

Genetic Regulation of Unsaturated Fatty Acid Composition in *C. elegans*

Trisha J. Brock, John Browse, Jennifer L. Watts*

Institute of Biological Chemistry, Washington State University, Pullman, Washington, United States of America

Delta-9 desaturases, also known as stearoyl-CoA desaturases, are lipogenic enzymes responsible for the generation of vital components of membranes and energy storage molecules. We have identified a novel nuclear hormone receptor, NHR-80, that regulates delta-9 desaturase gene expression in *Caenorhabditis elegans*. Here we describe fatty acid compositions, lifespans, and gene expression studies of strains carrying mutations in *nhr-80* and in the three genes encoding delta-9 desaturases, *fat-5*, *fat-6*, and *fat-7*. The delta-9 desaturase single mutants display only subtle changes in fatty acid composition and no other visible phenotypes, yet the *fat-5;fat-6;fat-7* triple mutant is lethal, revealing that endogenous production of monounsaturated fatty acids is essential for survival. In the absence of FAT-6 or FAT-7, the expression of the remaining desaturases increases, and this ability to compensate depends on NHR-80. We conclude that, like mammals, *C. elegans* requires adequate synthesis of unsaturated fatty acids and maintains complex regulation of the delta-9 desaturases to achieve optimal fatty acid composition.

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Introduction

Monounsaturated fatty acids (MUFAs) are key components of membrane phospholipids and triglycerides that play important roles in diverse cellular processes such as membrane function, energy storage, and signaling. MUFAs are synthesized from saturated fatty acids by delta-9 ($\Delta 9$) desaturases, also known as stearoyl-CoA desaturases (SCDs), which introduce a double bond between the 9th and 10th carbon of a saturated fatty acyl chain. Alterations in the ratio of MUFAs to saturated fatty acids are implicated in heart disease and cancer [1], the two leading causes of death in the United States [2]. The appropriate ratio between MUFAs and saturated fatty acids is maintained by the activity of the $\Delta 9$ desaturases, which are subject to complex regulation [3]. As a key control point in metabolic regulation, $\Delta 9$ desaturases could be therapeutic targets for treatment of obesity, diabetes, and cardiovascular disease.

The $\Delta 9$ desaturases are ubiquitous enzymes in eukaryotes, found in organisms from yeast to humans. Yeast have one $\Delta 9$ desaturase, Ole1p, and mutants that lack this activity are not able to survive without exogenous supplementation of unsaturated fatty acids [4]. Mice have four $\Delta 9$ desaturases, each having a unique expression pattern [5,6]. Mutant analysis has revealed distinct roles for SCD1 and SCD2. SCD1 is important for adult energy metabolism and lipid synthesis [7], while SCD2 is involved in lipid synthesis during embryonic development [8]. In humans, two SCD isoforms, hSCD1 and hSCD5, have been described [9,10]. A variety of environmental and physiological signals affect the expression of $\Delta 9$ desaturases. Diets rich in unsaturated fatty acids decrease $\Delta 9$ desaturase expression, while high carbohydrate consumption increases expression [3]. Decreased temperature leads to increases in $\Delta 9$ desaturase gene expression in poikilotherms [11]. In addition, endogenous hormones such as leptin and glucagon cause a decrease in $\Delta 9$ desaturase gene expression, while insulin has the opposite effect [3].

Sterol regulatory element binding proteins (SREBPs) and peroxisome proliferator-activator receptor protein-alpha

(PPAR α) have been identified as key transcriptional regulators of SCD1 gene expression in mammals [5]. The SREBP-1 gene encodes a transcription factor that stimulates expression of genes involved in fatty acid biosynthesis, including SCD1 [12], while the SREBP-2 gene product stimulates genes involved in cholesterol biosynthesis [13]. PPAR α is one of a family of nuclear hormone receptors (NHRs), that, upon ligand binding, acts as a heterodimer with the retinoid X receptor to induce transcription of target fat metabolism genes [14]. PPAR α , like all NHRs, contains a hydrophobic pocket for ligand binding and a DNA binding domain for interacting with the promoters of target genes. The targets of PPAR α include genes for the β -oxidation enzymes, SCDs, and other fatty acid desaturases [15,16]. The other members of the PPAR family, PPAR δ and PPAR γ are also involved in regulation of fat metabolism [17]. These regulators have unique roles due to differences in their gene expression patterns and regulatory activities.

Caenorhabditis elegans is becoming recognized as an important model for the study of fat metabolism. These animals synthesize a wide variety of fatty acids using a $\Delta 12$ desaturase, an $\Delta 3$ desaturase, a $\Delta 5$ desaturase, a $\Delta 6$ desaturase, and three $\Delta 9$ desaturases [18,19]. *C. elegans* can also incorporate dietary

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Abbreviations: GFP, green fluorescent protein; MUFA, monounsaturated fatty acid; NHR, nuclear hormone receptor; PPAR, peroxisome proliferator-activator receptor; QPCR, quantitative RT-PCR; RNAi, RNA interference; SCD, stearoyl-CoA desaturase; SREBP, sterol regulatory element binding protein

* To whom correspondence should be addressed. E-mail: jwatts@mail.wsu.edu

Synopsis

The ratio of saturated to unsaturated fatty acids has a profound effect on the fluidity and function of cellular membranes. Animals, plants, and microorganisms regulate the synthesis of unsaturated fatty acids during changing environmental conditions, as well as in response to dietary nutrients. In this paper the authors use a combination of genetic and biochemical approaches to address the regulation of unsaturated fatty acid synthesis in the roundworm *Caenorhabditis elegans*. They identify a new transcription factor, NHR-80, that activates the expression of genes encoding delta-9 fatty acid desaturases, the enzymes responsible for catalyzing the insertion of double bonds into saturated fatty acid chains. These unsaturated fatty acids are critical components of membranes, as well as fat storage molecules. Experiments presented here demonstrate that the worms require adequate synthesis of unsaturated fatty acids for survival and that they maintain intricate regulation of the three delta-9 desaturase genes in response to different nutrients. Abnormalities in lipid metabolism lead to obesity and diabetes in humans; this study contributes to our understanding of the regulation of this metabolic pathway.

fatty acids into lipids, allowing researchers to modify the fatty acid composition of live animals [20,21]. In an RNAi (RNA interference) screen, genes were identified that altered fat storage and many of these genes have mammalian counterparts known to function in fat metabolism [22]. In addition, mutant analysis offers insight into pathways known to regulate fat storage in both nematodes and mammals such as the insulin-signaling pathway [23]. A recent study established a role for NHR-49, as a regulator of lipid homeostasis [24]. The *nhr-49* mutants have increased levels of the saturated fatty acid 18:0, higher fat accumulation, and a shorter lifespan than wild-type animals. NHR-49 is also required for inducing $\Delta 9$ desaturase expression in well-fed animals [25].

