Functional Desaturase Fads1 (Δ5) and Fads2 (Δ6) Orthologues Evolved before the Origin of Jawed Vertebrates

Luís Filipe Costa Castro¹*, Óscar Monroig², Michael J. Leaver³, Jonathan Wilson¹, Isabel Cunha¹, Douglas R. Tocher³

1 CIIMAR – Interdisciplinary Centre of Marine and Environmental Research, CIMAR Associate Laboratory, University of Porto, Porto, Portugal, ² Instituto de Acuicultura Torre de la Sal (IATS-CSIC), Castellón, Spain, ³ Institute of Aquaculture, School of Natural Sciences, University of Stirling, Stirling, Scotland, United Kingdom

Abstract

Long-chain polyunsaturated fatty acids (LC-PUFAs) such as arachidonic acid (ARA), eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) are essential components of biomembranes, particularly in neural tissues. Endogenous synthesis of ARA, EPA and DHA occurs from precursor dietary essential fatty acids such as linoleic and α-linolenic acid through elongation and Δ5 and Δ6 desaturations. With respect to desaturation activities some noteworthy differences have been noted in vertebrate classes. In mammals, the Δ5 activity is allocated to the Fads1 gene, while Fads2 is a Δ6 desaturase. In contrast, teleosts show distinct combinations of desaturase activities (e.g. bifunctional or separate Δ5 and Δ6 desaturases) apparently allocated to Fads2-type genes. To determine the timing of Fads1-Δ5 and Fads2-Δ6 evolution in vertebrates we used a combination of comparative and functional genomics with the analysis of key phylogenetic species. Our data show that Fads1 and Fads2 genes with Δ5 and Δ6 activities respectively, evolved before gnathostome radiation, since the catshark Scyliorhinus canicula has functional orthologues of both gene families. Consequently, the loss of Fads1 in teleosts is a secondary episode, while the existence of Δ5 activities in the same group most likely occurred through independent mutations into Fads2 type genes. Unexpectedly, we also establish that events of Fads1 gene expansion have taken place in birds and reptiles. Finally, a fourth Fads gene (Fads4) was found with an exclusive occurrence in mammalian genomes. Our findings enlighten the history of a crucially important gene family in vertebrate fatty acid metabolism and physiology and provide an explanation of how observed lineage-specific gene duplications, losses and diversifications might be linked to habitat-specific food web structures in different environments and over geological timescales.

Introduction

Fish, like mammals and probably all other vertebrates, are unable to endogenously synthesize polyunsaturated fatty acids (PUFA) and so these compounds are required in the diet [1]. This requirement is met by the basic essential fatty acids, 18:2n-6 (linoleic acid, LOA) and 18:3n-3 (α-linolenic acid, ALA) which cannot be biosynthesized or interconverted in vertebrates, and so all PUFA are ultimately derived from the primary producers, largely plants [2]. Both LOA and ALA have vital functions in themselves, but they also act as precursors for the long chain-PUFA (LC-PUFA), 20:4n-6 (arachidonic acid, ARA), 20:5n-3 (eicosapentaenoic acid, EPA) and 22:6n-3 (docosahexaenoic acid, DHA) [1,2]. LC-PUFA are essential components of cell membranes, particularly in neural tissues, and they can generally be produced from dietary LOA and ALA in mammals, although evidence suggests that the biosynthesis of EPA and, particularly, DHA from ALA is very low in humans [3], and non-existent in high carnivores such as cats [4]. The biosynthesis of ARA and EPA from LOA and ALA, respectively, involves an initial Δ6 desaturation, followed by chain elongation, and a further Δ5 desaturation [5]. An alternative pathway to produce ARA and EPA from LOA and ALA involves an initial elongation and subsequent Δ5 desaturation (Figure 1). It has been shown that the same enzyme protein displayed both Δ6 and Δ5 desaturation activities [6,7]. It has been is generally accepted that biosynthesis of DHA from EPA in vertebrates requires two further elongation steps and Δ6 desaturation followed by a peroxisomal chain-shortening step [8] (Figure 1). The Δ5 and Δ6/8 enzymes are commonly termed “front-end” desaturases since they introduce the double bond by “counting” from the carboxyl end of the fatty acid molecule. They are encoded by genes denominated fatty acyl desaturases (Fads).

Functional Δ5 and Δ6 desaturases are found in a wide array of lineages including fungi, invertebrate protostomes and vertebrates [9,10]. On the basis of functional criteria, Δ5 genes are thought to have evolved from a Δ6 ancestor, since the action of the latter provides the substrate for Δ5 desaturase in the LC-PUFA
biosynthesis pathway [9] (Figure 1). However, the evolution of desaturase gene lineages and their respective specificities is far from understood. In humans the \( A5 \) and \( A6 \) desaturations are catalysed by the products of FADS1 and FADS2 genes, respectively. These are organized into a tight physical cluster in chromosome 11 [11]. An additional desaturase, FADS3, is also part of the cluster, although its function has not yet been elucidated [11,12]. In the nematode Caenorhabditis elegans, \( A5 \) and \( A6 \) desaturation activities are also encoded by different Fads-like genes, which are found in close proximity in chromosome IV [13,14]. Nevertheless, the nematode genes are more similar to each other than to any of the mammalian gene set. Furthermore, sequence phylogenies demonstrate that the fungi and nematode Fads genes are basal to the human Fads1, Fads2 and Fads3 clade [9,10,13,14]. This evolutionary pattern of gene orthology versus gene function indicates that the acquisition of separate functional \( A5 \) and \( A6 \) genes occurred at least twice in evolution.

