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**CYP-13A12 OF THE NEMATODE *C. ELEGANS* IS A PUFA-EPOXYGENASE INVOLVED IN BEHAVIOURAL RESPONSE TO REOXYGENATION**

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**SHORT TITLE:** CYP-13A12 of *C. elegans* is a PUFA-epoxygenase

## SYNOPSIS

A specific behavioural response of *Caenorhabditis elegans*, the rapid increase of locomotion in response to anoxia/reoxygenation called the O<sub>2</sub>-ON response, has been used to model key aspects of ischemia/reperfusion injury. A genetic suppressor screen demonstrated a direct causal role of CYP-13A12 in this response and suggested that CYP-eicosanoids, which in mammals influence the contractility of cardiomyocytes and vascular smooth muscle cells, might function in *C. elegans* as specific regulators of the body muscle cell activity. Here we show that co-expression of CYP-13A12 with the NADPH-CYP-reductase EMB-8 in insect cells resulted in the reconstitution of an active microsomal monooxygenase system that metabolised EPA (eicosapentaenoic acid) and also AA (arachidonic acid) to specific sets of regioisomeric epoxy and hydroxy derivatives. Main products included 17,18-EEQ (epoxyeicosatetraenoic acid) from EPA and 14,15-EET (epoxyeicosatrienoic acid) from AA. Locomotion assays showed that the defective O<sub>2</sub>-ON response of C20-PUFA-deficient,  $\Delta^{-12}$  and  $\Delta^{-6}$  fatty acid desaturase mutants (*fat-2* and *fat-3*, respectively) can be restored by feeding the nematodes AA or EPA, but not ETYA (eicosatetraenoic acid), a non-metabolisable AA-analogue. Already short-term incubation with 17,18-EEQ was sufficient to rescue the impaired locomotion of the *fat-3* strain. The endogenous level of free 17,18-EEQ declined during anoxia and was rapidly restored in response to reoxygenation. Based on these results, we suggest that CYP-dependent eicosanoids such as 17,18-EEQ function as signalling molecules in the regulation of the O<sub>2</sub>-ON response in *C. elegans*. Remarkably, the exogenously administered 17,18-EEQ increased the locomotion activity already under normoxic conditions and was effective not only with C20-PUFA mutants but to a lesser extent also with wild-type worms.

**KEYWORDS:** Eicosanoids; Polyunsaturated fatty acids; *Caenorhabditis elegans*; Cytochrome P450; Reoxygenation; 17,18-EEQ

**ABBREVIATIONS USED:** AA, arachidonic acid; CID, collision-induced dissociation; COD, carbon monoxide difference; CPR, NADPH-CYP reductase; CYP, cytochrome P450; DiHETE, dihydroxyeicosatetraenoic acid; DTT, dithiothreitol; EGL, egg laying defect; EGLN, EGL nine; EEQ, epoxyeicosatetraenoic acid; EET, epoxyeicosatrienoic acid; EPA, eicosapentaenoic acid; ETYA, eicosatetraenoic acid; GFP, green fluorescent protein; GPCR, G protein-coupled receptor; HEET, hydroxyepoxyeicosatrienoic acid; HEPE, hydroxyeicosapentaenoic acid; HETE, hydroxyeicosatetraenoic acid; HIF, hypoxia inducible factor; LC, liquid chromatography; MC, pharyngeal marginal cell; MS, mass spectrometry; NGM, nematode growth medium; PUFA, polyunsaturated fatty acid; RP, reversed-phase

## INTRODUCTION

Oxygen deprivation upon restriction of blood supply followed by reperfusion and concomitant reoxygenation causes tissue injury and is involved in the initiation of various human pathologies including ischemic stroke, myocardial infarction, and acute kidney injury [1]. The nematode *C. elegans* can survive at a wide range of oxygen concentrations, but exhibits an aerotaxis behaviour prompting the worms to migrate to their preferred oxygen concentration of about 10 % [2]. Upon anoxia/reoxygenation, *C. elegans* shows the so-called “O2-ON” behavioural response that is characterised by a rapidly increased locomotion speed [3, 4]. Recent studies suggest that evolutionarily conserved pathways contribute to ischemia-reperfusion injury in mammalian cells and to the O2-ON response in *C. elegans* [5].

Mammals and *C. elegans* share the expression of an evolutionarily conserved family of O<sub>2</sub>-dependent prolyl hydroxylases, EGLN2 and EGL-9, respectively. Prolyl hydroxylation regulates HIF (hypoxia-inducible transcription factor) levels in *C. elegans* as well as in mammals and, thus, links hypoxia to HIF-mediated physiological responses [6-10]. EGL-9 inactivation blocks the O2-ON response of *C. elegans* [3, 4]. A screen for mutations restoring the defective O2-ON response in an EGL-9 deficient strain, *egl-9(n586)*, identified a gain-of-function allele of the *cyp-13A12* gene that encodes a CYP (cytochrome P450) enzyme [5]. *C. elegans* harbours several CYP genes that are homologous to mammalian CYP isoforms [11]. CYP3A4, a CYP enzyme predominantly expressed in the liver, but also in the brain and other extrahepatic tissues [12, 13], is the most closely related human homolog of CYP-13A12 (32 % amino acid identity). Human CYP3A4 has been primarily known for its important role in liver microsomal drug metabolism but also contributes to the metabolism of a wide variety of endogenous substrates including the epoxidation of AA (arachidonic acid; C<sub>20:4, n-6</sub>) and anandamide [14, 15]. Further work revealed also the *emb-8* gene, encoding the worm’s homolog of mammalian CPRs (NADPH-CYP reductase) [16], as essential for the O2-ON response [5]. Taken together, these results of genetic analysis indicated that a microsomal monooxygenase system composed of a CYP and CPR component is involved in mediating the locomotion behaviour of *C. elegans*; however, the substrate specificity of this enzyme as well as the potential role of its metabolites in eliciting the O2-ON response remained unclear.

Suggesting an important role for C20-PUFAs (polyunsaturated fatty acids) and/or their metabolites in the O2-ON response,  $\Delta^{-12}$  and  $\Delta^{-6}$  fatty acid desaturase mutants (*fat-2* and *fat-3*, respectively) completely lack this behaviour [5]. EPA (eicosapentaenoic acid; C<sub>20:5, n-3</sub>) and AA are the main PUFAs in the wild-type strain, whereas neither EPA nor AA can be synthesized in the desaturase-deficient mutants. Concomitantly, only the wild-type, but not the *fat-2* and *fat-3* mutants, contain EPA- and AA-derived epoxy and hydroxy metabolites as produced by CYP monooxygenases when metabolizing these C20-PUFAs [17, 18]. We reported previously that also the EGL-9 deficient strain displayed very low levels of free CYP-eicosanoids and that this deficiency was partially overcome by the gain-of-function mutation in the *cyp-13A12* gene [5].

Based on these findings, we hypothesised an essential role for C20-PUFA-derived CYP-eicosanoids in mediating the O2-ON response and the locomotion behaviour of *C. elegans*. To address these questions, we cloned and heterologously co-expressed CYP-13A12 and EMB-8, analysed the substrate and reaction specificity of the recombinant monooxygenase system, and tested the effect of the major EPA-derived metabolite on the O2-ON response and locomotive activity of *C. elegans*.