To gain a deeper understanding of fatty acid metabolism in *C. elegans* we have characterized the three $\Delta 9$ desaturase mutants using biochemistry, gene expression, and phenotypic analysis. While the three $\Delta 9$ desaturase single mutants, *fat-5*, *fat-6*, and *fat-7* display few differences from wild type, we show that they compensate for loss of one isoform by regulated induction of the remaining $\Delta 9$ desaturase genes. This induction depends on NHR-80, a novel NHR that we have identified as a regulator of desaturase expression. Furthermore, the *fat-5;fat-6;fat-7* triple mutant is unable to survive, revealing that endogenous production of monounsaturated fatty acids is essential for survival under standard growth conditions. The $\Delta 9$ desaturase genes and their transcriptional regulators are vital for maintaining optimal fatty acid unsaturation and proper membrane composition.

Results/Discussion

Identification of NHR-80 as a Regulator of Fatty Acid Metabolism

In our search to identify the desaturases and elongases involved in generation of unsaturated fatty acids in *C. elegans*, we performed a genetic screen to identify mutants with altered fatty acid profiles [18]. In the process of identifying the molecular nature of one mutation, we used RNAi against 156 genes at the end of Chromosome III to determine the

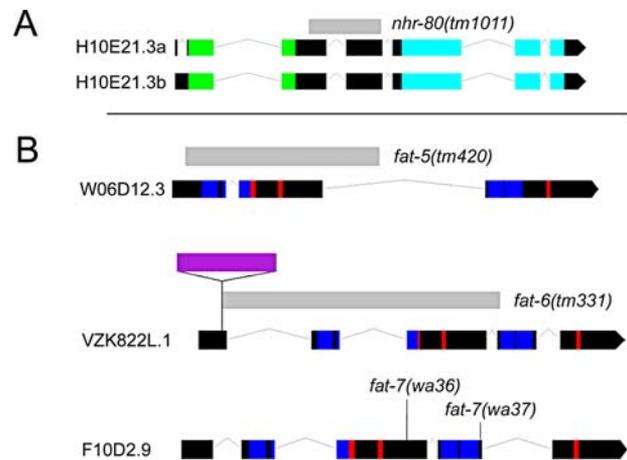


Figure 1. Diagram of *nhr-80*, *fat-5*, *fat-6*, and *fat-7* Genes and Mutations (A) *nhr-80* is composed of a zinc finger domain (green boxes) and a ligand-binding domain (light blue boxes). *nhr-80(tm1011)* contains a 446-bp deletion (light grey bar). (B) *fat-5*, *fat-6*, and *fat-7* all contain four trans-membrane domains (dark blue boxes) and three histidine boxes (red boxes). *fat-5(tm420)* consists of a 779-bp deletion (light grey bar). *fat-6(tm331)* contains a 1,232-bp deletion (light grey bar), and a 428-bp insertion (purple bar). The *fat-7* alleles are point mutations with *fat-7(wa36)*, creating a premature stop codon and *fat-7(wa37)* changing a conserved histidine into a tyrosine. DOI: 10.1371/journal.pgen.0020108.g001

fatty acid composition of animals when each of these genes was inactivated. We found an RNAi clone, *nhr-80*, that caused *C. elegans* to accumulate increased levels of 18:0. NHR-80 is a member of the NHR family of transcription factors in *C. elegans* [26]. To further examine this gene we obtained a deletion allele from the National BioResource Program for the Experimental Animal *C. elegans*, Japan. The *nhr-80(tm1011)* mutant carries a 446-bp deletion that eliminates approximately half of the nucleotides in the second exon and all of the third exon (Figure 1). Like the *nhr-80(RNAi)* worms, these mutants also showed an accumulation of 18:0 and reduction of 18:1 $\Delta 9$ (Figure 2) indicating that *nhr-80(tm1011)* is likely to be a loss of function mutation. In the *nhr-80* mutants, 18:0 accounts for about $10.2 \pm 0.3\%$ of the total fatty acids and 18:1 $\Delta 9$ accounts for $2.2 \pm 0.1\%$, as compared with $6.8 \pm 0.2\%$ and $3.2 \pm 0.1\%$, respectively, in the wild type. The difference between these fatty acids in the *nhr-80* mutants and wild-type animals is significant, with $p < 0.01$ for both fatty acids. The changes in fatty acid composition shown for the *nhr-80* mutants in Figure 2 are similar to those reported for the *nhr-49* mutants. In those mutants the ratio of 18:0 to 18:1 $\Delta 9$ was 4.3 compared to a ratio of 1.9 in wild-type animals [24]. In our analysis of the *nhr-80* mutants the 18:0 to 18:1 $\Delta 9$ ratio was 4.6 compared to the wild-type ratio of 2.2. The *nhr-80* mutants are viable and fertile indicating this change in fatty acid composition, though significant, does not affect essential functions of the animal.

Although two NHR mutant lines, *nhr-49* and *nhr-80*, show increased 18:0 as compared with wild-type worms, not all NHR mutants cause these changes in fat metabolism [24]. Both of these transcription factors are proposed to be derived from the same ancestral gene that also is the progenitor of the mammalian gene encoding hepatocyte nuclear factor 4 [27], which in mammals, binds to fatty acids as ligands and is a key activator of lipid and cholesterol metabolism genes [28].

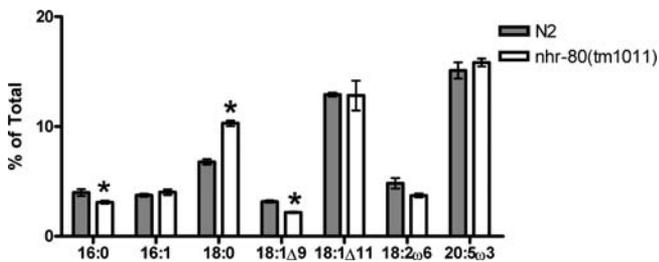


Figure 2. Fatty Acid Composition of *nhr-80*

Relative abundance of selected fatty acid species expressed as percentage of total fatty acid as determined by gas chromatography analysis. The *nhr-80* mutants have significantly higher levels of 18:0 and lower levels of 16:0 and 18:1 Δ 9 than wild type. Error bars represent the standard error. *significant differences between wild type and *nhr-80* mutant, $p < 0.01$.

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In addition to the change in fatty acid composition, the *nhr-49* mutants display an increase in fat storage based on staining of whole worms with the lipophilic dye Nile red [24]. In the *nhr-80* mutants, we observed no increase in Nile red staining as compared to wild type (unpublished data) indicating no increase in fat storage. To confirm this, we tested fat storage in the *nhr-80* mutants by measuring the percent triglycerides in the total lipids. In the *nhr-80* mutants triglycerides comprised $44 \pm 1\%$ of the total lipids as compared to $45 \pm 1\%$ in wild type signifying no increase in fat storage. Thus the increased 18:0 accumulation and increased 18:0 to 18:1 Δ 9 ratio does not cause increased triglyceride synthesis. However, the altered fatty acid profile of *nhr-80* mutants indicates a role for NHR-80 in the regulation of fatty acid metabolism in *C. elegans*.

