

Warning Concerning Copyright Restrictions

The Copyright Law of the United States (Title 17, United States Code) governs the making of photocopies or other reproductions of copyrighted materials.

Under certain conditions specified in the law, libraries and archives are authorized to furnish a photocopy or other reproduction. One of these specified conditions is that the photocopy or reproduction is not to be used for any purpose other than private study, scholarship, or research. If electronic transmission of reserve material is used for purposes in excess of what constitutes "fair use," that user may be liable for copyright infringement.

University of Nevada, Reno

Effects of Glycosylation on Cholesterol Metabolism in *Caenorhabditis elegans*

A thesis submitted in fulfillment of a degree of Bachelor of Science in Biology in the
Honors Program

Heather Wilson

Dr. Patricia Berninsone, Thesis Advisor

May 2011

UNIVERSITY

OF NEVADA

RENO

THE HONORS PROGRAM

We recommend that the thesis
prepared under our supervision by

Heather Michelle Wilson

entitled

Effects of Glycosylation on Cholesterol Metabolism in *Caenorhabditis elegans*

be accepted in partial fulfillment of the
requirements for the degree of

BACHELOR OF SCIENCE, BIOLOGY

Patricia Berninsone, Ph.D., Thesis Advisor

Tamara Valentine, Ph. D., Director, **Honors Program**

May 2011

Abstract

Transport of cholesterol within multi-cellular organisms is a vital life process. Research with cholesterol transport and metabolism using the *Caenorhabditis elegans* (*C. elegans*) is beneficial to the health science community because many biological processes are conserved between *C. elegans* and humans. Efficient cholesterol transport requires glycosylation, which is the modification of proteins with sugars. In nematodes, some of the glycosylated proteins (glycoproteins), called vitellogenins, play a role in cholesterol uptake in oocytes of *C. elegans*. There are a large number of glycosylation and cholesterol transport *C. elegans* mutants available. Furthermore *C. elegans* present an unusual research advantage since they cannot synthesize cholesterol and must take it in from their outside environment.

Since *C. elegans* must take in cholesterol from their outside environment, I hypothesized that abnormal glycosylation of vitellogenins impairs the transport of cholesterol in *C. elegans*. I tested this hypothesis with three approaches: quantifying cholesterol in *C. elegans*. I tested this hypothesis with three approaches: quantifying cholesterol incorporation into *C. elegans* oocytes, assaying viability of glycosylation defective mutants grown under low or no sterol conditions, and comparing the total cholesterol content of glycosylation-defective mutants vs. wildtype animals.

Through these studies, it was found that 1) *srf-8 C. elegans* have a significantly different accumulation of cholesterol than wildtype worms as determined by fluorescent cholesterol microscopy and, 2) *srf-8 C. elegans* have a slow hatching phenotype when grown in no or low-cholesterol conditions.

Table of Contents

Abstract.....	i
Table of Contents.....	iii
List of figures.....	iv-viii
Introduction.....	1
Materials and Methods.....	4
Results.....	8
Discussion.....	11
References.....	13

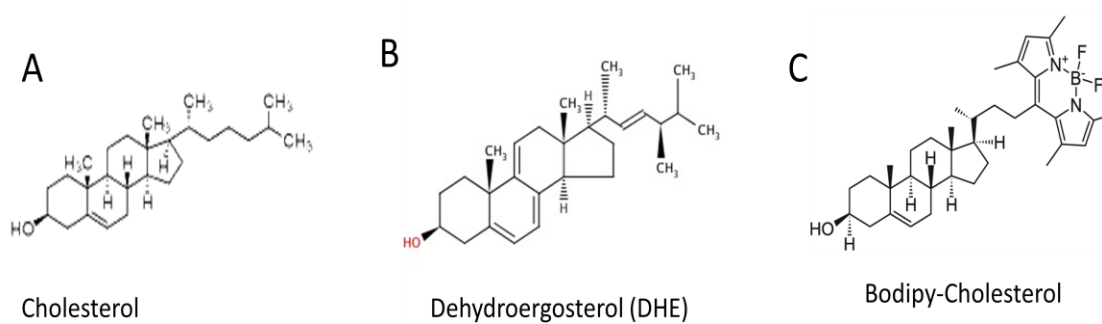


Figure 1. The structures of cholesterol and the fluorescent cholesterol analogs used in the studies.