## EXPERIMENTAL

### Nematode strain and cultivation condition

The *C. elegans* wild-type strain Bristol N2 and the mutant strains *fat-2(wa17)*, *fat-3(ok1126)* and *fat-3(wa22)* were used throughout this study. The nematodes were grown at 20°C on NGM (nematode growth medium) agar plates inoculated with *Escherichia coli* OP50 as food source [19] and were incubated under similar conditions as described before [17].

### Chemicals

Non-labelled AA, EPA, ETYA (eicosatetraenoic acid), and 17,18-EEQ (17,18-epoxyeicosatetraenoic acid) were purchased from Cayman Chemicals; all radio-labelled fatty acids were purchased from Hartman Analytical GmbH. The compound used as 17,18-EEQ agonist was synthesized as described previously [20]. To prevent autooxidation, all stock solutions were prepared in an oxygen evacuated nitrogen chamber. DMSO (dimethyl sulfoxide), purchased from Sigma, was used as vehicle.

### Amplification and cloning of *cyp-13A12* and *emb-8* cDNAs

To perform reverse transcription, 10 µg total RNA, isolated from a *C. elegans* culture according to [21], was mixed with 0.8 µl oligo dT and incubated for 5 min at 70 °C. Then, 3 µl 5x RT-buffer, 0.8 µl 10 mM dNTPs and, 0.4 µl M-MLV revertase (Promega) were added and incubated for 90 min at 42 °C. The reaction was stopped at 94 °C for 4 min. Subsequently, PCR amplification of the *cyp-13A12* and *emb-8* cDNAs were performed using the Phusion<sup>®</sup> Flash PCR Mastermix (New England Biolabs) and the following primer pairs: 5'-TAGGCTTACACGGTCCGATC-ATGGCAATTATATTTCTTGC-3' and 5'-CAGAAG-CTGCTCGAGTCAGTGATGAT-GGTGATGATTATCATCCCTCGGCGTC-3' for *cyp-13A12* and 5'-ATGCGTAGTA-CGGTCCGATCATGCTGGCGTGGATTGTGTC-3' and 5'-CTACGTTACCTCGAGTCAGTGATGATGATGATGGTGTGACCACACATCAGCTTGG-3' for *emb-8*. Both reverse primers contained a 6× histidine encoding codon block directly in front of the stop codon to allow an immunological detection of all heterologously expressed proteins using a 6-histidine epitope tag antibody. The obtained cDNAs were cloned into the pFastBac<sup>TM1</sup> vector (Invitrogen), while *RsrII* and *XhoI* (New England Biolabs) were used as corresponding restriction enzymes. The identity of all cloned cDNAs was confirmed by full length sequencing of both DNA strands, performed by LGC Genomics.

### Generation of recombinant baculoviruses

Recombinant baculoviruses containing the cloned cDNAs under control of the strong polyhedrin promoter were produced using the Bac-to-Bac<sup>®</sup> baculovirus expression system from Invitrogen. After re-amplification in *Spodoptera frugiperda* Sf9 cells, a virus titre of around  $1-3 \times 10^9$  was obtained.

### *cyp-13A12/emb-8* co-expression in insect cells and preparation of enzymatically active microsomes

Sf9 cells were grown in Insectomed SF express medium (Biochrom) supplemented with 50 U/ml penicillin, 50 µg/ml streptomycin, and 10 % heat-inactivated foetal bovine serum. To generate a complete CYP-CPR monooxygenase system, Sf9 cultures were co-infected with recombinant baculoviruses of *cyp-13A12* and *C. elegans*' CPR gene *emb-8* after they reached a cell density of  $2 \times 10^6$  cells/ml. Control groups were infected with an empty baculovirus or with either *cyp-13A12* or *emb-8* virus alone. Twenty-four h after infection, the Sf9 culture was supplemented with 5 µM haemin chloride and 100 µM riboflavin to support the production of CYP and CPR holoenzymes. The cells were harvested after 60 h, resuspended in ice-cold 0.1 M potassium phosphate buffer, pH 7.4, containing 20 % glycerol, 1 mM EDTA, 100 µM

PMSF, and 0.5 mM DTT. The insect cells were homogenised by sonication three times for 20 s on ice. The microsomal fraction was prepared at 4 °C through differential centrifugation: 5 min at  $3,000 \times g$ , 20 min at  $10,000 \times g$  and 65 min at  $100,000 \times g$ . Subsequently, the microsomes were resuspended and homogenized in 0.1 M potassium phosphate buffer pH 7.4 containing 20 % glycerol, 1 mM EDTA, and 0.5 mM DTT. Several aliquots were shock frozen in liquid nitrogen and stored at  $-80^{\circ}\text{C}$ .

The microsomal protein concentration was measured according to Lowry et al. [22]. The CYP content was determined by COD (carbon monoxide difference) spectra using a difference extinction coefficient of  $91 \text{ mM}^{-1} \text{ cm}^{-1}$  for the wavelength pair 450 nm minus 490 nm [23]. The CPR content was estimated as NADPH-cytochrome *c* reductase activity using an extinction coefficient at 550 nm of  $21 \text{ mM}^{-1} \text{ cm}^{-1}$  [24].

### **Western blot**

For Western blot analysis microsomal protein (3  $\mu\text{g}$  per lane) was separated by 10 % sodium dodecyl sulphate-polyacrylamide gel electrophoresis and transferred to Immobilon-P PVDF membrane (Millipore) [25]. A polyclonal 6-histidine epitope tag antibody raised in rabbits (Novus Biologicals) was used as primary antibody against recombinant tagged CYP-13A12 and EMB-8, respectively. The peroxidase conjugated anti-rabbit secondary antibody was purchased from Sigma. The blots were developed with Amersham ECL prime Western blotting detection reagent and finally visualised by using Amersham Hyperfilm<sup>TM</sup> ECL (GE Healthcare).

### **Microsomal fatty acid metabolism**

[1-<sup>14</sup>C]-labelled AA (53.8 mCi/mmol) and EPA (53.9 mCi/mmol) (Hartman Analytical GmbH) had radiochemical purities of >99 %. To analyse the whole range of primary and secondary metabolites produced by CYP-13A12, reactions were performed in 400  $\mu\text{l}$  0.1 mM potassium phosphate buffer, pH 7.2, containing 50 pmol recombinant CYP-13A12 and the substrate at a final concentration of 10  $\mu\text{M}$ . At first, the appropriate volume of microsomal sample was pre-incubated with the substrate for 10 min at 25 °C. Subsequently, the reaction was started with NADPH (1 mM final concentration) and terminated after 10 min shaking at 25 °C by adding 40  $\mu\text{l}$  of 0.4 M citric acid on ice. The reaction products were extracted with ethyl acetate, evaporated under nitrogen and re-dissolved in 50  $\mu\text{l}$  99.8% ethanol. Controls included omission of NADPH from the reaction mixtures and the use of microsomes lacking CYP-13A12 or EMB-8. The reactions were performed for each substrate at least in triplicate.