In vertebrate species, a complex evolutionary scenario is also emerging. The pattern reported in mammals is not similar to that found in teleosts. A combination of gene isolation and functional characterization has uncovered at least three distinct organizations in fish. A single bifunctional \( A5/A6 \) desaturase was described in zebrafish (Danio rerio), though this gene is more similar to the mammalian Fads2 [15]. A similar result was recently described in the rabbitfish (Siganus canaliculatus), a marine teleost from which two Fads2-related genes have been characterised, one having \( A5/A6 \) activity and the other with \( A5/A4 \) function [16]. Separate \( A5 \) and \( A6 \) genes were found in Atlantic salmon (Salmo salar), and again these are both of the Fads2-type [17,18,19]. Finally, in most other studied teleost species a single \( A6 \) Fads2-like gene and enzyme has been characterized [2,20,21,22].

In summary, Fads1-\( A5 \) appears to be mammalian specific, while teleosts have typically Fads2-\( A6 \), with some examples of bifunctional desaturases (e.g. zebrafish) or separate Fads2-like-\( A5 \) and Fads2-like-\( A6 \) (e.g. Atlantic salmon). Since the LC-PUFA products of “front-end” desaturases, particularly ARA and DHA, are essential components of complex vertebrate nervous systems, the key question is when in vertebrate history did the Fads1-\( A5 \) and Fads2-\( A6 \) linked activities evolve, and what is the diversity of the Fads gene portfolio in vertebrate classes.

At least two evolutionary scenarios can explain the described pattern of gene diversity versus FADS selectivity in vertebrate classes. First, Fads1-\( A5 \) and Fads2-\( A6 \) may have duplicated just before mammalian speciation. In that case, the phylogenetic analysis, which strongly groups teleost Fads genes with mammalian Fads2, is difficult to interpret unambiguously. An alternative explanation indicates that the duplication event generating both gene lineages, Fads1 and Fads2, is older as is the respective \( A5 \) and \( A6 \)-linked activities. In this case, teleosts would have lost the Fads1-\( A5 \) lineage, while in some species the \( A5 \) function was regained through duplication/diversification of a Fads2-\( A6 \) ancestor. To test these hypotheses we took two approaches. First, we determined the diversity and evolutionary history of Fads genes in various vertebrate classes where full genomes are available (teleosts, amphibians, birds, reptiles and mammals). Secondly, we isolated and characterized the Fads gene family in the basal gnathostome, the cartilaginous fish small-spotted catshark (Scyliorhinus canicula), formerly commonly known as the lesser spotted dogfish. Our findings show that clear orthologues of Fads1 and Fads2 arrived before gnathostome radiation. Furthermore, functional analysis demonstrates that the dogfish Fads1 and Fads2 are \( A5 \) and \( A6 \) desaturases, respectively. Thus, a complete Fads1 loss is observed.

Figure 1. Biosynthetic pathway of very long chain-PUFA (VLC-PUFA).
doi:10.1371/journal.pone.0031950.g001

PLoS ONE | www.plosone.org 2 February 2012 | Volume 7 | Issue 2 | e31950

...
in teleosts, with the occasional recruitment of the Δ5 function into Fads2 genes. Interestingly, we also find that some teleost species have completely lost the full set of “front-end” desaturases (e.g. the pufferfishes Takifugu rubripes and Tetraodon nigroviridis). Finally, a novel Fads gene was uncovered in some mammalian species, while in birds and reptiles the Fads1-like portfolio has specifically expanded, with yet unknown functional consequences. We argue that the overall Fads gene/function history in vertebrates involves selective dietary/nutritional pressures, combined with gene events of gain and loss.

Results

The Fads gene portfolio has different complements in vertebrate classes

To investigate the portfolio of Fads genes in vertebrate classes we searched the genomes of human, Rhesus macaque, mouse, dog, rabbit, opossum, platypus, green anole, chicken, western clawed frog, zebrafish, medaka, stickleback, pufferfish and green-spotted puffer (Table S1). This task involved the identification of genome annotated sequences but also direct searches through the Blastp algorithm (PSI Blast). In humans we found the previously described FADS1, FADS2 and FADS3 genes in chromosome 11 (Table S1). A similar Fads gene set was also uncovered in the opossum and the dog, though the Fads2 sequence in opossum was shorter due to the presence of a sequence genome gap that disallowed its analysis. In the Rhesus macaque, rabbit, mouse and platypus, apart from the classic desaturase gene set, a fourth Fads-like gene was found. This sequence contains the common FADS features such as the presence of three conserved histidine motifs (Figure S1). Birds and reptiles showed the highest number of Fads gene ORFs of the analysed species (Table S1), with the presence of four genes in the chicken and six in the green anole. The amphibian X. tropicalis has two clear Fads genes, while a third Fads-like sequence was found in a distinct scaffold, but was incomplete and could not be included in the analysis. In teleosts, several different scenarios were noted. The zebrafish has a single Fads gene as previously described [15], while none were found in the two pufferfish species (Table S1). In stickleback, three Fads-like gene annotations are present at distinct genomic regions. Nevertheless, examination of protein and nucleotide sequence (introns included) showed that they correspond to the exact same sequence and are most likely the result of poor genome assembly in the region. Three medaka Fads-like annotations were also uncovered, but two sequences were incomplete with several missing exons. Using the human FADS2 genomic organization and the genome sequence of the medaka we were able to deduce the complete sequence of the two extra genes (Figure S2). We conclude that there are three Fads-like genes in medaka. Finally, a single copy Fads gene was found in the invertebrate chordate amphioxus.

