THE PENNSYLVANIA STATE UNIVERSITY
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DEPARTMENT OF BIOCHEMISTRY AND MOLECULAR BIOLOGY

DE NOVO SYNTHESIS OF NAD+ CONRTIBUTES TO REPRODUCTIVE DEVELOPMENT IN *CAENORHABDITIS ELEGANS*

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ABSTRACT

NAD+ is a molecule that is crucial in many different biochemical pathways across all organisms as an electron transporter. NAD+ also interacts with other proteins, such as sirtuins, which have been linked to aging and stress. In *Caenorhabditis elegans*, there are three biosynthetic pathways of NAD+, but my focus will be on a newly discovered pathway present in *C. elegans*, the *de novo* pathway. In this study, I will show through manipulation of this pathway that it contributes to reproductive development in *C. elegans*. In previous studies, it has been shown that blocking the salvage pathway of NAD+ synthesis with *pnc-1* mutants results in a gonad delay phenotype. I have found that when knocking out both the *de novo* and salvage pathways with a *umps-1;pnc-1* double mutant, the gonad delay phenotype is more penetrant. In *pnc-1* mutants, supplementation with quinolinic acid (QA), a metabolite in the *de novo* pathway, has been shown to reverse the phenotype by increasing NAD+ levels. I show that supplementing *umps-1;pnc-1* worms with QA does not fully reverse the phenotype since *umps-1* is required to metabolize QA. In addition to the gonad delay phenotype, I focused on brood size, the number of progeny a single worm has, as an indicator of reproductive development. Also in the *de novo* pathway, *kynu-1* mutants have a lower brood size than wild type. I show that supplementation of *kynu-1* mutants with QA rescues the lowered brood size phenotype. I also supplement with nicotinic acid (NA), a metabolite in the salvage pathway. NA most likely increases NAD+, which partially reverses the NAD+-dependent phenotype.

TABLE OF CONTENTS

LIST OF FIGURES iii

LIST OF TABLES iv

ACKNOWLEDGEMENTS v

Chapter 1 Introduction 1

NAD+ Biosynthesis Pathways 1
Stages of Gonad Development 2
Gonad Delay Phenotype 3
NAD+ and *C. elegans* Development 5

Chapter 2 Results: Gonad Development 8

The effect of *umps-1* on *pnc-1* gonad delay 8
Quinolinic Acid Supplementation of *umps-1;pnc-1* 10

Chapter 3 Results: Brood Size 12

kynu-1 worms have a smaller brood size 12
Quinolinic Acid and Nicotinic Acid affect *kynu-1* brood size 14

Chapter 4 Discussion 16

The importance of NAD+ as a potential drug target 16
de novo NAD+ synthesis and *C. elegans* Development 16
Future Directions 17

Chapter 5 Materials and Methods 19

C. elegans Strains and Maintenance 19
Gonad Delay Assays 19
Brood Size Assays 20

BIBLIOGRAPHY 21

LIST OF FIGURES

Figure 1.1 Some NAD+ Biosynthesis Pathways in *C. elegans*.....1

Figure 1.2 Stages of Gonad Development.. 3

Figure 1.3 Gonad Delay Phenotype. 4

Figure 2.1 *umps-1;pnc-1* has gonad delay phenotype.....9

Figure 2.2 Quinolinic Acid Reversal of Gonad Delay in *pnc-1*, but not *umps-1;pnc-1* 11

Figure 3.1 The brood size of *kynu-1* worms is smaller than N2 worms 13

Figure 3.2 The Effect of NA and QA on *kynu-1* brood size 15

LIST OF TABLES

Table 1. Normal Gonad Development in N2 4

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Chapter 1

Introduction

NAD+ Biosynthesis Pathways

There are three different pathways to synthesize NAD+ (nicotinimide adenine dinucleotide) in *C. elegans*, but I will focus on two of those pathways, the *de novo* (also known as the kynurenine pathway) and salvage pathways (Figure 1.1).