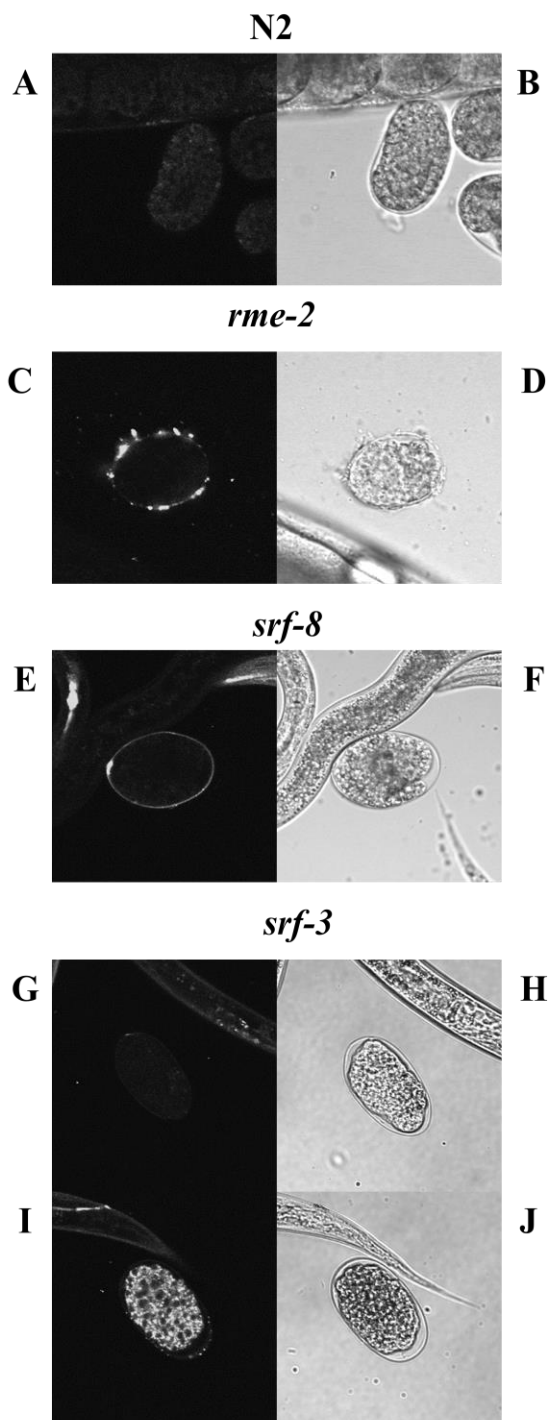


Figure 2. Bodipy-cholesterol was incorporated into *C. elegans* in replacement of cholesterol, this allowed cholesterol to be localized in the nematode. A confocal microscope was used to obtain images of gastrulated embryos. Fluorescence excitation occurred from 510nm to ~665 nm. The images were processed using Olympus Fluo View Software.

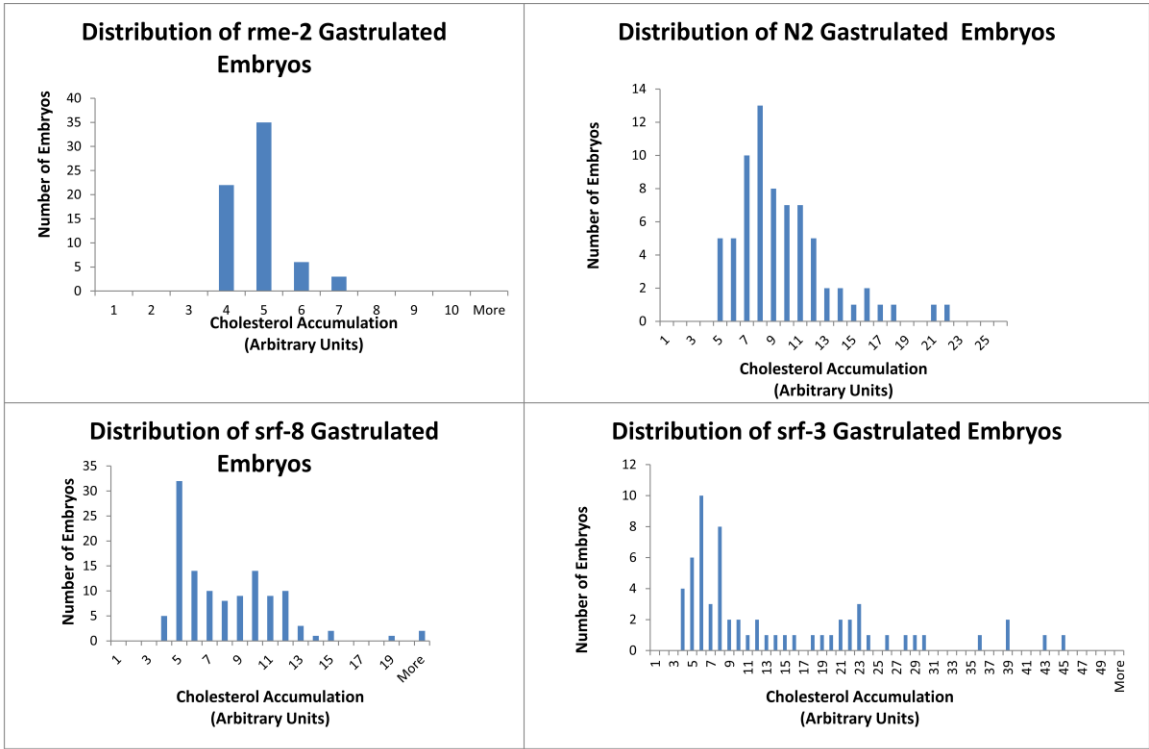


Figure 3. There is a different distribution pattern for gastrulated embryos of *srf-3* compared to other *C. elegans* strains. Images of gastrulated embryos were obtained using a confocal microscope, were processed with Olympus Fluo View Software and fluorescence quantity analyzed with ImageJ Software. Each graph displays a *C. elegans* strain's number of gastrulated embryos per accumulation of Bodipy-cholesterol (in arbitrary units).

