Dietary PUFA Ratios and their Effect on Cellular Stress in *C. elegans*

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ABSTRACT

Our Western diet, which is deficient in omega-3 fatty acids and has excessive omega-6 fatty acids, has been highly correlated with the central nervous system and metabolic disorders (Western diseases). However, it is unclear how dietetic omega fatty acids affect these disorders. In this study, I investigated whether omega 3:6 fatty acid ratios influence cellular stress and autophagy processes in the genetic model system *Caenorhabditis elegans*, as these processes are strongly linked to fat metabolism and have been reported to affect many of the same disorders. Unlike humans, *C. elegans* are able to synthesize their own omega-6:3 fatty acids. Therefore, to study the effects of dietary PUFAs and their ratios similarity to Western diets on autophagy in *C. elegans*, I used a strain deficient in the synthesis of both omega-6 and omega-3 fatty acids that carries an autophagy marker (GFP::*lgg-1*). To generate this strain, I created animals with mutations in *fat-1* (required for omega-3 synthesis) that carry the GFP::*lgg-1* transgene and conducted *fat-2* RNA interference (RNAi) on these animals (*fat-2* is required for omega-6 synthesis). I used state-of-the art imaging and analytical tools to visualize and quantify autophagy in *fat-1*; *fat-2(RNAi);* GFP::*lgg-1* animals when exposed to five different ratios of omega-6:3 fatty acids. I found that a dietary ratio of omega-3:6 fatty acids (5:1) produced the least amount of autophagy, whereas a dietary ratio of omega-3:6 fatty acids (1:15 or 1:1) produced the most autophagy in the *fat-1*; *fat-2(RNAi);* GFP::*lgg-1*worms. Further studies of a balanced ratio of omega-3:6 fatty acids may allow us to design strategies and ways to prevent Western diseases that are correlated to Western diets.

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INTRODUCTION

Western diseases correlated with Western diet

The Western diet consists of high saturated fats, junk foods, and foods with empty calories. This diet tends to lack fresh fruits and vegetables, wholegrain, seafood, and poultry. Western diet has become the norm in regions like Europe and North America, but this diet is also becoming prevalent in underdeveloped countries such as West Africa and New Guinea. Studies show that diets like the Western diet can have a negative effect on health and a high correlation with Western disease (Patterson 2007).

In the 21st century, non-communicable diseases like common Western diseases have become more prevalent in Western societies than ever before. Western diseases, including breast cancer, prostate cancer, type two diabetes, coronary heart disease, colon cancer, high cholesterol, cardiovascular disease, allergies, mental health problems, etc. are common non-communicable diseases prevalent in people living in Western societies (Pollard et al. 2008). One theory for the occurrence of such diseases is the change that humans experienced in Western societies due to a decrease in physical exercise and increase in food availability. Humans originally evolved in situations that were very different from Western societies today, where the earliest humans had to hunt and gather food that was found and grown in natural environments (Terrence et al. 1998).

When an environment is constant, stabilizing selection maintains genetic traits that correlate with the optimal average for a population. When environmental conditions change, evolutionary discordance arises between a species' genome and its environment, and directional selection replaces stabilizing selection, which moves the average population genome to a new normal (Terrence et al. 1998). "Discordance" is often expressed in species

as disease. Environmental changes may have occurred too recently for the human genome to adapt to such extravagant changes in agriculture and diet (Cordain et al. 2005). Human biology, in turn, is suffering greatly from not being able to evolve to modern environmental and dietary changes. Western diseases are considered to be "man-made" and became prevalent by this onset of increased food availability and a reduction of physical activity (Pollard et al. 2008).

Dietary PUFAS and their effect on human diet and disease

Polyunsaturated fatty acids (PUFAs) commonly exist in the Western diet in a 15-16.7 omega-6 (n6):1 omega-3 (n3) ratio. This is a high ratio that has been correlated with the incidence of Western diseases. However, as essential fatty acids, n6s and n3s are necessary in the diet of animals to provide a fluid cellular membrane environment. Mammals, in particular, require essential fatty acids like n6s and n3s in their regular diet. PUFA composition affects many different cellular processes including modulation of ion channels, endocytosis/exocytosis, and activities of membrane-associated enzymes (Watts et al. 2002). Some PUFAs can also be precursors of eicosanoid effectors like prostaglandins and leukotrienes. Desaturation and elongation of fatty acids produce C20 PUFAs that become membrane components and precursors for eicosanoids. A disruption of a balanced PUFA intake is associated with certain diseases like coronary artery disease, hypertension, diabetes, inflammatory disorders, and cancer (Watts et al. 2002).

Omega n6 and n3 PUFAs are fundamental to the human diet, but humans must obtain these from foods or dietary supplements because our bodies cannot make them (Sacks et al. 2013). N6 PUFAs in diet include meat and vegetable oils, like linoleic acid. N6 PUFAs are

thought to reduce diabetic neuropathy, rheumatoid arthritis, allergies, and high blood pressure. N6 PUFAs are known to lower LDL (bad) cholesterol and reduce inflammation. N3 PUFAs include wheat germ, fruit, vegetables, fish and fish oils, olive oil, and canola oil. N3 PUFAs are thought to reduce high blood pressure, high cholesterol, blood clotting, arthritis, lupus, osteoporosis, asthma, diabetes, irritable bowel disease, breast cancer, and colon cancer (Sacks et al. 2013). However, n6 fatty acids promote inflammation, and when n6 and n3 fatty acids are present in an increased amount together, they can compete for metabolization, which is the process of synthesizing and breaking down substances. Metabolization is vital to cell survival and is negatively impacted when there is competition between n6 and n3 PUFAs (Duttaroy et al. 2003).

Excessive omega n6 PUFAs in our diet has been correlated with the incidence, as well as morality, of Western diseases (Sachiko 1996).. Western diets tend to elevate plasma levels of sex hormones while decreasing the sex hormone binding globulin concentration, which increases bioavailability of steroids (Cordain et al. 2005). Western diet is also correlated to a low formation of mammalian ligans and isoflavonic phytoestrogens, which affects hormone metabolism and production, as well as cancer cell growth, through many different mechanisms. This process makes them candidates for a role as cancer protective substances.