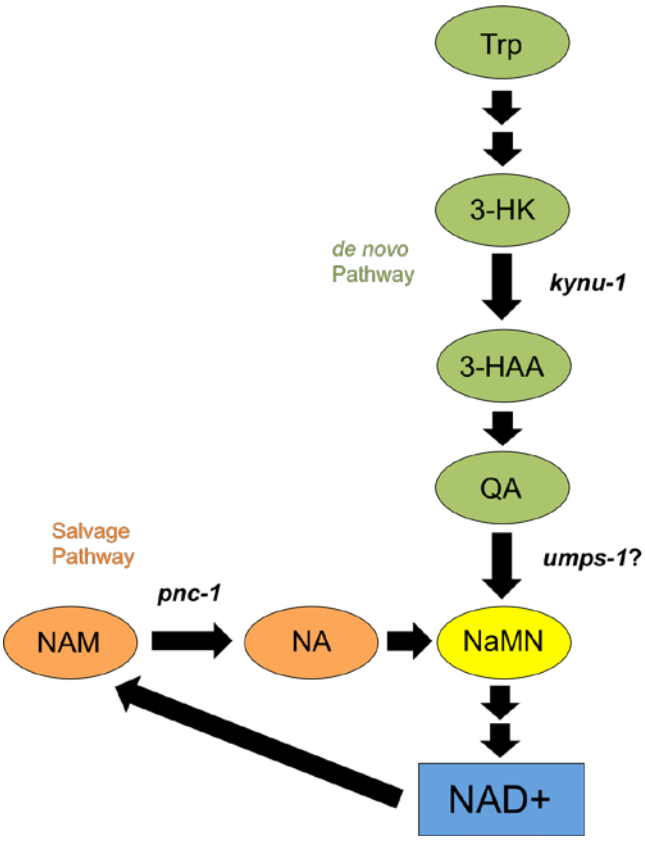

Figure 1.1 Two NAD+ Biosynthesis Pathways in *C. elegans*

The salvage pathway is based upon regenerating NAD+ from the NAM that is released upon hydrolysis of NAD+ (Magni, Amici, Emanuelli, Raffaelli, & Ruggieri, 1999). A key part of the pathway is the conversion from NAD+ to NAM, which is done by NAD+ consumers, such as PARPs (poly (ADP-ribose) polymerases) and sirtuins, which breakdown NAD+ into its two parts: NAM (nicotinamide) and ADP-ribose. The ADP-ribose is transferred to an acceptor protein while the NAM can be recycled back into NAD+ through other enzymes (Rongvaux, Andris, Van Gool, & Leo, 2003).

The *de novo* pathway is a linear pathway that starts with tryptophan and gets degraded into kynurenine-related metabolites, then into quinolinic acid (QA). At this point the QA is converted into NaMN, where it converges with the salvage pathway to become NAD+. Interestingly, the salvage pathway is the main contributor to the NAD+ pool, rather than *de novo* synthesis (Sporty et al., 2009).

Stages of Gonad Development

C. elegans has many distinct stages of development after hatching from the egg which include four larval stages (L1-L4) and an adult stage in which the worms are sexually mature and are able to lay eggs. The development of the gonad happens during the L4 stage, in which the vulva and uterine lumen open. The pinching and eventual closing of the vulva opening and uterus lumen marks the beginning of the adult worm (Greenwald, 1997). Within the L4 stage, there are several sub-stages that correspond to specific morphologies (Sharma-Kishore, White, Southgate, & Podbilewicz, 1999). A simplified view of this vulva/uterus development is in three

sub-stages: early L4, mid L4, and late L4. A representation of the gonad development in each sub-stage of an N2 (wild type) worm is shown in Figure 2.1.

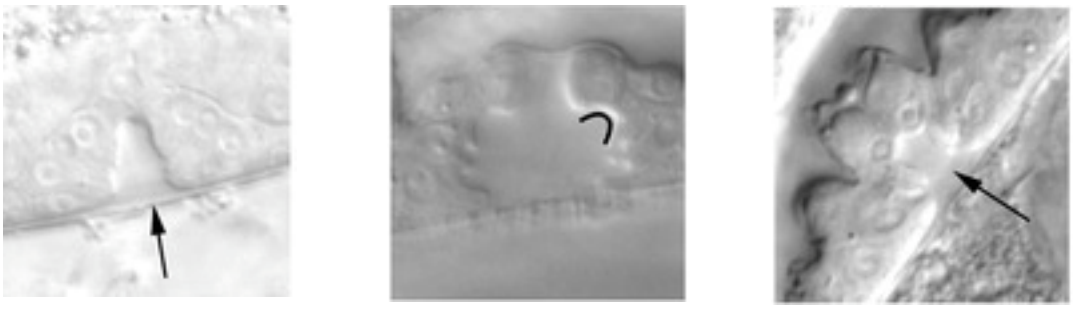

Figure 1.2 Stages of Gonad Development. (A) An early L4 N2 worm, arrow points to vulva opening. (B) A mid L4 N2 worm, curved line emphasizes vulval shape. (C) A late L4 worm N2 worm, arrow points to vulva opening. Used by permission from *BMC Developmental Biology* (Mok, Sternberg, & Inoue, 2015).

Normal uterine development of *C. elegans* happens during the mid-L4 stage, where there is an open lumen of the uterus present (see Figure 2.1B, lumen is above vulva). In the early L4 stage, the lumen has not yet formed, so the uterus appears as "closed." In the late L4 stage, the lumen begins to close, and is gone by the beginning of the adult stage (Sulston & White, 1980).

Gonad Delay Phenotype

In order to analyze correct gonad development, I have chosen to focus on the mid L4 stage because 100% of N2 worms have an open lumen. In the other L4 stages, this is not the case, as early L4 worms have 0% open lumens and late L4 worms have 80.77% open lumens (Table 1).

Table 1. Normal Gonad Development in N2

Sub-Stage	Open Uterus
Early L4	0%
Mid L4	100%
Late L4	80.77%

The gonad delay phenotype is when a mid L4 worm does not have an open lumen but goes on to develop one at a later stage. In a previous study, *pnc-1* mutants have been shown to have this phenotype (Huang & Hanna-Rose, 2006). In studies that investigate the gonad delay phenotype in *pnc-1*, the penetrance of the phenotype is based on the quality of the food source, OP50. When the worms are grown on UV-killed OP50, the phenotype is more apparent than when the worms are grown on live OP50.

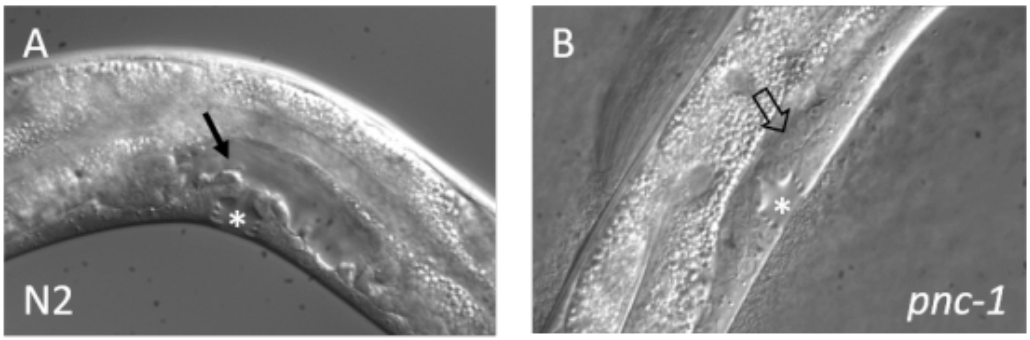

Figure 1.3 Gonad Delay Phenotype. (A) Mid L4 N2 worm with normal vulva (asterisk) and open uterine lumen (arrow). (B) Mid L4 *pnc-1* worm with normal vulva (asterisk), but no open lumen (arrow), showcasing the gonad delay phenotype.