### **Analysis of the metabolite profiles**

The reaction products formed from the [1-<sup>14</sup>C]-labelled substrates were analysed by RP (reversed-phase)-HPLC (Shimadzu LC 10 Avp) using a Nucleosil 100-5C18HD column (250  $\times$  4 mm; Macherey-Nagel, Germany) and a linear solvent gradient of acetonitrile/water/acetic acid (50:50:0.1, v/v/v) to acetonitrile/acetic acid (100:0.1, v/v) over 45 min at a flow rate of 1 ml/min. For detection and quantification of the metabolites, an online radio flow detector (LB 509; Berthold) was used. Authentic standard compounds were prepared and used as described previously [26, 27]. The AA and EPA hydroxylase activities were derived from the sum of 19-/20-HETE (19-/20-hydroxyeicosatetraenoic acid) and 19-/20-HEPE (19-/20-hydroxyeicosapentaenoic acid), respectively, and total AA and EPA epoxygenase activities as the sum of all detectable EETs (epoxyeicosatrienoic acids) and EEQs, respectively. All data were calculated per minute and nmol of CYP protein, as determined by the corresponding COD. The metabolite profiles were also analysed by LC-MS/MS (liquid chromatography-mass spectrometry) using a triple quadrupole tandem mass spectrometer Agilent 6460 combined with an Agilent 1200 HPLC-system as described before [28]. For these

experiments, microsomal incubations were performed exactly as described above; however, unlabelled AA and EPA were used as substrates.

### **Preparation of assay plates and treatment**

For long-term incubations, C20-PUFA stocks were mixed with living OP50 bacteria and seeded on NGM plates at a final concentration of 80  $\mu\text{mol}$  in the bacterial lawn. Plates were dried in the dark at room temperature. Worms from a mixed culture were chunked to assay plates and incubated for a couple of days to ensure that next generation was fed its whole life with dietary PUFA. Then, age-synchronised nematodes were obtained by bleaching gravid nematodes disintegrating all worms but eggs [29]. This synchronised progeny was incubated on freshly supplemented NGM/OP50 agar plates for three further days prior use in the locomotion assay. Vehicle control experiments were included by mixing only solvent, 0.3 % (v/v) DMSO, with the bacteria. Only in case of the multi-worm tracker experiments, C20-PUFA salts were dissolved in ethanol at 1 mg/ml and 50  $\mu\text{l}$  were spread evenly onto NGM plates before drying briefly and cultivating *E. coli* OP50 on the plates.

For short-term incubations, two synchronized young adults (>15 trials per strain) were spread onto NGM/OP50 plates supplemented with either 10  $\mu\text{M}$  of EPA, 17,18-EEQ, a 17,18-EEQ agonist, or only 0.3 % (v/v) DMSO as vehicle control. In this case, time of treatment was limited to 40 min.

### **Locomotive behaviour**

The locomotive behaviour was measured by two different methods based on experience and equipment of the involved laboratories. The O2-ON response of young adult *fat-2(wa17)* ( $n>50$  for each treatment) was continuously measured using a multi-worm tracker with a gas-flow chamber system and quantified by customized MatLab algorithms as previously described [4]. For characterizing the locomotive behaviour of wild-type and *fat-3(wa22)*, we determined the worm's speed and body bend frequency according to Hart, 2006 [30]. At least thirty worms per treatment were examined for each replicate by using a VHX-600 digital microscope (Keyence). To determine the move length, two nematodes per trial were transferred to a fresh plate and the length of a 20 s crawler lane leaved in the OP50 lawn was measured for each individual. In addition, body bends as change in the direction of *C. elegans* movement were counted. A binocular capable, custom built acrylic glass chamber equipped with an oxygen manometer (DO-100 from Voltcraft) and a gas-flow system of pure  $\text{N}_2$  was used for experiments involving anoxia condition. Reoxygenation was achieved by setting open plates back to ambient air for 2 min.

### **Determination of the endogenous EPA and 17,18-EEQ content**

The amount of free EPA and the CYP-derived metabolite 17,18-EEQ was determined by LC-MS/MS for the wild-type under normoxia, anoxia (5 min) and reoxygenation (2 min) conditions. To ensure anoxia conditions during the process of harvest as long as possible, liquid nitrogen was carefully filled at the bottom of the anoxia chamber, but without freezing the agar inside the Petri dishes. Then, nematodes were washed with ice-cold M9 buffer from the plates and prepared for LC-MS/MS analysis essentially as described previously [17, 18].

### **Statistical analysis**

The locomotion assay and the endogenous EPA and 17,18-EEQ datasets were analysed by one way analysis of variance to test for significant differences between treatments followed by the Bonferroni test to identify treatments that were significantly different from the control. All statistical tests were performed using Sigma Stat 3.5 (Systat Software, Inc., USA). The error bars in the figures represent standard deviations.

## RESULTS

### **Cloning and heterologous expression of *cyp-13A12* and *emb-8***

The coding sequences of *emb-8* and *cyp-13A12* were amplified from *C. elegans* N2 and showed 100 % identity with the corresponding mRNA sequences (NM\_065702.5 and NM\_067304.3) as available from NCBI Nucleotide. Heterologous expression of both components was successfully achieved in a baculovirus/Sf9 insect cell system. Microsomes isolated from insect cells transfected with the recombinant *cyp-13A12* baculovirus displayed reduced carbon monoxide difference spectra as characteristic for P450 proteins (Fig. 1A). The Soret peak at 450 nm indicated that the majority of the recombinant CYP protein retained the haem-thiolate co-ordination in the ferrous-CO complex. Only a very small amount of a P420 form, which could reveal an inactive form of the enzyme, was detectable. A maximal expression level of 0.59 nmol CYP-13A12 per mg microsomal protein was obtained 60 h after infection. Control microsomes, isolated after transfection with an empty baculovirus, were free of any spectrally detectable CYP protein (data not shown). Microsomes containing recombinant EMB-8 showed NADPH-dependent reduction of cytochrome *c* with a reductase activity of 43.3 mU/ mg protein.

For metabolic studies, the required co-expression of *cyp-13A12* with *emb-8* was achieved by co-infection of Sf9 cells with both recombinant baculoviruses. The microsomes obtained from these co-infected cells contained 0.34 nmol spectrally active CYP-13A12/mg protein and 53.1 mU EMB-8/mg protein. Successful co-expression of both the His-tagged recombinant CYP-13A12 and EMB-8 was also confirmed by Western blotting. The expression of both components became visible 48 h post infection and reached a maximum after 60 h (Fig. 1B). The samples showed the presence of a prominent 58 kDa protein band for CYP-13A12 and a band at 79 kDa for EMB-8, both enriched in the microsomal fraction (Fig. 1B).

### **Metabolism of AA by recombinant CYP-13A12/EMB-8**

The recombinant microsomal CYP-13A12/EMB-8 system metabolised AA to a complex product pattern predominantly consisting of epoxy and hydroxy metabolites (Fig. 2A). AA was metabolized only in the presence of NADPH and none of the metabolites occurred in incubations with control microsomes lacking CYP-13A12 or EMB-8. The main primary epoxy metabolite produced by CYP-13A12 was 14,15-EET, followed by 11,12-EET. The main primary hydroxy metabolites produced by CYP-13A12 co-migrated with 19-/20-HETE. A group of minor reaction products eluted in the RP-HPLC between 19-/20-HETE and 14,15-EET indicating the formation monohydroxy derivatives originating from CYP-13A12-catalysed mid-chain oxidations.

LC-MS/MS analysis was used to validate the identity of the obtained metabolites (Fig. 2B). The primary epoxy products were confirmed as 14,15-EET (47.4 % of total products) and 11,12-EET (13.9 % of total products). In addition, low amounts of 8,9-EET (1.0 % of total products) were detectable. 19-HETE (24.5 % of total products) was identified as the main primary monohydroxy product. In addition, minor amounts of 15-HETE, 12-HETE and 11-HETE (together 4.2 % of total products), but none of the other potential mid-chain HETEs were produced (Fig. 2B). The ratio of epoxy to monohydroxy products was about 2:1. Moreover, small amounts of 14,15- and 11,12-HEET (hydroxyepoxyeicosatrienoic acid) were detectable that were presumably formed as secondary metabolites, e.g. by secondary  $\omega$ -hydroxylation of the primary EETs. At a substrate concentration of 10  $\mu$ M, AA was metabolized by the CYP-13A12/EMB-8 monooxygenase system with a rate of 0.43 nmol/nmol/min to the primary epoxy metabolites and with a rate of 0.26 nmol/nmol/min to primary hydroxy metabolites.