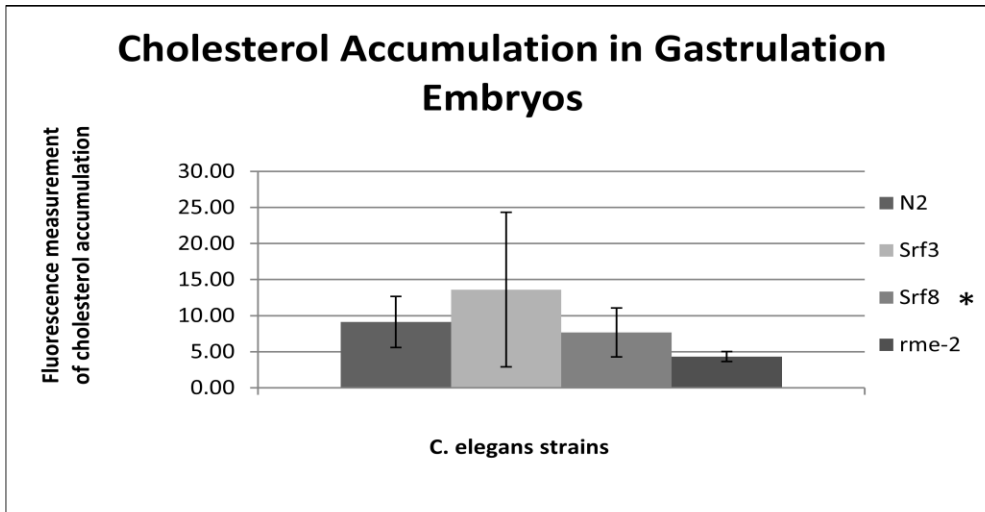


Figure 4. *srf-8* had a significantly different accumulation of cholesterol than N2 (wildtype) *C. elegans*. Images of gastrulated embryos were obtained using a confocal microscope and Olympus Fluo View Software for processing the images. The amount of cholesterol in the embryos was quantified using ImageJ Software. The Mann-Whitney test was used to test if there was a significant difference in the median cholesterol accumulation between N2 (wildtype) and the *srf* mutants' gastrulated embryos. The Mann-Whitney test was used instead of the T-test since there was not a normal distribution of fluorescence for each genotype. *Srf-3* mutants did not have a significant difference. *Srf-8* mutants did have a significant difference (* $P < 0.002$).

