

Is $\omega 6$ docosapentaenoic acid an essential fatty acid during early ontogeny in marine fauna?

Abstract—A thraustochytrid marine protist (*Schizochytrium* sp.) was fed to rotifers (*Branchionus plicatilis*), which in turn, were fed to cod larvae (*Gadus morhua*). Samples of larvae 1 and 11 d after hatch, the rotifer diet, and the enrichment were collected for molecular and isotope analyses of fatty acids. *Schizochytrium* sp. had unusually high proportions of $\omega 6$ DPA ($8.6\% \pm 0.6\%$), which was reflected in the rotifers fed this protist ($8.7\% \pm 0.2\%$). This fatty acid was also unusually ^{13}C -enriched in both the protists ($-11.63\text{‰} \pm 0.11\text{‰}$) and the rotifers ($-11.83\text{‰} \pm 0.39\text{‰}$). The proportions of $\omega 6$ DPA were very low in prefeeding cod larvae but they increased 30-fold by d 11; however, $\omega 6$ DPA showed the smallest $\delta^{13}\text{C}$ change from the protist source. This, combined with reports of significantly higher growth rates in cod and scallops fed diets rich in this fatty acid, provide strong evidence for $\omega 6$ DPA being essential at least in the early life stages of these two very different groups.

It is generally believed that arachidonic acid (ARA; 20:4 $\omega 6$), eicosapentaenoic acid (EPA; 20:5 $\omega 3$), and docosahexaenoic acid (DHA; 22:6 $\omega 3$) are the most important long-chain (C_{20} – C_{22}) polyunsaturated fatty acids (PUFA) in mammals (e.g., Simopoulos 2002), fish (Sargent et al. 1999; Montero et al. 2003, 2004), and aquatic food webs (Müller-Navarra et al. 2000, 2003). They must be supplied to animals in their diet, although some animals can synthesize at least some of them when sufficient quantities of the correct polyunsaturated precursors are available. In mammals, consumption of the $\omega 3$ fatty acids EPA and DHA has beneficial effects on several diseases including inflammatory and autoimmune diseases (Simopoulos 2002), and even on adipose tissue hypertrophy (Parrish et al. 1990). The main source of EPA and DHA is the aquatic food web, where they have very important functions as well, at various trophic levels (Müller-Navarra et al. 2000, 2003; Wacker et al. 2002). In finfish, ARA, EPA, and DHA are required for normal growth, survival, neural development, pigmentation, and reproduction (Sargent et al. 1999), whereas EPA and DHA are important in stress resistance (Montero et al. 2003) and immunity (Montero et al. 2004). Dietary DHA is also needed for finfish schooling behavior (Masuda and Tsukamoto 1999) and growth in shellfish (Wacker et al. 2002). Although ARA, EPA, and DHA are often quantitatively dominant, a number of $\omega 3$ and $\omega 6$ long-chain PUFA are commonly found in lipid extracts, especially from aquatic food webs, which may be nutritionally important as well. Indeed, $\omega 6$ DPA (docosapentaenoic acid; 22:5 $\omega 6$) has recently been shown to be important for growth in different scallop species (Milke et al. 2006 and references therein).

PUFA are usually synthesized by a series of desaturations and elongations, but the $\omega 3$ and $\omega 6$ series are not

interconvertible in animals except in transgenic animals (Kang et al. 2004). Other pathways for PUFA synthesis do not require desaturation and elongation of saturated fatty acids. *Schizochytrium* sp. is a thraustochytrid marine protist that synthesizes $\omega 3$ and $\omega 6$ long-chain PUFA de novo using polyketide synthases instead (Metz et al. 2001). It is a common marine microheterotroph, closely aligned with algae, with a wide geographic distribution (Lewis et al. 1999). This protist also forms the basis of a commercial product that is widely used as a feed in aquaculture where it is fed to rotifers (*Branchionus plicatilis*) and *Artemia* sp. that in turn are routinely used as live feeds for fish larvae. In the present study we determined the molecular and stable isotopic composition of long-chain fatty acids in *Schizochytrium* sp., rotifers, and cod (*Gadus morhua*) larvae to examine the effects of trophic transfer on PUFA.