The search of Fads front-end desaturases in vertebrate genomes uncovered an unexpected diversity of genes. To model the relative timing of duplication and evolutionary histories, we next examined their phylogenetic relationships (Figure 2). Apart from the desaturases identified above, we also included other reported Fads sequences in particular from teleosts. Two well-supported clades can be observed, one including Fads1-like sequences (aLRT 0.958) and a second with Fads2, Fads3 and the novel mammal-specific gene family which we name Fads4 (aLRT 0.990). The Fads1 clade includes sequences from tetrapod species but not teleosts. Furthermore, the unexpected high number of Fads sequences
found in reptiles and birds can now be unequivocally attributed to a lineage-specific expansion of the Fads1 gene group. We labelled each bird and reptile species gene (a to e) according to their position in the cluster (see below). In the Fads2/3/4 clade we find that mammalian species have single copy genes of Fads2, Fads3 and Fads4, though the entire group does not form an independent well supported clade. Fads3 and Fads4 are outgrouped by the chicken and anolis Fads2 but this is a poorly supported clade. This larger collection is outgrouped by the amphibian and teleost FADS2 genes. We opted to maintain the gene nomenclature, although the mammalian Fads2, Fads3 and Fads4 are probably co-orthologues of the remaining Fads2 sequences. Though some teleost species have more than one Fads gene these have clearly resulted from species-specific expansions as well (e.g. Atlantic salmon). Thus, the present phylogenetic tree suggests that teleosts have probably lost a Fads1 orthologue; an expansion of a Fads1-like gene took place in the ancestor of birds and reptiles, and the Fads2/3/4 duplications are specific to mammals.

The human “front-end” desaturase gene set is organized into a tight physical cluster in chromosome 11 [11]. To determine whether this represents a distinctive feature in the desaturase genomic organization, we analyzed the location of Fads genes in the various species (Figure 3). Synteny analysis can also assist in the discrimination between gene loss or incomplete genome sequence as causes for gene absence, such as we have shown in the pufferfishes, T. rubripes and T. nigroviridis.

In tetrapods, the Fads gene(s) locus arrangement is highly conserved with most of the flanking gene families being shared. One exception is found with the opossum Fads1 that maps at a distinct location (Figure 3). However, the presence of a gap between Fads2 and Fads3 precludes a final conclusion regarding the conservation of this gene cluster. The mouse Fads4 also maps at a different chromosome, a condition which is repeated in all the mammalian species having Fads4 (not shown). Therefore, this duplication probably involved a parallel translocation into a distinct genomic region. In teleosts, although the flanking gene families are different from those observed in the tetrapod Fads locus, their human orthologues map to chromosome 11. For example, Eps8l1 which flanks teleost Fads genes is also found at the human chromosome 11 (though distantly), while Lscr10b and Syt7 orthologues map very close to the human Fads gene cluster. The genomic loci investigation served also to address the failure to encounter Fads genes in the pufferfishes. While Fads genes are not found in these species, we find that this is not the result of an incomplete genome sequence, but gene deletion. Thus, we find that genes typically bordering the teleosts Fads gene(s) have a similar arrangement in pufferfishes, but without any intervening Fads ORF (Figure 3).

Cartilaginous fish have clear functional orthologues of Fads1 and Fads2/3/4

Our phylogenetic analysis suggests that the duplication timing of Fads1 and Fads2/3/4 pre-dates the divergence of teleosts and tetrapods, although teleosts have no Fads1 orthologue. Consequently, Fads1 and Fads2/3/4 gene lineages are presumably older than anticipated. To better define the timing of the Fads1/Fads2

![Figure 3. Synteny map of the Fads gene cluster in vertebrates.](image)

Mammals (Hs-Homo sapiens, Mm-Mus musculus, Md-Monodelphis domestica), birds (Gg-Gallus gallus), reptiles (Ac-Anolis carolinensis), amphibians (Xt-Xenopus tropicalis) and teleosts (Ga-Gasterosteus aculeatus, Ol-Oryzias latipes, Dr-Danio rerio, Tr-Takifugu rubripes, Tr-Tetraodon nigroviridiss). Double dash denotes gap. doi:10.1371/journal.pone.0031950.g003
duplication event, we examined the gene complement in an earlier diverging branch of the vertebrates, the chondrichthians. Through PCR with degenerate primers and RACE PCR we obtained two distinct full coding sequences of “front-end” desaturase genes. Both have all the distinctive features of Fads genes, namely the histidine boxes (Figure S1). To address the orthology of the new sequences found in catshark (S. canicula), we undertook phylogenetic analysis (Figure 2). We found that one of the catshark genes robustly groups within the Fads1 tetrapod group (though not basally as would be expected), while the other gene is basal to the mammalian and sauropsid Fads2 clade, together with the amphibian Fads2 orthologue. Thus, we named these ScaFads1 and ScaFads2, respectively. Whether this represents the full complement of Fads gene in the catshark is at present unknown.