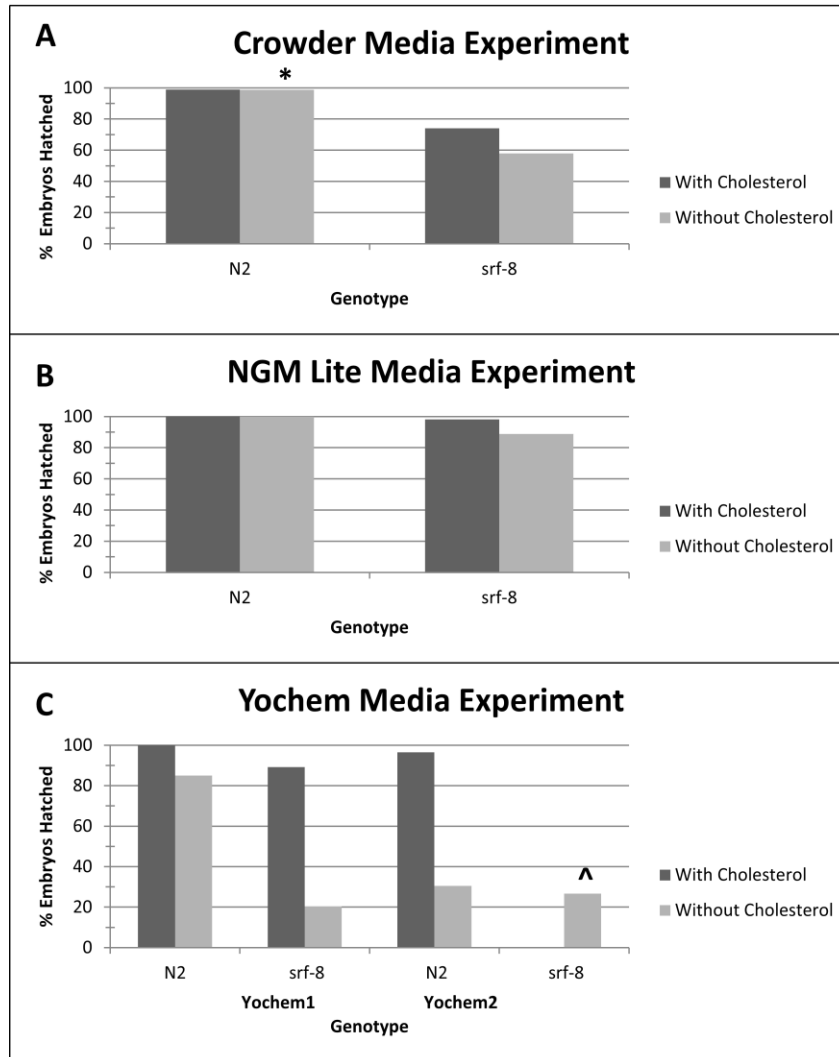


Figure 5. *Srf-8* had a decreased hatch rate compared to N2 across types of media and experimental runs. Eggs for each experiment with each media were obtained from an embryo flotation, grown on NGM stock plates until L4 stage then placed on experimental plates. (A) “Crowder” denotes experiments performed using no-sterol media prepared according to the method of Crowder³. Even with a decreased growth rate, the *srf-8* embryos had a lower hatch rate than N2. *The N2 Crowder no-cholesterol plate had an adult on it, the actual hatch rate may be higher than what was observed. (B) The bioassay performed on NGM-lite media had low-sterol conditions. Even though conditions were not as extreme as the Crowder media, it showed the *srf-8* embryos had a lower hatch rate than N2. (C) For the Yochem1 and Yochem2 results “Yochem” denotes experiments performed with low-sterol media prepared according to the method of Yochem¹⁵. Both Yochem experiments showed *srf-8* embryos had a lower hatch rate than N2. ^ The Yochem 2 *srf-8* with cholesterol could not be obtained and is not shown.

Introduction

Humans and the free-living nematode *Caenorhabditis elegans* (*C. elegans*) have many biological processes conserved between them. This makes *C. elegans* a useful model in the study of human physiology and disease. Cholesterol is an important component of eukaryotic cells. It can aid in the structure of the cell membrane by affecting the lipid bilayer permeability and making the membrane less fluid¹⁰. Cholesterol is also involved in signaling processes by modulating the cell's signaling through glycolipid-enriched membranes (rafts)¹⁰. Additionally, *C. elegans* has played a vital role as a model in the study of cholesterol. Cholesterol is thought to be involved in mediating membrane structure and raft functions in certain cells, as well as being involved in the covalent linkage of signaling proteins in *C. elegans*⁷. The complete genome of *C. elegans* is known; therefore, this enables researchers to disrupt the genes to see if there is an effect on cholesterol metabolism or function. Since *C. elegans* are not able to synthesize sterols within their body; they must therefore get all sterols from their outside environment⁷. This makes *C. elegans* an ideal model because they can be grown with a fluorescent cholesterol-analog with ease. Researchers can also specifically test the effects of cholesterol deprivation rather than just general nutrient deprivation.

Studies have shown that cholesterol plays a vital role in signaling within *C. elegans*⁷. Previous studies have shown that cholesterol accumulation occurs in *C. elegans* in the pharynx, nerve ring, excretory gland cells, oocytes, spermatozoa and gut of L1-L3 larvae¹⁰. The uptake and transport of cholesterol into the oocytes, a major accumulation site, of *C. elegans* occurs via an endocytotic pathway. This pathway uses

yolk proteins to aid in endocytosis¹⁰. The yolk is a lipoprotein that is made of lipids and lipid-binding proteins⁴. The yolk proteins are called vitellogenins. In order for cholesterol to be transported into the oocytes, vitellogenin-2 (VIT-2) must bind to the receptor, RME-2.

Fluorescent analogs of cholesterol can be used to image cholesterol spatial distribution within tissues or cells. Dehydroergosterol (DHE) (Figure 1B) is a naturally-occurring, fluorescent analog of cholesterol that mimics many of cholesterol's properties. Previous studies have shown that DHE, like cholesterol, accumulates in tissues such as the nerve ring, spermatheca and oocytes in *C. elegans*¹⁴. However, DHE has been shown to cause complications during fluorescent imaging of *C. elegans* due to the *C. elegans* having a high autofluorescence produced at the same spectrum that DHE is emitted at¹⁴.

Fluorescent boron dipyrromethene difluoride linked to sterol carbon-24 (BODIPY-cholesterol) (Figure 1C) is a synthetic cholesterol analog. This cholesterol analog has been used to observe sterol movement in mammalian cells⁵. Fluorescent BODIPY-cholesterol is useful due to its high photostability. In a study performed on Zebrafish, BODIPY-cholesterol allowed for growth and viability of the cell in the same way cholesterol and DHE did. Additionally, it was easier to visualize than DHE⁵.

