Deficiencies in C20 Polyunsaturated Fatty Acids Cause Behavioral and Developmental Defects in *Caenorhabditis elegans fat-3* Mutants

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ABSTRACT

Arachidonic acid and other long-chain polyunsaturated fatty acids (PUFAs) are important structural components of membranes and are implicated in diverse signaling pathways. The Δ6 desaturation of linoleic and linolenic acids is the rate-limiting step in the synthesis of these molecules. *C. elegans fat-3* mutants lack Δ6 desaturase activity and fail to produce C20 PUFAs. We examined these mutants and found that development and behavior were affected as a consequence of C20 PUFA deficiency. While *fat-3* mutants are viable, they grow slowly, display considerably less spontaneous movement, have an altered body shape, and produce fewer progeny than do wild type. In addition, the timing of an ultradian rhythm, the defecation cycle, is lengthened compared to wild type. Since all these defects can be ameliorated by supplementing the nematode diet with gamma-linolenic acid or C20 PUFAs of either the n6 or the n3 series, we can establish a causal link between fatty acid deficiency and phenotype. Similar epidermal tissue defects and slow growth are hallmarks of human fatty acid deficiency.

THE Δ6 fatty acid desaturase catalyzes the rate-limiting step in the conversion of the essential fatty acids, linoleic acid (18:2n6) and linolenic acid (18:3n3), into C20 polyunsaturated fatty acids (PUFAs) such as arachidonic acid (20:4n6) and eicosapentaenoic acid (20:5n3). (Fatty acid nomenclature used here is the following: X:YnZ refers to a fatty acid chain of X carbon atoms and Y methylene-interrupted *cis* double bonds; Z indicates the position of the terminal double bond relative to the methyl end of the molecule.) PUFAs play critical roles in regulating membrane structure, dynamics, and permeability. In mammals, C20 PUFAs are substrates for oxygenases that produce powerful short-range eicosanoid effector molecules, including prostaglandins, leukotrienes, and thromboxanes (Funk 2001). In response to mechanical, cytokine, or growth factor stimuli, phospholipase A_2 cleaves PUFAs from cell membranes so they can be acted on by cyclooxygenase, lipoxygenase, and P450 monooxygenase enzymes. The eicosanoids mediate a variety of processes in many cell types, including pain, inflammation, and reproductive processes. In addition to these roles, liberated free fatty acid forms of C20 PUFAs, most notably 20:4n6, display considerable biological activity, including activation of nuclear hormone receptors, modulation of ion channels, and as second messengers in signal transduction (Brash 2001). Studies examining the roles of specific fatty acids in these processes in animals are hampered by the difficulty of manipulating lipid composition *in vivo*.

Caenorhabditis elegans is an attractive animal model in which to investigate the physiological roles of specific fatty acids in growth, development, and the nervous system. Unlike mammals, *C. elegans* does not require essential fatty acids in its diet, but is capable of synthesizing 20:4n6 and 20:5n3 using only saturated and monounsaturated fatty acids from bacteria as precursors (Hutzell and Krusberg 1982). This is possible because *C. elegans* expresses the full range of desaturase activities found in plants (Δ12 and n3 desaturase) and animals (Δ5 and Δ6 desaturase) as well as PUFA elongase activities found in animals (Napier and Michaelson 2001).

To investigate the roles of various fatty acids in growth, development, and neurological function in an animal system, we recently isolated *C. elegans* mutants deficient in PUFA synthesis by direct analysis of fatty acid composition (Watts and Browse 2002). These mutants revealed that *C. elegans* does not require n3 or Δ5 unsaturated PUFAs for normal development under laboratory conditions. The n3 and Δ5 desaturase mutants are deficient in certain classes of C20 PUFAs but accumulate higher levels of precursor C20 PUFAs as a consequence of these deficiencies. In contrast, the *fat-3* mutants that lack Δ6 desaturase activity fail to produce any of the common C20 PUFAs and, as a result, their growth and behavior are compromised. Here we demonstrate that although the *fat-3* mutants are viable and fertile, they exhibit neuromuscular defects, cuticle abnormalities, reduced brood size, and altered biological rhythms. These defects can be biochemically comple-

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mented by dietary supplementation of various 20-carbon PUFAs.

MATERIALS AND METHODS

Culture and measurement of nematodes: Nematodes were cultured and maintained according to standard methods (Wood 1988). The strains were grown at 20° on nematode growth medium (NGM) plates unless otherwise indicated. The wild-type strain was N2 and the *fat-3* strain used in these studies was *fat-3(wa22)*. Body-length measurement of late L4 animals was obtained essentially as described in Reiner *et al.* (1999). For each genotype or fatty acid treatment, 10 animals were measured using an Alvin map wheel. Brood size and hatch rate was measured by placing 8–10 L4 animals onto individual plates. Worms were transferred to a new plate each day for 4 days, and the number of eggs and larvae on each were counted. Percentage viability was obtained by dividing the number of hatched L1 larvae by the total number of eggs laid for each worm. Cuticle disintegration was scored by directly observing gravid adult nematodes placed in 0.5 ml alkaline hypochlorite solution (1% sodium hypochlorite, 0.25 M NaOH) in 24-well culture plates and noting the time of the first major break in the cuticle. Plates were agitated gently every 30 sec during observation. Errors are SEM.

