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A Study of the Mechanism by which Nicotinamide and TRPV Channels Induce an Egg-Laying Defect in *C. elegans*

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ABSTRACT

Nicotinamide (NAM) is known to bind to heterotetrameric TRPV cation channels and cause them to be constitutively active. The OCR-4 and OSM-9 monomers of these channels are necessary for nicotinamide to induce uv-1 cell necrosis and an egg-laying defect (Egl) in which the animal cannot lay eggs. So, while NAM treatment induces these phenotypes in wild-type *C. elegans*, it induces very little or none of them in *osm-9* and *ocr-4* mutants lacking TRPV channels. While the cell necrosis and Egl phenotypes are always present or absent together in NAM-treated animals, we know that neither OLQ nor uv-1 cell necrosis causes Egl. I investigate this hypothesis by first inducing the Egl phenotype shown previously in my laboratory's research. To establish that NAM induces Egl in N2 wild-type worms but not *osm-9* worms, I did bagging assays on both strains treated with NAM. I have tested NAM age, NAM time on the plate, NAM concentration, and wild-type strain as causes for the inconsistent Egl phenotype. Thus far, NAM has induced Egl in only about half of wild-type animals regardless of concentration, and only 5.56% Egl in *osm-9* worms.

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ACKNOWLEDGEMENTS

I would like to thank Avni Upadhyay for her research and advice that made this study possible. I'd also like to thank Dr. Wendy Hanna-Rose for mentoring me for the last year and helping me thrive in independent research.

Chapter 1

Introduction

A wealth of research has been done on nicotinamide adenine dinucleotide (NAD^+); including studies on its biosynthesis, genetic predictors, role as an electron carrier, and effect on *C. elegans* development and function. Nicotinamide (NAM) is a key precursor to NAD^+ biosynthesis and byproduct of NAD^+ consumption. Our laboratory first uncovered the importance of moderating NAM levels when we found a mutant *C. elegans* strain lacking functional PNC-1 enzyme, a nicotinamidase whose substrate is NAM. These *pnc-1* worms displayed a few distinct phenotypes, OLQ and uv1 cell death, foraging and nose touch defects, and an egg-laying defect, all of which are caused by NAM accumulation[1,2]. Furthermore, we found that acute NAM treatment causes these exact phenotypes. More recently, our laboratory found that a subset of TRPV cation channels are necessary for nicotinamide to cause these phenotypes. We elucidated the causes and mechanisms by which OLQ and uv1 cell death and the foraging and nose touch defects occur, but one still remains: the egg-laying defect (Egl)[2]. In this chapter, I will provide background on the significance of NAM and NAD^+ biosynthesis in general and for this project, TRPV channel function and the specific ones focused on in this study, normal egg laying in *C. elegans*, the role of vulval muscles and uv1 cells in egg laying, the past data that lead me to this study, and my specific experimental hypothesis and strategies.

Nicotinamide and Nicotinamide Adenine Dinucleotide Biosynthesis

Nicotinamide acts as an NAD^+ precursor in the NAD^+ salvage biosynthesis pathway in all organisms[1]. While higher and lower organisms use different enzymes and intermediate metabolites to get there, they all create NAD^+ from NAM[1,3] and break down NAD^+ to restart the cycle (Figure 1.1). In fact, in humans, pellagra is a disease caused by NAM deficiency and treated by supplementation with vitamin B3, which includes niacin, nicotinamide riboside (NR), and NAM[4]. Vitamin B3 supplements have become increasingly popular over the years. NR specifically has gained attention for its apparent protection against unhealthy diet and neurodegenerative diseases[5,6], and vitamin B3 is often added to energy drinks to add another energy source besides caffeine and market them as healthier options. In *C. elegans*, both NAD^+ biosynthesis and NAM levels have been linked to development and health[1].

Given all of the benefits of NAM and consequences of its deficiency, it is interesting that excessive NAM causes deleterious phenotypes in *C. elegans*. Our laboratory began to study *pnc-1* mutants, which lack function of PNC-1, a nicotinamidase that converts NAM to nicotinic acid (NA) in the NAD^+ salvage biosynthesis pathway (Figure 1.1). *pnc-1* mutants have several phenotypes such as the inability to lay eggs, necrosis of two specific cell types, and neurobehavioral defects. More recently, we discovered that these phenotypes are caused by elevated NAM levels that result from the inability to convert it to NA. Specifically, NAM acts as an agonist for TRPV channels, making them constitutively active, and this causes the phenotypes[2].

TRPV Channels

TRPV channels are cation channels that are most commonly expressed in sensory neurons, and some other cell types as well[7]. They are found in both invertebrates and vertebrates and respond to a variety of endogenous and exogenous stimuli. They are homo- or heterotetramers, the monomers of which interact to open and close the channel as they receive signals to do so. In this study, I will focus on OSM-9/OCR-4 heterotetramers. These subunits were found to be the only two whose function are necessary for NAM-induced phenotypes to occur[2]. The majority of the literature on TRPV channels concerns their involvement in sensory function and neurons[8,9], not reproductive function. This research will begin to fill that gap by possibly identifying a new cell type where OSM-9 TRPV channel subunits are expressed and explaining how NAM and TRPV channels work together to prevent *C. elegans* hermaphrodites from laying eggs.

Egg Laying in C. elegans

As *C. elegans* hermaphrodites exit larval stage L4 and enter the young adult stage, they begin to accumulate self-fertilized eggs in the uterus. The hermaphrodite-specific neurons (HSNs), VC motorneurons (VCs), and sex muscles (including uterine and vulval muscles) all work together to direct egg-laying. The HSNs connect the egg-laying circuit to both the nerve cord and nerve ring, making it absolutely vital for the signaling involved in egg laying. They utilize multiple neurotransmitters, including serotonin[10] and acetylcholine[11]. While worms lacking functional HSNs do have an egg-laying defect, it is not the same one that I am screening for in this study. Lack of HSNs decreases the number of egg laying events a worm has, but does

not decrease the number of eggs or speed of egg laying during the events that do happen[12]. VCs are also hermaphrodite specific neurons and are located in the nerve cord; they receive signals from the HSNs as well as communicate with the vulval muscles through neuromuscular junctions. Unlike HSNs, VCs seem to inhibit egg laying, since mutants lacking functional VCs lay eggs more quickly and accumulate less eggs in the uterus at once. VC4 and VC5, specifically, seem to be the most actively involved with vulval muscles[13]. Finally, the vulval muscles play a role in egg laying. In particular, the four vm2 cells receive the most signals from neurons and are the strongest determinant of egg laying. Worms lacking functional vm2 cells cannot lay eggs at all[14], and therefore have the egg-laying defect that I am exploring in this study. The other vulval muscles as well as some uterine muscles are thought to be involved in egg-laying, but do not induce a defect upon removal. In a screen of 59 egg-laying abnormal (*egl*) *C. elegans* mutants, the majority of them also had somatic and/or sex muscle defects[15]. Given vulval muscles seem to be the most key players in *C. elegans* egg laying, it is valid to explore damaged or dysfunctional vulval muscles as a possible cause for the egg-laying present in NAM treated worms.