In mammals, FADS1 is a Δ5 enzyme, while FADS2 is a Δ6 desaturase. In contrast, in teleosts the exclusive FADS2 portfolio has diverse desaturase activities, including Δ5. Thus, we next performed functional characterization of the FADS proteins from catshark, by determining the fatty acid profiles of yeast transformed with ScaFads1 and ScaFads2 constructs (Figure 4). Transgenic yeast were grown in the presence of potential fatty acid substrates for Δ6, Δ5 and Δ4. The GC-MS analysis clearly indicated that yeast transformed with ScaFads1 was able to bioconvert the Δ5-desaturase substrates 20:4n-3 and 20:3n-6 to 20:5n-3 and 20:4n-6, respectively, with no activity observed as Δ6- and Δ4-desaturase (Figure 4; Table 1). Yeast expressing ScaFads2 actively transformed the Δ6-desaturase substrates 18:3n-3 and 18:2n-6 producing 18:4n-3 and 18:3n-6, respectively, with no activity detected towards Δ5- and Δ4-desaturase substrates (Figure 4; Table 1). Both ScaFads1 and ScaFads2 gene products exhibited higher activity towards n-3 PUFA substrates than the corresponding n-6 PUFA substrates (Table 1).

**Discussion**

In this work we aimed to elucidate some aspects of the evolutionary history and function of Fads genes in vertebrates. This gene family participates in the fundamental biochemical pathway of LC-PUFA biosynthesis (Figure 1). By taking a comparative genomics approach with the investigation of key phylogenetic species, we attempted to resolve the question of when in vertebrate evolution did Fads1-Δ5 and Fads2-Δ6 genes evolve, and whether the gene diversity is wider than previously anticipated. Our results support an evolutionary scenario that combines processes of gene duplication, gene loss and functional diversification (Figure 5).

We identified three noteworthy events of gene duplication. The isolation of clear Fads1 and Fads2/3/4 orthologues in the catshark indicates that the first duplication episode from an ancestral Fads occurred prior to the gnathostome radiation, but after invertebrate chordate divergence (Figure 5). However, based on the current evidence we cannot conclude whether this happened before or after the lamprey-gnathostome split. Although, a search of the sea
lamprey (*Petromyzon marinus*) genome found a single incomplete desaturase sequence, this was unsuitable for robust phylogenetic analysis (not shown). Although our data is equivocal regarding the duplication timing of *Fads2/3/4* (since the mammalian genes do not form an independent statistically supported group), we find more parsimonious that a single *Fads2* gene duplicated in the ancestor of mammals to originate *Fads2*, *Fads3*, and *Fads4*. Otherwise numerous events of gene loss would have to be taken into account. Therefore, these genes are probably co-orthologues of the *Fads2* from other vertebrate classes. However, a stable *Fads* gene set was not retained in mammals as a whole. *Fads4* was lost in various species including humans, dog and the opossum. Also, we find that the well-known very limited capacity for LC-PUFA biosynthesis observed in some extreme carnivores such as cats [4] is probably related with gene loss. Although the genome sequence is incomplete, we found partial *Fads1* and *Fads4* sequences in the cat genome, but no orthologues of *Fads2* or *Fads3* (L. Filipe C Castro, unpublished). These findings are consistent with previous biochemical characterizations, in particular, the apparent absence of *D6* activity in comparison to presence of *D5* activity in domestic cat (*Felix catus*) [23,24]. In the tetrapod clade, we demonstrated that a large and unique independent expansion of the *Fads1* lineage in chicken (three genes) and green anole (five genes) has taken place but, at present, with no clues on possible functional impacts (Figure 5).

The examination of catshark desaturases shows that the *Fads1-D5* and *Fads2-D6* activities evolved before gnathostome radiation.

---

**Table 1.** Functional characterization of catshark *Scyliorhinus canicula* FADS1 and FADS2 proteins in *Saccharomyces cerevisiae.*

| FA substrate | Product | Conversion (%) | Activity |
|--------------|---------|----------------|----------|
| 18:3n-3      | 18:4n-3 | 73             | Δ6       |
| 18:2n-6      | 18:3n-6 | 57             | Δ6       |
| 20:4n-3      | 20:5n-3 | 55             | Δ5       |
| 20:3n-6      | 20:4n-6 | 29             | Δ5       |
| 22:4n-6      | 22:5n-6 | 0              | Δ4       |

Results are expressed as a percentage of total fatty acid substrate converted to desaturated product.

doi:10.1371/journal.pone.0031950.t001

---

**Figure 5.** Evolutionary model of *Fads* gene diversification along the vertebrate lineage. Dotted line indicates physical linkage unknown. Gg- *Gallus gallus*, Ac- *Anolis carolinensis*, Xt- *Xenopus tropicalis*, Ga- *Gasterosteus aculeatus*, Ol- *Oryzias latipes*, Dr- *Danio rerio*, Tr- *Takifugu rubripes*, Tn- *Tetraodon nigroviridis*, Ss- *Salmo salar*, Sc- *Siganus canaliculatus*, and Sca- *Scyliorhinus canicula*.

