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Title

Essential fatty acid metabolism and requirements of the cleaner fish, ballan wrasse *Labrus bergylta*: Defining pathways of long-chain polyunsaturated fatty acid biosynthesis

Authors

Naoki Kabeya[1,2], Simon Yevzelman[1], Angela Oboh[1,3], Douglas R. Tocher[1], Oscar Monroig[1]*

Addresses

[1] Institute of Aquaculture, Faculty of Natural Sciences, University of Stirling, Stirling FK9 4LA, Scotland, UK

[2] Department of Aquatic Bioscience, The University of Tokyo, 1-1-1 Yayoi, Bunkyo-ku, Tokyo 113-8657, Japan

[3] Department of Biological Sciences, University of Abuja, P.M.B. 117, Nigeria

***Corresponding author**

Oscar Monroig

Institute of Aquaculture, Faculty of Natural Sciences, University of Stirling, Stirling FK9 4LA, Scotland, UK

Tel: +44 1786 467892; E-mail: oscar.monroig@stir.ac.uk

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Abstract

Ballan wrasse (*Labrus bergylta*) is an effective counter-measure against sea lice used by Atlantic salmon farmers, proving to be more effective and economical than drugs or chemical treatments alone. There are currently efforts underway to establish a robust culture system for this species, however, essential fatty acid dietary requirements are not known for ballan wrasse. In the present study, we isolated and functionally characterised ballan wrasse fatty acid desaturase (Fads) and elongation of very long-chain fatty acids (Elovl) protein to elucidate their long-chain polyunsaturated fatty acid (LC-PUFA) biosynthetic capability. Sequence and phylogenetic analysis demonstrated that the cloned genes were *fads2* and *elovl5* orthologues of other teleost species. Functional characterisations of *fads2* and *elovl5* were performed using the yeast (*Saccharomyces cerevisiae*) heterologous expression system. The Fads2 showed Δ6 desaturase activity towards 18:3n–3, 18:2n–6 and 24:5n–3, and Δ8 desaturase activity towards 20:3n–6 and 20:2n–6. The Elovl5 showed elongase activities towards various C_{18} and C_{20} fatty acids. Therefore, 20:4n–3 and 20:3n–6 can be synthesised from 18:3n–3 and 18:2n–6, respectively in ballan wrasse *via* two possible pathways, the Δ6 (Δ6 desaturation – elongation) and Δ8 (elongation – Δ8 desaturation) pathways. However, due to the absence of Δ5 desaturase activity and no other Fads2 in their genome, 20:5n–3 (eicosapentaenoic acid, EPA) and 20:4n–6 (arachidonic acid, ARA) cannot be synthesised from C18 PUFA precursors and they could consequently be regarded as dietary essential fatty acids for ballan wrasse. Since no Δ4 desaturase activity was detected in ballan wrasse Fads2, 22:6n–3 (docosahexaenoic acid, DHA) can only be synthesised from EPA *via* the Sprecher pathway comprising two sequential elongation steps to produce 24:5n–3 followed by Δ6 desaturation and chain shortening. Although ballan wrasse Elovl5 had no elongase activity towards C_{22}, other elongases such as Elovl4 exist in the ballan wrasse genome that may be able to produce 24:5n–3. Therefore, as

ballan wrasse Fads2 can desaturate 24:5n–3 to produce 24:6n-3, it can be assumed that ballan wrasse can synthesise DHA from EPA.

Highlights

The LC-PUFA biosynthesis capability of ballan wrasse showed a typical marine teleost pattern.

Ballan wrasse Fads2 showed Δ6 and Δ8 activities, but no Δ5 or Δ4 activities.

Ballan wrasse Elovl5 showed elongation activity towards C_{18} and C_{20}, but not for C_{22} PUFA.

Ballan wrasse Fads2 can also desaturate 24:5n–3 to synthesise 24:6n–3, an important intermediate in the production of DHA from EPA.

Introduction

Plant ingredients are commonly used nowadays in aquafeeds to replace marine ingredients including fishmeal and fish oil (FO) (Turchini et al., 2010). While this strategy has numerous advantages associated with lower cost, high availability and perceived environmental sustainability (Turchini et al., 2010), a major drawback derived from use of plant ingredients is related to their poorer nutritional profile in comparison to marine ingredients. With regards to the oil component of the diet, plant ingredients, unlike marine ingredients, completely lack long-chain ($\geq C_{20}$) polyunsaturated fatty acids (LC-PUFA) including eicosapentaenoic acid (EPA; 20:5n–3), docosahexaenoic acid (DHA; 22:6n–3) and arachidonic acid (ARA; 20:4n–6), physiologically critical compounds necessary for normal cellular function of all vertebrates including fish (Tocher, 2010, 2015). High inclusion of plant ingredients, particularly vegetable oils (VO), thus results in decreased accumulation of LC-PUFA such as EPA and DHA (Henriques et al., 2014; Sprague et al., 2016), which compromises the nutritional quality of farmed fish products for human consumers (Swanson et al., 2012; Calder, 2015). Further detrimental effects from VO use in aquafeed include those on the health of fish themselves, at least partly related to potentially limited provision of essential fatty acids (EFA) such as EPA, DHA and ARA in the diet (Tocher, 2015). While endogenous production (biosynthesis) of LC-PUFA from α-linolenic acid (ALA; 18:3n–3) and linoleic acid (LA; 18:2n–6), present in a range of VO sources, is possible in some fish species, EFA requirements in marine species typically cannot be satisfied by C_{18} polyunsaturated fatty acids (PUFA), ALA and LA, as they have limited LC-PUFA biosynthetic capability (Tocher, 2010; Castro et al., 2016). With diversification of finfish aquaculture often involving marine species, it is important to understand LC-PUFA biosynthesis capabilities so that nutritionally balanced diets including

plant ingredients can be designed without compromising the EFA requirements and health of marine fish species.