NHR-80 Is Required for Normal Expression of Δ 9 Desaturases

As NHR-80 is a transcription factor expressed in the intestine [26], the major site of fat metabolism in *C. elegans*, the increased 18:0 accumulation in the *nhr-80* mutants may be due to a reduced expression of the Δ 9 desaturase genes. To test this we used quantitative RT-PCR (QPCR) to measure gene expression with primers designed to amplify *fat-5*, *fat-6*, and *fat-7*, along with the control genes *tbb-2* (β -tubulin) and *ubc-2* (ubiquitin-conjugating enzyme, E2). Relative expression of these genes was examined in wild-type and *nhr-80* mutant adult populations and we found that expression of all three Δ 9 desaturases was decreased in the *nhr-80* mutants relative to wild type for eight experimental replicates (Figure 3). On average, *fat-5* and *fat-6* expression were reduced by 66% and 22% respectively, while *fat-7* expression was almost completely eliminated in the *nhr-80* mutants.

To determine if the expression pattern of *nhr-80* overlapped with the expression pattern of the Δ 9 desaturases we created two green fluorescent protein (GFP)-fusion expressing lines for each of the Δ 9 desaturase genes. Like *nhr-80*, all three Δ 9 desaturase genes were expressed in the intestine in adult worms (Figure 3B), and in all four larval stages (unpublished data). The *fat-5 promoter::GFP* expressing lines showed additional expression in the pharynx and tail cells after hatching and throughout the lifespan. The *fat-6 whole gene::GFP* expressing lines displayed additional expression in the hypodermis in all life stages. The overlapping intestinal

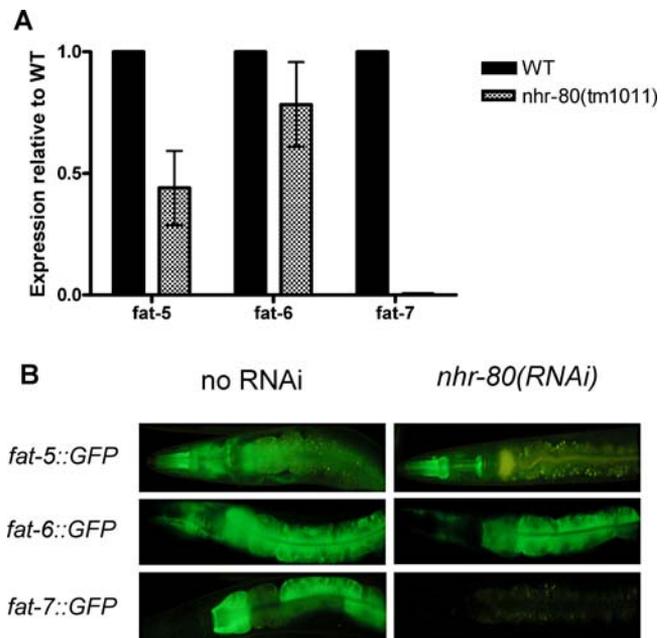


Figure 3. Expression of the Δ 9 Desaturase Genes in *nhr-80*

(A) Gene expression by QPCR in the *nhr-80* mutant reveals a decrease in expression of the Δ 9 desaturase genes relative to wild type. Error bars represent standard error.

(B) Transformed lines expressing Δ 9 desaturase gene GFP fusions grown to adulthood on empty vector control bacteria or *nhr-80(RNAi)* bacteria. Exposure times for photographs were adjusted due to different GFP expression in the three genes, although the exposure time for the two treatments was kept the same for each genotype. The exposure time for the *fat-5::GFP* worms was 1/4 s, for the *fat-6::GFP* worms was 1/30 s, and for the *fat-7::GFP* worms was 1/8 s. After 4 d, there is a dramatic reduction in Δ 9 desaturase gene expression in the intestine for *fat-5::GFP* and *fat-7::GFP* lines grown on *nhr-80(RNAi)*.

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expression for all three Δ 9 desaturase genes indicates possible functional redundancy. The potential role for *fat-5* in the pharynx and *fat-6* in the hypodermis remain to be determined; however, the constitutive expression of these genes in the intestine is consistent with a central role for Δ 9 desaturation in normal *C. elegans* function.

To confirm the regulation of the Δ 9 desaturases by NHR-80, lines expressing the GFP fusions were grown on *nhr-80(RNAi)* bacteria. Transformed adults were allowed to lay eggs on *nhr-80(RNAi)* and control bacteria. The adults were removed and about 20 of the progeny were examined for GFP expression after 4 d of growth. Representative samples are shown in Figure 3B. Expression of *fat-7 whole gene::GFP* was completely eliminated by the RNAi treatment. Expression of *fat-5 promoter::GFP* was decreased but only in the intestine, not in the pharynx. Expression of *fat-6 whole gene::GFP* was also slightly decreased. The reduction of *fat-5* and *fat-6* expression and the elimination of *fat-7* expression likely accounts for the changes in fatty acid composition observed in the *nhr-80* mutant. Similar to the *nhr-80* mutants, the *nhr-49* mutants exhibited an increased level of 18:0 accumulation and a decrease in expression of the Δ 9 fatty acid desaturase genes by QPCR with *fat-5* and *fat-7* as the most reduced [24]. However, the *nhr-49* mutants have increased fat storage, which is not seen in the *nhr-80* mutants, and show decreased expression of two genes that encode proteins that participate in the mitochondrial β -oxidation pathway, an enoyl-CoA

hydratase gene (C29F3.1, *ech-1*) and an acyl-CoA synthetase gene (F28F8.2, *acs-2*). We tested the expression of *ech-1* and *acs-2* in the *nhr-80* mutants by QPCR and found that there was no change in expression levels relative to wild-type expression. This is consistent with the normal level of fat storage seen in the *nhr-80* mutants. Though both NHR-49 and NHR-80 are required for $\Delta 9$ desaturase expression, their effects on fatty acid metabolism in *C. elegans* are not identical; NHR-49 appears to regulate a wider range of lipid homeostasis pathways.

nhr-80 Mutants Do Not Die Early like *nhr-49* Mutants

It has been suggested that shifts in the ratio of saturated fatty acids to MUFAs in *C. elegans* may lead to a decreased lifespan. For example, the change in the ratio of 18:0 to 18:1 $\Delta 9$ from 1.9 in wild type to 4.3 in *nhr-49* mutants has been proposed to cause a substantial reduction in lifespan from 15–18 d in wild type, to 6–8 d in *nhr-49* mutants [24]. We examined the lifespan of the *nhr-80* mutants (Figure 4) and found that they may have slightly shorter lifespans than wild type but live considerably longer than *nhr-49* mutants despite having a similar fatty acid composition. In this experiment, the average lifespan of the *nhr-80* mutant was 12.5 ± 0.5 d as compared to 13.9 ± 0.4 d in wild-type animals and 8.2 ± 0.2 d in *nhr-49* mutants when grown at 25 °C. These data indicate a 10% decrease in mean lifespan between wild type and *nhr-80* mutants, the difference between wild type and *nhr-49* mutants is much greater with a 41% reduction in mean lifespan. The early death of the *nhr-49* does not seem to be caused solely by an elimination of *fat-7* expression or an increase in the ratio of 18:0 to 18:1 $\Delta 9$ since *nhr-80* mutants also show these characteristics but do not have a dramatically shortened lifespan. It is possible that the shorter lifespan of the *nhr-49* mutants is caused by metabolic changes due to other targets of NHR-49 regulation.