Feeding experiment—Fertilized Atlantic cod eggs (*G. morhua*) were collected from broodstock kept at the Aquaculture Research and Development Facility of the Ocean Sciences Centre. Larvae (4.75 mm long) from the same batch were stocked in 3-m³ tanks at 50 larvae L⁻¹. Temperature was maintained at $10 \pm 1^\circ\text{C}$, salinity at 30 ± 0.5 , and light was gradually increased from 800 to 2000 lux over the first 4 d. Larvae were fed enriched rotifers (*B. plicatilis*) from d 3 to d 10 after hatch, three to four times a day, maintaining a prey concentration of 4,000 L⁻¹ (Puvanendran et al. unpubl.). Rotifers, which contain $\leq 0.2\%$ long-chain PUFA (Bell et al. 2003), were enriched with Algamac 2000 (100% spray-dried *Schizochytrium* sp.) in 300-liter cones at a density of 5×10^5 L⁻¹ at 25°C. Triplicate samples for molecular and isotope analyses of fatty acids were collected from larvae 1 d and 11 d after hatch (100–125 larvae sample⁻¹), from the rotifer diet, and from the enrichment.

Lipid extraction and derivatization—Lipids were extracted using a modified Folch method (Parrish 1999). A combination of chloroform, methanol, and purified water was added to each sample creating upper inorganic and lower organic layers. The lower, organic layer, which contains the lipids, was then removed using a double-pipetting technique. Samples were stored in a -20°C freezer under nitrogen. Total lipid was determined using the Chromarod-Iatroscan system (Parrish 1999), and a portion of each extract was then derivatized using a mixture of $\text{BF}_3/\text{CH}_3\text{OH}$ at 85°C for 90 min under nitrogen. We have found that this procedure esterifies more than 90% of acyl lipids. Purified water and hexane were then added. The upper, organic layer was transferred to a 2-mL vial, leaving behind the unwanted lower aqueous layer. The resulting methylated fatty acids (FAME) were stored at -20°C under nitrogen.

Table 1. Polyunsaturated fatty acid proportions (% total fatty acids \pm SD, $n = 3$) in the protist *Schizochytrium* sp., the rotifer *B. plicatilis*, and in cod (*G. morhua*) larvae at d 1 (Prefed) and d 11 after hatch (Fed), and their ratios (Rotifer:Protist, Fed:Prefed).

	Prefed cod	Protist	Rotifer	Fed cod	Rotifer:Protist	Fed:Prefed
ARA	1.3 \pm 0.0 ^{ab}	0.7 \pm 0.0 ^a	1.5 \pm 0.0 ^b	3.5 \pm 0.5 ^c	2.1	2.7
EPA	14.6 \pm 0.5 ^a	0.6 \pm 0.0 ^b	2.0 \pm 0.0 ^b	4.7 \pm 1.2 ^c	3.3	0.3
ω 6DPA	0.2 \pm 0.0 ^a	8.6 \pm 0.6 ^b	8.7 \pm 0.2 ^b	6.0 \pm 0.1 ^c	1.0	30
DHA	32.4 \pm 0.5 ^{ac}	25.0 \pm 0.3 ^b	27.2 \pm 0.3 ^{bc}	36.1 \pm 4.5 ^a	1.1	1.1
Σ PUFA	52.7 \pm 0.9 ^{ac}	36.8 \pm 1.1 ^b	48.0 \pm 0.3 ^c	55.1 \pm 4.5 ^a	1.3	1.0

Mean proportions in the same row with different superscripts are significantly different ($p < 0.05$) from all other means in the same column.

FAME analyses—The FAME were quantified on a Varian 3400 gas chromatograph (GC) equipped with a flame ionization detector (FID). Peak identifications were confirmed on a Varian Saturn 3800 GC connected to a Varian 2000 mass spectrometer. Samples were run in EI mode over a range of 40 to 650 m/z , scanning at a rate of 0.75 s scan⁻¹. The identity of ω 6DPA was confirmed by careful reference to retention data (Ackman 1986) and to mass spectral patterns (Christie 2006). Ackman (1986) shows chromatograms and gives retention times for gas chromatography of FAME, including those found in one of the standards (PUFA-1, Supelco Inc.) routinely used here, whereas Christie (2006) provides a comprehensive library of mass spectra for FAME including the two isomers of DPA and an adjacent ω 6 peak. By using the same phase (Omegawax) on the GC/FID column and the gas chromatography/mass spectrometry column the identification is straightforward. In addition to the normal characteristic ions (e.g., the McLafferty rearrangement ion at m/z 74 and the molecular ion at m/z 344), the key identifier in the FAME mass spectrum for the ω 6 isomer of DPA is the ion at m/z 150. In Algamac powder, rotifers enriched with Algamac powder, and cod fed these rotifers, the intensity of the m/z 150 ion ranged from 10% to 15% of the base peak, whereas the abundance of the ω 3 identifier, m/z 108, was 4%–5%.