Unknown cause of the NAM-induced egg-laying defect

In a previously published paper from our lab, we discovered that acute exposure to NAM induces OLQ and uv1 cell necrotic death, several behavioral phenotypes, and an egg-laying defect (Egl)[2]. The majority of NAM treated wild-type worms have dead uv1 and OLQ cells. However, no mutants for the heterotetratmeric OSM-9/OCR-4 TRPV channels implicated in this project show dead uv1 or OLQ cells. Both *osm-9* and *ocr-4* mutants show 0% dead uv1 and OLQ

cells compared to the majority of wild-type worms that do. Various other TRPV channel mutants still display these phenotypes (Figure 1.4). Thus, it is clear that NAM and OSM-9/OCR-4 TRPV channels work together to cause uv1 and OLQ cell death.

An egg-laying defect (Egl) also occurs in almost all wild-type worms treated with NAM. However, again, *osm-9* mutants show 0% Egl when treated with NAM (Figure 1.5). While *ocr-4* mutants do have reduced Egl with only 15% penetrance, the presence of any Egl is significant. *ocr-4* mutants completely eliminate uv1 cell death, but some Egl remains.

Uterine vulval uv1 cells are neuroendocrine cells that help control the uterus by signaling it when and when not to release eggs. Thus, uv1 cells initially seem like they may be a viable candidate for the cell type that NAM acts on to cause Egl. However, in addition to some Egl remaining while uv1 cell death is eliminated in the *ocr-4* mutants, the literature shows that dead or malfunctioning uv1 cells should not be expected to inhibit egg laying. In fact, uv1 cells are meant to inhibit egg laying. Uv1 cell mutant *C. elegans* actually lay eggs more quickly; they hold them for less time in the uterus and lay them while the embryos are at earlier developmental stages[16].

Experimental Rationale

I hypothesized that acute NAM treatment causes Egl in *C. elegans* by acting on TRPV channels in muscle cells because of the importance of vulval muscles in egg laying. I planned to investigate this hypothesis by focusing on OSM-9, one of the two TRPV subunits that proved to be necessary for Egl to occur in response to NAM. I predict that expression of OSM-9 exclusively in the muscle will rescue an *osm-9* mutant by restoring its sensitivity to NAM. I

designed a vector to clone the OSM-9 gene into such that it is fused with green fluorescent protein (GFP) and its expression is driven by the muscle-specific *myo-3* promoter. I also collected data to establish baselines for comparison once we obtain the transgenic animals. I measured whether or not worms have Egl by conducting assays in which worms are observed for the presence, or lack thereof, of "bagging", in which eggs hatch inside of the mother because they cannot be laid. Specifically, I conducted the following bagging assays.

1. Negative control: N2 wild-type worms with normal OP50 *E. coli* food. These worms should not be Egl.
2. Positive control: N2 wild-type worms with OP50 and NAM. These worms should be Egl.
3. Negative control: *osm-9* mutants with OP50 and NAM. These worms should not be Egl.

Most of my time was spent optimizing assay no. 2 because my experiments with NAM did not produce nearly as penetrant Egl as that in past publications[2], despite several different experimental conditions.

By knocking out any TRPV channels containing OSM-9 in the *osm-9* mutants, we deny NAM the opportunity to bind to the channels, render them constitutively active, and cause Egl. Therefore, I expected *osm-9* mutants to have 0% Egl on NAM.

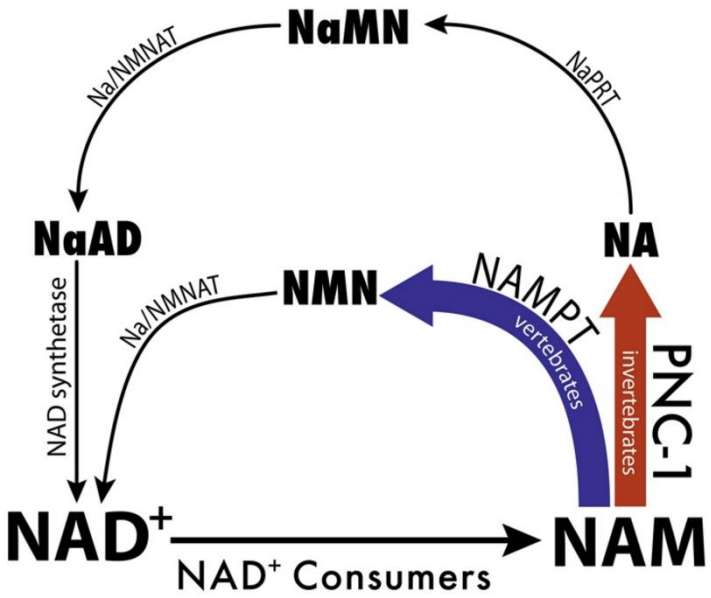

Figure 1.1. Salvage pathway for NAD^+ biosynthesis[1].

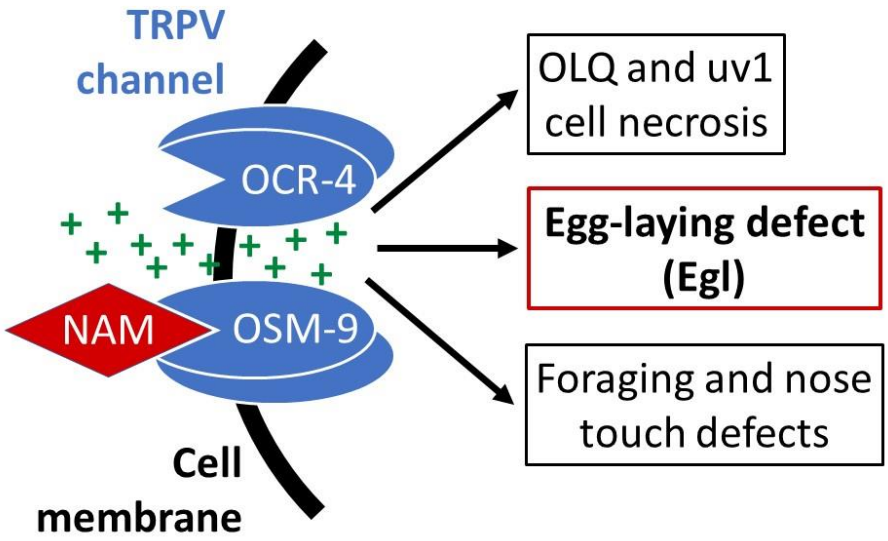

Figure 1.2. Interaction between NAM and heterotetrameric TRPV channels to induce the resultant phenotypes .

Figure 1.3. A bagged N2 wild-type worm on OP50 *E. coli* supplemented with NAM. The bagging is evident from the stiff straight shape of the worm's body and the small L1 larvae inside of the body.