C. elegans $\Delta 9$ Desaturases Are Redundant under Standard Growth Conditions

Previous studies revealed that the three *C. elegans* $\Delta 9$ desaturase isozymes display different substrate specificities. While FAT-6 and FAT-7 preferentially desaturate stearic acid (18:0), similar to most of the characterized SCDs, FAT-5 prefers palmitic acid (16:0) and has little or no activity on stearic acid [19]. We obtained $\Delta 9$ desaturase single mutants to further characterize the roles of these three desaturases. We obtained *fat-5(tm420)* and *fat-6(tm331)* deletion alleles from the National BioResource Program for the Experimental Animal *C. elegans*, Japan (Figure 1B). The *fat-5* allele has a 779-bp deletion early in the coding sequence that eliminates two of the conserved histidine boxes and two of the trans-membrane domains. The *fat-6* allele has a 1,232-bp deletion and a 428-bp insertion. The deletion is early in the coding sequence and also eliminates two of the conserved histidine-rich regions and two trans-membrane domains. Both of these mutations are likely null. The *fat-7(tm326)* deletion allele is available but molecular analysis of this allele led us to believe that a more extensive genetic disruption had occurred that affects other genes in addition to *fat-7*. Alternative *fat-7* alleles were isolated by TILLING (Targeting Induced Local Lesions IN Genomes) [29] and are single base pair changes (Figure 1B). The *fat-7(wa36)* allele is a C to T mutation that leads to a premature stop codon that eliminates two trans-membrane domains and

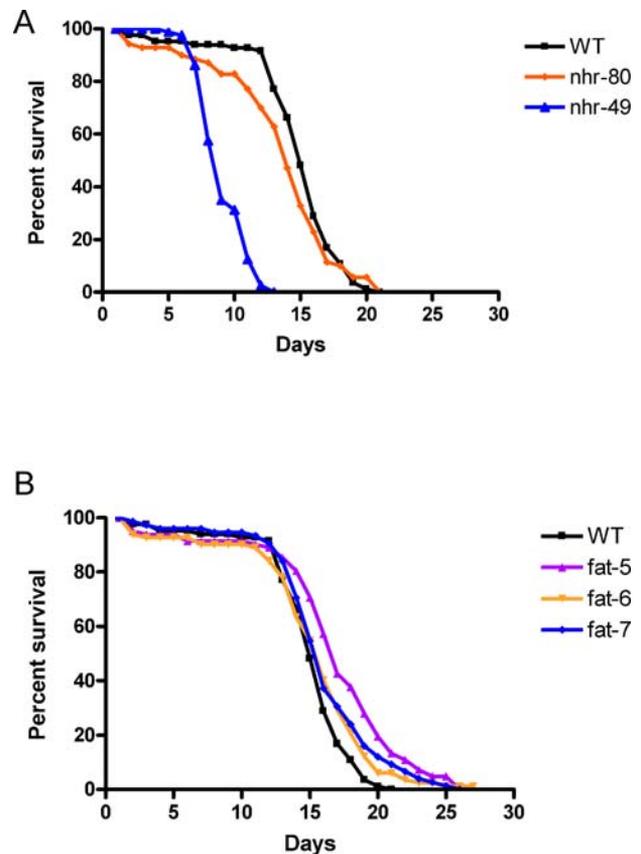


Figure 4. Aging of Adult Mutant Populations

(A) Life span of *nhr-49*, *nhr-80*, and wild type at 25 °C. The *nhr-80* and wild type display a considerably longer life than the *nhr-49* mutants. All lifespan data are presented as mean lifespan \pm standard error (total number of animals scored). Wild type: 13.9 ± 0.4 (83); *nhr-49*: 8.2 ± 0.2 (80); *nhr-80*: 12.5 ± 0.5 (70).

(B) Life span of *fat-5*, *fat-6*, *fat-7*, and wild type at 25 °C. The *fat-5*, *fat-6*, and *fat-7* mutants exhibit a lifespan similar to wild type. Wild type: 13.9 ± 0.4 (83); *fat-5*: 15.9 ± 0.6 (82); *fat-6*: 14.2 ± 0.5 (82); *fat-7*: 15.0 ± 0.5 (75). DOI: 10.1371/journal.pgen.0020108.g004

one of the conserved histidine boxes required for activity of the rat SCD enzyme [30], indicating that this allele is, at a minimum, a strong reduction-of-function allele. The *fat-7(wa37)* allele is a C to T mutation that replaces a conserved histidine with tyrosine [19]. Because these histidines are expected to be required for $\Delta 9$ desaturase activity we expressed this allele in mutant yeast that lack $\Delta 9$ desaturase activity (*ole1* mutants). The mutant *fat-7(wa37)* did not support growth of the *ole1* mutant yeast, whereas expression of wild-type *fat-7* did allow growth [19]. Phenotypic characterization including fatty acid composition and lifespan with *fat-7(wa37)* showed no difference from *fat-7(wa36)* therefore only data from *fat-7(wa36)* are reported here.

The *C. elegans* $\Delta 9$ desaturase mutants show subtle differences from wild type in their fatty acid profile when grown on an *Escherichia coli* lawn on NGM plates at 20 °C (Figure 5). Compared to wild type ($4.1 \pm 0.2\%$), the *fat-5* mutants display decreased 16:1 $\Delta 9$ ($3.4 \pm 0.1\%$), which is the product of FAT-5 desaturation based on the substrate specificity exhibited in yeast [19]. The *fat-6* mutants exhibit a significant increase in their accumulation of the predicted substrate of FAT-6, 18:0 ($9.6 \pm 0.2\%$), over wild type ($7.0 \pm 0.2\%$).