The FAME carbon isotope ratios ($\delta^{13}\text{C}$, ‰) were determined, relative to the Vienna PDB standard, after combustion at 850 °C, in a continuous flow isotope ratio mass spectrometer (Finnigan MAT 252; Veefkind, 2003). FAME were separated on a Supelco SPB-PUFA column (30 m \times 0.25 mm internal diameter \times 0.2 μm film) in a Varian 3400 GC with the outlet connected to the combustion chamber. Determination of natural abundance isotope ratios by GC-combustion-isotope ratio mass spectrometry is a refinement of the bulk isotope approach commonly used in food web studies (e.g., Canuel et al. 1995). The measured natural carbon isotope compositions for esters are reported as $\delta^{13}\text{C}$:

$$\delta^{13}\text{C}_{\text{sample}} = 1,000 \times \left\{ \left(\frac{^{13}\text{C}/^{12}\text{C}_{\text{sample}}}{^{13}\text{C}/^{12}\text{C}_{\text{PDB}}} \right) - 1 \right\}$$

$\delta^{13}\text{C}$ values, in parts per thousand, are used to describe the small variations in the relative isotope abundances. Standardization is accomplished by comparing integrated $^{13}\text{C}:^{12}\text{C}$ for each compound peak with similar ratios from pulses of reference CO_2 gas introduced before and after the sample chromatographic window. All fatty acid $\delta^{13}\text{C}$ data were corrected for the contribution made by the derivatiz-

ing agent, BF_3/MeOH , whose ratio, determined by bulk isotope ratio mass spectrometry (Finnigan Delta Plus XL Thermo Quest), was on average, -38.23% .

Results and discussion—Each step in the protist→rotifer→cod food chain is accompanied by an increase in Σ PUFA proportions (Table 1) showing how important these fatty acids are to each organism. ARA, EPA, ω 6DPA, and DHA comprised most of the PUFA with at least three of the four ranking within the top four most abundant PUFA. All the other PUFA together accounted for only 1.9–8.6% of total fatty acids. In the transfer from protist to rotifer, proportions of ARA, EPA, ω 6DPA, and DHA all increase in absolute and relative terms, but the largest relative increase was in EPA ($3\frac{1}{2}\times$) probably because of some retroconversion from DHA as seen in *Artemia* sp. (Han et al. 2001). The same β -oxidation pathway may have been operating on ω 6DPA to produce ARA, but to a lesser extent, because the absolute and relative increases were smaller for ARA (0.8% and $2\times$), although the difference is significant ($p = 0.02$; ANOVA, Tukey).

In long-term feeding experiments, comparisons between fatty acid proportions in diets and tissues can be used to infer the essential nature of fatty acids in early life history stages (e.g., Milke et al. 2006 and references therein); however, the PUFA transfer from rotifers to cod is complicated by the PUFA-containing yolk sac in newly hatched larvae. Although the larvae grew at $>1\%$ d⁻¹ and there was a significant ($p = 0.041$) increase in lipid content between d 1 and d 11 (5.64 ± 0.20 to $7.06 \pm 0.80 \mu\text{g}$ larva⁻¹), this difference amounts to only 25%. Thus, while EPA appears to have more than doubled its proportions in fed cod by comparison with its food, it has actually decreased to one-third of what was originally present in the larvae. Clearly, the other PUFA are more important for the growing larvae. The similarity in Σ PUFA proportions between d 1 and d 11 suggests a major effect of feeding on the cod larval lipids is a remodeling of membrane phospholipids.

Schizochytrium sp. had unusually high proportions of ω 6DPA by comparison with most algae (Milke et al. 2006), which are reflected in the rotifers that are fed this protist (Table 1). All the fatty acids in *Schizochytrium* sp. are also unusually ^{13}C -enriched, which is again reflected in the stable isotope ratios ($\delta^{13}\text{C}$) of the rotifer (Table 2). The weighted mean of $-8.22\% \pm 0.35\%$ for all fatty acids in the protist is probably the result of the use of the abbreviated polyketide synthase pathway for fatty acids

Table 2. $^{13}\text{C}:^{12}\text{C}$ ratios ($\delta^{13}\text{C}:\text{‰} \pm \text{SD}$, $n = 3$) in individual PUFAs and overall weighted means in the protist *Schizochytrium* sp., the rotifer *B. plicatilis*, and in cod (*G. morhua*) larvae at d 1 (Prefed) and d 11 after hatch (Fed), and the difference between *Schizochytrium* sp. and *G. morhua* (Protist-fed cod).