Studies aiming to elucidate LC-PUFA biosynthesis in a large variety of fish species have enabled us to establish that the LC-PUFA biosynthetic capacity of fish varies among species depending upon the repertoire and function of two types of enzymes, namely fatty acyl desaturases (Fads) and elongation of very long chain fatty acids (Elovl) proteins (Fig .1). All Fads enzymes characterised from teleosts, the fish group to which virtually all species farmed belong, are Fads2 (Castro et al., 2012; Kabeya et al., 2017). In agreement with mammalian FADS2, the majority of teleost Fads2 have Δ6 desaturase activity and thus can operate towards ALA and LA to produce 18:4n–3 and 18:3n–6, respectively (Fig. 1) (Castro et al., 2016; Kabeya et al., 2017). Recently it has been demonstrated that most teleost Fads2 can also catalyse the Δ6 desaturation that converts 24:5n–3 to 24:6n–3, a critical reaction for DHA biosynthesis *via* the so-called "Sprecher pathway" (Fig. 1) (Oboh et al., 2017a). In some species, Δ6 Fads2 have evolved by acquiring either further desaturase capacities to become bifunctional Δ6Δ5 desaturases (subfunctionalisation) or new activities like monofunctional Δ5 and Δ4 desaturases (Castro et al., 2016; Kabeya et al., 2017). With the exception of Δ4 desaturases, fish Fads2 exhibit Δ8 desaturase capability, which appears to be an inherent feature among vertebrate Fads2 enzymes (Monroig et al., 2011; Park et al., 2009).

Along with Fads, LC-PUFA biosynthesis requires the action of Elovl (or elongase) enzymes (Fig. 1). Three types of Elovl, namely Elovl5, Elovl2 and Elovl4, play major roles in LC-PUFA biosynthesis (Castro et al., 2016) and differ from each other according to their substrate specificity (Jakobsson et al., 2006). Elovl5 is found in virtually all teleosts and has preference for C_{18} and C_{20} PUFA, but not C_{22} substrates (Castro et al., 2016). In agreement with a shared evolutionary origin (Monroig et al., 2016), Elovl2 can elongate C_{20} PUFA like Elovl5 but, in

contrast, Elovl2 has the ability to elongate C_{22} PUFA substrates and only limited activity towards C_{18} PUFA (Castro et al., 2016). Importantly, the *elovl2* gene has been lost during the evolution of teleosts and, as a consequence, the vast majority of marine fish species currently farmed do not possess this type of elongase (Castro et al., 2016). It has been postulated that loss of elongation capacity towards C_{22} PUFA through the absence of Elovl2 in marine fish can be partly compensated by another elongase, Elovl4 (Monroig et al., 2010; 2011) since, in addition to the biosynthesis of very long-chain (> C_{24}) PUFA (Oboh et al., 2017b), fish Elovl4 can elongate C_{22} PUFA (Monroig et al., 2010; Jin et al., 2017). The above mentioned diversity of gene complement and substrate specificities in fish LC-PUFA biosynthesising enzymes demonstrates that species-specific studies on Fads and Elovl are required to unequivocally elucidate the capacity to utilise dietary VO of new fish species that are candidates for aquaculture diversification.

Ballan wrasse *Labrus bergylta* has been used in the biological control of the ectoparasite *Lepeophtheirus salmonis* in salmon aquaculture (Bjordal et al., 1991; Torrissen et al., 2013; Aaen et al., 2015). As the demand for ballan wrasse juveniles grows, husbandry practices including feeding and nutrition (Hamre et al., 2013) for this species are rapidly developing in order to guarantee supply and avoid exploitation of wild populations. With regards to essential lipids, it has been reported that higher dietary DHA:EPA ratio is positively correlated with growth performance of ballan wrasse (Kousoulaki et al., 2015), but little is known about their capacity to utilise dietary VO to meet EFA requirements. For that purpose, the present study aimed to clone and functionally characterise *fads2*- and *elovl5*-like cDNAs with putative roles in the LC-PUFA biosynthetic pathways of ballan wrasse.

Materials and Methods

Sample collection, RNA extraction and cDNA synthesis

All experiments were subjected to ethical reviewed and approved by the University of Stirling through the Animal and Welfare Ethical Review Body. The project was conducted under the UK Home Office in accordance with the amended Animals Scientific Procedures Act implementing EU Directive 2010/63. One single ballan wrasse juvenile (42.6 g) was obtained from Otter Ferry Seafish Ltd. (Otter Ferry, Scotland, UK) and humanely euthanised with an overdose of anaesthetic (metacaine sulphonate, MS-222; PHARMAQ, UK) prior to dissection. Tissues including brain, liver, intestine and eye were chosen for RNA extraction based on their roles in LC-PUFA biosynthesis (Hamid et al., 2016). Total RNA from each tissue sample (~30 mg) was extracted by TRIzol® (Thermo Fisher Scientific, UK) following the manufacturer's instructions. Subsequently, cDNA was synthesised from 2 μg of total RNA with a random primer using High Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific) following the manufacturer's instructions.