Behavioral assays: The defecation cycles of first-day adult animals were scored by measuring the time from one posterior body contraction to the next. The presence or lack of an enteric muscle contraction at the end of each cycle was noted as well. For each strain a minimum of six animals were scored for 10 cycles each. Unless otherwise noted, defecation cycles and enteric muscle contractions were scored with the petri dishes closed. Movement assays were performed as described (Miller *et al.* 1996) in M9 buffer. One "thrash" was defined as a change in direction of bending at the midbody. At least 10 animals for each genotype or fatty acid treatment were measured. Pharyngeal pumping was scored by direct observation of at least 10 animals for each genotype for 1 min. All behavioral assays were performed at room temperature (22°–23°).

Construction of the *fat-3*::GFP reporter gene: The full-length translational fusion was constructed by fusion PCR of the amplified *fat-3* promoter and coding sequences together with the green fluorescent protein (GFP) coding sequence amplified from pPD95.75 (Hobert *et al.* 1999). The upstream regulatory region included 1086 bases upstream of the *fat-3* ATG. This region included 97 bases of the last exon of the upstream *fat-4* gene. The constructs were microinjected into wild-type or *fat-3(wa22)* worms together with the *rol-6(su1006)* dominant marker plasmid pRF4 (Mello *et al.* 1991). Rolling transgenic worms were isolated and multiple independent lines that produced heritable rolling progeny were examined for GFP expression and analyzed for fatty acid composition.

Fatty acid supplementation and analysis: Fatty acid sodium salts were obtained from NuChek Prep (Elysian, MN) and stored at −20° in the dark. For each experiment, a fresh 0.1 M stock was prepared by dissolving fatty acids in sterile H_2O. NGM agar was prepared with the addition of 0.1% tergitol (NP-40). Agar was cooled to 45°–50° and fatty acid stock was added slowly and stirred for 1 min. Plates were poured immediately and then covered to dry in the dark for 24 hr. Plates were then seeded with *Escherichia coli* and allowed to dry for 2 days in the dark at room temperature before the addition of embryos. Embryos were prepared by alkaline hypochlorite treatment of adult nematodes to obtain a semisynchronized population of early embryos. After phenotypic analysis of adult worms, nematodes were washed off the plates in H_2O and centrifuged gently to pellet the worms. As much water as possible was removed and the worm pellets were frozen for determination of fatty acid composition as described in Watts and Browse (2002).

RESULTS AND DISCUSSION

Fatty acid composition of Δ6 desaturase mutants and growth phenotypes: The Δ6 desaturase mutants were isolated without selection using gas chromatography analysis of fatty acids derived from mutagenized nematodes (Watts and Browse 2002). The fatty acid composition of three independent *fat-3* lines revealed elevated levels of the Δ6 desaturase precursors 18:2n6 and 18:3n3 and a deficiency in C20 fatty acids, most notably undetectable levels of dihomogamma-linolenic acid (20:3n6), arachidonic acid (20:4n6), and eicosapentaenoic acid (20:5n3). We found that the phenotypes described in this work were indistinguishable among the three *fat-3* alleles (*wa22*, *wa23*, and *wa25*). Worms of all three genotypes display identical fatty acid compositions with undetectable Δ6 unsaturated PUFAs, implying that all three alleles represent loss of activity of the Δ6 desaturase. Detailed phenotypic characterization was carried out with *fat-3(wa22)*.

The *fat-3* homozygous worms are viable and fertile, indicating that C20 PUFAs are not essential for life in this organism. However, they grow at a slower rate than wild type, requiring one extra day of development at 20° before they become fertile adults (Watts and Browse 2002). In this study, we examined brood size and hatch rate of *fat-3* worms and embryos at three growth temperatures. We found that within a range of temperatures between 15° and 25° the *fat-3* worms consistently produced smaller broods than wild type did, with the largest difference at 15° (Table 1). In addition, at the relatively low growth temperature of 15°, ~20% of the *fat-3* embryos failed to hatch. These observations indicate that C20 PUFAs are necessary for optimal egg production at a range of temperatures and that at low temperature embryogenesis is compromised by the lack of C20 PUFAs. Mutations that affect the degree of fatty acid unsaturation in plants and cyanobacteria also result in cold-sensitive phenotypes, presumably because proper membrane fluidity and permeability at lower growth temperatures requires high levels of membrane unsaturation (Miquel and Browse 1994; Wada *et al.* 1994).

Even though development is delayed and brood size is reduced, we did not notice any apparent tissue or cell fate specification defects in *fat-3* worms. The pharyngeal, intestinal, hypodermal, muscular, neuronal, and reproductive tissues appear normal and their cell nuclei maintain their distinctive characteristics. We performed one trial to examine if *fat-3* worms exhibited shorter or longer life spans than wild type and found that their life span was very similar to that of wild type, in contrast to the long-lived control *age-1*(hx546) (data not shown).