Figure 2.2 shows that the opening of the lumen had not occurred for the *pnc-1* mutant, even though it entered the mid L4 stage. Because *pnc-1* is part of the salvage pathway of NAD+ synthesis, this pathway contributes to reproductive development in *C. elegans*.

NAD+ and *C. elegans* Development

In *C. elegans*, the lack of NAD+ salvage has a detrimental effect on reproductive development. The blocking of this pathway causes a delay in reproductive development, as demonstrated by using worms without *pnc-1*, an enzyme in the salvage pathway (Vrablik, Huang, Lange, & Hanna-Rose, 2009). The *pnc-1* gonad delay phenotype has been directly linked to lowered levels of NAD+ compared to N2. If the level of NAD+ is increased by supplementation with a different metabolite in the salvage pathway, then the gonad delay phenotype is effectively reversed (Wang et al., 2015). In addition to a gonad delay, *pnc-1* worms have a reduced brood size (Huang & Hanna-Rose, 2006).

In most organisms, including humans, there is a functioning *de novo* pathway to create NAD+ from tryptophan (Bender & Olufunwa, 1988). However, in *C. elegans*, the *de novo* pathway was thought to not exist because the genome does not contain a homolog for the gene that codes for quinolinic acid phosphoribosyltransferase (QPRTase) (Altschul et al., 1997). QPRTase is needed for the *de novo* pathway because it converts QA to NaMN, a precursor to NAD+. Curiously, the five other genes required for the *de novo* pathway are present in the *C. elegans* genome (Gossmann et al., 2012; Magni et al., 2004; Vrablik et al., 2009). The theory that *de novo* pathway is not present was put into question when it was found that the addition of

quinolinic acid to the animals' diets reversed the *pnc-1* gonad delay phenotype (Wang, 2014). This indirectly shows that when the salvage pathway is knocked out, increasing the input of the *de novo* pathway reverses the phenotype, suggesting an increase the amount of NAD+. Because quinolinic acid is a metabolite of the *de novo* pathway, this data implies that the *de novo* pathway is present in *C. elegans* and that there is some other gene coding for a QPRTase-like enzyme. Another possibility is that there are two genes that substitute for QPRTase activities.

I hypothesize that *umps-1* is responsible for the apparent QPRTase activity seen in *C. elegans*. *umps*-1 codes for uridine-5'-monophosphate synthase, which is a key part of pyrimidine synthesis (Kim, Park, Kim, Hwang, & Shim, 2009). UMPS-1 is a good candidate for the "missing enzyme" of the *de novo* pathway because it contains the same enzymatic domains as QPRTase, a phosphoribosyltransferase domain and a carboxylase domain (Eads, Ozturk, Wexler, Grubmeyer, & Sacchettini, 1997; Floyd & Jones, 1985).

Because lowered NAD+ levels from a block of the salvage pathway results in reproductive phenotypes, I hypothesize that a block of the *de novo* pathway can also lead to similar phenotypes. *kynu-1* is present in the *C. elegans* genome and codes for KYNU-1, which converts 3-hydroxy-L-kynurenine (3-HK) to 3-hydroxy-anthranilic acid (3-HAA). This step is in the *de novo* synthesis of NAD+, so knocking out *kynu-1* leads to lower levels of NAD+. Importantly, both *pnc-1* and *kynu-1* lead to less NAD+, due to a block of one of the NAD+ biosynthesis pathways (McReynolds, unpublished). However, *kynu-1* worms do not have the gonad delay phenotype present in *pnc-1* worms (Wang, 2014).

By manipulation of these pathways using worms with mutated genes, one can alter the level of NAD+ in the worms. A *pnc-1* worm blocks the salvage pathway while a *kynu-1* worm blocks the *de novo* pathway, both leading to less NAD+. Theoretically, phenotypes caused by

NAD+ can be reversed by adding a metabolite that is in a different NAD+ pathway or adding a metabolite that is after the step in which the enzyme is missing from a mutant worm. I carry out this experimental design with separate experiments analyzing the gonad delay and smaller brood size phenotypes.

Chapter 2

Results: Gonad Development

The effect of *umps-1* on *pnc-1* gonad delay

The *C. elegans* genome contains all of the genes necessary for the *de novo* pathway of NAD+ synthesis, except for the gene quinolinic acid phosphoribosyltransferase (QPRTase), which converts QA into Nicotinic Acid Mononucleotide (NaMN), which becomes NAD+. I hypothesized that *umps-1* was responsible for the apparent QPRTase function in *C. elegans*, because it contains the same enzymatic domains as QPRTase (phosphoribosyltransferase and carboxylase).

I hypothesize that if the *de novo* pathway is contributing to NAD biosynthesis, the gonad delay phenotype (a NAD+-dependent phenotype) of *pnc-1* would get worse if the *de novo* pathway was blocked. In order to test this, I used a double mutant that knocked down both genes: *umps-1;pnc-1* worms and observed the gonad development (Figure 2.1).