### **Metabolism of EPA by recombinant CYP-13A12/EMB-8**

The CYP-13A12/EMB-8 system accepted also EPA as substrate and converted this n-3 PUFA to a set of epoxy as well as monohydroxy metabolites. 17,18-EEQ represented the main primary epoxy metabolite, whereas the main primary monohydroxy product co-migrated with 19-/20-HEPE (Fig. 2C). No product formation occurred in control experiments, when either NADPH was omitted or microsomes lacking CYP-13A12 or EMB-8 were used.

The identities of the EPA metabolites produced by CYP-13A12 were also confirmed by LC-MS/MS analysis (Fig. 2D). The main primary epoxy products were identified as 17,18-EEQ (64.5% of total product) and 11,12-EEQ (10.7% of total product); Fig. 2D. Including also 14,15-EEQ and 8,9-EEQ, the epoxy metabolites represented together 89.2% of total products. As the main primary monohydroxy product, 18-HEPE was about 2.4% of total products; 20-, 19-, 15-, 12-, 9-, 8- and 5-HEPE represented together about 5.6% of total products (Fig. 2D). The ratio of epoxy and monohydroxy metabolites was about 11:1. In addition, 17,18-DiHETE (17,18-dihydroxyeicosatetraenoic acid) originating from the hydrolysis of 17,18-EEQ was present in minor amounts (2.1% of total product). Formation of total primary metabolites from EPA occurred with a rate of 0.45 nmol/nmol/min for epoxy metabolites and 0.06 nmol/nmol/min for hydroxy metabolites.

### **A C20-PUFA-metabolite is required for the normal O2-ON response**

In line with previous studies [5], the O2-ON response was well pronounced in the *C. elegans* N2 wild-type strain, but did not occur in the C20-PUFA-deficient *fat-2(wa17)* and *fat-3(ok1126)* mutants (Figs. 3A-C). As shown for the *fat-2(wa17)* mutant, this phenotypic impairment was rescued after feeding the worms AA or EPA for several days using the long-term incubation protocol with 80  $\mu$ M C20-PUFAs as described in the Experimental section (Figs. 3D and 3E). In contrast, ETYA, a non-metabolisable AA-analogue, failed to restore the O2-ON response under otherwise identical conditions (Fig. 3F).

The normal O2-ON response of the wild-type strain (Fig. 4A) was accompanied by significant changes in the endogenous levels of free EPA (Fig. 4C) and its CYP-dependent metabolite 17,18-EEQ (Fig. 4 D) as revealed by LC-MS/MS analysis of worms harvested during normoxia, 5 min after anoxia, and 2 min after reoxygenation. Free EPA levels were already slightly higher after anoxia compared to normoxia and then increased almost 5-fold in the immediate reoxygenation phase. Free 17,18-EEQ levels declined during anoxia and were then rapidly restored in response to reoxygenation. Similar, but less pronounced, changes were observed regarding the release of free AA and the formation of its CYP-dependent metabolite 14,15-EET. However, the maximal levels of free AA and 14,15-EET reached only about 30 and 10 % of the corresponding EPA and 17,18-EEQ levels (data not shown).

To test the hypothesis that 17,18-EEQ is required for the O2-ON response, we pre-treated worms carrying the *fat-3(wa22)* mutation with this EPA-derived epoxy metabolite. In the presence of 10  $\mu$ M 17,18-EEQ, we observed an almost two-fold increase of the nematode's speed and number of body bends after a 5 min/2 min anoxia/reoxygenation event, compared to the non-treated control group (Fig. 4B, right panel). However, the exogenously applied 17,18-EEQ was already effective during normoxia (Fig. 4B, left panel), indicating that 17,18-EEQ has the capacity of increasing the locomotion speed also independent of anoxia/reoxygenation as shown in detail below.

### **17,18-EEQ increases the locomotive activity of *C. elegans***

In the last part of this study, we compared the effects of C20-PUFA- and 17,18-EEQ-supplementation on the locomotion behaviour of the *fat-3(wa22)* mutant and N2 wild-type strains; the chemical structures of compounds used for treatment are shown in Fig. 5A. In these experiments, we took advantage of the fact that C20-PUFA deficiency does not only abolish the O<sub>2</sub>-ON response, but already impairs locomotion under normoxic conditions. Compared to their wild-type counterparts, untreated *fat-3(wa22)* worms showed an almost 50 % reduction of migration speed and number of body bends per min when kept on agar plates in ambient air (Fig. 5 B and C, left panels). The impaired locomotion behaviour of the C20-PUFA-deficient *fat-3(wa22)* mutant strain was largely improved upon long-term incubation (3 d, 80  $\mu$ M) with exogenous EPA or AA (Fig. 5B, central panel). In contrast, neither EPA- nor AA-supplementation increased the normal locomotion activity of the N2 wild-type strain that is self-sufficient in producing C20-PUFAs (Fig. 5C, central panel). ETYA, the non-metabolisable AA analogue, significantly reduced the speed and body bending of wild-type worms, but did neither rescue nor further decrease the impaired locomotion activity of the *fat-3(wa22)* mutant strain (Fig. 5 B and C, left panels).

17,18-EEQ clearly rescued the *fat-3(wa22)* mutant strain from locomotion impairment already when administered for 40 min at a concentration of 10  $\mu$ M (Fig. 5B, right panel). In contrast, the same treatment regime was not sufficient for achieving any effects with EPA, the precursor of 17,18-EEQ. Remarkably, however, the short-term exposure to 17,18-EEQ was almost as effective as long-term EPA or AA feeding. Like 17,18-EEQ, a synthetic 17,18-EEQ agonist also accelerated the migration of the worms (Fig. 5B, right panel). Complementing studies with the N2 strain revealed that also wild-type worms respond with increased locomotion activity when exposed to 17,18-EEQ or its synthetic agonist (Fig. 5C, right panel).

## DISCUSSION

The present study demonstrates that CYP-13A12 and EMB-8 constitute a microsomal monooxygenase system that metabolizes C20-PUFAs and, thereby, generates signalling molecules regulating the locomotion behaviour of *C. elegans*. The identity of the monooxygenase system and its function are in line with and directly explain previous genetic data implicating the *cyp-13A12* and *emb-8* genes as well as C20-PUFAs into the O<sub>2</sub>-ON response of *C. elegans* [5].