Molecular cloning of L. bergylta elovl5 and fads2

Primers for amplification of the first fragment of *elovl5* cDNA were designed on conserved regions of the following *elovl5* sequences: *Sparus aurata* (NCBI accession: AY660879), *Oreochromis niloticus* (NM_001279460), *Lates calcarifer* (GQ214180), *Clarias varegatus* (XM_015374479), *Dicentrarchus labrax* (FR717358), *Pagrus major* (HQ415605), *Siganus canaliculatus* (GU597350), *Siniperca chuatsi* (EU683736), and *Larimichthys crocea* (NM_001303374). The first fragment was amplified using LBElovlF2 and LBElovlR2 (Table 1) with GoTaq® Green Master Mix (Promega, USA). The PCR for the first fragment amplification consisted of 95 ºC for 2 min, 40 cycles of 95 ºC for 30 s, 54 ºC for 30 s and 72

ºC for 1 min, and 72 ºC for 5 min. The amplified fragment was purified on an agarose gel using the Illustra™ GFX™ PCR DNA and Gel Band Purification Kit (GE Healthcare UK Limited, UK) then sequenced (GATC Biotech, Germany). The obtained sequence was subsequently used for designing primers to perform 5' and 3' Rapid Amplification of cDNA Ends (RACE) (Table 1). The RACE cDNA was synthesised using the FirstChoice™ RLM-RACE Kit (Thermo Fisher Scientific) following the manufacturer's instructions. All the PCR amplifications (first and nested) were performed with 95 ºC for 2 min, 35 cycles of 94 °C for 30 s, 60 °C for 30 s and 72 °C for 30 s, and 72 ºC for 5 min using appropriate primers (Table 1) and adaptor primers provided from the RACE kit. The nested PCR products were purified and sequenced as described above. After isolation of *elovl5* sequence, the genomic assembly of ballan wrasse became publicly available in NCBI (GenBank assembly accession; GCA_900080235.1) and thus we obtained the putative *fads2* full-length sequence from the assembly (NCBI Gene ID; 109982230).

Phylogenetic analysis

The deduced amino acid (aa) sequences of the *fads* and *elovl* cDNAs from various species were collected from NCBI and the alignment of each gene was generated by MAFFT version 7 with the L-INS-i method (Katoh and Standley, 2013). The 429 aa (Fads) and 257 aa (Elovl) gap-free sites from each alignment were then used for maximum likelihood phylogenetic analyses carried out using PhyML v3.0 server (Guindon et al., 2010). The protein evolutionary model of both Fads and Elovl was selected as JTT+G+I+F by Smart Model Selection (SMS) with Akaike's information criterion (AIC) (Lefort et al., 2017). The number of bootstrap replicates was set to 1000 and the resulting trees were visualised in FigTree V1.3.1 available at http://tree.bio.ed.ac.uk/software/figtree/.

Functional characterisation of ballan wrasse Fads2 and Elovl5 in yeast

The full-length open reading frame (ORF) sequences of ballan wrasse *fads2* and *elovl5* were amplified from a mixture of brain and liver cDNAs using the high fidelity *Pfu* polymerase (Promega, USA). The primer sets were designed to anneal to the start and stop codon with restriction sites for *Hin*dIII and *Xba*I (*fads2*), and *Bam*HI and *Xho*I (*elovl5*). Both PCR amplifications were performed with 95 ºC for 2 min, 35 cycles of 94 °C for 30 s, 60 °C for 30 s and 72 °C for 30 s, and 72 ºC for 5 min. Whole ORF PCR products were purified as above, and subsequently digested with the corresponding restriction enzymes and ligated into similarly restricted the yeast expression vector pYES2 (Thermo Fisher Scientific) using T4 DNA ligase (Promega). Yeast transformation and culture were carried out following a previously described method (Oboh et al., 2016). In order to determine the activity of the ballan wrasse Fads2 and Elovl5, transgenic yeast expressing their ORF were grown in the presence of one of the following fatty acid (FA) substrates (98–99 % pure): 18:2n–6, 18:3n–3, 20:2n–6, 20:3n–3, 20:3n–6, 20:4n–3, 22:4n–6 and 22:5n–3 for Fads2; and 18:2n–6, 18:3n–3, 18:3n–6, 18:4n–3, 20:4n–6, 20:5n–3, 22:4n–6 and 22:5n–3 for Elovl5. In addition, to test desaturase activity towards 24:5n–3, yeast co-expressing the zebrafish *elovl2* (Monroig et al., 2009) and the ballan wrasse *fads2* were grown in the presence of 22:5n–3 following the method described previously in detail by Oboh et al. (2017a). Exogenous substrates were added at concentrations of 0.5 mM (C_{18}), 0.75 mM (C_{20}) and 1 mM (C_{22}) as uptake efficiency decreases with increasing chain length (Lopes-Marques et al., 2017). After culture, the yeast cells were harvested, washed and lipid extracted by homogenisation in chloroform/methanol (2:1 v/v) containing 0.001 % butylated hydroxytoluene as antioxidant (Hastings et al., 2001).

Fatty acid analysis

Total lipid extracts from yeast samples (Folch et al., 1957) were used to prepare fatty acid methyl esters (FAME) as described previously (Hastings et al., 2001). FAME samples were analysed using a Fisons GC-8160 (Thermo Fisher Scientific, UK) gas chromatograph equipped with a 60 m x 0.32 mm i.d. x 0.25 μm ZB-wax column (Phenomenex, UK) and flame ionisation detector. The conversion efficiencies of FA substrates added to yeast cultures to FA products were calculated as [all product area / (all product area + substrate area)] × 100. Substrate FA conversion for the Δ6 desaturase activity towards 24:5n−3 was calculated using the same formula as above considering the area of 24:5n−3 produced endogenously by the zebrafish Elovl2 as substrate for calculations.

