Betancor M, Ortega A, de la Gandara F, Tocher DR & Mourente G (2017) Molecular aspects of lipid metabolism, digestibility and antioxidant status of Atlantic bluefin tuna (T. thynnus L.) larvae during first feeding, *Aquaculture*, 479, pp. 357-369.

DOI: 10.1016/j.aquaculture.2017.06.011

Molecular aspects of lipid metabolism, digestibility and antioxidant status of Atlantic bluefin tuna (*T. thynnus* L.) larvae during first feeding

Mónica B. Betancor[a*], Aurelio Ortega[b], Fernando de la Gándara[b], Douglas R. Tocher[a] and Gabriel Mourente[c]

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

[b] *Planta Experimental de Cultivos Marinos, Instituto Español de Oceanografía (IEO), 30860 Puerto de Mazarrón (Murcia), Spain*

[c] *Departamento de Biología, Facultad de Ciencias del Mar y Ambientales, Universidad de Cádiz, 11510 Puerto Real, Cádiz, Spain*

* Corresponding author:

Tel.: +44-1786-467993

E-mail address: m.b.betancor@stir.ac.uk

Abstract

Atlantic bluefin tuna (*Thunnus thynnus* L.; ABT) larvae were fed on enriched rotifers *Brachionus rotundiformis* and copepod nauplii *Acartia tonsa* from first feeding to 15 days post hatching. Rotifers were enriched with five different commercial products: OG, MG, AG and RA plus selenium and vitamin E. Copepods (COP) were cultured with the algae *Rhodomonas baltica*. Metabolic processes were studied by determining the expression of 30 genes related to lipid metabolism (transcription factors, fatty acid metabolism and lipid homeostasis), antioxidant enzymes, myogenesis and digestive enzymes. Growth and development parameters and high expression of myogenesis genes *myhc2* and *tropo* indicated that COP were better than enriched rotifers as live prey for first feeding ABT. COP and AG-fed larvae showed the lowest values for the transcription factors *pparγ* and *srebp2*. The expression of *fas* showed differences among treatments, with highest relative expression in COP-fed larvae and those fed with RA rotifers. In relation to fatty acid catabolism, larvae fed RA had the highest *aco* expression levels, with the lowest observed in those fed COP. The expression profiles of lipid homeostasis genes showed that larvae fed COP had higher *fabp2* and *4* expressions. Larvae fed AG showed the lowest *lpl* expression levels, with highest values observed in larvae fed OG. Regarding antioxidant enzyme gene expression, *sod* showed highest values in larvae fed COP and RA, with larvae fed MG rotifers showing lowest expression levels. A similar pattern was observed for the expression of *cat* and *gpx1* and *4*. The expression of genes for digestive enzymes showed that *tryp* expression levels were highest in COP-fed larvae but, in contrast, COP-fed larvae showed the lowest *anpep* and *alp* levels. ABT larvae fed AG displayed the lowest expression level of *pla2*. *bal1* and *bal2* presented similar expression patterns, with highest values in COP-fed ABT and lowest expression in larvae fed AG rotifers. Copepods were a superior live prey for first feeding ABT larvae compared to enriched rotifers, as indicated by the higher growth and flexion index achieved by COP-fed larvae, possibly reflecting the higher protein content of the copepods.

Keywords: Bluefin tuna, larvae, rotifers, copepods, lipid metabolism, gene expression.

Abbreviations: ABT, Atlantic bluefin tuna; *aco*, acyl coA oxidase; *alp*, alkaline phosphatase; *amy*, amylase; *anpep*, amino peptidase; ARA, arachidonic acid (20:4n-6); *bal1*, bile salt activated lipase 1; *bal2*, bile salt activated lipase 2; C, free cholesterol; *cat*, catalase; *cpt1*, carnitine palmitoyl transferase I; dah, days after hatch; DHA, docosahexaenoic acid (22:6n-3); EPA, eicosapentaenoic acid (20:5n-3); *elovl5*, fatty acyl elongase 5; *fabp2*, fatty acid binding protein 2 (intestinal); *fabp*4, fatty acid binding protein 4 (adipocyte); *fabp7*, fatty acid binding protein 7 (brain-type); *fads2d6*, delta-6 fatty acyl desaturase; FAME, fatty acid methyl ester; *fas*, fatty acid synthase; *gpx1*, glutathione peroxidase 1; *gpx4*, glutathione peroxidase 4; *hmgcl*, 3-hydroxy-3-methylglutaryl-CoA lyase; HPLC, high-pressure liquid chromatography; HPTLC, high performance thin-layer chromatography; LC-PUFA, long-chain polyunsaturated fatty acid; *lpl*, lipoprotein lipase; *lxr*, liver X receptor; *myhc*, myosin heavy chain; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; *pl*, pancreatic lipase; *pla2*, phospholipase A_2; *pparα,* peroxisome proliferator-activated receptor alpha; *pparγ,* peroxisome proliferator-activated receptor gamma; PS, phosphatidylserine; qPCR, quantitative real-time PCR; *rxr*, retinoid X receptor; SE, steryl ester; *sod*, superoxide dismutase; *srebp1*, sterol regulatory element-binding protein 1; *srebp2*, sterol regulatory element-binding protein 2; TAG, triacylglycerol; *tropo*, tropomyosin; *tryp*, trypsin.

1. Introduction

There are still many issues that require to be investigated and solved in the rearing of larvae and juveniles of Atlantic bluefin tuna (*Thunnus thynnus*; ABT) to prevent "mass-mortality" during these early developmental stages. The supply of viable eggs and optimising the nutritional value of live prey (e.g. rotifers, *Artemia*, copepods, fish yolk-sac larvae), larval and juvenile diets are essential to establish full-cycle culture technology for ABT. To date, standard live feeds and artificial diets feeding protocols for larval and juvenile of ABT are associated with poor survival, growth and stress resistance. Moreover, low swimbladder inflation rates, surface and sinking deaths, dispersed sizes, malformations and tank wall collisions are common issues, not only in ABT culture, but also in other tuna species (Sawada et al., 2005; Yasunori, 2012). Initial data related to the feeding sequence of ABT larvae suggested that mortality observed during the first stages of life could be due partly to nutritional deficiencies (De La Gandara et al., 2010; Partridge, 2013; Betancor et al., 2016). In this respect, researchers have described three critical periods of mortality in Pacific bluefin tuna larvae: the first during the initial 10 days after hatching (dah), the second between 14 and 30 dah, and the third from 30 to 60 dah (Partridge, 2013). The causative factors of mortality at each stage included physical and nutritional factors during the first stage, cannibalism and nutritional factors in the second stage and collision mortality in the third (Partridge, 2013).