Cellular stress or "Autophagy"

Stress impacts cells under non-optimal conditions, including Western diets with excessive omega n6 PUFAs but deficient in omega n3. Cellular stress is also termed "autophagy," which generally describes degradation of cytoplasmic components within

lysosomes and usually refers to macroautophagy unless specified otherwise. Autophagy can occur for many reasons, but it commonly occurs when cells lack essential nutrients and put under duress, which evokes a survival response within the cell (Mizushima et al. 2007). The process of autophagy consists of sequestration, transport to lysosomes, degradation, and utilization of degradation products within a cell. Proteins, part of a cell membranes, bacteria, or viruses are presented and isolated from the cell and contained in the autophagosome. Autophagosomes are then fused with lysosomes and degraded or recycled. Autophagy serves as a process for degrading dangerous proteins or degrading cellular components to use as energy source during times of nutrient deprivation (**Figure 1**).

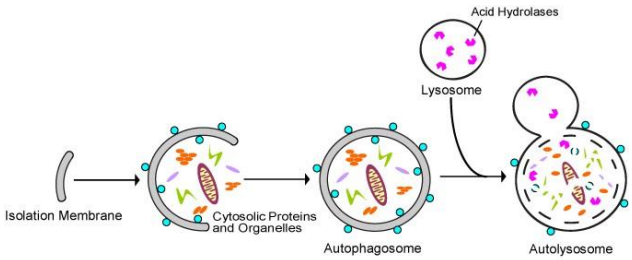

Figure 1: The process of autophagy including its cellular components

Dietary PUFAs and their effect on autophagy

Western diets were thought to have evolved from "a decrease of civilization" that was the result of a change in glycemic load, fatty acid composition, macronutrient composition, micronutrient density, acid-base balance, sodium-potassium ratio, and fiber content. Diets of people in the United States have their percentage of total food-energy derived from three major macronutrients of carbohydrate (51.8%), fat (32.8%), and protein (15.4%) (Cordain et al. 2005). Western diets tend to be deficient in n3 PUFAs and have an excessive amount of n6 PUFA, resulting in an unbalanced ratio of n6:n3 PUFAs of 15-16.7:1. Recent studies have

shown that high ratios of n6:n3 PUFAs can be harmful, so it may be better to regulate the intake of these essential fatty acids (Simopoulos 2002). There has been a great increase in n6 PUFAs in the Western diet from consumption of linoleic acid or soybean oil, which has impacted humans negatively (Blasbalg et al. 2011). A more balanced ratio of n3:n6 PUFAs could potentially be a more desirable ratio for the human diet, reducing risks of chronic Western diseases (Simopoulos et al. 2002). In this thesis, I use the nematode *Caenorhabditis elegans* as a model organism to study autophagy and its correlation with dietary PUFAs.

Using *Caenorhabditis elegans* to study cellular stress and the effects of dietary PUFAs

The nematode *C. elegans* was introduced in 1963 by Sydney Brenner who understood the importance of simple organisms for research in developmental and neurobiology (Adams et al. 2008). *C. elegans* are often used as model organisms for human studies because they have similar nervous and developmental systems. In addition, *C. elegans* are easy to grow in the lab, have a short generation time of only three days, have a completely sequenced genome consisting of about 19,800 genes, and have a comprehensive description of their anatomy. Importantly, 35% of *C. elegans* genes have human orthologs (Shaye et al. 2011).

Although humans cannot synthesize their own n6 and n3 PUFAs, *C. elegans* are able to synthesize omega n6 and n3 PUFAs through a series of metabolic processes. There are different pathways of PUFA synthesis within different species (**Figure 2**). In order to study PUFA synthesis in humans, *C. elegans* can be easily manipulated to reflect the same PUFA synthesis pathway that occurs in humans.

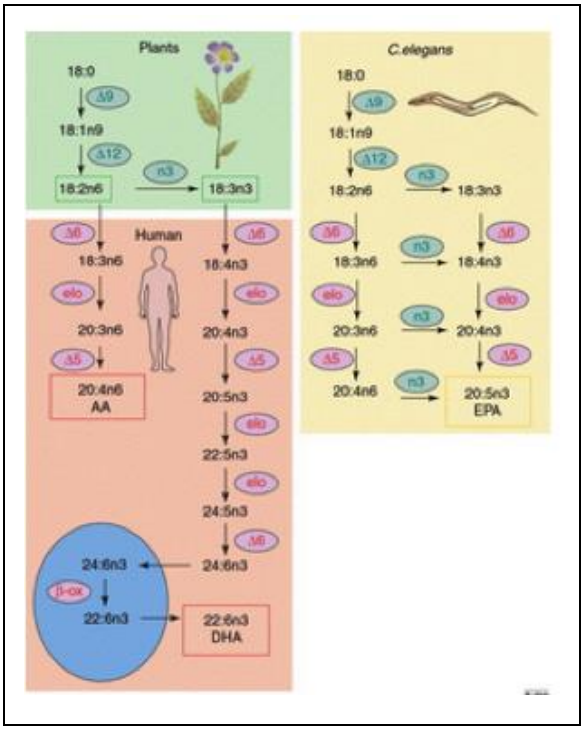

Figure 2: Differences in pathways of PUFA synthesis in plants, humans, and *C. elegans*. Plants are able to produce linoleic acid (18:2n6) and linoleic acid (18:3n3) through delta 12 and n3 desaturase activities. Mammals, represented here as a human, do not possess delta 12 and n3 desaturase activities, and must receive 18:2n6 and 18:n3n from their diets or from supplements. *C. elegans* possess all desaturase activities required, but it is not able to elongate C20 PUFAs (Watts et al. 2002).

To investigate roles that fatty acids have on the growth, development, and neural function in an animal system, Jennifer L. Watts and John Browse studied the effects of PUFAs on the membrane components of *C. elegans*. Watts and Browse isolated mutations in *fat-1, fat-4, fat-3,* and *elo-1* genes in *C. elegans* that are deficient in PUFA synthesis by direct analysis of fatty acid composition. The fatty acid composition of these mutants revealed the substrate preferences of the desaturase and elongation step of the PUFA biosynthetic pathway (Watts et al. 2002).