The recombinant CYP13A12/EMB8 monooxygenase system functioned predominantly as an epoxygenase when metabolizing AA and EPA. The main product produced from AA was 14,15-EET, whereas EPA was preferentially epoxidised at its ( $\omega$ -3) double bond to yield 17,18-EEQ as the main metabolite. Moreover, CYP-13A12 showed ( $\omega$ -1)-hydroxylase activity with AA, and also catalysed mid-chain oxidations resulting in a series of regioisomeric hydroxy metabolites as minor products. The metabolite patterns of CYP-13A12 resemble those produced by CYP-33E2 from *C. elegans* [18], CYP2J2, an epoxygenase highly expressed in the human heart [31, 32], and also human CYP3A4 [14]. CYP-13A12, CYP-33E2 and CYP2J2 have in common that they prefer EPA over AA as substrate and show a regioselectivity in favour of 17,18-EEQ as the main EPA-derived metabolite. Compared with CYP-13A12, CYP3A4 displays a lower regioselectivity and produces considerably higher amounts of various mid-chain oxidation products [33]. Among the human counterparts of CYP-13A12, CYP2J2 has attracted particular interest for its protective role in ischemia/reperfusion injury. CYP2J2 overexpression in transgenic mice mediates improved postischemic functional recovery of the heart and reduces infarction size [34, 35]. CYP2J2 overexpression also protects against hypoxia-reoxygenation injury in cultured endothelial

cells [36]. Most recently, CYP2J2 was shown to exert marked neuroprotective effects in a mouse model of cerebral ischemia [37].

EPA and AA are the main C20-PUFAs in *C. elegans*, whereby EPA represents about 30 % and AA about 5 % of total fatty acids [17]. As exemplified in the present study by the *fat-2* and *fat-3* mutant strains, any inability of synthesising C20-PUFAs is associated with impaired locomotion activity already under normoxic conditions and a lack of the O<sub>2</sub>-ON response to anoxia/reoxygenation. Long-term feeding with EPA or AA, but not with ETYA, rescued this impaired locomotion behaviour. ETYA harbours triple instead of double bonds [38] and has been used as a non-metabolisable analogue of AA and inhibitor of AA-derived eicosanoid formation [39-41]. Noteworthy, C20-PUFAs are also essential for touch sensation of *C. elegans*. Unlike impaired locomotion, this phenotype can be rescued both by AA and its non-metabolisable analogue ETYA [42]. These findings suggested that C20-PUFAs modulate touch sensation while being incorporated into membrane phospholipids [42], whereas EPA- and AA-derived metabolites, rather than the parental C20-PUFAs themselves, regulate the locomotion activity of the nematodes.

Indeed, our subsequent experiments revealed that 17,18-EEQ, the major EPA-derived metabolite produced by the CYP-13A12/EMB-8 monooxygenase system, was alone sufficient to improve the impaired locomotion activity of the C20-PUFA deficient *fat-3(wa22)* strain. This effect occurred already after short-term exposure of the worms to 10  $\mu$ M 17,18-EEQ, whereas long-term feeding and much higher concentrations were required to achieve similar effects with AA or EPA. Also other authors found that high concentrations and a minimal time of about 24 h are required to restore the phenotype of *fat-3* mutants by C20-PUFA supplementation [43-48]. Remarkably, the exogenously administered 17,18-EEQ increased the locomotion activity already under normoxic conditions and was effective not only with the mutant but to a lesser extent also with wild-type worms. According to our LC-MS/MS data, 17,18-EEQ is also the leading candidate for mediating the O<sub>2</sub>-ON response of the wild-type strain. These data show that free 17,18-EEQ levels decline during anoxia and are rapidly restored upon reoxygenation. Providing the substrate for increased *de novo* biosynthesis of 17,18-EEQ, free EPA levels were strongly increased upon anoxia/reoxygenation. Taken together, these results suggest a direct correlation between the free 17,18-EEQ levels and the locomotive activity of *C. elegans*. This correlation also explains that exogenous 17,18-EEQ was already effective under normoxic conditions, whereas the O<sub>2</sub>-ON response is obviously mediated by enhanced endogenous 17,18-EEQ biosynthesis following anoxia/reoxygenation. We cannot exclude the possibility that other EPA- and also AA-derived metabolites share the locomotion-promoting capacity of 17,18-EEQ. In particular, AA-derived metabolites such as 14,15-EET may take over the function of 17,18-EEQ, if EPA is not available as suggested by the normal locomotion behaviour of *fat-1* mutants that are unable to synthesise n-3 PUFAs [49]. Moreover, considering that the substrate and reaction specificities of CYP-13A12 and CYP-33E2 largely overlap, further genetic analysis is required to dissect the individual roles of these enzymes in the O<sub>2</sub>-ON response.

The postulated role of 17,18-EEQ as a mediator of the O<sub>2</sub>-ON response in *C. elegans* (compare Fig. 6) is in line with the general concept of CYP-eicosanoid formation and action as established in mammalian cells [50-53]. CYP enzymes require molecular oxygen, NADPH, and free C20-PUFAs to catalyse epoxidation and hydroxylation reactions [54]. Accordingly, the biosynthesis of epoxy and hydroxy metabolites proceeds under normoxic conditions and is limited by the availability of free EPA and AA as substrates. Under basal conditions, the majority of intracellular EPA and AA are esterified into membrane phospholipids. Thus, *de novo* synthesis of CYP-eicosanoids is in general strictly coupled to the activation of

phospholipases as triggered by diverse hormones and growth factors [51]. Importantly, also hypoxia results in phospholipase activation and thus provides free C20-PUFAs for CYP-dependent metabolite formation in the subsequent reoxygenation phase. In mammalian cells, ischemia activates the cytosolic calcium-dependent phospholipase A2 that liberates AA and other PUFAs from the sn-2 position of glycerophospholipids [55-57]; however, the identity and substrate specificity of the phospholipases activated in the nematode during anoxia/reoxygenation remain to be elucidated. Once produced, the epoxides of C20-PUFAs can be rapidly further metabolised and inactivated by soluble epoxide hydrolases [58]. *C. elegans* harbours two genes encoding soluble epoxide hydrolases [59]; however, their role in modulating the locomotive activity remains to be shown.

The cellular and molecular mechanisms of how 17,18-EEQ increases the locomotive activity of *C. elegans* are unknown. In mammals, CYP-eicosanoids act in an autocrine or paracrine manner and serve as second messengers of diverse hormones regulating the contractility of vascular smooth muscle cells and cardiomyocytes [20, 60, 61]. In *C. elegans*, 17,18-EEQ mediates the O<sub>2</sub>-ON response by increasing the activity of body muscle cells. Surprisingly, we found that CYP-13A12 and also CYP-33E2, another 17,18-EEQ-generating CYP isoform, are predominantly located in the MCs (marginal cells) of the worm's pharynx [5, 18]. MCs intercalate with pharyngeal muscles and might, on the one hand, structurally reinforce these muscles [62]. On the other hand, MCs contain abundant mitochondria, suggesting that these cells might perform active non-structural roles [63], too. Nonetheless, it is currently largely unclear how CYP-eicosanoids produced in the pharynx are able to modulate the muscle activity in the worm's body. Possibly, 17,18-EEQ is secreted by the marginal cells and then recognized as a signalling molecule by nearby sensory neurons that in turn trigger the O<sub>2</sub>-ON response via neural circuits [64, 65] known to control forward/backward locomotion. Within this process, 17,18-EEQ might also stimulate the release of neuropeptides that act as neurohormones onto body muscles. Providing a first link between C20-PUFA deficiency and neurotransmission, *fat-3(lg8101)* worms were shown to release abnormally low levels of neurotransmitters at cholinergic and serotonergic neuromuscular junctions and to be depleted of synaptic vesicles [43].