In fish, lipids and their constituent fatty acids (FA), play key roles in promoting and/or maintaining optimum growth, survival, feed eficiency, health, neural and visual development, and response to stressors in addition to generally being the main energy source (Sargent et al., 1989; 2002; Tocher, 2003; 2010). Among the lipids and their constituents, phospholipids and long-chain polyunsaturated fatty acids (LC-PUFA) are particularly important due to their critical roles in the physiological processes above mentioned. Appropriate uptake and accumulation of lipids improve growth and survival of all fish but are particularly important in highly active migratory predator fish species such as tuna (Mourente and Tocher, 2003; 2009). Additionally, omega 3 (n-3) LC-PUFA, such as eicosapentaenoic acid (EPA; 20:5n-3) and docosahexaenoic acid (DHA; 22:6n-3), are

required by most marine fish and are essential fatty acids for survival, normal growth and development (Tocher, 2010). There has been considerable research interest in the key enzymes of the LC-PUFA biosynthetic pathway, fatty acyl desaturases (Fads) and elongases of very long chain fatty acids (Elovl) in fish species including tuna (Gregory et al., 2010; Morais et al., 2011; Betancor et al., 2016). Furthermore, the capacity for endogenous synthesis of EPA and DHA is limited in ABT and so the lipid biochemistry underpinning the high tissue DHA and DHA/EPA ratio is unclear (Gregory et al., 2010; Morais et al., 2011; Scholefield et al., 2015).

The regulation of lipid homeostasis in fish is a complex balance between lipid uptake, transport, storage, energy utilization and biosynthesis with each process being controlled independently and also in conjunction with other processes (Tocher, 2003). Thus, studying the impact of dietary lipid on lipid and FA metabolism, including effects on lipid and FA compositions and the expression of genes of major lipid metabolic pathways including lipogenesis, lipid deposition, FA β-oxidation as well as LC-PUFA biosynthesis is highly relevant in ABT. Furthermore, key to this understanding is knowledge of lipid-regulated transcription factors and nuclear receptors controlling and regulating the expression of genes involved in FA/lipid metabolic pathways.

However, not only lipids and essential fatty acids, but other nutrients such as antioxidants (vitamin E, vitamin C and Se) and taurine, have been proposed as essential components for fast growing marine fish species, particularly in tuna which cannot synthesize these compounds (Yokohama et al., 2001; Waagbø, 2010; NRC, 2011; Izquierdo and Betancor, 2015; Katagiri et al., 2016). Indeed, growth in fish is primarily due to protein deposition with most marine fish larvae having a high requirement for essential amino acids (Rønnestad et al., 2003). Additionally, fast growth in larvae requires significant amounts of pro-oxidants such as n-3 LC-PUFA to be included in live prey during larval stages and in feeds for juvenile fish. Moreover, high culture temperature conditions (about 28 ºC) and strong aeration/oxygenation in live food enrichment protocols and rearing tanks, promotes high pro-oxidative conditions. As a consequence, during early

development, sufficient amounts of antioxidant nutrients should be included in live prey through appropriate enrichment protocols in order to protect larvae from oxidative stress and promote good growth and survival rates. Live foods that are commonly used for larval marine fish rearing (including tunas), such as rotifers (*Brachionus sp.*), have naturally low levels of essential fatty acids (Mæhre et al., 2013; Takeuchi, 2014; Kostopoulou et al., 2015), vitamins E and C, Se, iodine (Hamre et al., 2008; 2013), taurine (Takeuchi, 2014; Katagiri et al., 2016) and essential amino acids (Rajkumar and Kumaraguru vasagam, 2006), especially when compared with copepods, the natural prey of tuna larvae (van der Meeren et al., 2008; Mæhre et al., 2013). In this context, successful larval production of marine fish depends upon the supply of live feed fortified with the essential nutrients that are insufficient in the live feeds.

Our overarching aim is to gain knowledge to better understand the molecular basis of ABT larval nutrition during first feeding. In order to achieve this goal, the effects of four enrichment protocols for rotifer *B. rotundiformis* as well as copepod (*Acartia*) nauplii on survival, growth performance and development, and expression of key genes of lipid metabolism, antioxidant defence and digestive capabilities were investigated in early ABT larvae at the outset of exogenous feeding period. Growth performance was evaluated by biometry (total length and total dry mass), and development by notochord flexion index, as well as the expression of myosin heavy chain (*myhc*) and tropomyosin (*tropo*) genes, both implicated in myogenesis (Johnston et al., 2011). Key genes involved in major lipid pathways including fatty acid and LC-PUFA biosynthesis (*fas*, *fads2* and *elovl5*), lipid transport (*fabp2*, *4* and *7*), deposition (*lpl*) and β-oxidation (*cpt1* and *aco*) and their control and regulation (transcription factors *ppara*, *pparγ*, *lxr*, *rxr*, *sreb1* and *2*), were evaluated in ABT larvae fed different live prey. Additionally, ABT antioxidant protection status was evaluated by determining the expression of genes encoding antioxidant enzymes superoxide dismutase (*sod*), catalase (*cat*) and glutathione peroxidase (*gpx1* and *4*). Whereas digestive capabilities were assessed by the relative expression of the genes codifying for the enzymes trypsin (*tryp*), aminopeptidase (*anpep*), alcaline phosphatase (*alp*), amylase (*amy*), pancreatic lipase (*pl*),

bile salt-activated-lipases (*bal1* and *bal2*) and phospholipase A_2 (*pla2*).