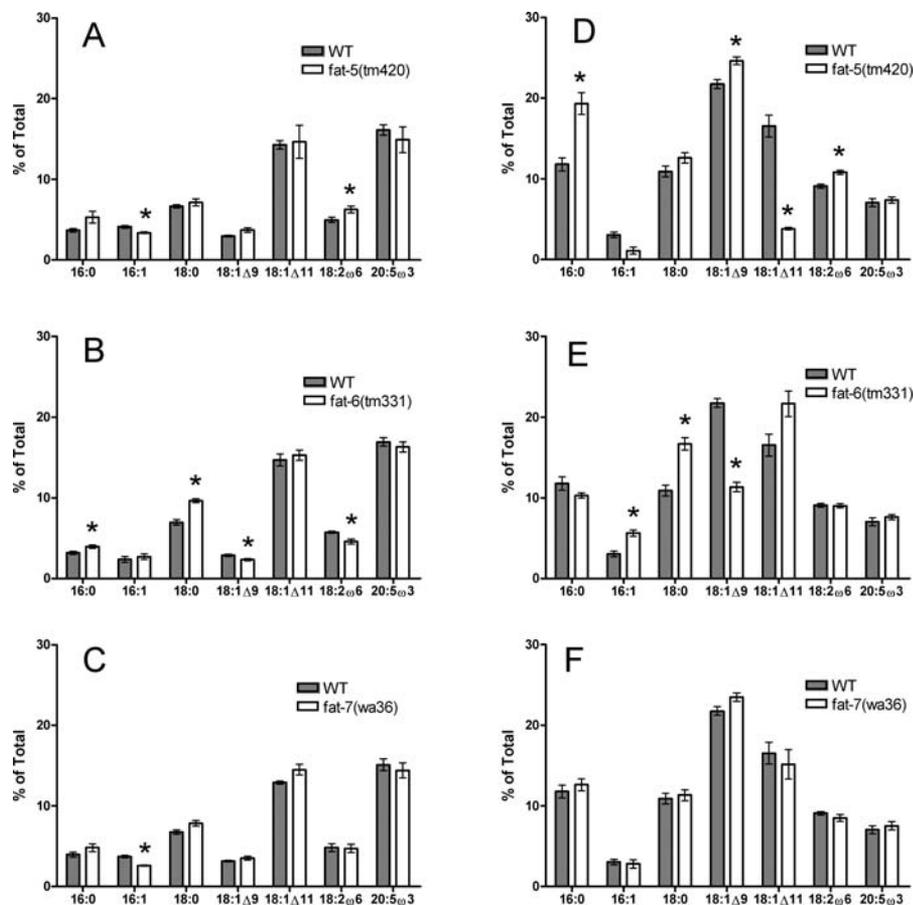


Figure 5. Fatty Acid Composition of the Δ 9 Desaturase Mutants

(A–C) There is little change in fatty acid composition for *fat-5* (A), *fat-6* (B), and *fat-7* (C) mutants compared to wild-type worms when grown under standard growth conditions with OP50 *E. coli* as the sole food source.

(D–F) Axenic growth conditions for wild-type worms and *fat-5* (D), *fat-6* (E), and *fat-7* (F) mutants reveal major changes in fatty acid composition for *fat-5* and *fat-6* mutants compared to wild-type worms. In all graphs relative abundance of selected fatty acid species is expressed as percentage of total fatty acid as determined by gas chromatography analysis. Error bars represent the standard error. *significant difference from wild type, $p < 0.01$

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The Δ 9 desaturase mutants are indistinguishable from wild type in other characteristics tested including growth rate, reproduction, and behavior. The lack of phenotype indicates that subtle changes in fatty acid composition have no apparent effect and that the desaturases are functionally redundant. To determine if gene expression changes are involved in compensating for the lack of one isozyme, we examined expression of the Δ 9 desaturase genes in the *fat-5*, *fat-6*, and *fat-7* mutants (Figure 6). In the *fat-6* mutants, *fat-7* expression is increased approximately 4-fold over wild type and *fat-5* expression is increased 2–3-fold over wild type. In the *fat-7* mutant, expression of *fat-6* and *fat-5* is also slightly increased over wild type. The *fat-5* mutant shows little difference from wild type in *fat-6* and *fat-7* expression.

Axenic Growth Reveals Substrate Specificity of the Δ 9 Desaturases

The standard strain of *E. coli* on which *C. elegans* are maintained in the laboratory contains palmitic (16:0), palmitoleic (16:1 Δ 9), and vaccenic (18:1 Δ 11), but not oleic acid (18:1 Δ 9) or polyunsaturated fatty acids [31]. When worms eat these bacteria they incorporate the fatty acids in their lipids. To test the fatty acid composition of the Δ 9

desaturase mutants grown on a different food source we grew the *C. elegans* strains in axenic media devoid of bacteria. This liquid media provides amino acids, vitamins, growth factors, and heme [32]. Our measurements reveal that the axenic

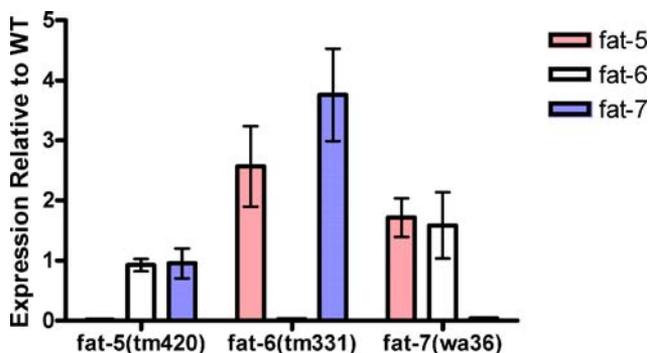


Figure 6. Expression of the Δ 9 Desaturase Genes in the *fat-5*, *fat-6*, and *fat-7* Mutants

Gene expression by QPCR in the *fat-5*, *fat-6*, and *fat-7* mutants relative to wild type reveals an increase in Δ 9 desaturase gene expression in the *fat-6* and *fat-7* mutants, relative to wild type. Error bars represent standard error of 7–12 experiments.

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media contains palmitic, palmitoleic, oleic and linoleic acids, but no vaccenic acid (unpublished data). Wild-type worms grow considerably more slowly under the axenic growth conditions, and the fatty acid profile is also dramatically different. In axenic culture, wild-type worms accumulate higher levels of 16:0, 18:0, and 18:1 $\Delta 9$, while they produce lesser amounts of 20:5 (Figure 5A and 5D).

The $\Delta 9$ desaturase mutants show greater differences in fatty acid composition when grown axenically than when grown on *E. coli* plates (Figure 5D–5F). Comparing the fatty acid composition of the *fat-5* mutant with wild type we observe an increase in 16:0 ($19 \pm 1\%$ versus $12 \pm 1\%$) and a decrease in 16:1 $\Delta 9$ ($1.1 \pm 0.4\%$ versus $3.0 \pm 0.3\%$) and 18:1 $\Delta 11$ ($3.8 \pm -0.1\%$ versus $17 \pm 1\%$) in the *fat-5* mutants. The *fat-6* mutants also display dramatic differences from wild type, with an increase in 18:0 ($16.7 \pm 0.8\%$ versus $10.9 \pm 0.7\%$) and a decrease in 18:1 $\Delta 9$ ($11.3 \pm 0.6\%$ versus $21.8 \pm 0.5\%$) in the *fat-6* mutants. The fatty acid composition of *fat-7* mutants does not differ significantly from wild type, indicating that *fat-6* can completely compensate for *fat-7* in axenic culture and therefore that FAT-7 does not play an important role in maintaining proper fatty acid composition under axenic conditions. The dramatic reduction of 16:1 $\Delta 9$ and 18:1 $\Delta 11$ fatty acids in *fat-5* mutants and 18:1 $\Delta 9$ in *fat-6* mutants grown in axenic culture is the first evidence that these enzymes have the same substrate specificity in *C. elegans* as they do when expressed in yeast [19].