The O<sub>2</sub>-ON response of *C. elegans* and ischemia/reperfusion injury of mammalian organs are quite different in terms of their final outcomes. However, as elaborated in the present study, they may share common mechanisms of CYP-eicosanoid formation and action. In particular, it appears that the epoxy metabolites, known to play a protective role in ischemia reperfusion injury, serve in *C. elegans* as mediators of the O<sub>2</sub>-ON response. A major open question concerning both research fields is the identity of the primary cellular targets of CYP-eicosanoids. Increasing evidence obtained in mammalian systems suggests the existence of GPCRs (G protein-coupled receptors) that specifically interact with selected sets of epoxy and hydroxy metabolites derived from n-6 and n-3 PUFAs [52, 66, 67]. Supporting the hypothesis that 17,18-EEQ may interact with similar targets in mammals and *C. elegans*, we found that a metabolically robust synthetic analogue, developed to mimic the effect of 17,18-EEQ on cardiomyocyte contractility [20], was also effective in modulating the locomotive activity of *C. elegans*. Further genetic analysis of the components mediating the 17,18-EEQ effects in *C. elegans* may facilitate the search for the thus far unknown receptors of CYP-eicosanoids.

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## AUTHOR CONTRIBUTIONS

R.M. and W.-H.S. designed the study; A.E. and J.K. performed heterologous expression and metabolism experiments, A.K. supervised this experimental work; D.M., J.J. and E.N. performed the locomotion assays; J.F. performed the chemical synthesis of the 17,18-EEQ agonist. R.M., J.K., and W.-H.S. wrote the manuscript, J.F. and A.K. performed proof-reading.

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## FIGURE LEGENDS

### Figure 1 Detection of recombinant CYP-13A12 and CPR

(A) Carbon monoxide difference spectra of sodium dithionite reduced microsomal samples isolated from 60 h old Sf9 insect cell cultures, expressing CYP-13A12 (0.59 nmol/mg protein). (B) Representative Western blot analysis showing recombinant His-tagged CYP-13A12 and EMB-8 in single and co-expression. Shown are microsomal preparations and three corresponding homogenate samples isolated at the indicated times. Each lane contains 3 µg protein. Besides the marker, recombinant His-tagged *cyp4a12* served as control. A 6-histidine epitope tag antibody was used as primary antibody.

### Figure 2 Metabolism of AA and EPA by recombinant CYP-13A12

(A) Representative RP-HPLC chromatogram showing the metabolite pattern formed after incubating 10 nmol AA with 50 pmol of CYP-13A12 in a total volume of 400 µl for 10 min at 25°C in the presence of 1 mM NADPH (upper line). None of the metabolites were produced in control samples, where NADPH was omitted (lower line). Main metabolites co-migrated with authentic 19/20-HETE (Rt=15.4 min) and a set of regioisomeric monoepoxides at 23.5 min (14,15-EET) and, e.g., 24.9 min (11,12-EET). Metabolites with uncertain or unknown identity are marked (\*). (B) The metabolite pattern was analysed by LC-MS/MS analyses (n=3). The product distribution indicates the relative contribution of the respective individual metabolites in percent; moreover the metabolite class is indicated. The error bars represent the standard deviation. (C) Representative RP-HPLC chromatogram showing the metabolite pattern formed after incubating 10nmol EPA with 50pmol of CYP-13A12 in a total volume of 400 µl for 10 min at 25 °C in the presence of 1 mM NADPH (upper line). None of the metabolites were produced in control samples, where NADPH was omitted (lower line). Main metabolites co-migrated with authentic 19/20-HEPE (Rt=13.9 min), 17,18-EEQ (Rt=19.5 min) and a set of other regioisomeric EEQs (Rt=21-23 min). Metabolites with uncertain or unknown identity are marked (#). (D) The metabolite patterns were analysed by LC-MS/MS analyses (n=3). The product distribution indicates the relative contribution of the respective individual metabolites in percent, moreover the metabolite class indicated. The error bars represent the standard deviation.

### Figure 3 A metabolisable PUFA is required for the normal O<sub>2</sub>-ON response

(A) Speed graph of N2 wild type showing an intact O<sub>2</sub>-ON response, whereas (B) *fat-2(wa17)* and (C) *fat-3(ok1126)* mutants show in each case a defective O<sub>2</sub>-ON response. Average speed values ± 2 SEMs (light grey) of animals (n > 50) are shown with step changes of O<sub>2</sub> between 20 % and 0 % at the indicated times. (D) Speed graph of *fat-2(wa17)* mutants with the O<sub>2</sub>-ON response rescued by AA supplementation, and (E) Speed graph of *fat-2(wa17)* mutants with the O<sub>2</sub>-ON response rescued by EPA supplementation. (F) Speed graph of *fat-2(wa17)* mutants with the defective O<sub>2</sub>-ON response not rescued by ETYA supplementation. Only in case of wild-type as well as AA and EPA exposure of *fat-2(wa17)*, the mean speed within 0 to 120 s after O<sub>2</sub> restoration is increased relative to that before O<sub>2</sub> restoration ( $P < 0.01$ , one-sided unpaired t-test).

### Figure 4 Impact of 17,18-EEQ on the O<sub>2</sub>-ON response

(A) Nematode's speed and number of body bends increase in the wild-type in response to a 5 min anoxia/ 2 min reoxygenation stimulus. (B) Supplementation with 10  $\mu$ M 17,18-EEQ restored the locomotion of *fat-3(wa22)* mutants both in anoxia/reoxygenation and normoxia conditions. A+B:  $n \geq 30$  ( $\geq 15$  trials with  $n=2$ ),  $\pm$ SD ( $***P < 0.001$ , One way ANOVA). (C) An anoxia/reoxygenation stimulus caused in the wild-type a strong increase in the amount of free EPA. (D) The free 17,18-EEQ content is reduced during anoxia but significantly restored in response to reoxygenation. C+D: LC-MS/MS analysis of in each case 5,000 synchronized worms (24 h post L4),  $n=3$ ,  $\pm$ SD ( $*P < 0.05$ ,  $***P < 0.001$ , One way ANOVA).

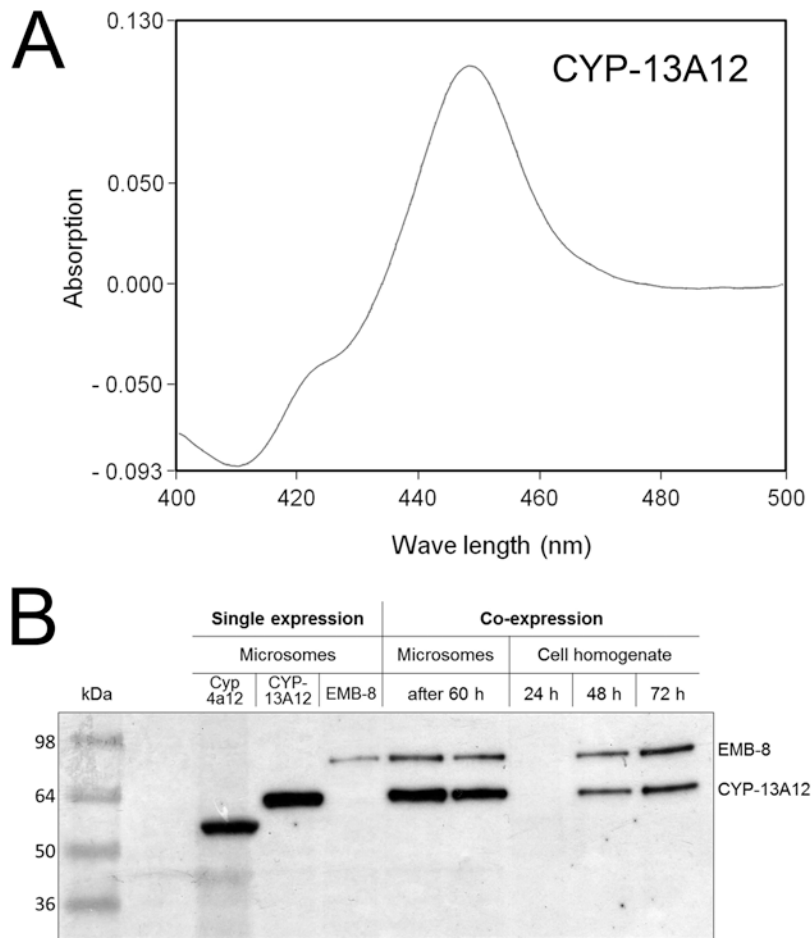
**Figure 5      17,18-EEQ rescues *fat-3(wa22)* mutants from movement restriction under normoxia**