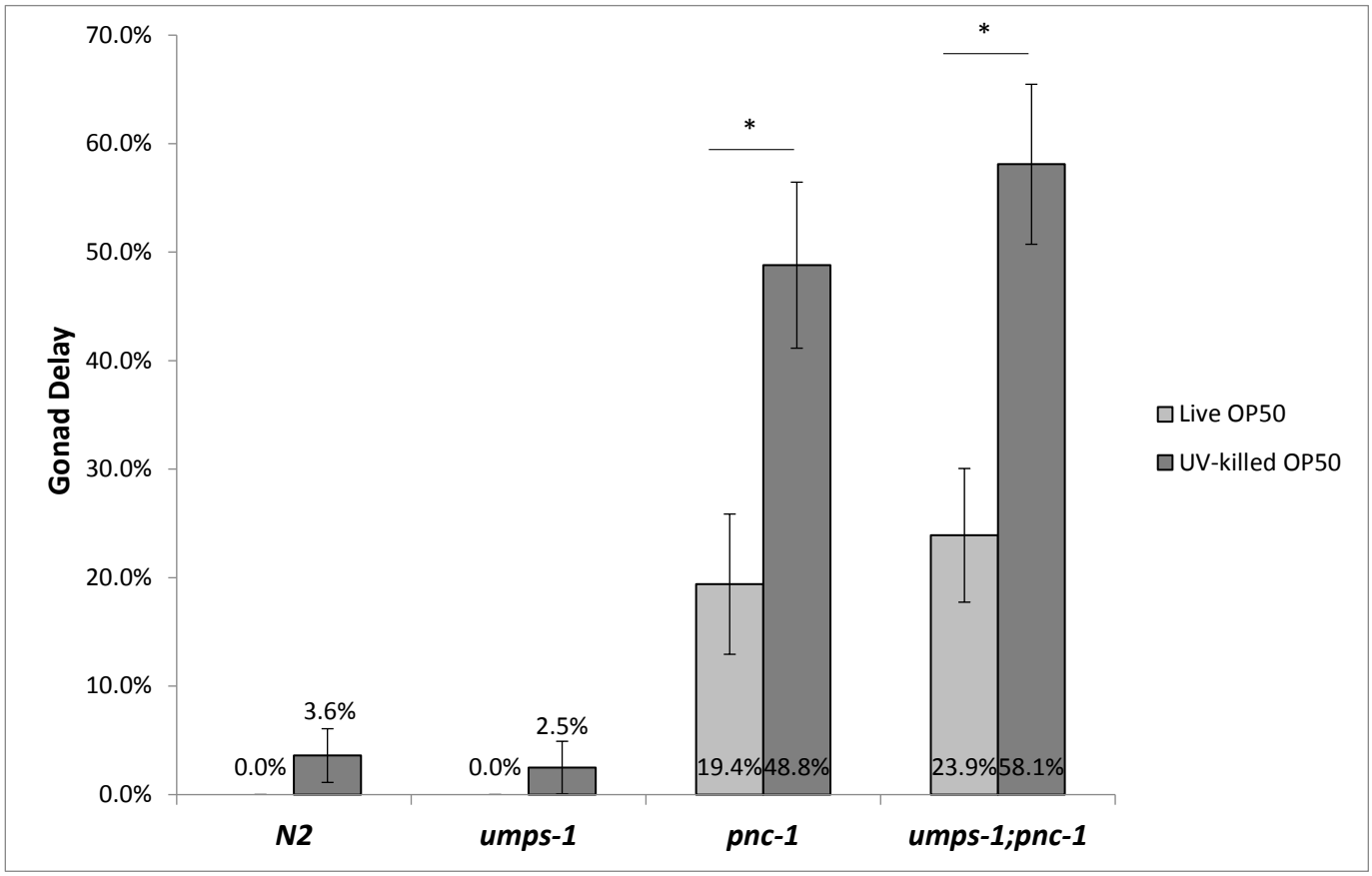

Figure 2.1 *umps-1;pnc-1* has gonad delay phenotype. No significant gonad delay seen in N2 live OP50 (n=56) and UV-killed OP50 (n=55) or in *umps-1* (ok2703) live OP50 (n=39) or UV-killed OP50 (n=40). Phenotype present to a small degree in *pnc-1* (pk9605) live OP50 (n=36) and *umps-;pnc-1* live OP50 (n=46). Phenotype present to strong degree in *pnc-1* UV-killed OP50 (n=41) and *umps-1;pnc-1* UV-killed OP50 (n=43). * = p<.05 using Fisher's exact test; error bars are 95% confidence interval.

When I scored the gonad phenotype of *umps-1* worms, there was no significant gonad delay in the mid L4 animals of *umps-1*; essentially no delay, just like N2 worms. However, in *umps-1;pnc-1* worms, the gonad delay phenotype appeared to became more penetrant, although not statistically significant (p=.5118, using Fisher's exact test).

Agreeing with previous data (Wang 2014), the gonad delay phenotype is much more apparent when grown on UV-killed OP50. This happens because when the worms are grown on

live OP50, the bacteria creates its own metabolites, which likely helps the metabolism of *C. elegans*. By using UV-killed OP50, the bacteria can no longer create extra variables that alter the metabolism.

Quinolinic Acid Supplementation of *umps-1;pnc-1*

After seeing the effect *umps-1;pnc-1* had on the gonad delay phenotype, I performed a QA supplementation experiment to see whether QA would rescue the phenotype. As mentioned before, QA supplementation was able to reverse the gonad phenotype in *pnc-1* mutants, most likely due to NAD+ compensation through the *de novo* pathway. If *umps-1* is needed to metabolize QA into NaMN, then I hypothesized that a *umps-1;pnc-1* mutant would block the QA rescue. I performed a QA supplementation experiment to see whether QA would rescue the phenotype. The results are shown in Figure 2.2, which used N2 and *umps-1* as controls (data not shown).