Results

Sequence and phylogenetic analysis of ballan wrasse fads2 and elovl5

The ORF of the ballan wrasse putative *fads2* and *elovl5* cDNA consisted of 1329 bp and 885 bp, respectively, encoding proteins of 442 aa and 294 aa, respectively. The ballan wrasse putative Fads2 sequence contained all typical features of "front-end" desaturases, namely a cytochrome *b*5-like region, a heme-binding domain (HPGG) and three histidine boxes (HXXXH, HXXHH, QXXHH) (Hashimoto et al., 2008). The putative Elovl5 sequence also contained typical features of the Elovl gene family, including a histidine box (HXXHH) and an endoplasmic reticulum (ER) retrieval signal (KKXRK) at the carboxyl terminus (Jakobsson et al., 2006). Phylogenetic analysis revealed that the ballan wrasse putative Fads2 was strongly grouped (bootstrap value, 91 %) within other Fads2 from Euteleostei species (Fig. 2A). All Teleostei Fads2 were also strongly clustered with a high bootstrap value (97 %) (Fig. 2A). Fads2 from all species included in the analysis were clearly separated from Fads1 of mammals

and cartilaginous fish (bootstrap value, 100 %) (Fig. 2A). The putative Elovl5 was strongly grouped (bootstrap value, 96 %) within other Elovl5 from all gnathostomes (jawed-vertebrates) including humans and cartilaginous fish (elephant shark) (Fig. 2B). Elovl5 and Elovl2 clades were clearly separated from an Elovl4 clade (bootstrap value, 100 %) (Fig. 2B).

Functional characterisation of ballan wrasse Fads2 and Elovl5

The ballan wrasse Fads2 and Elovl5 were functionally characterised by heterologous expression in yeast *S. cerevisiae*. Control transgenic yeast carrying empty pYES2 had no activity towards any of the PUFA substrates tested (data not shown) and showed typical yeast FA profiles comprising of 16:0, 16:1 isomers (16:1n−9 and 16:1n−7), 18:0 and 18:1n–9 (Monroig et al., 2010). Transgenic yeast expressing the ballan wrasse *fads2* was able to desaturate 18:2n–6 and 18:3n–3 to 18:3n–6 and 18:4n–3, respectively, indicating that the encoded enzyme possessed Δ6 desaturase activity (Fig. 3A, Table 2). Moreover, Δ8 desaturase activity towards 20:2n–6 and 20:3n–3 was also detected since these substrates were desaturated to 20:3n-6 and 20:4n-3 (Fig. 3B, Table 2). In addition, the ballan wrasse Fads2 showed Δ6 desaturase activity towards 24:5n–3 to produce 24:6n-3 (Fig. 3C) with a 6.0 % conversion efficiency, whereas that of the control for the co-expression assay (desaturation of 18:3n-3 to 18:4n-3) was 11.6 % (Table 2).

With regards to the ballan wrasse *elovl5*, transgenic yeast showed elongase activity towards C_{18} (18:3n–3, 18:2n–6, 18:4n–3 and 18:3n–6) (Fig. 3A,) and C_{20} (20:4n–6 and 20:5n–3) (Fig. 3B) FA substrates. However, no activity towards C_{22} (22:4n–6 and 22:5n–3) substrates was detected (Fig. 3C). The conversion efficiencies towards both C_{18} and C_{20} PUFA substrates are reported in Table 3.

Discussion

Ballan wrasse is rapidly becoming an important aquaculture species because of its delousing behaviour against sea lice affecting the Atlantic salmon industry in Europe. Diet formulations for ballan wrasse have been developed based on those for other marine species such as Atlantic cod (*Gadus morhua*), with determination of nutrient requirements being mostly estimated by studying the biochemical composition of wild specimens (Hamre et al., 2013). While it was established that dietary DHA:EPA ratio positively correlated with growth performance (Kousoulaki et al., 2015), no studies identifying the EFA requirements for ballan wrasse have been published to date. Therefore, the results herein on the molecular and functional characterisation of two genes encoding key enzymes in the LC-PUFA biosynthetic pathways provide key insight into which EFA will require to be provided in diets for ballan wrasse.

Sequence analysis and phylogenetics confirmed that the two herein cloned cDNAs corresponded to *fads2* and *elovl5* orthologues present in ballan wrasse. Thus, the deduced aa sequence of ballan wrasse *fads*-like cDNA contained several conserved features of all front-end desaturases, which would be important and essential for their function (Hashimoto et al., 2008; Shanklin et al., 2009). In addition, the location of ballan wrasse Fads2 in the phylogenetic tree confirmed that this gene is a *fads2* orthologue. Similarly, the ballan wrasse Elovl5 contained key structural features from this protein family including a histidine box and ER retrieval signal (Jakobsson et al., 2006). Its position in the phylogenetic tree in a cluster containing known Elovl5 from a range of fish (Castro et al., 2016) and non-fish vertebrates (Leonard et al., 2002; Monroig et al., 2016), established that the newly cloned *elovl* cDNA was indeed an *elovl5*.