2. Materials and Methods

2.1 Atlantic bluefin tuna larvae rearing conditions

The ABT eggs used in this study were obtained in June 2016 from ABT broodstock fish maintained in captivity in a floating net cage located at El Gorguel, off the Cartagena coast, SE Spain. Captive-reared ABT broodstock fish spawned naturally and spontaneously and floating eggs were collected inside the cage by means of a net of 500 µm mesh screen size. A 1.5 m polyvinyl sheet was also placed around the inside of the cage to avoid eggs drifting away from the cage by means of currents and/or waves. Collected eggs were transported in a 500 L plastic tank supplied with pure oxygen to the Spanish Institute of Oceanography (IEO) Planta Experimental de Cultivos Marinos (Puerto de Mazarrón, Murcia, Spain) aquaculture facilities and placed in 100 L tanks with gentle oxygenation and flow-through sterilized seawater. After 1 h, aeration and water flow were stopped to separate buoyant (viable) from non-buoyant (non-viable) eggs. After washing and counting, the fertilized eggs were incubated in 1500 L cylindrical tanks at a density of 10 eggs L^{-1}. Incubation was carried out at 25 - 26 ºC, 37 ‰ salinity and continuous photoperiod, with a light intensity of 1000 lux. An upwelling flow-through with gentle aeration was employed in order to maintain oxygen levels near to saturation. Larvae hatched approximately 32 h after fertilization, with a hatching rate of almost 90 %, and were fed with enriched rotifers or copepod nauplii from 2 dah. A mixture of the microalgae *Isochrysis* sp. (T-Iso) and *Chlorella* (V12 DHA-enriched, Pacific Trading Co., Japan) were added to tanks at a density of 2 - 3 $x10^5$ cells mL^{-1} as green water. During the trial, photoperiod was maintained at 16 h / 8 h light/dark (light intensity about 500 lux), temperature ranged between 24 - 26 ºC and daily water renewal was 50 -70 %. Incoming seawater was filtered at 10 µm and UV sterilized. An upwelling current was created to avoid larvae sinking (mainly at night) and maintain oxygen level (Ortega, 2015; De la Gándara et al., 2016).

168

169 *2.2 Dietary trial with live prey at first feeding: rotifers and copepod nauplii*

170

171 The feeding trial of ABT larvae was performed in triplicate tanks and rotifer *Brachionus*
172 *rotundiformis* with 4 different enrichment protocols (Origreen Skretting® (OG), Multigain Biomar®
173 (MG), Algamac 3050® (AG) and Red Algamac® (RA) and one treatment with *Acartia tonsa*
174 copepod nauplii (COP). To maintain constant live prey concentration (10 rotifer mL^{-1} or 5 copepod
175 nauplii/copepodite mL^{-1}) within each experimental tank, three water samples (10 mL) from each
176 tank were sampled and counted twice per day before supplying new feed (Ortega, 2015; De la
177 Gándara et al., 2016).

178

179 *2.2.1 Rotifer Brachionus rotundiformis culture and enrichment protocols.*

180

181 S-type rotifers *B. rotundiformis* were continuously cultured with commercial DHA enriched
182 algal paste (Chlorella V-12; Chlorella Industry, Kyushu, Japan), at a concentration of 3 mL
183 Chlorella paste per 10^6 rotifers per day, in four 2000 L cylindro-conical tanks supplied with filtered
184 and sterilized sea water at 24 – 26 ºC, 38 ‰ salinity, dissolved oxygen at saturation level and 24 h
185 continual illumination.

186 In addition to the enrichment products, rotifers were supplemented with taurine (Andrés
187 Pintaluba S.A., 0.5 g per 10^6 rotifers) 18 h before the enrichment treatment, and organic Se (Sel-
188 Plex® Alltech Spain SL; 3.0 mg per 10^6 rotifers), and vitamin E as dl-α tocopheryl acetate Lutavit
189 E50 (BASF; 0.9 mg per 10^6 rotifers) were added with the enrichment treatment. The enrichment
190 protocols were performed in 100 L cylindro-conical tanks at a density of 1000 rotifers mL^{-1} with the
191 dose of enricher added over a period of 3 h for OG and MG, and 6 h for AG and RA according to
192 manufacturer's recommendations.

193

194 *2.2.2 Cultivation of copepod Acartia tonsa*

195

196 The copepods (*A. tonsa*) were cultivated in 4000 L cylindrical tanks with seawater of 34 ‰ salinity
197 and 20 ºC and were continuously fed with algae *Rhodomonas baltica* at a concentration not below 3
198 x 10^4 cells mL^{-1}. *Acartia* eggs were harvested every day with a harvesting arm to collect the eggs
199 deposited on the flat bottom of the tanks. The eggs were washed thoroughly and stored in flasks at 2
200 ºC. Egg harvest started 3 months in advance the experiment and continued until the end of the trial.
201 The water in the flasks was renewed every 2 weeks and the number of eggs counted. The copepod
202 eggs were incubated at 23.0 ± 1.0 °C in 100 L tanks at a maximum density of 150 eggs mL^{-1}. From
203 2 days after hatch the nauplii were fed *ad libitum* with *R. baltica* and before harvesting the nauplii
204 density in the tanks was counted, harvested with a siphon, concentrated in a 60 µm sieve and then
205 transferred to ABT larval tanks.

206

207 *2.2 Sampling for biometrical, biochemical and molecular analysis.*

208

209 Thirty randomly caught ABT larvae per replicate treatment were anaesthetized (0.02 % 2-
210 phenoxyethanol, Sigma, Spain), total lengths measured and individual larvae were photographed
211 while measuring. Developmental stage was assessed by counting the number of ABT larvae which
212 had attained full flexion of the notochord by the end of the feeding trial (15 dah) in each replicate
213 set of samples. Individual larvae dry mass was determined in a precision balance after maintaining
214 samples at 110 °C for 24 h and cooling *in vacuo* for 1 h. Final survival (%) was calculated by
215 counting individual live larvae at the beginning and the end of the trial.

216 Triplicate samples of rotifers nutritionally boosted with the different enrichers and copepods
217 (*Acartia*) were washed and filtered, excess water drained and blotted with filter paper, and
218 immediately frozen in liquid N_2 and stored at -80 ºC prior to analysis. Two subsets of triplicate
219 samples (15 larvae per sample) of 15 dah ABT larvae fed the different live prey treatments were

collected: i) one subset of samples in 1 mL of RNA*later*® (Ambion, Madrid, Spain) for RNA extraction and molecular analysis, and ii) a second subset frozen in liquid N_2 and stored at -80 ºC for lipid analysis. All procedures were carried out according to the current national and EU legislation on the handling of experimental animals.