It is known that *C. elegans* must take in all sterols from their outside environment. We hypothesized that glycosylation defects may alter cholesterol endocytosis in *C. elegans* for several reasons. First, other studies have shown that some of the phenotypes of the *srf-3* and *srf-8* mutants have an overlap with the phenotypes of cholesterol-deprived wildtype worms. Previous studies showed that *srf-3* and *srf-8* dauer animals

grown under regular cholesterol conditions showed a SDS-sensitivity resembling the one that is seen in cholesterol deprived wildtype dauers⁹. *srf-8 C. elegans* grown under regular cholesterol conditions also express gonadal arm migration (distal tip cell migration) defects⁹. This distal tip cell migration defect is expressed in cholesterol deprived wildtype animals¹¹. Niemann-Pick proteins are involved in cholesterol transport. *C. elegans* that exhibit defects in the Niemann-Pick proteins appear to have distal tip cell migration defects as well⁸. Data from the Berninsone lab suggests that *srf-3* animals have differently glycosylated VIT-2 and VIT-6 than wildtype animals (Data came from an azido-GalNAc metabolic labeling followed by 2D gel electrophoresis and mass spectrometry identification of the candidate proteins, performed by Amanda Marusich).

This study looks at the total cholesterol incorporation and distribution in wildtype and glycosylation-defective mutant *C. elegans*. It utilized previous methods for spatial cholesterol imaging and quantitation of cholesterol incorporation using DHE. In addition, we developed a method for using BODIPY-cholesterol to track cholesterol accumulation and transport in *C. elegans* with fluorescent microscopy. The study specifically looked at the distribution of cholesterol in the oocytes of the nematode. Bioassays were also performed to compare the viability of glycosylation mutants that were grown under no cholesterol conditions. Lastly, the study set up pilot tests for a biochemical approach to quantify cholesterol within different glycosylation-defective mutants by using Amplex Red and the Bradford Assay to compare the milligrams of cholesterol per milligrams of total protein in worms.

The studies determined that *srf-8 C. elegans* had significantly different accumulation of cholesterol than compared to wildtype worms. They also indicated that the *srf-8 C. elegans* have a slow hatching phenotype when grown under low cholesterol conditions. This is due to the *srf-8* worms having a decreased hatch rate after 26 hours compared to wildtype; however, all *srf-8* eggs had hatched within 72 hours.

Materials and Methods

C. elegans maintenance and strains used

The wildtype strain and glycosylation mutants (*srf-3(yj10)*, *srf-3 (br6)*, *rme-2 (b1008)* and *srf-8*) were obtained from the Caenorhabditis Genetics Center (CGC) and maintained on NGM (Nematode Growth Media) plates as described ¹.

Labeling C. elegans with DHE

Bacteria containing DHE was prepared as directed ¹⁰. An equal volume of *E. coli* and 5mM DHE in ethanol were mixed. The mixture was dialyzed in water twice; the first time was for 3-4 hours, then the cassette was placed in fresh water to dialyze overnight. After dialyzing, the mixture was combined with live OP50 strain *E. coli*. Concentrations of 1:5 and 1:25 bacteria containing DHE and live *E. coli* of the OP50 strain were used. Bacterial lawns were grown overnight at 16°C. Gravid adults from each strain (wildtype (N2), *srf-3*, *srf-8* and *rme-2*) were put on plates and left to mature at 20°C for 3 days. The worms were washed off the plates with M9 and pelleted by spinning for 3 minutes at 250 x g and 10°C. Supernatant was removed, and the pellet of worms was resuspended in 1ml of 10mM sodium azide in M9. Worms were placed on top of agarose pads on microscope

slides, and a cover slip was placed over them. It was important to prepare and keep the worms in a dark environment so the DHE fluorescence did not fade. Worms were analyzed using an Olympus BX60 microscope fitted with an Olympus DP-70 camera. A UV mercury lamp was used with a DAPI (N31000) filter cube. The image acquisition software used was DP Controller Software (Olympus).

Preparation of BODIPY-cholesterol and Labeling of C. elegans

NGM plates were made and either cholesterol solubilized in ethanol or no cholesterol (i.e., ethanol alone), was added. NGM plates were seeded with a drop of OP50 bacteria, as is done with mating plates. BODIPY-Cholesterol was obtained from Avanti Polar Lipids Inc. The desired dilution of BODIPY-Cholesterol in DMSO was a concentration of 50 μ M for the experiment.

250 μ L of 50 μ M BODIPY-cholesterol/DMSO solution was placed on top of the OP50 bacterial lawns of each agarose plate and allowed to dry in the dark. Mixed stage worms from normal NGM-lite plates were put onto plates containing the BODIPY-Cholesterol. The plates were kept in the dark at 20°C for 24 hours. Once the worms had grown and incorporated the BODIPY-Cholesterol, they were ready for imaging.