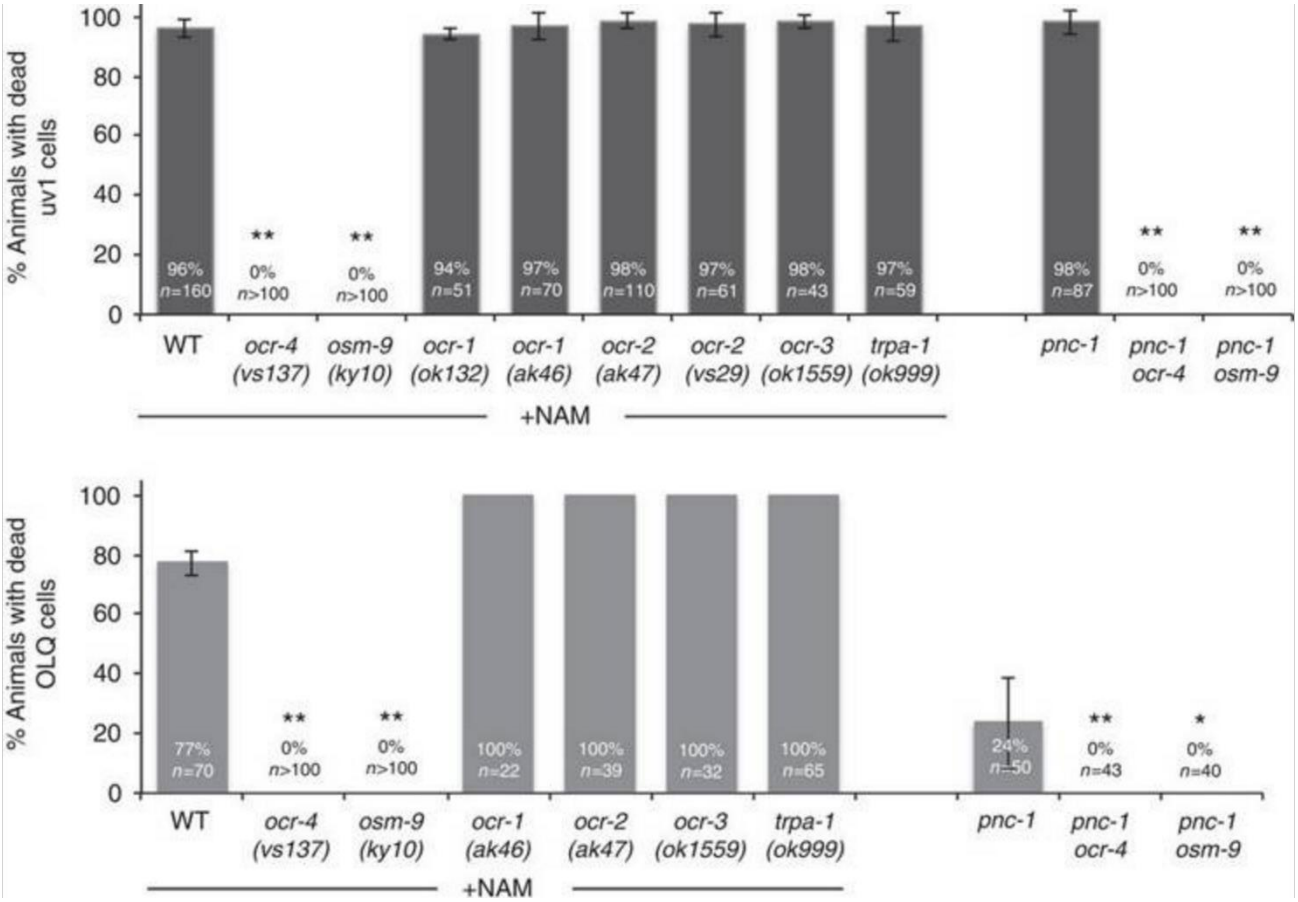

Figure 1.4. Differential *C. elegans* strain uv1 and OLQ cell death in the presence of nicotinamide. 96% of N2 wild-type worms on NAM have dead uv1 cells, while 0% of *ocr-4* and *osm-9* mutants have dead uv1 cells. All other mutants for TRPV channels had no effect on the phenotypes. Note: this figure was obtained from "Nicotinamide is an endogenous agonist for a C. elegans TRPV OSM-9 and OCR-4 channel," Upadhyay et al, 2016[2].