The functions demonstrated herein by the ballan wrasse Fads2 and Elovl5 suggest that both enzymes operate in a coordinated manner, enabling all the reactions converting 18:2n-6 (LA) and 18:3n-3 (ALA) to 20:3n–6 and 20:4n–3, respectively (Fig. 1). Those conversions can occur through two possible pathways: the "Δ6 pathway" (Δ6 desaturation - elongation) or the "Δ8 pathway" (elongation - Δ8 desaturation) (Fig. 1). For the Δ6 pathway, the ballan wrasse Fads2 exhibited Δ6 desaturase since it was able to convert LA and ALA to 18:3n-6 and 18:4n-3, respectively, the latter two FA being preferred substrates for the ballan wrasse Elovl5 that converts them to 20:3n–6 and 20:4n–3. Alternatively, the Δ8 pathway enabling the biosynthesis of 20:3n–6 and 20:4n–3 can also occur in ballan wrasse with an initial elongation of LA and ALA to 20:2n-6 and 20:3n-3, respectively, the latter two FA being subsequently desaturated by ballan wrasse Fads2 acting at the Δ8 position. It is unclear which of these two routes is more dominant but, according to the conversion efficiencies of both Fads2 and Elovl5 described above for both pathways, it can be assumed that the Δ6 pathway appears to be the preferred route for biosynthesis of 20:3n–6 and 20:4n–3 in this species. Nevertheless, the Δ8 pathway can make significant contributions as reported previously in other marine species (Monroig et al., 2011). Comparing the relative Δ6/Δ8 activities towards n-3 PUFA substrates among Fads2 from a range of fish species, Monroig et al. (2011) postulated that Fads2 from marine fish species had relatively low Δ6/Δ8 desaturation ratios compared to freshwater/salmonid species, thus suggesting that marine fish had greater Δ8 desaturase capability. In the present study, the Δ6/Δ8 activity towards n-3 PUFA substrates, i.e. $\Delta6_{18:3n-3}/\Delta8_{20:3n-3}$, was 5.1, within the range of marine species (Monroig et al., 2011) and therefore confirming that the Δ8 pathway could operate in ballan wrasse with desaturase and elongase capabilities reported herein.

In spite of the two alternative pathways enabling the biosynthesis of 20:3n–6 and 20:4n–3 in ballan wrasse, no evidence for Δ5 desaturation capability, required for the biosynthesis of

20:4n-6 (ARA) and 20:5n-3 (EPA), was found. Consequently, both ARA and EPA can be regarded as EFA for ballan wrasse, and provision in the diet must be ensured to guarantee normal and/or optimum growth. Lack of Δ5 desaturase activity is not uncommon in fish, particularly among teleosts due to their loss of *fads1*, a gene encoding a front-end desaturase with Δ5 activity in other vertebrate groups (Castro et al., 2012; 2016; Kabeya et al., 2017). Some teleost fish have evolved to acquire Δ5 desaturase capacity within their Fads2 (Castro et al., 2016; Kabeya et al., 2017) and thus compensating for the abovementioned loss of the *fads1* gene. However, this is not the case in ballan wrasse as its Fads2, being the sole *fads*-like gene in the genome assembly available for this species (NCBI: GCA_900080235.1), did not shown Δ5 desaturase activity in the yeast expression system.

Our results however demonstrated that the ballan wrasse has some ability to produce DHA from endogenous EPA *via* the so-called "Sprecher pathway", first reported in rats (Sprecher, 2000) and further confirmed in rainbow trout (Buzzi et al., 1997) and zebrafish (Tocher et al., 2003). More recently, Oboh et al. (2017a) demonstrated that this pathway is widespread among fish given that their Fads2 have the ability to desaturate 24:5n-3 to 24:6n-3, a key reaction preceding the final chain-shortening step leading to DHA (Tocher et al., 2003). Consistent with the above, the ballan wrasse Fads2 has the same ability. It is important to note that the Sprecher pathway is dependent upon the supply of the Fads2 substrate 24:5n-3 (Fig. 1). As we have demonstrated here that ballan wrasse Elovl5 is not able to elongate 22:5n-3 to 24:5n-3, and the genome assembly available for ballan wrasse confirmed the lack of an *elovl2*, another mechanism would be required for the provision of the 24:5n-3 required for the Sprecher pathway. This will likely be *via* elongation of 22:5n-3 by Elovl4, enzymes that can be identified in ballan wrasse genomic and transcriptomic databases (data not shown), and that have been demonstrated to be capable of elongating 22:5n-3 in all fish species investigated (Monroig et

al., 2010; Jin et al., 2017; Oboh et al., 2017b). Since the ballan wrasse Fads2 characterised herein did not show Δ4 desaturase capability, it can be deduced that DHA biosynthesis in ballan wrasse can only occur through the Sprecher pathway. Our findings thus suggest that DHA is technically not an essential fatty acid in ballan wrasse since it can be produced endogenously provided the diet contains EPA. However, it is likely that physiological demands for DHA in ballan wrasse exceed its biosynthetic capability, particularly in early life-cycle stages with rapidly forming neural tissues accumulating DHA (Monroig et al., 2009), and partly explaining why high dietary DHA:EPA positively correlated with growth performance of ballan wrasse juveniles (Kousoulaki et al., 2015).

Based on the complement and function of enzymes involved in the biosynthesis of physiologically critical fatty acids, the present study has enabled us to confirm that ARA and EPA, and to some extent also DHA, are required in diets for ballan wrasse. As hypothesised for carnivorous marine fish species, the limited biosynthetic capability observed herein for ballan wrasse is probably the result of an evolutionary adaptation to natural diets providing sufficient levels of these key biologically active LC-PUFA. The major natural food items for ballan wrasse include benthic invertebrates such as molluscs, crabs and sea urchins (Deady and Fives, 1995; Figueiredo et al., 2005). Consistent with this hypothesis, the fatty acid profiles of *Carcinus maenas* (green crab) and *Mytilus edulis* (blue mussel), important natural preys for the ballan wrasse, showed high levels of LC-PUFA, particularly EPA and DHA, although they have relatively low lipid content (Naczk et al., 2004; Fernández et al., 2015). Interestingly, sea lice contain some essential nutrients including LC-PUFA (Tocher et al., 2010) suggesting that ballan wrasse can obtain those nutrients from preying on sea lice when co-cultured in salmon cages. However, supply of additional feed is still required to satisfy the ballan wrasse stocks

and, according to the present results, such diets must include pre-formed ARA, EPA and DHA to ensure health status of fish.