In addition to studying PUFA synthesis, *C. elegans* is a great model system to study cellular stress. The gene *lgg-1* in *C. elegans* is a key component required for the degradation of cellular components via the process of autophagy. When the coding region of *lgg-1* is fused to Green Fluorescent Protein (GFP) (further referred as GFP::*lgg-1*) under control of its own promoter, this *gfp*-reporter can be successfully used to examine autophagy *in vivo* in *C.*

elegans. Transgenic animals expressing GFP::*lgg-1* show a marked increase in punctae staining in hypodermal seam cells that reflect an increase in the number of pre-autophagosomal and autophagosomal structures (WormBase 2013) (**Figure 3**).

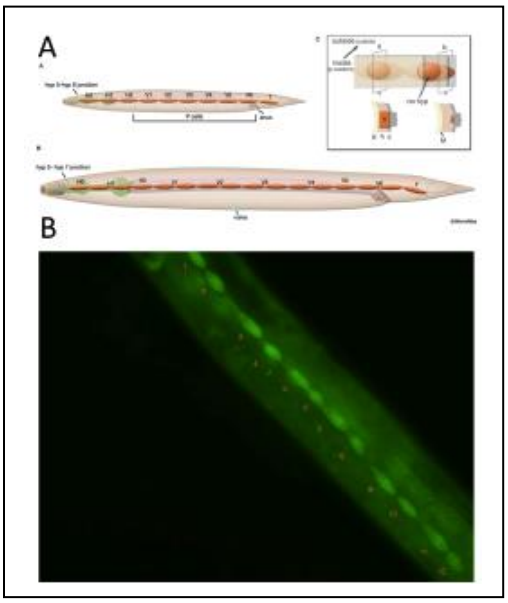

Figure 3: (**A**) Seam cells are stem cell-like epithelial cells that are arranged in longitudinal rows. These cells lie along the apical midline of the hypodermis all the way from the nose to the tail of the *C. elegans* (Wormatlas 2004). (**B**) Seam cells are numbered in *C. elegans* containing the transgene GFP::*lgg-1*. Autophagy is shown as punctae inside those seam cells. These punctate are counted to determine the amount of autophagy or "self-eating" taking place (see Methods).

Hypothesis of this thesis

Do dietetic PUFAs cause autophagy in *C. elegans*? and, if this is the case, is there a relationship between autophagy and the ratio of omega n6:n3 PUFAs in *C. elegans*? As described above, *C. elegans* are capable of synthesizing n6 and n3 PUFAs from metabolic precursors, and do not have to obtain PUFAs from their diet *Escherichia coli* (*E. coli*) (Simopoulos 2002). There are several mutants that are incapable of completing various steps in the *C. elegan* fat synthesis pathway. Mutations in *fat-2* result in animals that are unable to synthesize omega n6 PUFAs, while mutations in *fat-1* results in animals that cannot make omega n3 PUFAs. Therefore, animals mutant for both *fat-1* and *fat-2* would be unable to synthesize n3 and n6 PUFAs, and would require supplementation through diet. The goal of

this thesis is to examine the effects of dietary PUFAs on autophagy in *C. elegan* strains that lack both the function of *fat-1* and *fat-2*, which carries an *in vivo* marker for autophagy (GFP::*lgg-1*).

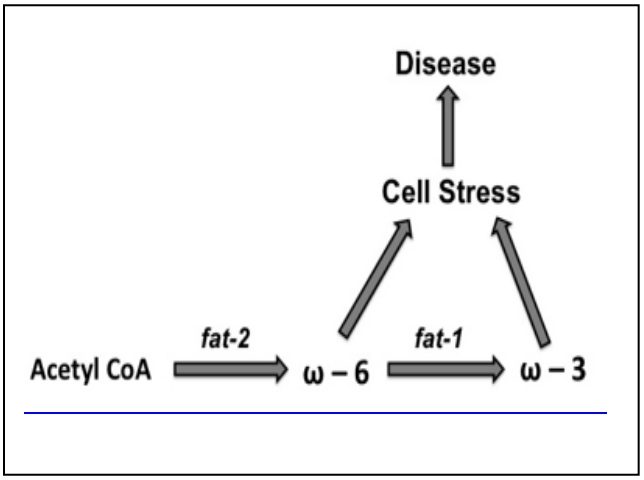

Figure 3: Hypothetical model of how omega n6:n3 PUFA ratios could cause cell stress in *C. elegans*. In *C. elegans*, the *fat-1* and *fat-2* genes are responsible for converting metabolic products into omega n6 and n3 PUFAs. Various ratios of those fatty acids could cause cell stress.

RESULTS AND DISCUSSION

To study the effects of dietary PUFAs on autophagy and to be entirely dependent on the diet of *C. elegans*, I first created a *fat-1(wa9)* mutant strain that carries the autophagy marker GFP::*lgg-1* using standard genetic crosses (see Methods). To confirm the mutation was present, I sequenced the *wa9* allele using standard sequencing methods (**Figure 4**). Next, I made 5 different sets of plates that contain different ratios of n3:n6 PUFAs (1:1, 5:1, 1:5, 1:15, and

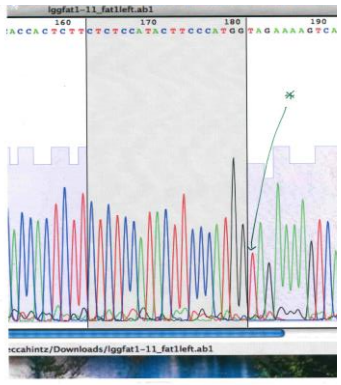

Figure 4: Confirmation of the *wa9* mutation in *fat-1* (indicated by a star).

tergitol only as the control), and I exposed both GFP::*lgg-1* animals and *fat-1(wa9)*; GFP::*lgg-1* animals to the different n3:n6 ratios. Following exposure, I counted the number of GFP punctate that were inside the seam cells of the of L3 larvae.