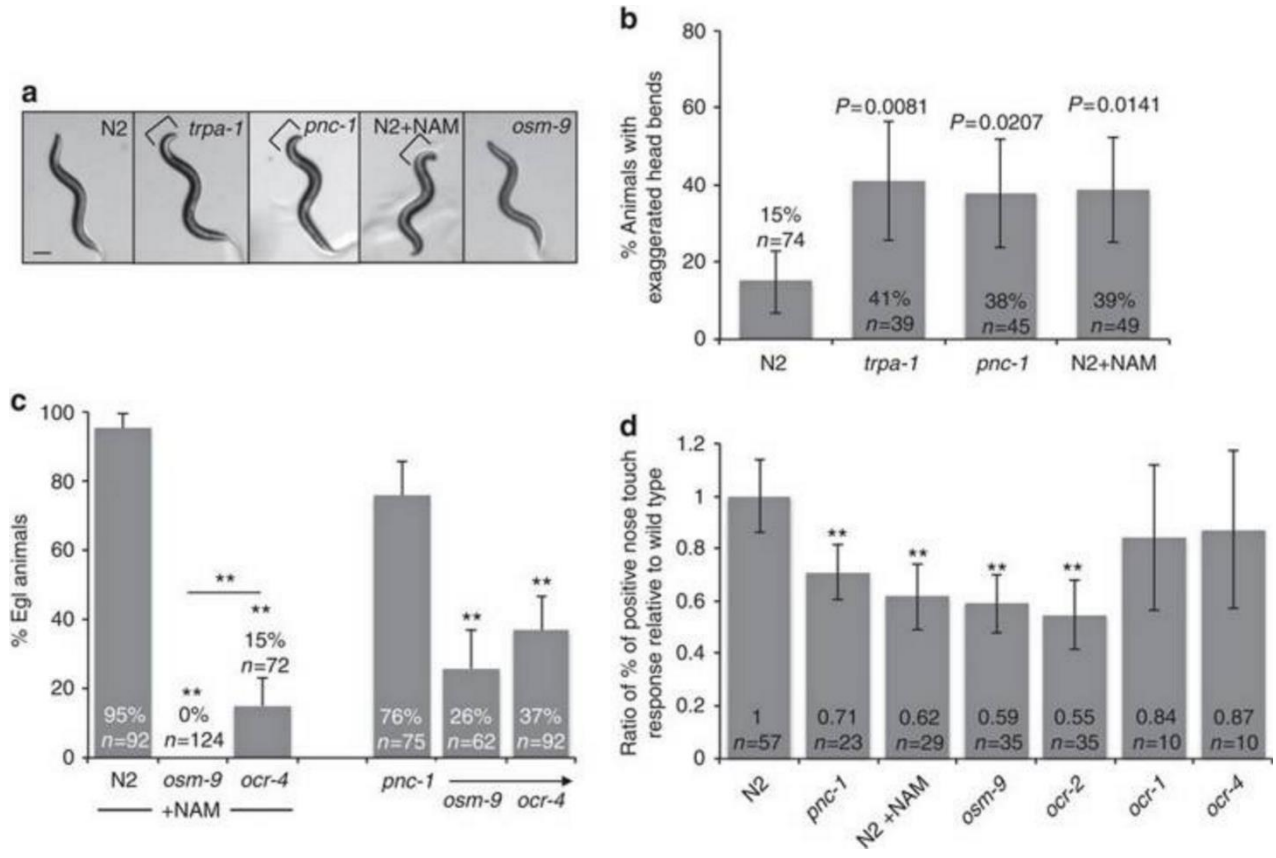

Figure 1.5. Differential *C. elegans* strain foraging behavior, nose touch response, and Egl in the presence of nicotinamide. Panel C shows that when treated with NAM, *osm-9* mutants are 0% Egl and *ocr-4* mutants are 15% Egl; both are greatly reduced compared to the 95% of N2 wild-type worms that have Egl[2]. Note: this figure was obtained from "Nicotinamide is an endogenous agonist for a C. elegans TRPV OSM-9 and OCR-4 channel," Upadhyay et al.

Chapter 2

Materials and Methods

The egg-laying defect observed in this study consists of the *C. elegans* adult hermaphrodite "bagging" instead of laying eggs. Bagging is when the eggs hatch inside of the mother's body, killing the mother and allowing the L1 larvae to survive. To measure bagging in different scenarios, I conducted bagging assays[1].

C. elegans Culture

I grew all worms on nematode growth medium (NGM) plates spotted with OP50 *E. coli* food. All worms were grown in an incubator at 20ºC. If NAM solution was supplemented, it was added to plates after they were spotted. OP50 was allowed at least two days to dry, and NAM was allowed at least one day to dry.

I produced two separate sterile 1 M NAM liquid solutions by dissolving solid NAM (MW=122.12 g/mol) in ddH_2O and filter sterilizing the solution. One stock solution contained NAM obtained years ago, and one contained NAM obtained in March 2017. To treat worms with NAM, I added the 1 M stock solution directly to 8 ml NGM plates spotted with OP50. I added 200 µl or 400 µl of 1 M stock solution to each plate to make a final concentration of 25 mM or 50 mM, respectively.

Bagging Assays for Assessing the Egg-laying Defect

Experiments were conducted with different strains of *C. elegans*, and with NAM at different concentrations and using different batches of NAM. I placed a single larval stage L4 (last larval stage before molting) animal onto an NGM plate spotted with OP50 food. For five days after, I recorded the state of the worm daily: whether it had laid eggs or had any live progeny, and whether or not it had bagged (Figure 2.1). Since the NAM did not cause a nearly as penetrant or fast-acting phenotype as expected, the majority of the worms had tens of progeny before they bagged (if they bagged at all). As the progeny aged, some of them bagged too; so it was difficult to tell if the original worm or a progeny bagged. In later experiments, the original worm was transferred to a new plate on day 3 so it was not confused with its own progeny.

For each experiment, I will explicitly state which *C. elegans* strain was used, whether or not NAM was added, at which concentration, and if it was the old or new batch.

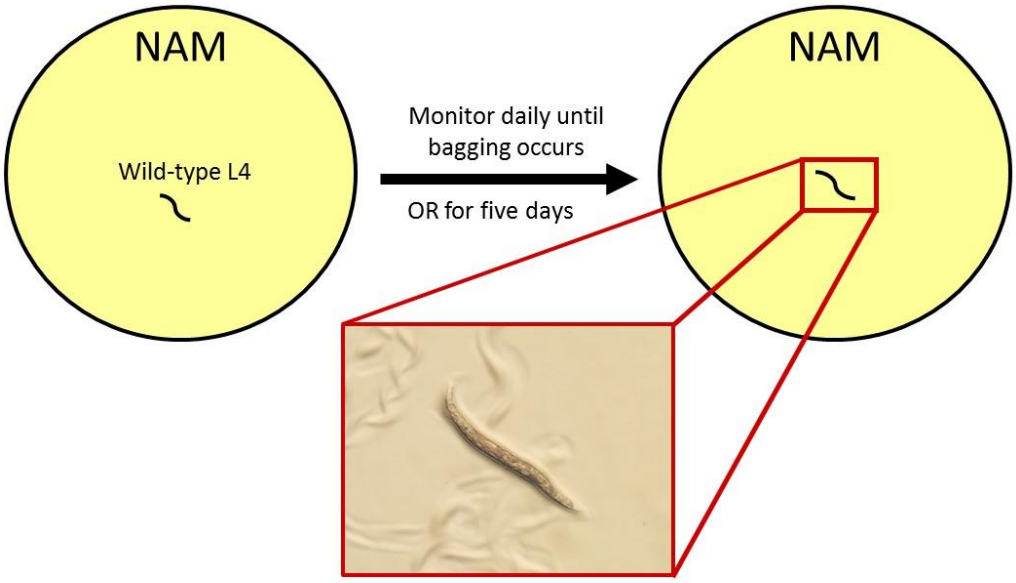

Figure 2.1. Diagrammatic representation of a bagging assay, and the bagging/Egl phenotype.

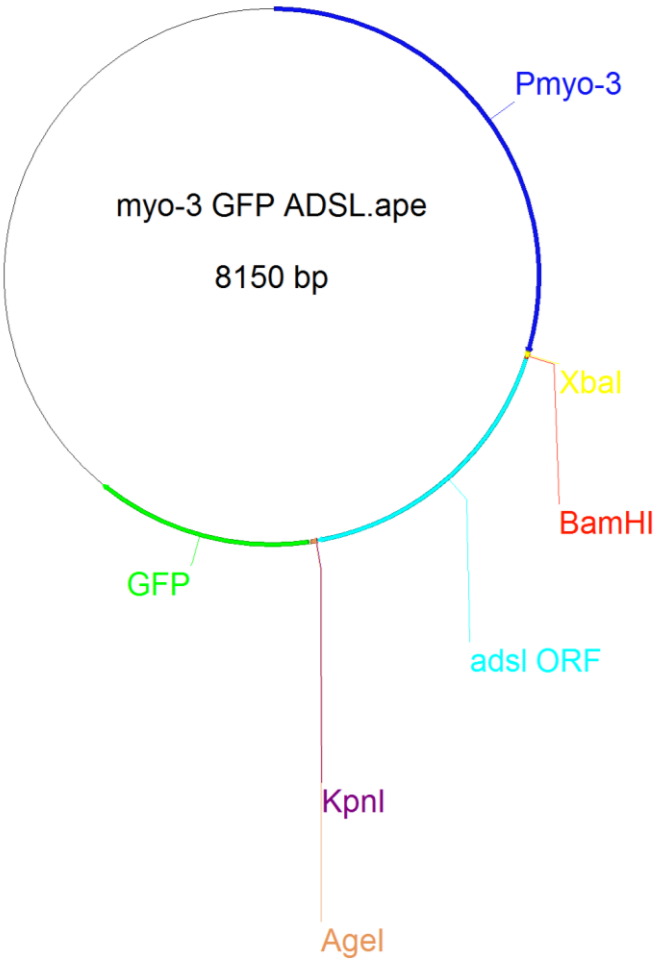

Figure 2.2. Plasmid map of P*myo-3*::adsl-1::GFP, the plasmid that I will modify for my experiment. The *adsl* gene was originally inserted using KpnI and BamHI, but I will remove it using AgeI and XbaI.