doi:10.1371/journal.pone.0031950.g005
and were retained in cartilaginous fish and mammals (and probably in all the tetrapod clade) (Figure 5). The findings in the catshark imply also that Fads1-65 was lost in the teleost ancestor. Consistently, the vast majority of teleost species examined to date have only Fads2-66 gene(s) [23,25]. Nevertheless, although the archetypal A5 desaturase, FADS1, has been lost in teleost ancestry, the A5 activity was selectivity regained within specific teleost lineages. The zebrafish Fads2 gene, codes for a bifunctional A5/A6 enzyme [13], and the two Fads2-like genes in rabbitfish encode bifunctional A5/A6 and A14/A5 enzymes [16]. In Atlantic salmon, A5 activity is encoded by a Fads2-type gene that shows >95% deduced amino acid identity with the salmon A6 having arisen from a salmonid-specific duplication [17,18,19]. Thus, the various teleost species with Fads2-65 represent secondary and lineage specific events. These teleost lineage-specific events and the resulting Fads functional plasticities parallel a similar scenario to that observed in the invertebrate protostome C. elegans [14]. Here, two separate Fads-65/Fads-66 genes are also present and have probably evolved from a A6 ancestor [9]. This interchange of functions between A6 to A5 represents an unusual case of functional plasticity. Theoretically, the capacity to change desaturase specificity in this way must be dependent on a minimal number of mutations. Otherwise, re-evolution of a A5 phenotype on a typically A6 genotype (Fads2) would be probabilistically unlikely. At present, no study has yet detailed the crucial domains or amino acid residues of animal FADS proteins responsible for their catalytic properties. However, in the ancestrally related A9 desaturase enzyme, coded by Stearyl CoA Desaturase (Scd), a recent study has discriminated three single amino acids as responsible for change of specificities [26]. We would predict a similar behaviour in the case of FADS desaturases.

It has been suggested that the teleost Fads gene(s) portfolio and their functional activities have been modelled by habitat-specific LC-PUFA abundance [1,2,27]. Thus, species, for example zebrafish and Atlantic salmon, inhabiting some freshwater environments have diversified their gene and catalytic complement in response to the lack or reduced levels of LC-PUFA in their natural diets [25]. This lack of LC-PUFA is also evident in terrestrial habitats that are fuelled by green plants [27,28], and is an explanation for the retention of both A5 and A6 desaturase genes in terrestrial tetrapods. In contrast, carnivorous marine and eutrophic freshwater species would have no need for endogenous synthesis of LC-PUFA, given the luxus of diatom and dinoflagellate-derived LC-PUFA [25,27,28]. In this regard, two groups of marine fish are particularly interesting. In the case of the pullerfish T. rubripes and T. nigroviridis, a complete absence of “front-end” desaturase genes is observed, which we would argue results from very low environmental pressure to maintain A6 and A5 desaturase genes. In contrast the marine species rabbitfish has duplicated and diversified Fads2-like genes, resulting in a unique diversity of desaturase specificity [16]. This might be explained by the fact that this species has specialised in feeding on seagrasses, a group of marine flowering plants including eelgrass, which have evolved from terrestrial plants that re-colonized the sea and, like terrestrial plants, lack LC-PUFA [29].

Despite the exceptions discussed above it is clear that the vast number of teleosts examined to date retain a Fads2-66 even if they inhabit marine environments. Although A6 and A5 activities are interdependent in the pathway of EPA biosynthesis, A6 activity is also required for the production of DHA from EPA whereas A5 is not and so retention of A6 may be due to specific biochemical/metabolic/physiological factors albeit still related to dietary supply of LC-PUFA. Comparative analysis of A6 gene expression between salmonids and marine fish (e.g. Atlantic cod, cobia, Asian sea bass) shows some remarkable differences. While the highest levels of Fads2-A6 expression and desaturation activity are in liver and intestine in salmon, the Fads2-A6 expression and desaturation activity is low in marine fish liver and intestine, but high in the brain [30] (Tocher et al., 2006), which, as with all neural tissues, has a fatty acid composition with a very high DHA:EPA ratio [1]. Thus, it has been suggested that retention Fads2-A6 in marine fish may be to maintain membrane DHA levels (by metabolism of EPA) in neural tissues at times of high physiological demand including during embryonic and larval development [20,22,30]. Despite the abundance of LC-PUFA in the marine environment and likely in the natural diet of the catshark, we could speculate that a functionally similar role, that is the ability to precisely regulate tissue DHA:EPA ratios, may explain the retention of A6 enzymes. However, the high levels of EPA and DHA in the present marine environment would make the retention of A5 desaturase activity more difficult to account for, since, according to the arguments above, there would be little pressure and consequently no requirement for endogenous LC-PUFA biosynthesis in marine habitats. However it is well known that that the early ocean, in which the basal gnathostomes evolved, was a very different habitat to that we see today. Particularly relevant is the paleontological evidence indicating that the major LC-PUFA producing phytoplankton, the diatoms and dinoflagellates, did not come to prominence until the Triassic (i.e. not more than 250 Ma ago) [31]. Prior to this primary productivity in the early oceans appears to have dominated by green algae and cyanobacteria. Since the appearance of sharks occurred more than 400 Ma years ago, predating the emergence of diatoms and dinoflagellates, the acquisition of both Fads1 and Fads2 genes may have been necessary to enable survival in a relatively LC-PUFA-poor ecosystem. In contrast the appearance and subsequent enormous radiation of the Teleostei is coincident with the rise to domination of the diatoms and dinoflagellates.