Because *fat-1* and *fat-2* genes are physically close (adjacent genes and less than 5kb from each other), it would be very difficult, if not impossible, to create *fat-1(wa9);fat-2(wa17)* double mutants by standard genetic crosses. To circumvent this problem, I decided to use RNA interference (RNAi) protocol in order to knock down *fat-2* function in a *fat-1;* GFP::*lgg-1* background. RNAi has been used to inhibit the function of approximately 86% of 19,427 predicted genes of *C. elegans* (Kamath et al. 2003). RNAi silences gene expression after transcription. RNAi is an antiviral response that can be exploited in order to allow specific inhibition of the function of any chosen target gene. RNAi uses double stranded DNA that is processed by a Dicer enzyme to cleave a portion off the end of the RNA before it is able to get translated (**Figure 5**). The RNAi induced silence complex (RISC), a multi-protein complex, to incorporate a small interfering RNA (siRNA) to be used as a template for recognizing complementary messenger RNA mRNA. Because a portion of the RNA was cleaved, the mRNA cannot be read properly and gets degraded. I used the RNAi method to knock down *fat-2* function in both GFP::*lgg-1* and *fat-1(wa9);*GFP::*lgg-1* animals. Following exposure to the different omega n3:n6 PUFA ratios as described above, I took pictures of GFP::*lgg-1* and *fat-1(wa9);*GFP::*lgg-1* in order

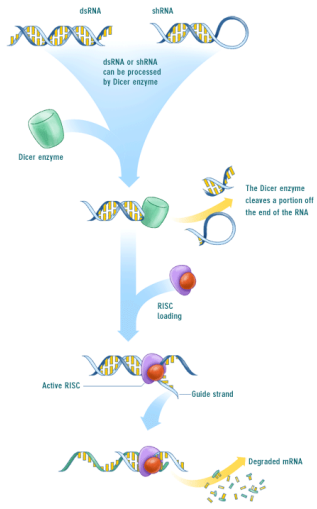

Figure 5: A step-by-step process of RNA

to count autophagy punctae in the seam cells, and observed the effect of omega n3:n6 PUFA ratios on these animals.

In the n6:n3 (15:1) PUFA ratio, *fat-1(wa9)*; GFP::*lgg-1* animals with *fat-2* RNAi, were found to have the most autophagy, with an average number of 12.63 punctae/seam cell. This result indicates that in a *fat-1(wa9)*; *fat-2* RNAi background, a high number of omega n6 PUFA causes more cellular stress. The n6:n3 (15:1) PUFA ratio is expected to be similar to the ratio of omega fatty acids that humans consume in Western diets. This suggests that this diet with excessive omega n6 PUFAs could cause more cellular stress in humans. With only omega n3 PUFA synthesis inhibited, *fat-1(wa9)*; GFP::*lgg-1* animals contained the least amount of autophagy with an average number of 4.5 punctae/seam cell. For the n3:n6 (1:5) PUFA ratio, *fat-1(wa9);*GFP::*lgg-1* with *fat-2* RNAi had an average number of 8.60 punctae/seam cell, which showed that omega n6 PUFAs might play a greater role in effecting autophagy abundance than omega n3 PUFAs. Animals of *fat-1(wa9)*; GFP::*lgg-1* had the least amount of autophagy with the average number of 5.06 punctate/cell. *C. elegans* supposedly makes a sufficient amount of n6:n3 PUFAs on its own, which could be the reason for the least amount of autophagy within the control group. For the n3:n6 (5:1) PUFA ratio, *fat-1(wa9);* GFP::*lgg-1* with only omega n3 PUFA synthesis inhibited contained the most cellular autophagy with an average number of 9.52 autophagy punctae/seam cell, which was a surprising result having observed a low autophagy with the omega n3:n6 (5:1) PUFA ratio. For the n6:n3 (1:1) PUFA ratio, *fat-1(wa9)*; GFP::*lgg-1* after *fat-2* RNAi contained the most autophagy punctae/seam cell with an average number of 12.55 autophagy punctae/seam cell. Interestingly, with equal amounts of omega n6s and n3 PUFAs in the *C. elegans* diet, I observe more autophagy. For the control of only tergitol, *fat-1(wa9)*;GFP::*lgg-1* animals

exposed to contained the most amount of autophagy with 8.24 autophagy punctae/seam cell, while GFP::*lgg-1* after *fat-2* RNAi contained the least amount of autophagy punctate with 3.13 autophagy punctae/seam cell (**Figure 6**).

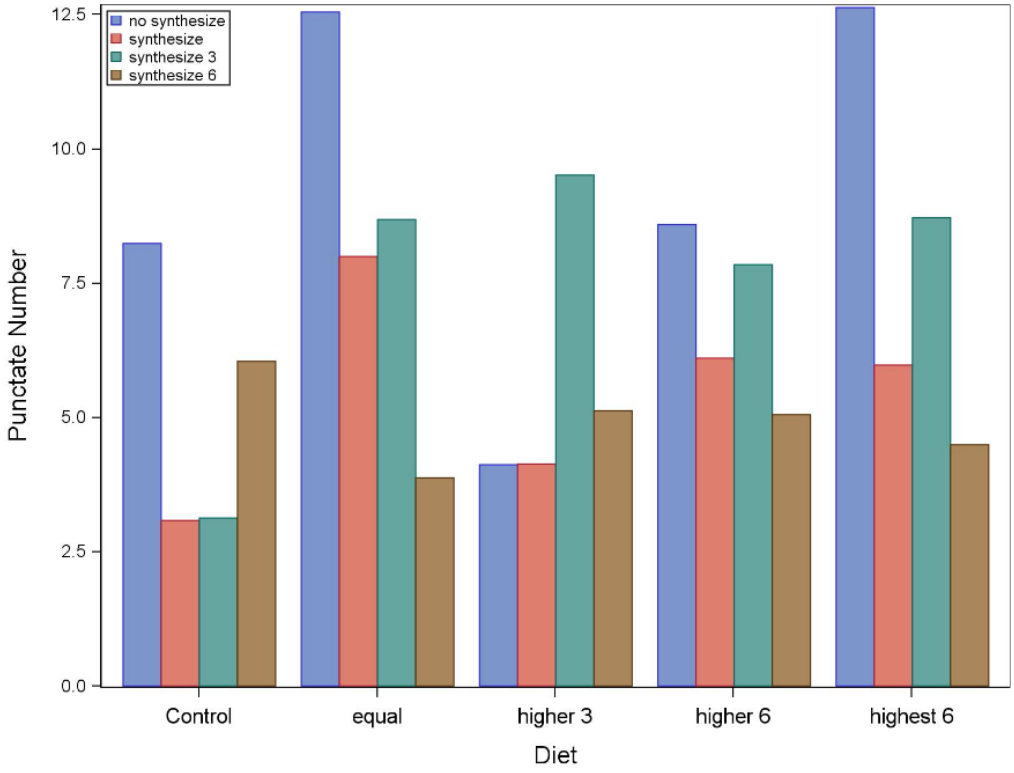

Figure 6: Interaction plot between genotype represented on the X-axis, PUFA ratios color coated at the bottom, and punctate represented on the graph. 'No synthesize' represents the *C. elegans* strain *fat-1(wa9)*; GFP::*lgg-1* after *fat-2* RNAi. 'Synthesize' represents the control group of GFP::*lgg-1,* 'Synthesize 3' represents GFP::*lgg-1* after *fat-2* RNAi, and 'Synthesize 6' represents *fat-1(wa9)*;GFP::*lgg-1.* Each label on the y-axis represents the control group of no PUFAs, an equal ratio of n6:n3 (1:1), a higher n3 of n6:n3 (1:5), a higher n6 of n6:n3 (5:1), and highest n6 of n6:n3 (15:1).