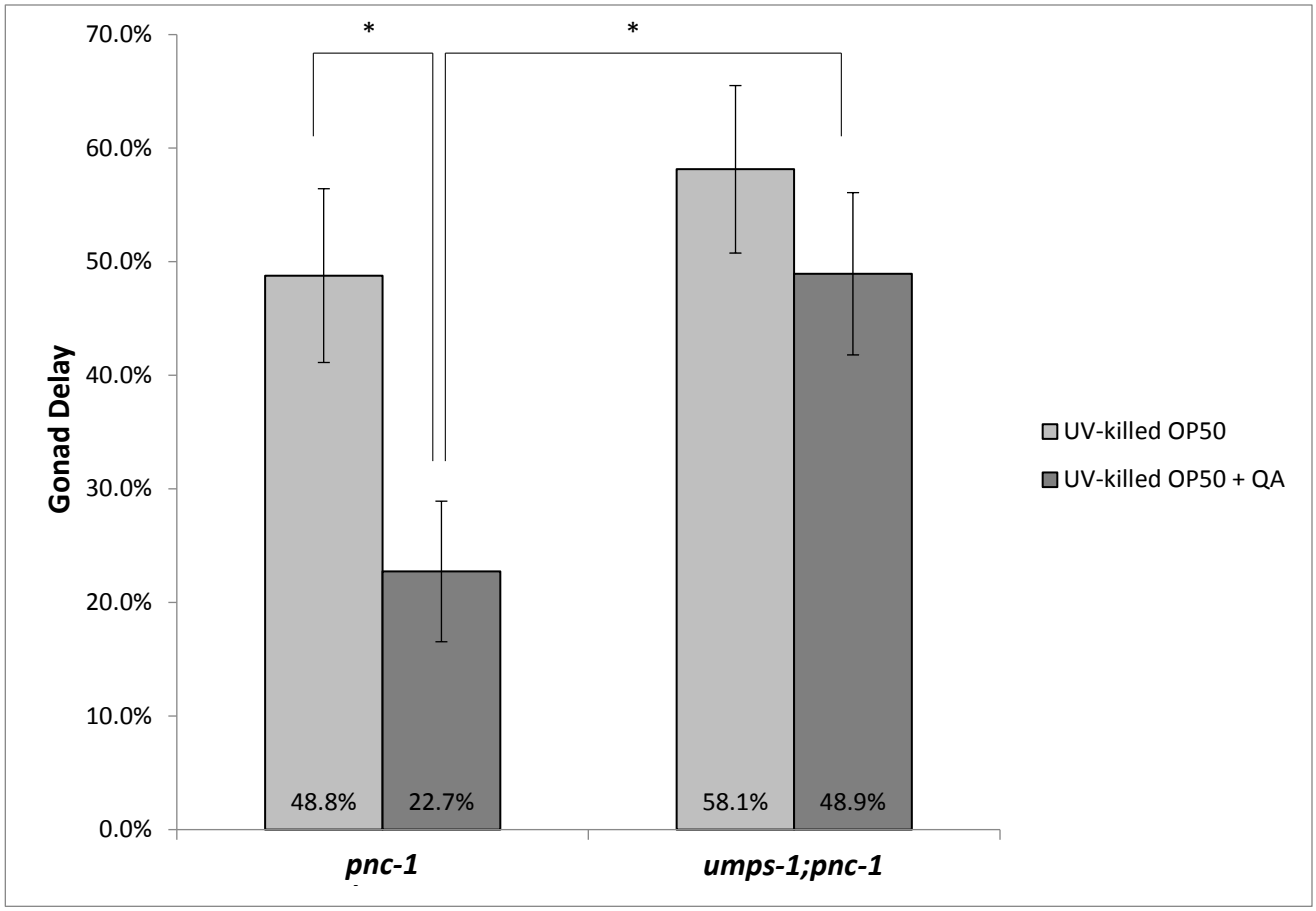

Figure 2.2 Quinolinic Acid Reversal of Gonad Delay in *pnc-1*, but not *umps-1;pnc-1*. The addition of 25 mM QA lessened the gonad delay phenotype in *pnc-1* (*pk9605*) grown on UV-killed OP50 (n=44). In *umps-1(ok2703);pnc-1*, the QA did not significantly affect the gonad delay phenotype (n=47). * = p<.05 using Fisher's exact test; error bars are 95% confidence interval.

In *pnc-1* worms, the gonad delay phenotype was effectively lowered from 48.78% of the worms to only 22.7% having gonad delay with the addition of QA to their food. However, the QA did not affect the *umps-1;pnc-1* worms as much, going from 58.1% to 48.9%. This supports my hypothesis that *umps-1* is needed for QA breakdown.

Chapter 3

Results: Brood Size

kynu-1 worms have a smaller brood size

As mentioned earlier, *pnc-1* worms had other reproductive phenotypes besides the gonad delay, one of these being a reduced brood size. Brood size refers to the number of progeny a single worm has over its lifespan. After seeing that the *de novo* pathway affected the reproductive development of *C. elegans*, I wanted to see if the brood size phenotype was present in *de novo* mutants. KYNU-1 is an enzyme responsible for converting kynurenine into anthranilic acid, which is an earlier step in the *de novo* pathway of NAD+ synthesis. When worms with a mutated *kynu*-1 gene were checked for gonad delay, there was no phenotype evident (Wang 2014). Although *kynu-1* worms do not share this phenotype with *pnc-1* worms, they displayed a different phenotype, a decreased brood size. The brood size between N2 and *kynu-1* worms are compared in Figure 3.1