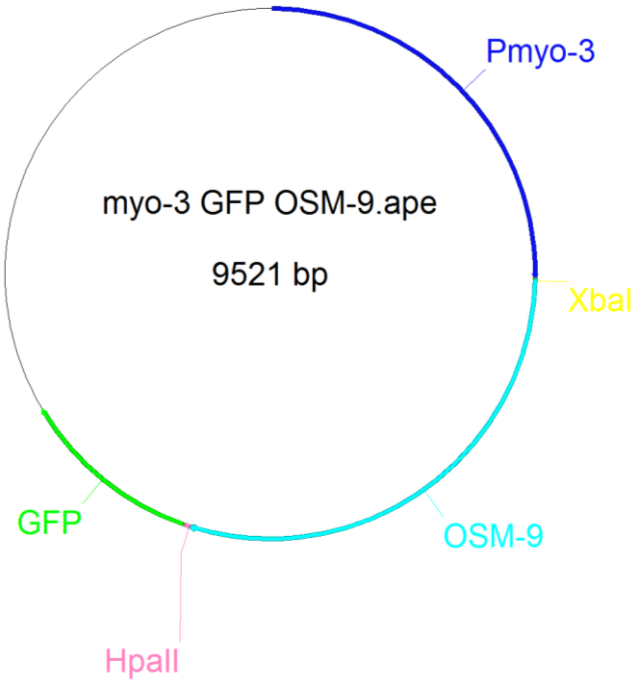

Figure 2.3. Plasmid map for P*myo-3*::osm-9::GFP, the plasmid that I will create from P*myo-3*::adsl-1::GFP.

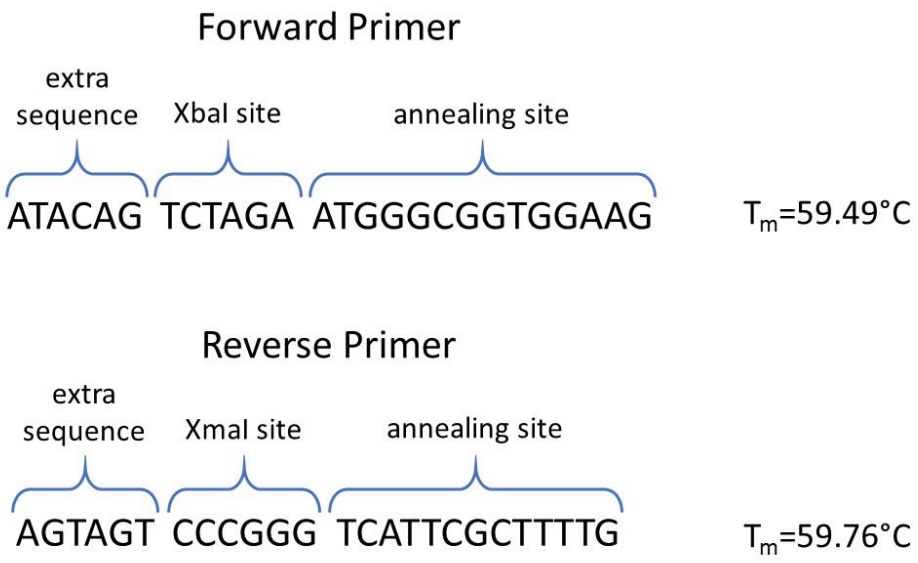

Figure 2.4. Primers that I designed to amplify the OSM-9 gene from OSM-9 cDNA. T_m values were calculated by nearest neighbor.

Chapter 3

Results

I experimented with improving the penetrance of NAM-induced Egl in N2 wild-type animals, the prevalence of bagging in *osm-9* mutants, and creating a recombinant plasmid to reintroduce functional OSM-9 subunits into the muscle cells of the mutants.

Establishing and Optimizing NAM-induced Egl

I first set out to induce Egl in N2 wild-type animals by comparing the percent Egl animals of N2 worms on plain OP50 plates and those on OP50 plates with 25 mM NAM added. In my first round of these experiments, I found that 60% of animals treated with NAM were Egl, while 0% of animals without NAM were Egl (Figure 3.1). According to Fisher's exact test, this is statistically significant. However, it is not as high as the 96% Egl previously observed in our lab[2] (Figure 1.4). The bagging was also delayed and almost always occurred after the worm had already laid some eggs. In fact, the majority of the worms that bagged did so on day five, after they had laid eggs for three or four days (Figure 3.2).

To reproduce the penetrant phenotype observed by previous researchers, I explored possible factors that could affect the efficacy of the NAM. First, I hypothesized that the longer NAM is left on the plate, the less effective it is. Thus, I did bagging assays comparing worms that had been placed on plates where NAM had been left on it for 3 or 7 days prior to the assay. Worms on 3 day old NAM had 70% Egl, while 7 day old NAM had 100% Egl (3.3). Both groups

oddly had higher Egl rates than the original bagging assay on NAM, and the percent Egl increased with the NAM that had been left on the plate for longer. So while both Egl phenotypes were statistically significant, something did not seem right.

The previous results seemed suspect because they both increased compared to the original control experiment, and older NAM produced a higher bagging rate. These significantly higher Egl phenotypes and the fact that the majority of the worms in the first experiment had bagged on day 5 led me to change my protocol. Since many of the worms laid eggs before they bagged, the ones that bagged on day 5 laid almost a full brood size. Thus, some of their progeny had become adults and bagged themselves. To avoid confusing the original worm with its progeny in bagging assays, I decided to transfer the original worm to a new plate on day 3 of each assay. Furthermore, I decided to test four more factors in an attempt to increase the penetrance of Egl: N2 strain, NAM concentration, NAM stock age, and progeny of worms grown on NAM.

To test different wild-type N2 strains, I compared the N2 strain I had been using to an N2 stock from the Caenorhabditis Genetics Center (CGC) (N2 cgc) and a thawed stock of our N2 lab strain from the 80°C freezer (new N2). First, the N2 worms had only 40% Egl, less than the original 60% I found, indicating that confusing progeny for the original worm likely caused an inflated percent Egl before. N2 cgc worms had 50% Egl and new N2 worms had only 10%. I conclude that N2 strain does not affect Egl penetrance because the N2 and N2 cgc results were so similar. The new N2's 10% Egl was statistically insignificant compared to the N2 without NAM control, but I hypothesize this is because of the small sample size, and that if I did more bagging assays on new N2 the overall Egl penetrance would increase to that of N2 and N2 cgc.

Next, I tested the effects of increasing NAM concentration and using newly bought NAM for my stock solution. I continued using the original NAM that was bought several years ago, and compared it to the new NAM bought in March 2017. For each NAM stock I used 25 and 50 mM concentrations on the plate. For 25 mM of old and new NAM, the worms had 18.75% and 12.5% Egl, respectively (Figure 3.5). For 50 mM of old and new NAM, the worms had 50% Egl (Figure 3.5). Thus, increasing the concentration of NAM did correlate with more penetrant Egl, but the stock NAM used did not.