To determine whether the levels of $\Delta 9$ desaturase gene expression are modulated in response to diet we examined the expression of *fat-5*, *fat-6*, and *fat-7* genes in axenic media and on *E. coli* seeded plates using QPCR. We found that compared to worms grown on *E. coli*, *fat-5* expression increases about 6-fold in axenic media. In contrast, *fat-6* expression is maintained at similar levels while *fat-7* expression is dramatically decreased in axenic media (Figure S1).

Single $\Delta 9$ Desaturase Mutants Have No Early-Death Phenotype

Previous studies investigating the *C. elegans* $\Delta 9$ desaturases have used RNAi to deplete *fat-7* expression and have suggested that *fat-7* expression is required to maintain a normal lifespan [23,24]. Based on these results, it was proposed that the reduced expression of *fat-7* was the cause of the short lifespan in the *nhr-49* mutants [24]. However, the *fat-7* mutants used in our experiment as well as the other $\Delta 9$ desaturase mutants, *fat-5* and *fat-6*, do not exhibit an early death phenotype (Figure 4B). The average lifespan of the *fat-5* mutants is 15.8 ± 0.6 d, the *fat-6* mutant is 14.2 ± 0.5 d, and the *fat-7* mutant is 15.0 ± 0.5 d, as compared with a lifespan of 13.9 ± 0.4 d in wild-type animals. In this experiment, the *fat-5* mutant displayed a slight but significant ($p < 0.01$) increase in lifespan over wild type, while the *fat-6* and *fat-7* mutants were not significantly different from wild type in average lifespan.

Our experiments with the *fat-7* mutant do not support the requirement for *fat-7* for normal lifespan as proposed from studies using *fat-7(RNAi)* [23,24]. Additionally, *fat-7(RNAi)* revealed major changes in fatty acid composition and a reduction of fat storage [24] that was not observed in the *fat-7* mutants. The RNAi phenotype observed could be due to transitive secondary RNAi effect [33] as *fat-7* has 84% nucleotide identity with *fat-6* including eight regions of 21–

44 nucleotides with 100% identity. Van Gilst et al. report that *fat-7(RNAi)* did not reduce *fat-6* expression when measured by QPCR [24]; however, we observe an elimination of *fat-6* expression when *fat-6 whole gene::GFP* lines were grown on *fat-7(RNAi)* (unpublished data). In addition, it is possible that compensation by the third $\Delta 9$ desaturase, *fat-5*, is inhibited in the *fat-7(RNAi)*. Because the *fat-7* loss-of-function mutant is wild type for fatty acid composition and lifespan it must be concluded that *fat-7(RNAi)* is having off-target effects on the worm.

$\Delta 9$ Desaturase Activity and Monounsaturated Fatty Acids Are Required for Survival

Because the $\Delta 9$ desaturase genes appear to compensate for each other, we constructed a *fat-5;fat-6;fat-7* triple mutant lacking all three $\Delta 9$ desaturases. We expected these mutants would be unable to survive under standard growth conditions, so we supplemented the worms with a combination of 18:1 $\Delta 9$, 18:2 $\omega 6$, and 20:5 $\omega 3$ dietary fatty acids. After identifying the *fat-5;fat-6;fat-7* triple mutant, we moved the worms to plates without fatty acid supplementation and found that indeed these worms could not survive. Larvae that hatch from eggs laid on unsupplemented plates arrest in the L1 stage, while L3 and L4 stage larvae that are moved from supplemented to unsupplemented plates develop into thin, sterile adults with reduced movement and early death. The MUFAs provided by the standard *E. coli* diet are not sufficient for survival in the *fat-5;fat-6;fat-7* triple mutant. Thus *C. elegans* have a requirement for a certain level of $\Delta 9$ desaturation that cannot be met by the standard *E. coli* diet. The yeast $\Delta 9$ desaturase mutant, *ole1*, is also unable to grow without supplementation [4]. The *fat-5;fat-6;fat-7* triple mutant is the first multicellular organism generated that lacks all endogenous $\Delta 9$ desaturase activity.

To examine genetic interaction between *nhr-80* and *fat-6*, the most highly expressed $\Delta 9$ desaturase, we constructed the *fat-6;nhr-80* double mutant using plates supplemented with dietary fatty acids. When we removed the *fat-6;nhr-80* double mutants to unsupplemented plates we found that these worms also did not survive. Since the *nhr-80(RNAi)* phenotype resembles the *nhr-80* mutants, we used RNAi in combination with the $\Delta 9$ desaturase mutants to study this interaction further. The *fat-6* mutants, when grown on *nhr-80(RNAi)* from eggs, become thin, slow growing, and reproductively inviable after 4 d of growth (Figure 7). They also accumulate very high levels of 18:0 (Figure 7B). The *fat-6* mutants grown on *nhr-80(RNAi)* accumulate $34 \pm 1\%$ of their fatty acids as 18:0 as compared to $9.1 \pm 0.1\%$ when *fat-6* is grown on control bacteria or $14 \pm 2\%$ when wild-type worms are grown on *nhr-80(RNAi)* bacteria. Although 18:0 also accumulates in the *fat-5* and *fat-7* mutants grown on *nhr-80(RNAi)*, the extent of 18:0 accumulation is not as dramatic as observed in *fat-6* (Figure 7B) and they do not show a synthetic lethality (Figure 7A).

One explanation for the synthetic lethality of *fat-6;nhr-80* double mutants is that NHR-80 is required for the increased *fat-5* and *fat-7* expression in the *fat-6* mutant. To test this we examined the expression of the $\Delta 9$ desaturase genes in the *fat-5*, *fat-6*, and *fat-7* mutants grown on *nhr-80(RNAi)*. We found that expression of *fat-7* in the *fat-6;nhr-80(RNAi)* is less than 10% of the expression of *fat-7* in the *fat-6* mutants grown on control bacteria, consistent with the notion that NHR-80 is required to induce the expression of *fat-7* (Figure 7C). We