In order to image, a sucrose flotation was performed to separate the worms from the bacteria. The sucrose flotation was modeled after the Koelle lab protocol⁶. The worms were washed off the plates with M9 and pelleted by spinning for 3 minutes at 250 x g and 10°C. The supernatant was poured off, and the pellets were chilled on ice for 5 minutes. The pellet of worms was resuspended in 4-5mL of 0.1 M NaCl and spun at 250

x g for 3 minutes. Supernatant was poured off after spinning, leaving a little liquid behind. The pellet of worms was chilled on ice for 5 minutes. The tube was swirled to resuspend the pellet, and a solution of one part 0.1M NaCl and one part 60% sucrose was added to the tube. Once the solution was added, the worms were evenly mixed into solution, and the tube was spun at 250 x g for 3 minutes with a slowdown period of 2 minutes. After spinning, the pellet was chilled on ice for 5 minutes. There was a dark pellet at the bottom of the tube containing bacteria, debris and dead worms. Furthermore, there was a light brown layer at the top of the tube which was comprised of live worms. The layer of live worms was removed and placed in a new centrifuge tube. The worm suspension was diluted four-fold in ice cold 0.1M NaCl and spun at 250 x g for 3 minutes. The supernatant was removed, and the worms were ready for imaging.

Imaging BODIPY labeled C. elegans

The worms were imaged using a confocal microscope, fluorescence excitation ranged from 510nm to ~665NM¹². The images were processed with Olympus Fluo View Software and quantitatively analyzed with ImageJ Software.

Low Cholesterol v. High Cholesterol Bioassay

Agarose plates were made following the procedure described for Crowder³ or Yochem¹⁵ media. Agarose plates were seeded with 75µL of OP50 bacteria prepared according to the Crowder or Yochem procedure. Synchronized worms were used for the assay. Worms were synchronized by an embryo flotation as described¹³. Alterations to the flotation procedure for these experiments included: replacing the egg buffer with M9,

using a 60% sucrose solution, and rinsing the eggs collected from the sucrose flotation. The 15mL tube containing the collected eggs had sterile water added to the tube, and the eggs were pelleted by spinning at 210 x g for 4 minutes. The spinning allowed all the eggs to sediment to the bottom. After the supernatant was pipetted off and the eggs were resuspended in a small amount of M9, the eggs were then put onto NGM lite plates with 5µg/ml cholesterol.

Once the eggs were put on plates they were grown at 16°C for 48 hours and became L4 stage *C. elegans*. 20-30 worms from each strain were put onto unseeded plates for 30 minutes. This allowed them to move around the plates and remove any bacteria that may have been on the worms. 10-15 worms from each strain were placed on experimental and control plates. The experimental plates were made with Crowder or Yochem media and did not contain cholesterol. The control plates were made from Crowder or Yochem media as well, but were supplemented with 5µg/ml cholesterol. The worms were grown at 16°C for 48 hours to become gravid adults. Once they were gravid adults, they were placed on new experimental and control plates. The worms laid eggs for 5 hours, and all the gravid adults were removed from the plates. Then, the numbers of eggs on the plates were counted. Plates were stored at 16°C for 26 hours to allow the eggs to hatch. After 26 hours, the eggs that had not hatched were counted, and the numbers were compared to determine the hatch rate of the different strains under the different cholesterol conditions.

Cholesterol Extraction from Agarose

Cholesterol was extracted from the agarose in 10X volume of 1:2 mixture of CHCl_3 ³. The agarose extraction was performed 3 times. The extracted agarose was left sitting in the fume hood overnight to dry in glass containers with kimwipes covering them.

Cholesterol Quantitation Assay

For each strain synchronized populations of worms were obtained by washing all the worms off of plates that had eggs laid on them using M9. The eggs were left at 16°C to allow the worms to hatch. Once all eggs had hatched and become L1 stage worms, after approximately 24 hours, they were washed off the plates with M9, pelleted, and placed on fresh plates. On the fresh plates, the worms were allowed to grow into gravid adults. Once mature, they were washed off the plates with M9 and pelleted. The worms were rinsed in large volumes of ice-cold M9 5 times. The supernatant of the last wash was saved for a blank in quantitation. The cholesterol quantitation assay was performed as described², using Amplex Red as a fluorescent reporter of cholesterol content.

Results

Cholesterol localization and accumulation in C. elegans embryos as determined by DHE fluorescence

The first part of this study used fluorescent analogs of cholesterol to visualize the amount and spatial incorporation of cholesterol in wildtype and mutant strains of *C. elegans*. DHE is a naturally occurring fluorescent analog of cholesterol. Previous studies suggested that DHE was shown to accumulate in the oocytes of *C. elegans*¹⁰. Upon

imaging, the worms that had DHE incorporated in them emitted too much autofluorescence at the same wavelength DHE was emitted (Data not shown). Due to the abundance of autofluorescence, other cholesterol visualization methods were pursued.