2.3 Biochemical analysis

2.3.1 Proximate composition

Proximate compositions of live feeds (protein and lipid) were determined according to standard procedures (AOAC, 2000). Three technical replicates of feeds (single batch production) were freeze-dried and at a subsequent time analysed. Moisture contents were obtained after drying in an oven at 110 °C for 24 h and ash content determined after incineration at 600 °C for 16 h. Crude protein was measured by determining nitrogen content (N × 6.25) using automated Kjeldahl analysis (Tecator Kjeltec Auto 1030 analyser, Foss, Warrington, UK) and crude lipid content determined gravimetrically after Soxhlet lipid extraction (Tecator Soxtec system 2050 Auto Extraction apparatus).

2.3.2. Total lipid, lipid class composition and fatty acid analysis

Total lipid of live feeds (enriched rotifers and copepods) and ABT larvae fed the different dietary regimes was extracted from triplicate pooled samples according to the method of Folch et al. (1957). Approximately 200 mg of ABT larvae was placed in 10 mL of ice-cold chloroform/methanol (2:1, by vol) and homogenized with an Ultra-Turrax tissue disrupter (Fisher Scientific, Loughborough, U.K.). The non-lipid and lipid layers were separated by addition of 5 mL of 0.88 % (w/v) KCl and allowed to separate on ice for 1 h. The upper aqueous layer was aspirated

and the lower organic layer dried under oxygen-free nitrogen. The lipid content was determined gravimetrically after drying overnight in a vacuum desiccator.

Lipid class composition was determined by high-performance thin-layer chromatography (HPTLC) using 10 x 10 cm plates (VWR, Lutterworth, England). Approximately 1 µg of total lipid was applied as a single spot and the plates developed in methyl acetate/isopropanol/ chloroform/methanol/0.25 % aqueous KCl (25:25:25:10:9, by vol.) to two-thirds up the plate. After drying for 20 min, the plate was fully developed with isohexane/diethyl ether/acetic acid (85:15:1, by vol.). The lipid classes were visualized by charring at 160 ^{o}C for 15 min after spraying with 3 % (w/v) aqueous cupric acetate containing 8 % (v/v) phosphoric acid and quantified by densitometry using a CAMAG-3 TLC scanner (version Firmware 1.14.16). Scanned images were recorded automatically and analyzed using winCATS Planar Chromatography Manager software (version 1.2.0) (Henderson and Tocher, 1992).

Fatty acid methyl esters (FAME) were prepared from total lipid by acid-catalyzed transesterification at 50 ^{o}C for 16 h according to the method of Christie (1993). The FAME were separated and quantified by gas-liquid chromatography (Carlo Erba Vega 8160, Milan, Italy) using a 30 m x 0.32 mm i.d. capillary column (CP Wax 52CB, Chrompak, London, U.K.) and on-column injection at 50^{o}C. Hydrogen was used as carrier gas and temperature programming was from 50 ^{o}C to 150 ^{o}C at 40 ^{o}C min^{-1} and then to 230 ^{o}C at 2.0 ^{o}C min^{-1}. Individual methyl esters were identified by comparison with known standards and by reference to published data (Ackman, 1980; Tocher and Harvie, 1988). Data were collected and processed using Chromcard for Windows (version 1.19)

2.3.3. Determination of alpha-tocopherol (vitamin E) content

Alpha-tocopherol concentrations in enriched rotifers and copepods were determined using high-pressure liquid chromatography (HPLC) with UV detection. Samples were weighed, homogenized in pyrogallol, and saponified as described by McMurray et al. (1980) and according to Cowey et al. (1981). HPLC analysis was performed using a 150 x 4.60 mm, reverse-phase Luna

5 lm C18 column (Phenomenox, CA, USA). The mobile phase was 98 % methanol pumped at 1.0 mL min^{-1}. The effluent from the column was monitored at a wavelength of 293 nm and quantification achieved by comparison with alpha-tocopherol (Sigma-Aldrich) as external standard.

2.3.4. Selenium determination

Total selenium concentration was measured in feeds according to the method established in Betancor et al. (2012). Dried samples were weighed in three replicates of between 0.04 and 0.1 g and digested in a microwave digester (MarsXpress, CEM, USA) with 5 % of 69 % pure nitric acid in three steps as follows; 21° C to 190° C for 10 min at 800 W then 190° C for 20 min at 800 W and finally a 30 min cooling period. The digested solution was poured into a 10 mL volumetric flask and made up to volume with distilled water. A total of 0.4 mL of this solution was added to 10 mL tubes, 10 µL of internal standard (Gallium and Scandium, 10 ppm, BDH, UK) included and 0.2 mL of methanol added. The tube was made up to volume with distilled water and total selenium was measured in a reaction cell by Inductively Coupled Plasma Mass Spectrometry (Thermo Scientific, XSeries2 ICP-MS, USA) using argon and hydrogen as carrier gas.

2.4. Tissue RNA extraction and cDNA synthesis

Approximately 100 mg of pooled larvae (n = 2 per tank; 6 per dietary treatment) were placed in RNA*later*® (Sigma – Aldrich, Dorset, UK) and processed according to manufacturer's instructions, before being frozen at -20 °C prior to total RNA extraction. Samples were homogenized in 1 mL of TriReagent® (Sigma-Aldrich) RNA extraction buffer using a bead tissue disruptor (Bio Spec, Bartlesville, Oklahoma, USA). Total RNA was isolated following manufacturer's instructions and quantity and quality determined by spectrophotometry using a Nanodrop ND-1000 (Labtech Int., East Sussex, UK) and electrophoresis using 200 ng of total RNA in a 1 % agarose gel. cDNA was synthesised using 2 µg of total RNA and random primers in 20 µl

294 reactions and the high capacity reverse transcription kit without RNase inhibitor according to the
295 manufacturer's protocol (Applied Biosystems, Warrington, UK).