TABLE 1

Brood size and hatch rate of wild type and *fat-3(wa22)* at 15°, 20°, and 25° growth temperature

	15°	20°	25°
Wild type (no. of eggs laid/worm)	281 (±11)	268 (±9)	219 (±11)
fat-3 (no. of eggs laid/worm)	124 (±6)	160 (±6)	131 (±10)
Wild type (% hatch)	99 (±0.3)	99 (±0.1)	99 (±0.4)
fat-3 (% hatch)	79 (±3)	94 (±1)	97 (±0.4)
fat-3 progeny (% of wild type)	35%	57%	59%

Values represent the average brood and hatch rate of 10 individual hermaphrodites at each temperature (±SEM).

Thus, although the *fat-3* worms require one extra day to develop from embryo to fertile adult, their overall life span is not significantly different from wild-type worms.

***fat-3* worms exhibit neuromuscular defects:** *C. elegans* has four major muscle groups: the body-wall muscles used for locomotion, the pharyngeal muscles used for feeding, the vulval and uterine muscles used for egg laying, and the enteric muscles used for defecation (Waterston 1988). The *fat-3* worms exhibit defects in three out of four of these muscle groups. The body-wall muscles are the most obviously affected, with mutant worms displaying much less spontaneous movement and tending to adopt a straighter body posture than that of wild type. Unlike severe muscle-structure mutants, the *fat-3* worms are capable of movement in response to touch. We quantified the movement defect by counting the number of body bends of worms placed in M9 buffer. Wild-type worms placed in liquid thrash rapidly, while *fat-3* worms exhibit only 30% of wild-type thrashing motion (Table 2).

The pharyngeal and enteric muscle groups are also affected by a lack of C20 PUFAs. We found that although the *fat-3* worms exhibit a regular pharyngeal pumping pattern, the rate is reduced to 70% of that of wild type (Table 2). In addition, the enteric muscle contraction, which expels gut contents during defecation, is reduced in *fat-3* mutants. Young adult wild-type animals exhibit a contraction during 99% of defecation cycles, while the enteric muscle contraction fails in 31% of defecation cycles in *fat-3* animals. The *fat-3* mutants lay eggs at approximately half the rate of wild type (2.9 eggs/hr *vs.* 5.6 eggs/hr at their peak egg-laying period, ~40 hr after the L4 to adult molt). However, newly laid eggs are at similar developmental stages as those laid by wild type. Mutants with hyperactive egg-laying muscles lay eggs at very early stages, while egg-laying defective mutants lay eggs that have developed to late stages of embryogenesis or fail to lay eggs and the retained embryos often hatch inside the parent (Thomas and Lockery 1999). We did not observe very early stage embryos or late stage embryos among newly laid eggs of young adult *fat-3* hermaphrodites (90% were multicellular, premorphogenic stage), indicating that egg-laying muscles and nerves that activate them are functioning relatively normally. The reduced movement, pharyngeal pumping, and enteric muscle contractions could be explained by nervous system defects, muscle structure defects, or muscle activation defects. Muscle activation results from the depolarization of the muscle cell membrane in response to coordinated input by motor neurons. The depolarization is coupled to mechanical contraction by release of calcium from intracellular stores. Muscle activation mutants, many of which show dominant, gain-of-function phenotypes, have been described (Reiner *et al.* 1995), and the Unc phenotypes have subsequently been

TABLE 2

Summary of *fat-3* mutant defects

Phenotype	Wild type (N2)	*fat-3(wa22)*
Thrashing (body bends/min)	225 (±5.0)	67 (±9.0)
Pharyngeal pumping (pumps/min)	180 (±1.9)	128 (±1.7)
Enteric muscle contraction (%)	98.8	69.3
Relative body length (%)	100 (±1.9)	79.8 (±1.9)
Cuticle disintegration (min)	5.4 (±0.1)	2.0 (±0.1)
C20 PUFAs (% of total lipid)	34.7	2.6

Thrashing, pharyngeal pumping, enteric muscle contractions, and cuticle disintegration were scored in young adult animals. Relative body length was measured for L4 hermaphrodites. Errors are SEM.