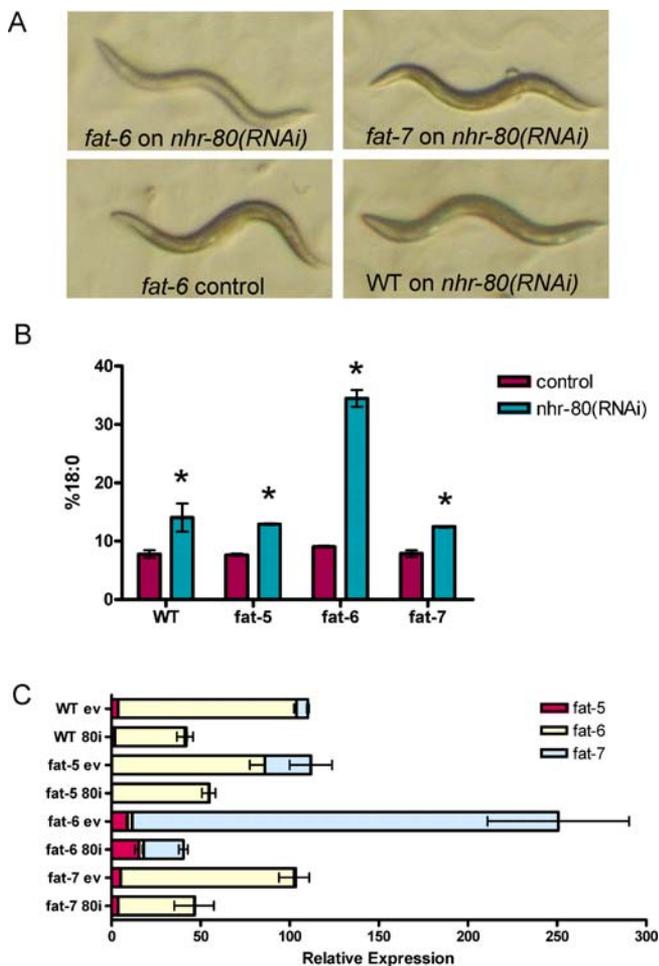


Figure 7. Effects of *nhr-80(RNAi)* in the $\Delta 9$ Desaturase Mutant Background

(A) Photographs showing adult worms after 4 d of growth on *nhr-80(RNAi)* and empty vector control bacteria. The *fat-6* mutants grown on *nhr-80(RNAi)* are thin, pale, and produce no viable progeny.

(B) Relative abundance of 18:0 expressed as a percentage of total fatty acid as determined by gas chromatography analysis. The *fat-6* mutants grown on *nhr-80(RNAi)* ($n = 5$) accumulate much higher levels than *fat-6* mutants grown on control ($n = 7$) and wild type grown on *nhr-80(RNAi)* ($n = 6$). *significant differences from growth on control bacteria, $p < 0.01$.

(C) Effects of *nhr-80* on $\Delta 9$ desaturase gene expression in *fat-5*, *fat-6*, and *fat-7* mutants. QPCR in *fat-5*, *fat-6*, *fat-7*, and wild type for worms grown on empty vector control bacteria (ev) and *nhr-80(RNAi)* (80i) ($n = 6$). Values are expressed relative to *fat-6* expression in wild-type worms grown on control bacteria. For all graphs error bars represent standard error.

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graphed the relative expression values, setting *fat-6* expression in wild-type worms grown on control bacteria as 100%. In wild-type worms on control bacteria *fat-6* is the most highly expressed $\Delta 9$ desaturase gene and *fat-5* and *fat-7* are expressed at $3.6 \pm 0.2\%$ and $6.5 \pm 0.6\%$ of the level of *fat-6* respectively. When the wild-type worms are grown on *nhr-80(RNAi)* we observe a similar relative decrease in $\Delta 9$ desaturase gene expression seen in the *nhr-80* mutants (Figure 3A). Comparing the *fat-5* and *fat-7* mutants grown on control with those grown on *nhr-80(RNAi)* reveals a decrease in $\Delta 9$ desaturase gene expression. However, the biggest difference is seen in the *fat-6* mutants. When these animals are grown on control bacteria *fat-7* is increased in expression 37-fold over

wild type. When *fat-6* is grown on *nhr-80(RNAi)* the *fat-7* relative expression is only a 3-fold increase over wild type.

The overall amount of $\Delta 9$ desaturase gene expression is approximately equal for all worms grown on *nhr-80(RNAi)*, but only *fat-6* displays synthetic lethality with *nhr-80*. This could be due to the composition of the $\Delta 9$ desaturase gene expression. When wild type, *fat-5*, or *fat-7* are grown on *nhr-80(RNAi)* *fat-6* is the major gene expressed suggesting its central importance to $\Delta 9$ desaturation activity. The *fat-6* mutants lack *fat-6* expression and compensate by substantially increasing *fat-7* expression when grown on control bacteria. It is noteworthy that under these conditions *fat-7* expression is increased 37-fold, perhaps indicating that *fat-7* is not as effective at $\Delta 9$ desaturation as *fat-6* due to differences in tissue specific expression, translation efficiency or protein stability. When the *fat-6* mutants are grown on *nhr-80(RNAi)* they are unable to compensate with an increase in *fat-7* expression to an appropriate level and this may cause their reduced survival. Thus NHR-80 is required for increasing *fat-7* expression in situations where higher *fat-7* levels are necessary and consequently defines a critical regulator of fatty acid metabolism.

Our characterization of the novel NHR-80 and the family of *C. elegans* $\Delta 9$ desaturase mutants enhances our understanding of the regulation of lipid homeostasis. Maintaining appropriate fatty acid composition is essential and without sufficient $\Delta 9$ desaturase activity both the *fat-5;fat-6;fat-7* triple mutants and the *fat-6;nhr-80* double mutants are unable to survive. The integration of endogenous and environmental signals by NHRs such as NHR-80 precisely regulates the expression of the $\Delta 9$ desaturase genes and the production of monounsaturated fatty acids leads to optimal membrane fluidity and fat storage.

Materials and Methods

Culture of nematodes. Unless otherwise noted, *C. elegans* were grown on nematode growth media (NGM) plates with OP50 strain of *E. coli* as a food source [34]. The wild-type strain used is strain N2. Mutant strains obtained from Shohei Mitani and Edwin Cuppen were outcrossed at least four times to the N2 strain. The *nhr-80(RNAi)* construct, as well as the others used in the screen of Chromosome III, are from the Ahinger RNAi library [35] and were used as described [36]. As a control for RNAi experiments, nematodes were grown on NGM plates with the HT115 strain of *E. coli* transformed with pPD129.36 (L4440) empty vector plasmid. The axenic culture media consisted of 3% soy peptone, 3% yeast extract, 0.5 mg/ml hemoglobin in 1M KOH, and 20% ultra-high temperature pasteurized skim milk [32]. Worms were grown in this liquid culture at room temperature (22–23 °C) with constant shaking. To make plates supplemented with dietary fatty acids a 0.1 M stock solution of fatty acid sodium salts (NuCheck Prep, Elysian, Minnesota, United States) in water was prepared fresh for each supplementation experiment. The fatty acid stock was added slowly to NGM containing 0.1% tergitol. Plates were poured, covered and allowed to dry in the dark at room temperature overnight. The OP50 strain of *E. coli* was added to each plate and allowed to dry for at least one night [21].

