progeny	Total Progeny (no eggs)	Total L3,L4, Adult
1	6	0	7	30	15	7	65	59	52
2	12	2	23	12	7	0	56	44	19
3	0	0	0	0	0	0	0	0	0
4	7	8	11	7	0	0	33	26	7
5	2	0	11	19	10	1	43	41	30
6	8	5	19	14	1	0	47	39	15
7	21	1	6	13	12	4	57	36	29
8	11	9	18	7	0	0	45	34	7
9	7	0	1	26	21	14	69	62	61
10	3	0	14	50	16	4	87	84	70
11	4	4	21	9	0	0	38	34	9
12	3	1	1	0	0	0	5	2	0
Total	84	30	132	187	82	30	545	461	299

Trial 4. Trial 3 blinded.
48 H
EXPT

	EGG	L1	L2	L3	L4	ADULT	Total Progeny	Total Progeny (no eggs)	Total L3,L4, Adult
1	7	0	0	0	0	0	7	0	0
2	26	0	0	0	0	0	26	0	0
3	11	0	0	0	0	0	11	0	0
4	5	0	0	0	0	0	5	0	0
5	0	0	0	0	0	0	0	0	0
6	14	0	0	0	0	0	14	0	0
7	10	0	0	0	0	0	10	0	0
8	23	3	0	0	0	0	26	3	0
9	5	0	0	0	0	0	5	0	0
10	28	0	0	0	0	0	28	0	0
11	0	0	0	0	0	0	0	0	0
12	3	0	0	0	0	0	3	0	0
Total	132	3	0	0	0	0	135	3	0

48 H
CONT

	EGG	L1	L2	L3	L4	ADULT	Total Progeny	Total Progeny (no eggs)	Total L3,L4, Adult
1	0	0	0	0	0	0	0	0	0
2	0	0	0	0	0	0	0	0	0
3	0	0	0	0	0	0	0	0	0
4	19	0	0	0	0	0	19	0	0
5	32	0	0	0	0	0	32	0	0
6	0	0	0	0	0	0	0	0	0
7	14	0	0	0	0	0	14	0	0
8	21	0	0	0	0	0	21	0	0
9	28	0	0	0	0	0	28	0	0
10	21	0	0	0	0	0	21	0	0
11	4	0	0	0	0	0	4	0	0
12	0	0	0	0	0	0	0	0	0
Total	139	0	0	0	0	0	139	0	0

72 H
EXPT

	EGG	L1	L2	L3	L4	ADULT	Total Progeny	Total Progeny (no eggs)	Total L3,L4, Adult
1	1	6	0	0	0	0	7	6	0
2	4	16	5	0	0	0	25	21	0
3	1	5	4	0	0	0	10	9	0
4	1	3	1	0	0	0	5	4	0
5	0		0	0	0	0	0	0	0
6	3	12	0	0	0	0	15	12	0
7	1	5	5	3	0	0	14	13	3
8	3	10	19	3	0	0	35	32	3
9	1	1	3	0	0	0	5	4	0
10	3	11	10	2	0	0	26	23	2
11	0	3	0	0	0	0	3	3	0
12	0	0	0	0	0	0	0	0	0
Total	18	72	47	8	0	0	145	127	8

72 H
CONT

	EGG	L1	L2	L3	L4	ADULT	Total Progeny	Total Progeny (no eggs)	Total L3,L4, Adult
1	0	0	0	0	0	0	0	0	0
2	0	0	0	0	0	0	0	0	0
3	0	0	0	0	0	0	0	0	0
4	3	13	12	1	0	0	29	26	1
5	1	11	15	8	0	0	35	34	8
6	0	0	0	0	0	0	0	0	0
7	3	7	5	0	0	0	15	12	0
8	2	6	11	5	0	0	24	22	5
9	2	10	11	3	0	0	26	24	3
10	2	8	9	2	0	0	21	19	2
11	0	1	3	0	0	0	4	4	0
12	0	0	0	0	0	0	0	0	0
Total	13	56	66	19	0	0	154	141	19

Trial 5. Trial 4 blinded.
48 H
EXPT

	EGG	L1	L2	L3	L4	ADULT	Total Progeny	Total Progeny (no eggs)	Total L3,L4, Adult
1	24	17	1	0	0	0	42	18	0
2	34	17	1	0	0	0	52	18	0
3	26	12	1	0	0	0	39	13	0
4	17	30	0	0	0	0	47	30	0
5	19	13	7	0	0	0	39	20	0
6	30	12	0	0	0	0	42	12	0
7	23	26	0	0	0	0	49	26	0
8	53	16	2	0	0	0	71	18	0
9	21	14	0	0	0	0	35	14	0
10	40	12	0	0	0	0	52	12	0
11	39	13	0	0	0	0	52	13	0
12	51	15	3	0	0	0	69	18	0
Total	377	197	15	0	0	0	589	212	0

48 H
CONT

	EGG	L1	L2	L3	L4	ADULT	Total Progeny	Total Progeny (no eggs)	Total L3,L4, Adult
1	53	11	0	0	0	0	64	11	0
2	30	22	3	0	0	0	55	25	0
3	31	13	0	0	0	0	44	13	0
4	21	8	0	0	0	0	29	8	0
5	32	15	0	0	0	0	47	15	0
6	46	9	0	0	0	0	55	9	0
7	30	14	0	0	0	0	44	14	0
8	39	20	0	0	0	0	59	20	0
9	16	16	0	0	0	0	32	16	0
10	26	8	0	0	0	0	34	8	0
11	36	15	0	0	0	0	51	15	0
12	25	7	0	0	0	0	32	7	0
Total	385	158	3	0	0	0	546	161	0

72 H
EXPT

	EGG	L1	L2	L3	L4	ADULT	Total Progeny	Total Progeny (no eggs)	Total L3,L4, Adult
1	6	28	25	9	0	0	68	62	9
2	5	7	33	20	4	0	69	64	24
3	1	1	17	27	14	5	65	64	46
4	4	9	24	16	2	0	55	51	18
5	9	0	1	9	14	10	43	34	33
6	4	18	41	8	0	0	71	67	8
7	4	32	42	7	0	0	85	81	7
8	1	0	17	23	10	7	58	57	40
9	7	3	18	19	1	0	48	41	20
10	6	11	37	14	3	0	71	65	17
11	5	9	29	8	0	0	51	46	8
12	22	1	31	23	3	3	83	61	29
Total	74	119	315	183	51	25	767	693	259

72 H
CONT

	EGG	L1	L2	L3	L4	ADULT	Total Progeny	Total Progeny (no eggs)	Total L3,L4, Adult
1	13	7	30	12	5	0	67	54	17
2	8	1	17	22	21	15	84	76	58
3	8	24	21	1	0	0	54	46	1
4	1	0	29	31	17	0	78	77	48
5	5	3	29	20	7	1	65	60	28
6	13	0	25	23	7	0	68	55	30
7	4	7	39	16	6	0	72	68	22
8	6	3	44	24	6	0	83	77	30
9	0	0	26	27	9	0	62	62	36
10	9	0	28	21	3	0	61	52	24
11	13	2	39	26	3	0	83	70	29
12	8	27	20	1	0	0	56	48	1
Total	88	74	347	224	84	16	833	745	324