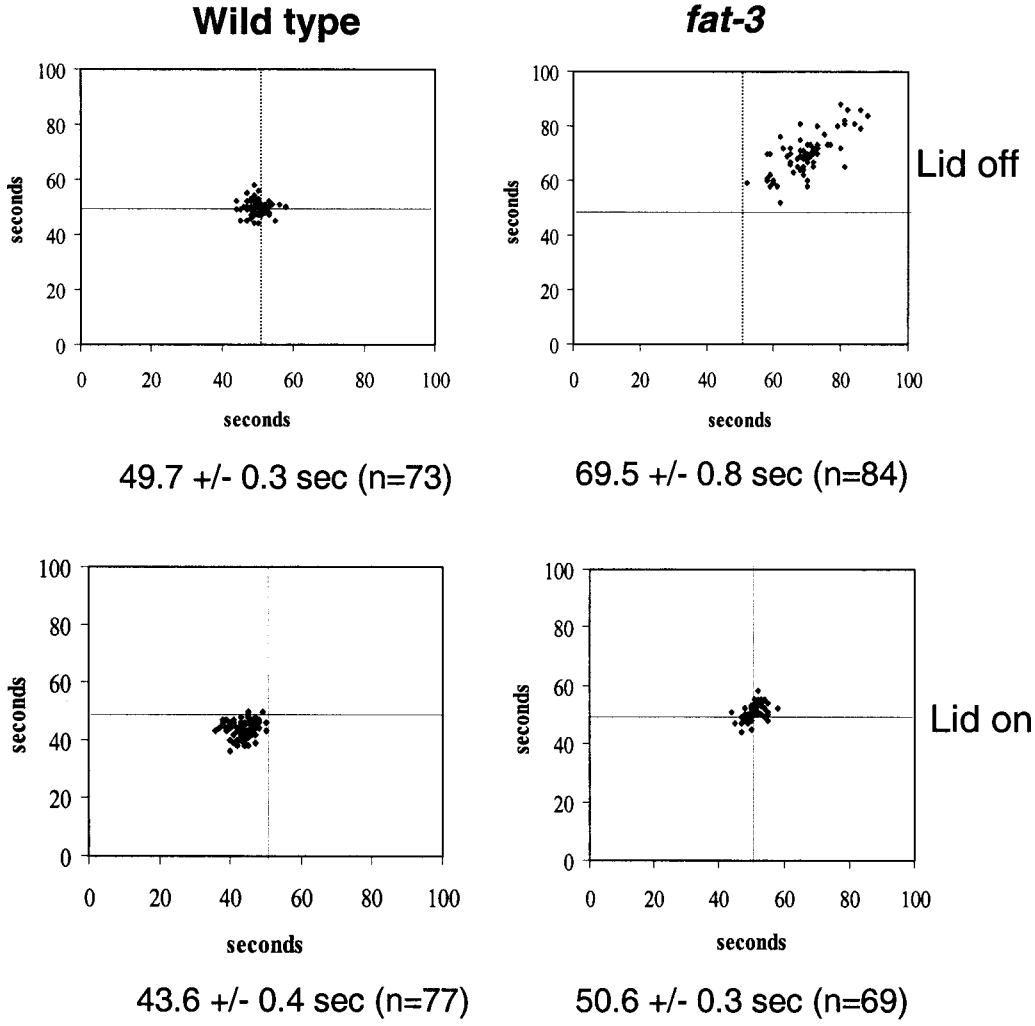

FIGURE 1.—Defecation interval length in wild type and *fat-3*. The graph shows the relationship between the length of each defecation interval (abscissa) and that of the next interval (ordinate). The numbers below the graph show the average defecation cycle period ±SEM.

shown to be caused by mutations in genes encoding ion channels (DAVIS *et al.* 1999) and signaling proteins such as CaM Kinase II (REINER *et al.* 1999). A considerable body of literature describes the modulation of various ion channels by C20 PUFAs such as arachidonic acid (MEVES 1994; CHYB *et al.* 1999; BRASH 2001; XIAO *et al.* 2001). One explanation for our observations is that the deficiency of C20 PUFAs in the *fat-3* mutants may affect ion channel function and result in decreased activation of body-wall muscles, pharyngeal muscles, and enteric muscles. Alternatively, C20 PUFA could be required to promote the formation of cone-shaped nonbilayer lipids required for synaptic vesicle formation or fusion (SCHMIDT *et al.* 1999). Mutants with defects in synaptic transmission display reduced neuromuscular activity (MILLER *et al.* 1996). Further experiments will be necessary to distinguish these and other explanations for decreased neuromuscular function in *fat-3* mutants.

Dumpy body shape of *fat-3* is likely due to cuticle defects: The *fat-3* worms exhibited a somewhat Dumpy (short and fat) body shape. The average body length of *fat-3(wa22)* L4 animals is ~80% that of wild-type L4s (Table 2). The Dumpy (Dpy) phenotype can result from disruption of several unrelated systems. Dpy body shape occurs in animals carrying mutations in genes with roles in dosage compensation or mutations that result in hypercontraction of muscles. It is unlikely that *fat-3* mutants have abnormal dosage compensation as *fat-3* males and hermaphrodites do not display sex-specific phenotypic differences common in dosage-compensation mutants (PLENEFISCH *et al.* 1989). Dominant Unc mutants, such as *unc-105*, are Dpy due to hypercontraction of muscles (REINER *et al.* 1995). This was demonstrated by the observation that the *unc-105 unc-54* double mutant was no longer Dpy. The *unc-54* gene encodes the major myosin isoform for the body-wall muscle and muscles of *unc-54* mutants are incapable of contraction (MOERMAN and FIRE 1997), so the *unc-105 unc-54* double mutants that were unable to contract their muscles lost their Dpy shape. We constructed *fat-3; unc-54* double mutants and found that they were shorter than *unc-54 fat-3*/+ or *unc-54*, indicating that the Dpy phenotype is not due to hypercontraction of body-wall muscles.