296

297 *2.5. qPCR analysis*

298 Several genes related to lipid and fatty acid metabolism, antioxidant and digestive enzymes,
299 as well as growth markers were evaluated in the present study. Quantitative real-time PCR (qPCR)
300 was carried out on transcription factors *pparα*, *pparγ*, *lxr*, *rxr*, *srebp1* and *srebp2*; LC-PUFA
301 biosynthesis genes *fads2d6* and *elovl5* and fatty acid metabolism genes *fas, cpt1, aco, fabp2, fabp4,*
302 *fabp7, lpl* and *hmgcl,* the antioxidant enzymes *sod, cat, gpx1* and *gpx4,* growth indicators *myhc* and
303 *tropo,* and digestive genes *tryp, anpep, alp, amy, pl, pla2, bal1 and bal2* (Supplementary Table 1).

304 Primers for *fads2d6, elovl5, fabp2, 4* and *7, rxr, hmgcl, pparγ, aco, fas, lpl, myhc, anpep,*
305 *amy, try* and *cat* were already available for ABT (Morais et al., 2011; Mazurais et al., 2015;
306 Betancor et al., 2016). Primers for *alp* and *tropo* were designed on sequences of *Thunnus thynnus*
307 (FM995226.1 and AB109447.1 respectively). Primers for *gpx1 and gpx4* were designed on the
308 sequences of *Thunnus maccoyii* (EF452497.1 and EF452498.3 respectively; Thompson et al.,
309 2010). Primers for *pl, bal1* and *bal2* were designed on the sequences of *Thunnus orientalis*
310 (AB859991.1, AB859992.1 and AB859993.1, respectively). Primers for *pla*2 and *sod* were
311 designed on the sequence read archive (SAR) SRX2255758 by identifying and assembling the
312 sequences.

313 Expression of genes of interest was determined by qPCR of all the RNA samples.
314 *Elongation factor-1α* (*elf1α*) and *β-actin* were used as reference genes. The cDNA was diluted 20-
315 fold with milliQ water. The efficiency of the primers for each gene was previously evaluated by
316 serial dilutions of cDNA pooled from the samples to guarantee it was > 85 % for all primer pairs.
317 qPCR was performed using a Biometra TOptical Thermocycler (Analytik Jena, Goettingen,
318 Germany) in 96-well plates in duplicate 20 µL reaction volumes containing 10 µL of Luminaris

Color HiGreen qPCR Master Mix (Thermo Scientific, Hemel Hempstead, UK), 1 µL of the primer corresponding to the analyzed gene (10 pmol), 3 µL of molecular biology grade water and 5 µL of cDNA (1/20 diluted). In addition, amplifications were carried out with a systematic negative control (NTC, no template control) containing no cDNA. Standard amplification parameters contained an UDG pre-treatment at 50 °C for 2 min, an initial denaturation step at 95 °C for 10 min, followed by 35 cycles: 15 s at 95 °C, 30 s at the annealing Tm and 30 s at 72 °C.

2.7 Statistical analysis

Results for biometry, lipid class and fatty acid compositions are presented as means ± SD (n = 20 for biometry, and n = 3 for survival, lipid class and fatty acid compositions with tank being the experimental unit). The data were checked for homogeneity of the variances by the Bartlett test and, where necessary, arc-sin transformed before further statistical analysis. Differences between mean values were analyzed by t-test and one-way analysis of variance (ANOVA), followed when pertinent by a multiple comparison test (Tukey). Differences were reported as statistically significant when $P < 0.05$ (Zar, 1999). All statistical analyses were performed using SPSS software (IBM SPSS Statistics 19; SPSS Inc., Chicago, IL, USA).

Gene expression results were analyzed using the relative expression software tool (REST 2009), which employs a pairwise fixed reallocation randomization test (10,000 randomizations) with efficiency correction (Pfaffl et al., 2002) to determine the statistical significance of expression ratios (gene expression fold changes) between two treatments.

3. Results

3.1 ABT larvae biometry and survival at 15 dah

343 Growth performance of 15 dah ABT larvae fed on enriched rotifers *B. rotundiformis* (OG,
344 MG, AG, RA) or 3 days post hatch nauplii of the copepod *A. tonsa* fed *R. baltica* (COP) are shown
345 in Table 1. Highest total length, total dry mass and flexion index were shown in ABT larvae fed on
346 nauplii of the copepod *Acartia* followed by larvae fed on rotifers enriched with AG. Lower
347 performance parameters were observed in ABT larvae fed on rotifers enriched with MG > RA >
348 OG. However, no significant differences were detected in final survival, which was around 8 % in
349 all the treatments.
350
351 *3.2. Macronutrient, vitamin E and Se content of enriched rotifers B. rotundiformis and Acartia*
352 *nauplii*
353 The contents of macronutrients, protein and total lipid (dry mass %), and vitamin E and Se
354 in live feeds are shown in Table 2. Total protein content of *Acartia* nauplii was 63.8 % of dry mass,
355 and higher than in enriched rotifers at about 53 % of dry mass. Conversely, total lipid content was
356 higher in enriched rotifers at about 11 % of dry mass, whereas *Acartia* nauplii had a lower lipid
357 content of 5.6 % of dry mass. Vitamin E content in enriched rotifers showed values ranging from
358 232 mg kg^{-1} in AG-enriched rotifers to 593 mg kg^{-1} in rotifers enriched with RA. *Acartia* nauplii
359 had a lower level of vitamin E at only 170.1 mg kg^{-1}. Selenium content in rotifers ranged from 4.8
360 µg g^{-1} in those enriched with AG to 10.6 µg g^{-1} in those enriched with RA, while *Acartia* nauplii
361 only contained selenium at 0.4 µg g^{-1}.
362
363 *3.2 Lipid class compositions of enriched rotifers B. rotundiformis and Acartia nauplii*
364 The lipid class composition of dietary live preys was predominantly neutral lipids with
365 triacylglycerol (TAG) the predominant class (Table 3). However, class compositions reflected lipid
366 content and, thus, copepods, with the lowest lipid content showed the lowest levels of neutral lipids
367 and TAG, whereas all enriched rotifers had higher neutral lipids and TAG. Polar lipids were
368 dominated by phosphatidylcholine (PC) and phospahtidylethanolamine (PE) and were higher in

rotifers than in copepods. Cholesterol did not show this tendency and values ranged between 0.66 and 1.04 μg mg^{-1} in rotifers enriched with OG and AG, respectively.