(A) Chemical structures of compounds used for treatment. (B) Long-term exposure with 80  $\mu$ M EPA and AA, but not ETYA, a non-metabolisable AA-analogue, rescued the impaired locomotion of the *fat-3(wa22)* strain, here detected under normoxic conditions. Even added for only 40 min (short-term exposure), 10  $\mu$ M 17,18-EEQ significantly increased the locomotion of *C. elegans* young *fat-3(wa22)* adults and (C) in N2 wild-type. Note also the impairment in locomotive behaviour in response to ETYA treatment of the wild-type. Vehicle: 0.3% DMSO;  $n \geq 30$  (3-5 trials with  $n=10$ ),  $\pm$ SD ( $*P < 0.05$ ,  $**P < 0.01$ ,  $***P < 0.001$ , One way ANOVA).

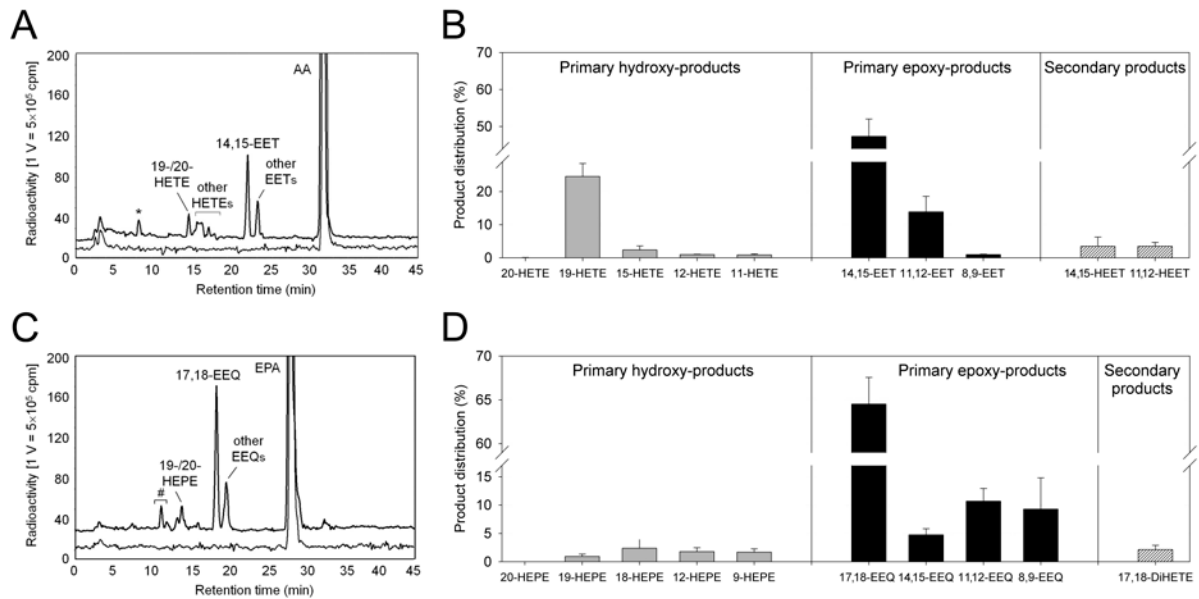
**Figure 6      Proposed role of CYP-eicosanoids in mediating the O<sub>2</sub>-ON response**

As shown in the present study CYP-eicosanoids such as 17,18-EEQ activate the locomotion behaviour of *C. elegans*. To produce the corresponding metabolites, CYP enzymes require molecular oxygen, NADPH, and free C20-PUFAs. Under normoxic conditions CYP-eicosanoid formation is limited by the availability of free C20-PUFAs. Anoxia results in decreased CYP-eicosanoid synthesis due to oxygen-limitation; however, anoxia activates phospholipases releasing free C20-PUFAs from membrane stores that are then available for enhanced CYP-eicosanoid formation in the subsequent reoxygenation phase resulting in a rapidly increased locomotion speed of the nematodes.