In conclusion, the present study demonstrated that ballan wrasse possess a *fads2* gene encoding an enzyme with Δ6 and Δ8 desaturase capabilities. The ballan wrasse Fads2 also had the ability to desaturate 24:5n-3 to 24:6n-3, a key reaction required for DHA biosynthesis from EPA *via* the Sprecher pathway. Moreover, it was confirmed that ballan wrasse have an Elovl5 that enables elongation of a range of PUFA substrates with chain lengths of C_{18} and C_{20}. Overall, the functional characterisation results of the ballan wrasse Fads2 and Elovl5 established that dietary EPA and ARA will be required to satisfy essential fatty acid requirements for this species. Furthermore, although the data confirmed that ballan wrasse has the ability to biosynthesise DHA from endogenous EPA, the fact that high dietary DHA:EPA ratio improved growth performance suggested that physiological demands for DHA in ballan wrasse exceed biosynthetic capability and so dietary DHA is also required.

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Figures and legends

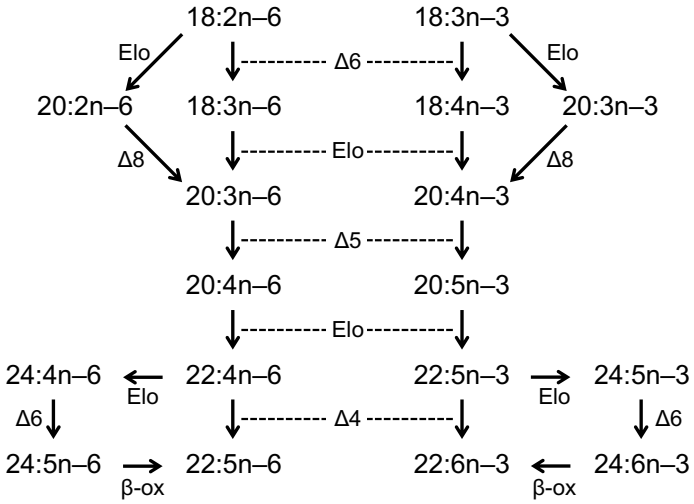

Fig 1. The biosynthetic pathways of LC-PUFA in vertebrates. "Δx" refers to desaturation activities by Fads enzymes, whereas "Elo" are reactions catalysed by Elovl enzymes.