I used the Statistical Analysis Software (SAS) to analyze the data set (**see Appendix**), using the predictor values of strain (or genotype) and fat (ratio of PUFAs) and the response variable as autophagy punctate. I ran a two-way ANOVA and used tukey grouping in order

to assess if the data was statistically significant and whether the dietetic groups and genotype groups were statistically similar or dissimilar from one another. For the strain (or genotype) predictor variable, I received a p-value < 0.0001, with an F-value of 88.66, meaning that genotype had a significant role in the amount of autophagy expressed in *C. elegans*. With such a high F-value, it expressed the most significance in determining how much autophagy would be present. For the PUFA ratios (fat), the p-value was < 0.0001, with an F-value or 18.76, meaning that there was statistical significance between the PUFA ratios and the amount of autophagy observed. An interaction of both PUFA ratios and genotype effecting overall autophagy abundance was statistically significant with a p-value < 0.0001 and an F-value of 17.43 (**Table 2**).

Based on the statistical analysis, I found that the ratios omega n6:n3 PUFA ratios 1:1, 15:1, and 5:1 showed the most autophagy, and were statistically similar to one another after running a tukey grouping test (**Table 4**). I also found that n3:n6 5:1 and the control showed the least amount of autophagy, and were statistically similar (**Table 4**). Since the p-values for the different strains, PUFA ratio, and interaction were < 0.0001, this data was statistically significant, and therefore, I can accept the hypothesis that the different strains and five different ratios of PUFAs show a difference in the displayed amount of autophagy. That being said, I observed the least amount of autophagy in the control (the control strain has still a functioning PUFA synthesis pathway) when no PUFAs are added. If they use pathways similar to humans and cannot produce their own PUFA synthesis internally, then a omega n3:n6 PUFA ration of 5:1 results in the least amount of autophagy. However, if *C. elegans* are exposed to omega n6:n3 PUFA 1:1 or 15:1 PUFA ratio, their punctae increases, which suggests increased cellular stress. This is an interesting observation and could be due to the

fact that *C. elegans* are making their own PUFAs in a balanced ratio, so adding the same amounts of omega n6 and n3 PUFAs puts *C. elegans* under much duress, possibly causing more autophagy to be present.

In summary, it is important to examine the potential link between dietary PUFAs and stress-induced autophagy, which would allow us to further understand the correlation between Western diet and disease. It would also provide understanding of the etiology of these diseases for the development of preventative and treatment methods for Western diseases and diets for the future.

Methods and techniques used in this study

Strain and maintenance: C. elegans were cultured and grown on Nematode Growth Medium (NGM) agar according to standard methods at 20°C. The diet used was *E. coli* OP50. Strains used in this study were: wild-type (N2), BX24 *fat-1(wa-9)* and BX26 *fat-2(wa17),* and DA2123 with the autophagy marker *adIs2122*[GFP::*lgg-1+rol-6(su1006)*]. The *adIs2122* transgenic strain can be easily visualized, as animals homozygous for the GFP::*lgg-1* transgenic array show a roller phenotype.

fat-1(wa9) mutants: Mutations in *fat-1* result in animals that are unable to synthesize omega n3 PUFAs. The *fat-1* gene is located on chromsome IV. *fat-1(wa9)* was crossed with the GFP::*lgg-1* transgenic line (autophagy marker) in order to obtain the *wa9* mutation that carries the autophagy marker. The *wa9* allele is a substitution mutation that causes a nonsense mutation in *fat-1*. To validate the presence of the *wa9* mutation, I performed PCR and sequencing (Nevada Genomics Center), and I was able to obtain a homozygous animal with the *wa9* mutation and the GFP::*lgg-1* transgene (see Results).

fat-2 RNAi: Mutations in *fat-2* function result in animals that are unable to synthesize omega n6 PUFAs, and these animals are exhibit a slow and sickly phenotype. *fat-2* is located on chromosome IV, and physically close to *fat-1* (Wormbase 2013). Since it would be very difficult to combine mutations between *fat-1* and *fat-2* (see Results), I used *fat-2* RNAi on *fat-1*; GFP::*lgg-1* animals in order to create a *fat-1*; *fat-2* double carrying the GFP::*lgg-1* transgene. For RNAi experiments and to knock down *fat-2* function, I streaked bacterial food that contain an RNAi construct directed againsts the *fat-2* gene onto standard LB and tetracycline (final 12.5 µg/ml) and amplicilin (final 100 µg/ml) agar plates to get single colonies on the plates. I grew up the RNAi food from a single colony in 5 ml LB liquid culture containing only ampicilin (final 100 µg/ml). I diluted 1:100 in LB broth and ampicilin (final 100 µg/ml) and grew for 4-6 hour. I inoculated six cm plates containing NGM agar and IPTG (final 1 mM) and ampicilin (final 50 µg/ml) with about 250 µl of the diluted bacterial culture (RNAi food) and grew overnight at room temperature for the *fat-2* gene. I then placed five L4 staged hermaphrodites of *fat-1;*GFP::*lgg-1* and GFP::*lgg-1* animals onto the RNAi seeded plates and incubated them for 40-48 hours at 15 degree. Next, two animals were independently replica plated onto plates seeded with the same RNAi food and allowed to lay eggs for 24 hours at 20C before the mothers were removed and killed. I let the eggs hatch and grow on the plate until they grew into larval stage 3 (L3). In addition, I synchronized L1 staged larvae on RNAi seeded plates and let develop to adults at 20C. I was able to watch the animals develop a *fat-2* sickly phenotype similar to the *fat-2(wa17)* mutants. I also used a positive control of bacterial food containing a RNAi construct directed against the *cbp-1* gene, which causes embryonically lethality.