Fatty acid and lipid analysis. For fatty acid analysis, adult nematodes were washed from plates and allowed to settle. The excess water was removed from the worm pellet and 1 ml of 2.5% methanolic H_2SO_4 was added and incubated at 80 °C for 1 h to generate fatty acid methyl esters, which were extracted by adding 1.5 ml water and 0.2 ml hexane. The hexane was sampled for determination of fatty acid composition by gas chromatography on an SP-2380 fused silica capillary column (Supelco, Bellefonte, Pennsylvania, United States) using an Agilent (Palo Alto, California, United States) 6890 series gas chromatograph [18].

For lipid analysis, about 0.5 ml of adult nematodes were collected in a glass tube and frozen. Lipids were extracted by incubation in (1:1)

chloroform/methanol overnight at -20°C . The samples were washed with 2.2 ml Hajra's solution (0.2M H_3PO_4 , 1M KCl) and the chloroform phase containing the lipids was isolated. The silica gel HL plates (Analtech, Newark, Delaware, United States) were activated by incubation at 110°C for 1 h and 15 min. The samples were loaded onto the thin layer chromatography plates along with lipid standards (Sigma, St. Louis, Missouri, United States). The plates were run with a 65:43:3:2.5 chloroform/methanol/water/acetic acid solvent mixture until the solvent front was three-fourths of the way up the plate. The plate was dried, a new solvent mixture of 80:20:2 hexane/diethyl ether/acetic acid was added, and the plate was run until the solvent front reached the top of the plate. The marker lanes were visualized using iodine vapor and the corresponding bands for triglycerides and individual phospholipids in the silica gel were scraped into individual tubes. To quantitate, 50 μg of 15:0 free fatty acid was added to each tube as an internal standard and fatty acid analysis was performed by gas chromatography as described above [22].

QPCR analysis. Adult nematodes were harvested and frozen in liquid nitrogen. RNA was prepared using TRIzol Reagent (Invitrogen, Carlsbad, California, United States). A DNA-FREE RNA kit (Zymo Research, Orange, California, United States) was used for Dnase treatment and purification. After quantification, 1 μg of RNA was used in a reverse-transcription reaction with SuperScriptIII (Invitrogen) to generate cDNA. Primer sequences for the $\Delta 9$ desaturase genes and the reference genes were designed using PrimerQuest software at <http://www.idtdna.com>. Other primer sequences were obtained from Dr. Marc Van Gilst [24]. Primer sequences are listed in Table S1. The PCR mixture consisted of 0.3 μM primers, cDNA, ROX, and 1 \times SYBR green mix (Invitrogen Platinum SYBR green qPCR Supermix UDG). The QPCR was run and monitored on a MX3000P (Stratagene, La Jolla, California, United States). Relative abundance was determined using the $\Delta\Delta\text{Ct}$ method and an average of the expression of the reference genes *tbb-2* and *ubc-2* to control for template levels [37].

Construction of GFP fusions and microinjection. Fusion PCR was used to create translational *fat-5*, *fat-6*, and *fat-7* GFP constructs. The promoters and coding sequences of *fat-6* and *fat-7* and the promoter and first exon of *fat-5* were amplified from genomic DNA. The upstream regulatory region for *fat-5* was 4 kb, for *fat-6* was 2.6 kb, and for *fat-7* was 3.0 kb. GFP was amplified from the Fire vector pPD95.75 including the entire coding sequence and a termination sequence. These PCR products were fused together in a final PCR using nested primers [38]. These fusions were microinjected into *lin-15* mutant *C. elegans* along with a rescuing plasmid, pJM23, containing the wild-type *lin-15* gene [39,40]. Multiple independent lines of nematodes without the *lin-15* phenotype were selected and examined for GFP expression using fluorescence microscopy on an Olympus IX70 microscope.

Lifespan analysis. Aging experiments were performed on adult nematodes grown at 25°C . Worms were moved to plates containing 5-fluoro-2'-deoxyuridine (Sigma) at the fourth larval stage of development (L4). Live animals were assayed for movement in response to touch every 1–2 d [41].

Generation of *fat-5;fat-6;fat-7* triple mutants and *fat-6;nhr-80* double mutants. The *fat-6(tm331);fat-7(wa36)* hermaphrodites were crossed with *fat-5(tm420);fat-7(wa36)* males on plates supplemented with 18:1 $\Delta 9$. The F1 generation was moved to new 18:1 $\Delta 9$ supplemented plates and their progeny were moved to plates supplemented with a combination of 18:1 $\Delta 9$, 18:2 $\omega 6$, and 20:5 $\omega 3$. After the F2 generation reproduced, the adults were harvested for single worm PCR to determine the genotype [42]. The *fat-5* and *fat-6* mutations were monitored using the difference in amplicon size between wild-type and mutant alleles due to the large deletions. The wild-type products

were 1,100 bp for *fat-5* and 1,457 bp, for *fat-6* compared with the mutant products of 321 bp and 652 bp, respectively. All cross-progeny were homozygous for the *fat-7* single base pair mutation.

To generate *nhr-80;fat-6* double mutants we crossed *fat-6* males with *nhr-80* hermaphrodites on 18:1 $\Delta 9$ supplemented plates and isolated the F1 generation onto new supplemented plates. The F2s were moved to fresh 18:1 $\Delta 9$ supplemented plates and allowed to reproduce then single worm PCR was used to identify *nhr-80;fat-6* double mutants. The *nhr-80* wild-type allele generated a PCR product of 745 bp, whereas the *nhr-80(tm1011)* mutant allele generated a product 298 bp in length.

Supporting Information

Figure S1. Expression of $\Delta 9$ Desaturase Genes in Wild-Type Worms Grown on *E. coli* (OP50) Seeded Plates or Axenic Liquid Media

The percent expression shown is relative to *fat-6* expression on *E. coli* plates, which is set at 100%. In wild-type worms grown in axenic culture the expression of *fat-5* is increased and the *fat-7* expression is nearly eliminated relative to expression in wild-type worms grown on *E. coli* (OP50) plates. Relative to *fat-6* expression, *fat-5* and *fat-7* expression is higher in wild-type worms grown on *E. coli* (OP50) compared to wild-type worms grown on *E. coli* (HT115) (Figure 7C). Error bars are SEM, $n = 3$ replicates for plate grown and $n = 6$ replicates for axenic cultured nematodes.

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Table S1. Sequence of DNA Primers Used in These Studies

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Accession Numbers

The GenBank (<http://www.ncbi.nlm.nih.gov/Genbank>) accession numbers for genes used in this study are *nhr-80* (H10E21.3) (AY204179), *fat-5* (W06D12.3) (AF260242), *fat-6* (VZK822L.1) (AF260244), and *fat-7* (F10D2.9) (AF260243).

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Author contributions. TJB, JB, and JLW conceived and designed the experiments. TB performed the experiments and analyzed the data. TJB, JB, and JLW wrote the paper.

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Competing interests. The authors have declared that no competing interests exist.

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