Finally, I did bagging assays on worms that hatched and grew up on 25 mM old and new NAM plates. Only the N2 worms grown on 25 mM new stock NAM had a statistically significant phenotype of 71.43% (Figure 3.6) compared to the N2 without NAM control. The N2 worms grown on 25 mM old stock NAM had a statistically insignificant phenotype of 42.86% Egl compared to the N2 without NAM control. I hypothesize that using progeny of worms grown on NAM plates does increase Egl penetrance, but that more bagging assays need to be done to increase sample size, strengthen the correlation, and establish if new vs old stock NAM makes a difference in this case.

Establishing Lack of Egl in *osm-9* Mutants

Since 50 mM had been shown to induce a statistically significant percent Egl in N2 worms, I proceeded to reproduce the the failure of *osm-9* mutants to display the phenotype, as shown in previous research[2]. *osm-9* mutants were only 0.5% Egl upon treatment with 50 mM new stock NAM (Figure 3.5). This was statistically significant compared to the 46.67% Egl observed for N2 worms on 50 mM new NAM (Figure 3.5).

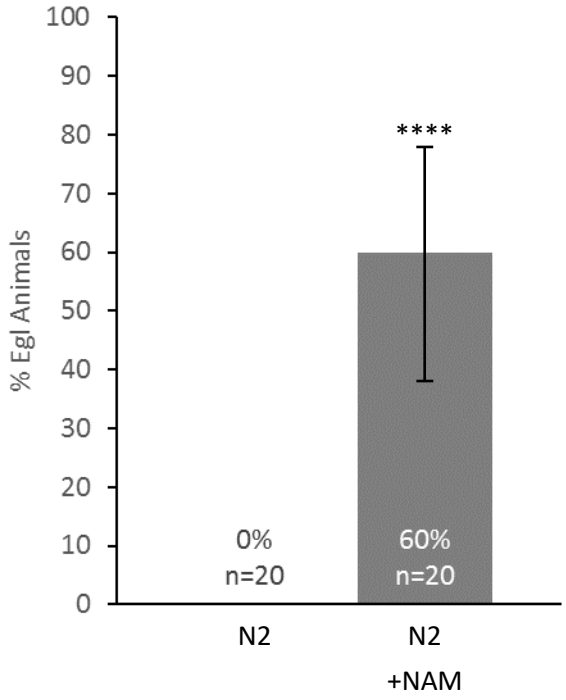

Figure 3.1. Results of bagging assays on N2 worms treated with and without 25 mM NAM. Error bar shows 95% confidence interval and asterisks represent p value according to Fischer's exact test. ****<0.0001.

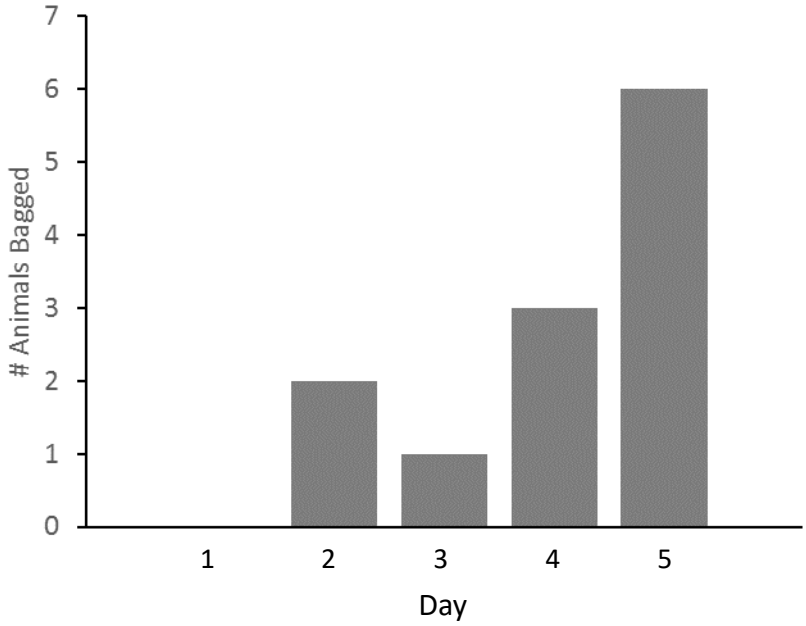

Figure 3.2. The day that each worm bagged in a series of bagging assays were recorded and compared.

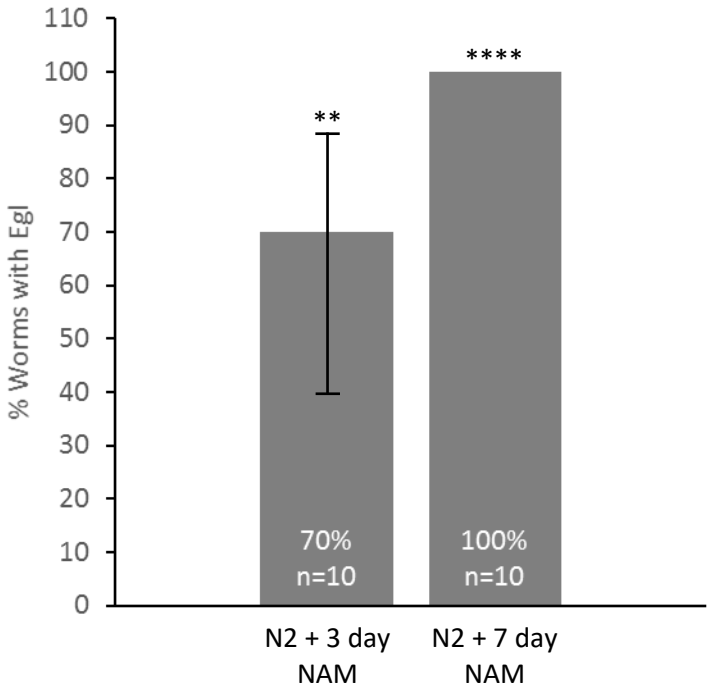

Figure 3.3. Results of bagging assays on worms treated with 25 mM NAM that had been left on plates for 3 and 7 days before the assays began. Error bar shows 95% confidence interval and asterisks represent p values according to Fischer's exact test. **=0.001-0.01, ****<0.0001.