3.3 Total lipid fatty acid compositions of enriched rotifers B. rotundiformis and Acartia nauplii

Total lipid of enriched rotifers contained higher levels of saturated fatty acids than copepods (Table 4). Absolute values of monoenes in rotifers varied between 4.4 and 8.9 μg mg^{-1} of total fatty acids with the highest value in OG-rotifers, with COP showing the lowest value (2.8 μg mg^{-1}). Total n-3 PUFA was the predominant fatty acid group ranging from 11.1 to 34.3 μg mg^{-1} of total fatty acids with DHA being predominant, ranging from 23.1 μg mg^{-1} in AG-enriched rotifers to 5.1 μg mg^{-1} in COP, which also showed the lowest DHA content (Table 4). The EPA levels ranged from 1.8 to 2.8 μg mg^{-1} of total fatty acids, with the lowest value in MG-enriched rotifers. However, when the data were expressed as percentage of total fatty acid, COP had the highest EPA content (10.4 %), double the amount found in any enriched rotifers (Supplementary Table 2). The DHA/EPA ratio was lowest in COP at 1.9, and ranged from 2.8 (OG) to 8.3 (AG) in enriched rotifers. Total n-6 PUFA (primarily 18:2n-6) were higher in all enriched rotifer treatments (ranging from 6.5 to 10.3 μg mg^{-1}) and much higher than in COP at around (1.4 μg mg^{-1}). Arachidonic acid (ARA; 20:4n-6) was highest in RA-enriched rotifers (1.1 μg mg^{-1}) with COP displaying only 0.6 μg mg^{-1} of this fatty acid.

3.4. Total lipid fatty acid compositions of 15 dah ABT larvae.

The fatty acid composition (% of weight) of total lipid of ABT larvae fed enriched rotifers or copepods is presented in Table 5. Total lipid of ABT larvae fed copepods had the highest proportion of total n-3 PUFA with the highest level of DHA at > 24 %, with larvae fed enriched rotifers showing values of DHA between 14 (OG) and 20 % (AG) (Table 5). The proportion of EPA

was more similar between all groups of larvae with highest levels in those fed OG-rotifers and copepods 3.5-3.6 %, and lowest in larvae fed RA-rotifers (2.7 %). The DHA/EPA ratio varied between 4.0 and 6.9 %and was highest in ABT larvae fed copepods and lowest in larvae fed OG-rotifers. Larvae fed copepods had the lowest percentages of 18:2n-6, ARA and total n-6 PUFA at 3.9, 1.2 and 12.7 %, respectively. In contrast total n-6 PUFA ranged from 16 to 21 % in larvae fed enriched rotifers with 18:2n-6 ranging from 8-11 %, and ARA from 1.4 to 2.5 %. Total saturated fatty acids ranged from 34 – 41 % in larvae fed enriched rotifers with larvae fed copepods showing an intermediate value of around 38 %. Similarly, total monoene content of ABT larvae ranged from around 9 – 14 % in larvae fed enriched rotifers with larvae fed copepods showing an intermediate value of around 12 %.

3.5. Expression of myogenic genes in 15 dah ABT larvae

The expression of *myhc* was higher in COP-fed larvae than in fish fed any enriched rotifer (Fig. 1). The same pattern was observed in the expression of *tropo*, although the differences were not statistically significant between larvae fed COP and AG-rotifers (Fig. 1).

3.6. Expression of lipid metabolism and transcription factor genes

Regarding fatty acid synthesis, the expression of *fas* showed differences among treatments, that likely reflected the lipid content of the live feeds. Thus, relative expression of *fas* was higher in larvae fed COP, which had the lowest lipid content, than in larvae fed enriched rotifers, significantly so in all cases other than RA-rotifers, while larvae fed AG-rotifers, which had highest lipid content, presented the lowest value (Fig.2). No significant differences were observed in the expression levels of *fads2d6* and *elovl5* among 15 dah ABT larvae fed the different live preys (Fig. 2).

In relation to fatty acid catabolism, the expression of *cpt1* did not show significant differences among treatments (Fig. 3). In contrast, *aco* displayed differences in relative expression levels in ABT larvae among dietary treatments, with larvae fed on RA-rotifers showing higher

418 expression than those fed COP, with larvae fed the other enriched rotifers showing intermediate
419 values (Fig. 3). The expression of *fabp*2 showed a similar pattern to *aco*, with larvae fed on RA-
420 rotifers showing higher expression than those fed COP, with larvae fed the other enriched rotifers
421 showing intermediate values (Fig. 4). Expression of *fabp4* was also lowest in larvae fed COP,
422 significantly so compared to rotifers enriched with MG and AG, although expression of *fabp7*
423 showed no significant differences. In contrast, larvae fed AG-rotifers showed lower *lpl* expression
424 than larvae fed OG-rotifers, with larvae fed the other live feeds showing intermediate values (Fig.
425 4). No differences among treatments were found in the expression of *hmgcl*.
426 Concerning transcription factors, larvae fed COP displayed lower expression of *ppar*γ than
427 larvae fed OG- and MG-rotifers with larvae fed the other live feeds showing intermediate
428 expression levels (Fig. 5). Similarly, larvae fed COP displayed lower expression of *srebp2* than
429 larvae fed OG- and RA-rotifers with larvae fed the other live feeds showing intermediate expression
430 levels. No differences in expression levels were observed among larvae fed the different dietary
431 treatments for *ppar*α, *srebp1*, *rxr* or *lxr* (Fig. 5).
432
433 *3.7. Expression of antioxidant defence enzyme genes*.
434 Expression of superoxide dismutase (*sod*) was higher in ABT larvae fed COP and RA-
435 rotifers than in MG-rotifers with larvae fed OG- and AG-rotifers displaying intermediate values
436 (Fig. 6). A similar pattern was observed for catalase (*cat*) with higher expression in ABT larvae fed
437 COP and RA-rotifers than larvae fed AG-rotifers with larvae fed the other live feeds showing
438 intermediate values. In contrast, larve fed COP had low expression of both glutathione peroxidase
439 genes (*gpx1* and *gpx4*), significantly lower than RA-rotifers in both cases with larve fed the other
440 enriched rotifers generally showing intermediate expression levels (Fig. 6).
441
442 *3.8. Expression of digestive enzyme genes.*
443 Trypsin (*tryp*) showed highest expression levels in ABT larvae fed COP, whereas