Cholesterol localization and accumulation in C. elegans embryos as determined by BODIPY-cholesterol fluorescence

BODIPY-cholesterol is a synthetic fluorescent analog of cholesterol. Previous studies used BODIPY-cholesterol to visualize and monitor cholesterol movement in mammalian cells. It was also shown to be highly photostable and fluorescence was emitted at a different wavelength than *C. elegans* autofluorescence. A protocol was developed to incorporate the BODIPY-cholesterol in place of cholesterol in the wildtype and mutant strains of *C. elegans*. When the worms were imaged, BODIPY-cholesterol was incorporated in the worms and could be visualized (Figure 2). Looking at the wildtype worms, BODIPY-cholesterol was seen in the oocytes and spermatheca. This is where other studies had suggested cholesterol was localized (Figure 2A, B). In contrast, *rme-2 C. elegans* mutants showed very little BODIPY-cholesterol incorporated in their oocytes and spermatheca (Figure 2C, D). This is consistent with the fact these mutants have a disruption in the vitellogenin receptor (RME-2) and have been shown to have very little cholesterol accumulation¹⁰. The mutant strain (*srf-3* and *srf-8*) worms also showed incorporation of cholesterol in their oocytes and spermatheca (Figure 2E-J).

After images were taken, the fluorescence in the oocytes was quantitated using imageJ software. Wildtype, *rme-2*, and *srf-8 C. elegans* had a similar distribution pattern for the total amount of BODIPY-fluorescence incorporated into each oocyte (Figure 3, A-

C). The distribution of the fluorescence intensity of the oocytes was different between the wildtype and *srf-3 C. elegans* (Figure 3, B and D). There was a wide variation in fluorescent intensity of gastrulated embryos for *srf-3* worms (Figure 2, I-J; Figure 3D). Some *srf-3* gastrulated embryos showed much greater fluorescence than the wildtype gastrulated embryos.

The mean fluorescence of gastrulated embryos in *C. elegans* strains was compared (Figure 4). The *srf* mutant strains were compared to the wildtype worms using the Mann-Whitney test. The test showed a significant difference of median fluorescence between the *srf-8* and wildtype *C. elegans* (Figure 4). This suggests that the *srf-8* mutation, which leads to an abnormal glycosylation profile in *srf-8* worms, also leads to an abnormal cholesterol-loading phenotype.

Slow-hatch rate phenotype as determined by C. elegans bioassays

The second part of the study used bioassays to determine if cholesterol is necessary for the viability of *C. elegans*. A variety of media was tested to find the best conditions on which to grow the *C. elegans*. The first media used was the Crowder media. Literature had shown *C. elegans* to grow successfully on Crowder media plates³. When the Crowder media was used for the bioassay, there was an extreme decrease in growth of the worms; the *C. elegans* took longer to become gravid adults and appeared smaller than gravid adults grown on NGM stock plates. Crowder plates also had a lower survival and hatch rate. Surprisingly, in experiments using animals grown on Crowder plates for additional generations, the animals on the Crowder plates with cholesterol had a lower hatch rate than the Crowder plates without cholesterol (Data not shown).

In the next pilot trial, a variety of media was tested. Media included Yochem media with OP50 bacteria, NGM-lite media with Crowder OP50, and NGM-lite media with regular OP50. Yochem media allowed for normal growth of *C. elegans*, and further bioassays were performed using Yochem media with regular OP50 bacteria.

Preliminary data for the hatch rate bioassay shows that *srf-8* had a lower hatch rate after the first 26 hours, as compared to wildtype *C. elegans* (Figure 5). Even when Crowder plates were used for the bioassay and complications occurred, there was a decrease in the hatch rate after the first 26 hours (Figure 5A). NGM-lite media without cholesterol also showed a decrease in the hatch rate for *srf-8* compared to wildtype after the first 26 hours (Figure 5B). The bioassays that were performed using Yochem media showed an even greater decreased hatch rate than NGM-lite after the first 26 hours for *srf-8* compared to wildtype (Figure 5C). The hatch rate decrease between NGM-lite media and Yochem media is most likely due to Yochem media having less cholesterol than NGM-lite media (Figure 5B, C). After 72 hours the plates were checked. At this time, all the eggs that were laid on the *srf-8* plates had hatched. This showed that there is not a viability phenotype in the *srf-8*. Rather, there is a slow hatching phenotype that occurs when *srf-8*, but not wildtype, *C. elegans* are deprived of cholesterol. Further bioassays are being performed to expand our data on the slow hatching phenotype in order to test the statistical significance of these findings.

Discussion

Since *C. elegans* must take in sterols from the outside environment, there is a possibility that glycosylation defects may alter the nematodes ability to endocytose

cholesterol. This was analyzed by studying the incorporation and distribution of cholesterol in *C. elegans*. There appeared to be a cholesterol incorporation phenotype in the *srf-8 C. elegans*. This was evidenced by the significant difference of cholesterol accumulation in gastrulated embryos of the *srf-8* and wildtype strains. The phenotype was also evidenced in the bioassay experiments. The *srf-8* strain had a lower hatch rate than wildtype after the first 26 hours; however, within 72 hours all eggs had hatched. This suggested that cholesterol deprivation did not result in a viability difference but rather a slower hatch rate phenotype.

The studies showed cholesterol accumulation in the oocytes and spermatheca. This is in concurrence with previously published literature. Since there was a lower accumulation of BODIPY-cholesterol in *srf-8*, and the bioassay showed a cholesterol-deprivation phenotype in *srf-8*, it appears that defects in this mutant *C. elegans* lead to an altered ability to endocytose cholesterol.