The appearance of morphological and physiological innovations in the vertebrate clade has long been linked with gene duplication, namely genome duplications [32]. Two rounds of genome duplications (2R) are now an established event in vertebrate evolution [33]. The vertebrate Fads evolutionary pattern indicates that an increase in gene number took place just before the emergence of vertebrates. In effect, we find that a tandem gene duplication of Fads gene precursor originated separate A5 and A6 genes, with further genes increases in particular lineages. Our analysis of the Fads history shows that the increase in number of the front-end desaturase vertebrate set was linked with tandem duplications and not 2R. This observation contrasts with what is found in other desaturase gene families. The Scd genes, which catalyse the desaturation of saturated fatty acyl-CoA substrates at the A9 position, were recently showed to have specifically duplicated in the vertebrate lineage as a result of 2R [34,35].

A discussion of the LC-PUFA biosynthetic pathway cannot be complete without considering the other main enzymatic steps, those performed by fatty acid elongases, Elov2 and Elov5 (Figure 1). The partial or complete loss of front-end desaturases in teleost species indicates the disruption of the LC-PUFA synthesis pathway (Figure 1). For example, absence of a A5 gene implies the inability to synthesize either EPA or ARA from 20:4n-3 and 20:3n-6 substrates, respectively. Curiously, the presence of Fads2-65 genes in teleosts is apparently correlated with the retention Elov2 orthologues (e.g. zebrafish and Atlantic salmon) [36,37]. In contrast, medaka, stickleback, fugu and pufferfish have no obvious Elov2 sequences [36]. Although the Fads2 genes from these species have not yet been functionally characterized it is tempting to suggest that they are functionally A6. This apparent
correlation between the genome complement of desaturases and elongases genes would imply that cartilaginous fish should have an Elovl2 since we have now shown the presence of a Fads1-<I>A5</I> gene. Searches to the elephant shark genome confirm that Elovl2 like sequences are present (not shown). Exactly why teleosts that possess no desaturase gene complement still retained an Elovl3 and Elovl4 such as: T. rubripes and T. nigroviridis remains to be investigated.

In summary, we provide a clear framework of “front-end” desaturase evolution in vertebrate history. We present an explanation of how observed lineage-specific gene duplications, losses and diversifications might be linked to habitat-specific food web structures in different environments and over geological timescales.

Materials and Methods

The animals used in the research described in this paper were treated in accordance with the Portuguese Animals and Welfare Law (Decreto-Lei n° 197/96) approved by the Portuguese Parliament in 1996. Institutional animal approval by CIIMAR/UP and DGV (Ministry of Agriculture) was granted for this study.

Mining of FADS genes, phylogenetic and synteny analysis

The Fads gene portfolio was identified in release 6 of the Ensembl database (www.ensembl.org) and Genbank from the following species: Homo sapiens (human-placental mammal), Macaca mulatta (Rhesus macaque-placental mammal), Mus musculus (mouse-placental mammal), Canis familiaris (dog-placental mammal), Ornithorhynchus anatinus (platypus-mono-treme), Anolis carolinensis (green anole-reptile), Gallus gallus (chicken-bird), Xenopus tropicalis (western clawed frog-amphibian), D. rerio (teleost), Gasterosteus aculeatus (three-spined stickleback-teleost), Oryzias latipes (medaka-teleost), T. rubripes (Fugu) pufferfish-teleost), and T. nigroviridis (chondrichthyes). The search of the cartilaginous fish Colobocentrus nudivum (elephant shark-chondrichthyes) genome was performed using the human FADS1 and FADS2 sequences with TBLASTN at http://sharkgenome.imb.c-ast. edu.sg/Blast/ [38].

To identify sequences that were poorly annotated or non-annotated in databases we also performed a BLAST search Blastp (PSI-BLAST). Some sequences were manually curated using the human gene FADS1 or FADS2 gene structure as a model.

Protein sequences from Fads genes were aligned using MAFFT (http://mafft.cbrc.jp/alignment/server/) with the L-INS-i method [39]. The alignment was further adjusted manually with gap removal. The final dataset had a total of 55 sequences and 297 characters. For tree reconstruction, we first applied ProtTest [40] to estimate the optimal model of amino acid substitution (JTT+H+G+F). A maximum likelihood tree was constructed using PHYML (online) [41] with the JTT model. The amino acid frequency (equilibrium frequency), proportion of invariable sites and gamma-shape (4 rate substitution categories) for the amino acid substitution rate heterogeneity parameters were estimated from the dataset. Confidence in each node was assessed by aLRT non-parametric branch support (SH-like) [42]. The phylogenetic tree was rooted with the amphioxus sequence using NJPlot (version 2.3) [pbil.univ-lyon1.fr/software/njplot.html] [43].

Genomic regions containing Fads genes were identified in Ensembl and Genbank databases. The two closest gene families flanking Fads genes were identified.