We hypothesize that the *fat-3* Dpy phenotype is due to defects in cuticle composition resulting from C20 PUFA deficiency. We found that *fat-3* worms are more

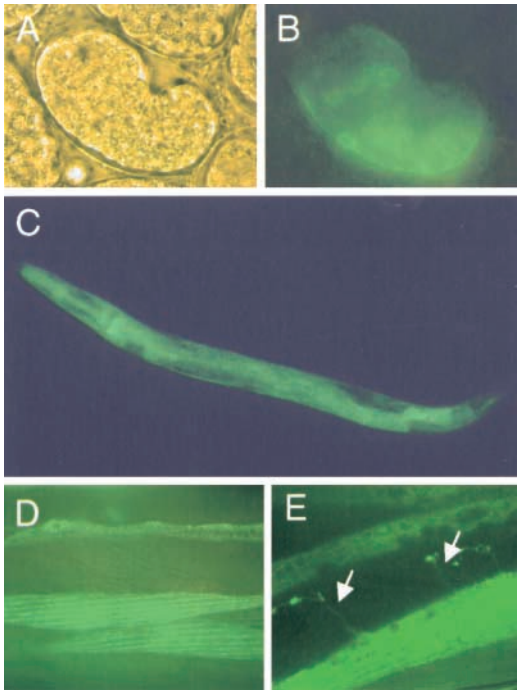

Figure 2.—GFP expression of *fat-3(wa22)* transgenic worms carrying a full-length *fat-3::GFP* construct. (A) Comma-stage embryo. (B) Same embryo as A. (C) L3 larva with pharyngeal and intestinal fluorescence. (D) Adult worm body-wall muscle fibers. (E) Adult worm with processes extending between muscle fibers and neurons indicated by white arrows.

sensitive than wild type to a chemical treatment that disrupts the nematode cuticle. The *fat-3* worms showed major cuticle disruption after an average of 2.0 (±0.1) min in alkaline hypochlorite solution, while wild type did not show a break in the cuticle until an average of 5.4 (±0.1) min. The lipid components of the *C. elegans* cuticle are complex and include polar phospholipids, unesterified fatty acids, triacylglycerides, and complex glycolipids (Blaxter 1993). The fatty acid composition of the *C. elegans* cuticle lipid components has not been determined. However, the surface lipids of the parasitic nematode *Brugia malayi* have been shown to contain nearly 15% 20:3n6 plus 20:4n6 (Smith *et al.* 1996). One of the first symptoms of essential fatty acid deficiency in mammals is the manifestation of scaly dermatosis and increased transepidermal water loss (Chapkin 1992). A recent study of a human patient with a Δ6 desaturase deficiency reported skin and hair abnormalities in the patient as well as slow growth (Williard *et al.* 2001). Thus, C20 PUFAs play important roles in the function of epidermal tissues in both humans and *C. elegans*.

***fat-3* mutants display an abnormal defecation rhythm:** Defecation is an ultradian rhythm that occurs every 45 sec in wild-type hermaphrodites (Thomas 1990). The defecation motor program consists of a posterior body-wall muscle contraction, an anterior body contraction, and an enteric muscle contraction (EMC) that expels gut contents. We found that *fat-3* worms displayed a longer defecation cycle period than did wild type (Figure 1). During the course of these studies, we also noticed a difference in defecation cycle intervals, depending on whether the petri plate lids were open (lid removed) or closed. Wild-type rhythms were slightly longer when plates were assayed with the lid off, displaying a rate of 43.6 sec when assayed with the lid on *vs.* 49.7 sec when assayed with the lid removed. The two assay conditions resulted in much larger differences in the *fat-3* worms, with an average cycle rate of 50.6 sec with the lid on and 69.5 sec with the lid off. Assaying with the lid off resulted in a more irregular cycle in *fat-3* as well (Figure 1). We do not have a definitive explanation for these differences in cycle rate. One possibility is that the worms are sensitive to air currents or humidity changes when petri dish lids are removed. Given the cuticle defects in *fat-3* described above, this hypothesis is consistent with the much greater effect of open conditions on *fat-3* worms.

The length of the defecation cycle is regulated by periodic calcium release in the intestine that is mediated by an inositol triphosphate (IP_3) receptor, an intracellular calcium channel (Dal Santo *et al.* 1999). This IP_3 receptor is encoded by the *itr-1* gene. Mutations in this gene result in worms with slow or no calcium oscillations or posterior body contractions, while overexpression of this gene results in a shorter cycle. Other mutants with altered defecation cycle lengths have also been reported, including CaM kinase II mutants, which cause an irregular cycle (Reiner *et al.* 1999); *flr1*-degenerin/epithelial sodium channel mutants, which exhibit a shortened cycle period (Take-Uchi *et al.* 1998); and *clk-1* mutants defective in the synthesis of ubiquinone (Miyadera *et al.* 2001), which have slow defecation cycles, slow development, and long life spans (Branicky *et al.* 2001). It is possible that the lack of C20 PUFAs in *fat-3* mutants affects the regulation of the ion channels involved in maintaining this rhythm. Alternatively, the fatty acid components of phosphatidyl inositol and diacylglycerol molecules have been shown to affect the activation of protein kinase C (Carricaburu and Fournier 2001; Madani *et al.* 2001). Therefore, abnormal fatty acid composition of phosphatidyl inositol and diacylglyceride molecules in the *fat-3* mutants could affect PKC activity and subsequent signaling events.