The future goals of this study would be to complete double mutant *C. elegans* crosses and quantitate the amount of cholesterol per protein found in each strain of *C. elegans* studied. Steps have already been made to cross *srf-3(yj10)*, *srf-3 (br6)*, and *srf-8* worms with a *vit-2::GFP* worm, which carries a translational VIT-2-GFP fusion construct. The cross of these strains would allow the VIT-2 protein to be localized in the *srf* strains. Vitellogenin 2 would fluoresce in each strain once crossed with *vit-2::GFP*, and images could be taken. If Vitellogenin 2 is mislocalized in the *srf* mutants, it would explain at least part of the mechanism by which those *C. elegans* have defects with cholesterol endocytosis.

Another part of the study that is in progress is the quantitation of cholesterol in *C. elegans* strains. An Amplex Red Cholesterol Kit, provided by Invitrogen, is used to quantitate the cholesterol. By coupling this kit with the Bradford total protein quantitation method, the amount of cholesterol per amount of total protein in each strain of worm will be able to be quantified. Once the amount of cholesterol is quantitated there will be a better understanding as to if there is a deficiency in cholesterol endocytosis due to mutations in the *srf* genes.

The mechanism behind cholesterol transport within *C. elegans* will be beneficial to the health science community. A better understanding of cholesterol transport in *C. elegans* leads to knowledge of cholesterol transport and function in Eukaryotic cells. Studying cholesterol transport and the mechanics of it will help to control metabolic disorders that lead to cholesterol problems in humans.

Reference

1. Brenner, S. (1974). The genetics of *Caenorhabditis elegans*. *Genetics*, 77, 71-94.
2. Cheong, M.C., Na, K., Kim, H., Jeong, S.K., Joo, H.J., Chitwood, D.J., et al. (2010) A potential biochemical mechanism underlying the influences of sterol deprivation stress on the *Caenorhabditis elegans* longevity. *The American Society for Biochemistry and Molecular Biology Inc*. Retrieved February 20, 2011, from www.jbc.org
3. Crowder, C.M., Westover, E.J., Kumar, A.S., Ostlund E. Jr., Covey, D.F. (2001) Enantiospecificity of cholesterol funcon in vivo. *The Journal of Biological Chemistry*, 27, (48), 44369-44372.
4. Grant, B., Hirsh, D. (1999). Receptor-mediated Endocytosis in the *Caenorhabditis elegans* oocyte. *Molecular Biology of the Cell*, 10, 4311-4326.
5. Hölttä-Vuori, M., Uronen, R.L., Repakova, J., Salonen, E., Vattulainen, I., Panula, P., et al. (2008). BODIPY-cholesterol: A new tool to visualize sterol trafficking in living cells an organism. *Traffic*, 9, 1839-1849.

6. Koelle, M., Herman, T., Hengartner, M. (1994) Liquid culture of worms: After the culture has grown. Retrieved June 30, 2010, from http://medicine.yale.edu/labs/koelle/Site/Protocols_files/protocol_liquid_culture.html
7. Kurzchalia, T.V., Ward, S. (2003). Why do worms need cholesterol. *Nature Cell Biology*, 5(3), 684-688.
8. Li, J., Brown, G., Ailion, M., Lee, S., Thomas, J.H. (2004). NCR-1 and NCR-2, the *C. elegans* homologs of the human Niemann-Pick type C1 disease protein, function upstream of DAF-9 in the dauer formation pathways. *Development*, 131(22), 5741-5752.
9. Link, C.D., Silverman, M.A., Breen, M., Watt, K.E., Dames, S.A. (1992) Characterization of *Caenorhabditis elegans* lectin-binding mutants. *Genetics Society of America*, 867-881.
10. Matyash, V., Geier, C., Henske, A., Mukherjee, S., Hirsh, D., Thiele, C., et al. (2001). Distribution and transport of cholesterol in *Caenorhabditis elegans*. *Molecular Biology of the Cell*, 12, 1725-1735.
11. Merris, M., Wadsworth, W.G., Khamrai, U., Bittman, R., Chitwood, D.J., Lenard, J. (2003). Sterol effects and sites of sterol accumulation in *Caenorhabditis elegans*: developmental requirement for 4 α -methyl sterols. *Journal of Lipid Research*, 44, 172-181.
12. Molecular Probes Inc. (2003) Product information: Bodipy lipid probes. Retrieved June 11, 2010 from www.probes.com
13. Shaham, S. Preparation of *C. elegans* eggs. *Methods in Cell Biology*. Retrieved June 30, 2011, from www.wormbook.org
14. Wüstner, D., Larson, A.L., Faergeman, N.J., Brewer, J.R., Sage, D. (2010). Selective visualization of fluorescent sterols in *Caenorhabditis elegans* by bleach-rate-based image segmentation. *Traffic*, 11, 440-454.
15. Yochem, J., Tuck, S., Greenwald, I., Han, M. (1999). A gp330/megalín-related protein is required in the major epidermis of *Caenorhabditis elegans* for completion of molting. *Development*, 126, 597-606.