Cloning and sequencing of the S. canicula Fads1 and Fads2 cDNA

Catshark specimens in May 2010 were given an overdose of tricane methylsulfonate (1:5000 MS-222 Aquapharm UK) and killed by cervical transection. Total RNA was isolated from the collected tissues using the Illustra RNAspin minikit from GE Healthcare (Little Challont, UK) with on-column DNase I treatment. RNA concentration was measured with a Qubit fluorometer platform (Invitrogen, Carlsbad CA). Conversion of total RNA into first strand cDNA was performed using the iScript cDNA synthesis following the manufacturer recommendations (Bio-Rad). Polymerase chain reaction (PCR) used cDNA from catshark (S. canicula) and degenerate primers for Fads1 and Fads2 (Table S2). Rapid amplification of cDNA ends (RACE) PCR was used to obtain the full open reading frames (ORF) from the original sequence fragments (Clontech, USA). For all PCR protocols, the Phusion Flash hot start high fidelity polymerase mix with the manufacturer recommended conditions was used (Finzyme, Helsinki FI). Amplicons of the appropriate size were isolated from the agarose gel (GFX cleaning kit, GE Healthcare), and sequenced directly with one of the flanking primers (Stabvida, Portugal).

Functional assay of the Fads1 and Fads2 in S. canicula

Primers with restriction sites of HindIII and EcoRI flanking the predicted longest ORF of SeaFads1 and SeaFads2 were used for PCR with Phusion Flash hot start high fidelity polymerase mix (Table S2). Bands of the appropriate size were cloned into pGEMT-easy. Four clones were sequenced and compared with the cDNA assembled contig. After restriction digestion, the insert was cloned into pYES2 yeast expression vector (Invitrogen), and sequenced. The constructs pYES2-SeaFads1 and pYES2-SeaFads2 were transferred into Saccharomyces cerevisiae competent cells (strain InvSc, Invitrogen). Transformation and selection of yeast with recombinant pYES2 plasmids and yeast culture and fatty acid (FA) analyses were performed as described in detail previously [15,44]. Transgenic yeast were grown in the presence of potential fatty acid substrates for Δ6 (18:3n-3 and 18:2n-6), Δ5 (20:4n-3 and 20:3n-6) or Δ4 (22:5n-3 and 22:4n-6) activities. The FA were added to the yeast cultures at final concentrations of 0.5 (C18), 0.75 (C20) and 1.0 (C22) mM as uptake efficiency decreases with increasing chain length. Yeast transformed with empty pYES2 were also grown in presence of PUFA substrates as control treatments. After 2-days culture at 30°C, yeast were harvested and washed, and lipid extracted by homogenization in chloroform/methanol (2:1, v/v) containing 0.01% butylated hydroxy toluene as antioxidant. Methyl esters of FA were prepared, extracted, purified, and analyzed by GC in order to calculate the proportion of substrate FA converted to desaturated FA product as [product area/(product area+substrate area)]×100. Identities of FA peaks were based on GC retention times and confirmed by GC-MS as described previously [15,16].

Supporting Information

Figure S1 Alignment of amino acid sequences of FADS proteins from H. sapiens (FADS1, FADS2 and FADS3), M. musculus (FADS1, FADS2, FADS3 and FADS4) and S. canicula (FADS1 and FADS2). The “HPG” characteristic of cytochrome b5 domain is underlined in black. The three conserved histidine motifs “HXXXH”, “HXXHH”, and “QXXHH” is underlined in red.

(BOX)}
**Table S1** List of identified Fads sequences and the accession numbers for all the sequences used in the phylogenetic analysis. (DOC)

**References**

1. Tocher DR (2003) Metabolism and functions of lipids and fatty acids in teleost fish. Rev Fisheries Sci 11: 107–104.

2. Tocher DR (2010) Fatty acid requirements in oogenesis of marine and freshwater fish. Aquaculture Res 41: 717–732.

3. Burdge GC, Calder PC (2005) Conversion of alpha-linolenic acid to longer-chain polyunsaturated fatty acids in human adults. Reprod Nutr Dev 45: 449–464.

4. Rivers JPW, Sinclair AJ, Crawford MA (1975) Inability of the cat to desaturate essential fatty acids. Nature 258: 171–173.

5. Cook HW, McMaster RCR In:, Biochemistry of Lipids, Lipoproteins, and Membranes. Elsevier, Amsterdam: Elsevier. pp 191–204.

6. Park WJ, Kothapalli KS, Lawrence P, Tyburczy C, Brenna JT (2009) An intron-exon organization of fatty acyl desaturase and elongase gene for Atlantic salmon (Salmo salar). FEBS Lett 439: 215–221.

7. Lopez Alonso D, Lopez Alonso F, Garcia-Maroto J, Rodriguez-Ruiz JA, Monroig O’ (2011) Delta-8 desaturation activity varies among fatty acyl desaturases of teleost fish: high activity in delta-6 desaturases of marine species. Comp. Biochem. Physiol. 159B: 206–213.

8. Sprecher H (2000) Metabolism of highly unsaturated n-3 and n-6 fatty acids. Biochim. Biophys. Acta 1486: 219–251.

9. Munroig O, Li Y, Tocher DR (2011) Delta-6 desaturation activity varies among fatty acyl desaturases of teleost fish: high activity in delta-6 desaturases of marine species. Comp. Biochem. Physiol. 159B: 206–213.