Fatty acid analysis. To examine the effects of omega n3:n6 PUFAs on the created strains, I made NGM agar plates containing tergitol and varied the amount of 0.1 M omega n3 and n6 PUFAs in each plate (**Table 1**).

Table 1: Different ratios of omega n3:n6 PUFAs used in the tergitol NGM plates

Omega n3 PUFA	**Omega n6 PUFA**
1 (167 µL)	1 (167 µL)
1 (167 µL)	15 (2.505 mL)
1 (167 µL)	5 (835 µL)
5 (835 µL)	1 (167 µL)
0 (Tergitol only)	0 (Tergitol only)

After making the tergitol NGM plates containing various omega n3:n6 PUFA ratios, I took at least five plates of adults full of eggs on *fat-1*; GFP::*lgg-1* and five plates of GFP::*lgg-1* and synchronized the animals by bleaching. This bleaching process included washing each plate with 1x M9 solution so they were free from bacteria. Next, I pipetted the liquid to 100 µL and used 500 µL of 2x bleach (containing 20 mL M9, 10 mL 5M KOH, and 15 mL NaOCl) to agitate them for 2-4 minutes. When I saw the animals had ruptured under the microscope, I added 1 mL of M9 solution three times until there were clean eggs in the liquid. I removed excess liquid, and pipetted the eggs onto three plates containing the different ratios of omega n3 and n6 PUFAs.

Since the omega n3 and n6 PUFAs are sensitive to light, I covered the plates and left the plates at room temperature until the eggs of the created strains reached the third stage of larvae (L3) development, which took about 40-48 hours. The seam cell of a L3 larvae has not yet morphed together as one large seam cell, as they do when they reach larval stage four

(L4). Because their seam cells can be individualized, I was able to photograph the seam cells under high magnification and fluorescence, and count the autophagy punctate inside of the seam cells (see below measurement and quantification).

Measurement and quantification of autophagy: A fluorescent microscope was used in order to locate and identify autophagy punctate within seam cells of the *C. elegans*. A Leica confocal fluorescent microscope was used for suitable 3-dimensional imaging (Kimura et al. 1989). For each strain under following different exposures of omega n3 and n6 PUFA ratios, I transferred 25 animals on a slide and observed them under the fluorescent microscope. I identified autophagy punctate in the seam cells and photographed animals (**Figure X**). Seam cells were labeled and the punctate within them were counted by hand and recorded in a Microsoft Excel spreadsheet. I used a two-way ANOVA using SAS and tukey grouping in order to determine if the data was statistically significant.

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APPENDIX

Statistical Analysis of Data Sets

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	19	6467.59295	340.39963	28.96	<.0001
Error	821	9650.40705	11.75445		
Corrected Total	840	16118.00000			

R-Square	Coeff Var	Root MSE	punctate Mean
0.401265	52.88608	3.428477	6.482759

Source	DF	Type I SS	Mean Square	F Value	Pr > F
genotype	3	3126.439066	1042.146355	88.66	<.0001
fat	4	881.987817	220.496954	18.76	<.0001
genotype*fat	12	2459.166068	204.930506	17.43	<.0001

Source	DF	Type III SS	Mean Square	F Value	Pr > F
genotype	3	2272.249980	757.416660	64.44	<.0001
fat	4	1021.946757	255.486689	21.74	<.0001
genotype*fat	12	2459.166068	204.930506	17.43	<.0001

Table 2: Statistics from the two-way ANOVA ran from the GLM procedure. Figures 9, 10, and 11 represent graphs obtained from the two-way ANOVA showing how autophagy, genotypes, and PUFA ratios are statistically significant to one another.

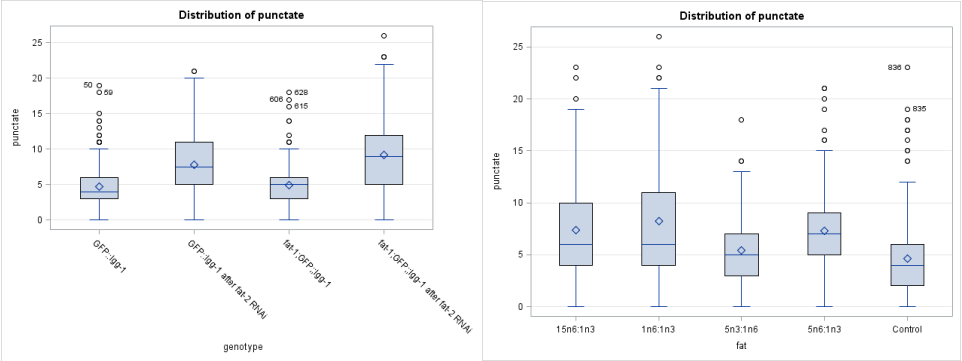


Figure 7: (A) A box-plot of the distribution of punctate expressed in four different genotypes, expressed in a 95% confidence interval, showing variation and outliers A box-plot of the distribution of punctate expressed in five ratios of PUFAs, expressed in a 95% confidence interval, showing variation and outliers.

Means with the same letter are not significantly different.

Tukey Grouping	Mean	N	genotype
A	9.1866	209	*fat-1*;GFP;;*lgg-1* after *fat-2* RNAi
B	7.8049	164	GFP::*lgg-1* after *fat-2* RNAi
C	4.9120	250	*fat-1*;GFP;;*lgg-1*
C			
C	4.6972	218	GFP::*lgg-1*

Table 3: Represents tukey grouping, where A, B, and C are represented as statistically dissimilar. The *fat-1*;GFP;;*lgg-1* after *fat-2* RNAi expresses the highest mean of autophagy and does not synthesize n6s or n3s. GFP::*lgg-1* after *fat-2* RNAi has the next highest amount of autophagy and does not have

the ability to synthesize n6s only. *fat-1*;GFP;;*lgg-1*, unable to synthesize n3s, and GFP::*lgg-1*, able to synthesize n3s and n6s have similar significant data with low amounts of autophagy.