***fat-3* is expressed in multiple tissues throughout the life of the worm:** To determine the tissues where the *fat-3* gene is expressed, we constructed a gene fusion between *fat-3* and the GFP gene sequences. The fusion included the upstream regulatory region and the entire *fat-3* coding sequence fused to GFP. Both N2 and *fat-3 (wa22)* were transformed with this construct and the GFP fluorescence pattern was similar in both genotypes. Normal body shape and movement were restored to the transgenic *fat-3* worms and lipid analysis revealed that 19% of the total fatty acids consisted of 18:3n6, 20:3n6,

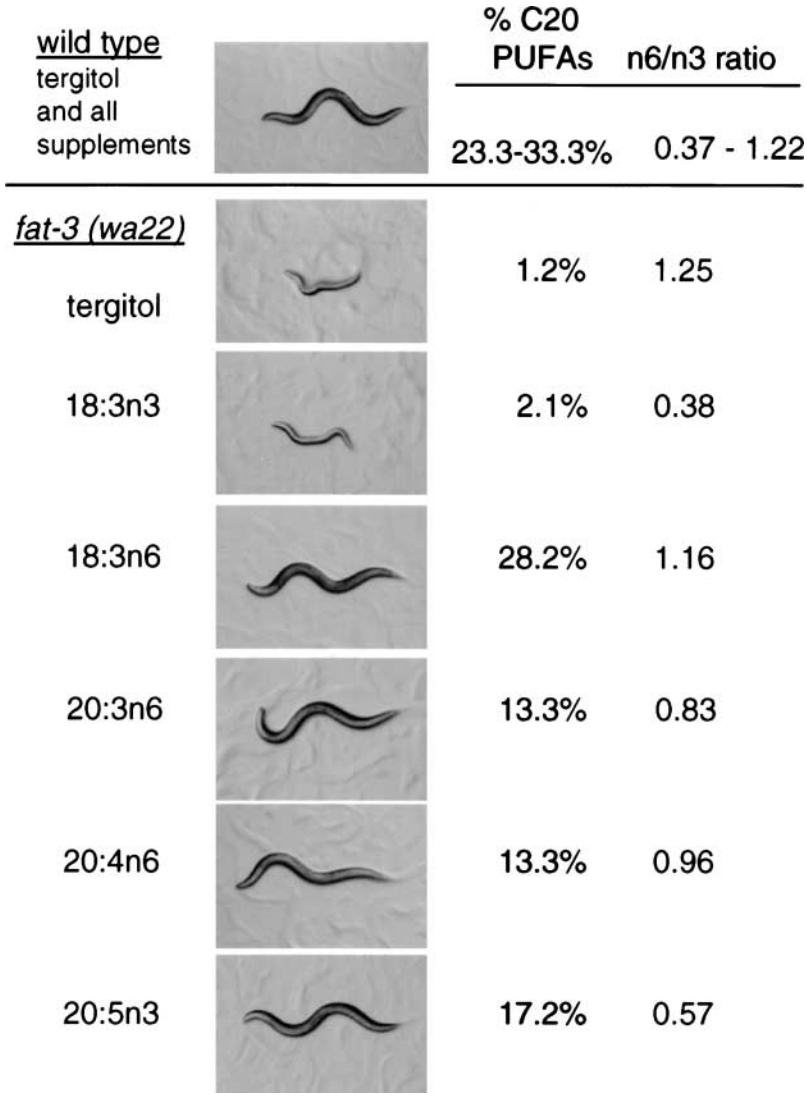

FIGURE 3.—Biochemical complementation of *fat-3* defects by dietary fatty acids. Photographs depict typical *fat-3* animals grown from early embryos on the indicated fatty acid supplements (80 μM) for 3 days at 20°. C20 PUFA values are the average of eight fatty acid determinations from three separate supplementation experiments and n6/n3 ratios of C18 and C20 PUFAs are indicated. Standard errors were <8% of the mean.

20:4n6, and 20:5n3, PUFAs that are undetectable in untransformed *fat-3* animals.

GFP expression is first apparent in comma-stage embryos in intestinal cells and continues throughout all larval stages and into adulthood (Figure 2). L1 larvae carrying the *fat-3::GFP* constructs showed GFP expression in the intestine, pharynx, and body-wall muscles. In L2–L4 larvae and adults, in addition to intestinal, pharyngeal, and body-wall muscle expression, faint expression is observed in several head and tail neurons. This wide range of expression underscores the importance of lipids for storage fuel and as components of membranes critical to cell function. In *C. elegans*, the intestine is the organ responsible for nutrient uptake, digestion, nutrient distribution, and fat storage. The high level of intestinal expression of the FAT-3 Δ6 desaturase suggests that significant fatty acid modifications occur in this organ as well. In addition, the muscular and neuronal expression is consistent with defects in these tissues that are observed in *fat-3* mutants.