10. Li Y, Monroig O, Zhang L, Wang S, Zheng X, et al. (2010) Vertebrate fatty acid desaturase with Delta 5 and Delta 6 activities. Proc Natl Acad Sci U S A 97: 4449–52.

11. Evans H, De Tomaso T, Quail M, Rogers J, Gracey AY, et al. (2008) Ancient and modern duplication events and the evolution of stearoyl-CoA desaturases in teleost fish. Physiol Genomics 35: 18–29.

12. Shimeld SM, Holland PW (2000) Vertebrate innovations. Proc Natl Acad Sci U S A 97: 4449–52.

13. Castro LF, Wilson JM, Goncalves O, Galante-Oliveira S, Rocha E, et al. (2011) Functional identification of a fatty acid delta5 desaturase gene from Caenorhabditis elegans. FEBS Lett 439: 215–221.

14. Anisimova M, Gascuel O (2006) Approximate Likelihood-Ratio Test for varying number of rate categories across sites. Systematic Biol 55: 536–545.

15. Katoh K, Toh H (2008) Recent developments in the MAFFT multiple sequence alignment program. Brief Bioinform 9: 286–98.

16. Venkatesh B, Kirkness EF, Loh YH, Halpern AL, Lee AP, et al. (2007) The amphiarch genome and the evolution of the chordate karyotype. Nature 453: 1064–1071.

17. Evans H, De Tomaso T, Quail M, Rogers J, Gracey AY, et al. (2008) Ancient and modern duplication events and the evolution of stearoyl-CoA desaturases in teleost fish. Physiol Genomics 35: 18–29.

18. Anisimova M, Gascuel O (2006) Approximate Likelihood-Ratio Test for varying number of rate categories across sites. Systematic Biol 55: 536–545.

19. Venkatesh B, Kirkness EF, Loh YH, Halpern AL, Lee AP, et al. (2007) The amphiarch genome and the evolution of the chordate karyotype. Nature 453: 1064–1071.

20. Knoll HA, Simmons RE, Waldbauer JR, Zumberge JE (2007) The geological succession of primary producers in the oceans. In: Evolution of Primary Producers in the Sea, Falconski PG, Knoll AH, eds. Academic Press, New York.

21. Shmilovitz M (1980) Vertebrate innovations. Proc Natl Acad Sci U S A 76: 4499–502.

22. Knoll HA, Simmons RE, Waldbauer JR, Zumberge JE (2007) The geological succession of primary producers in the oceans. In: Evolution of Primary Producers in the Sea, Falconski PG, Knoll AH, eds. Academic Press, New York.

23. Knoll HA, Simmons RE, Waldbauer JR, Zumberge JE (2007) The geological succession of primary producers in the oceans. In: Evolution of Primary Producers in the Sea, Falconski PG, Knoll AH, eds. Academic Press, New York.

24. Sinclair AJ, McLean JJ, Monger EA (1979) Metabolism of linoleic acid in the cat. Lipids 14: 932–6.

25. Sinclair AJ, Slattery W, McLean JJ, Monger EA (1981) Essential fatty acid deficiency and evidence for arachidonate synthesis in the cat. Br J Nutr 46: 93–6.

26. Leaver MJ, Basista JM, Bjorjonsen T, Jonsson E, Krey G, et al. (2000) Towards fish lipid nutrigenomics: current state and prospects for fin-fish aquaculture. Rev Fisheries Sci 10S1: 71–92.

27. Anisimova M, Gascuel O (2006) Approximate Likelihood-Ratio Test for varying number of rate categories across sites. Systematic Biol 55: 536–545.

28. Vinayak G, Gouy M (1996) WWW-Query: an on-line retrieval system for protein sequences. Bioinformatics 12: 1055–1062.

29. Guindon S, Dufayard JF, Lefort V, Anisimova M, Hordijk W, et al. (2010) New algorithms and methods to Estimate Maximum-Likelihood Phylogenies: Assessing the Performance of PhyML 3.0. Systematic Biol 59: 307–21.

30. Anisimova M, Gascuel O (2006) Approximate Likelihood-Ratio Test for Branches: A Fast, Accurate, and Powerful Alternative. Systematic Biology 55: 539–552.

31. Anisimova M, Gascuel O (2006) Approximate Likelihood-Ratio Test for Branches: A Fast, Accurate, and Powerful Alternative. Systematic Biology 55: 539–552.

32. Perez-Rey G, Gouy M (1996) WWW-Query: an on-line retrieval system for biological sequence banks. Bioinformatics 12: 364–369.

33. Agaba M, Tocher DR, Dickson C, Dick J, Teale AJ (2004) Zebrafish cDNA encoding multifunctional fatty acid elongase involved in production of eicosapentaenoic (20:5n-3) and docosahexaenoic (22:6n-3) acids. Mar Biotechnol 6: 251–261.

**Table S2** List of primers used to isolate and characterize Fads1 and Fads2 genes in Scoliodon canalicula. (DOC)

**Author Contributions**

Conceived and designed the experiments: LFCC OM DRT. Performed the experiments: LFCC OM MJL IC DRT. Contributed reagents/materials/analysis tools: LFCC MJW DRT. Wrote the paper: LFCC OM MJL DRT.