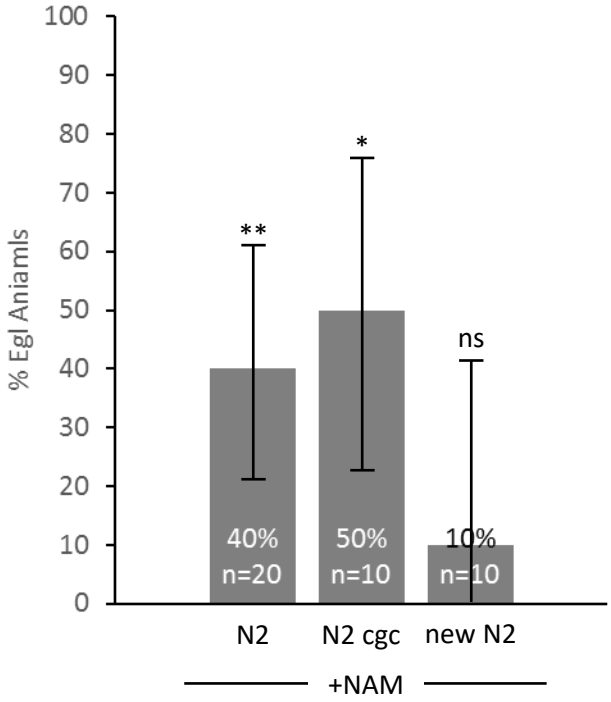

Figure 3.4. Results of bagging assays on our current lab strain N2 worms (N2), N2 worms from the CGC (N2 cgc), and our lab strain N2 thawed from a -80ºC stock (new N2) treated with 25 mM NAM. Error bars show 95% confidence intervals and asterisks represent p values according to Fischer's exact test. *=0.01-0.05, **=0.001-0.01, ns=not significant.

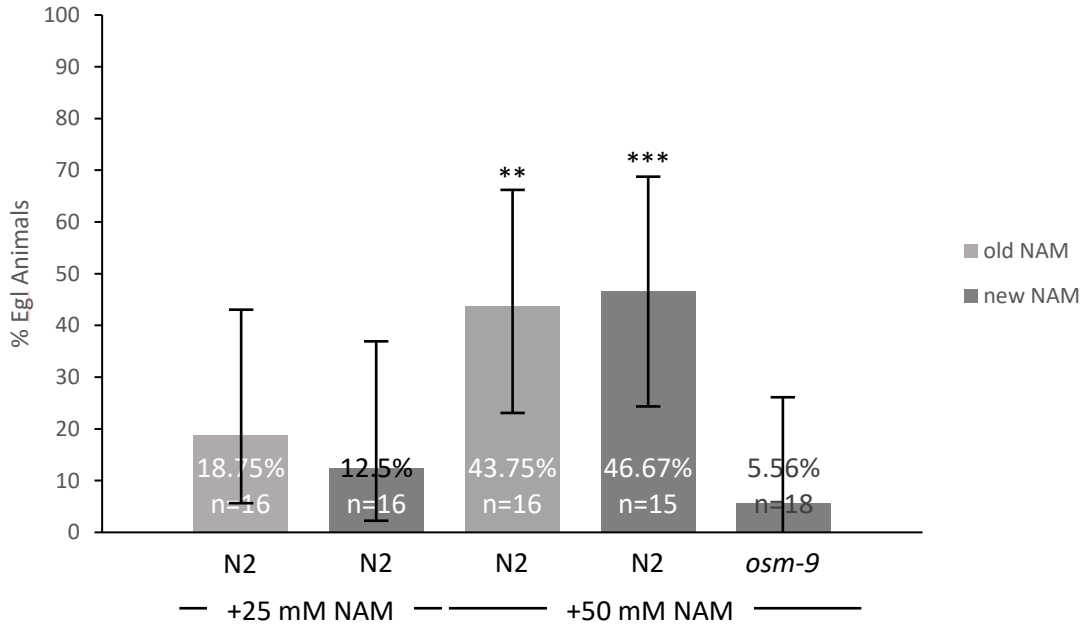

Figure 3.5. Results of bagging assays on the progeny of worms that laid eggs on plates with 25 mM old and new NAM. Error bars show 95% confidence intervals and asterisk represent p values according to Fischer's exact test. *=0.05-0.01, ns=not significant.

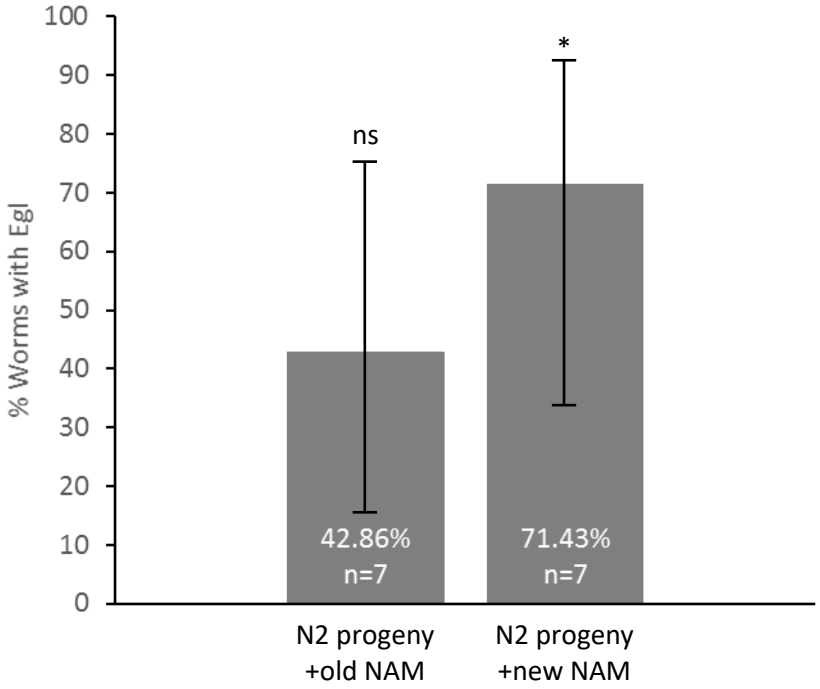

Figure 3.6. Results of bagging assays on N2 worms treated with 25 and 50 mM old and new NAM, as well as *osm-9* worms treated with 50 mM new NAM. Error bars show 95% confidence intervals and asterisks represent p values according to Fischer's exact test. **=0.01-0.001, ***=0.001-0.0001.

Chapter 4

Discussion

The bulk of my research has centered on finding a way to induce Egl with NAM to the extent as previously found in our laboratory. I experimented with leaving the NAM on the plate for different amounts of time, using N2 wild-type strains from different sources, comparing NAM bought years ago to new stocks purchased in March 2017, increasing NAM concentration from 25 mM to 50 mM, and doing bagging assays with worms that hatched and matured on NAM plates. Once I found the conditions under which I could consistently induce Egl (although still at a lower penetrance than expected), I proceeded to show that *osm-9* mutants have no Egl on NAM.

Establishing and Optimizing NAM-Induced Egl

In the initial experiment to establish that 25 mM NAM induces Egl, I found that 60% of N2 wild-type worms treated with NAM had Egl (Figure 3.1). Of these worms that bagged, the majority of them bagged on day 5 after they had laid tens or hundreds of eggs (Figure 3.2). In the past, NAM caused a highly penetrant Egl phenotype[2]. As mentioned in the results section, 60% Egl is much lower than expected. I theorized that the first experiment yielded an unusually low Egl rate because the NAM had been on the plate too long. Perhaps it had degraded or been metabolized by the OP50 on the plate. To test this hypothesis, I made two sets of NAM plates; in one set the NAM had been allowed to soak in for three days, and in the other set for seven days. In this experiment, I found surprisingly high results compared to the previous experiment. Worms on the three day NAM plates had 70% Egl, and those on the seven day NAM plates had

100% Egl (Figure 3.3). Again, the majority of the worms bagged after they had laid many eggs and on day 4 or 5. It seemed unusual that as the worms were bagging very late, they were somehow bagging at a higher rate than the worms in the first experiment which were grown at similar conditions (Figure 3.1).