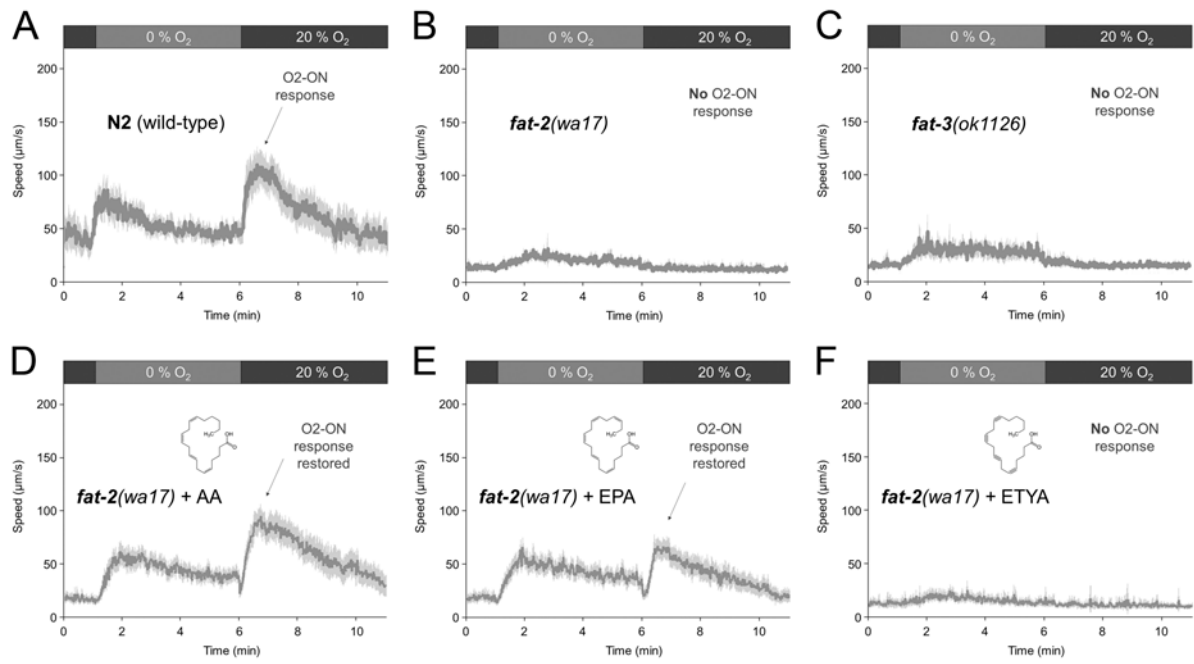
**Figure 1**



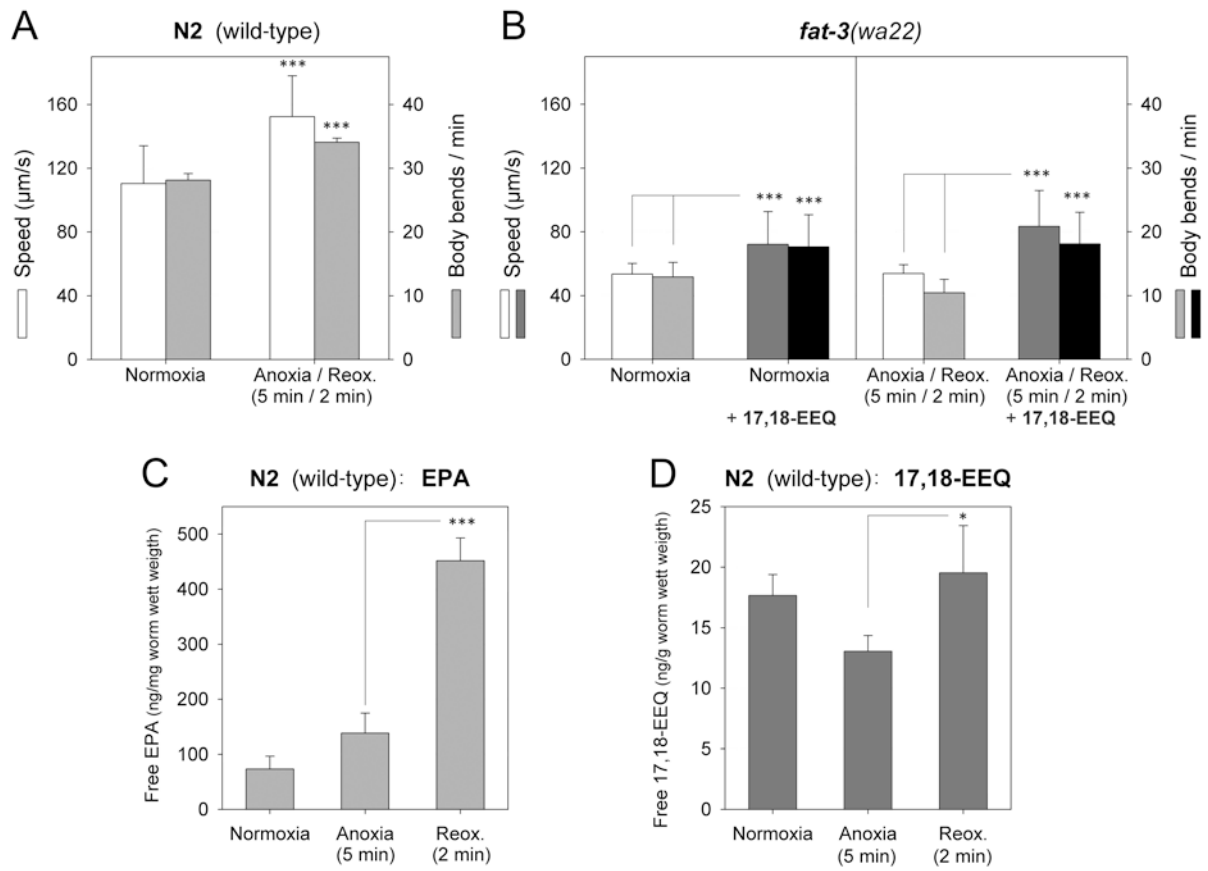
**Figure 2**



**Figure 3**



**Figure 4**



**Figure 5**

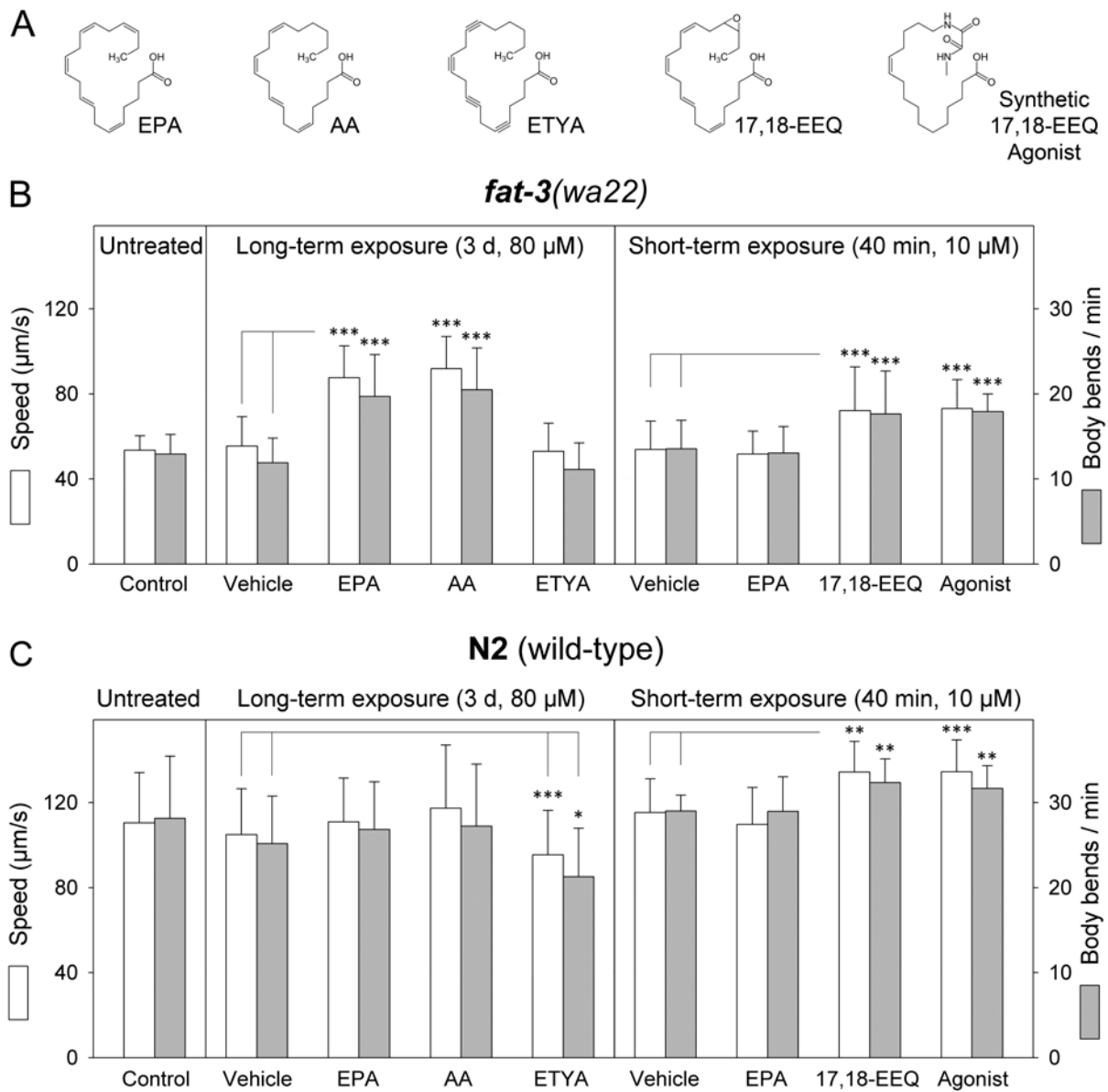




Figure 6