B-ox, beta-oxidation

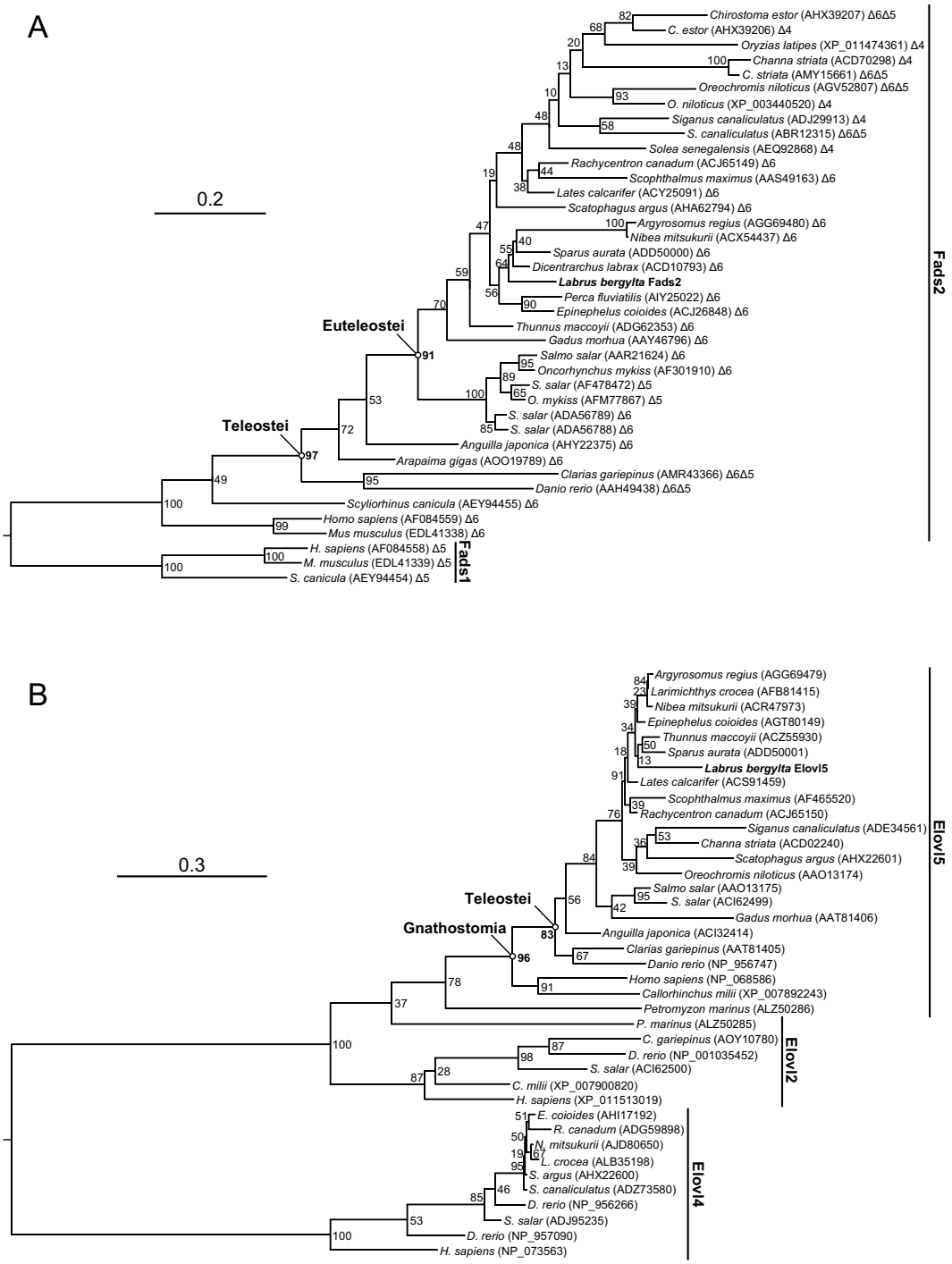

Fig 2. Maximum likelihood phylogenetic tree of Fads (A) and Elovl (B) sequences. Types of Fads and Elovl enzymes are indicated accordingly in each panel. The analyses were performed in PhyML v3.0 server (Guindon et al., 2010). The protein evolutionary model was calculated in PhyML using smart model selection resulting in JTT +G +I +F and the number of bootstrap replicates was set to 1000. The bootstrap values are presented as %.

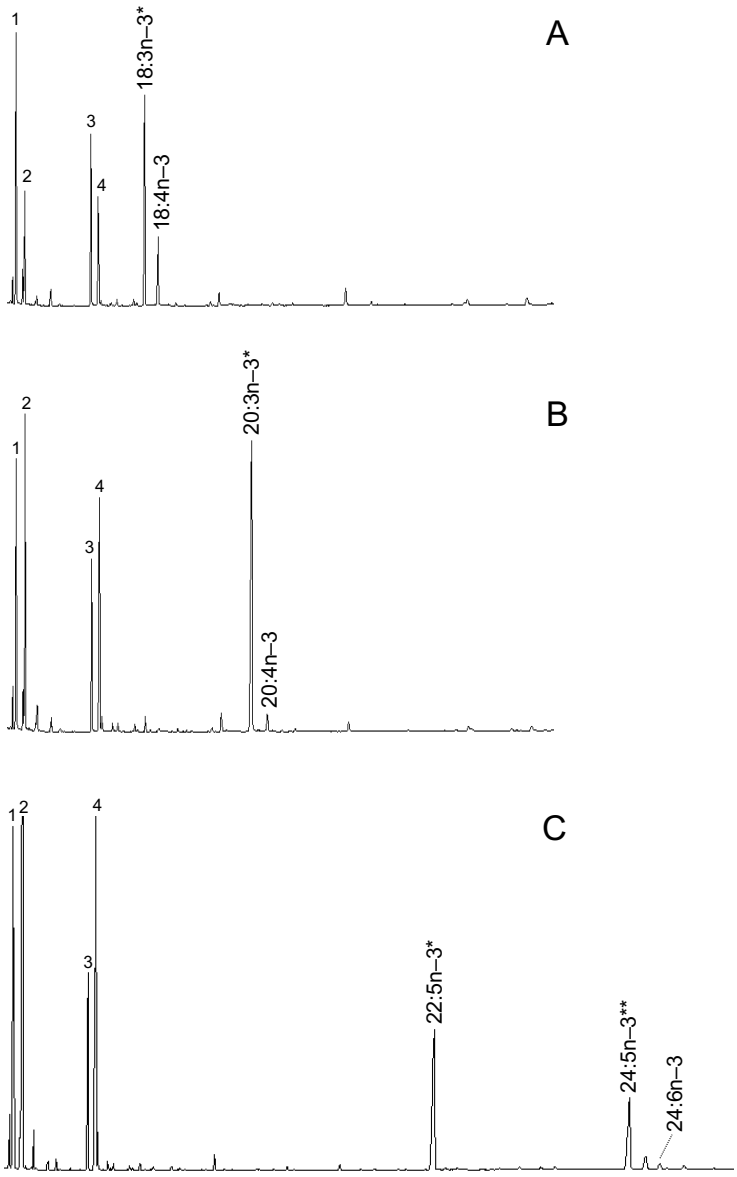

Fig 3. Functional characterisation of the *Labrus bergylta* Fads2 in yeast *Saccharomyces cerevisiae*. Fatty acid (FA) profiles of yeast transformed with ballan wrasse pYES2-Fads2 and grown in the presence of FA substrates 18:3n–3 (A) and 20:3n–3 (B). (C) FA profile of yeast co-expressed of *Danio rerio elovl2* and ballan wrasse *fads2* and grown in the presence of 22:5n–3. All exogenously added FA peaks are indicated as *. The elongated FA peak by Elovl2 is indicated as ** (C). Peaks 1-4 in all panels are the yeast endogenous FA, namely 16:0 (1), 16:1 isomers (2), 18:0 (3) and 18:1n-9 (4). Horizontal axis represents retention time, vertical axis represents FID response.