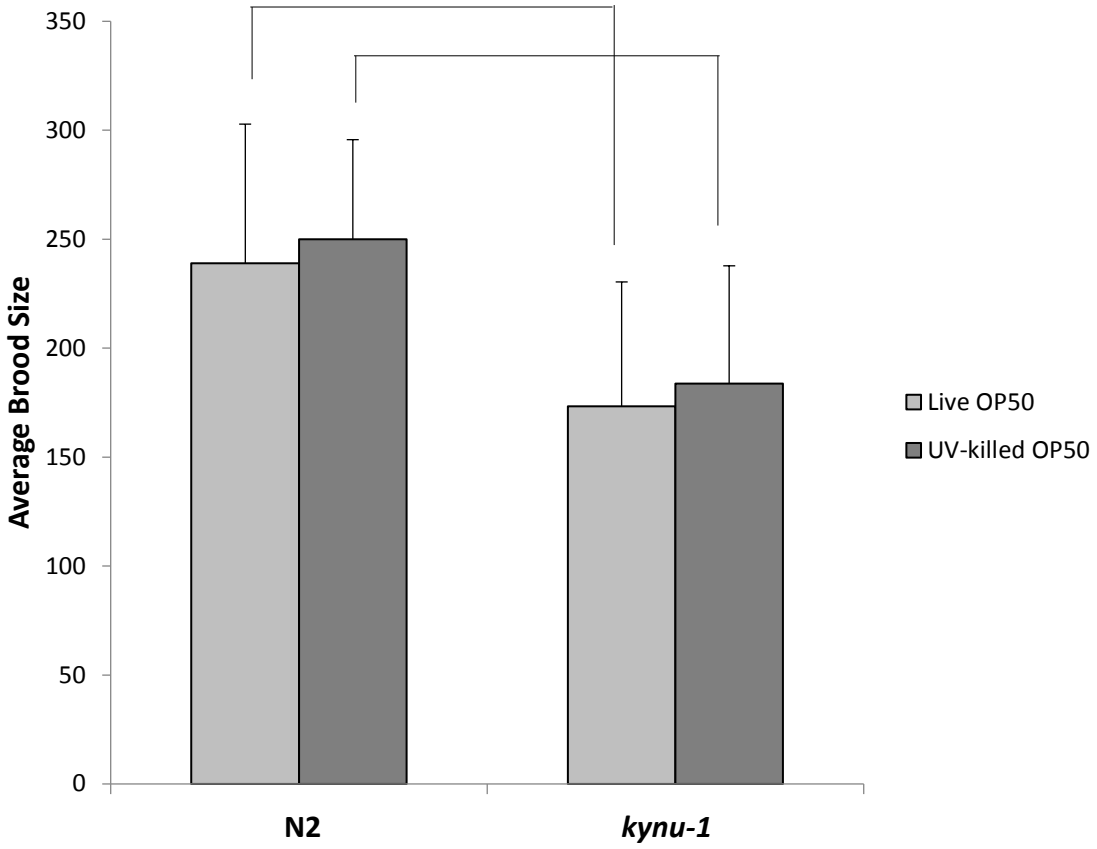

Figure 3.1 The brood size of *kynu-1* worms is smaller than N2 worms. The brood size of N2 on live OP50 (n=8) was greater than that of *kynu-1* on live OP50 (n=8). The brood size of N2 on UV-killed OP50 (n=8) was also greater than that of *kynu-1* on UV-killed OP50 (n=8). In both strains, there was no difference between live and UV-killed OP50. * = $p<.05$ using a t-test; error bars are standard deviation.

In this experiment, the effect of live OP50 vs UV-killed OP50 is not present like it was in the gonad delay experiments. However, the important conclusion here is that by blocking synthesis of NAD+ via the *de novo* pathway with *kynu-1* mutants, there is a clear reproductive phenotype present.

Quinolinic Acid and Nicotinic Acid affect *kynu-1* brood size

I hypothesized that *kynu-1* worms have a smaller brood size due to lower NAD+ levels and that supplementing them with a metabolite that allows production of NAD+ via another pathway could reverse the brood size phenotype. First, I used Nicotinic Acid (NA) as a supplement, which is a metabolite of the salvage pathway of NAD+ synthesis. I hypothesized that adding more NA would reverse the brood size phenotype, which is shown in Figure 3.2. I also added QA as a supplement, which is several steps down the *de novo* pathway from which *kynu-1* is active. Similar to NA, I hypothesized that increasing QA levels would also reverse the brood size phenotype, but using the *de novo* pathway, shown in Figure 3.2.