Biochemical complementation of *fat-3* defects by dietary supplementation of PUFAs: To determine if the physiological effects of PUFA deficiency could be reversed by dietary supplementation of fatty acids, we included fatty acids in the nematode culture plates. Early embryos were plated onto media containing various PUFAs solubilized with tergitol. Growth, movement, and defecation were characterized after 3–4 days, when the embryos developed into young adults. Supplementation with 18:3n3, a fatty acid substrate of the Δ6 desaturase that already accumulates in *fat-3* mutants, had little effect on growth rate, and the supplemented worms performed only slightly better than unsupplemented *fat-3* controls in the movement assay. However, the addition of 80 μM of 18:3n6, 20:3n6, 20:4n6, or 20:5n3 completely rescued the slow growth and Dpy body-shape defects caused by the Δ6 desaturase deficiency (Figure 3). The rescue of the Dpy body shape also correlated with the rescue of the sensitivity of the cuticle to alkaline hypochlorite treatment (Figure 4A). In addition, the defecation cycle of the rescued worms was similar to wild type for all of these fatty acids (Figure 4C). Finally, the body-wall, pharyngeal, and enteric muscle functions were also rescued with a range of dietary fatty acids in the *fat-3*

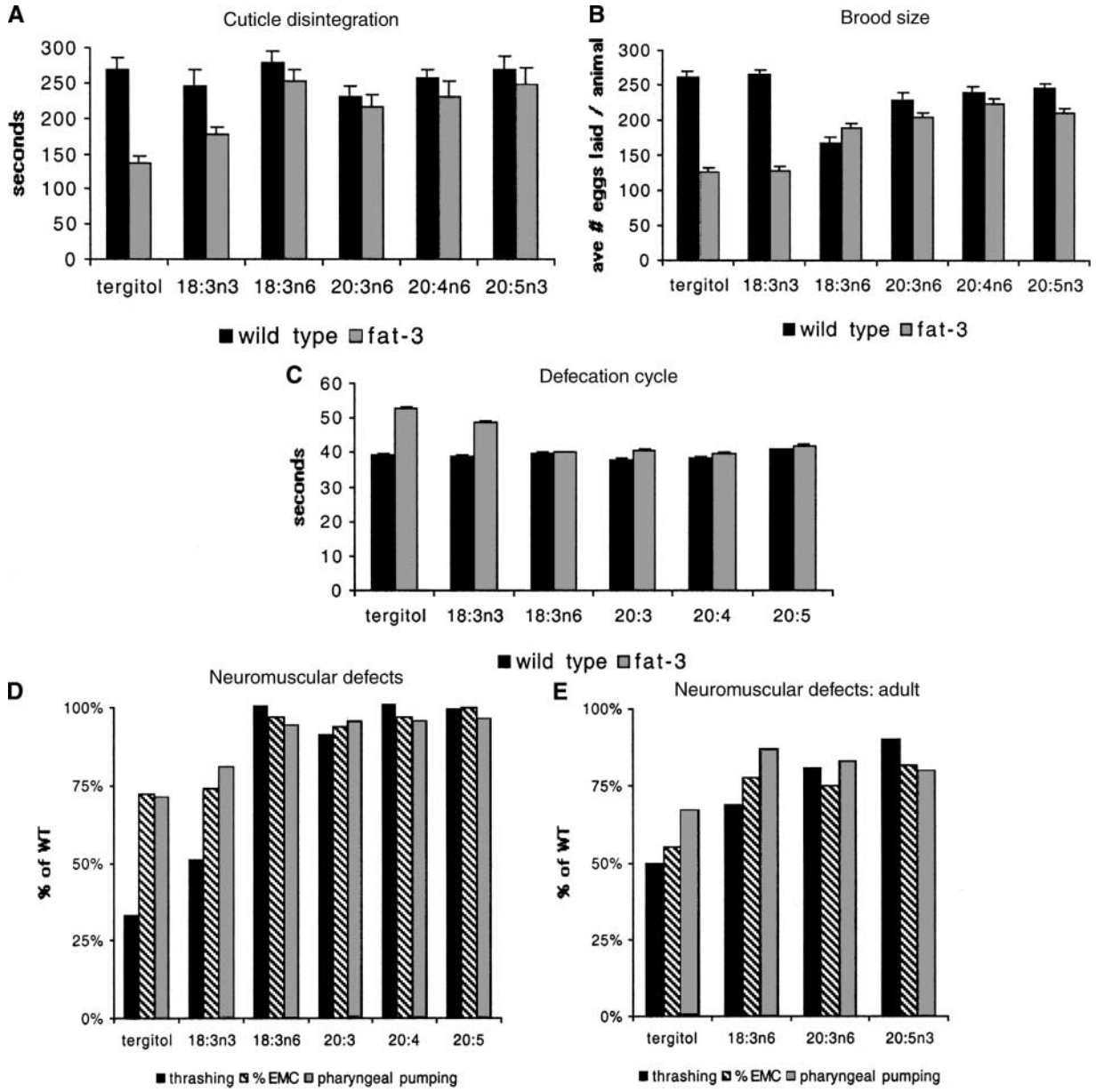

FIGURE 4.—Complementation of *fat-3* defects by dietary fatty acids. For all graphs, error bars are SEM. (A–D) Eggs were placed on agar media containing fatty acids at a concentration of 80 μM. (A) Sensitivity to alkaline hypochlorite treatment. Supplemented adults and unsupplemented controls were transferred to alkaline hypochlorite solution and observed directly. The average time until the first major break in the cuticle was calculated ($n = 8$). (B) Comparison of brood size of supplemented wild type and *fat-3*. The values represent the average number of eggs laid/worm ($n = 6$ for each). (C) Complementation of the defecation cycle length. At least 10 cycles were observed for 6 worms; cycles were scored with the petri dish lids on. (D) Complementation of neuromuscular defects of *fat-3* worms by supplementation with dietary fatty acids, shown as percentage of wild type. Thrashing and pharyngeal pumping represent the average determinations of 10 worms; enteric muscle contractions were determined from the observation of 10 defecation cycles for each of 6 worms. (E) Partial rescue of neuromuscular defects of 24-hr-supplemented *fat-3* adult worms ($n = 8$ worms for thrashing and pharyngeal pumping and at least 50 defecation cycles for percentage EMC).

worms (Figure 4D). Rescue with dietary fatty acids assures us that these defects arise solely as a result of a 20-carbon PUFA deficiency. Similarly, the human patient with a Δ6 desaturase deficiency was given dietary supplements of 20:4n6 and 20:5n3, which cured her growth failure and greatly improved her skin condition (WILLIARD *et al.* 2001).

Biochemical complementation with Δ6-desaturated dietary fatty acids also restored, or nearly restored, normal brood size in the *fat-3* mutants (Figure 4B). However, fatty acid supplements had an adverse affect on wild-type brood size. The most severe effect was observed with supplementation with 18:3n6, which shifted the n6/n3 ratio from 0.47 to 1.22 and resulted in only 66% as many eggs as produced by unsupplemented wild-type worms. This suggests that the proper balance of C20 n6 and n3 PUFAs may be a prerequisite for optimal egg production.