The fact that the majority of the animals bagged quite late and the majority of animals had laid many eggs before bagging lead me to the conclusion that the percent Egl I found in both experiments might be due to an experimental error. For each assay, I left a single worm on the same plate for five days straight. For the ones that laid many eggs, this meant that the progeny matured on that same plate. Some of the progeny reached adulthood and became fertile. This was evident from some of the plates containing several bagged worms. I recorded the worm as bagged when I saw several worm bags on the plate. This was likely cause for the unusual result because there is no way to discern the corpse of the original worm from those of its progeny.

To assess the possible effects of miscounting late bagged worms, I hypothesized that all worms recorded to have bagged on day 5 in the first experiment did not actually bag. Under this assumption, NAM would have only induced a 30% Egl rate. Under this assumption it suggested that NAM may be even less effective at causing Egl than it originally seemed, and that these experiments need to be repeated. Thus, for future experiments, I transferred the original worm to a new plate on day 3 to prevent confusion with progeny. I also repeated the original experiment to find a more accurate % Egl induced by the two-year-old NAM.

After improving my bagging assay protocol, I proceeded to test some more factors: N2 vs N2 cgc and new N2, old (several years old) NAM vs new (bought March 2017) NAM, 25 mM vs 50 mM, and N2 worms that grew up on 25 mM NAM. In the assays testing various strains, N2 worms have 40% Egl, N2 cgc had 50% Egl, but new N2 had only 10% Egl (Figure 3.3). Since

40% Egl is lower than the original 60% Egl I found with N2 worms, this confirms that transferring worms to new plates on day 3 would prevent the inflation of the Egl rate. I hypothesize that the new N2 having a low and statistically insignificant 10% Egl is because of the small sample size, but that needs to be confirmed with additional experiments.

In the next experiment, I tested old and new NAM stocks along with 25 mM and 50 mM concentrations. Old and new NAM did not produce significantly different results. This makes sense because NAM is not very susceptible to degradation over time. However, 50 mM NAM did produce higher percent Egl. 25 mM old and new NAM produced 18.75% and 12.5% Egl, respectively. 50 mM old and new NAM produced 43.75% and 46.67% Egl, respectively. Neither 25 mM group was statistically significant compared to N2 worms without NAM. However, both 50 mM groups showed statistically significant effects compared to N2 worms without NAM, but not compared to the 25 mM groups. I hypothesize that with larger sample sizes, the trends for the 50 mM groups and the 25 mM groups would show a significant difference. Overall, I found that 50 mM NAM induces a more penetrant and consistent Egl phenotype than 25 mM NAM, but old and new NAM stocks make no difference.

Finally, I tested the progeny of worms on 25 mM old and new NAM plates. To do this, I allowed N2 worms to lay eggs on 25 mM NAM plates. Once the larva had had time to age, I picked several L4 worms from each plate and put them onto new separate NAM plates for bagging assays. Worms on old NAM had 42.86% Egl, while those on new NAM had 71.43% Egl. Only the new NAM phenotype was statistically significant, but this is likely because the sample size was only n=7 for each group. Thus, it is hard to tell if the progeny of NAM-treated worms will always show around 70% Egl, or if the penetrance will go down as I increase sample

size. Regardless, both samples showed higher penetrance of Egl than the N2 on 25 mM NAM with 40% Egl.

For future researchers who work on this project, I recommend the following:

1. Use 50 mM NAM as your new standard.
2. Do not worry about which stock NAM you use as long as you consistently use one, as my data shows there is no significant difference between the new and old NAM, but use of different stocks may introduce additional experimental variation.
3. Do bagging assays on the progeny of N2 worms grown on 50 mM NAM plates (I only did so with 25 mM NAM).
4. Use larger numbers of individuals for bagging assays on N2, N2 cgc, and new N2 on 25 mM and 50 mM NAM to see if there are significant differences between the three strains.
5. Try to accumulate larger sample sizes for each strain and treatment; our lab's previous research had around n=100 for each group[2].

Confirming Lack of Egl in osm-9 Mutants

Since 50 mM NAM produced a consistent Egl phenotype with above 40% penetrance, I did bagging assays on *osm*-9 mutants on 50 mM NAM. These mutants lack the OSM-9 TRPV subunit, and thus are unable to form OSM-9-OCR-4 TRPV channels that are required for NAM to induce Egl. Thus, they should not have Egl, and our lab previously showed that they have 0% Egl[2]. I was also able to show that *osm-9* mutants have 5.5% Egl on 50 mM NAM.

Conclusions and Future Plans

Overall, I have been unable to reproduce the nearly 100% penetrant Egl phenotype that was previously shown in our lab. However, I was able to achieve a consistent Egl phenotype with over 40% penetrance, and confirm that *osm-9* mutants are 0% Egl. Future research will utilize the primers I made (Figure 2.4) to amplify OSM-9. Then, restriction enzymes will be used to cut ADSL out of P*myo-3*::adsl-1::GFP (Figure 2.2) and put OSM-9 into the cut plasmid to produce P*myo-3*::osm-9::GFP (Figure 2.3). Once this plasmid is constructed, an attempt should be made to replace OSM-9 function in only the muscle cells of *osm-9* mutants and see if they are then capable of having Egl when treated with NAM. This hypothesis can be tested by using microinjections to introduce the plasmid into *osm-9* mutants. If *osm-9* mutants containing the plasmid do show Egl, that will support my hypothesis.

While I was unable do my cloning experiments and inject *osm-9* mutants with P*myo-3*::osm-9::GFP I have suggestions for future experiments. If replacing OSM-9 in the muscle cells with the *myo-3* promoters does not restore Egl, this does not necessarily mean that muscle cells are not responsible for NAM-induced Egl. Vulval muscles are so integral to egg laying that it could be them specifically, and not all muscle cells, that are affected by NAM and cause Egl. Some mechanism in the vulval muscles may block the OSM-9 in the recombinant plasmid from being expressed in them. In that case, Egl would not be restored. To mitigate this, we could drive expression of OSM-9 using vulval muscle-specific promoters.

If Pmyo-3::osm-9::GFP does not restore Egl in *osm-9* mutants, this may also mean that NAM-induced Egl is not, in fact, caused by muscle cells. It could possibly be caused by uterine vulval cells other than uv1 (uv3, uv4), or other parts of the egg-laying machinery and general reproductive system. In this case, OSM-9 could be expressed by a promoter specific to uv3 or

uv4 cells. Or, it could be expressed by uterus, vulval, or gonad specific promoters to test if a cell type in proximity to the eggs themselves is responsible for the phenotype.

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