expression of phosphatase alkaline (*alp*) presented exactly the opposite pattern of expression than *tryp*, with larvae fed COP showing the lowest expression level (Fig. 7). Similar to the pattern for *alp*, lower amino peptidase (*anpep*) expression was observed in larvae fed COP than larvae fed OG-rotifers with larvae fed the other enriched rotifers showing intermediate expression levels. The expression patterns of phopholipase A2 (*pla2*) and bile salt-activated lipase genes (*bal1* and *bal2*) were similar to that for *tryp*. Thus, larvae fed COP showed the highest expression, significantly higher than in larve fed all other live feeds in the case of *bal1*, and significantly higher than larve fed AG-rotifers in the cases of *pla2* and *bal2*, with other treatments showing intermediate levels (Fig. 7). The expression of amylase (*amy*) and pancreatic lipase (*pl*) showed no significant differences among larvae fed the different live feeds (Fig. 7).

4. Discussion

It has been often demonstrated that copepods are superior live prey than rotifers and *Artemia* for rearing of marine fish larvae (Hamre, 2006; Toledo et al., 1999; Witt el al., 1984). Moreover, copepods are among the natural prey in the wild for tuna (Uotani et al., 1990), and previous studies have indicated that copepods support better growth performance than other types of live prey for larvae of ABT (Yufera et al., 2014; Betancor et al., 2016). In the present study, this is confirmed by biometric data (highest weight and total length), advanced stage of development (highest flexion index) and highest expression of the myogenic genes, *myhc2* and *tropo* (Table 1 and Fig. 1). In this respect, in a previous study in ABT from hatching to 20 dah, exponential somatic growth of the larvae was confirmed by the expression of *myhc2* (Mazurais et al., 2015), given that this gene can be considered as a molecular marker of somatic growth as its expression has been shown to increase with muscular tissue formation throughout fish larvae development (Imsland et al., 2006).

Likely contributing to the growth enhancement in ABT larvae fed copepods is the fact that *Acartia* nauplii (COP treatment) presented a higher protein content, more than 15 % higher than enriched rotifers as previously shown (Karlsen et al., 2015), with quantitatively more amino acids

available for protein synthesis and growth. Nutrition is a key modulator of protein synthesis in fish larvae which increases with dietary protein levels (Fauconneau et al., 1986), which in turn could explain the high *myhc* and *tropo* gene expression in COP-fed larvae. Indeed, expression of *myhc*, a late marker of myogenesis, can be an indicator of the effect that nutritional status has on muscle growth (Bower et al., 2008).

Regarding lipid content in live prey used in this study, copepod nauplii showed the lowest content in comparison to enriched rotifers (about 50 %) although its lipid class composition was richer in polar lipids and the fatty acid profile more balanced (Tables 3 and 5). Composition data of live preys used in the present study are similar to those reported recently (Hamre, 2016). However, there are large variations in nutrient composition of commercial enrichers and enriched live prey resulting in some values below those recommended for larvae of other teleost species (NRC, 2011).

In a previous study with ABT larvae at first feeding, a modulation of *fads2d6* expression, with differing direction in two consecutive years, accompanied by no significant regulation in the expression of *elovl5* was observed (Betancor et al., 2016). This appeared to be driven by differences in the fatty acid profiles of the live prey used as differences in the levels of n-3 LC-PUFA were observed between the different trials. In contrast, the expression of *fads2d6* and *elovl5* in ABT larvae in the present study (Fig. 2) showed no significant dietary regulation despite different levels of dietary n-3 LC-PUFA (ranging from 23 to 35 % of the total lipids or from 8 to 28 μg fatty acid mg^{-1} dry mass). Generally up-regulation of *fads2d6* expression has been observed previously in fish fed low dietary levels of n-3 LC-PUFA, whereas high dietary levels of these fatty acids was associated with reduced expression (Morais et al., 2012; Betancor et al., 2015). Therefore, the lack of regulation of *fads2d6* and *elovl5* in the present trial could reflect that the fatty acid composition of all dietary treatments possibly provided sufficient n-3 LC-PUFA to satisfy minimum requirements and did not significantly affect gene expression.

Moreover, high levels of dietary n-3 LC-PUFA, particularly DHA, can act as ligands for transcription factors such as *pparα* and *srebp1* down-regulating the biosynthesis of LC-PUFA

(Worgall et al., 1998; Hihi et al., 2002; Cunha et al., 2013). Although the transcription factor genes did not show regulation in the present trial, *fas*, a direct target of *srebp1*, was strongly regulated by the different dietary treatments and followed a similar pattern as *srebp1*. Indeed, *fas* expression levels were lowest in ABT larvae fed AG enriched rotifers, which contained the highest lipid level, and highest in larvae fed COP, which contained the lowest total lipid content (Fig. 2). Previous studies in teleosts have described an inhibition in hepatic *fas* expression when fish were fed in a restricted manner (Tian et al., 2013; He et al., 2015; Gong et al., 2017) or with increasing dietary fat levels (Leng et al., 2012), which is consistent with our findings. Another transcription factor involved in lipid storage and lipogenesis, as well as osteogenesis (Nedergaard et al., 2005; Ji et al., 2011; Agawa et al., 2012), *pparγ*, was regulated in the present trial, showing a pattern opposite to *fas*, with COP-fed ABT larvae displaying the lowest expression level (Fig. 5). An up-regulation in *pparγ* expression was also observed in rotifer-fed ABT in a previous trial (Betancor et al., 2016) and this was apparently associated with dietary PC content, which was in agreement with results found in blunt snout bream (*Megalobrama amblycephala*) where *pparγ* expression in liver was significantly affected by dietary phospholipid supplementation (Li et al., 2015).