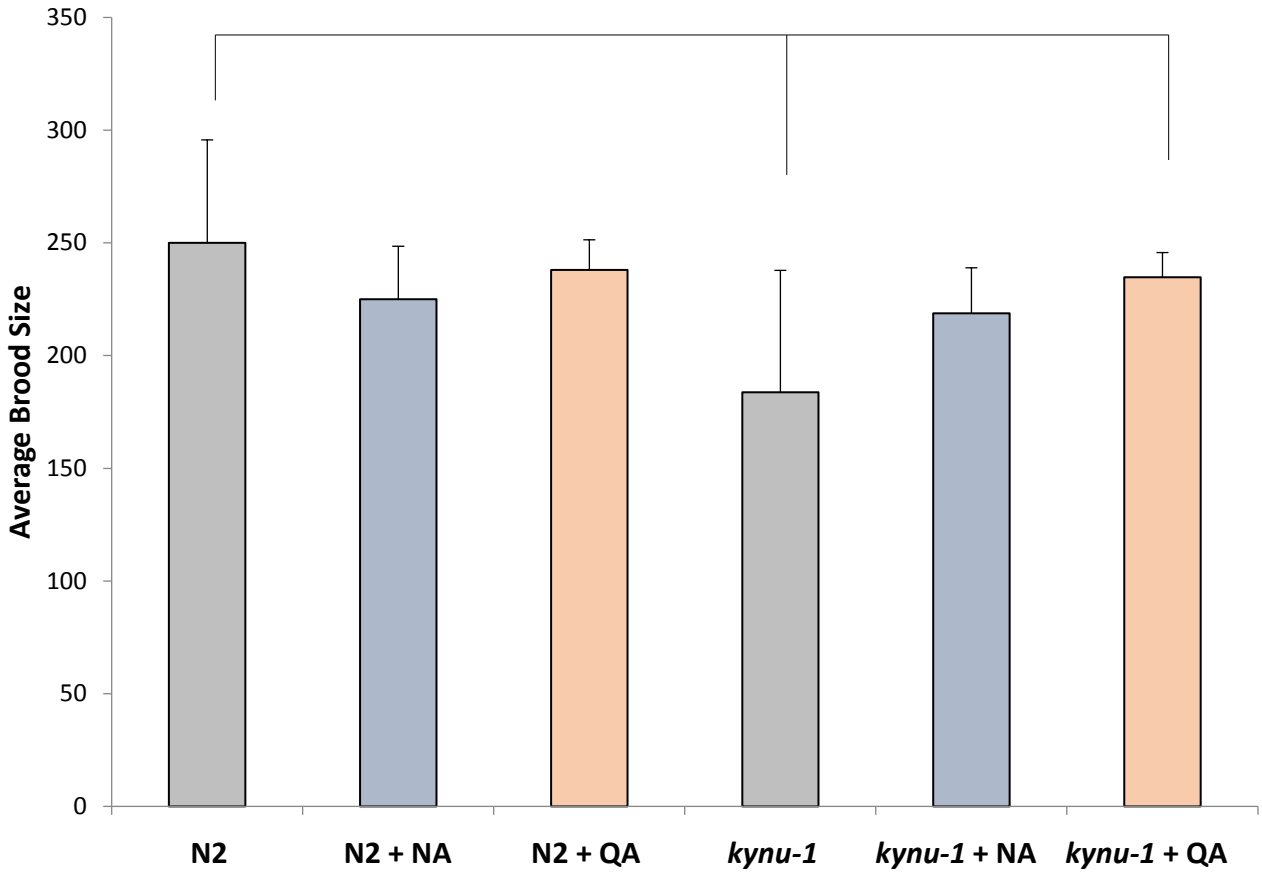

Figure 3.2 The Effect of NA and QA on *kynu-1* brood size. The brood sizes between N2 and the supplements NA and QA did not differ significantly. The brood size seemed to increase in *kynu-1* with the addition of 25 mM NA, but not significantly. The brood size had a significant increase in *kynu-1* with the addition of 25 mM QA. * = $p<.05$ using a t-test; error bars are standard deviation.

For the supplementation with NA, this data displays a clear, but not statically significant upward trend for the average *kynu-1* brood size. As for the supplementation of QA, there is a greater increase. These outcomes make sense because NA is a part of the salvage pathway, so it seems to increase the amount of NAD+ in an indirect way. On the other hand, the QA helped more than NA (QA has significant increase while NA does not) because it is able to directly increase NAD+ through later steps in the *de novo* pathway, from which *kynu-1* is a part of.

Chapter 4

Discussion

de novo NAD+ synthesis and *C. elegans* Development

The results from both the gonad delay and brood size experiments point to a conclusion that the *de novo* pathway is active in *C. elegans*, and has an effect on the reproductive development. The fact that supplementation with quinolinic acid on *umps-1;pnc-1* animals had no significant effect shows that *umps-1* is likely needed for metabolism of quinolinic acid into nicotinic acid mononucleotide, and then into NAD+. This also shows that although salvage synthesis of is the main contributor of NAD+ levels and has a larger effect on *C. elegans* reproductive development, the *de novo* pathway also contributes to development. Previous data has shown that *kynu-1* worms have less NAD+ than wild-type, and since *kynu-1* worms have a smaller brood size, this implies that NAD+-dependent reproductive phenotypes exist for the *de novo* pathway as well.

The importance of NAD+ as a potential drug target

NAD+ is a coenzyme present across all organisms and is highly involved in metabolic pathways related to energy, such as the TCA cycle (Krebs & Veech, 1969). In addition to its well-known role as an electron transporter, NAD+ is a substrate of the effectors sirtuins, PARPs, and CD38 (Blander & Guarente, 2004; Chambon, Weill, Doly, Strosser, & Mandel, 1966; Chambon, Weill, & Mandel, 1963). The level of NAD+ has a direct effect on the function of

these protein families and can lead to beneficial outcomes such as oxidative stress resistance and weight loss, specifically with sirtuins (Merksamer et al., 2013; Rappou et al., 2016). In more recent studies, the potential for NAD+ as a drug target to treat an array of human diseases has become more apparent, including mitochondrial disorders, Alzheimer's disease, and hepatic carcinoma (Bai et al., 2011; Gong et al., 2013; Tummala et al., 2014). By understanding more on the different biosynthetic pathways of NAD+ and how they interact with each other, it can provide insight on how to construct possible drugs that affect NAD+ levels.