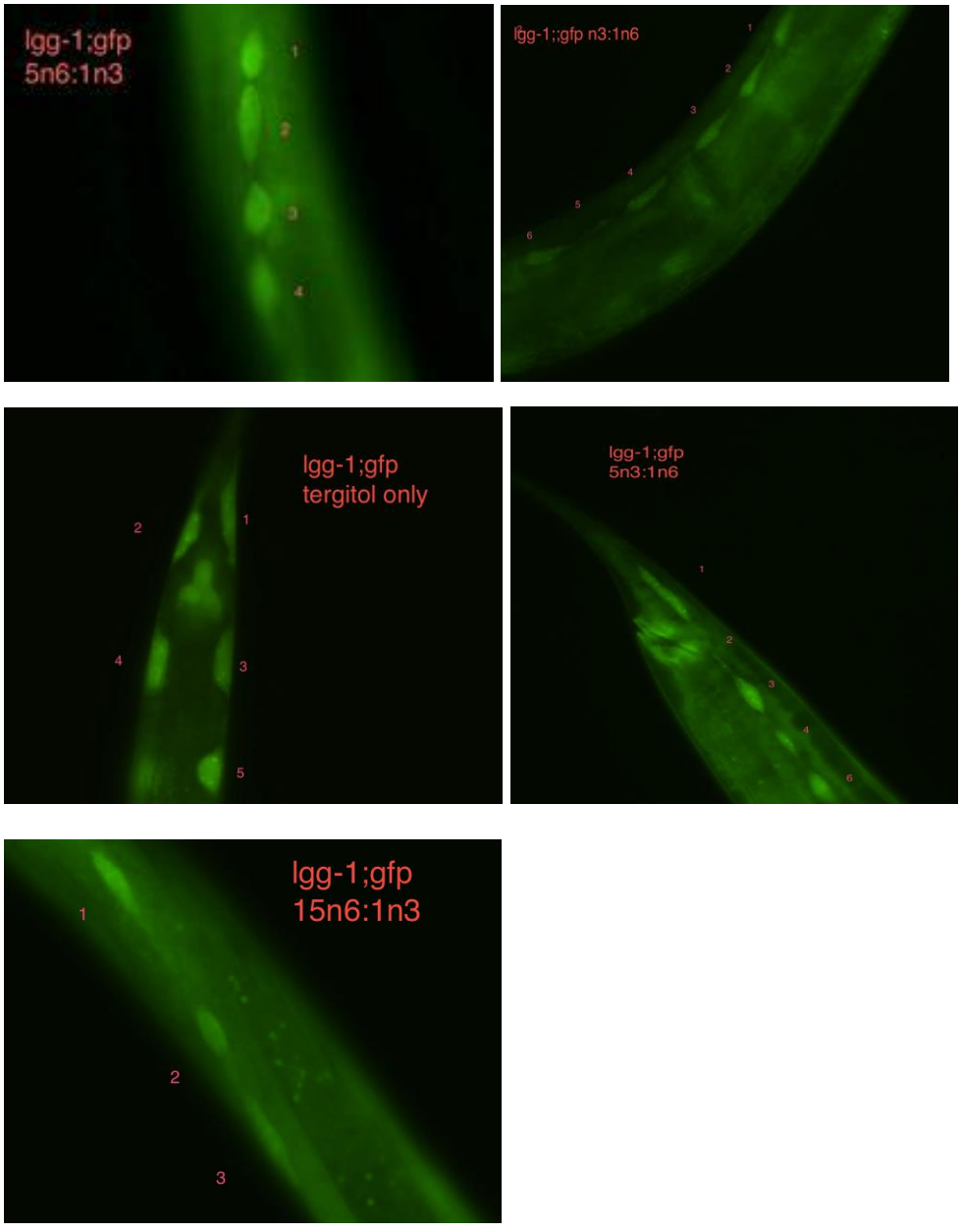
Means with the same letter are not significantly different.

Tukey Grouping	Mean	N	fat
A	8.2376	101	1n6:1n3
A			
A	7.3902	205	15n6:1n3
A			
A	7.2649	185	5n6:1n3
B	5.4463	177	5n3:1n6
B			
B	4.6069	173	Control

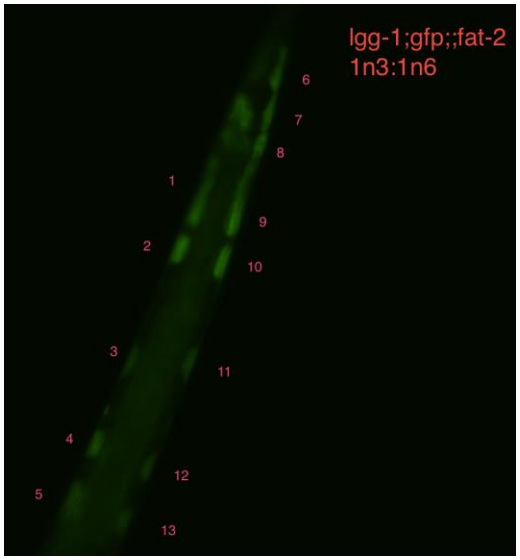
Table 4: Represents tukey grouping where A and B are statistically dissimilar. The n6:n3 (1:1), (15:1), and (5:1) are all statistically similar and express a higher mean about of autophagy punctae. The n6:n3 (1:5) and control are statistically similar and different from the other ratios, with a fairly low amount of punctae.