	Prefed cod	Protist	Rotifer	Fed cod	Protist-fed cod
ARA	-27.26 ^a	-12.42 \pm 0.61 ^b	-14.23 \pm 0.22 ^c	-18.68 \pm 0.73 ^d	6.26
EPA	-27.02 \pm 0.17 ^a	-11.93 \pm 0.46 ^b	-13.56 \pm 0.21 ^c	-21.29 \pm 0.20 ^{d*}	9.36
ω 6DPA	n.d.	-11.63 \pm 0.11 ^a	-11.83 \pm 0.39 ^a	-12.40 \pm 1.17 ^{a*}	0.77
DHA	-26.24 \pm 0.25 ^a	-10.50 \pm 0.29 ^{b*}	-11.07 \pm 0.20 ^b	-15.19 \pm 0.32 ^{c*}	4.69
Mean	-26.11 \pm 0.12 ^a	-8.22 \pm 0.35 ^{b*}	-12.44 \pm 0.99 ^c	-17.70 \pm 1.33 ^d	9.48

Mean ratios in the same row with different superscripts are significantly different ($p < 0.05$). Mean ratios in the same column with asterisks are significantly different ($p < 0.05$).

(Metz et al. 2001), with fewer synthetic steps causing less kinetic fractionation of the isotope. A bulk determination of $-9.06\text{‰} \pm 0.10\text{‰}$ was made on three total lipid extracts of the protist to confirm the lack of isotopic segregation. This makes this commercially available powder that is widely used in aquaculture extremely useful for trophic studies and especially those involving ω 6DPA. This fatty acid showed a remarkable 30-fold increase in cod larvae between d 1 and d 11 (Table 1) and it showed the smallest $\delta^{13}\text{C}$ change from the protist source (Table 2). In fact, ω 6DPA showed the smallest differences among 17 fatty acids for which stable isotope data were available in all three sample types. Other fatty acids (ARA and DHA) were also proportionally enriched in the larvae (Table 1), but to a much lesser extent (less than 3-fold), and overall PUFA levels increased by $<3\%$ of total fatty acids. All PUFA in d 11 larvae except ω 6DPA had isotope ratios that were intermediate between d 1 larvae and the rotifer food, although with the exception of EPA, they were closer in composition to that of the food.

The small change in the stable isotope ratio ($\Delta\delta^{13}\text{C}$) of ω 6DPA in the protist \rightarrow rotifer \rightarrow cod larva food chain ($p > 0.4$) combined with the large difference in the ω 6DPA $\delta^{13}\text{C}$ compared with the mean and other PUFA in fed cod ($p < 0.02$) shows they do not synthesize ω 6DPA from other fatty acids (Table 2). When it is made available in the diet, larval cod take it up rapidly and show significantly higher growth rates (Garcia et al. 2005; Puvanendran et al. unpubl. data). Larvae fed *Schizochytrium*-enriched *Artemia* had significantly higher growth rates at d 59 (13.7% d^{-1} ; $p < 0.001$) than larvae fed other diets or diet combinations in duplicate tanks (Garcia et al. 2005). In an experiment conducted in parallel to that described here, the larvae fed the *Schizochytrium*-enriched rotifers had a higher standard length (5.49 ± 0.20 mm) after 15 d than those fed *Isochrysis galbana*-enriched diets in duplicate (Puvanendran et al. unpubl. data). The difference became significant after 29 d ($p < 0.002$). Add to this extensive bioaccumulation of ω 6DPA linked to improved growth in scallop species fed diets rich in this fatty acid (Milke et al. 2006 and references therein) and there is strong evidence for ω 6DPA being essential at least in the early life stages of these two very different groups. The stable isotope data point to this fatty acid as being an essential nutrient rather than an essential metabolite, but it may be conditionally essential according to life stage or availability of other fatty acids. This PUFA may play an important structural role in

membranes or it may be a precursor of bioactive docosanoids (or both). Recently, the C_{22} DHA has been found to be a precursor of bioactive compounds generated via enzymatic oxygenations (Hong et al. 2003). The same enzymes could work on the C_{22} ω 6DPA to form a parallel series of competitive products as found with the C_{20} EPA and ARA (Simopoulos 2002).

It has been speculated that PUFA originally synthesized by the polyketide synthase pathway may be a significant contributor to PUFA in fish (Metz et al. 2001). We show here a direct connection between PUFA derived from the polyketide synthase pathway and fish PUFA and provide a molecular marker (ω 6DPA) and an isotopic marker (high $\delta^{13}\text{C}$) of the transfer. It is important that we determine other sources of and requirements for ω 6DPA in aquatic food webs, as well as the $\delta^{13}\text{C}$ of individual fatty acids to determine the influence of the polyketide synthase pathway in general.

Christopher C. Parrish

Ocean Sciences Centre
Memorial University of Newfoundland, St. John's
Newfoundland, A1C 5S7, Canada

Michael Whitticar

School of Earth and Ocean Sciences
University of Victoria, Victoria
British Columbia V8W 2Y2, Canada

Velmurugu Puvanendran

Ocean Sciences Centre
Memorial University of Newfoundland, St. John's
Newfoundland A1C 5S7, Canada

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