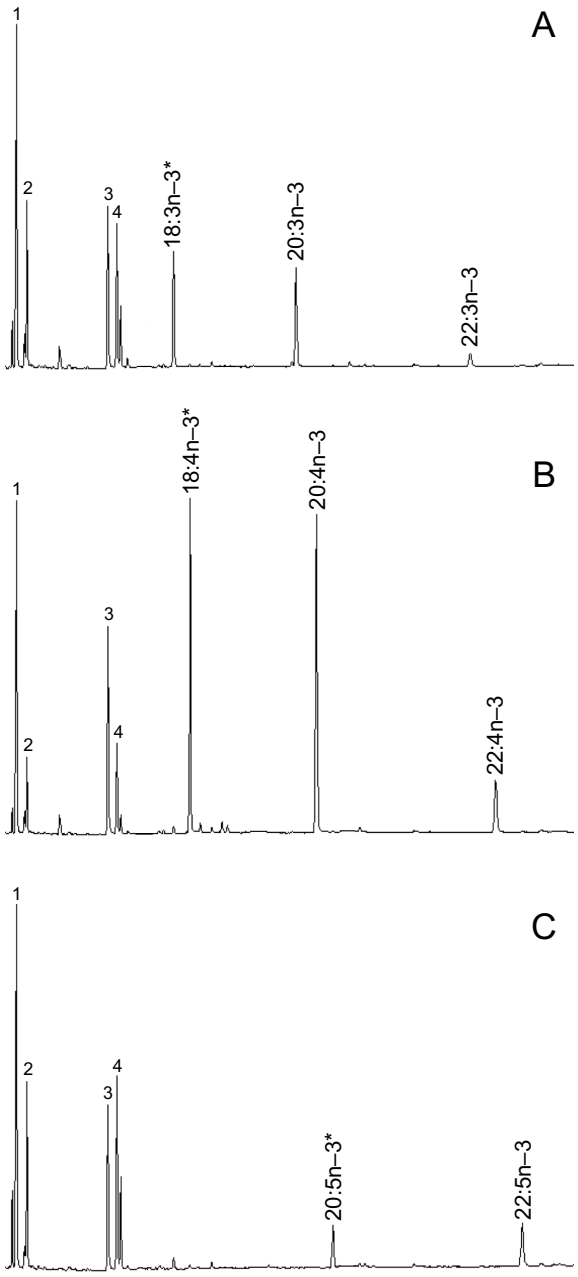

Fig 4. Functional characterisation of *Labrus bergylta* Elovl5 in yeast *Saccharomyces cerevisiae*. Fatty acid (FA) profiles of yeast transformed with ballan wrasse pYES2-Elovl5 and grown in the presence of FA substrates 18:3n–3 (A), 18:4n–3 (B) and 20:5n–3 (C). All exogenously added FA peaks are indicated as *. Peaks 1-4 in all panels are the yeast endogenous FA, namely 16:0 (1), 16:1n-7 and 16:1n-9 (2), 18:0 (3) and 18:1n-9 (4). Horizontal axis represents retention time, vertical axis represents FID response.

Tables

Table 1. Sequences for the primers designed for the molecular cloning of *Labrus bergylta fads2* and *elovl5*. In primers used for amplification of the whole open reading frame (ORF) sequence, restriction sites are underlined, while the start and stop codons are in bold.

Target	Name	Sequence
First fragment	LBElovlF2	5'-TGGATGGGGCCCAAGTACATGAA-3'
	LBElovlR2	5'-ACCGACATGAAGGTTTATTCAATGTAC-3'
5' RACE	LBElovlR4	5'-GGCCCAGATTGTAGAGCACC-3'
	LBElovlR6	5'-CCACCACAGGACGTGTGCG-3'
3' RACE	LBElovlF3	5'-TACCGACTCTCCGGCATCATA-3'
	LBElovlF4	5'-CCGAGTCGACATTGACACAC-3'
ORF	LBE5VF	5'-CCCGGATCCAAA**ATG**GAGACCTTCAATCATAAAC-3'
	LBE5VR	5'-CCGCTCGAG**TCA**ATCAAATGTCAATCCATC-3'
	LBF2VF	5'-CCCAAGCTTAGG**ATG**GGGGGTGGAGG-3'
	LBF2VR	5'-CCGTCTAGA**TCA**TTTGTGGAGATACGCATCCA-3'

Table 2. Substrate conversions by the *Labrus bergylta* Fads2 expressed in *Saccharomyces cerevisiae* and grown in the presence of exogenously added fatty acid substrates (18:3n–3, 18:2n–6, 20:3n–3, 20:2n–6, 20:4n–3, 20:3n–6, 22:5n–3 and 22:4n–6). Conversion percentages were calculated by the formula [(all product peak areas / all product peak areas + substrate peak area) × 100].

Substrate	Product	Conversion (%)	Activity
18:2n–6	18:3n–6	12.6	Δ6
18:3n–3	18:4n–3	26.1	Δ6
20:2n–6	20:3n–6	1.9	Δ8
20:3n–3	20:4n–3	5.1	Δ8
20:3n–6	20:4n–6	ND	Δ5
20:4n–3	20:5n–3	ND	Δ5
22:4n–6	22:5n–6	ND	Δ4
22:5n–3	22:6n–3	ND	Δ4
Expressed with Elovl2			
18:3n–3	18:4n–3	11.6	Δ6
24:5n–3	24:6n–3	6.0	Δ6*

Table 3. Substrate conversion capability of *L. bergylta* Elovl5 expressed in *Saccharomyces cerevisiae* and grown in the presence of exogenously applied PUFA substrates (18:3n–3, 18:2n–6, 18:4n–3, 18:3n–6, 20:5n–3, 20:4n–6, 22:5n–3 and 22:4n–6) one substrate per culture. Conversion percentage was calculated by the formula [(Product peak areas/product peak areas + substrate peak area) × 100].

FA substrate	FA product	% conversion
18:3n–3	20:3n–3	55.94
18:2n–6	20:2n–6	41.72
18:4n–3	20:4n–3	63.67*
18:3n–6	20:3n–6	69.76*
20:5n–3	22:5n–3	60.35
20:4n–6	22:4n–6	37.87
22:5n–3	24:5n–3	Not detected
22:4n–6	24:4n–6	Not detected

*Elongation FA products 20:4n–3 and 20:3n–6 consist of 18:4n–3 and 18:3n–6, respectively, were themselves elongated to secondary products 22:4n–3 and 22:3n–6, respectively.