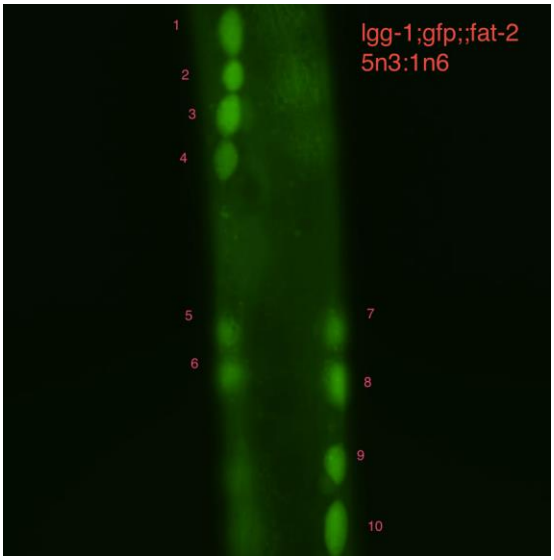
Figure 8: Photographed Pictures of each strain and each PUFA ratio

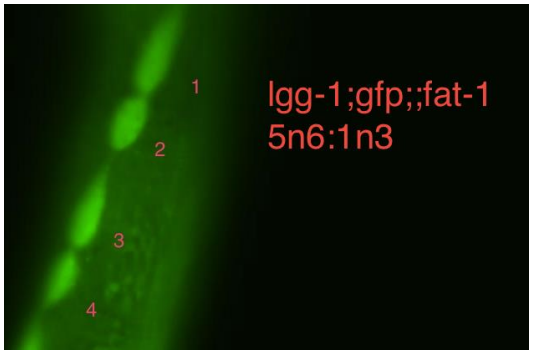
GFP::*lgg-1* on n3 and n6 tergitol plates (Control)

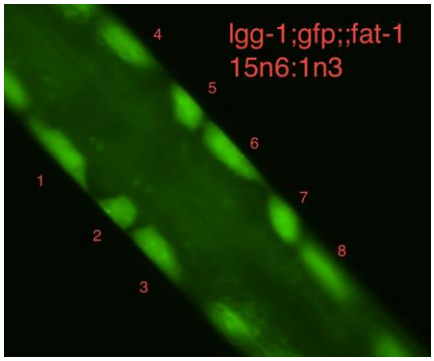


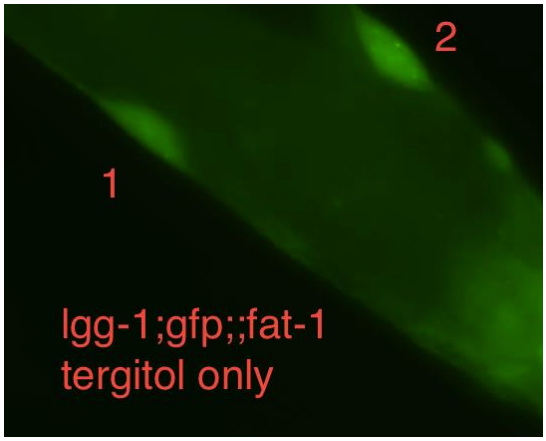
*fat-1(wa9);*GFP*::lgg-1* on omega n3 and n6 fatty acid plates with tergitol



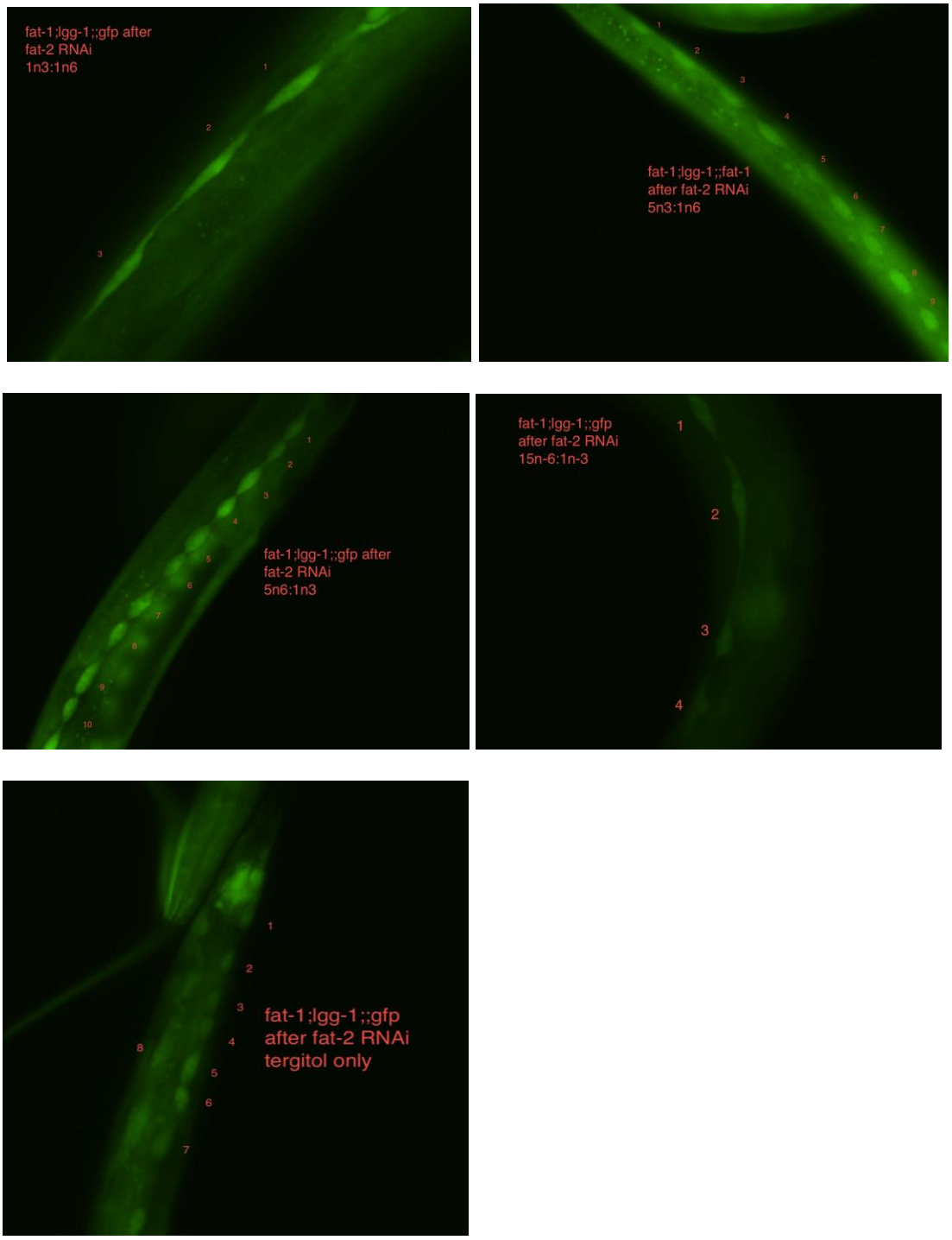








*fat-1(wa9);*GFP::*lgg-1* after *fat-2* RNAi



GFP::*lgg-1 C. elegans* after *fat-2* RNAi