Finally, to test if dietary fatty acids could rescue the various defects in adult worms that had already completed development, we placed 1-day adults that had commenced laying eggs onto plates containing 0.1 mM 18:3n6, 20:3n6, or 20:5n3 supplements. The defecation cycle, enteric muscle contractions, thrashing, and pharyngeal pumping were scored after 24 hr. The worms retained their Dpy body shape, since their final cuticle molt had already occurred, but they were visibly more active on the plates than the control worms. The fatty acid composition of the 24-hr-supplemented adults was similar to worms that were grown on supplements for their entire lives (data not shown). After 24 hr the length of the defecation cycle was restored to wild type in the animals fed 18:3n6, 20:3n6, or 20:5n3 (average of 44 sec for all). Quantitation of movement, pharyngeal pumping, and enteric muscle contraction revealed significant improvement over *fat-3* control worms with both fatty acids, but not complete rescue (Figure 4E). Therefore, dietary fatty PUFAs are capable of restoring biological rhythms and neuromuscular functions even in worms that have completed development without them.

Determination of the fatty acid composition of supplemented worms reveals a significant uptake of dietary fatty acids (Figure 3). Supplementation of *fat-3* mutants with fatty acids normally synthesized late in the PUFA biosynthetic pathway, such as 20:5n3, result in dramatically altered fatty acid composition compared to wild type, since nematodes cannot rehydrogenate double bonds to convert 20:5n3 to 20:3n6 or 20:4n6. In wild type, supplementation with 18:3n6, 20:3n6, or 20:4n6 resulted in an increased n6/n3 ratio, while supplementation with 20:5n3 resulted in a decrease in this ratio. Despite these alterations in fatty acid composition, growth on these supplements was sufficient to rescue the *fat-3* defects and the altered n6/n3 ratio had few adverse effects on wild-type worms. Therefore, the precise fatty acid composition observed in wild-type worms is not a requirement for optimal neuromuscular function, growth, and body-shape determination; rather, our data show that the presence of a combination of the fatty acids 20:3n6, 20:4n6, and 20:5n3 is sufficient for these functions.

In mammals, eicosanoid products derived from C20 PUFAs are effective short-range signaling molecules that mediate pain, inflammation, and reproductive processes. It is not known whether *C. elegans* produces eicosanoid effectors from C20 PUFAs. The *fat-3* defects described in this work could arise from a deficiency of eicosanoids derived from C20 PUFAs by cyclooxygenase and P450 monooxygenase enzymes (*C. elegans* apparently lacks lipoxygenase-like genes). The ability of 20:5n3 to rescue most defects as well as 20:4n6 argues against the importance of cyclooxygenase products, since in mammals 20:5n3 is a poor substrate for these enzymes. However, *C. elegans* contains 80 cytochrome P450 genes, some of which may be capable of forming epoxy (EET), hydroxy (HETE), and lipoxin products from PUFAs (Menzel *et al.* 2001). More studies will be necessary to explore the importance of these products in *C. elegans* development and behavior.

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