Future Directions

Although the results from the gonad delay experiments show that *umps-1* is needed for *de novo* synthesis of NAD+ from QA, there needs to be more experiments done to conclude that UMPS-1 is the enzyme acting as a QPRTase in *C. elegans*. Another gonad delay experiment could be done by supplementing the *umps-1;pnc-1* worms with nicotinic acid (NA) instead of QA. Because NA is after the *pnc-1* step of the salvage pathway and would be unaffected by a lack of UMPS-1 if it is the QPRTase, the expected result would be that NA would rescue the gonad delay phenotype by raising NAD+ levels. This would help support the data from the failed QA rescue of *umps-1;pnc-1* by showing that there is not another factor causing the lack of rescue and that the worms are actually capable of reversing the gonad delay.

Even more direct than more phenotypic assays would be a simple enzyme assay on UMPS-1. If purified UMPS-1 was added to QA, then NaMN would be detected after some time if UMPS-1 is acting as a QPRTase. This assay would ultimately prove that UMPS-1 is the

"missing" enzyme and *C. elegans* would be shown to have a full complement of all genes required for *de novo* synthesis of NAD+.

Chapter 5

Materials and Methods

C. elegans Strains and Maintenance

All *C. elegans* strains were maintained under standard conditions at 20^{o}C on *E. coli* OP50 (Brenner, 1974). The strains used were N2 (wildtype), *pnc-1*(*pk9605*), *umps-1*(*ok2703*), *umps-1;pnc-1*, and *kynu-1*(*tm4924*). *pnc-1*(*pk9605*) and *umps-1*(*ok2703*) were obtained from the CGC and *kynu-1*(*tm4924*) was obtained from Mitani Lab at National BioResource Project, Japan. The double mutant, *umps-1;pnc-1* was created in the Hanna-Rose lab by Melanie McReynolds crossing the *pk9065* and *ok2703* strains.

Gonad Delay Assays

The gonad delay assays required plating several mid L4 stage worms on a microscope slide, and then observing the morphology of the vulva and uterine lumen under a DIC microscope at 40x to 100x magnification. If there were any abnormalities with the vulva, as compared to an N2 worm, the data was not used. Each worm was scored as having the gonad delay phenotype if the vulva was at the correct mid L4 stage shape and there was no uterine lumen present. Any presence of lumen was counted as normal development with no gonad delay phenotype present.

For the quinolinic acid supplementation experiments, 200uL of 25 mM filter-sterilized QA was spotted onto 400 μL of OP50, using nematode growth medium (NGM) plates. Stage

L2-L3 worms were plated and allowed to grow on QA plates until the second generation of worms were at the L4 stage, in which they were put onto microscope slides and observed.

Brood Size Assays

For the brood size assays, a single L3 worm was plated on 400μL of OP50 on NGM plates and allowed to grow. Each day after, the progeny of the original worm would be counted and removed from the plate. Once the original worm stopped laying eggs, the assay was over (about 5 days).

For the nicotinic acid supplementation, 200 μL of 25 mM filter-sterilized NA was spotted onto 400μL of OP50, using NGM plates. Stage L2-L3 worms were allowed to grow for 2 generations on the NA plates, and then a single L3 worm would be plated on a new NA plate to be assayed. The quinolinic acid supplementation followed the same procedure, using 200 μL of 25 mM filter-sterilized QA.

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Academic Vita

Lauren Holleran

Education

The Pennsylvania State University – University Park
Schreyer Honors College
Bachelor's of Science in Biochemistry and Molecular Biology
Anticipated Graduation – Spring 2017
Thesis: de novo *synthesis of NAD+ contributes to reproductive development in* C. elegans
Relevant Courses: Immunology, Physiology, Developmental Biology, Functional Genomics

Education Abroad **May 2015**
Fudan University – Shanghai, China
Introductory Biochemistry II

Work & Volunteer Experience

Pre-Med Program Volunteer; Saint Mary Medical Center – Langhorne, PA **June-Aug 2016**
- Shadowed various physicians and observed medical procedures
- Volunteered at the hospital for 120 hours helping with patients' mobility

Research Assistant; Developmental Biology Lab – University Park, PA **July 2015-present**
- Spent 10 hours per week investigating developmental phenotypes using *C. elegans* as a model system
- Co-authored a publication being sent to the Journal of Biochemistry
    - *Eukaryotic de novo NAD+ biosynthesis from tryptophan in the absence of a QPRTase homolog*
- Presented research project on NAD+ in 2016 Undergraduate Research Exhibition

Research Volunteer; Fudan University Cell Biology Lab – Shanghai, China **May 2015**
- Worked 5 hours per week for 5 weeks during study abroad program
- Helped performed laboratory techniques such as Western Blots

Activities Promoter; Epilepsy Foundation of Eastern PA **Sept 2011-June 2014**
- Spread awareness of epilepsy through media and guest speakers

Camp Counselor; Bucks Community College Kids on Campus – Newtown, PA **June 2013-Aug 2014**
- Supervised ~15 kids each week through different activities

Hospital Volunteer; St. Mary Medical Center – Langhorne, PA **June 2009-Aug 2012**
- Worked over 150 hours over 4 summers of assisting families and patients

Leadership Experience

Undergraduate Research Society, Social Chair **Oct 2015-May 2016**
- Developed new ideas to raise money and involve more members
- Facilitated freshmen and sophomores' entrance into various research labs

THON Rules and Regulations Committee, Fundraising Leader **Oct 2015-Feb 2016**
- Worked with co-chairs to implement alternative fundraisers
- Gave weekly presentations to educate committee on fundraising safety

Biomedical Engineering Society, Fundraising Chair **Jan 2014-Dec 2015**
- Contacted local businesses to organize fundraising events
- Organized trips to national biomedical engineering meetings

Schreyer Honors College, Tour Guide **Nov 2013-May 2014**
- Led small groups of parents and students around honors college buildings
- Held personal Q&A sessions with prospective students