Additionally, *pparγ* plays an important role in regulating lipid metabolism in mature adipocytes (Lehrke and Lazar, 2005). Up-regulation of *pparγ* expression has been observed in grass carp (*Ctenopharyngodon idellus*) as an adaptive mechanism increasing adipocyte differentiation and lipolysis when fish were fed high lipid feeds (Yuan et al., 2016). In the present trial, a down-regulation of *pparγ* was observed in COP-fed larvae, concomitantly with an up-regulation of *fas* which appeared to be related to the low lipid content of copepods (5.6 %) compared to the enriched rotifers (9.8 - 12.9 %) (Figs. 5 and 2). Given that copepods, including *Acartia* spp., are among the natural prey of ABT larvae in the wild, it is feasible to suggest that the high lipid content in all the enriched rotifer treatments triggered a response in ABT larvae by adjusting lipogenetic/lipolytic mechanisms in order to adapt to energy-dense feeds. In mammals, targets directly regulated by *pparγ* include genes that favour uptake of circulating fatty acids by adipocytes (Schoonjans et al.,

1996; Frohnert et al., 1999; Chui et al., 2005) and others that promote recycling rather than export of intracellular fatty acids (Guan et al., 2002; Hibuse et al., 2005). These paradoxical effects on adipocyte biology mean that, apart from enhancing fatty acid deposition similar to *pparα*, *pparγ* can lead to increased fatty acid oxidation (Lehrke and Lazar, 2005). This may explain why higher *pparγ* expression in larvae fed enriched rotifers was associated with up-regulation of *aco* expression, an oxidoreductase that participates in peroxisomal β-oxidation. However, no significant effect was noticed on the expression of *cpt1* related to mitocondrial fatty acid β-oxidation (Figs. 5 and 3). Although rotifer-fed larvae displayed higher lipid content they also had a smaller size compared to ABT larvae fed copepods, which could mean higher energy requirements for growth that could, in turn, explain up-regulation of *pparγ*. Similarly, *pparγ* was correlated with *de novo* fatty acid synthesis (*fas*) and phospholipid hydrolysis (*hepatic lipase*) in unfed turbot larvae (Cunha et al., 2013).

Whereas *srebp1* preferentially regulates fatty acid and LC-PUFA synthesis, *srebp2* regulates the expression of genes involved in cholesterol synthesis (Jeon and Osborne, 2012; Carmona-Antoñanzas et al., 2014) and is up-regulated in response to reduced cholesterol (Minghetti et al., 2011; Carmona-Antoñanzas et al., 2014). Consistent with this, in the present study, there was a negative correlation between cholesterol levels in rotifers and *srebp2* expression in ABT larvae ($y = -2.3296x + 0.8105$; $R^2 = 0.8594$), with the lowest expression level found in fish fed COP, which contained the highest cholesterol level. Conversely, *lxr*, a transcription factor which acts to regulate the formation of bile acids from cholesterol, was not modulated in the present trial. These results agree with a previous trial in ABT larvae comparing copepods with enriched rotifers or co-fed copepods and rotifers (Betancor et al., 2016), and in Atlantic salmon fed differing n-3 and n-6 LC-PUFA levels (Betancor et al., 2014). The explanation to this could be that LXR is activated by several sterols, including intermediates in the synthesis of cholesterol (Carmona-Antoñanzas et al., 2014) and, although the level of cholesterol differed among treatments, the levels of other sterols

547 that may activate *lxr* might be similar. In this sense, it was shown that oxysterols and not cholesterol
548 activated salmon *lxr* in a heterologous *in vitro* system (Carmona-Antoñanzas et al., 2014).

549 In agreement with our previous study (Betancor et al., 2016), *fabp4* and *fabp2*, carrier
550 proteins involved in fatty acid uptake, transport and metabolism (Glatz and van der Vusse, 1996),
551 were also up-regulated in rotifer-fed larvae, possibly reflecting increased uptake and accumulation
552 of lipid into larval tissues (Fig. 4). This increased lipid accumulation in rotifer-fed larvae could be
553 directly related to the higher lipid content of rotifers compared to copepods. Similarly, Senegalese
554 sole (*Solea senegalensis*) larvae showed differential regulation of *fabp2* expression when fed
555 different levels of n-3 LC-PUFA, particularly EPA (Darias et al., 2012), which in turn translated
556 into higher liver lipid deposition (Boglino et al., 2012). However, a recent study in Senegalese sole
557 larvae showed no regulation of *fabp2* expression when larvae were fed enriched *Artemia*, whereas
558 up-regulation of *fabp1* and *fabp3* was observed in larvae fed high levels of n-3 LC-PUFA (Bonacic
559 et al., 2016), which may indicate differential regulation of *fabp* at different developmental stages
560 (André et al., 2000). In the present study, rotifer-fed larvae, also showed up-regulation of *fabp4* and
561 *fabp2* and down-regulation of *fas* expression. However, ABT larvae showed an unusual pattern of
562 expression of *lpl*, a lipase highly expressed in muscle and liver of ABT (Betancor et al., 2016) that
563 hydrolyzes TAG in plasma lipoproteins and supplies free fatty acids for deposition in adipose tissue
564 or for oxidation in other tissues (Nilsson-Ehle et al., 1980; Kersten, 2014). High levels of
565 expression and activity of *lpl* have been associated with increased lipid utilization in darkbarbel
566 catfish (*Pelteobagrus vachelli*) larvae fed high-lipid diets (Zheng et al., 2010). Thus, up-regulation
567 of *fabp4* and *fabp2* expression together with down-regulation of *fas* expression in rotifer-fed ABT
568 larvae might indicate enhanced lipid utilisation in order to compensate for reduced growth, as
569 indicated by lower growth rates as well as lower expression of *myhc* and *tropo* genes (Table 1 and
570 Fig. 1).

571 The antioxidant system, protecting cells against reactive oxygen species, includes several
572 enzymes, such as superoxide dismutase, catalase, glutathione peroxidases, glutathione reductase

573 and glutathione-S transferase, associated with antioxidant vitamins, such as alpha tocopherol
574 (vitamin E), retinoic acid (vitamin A), ascorbic acid (vitamin C) and Se as cofactor of glutathione
575 peroxidase (Mourente et al., 2007; Izquierdo and Betancor, 2015). Live feed production and
576 enrichment is performed under highly pro-oxidative conditions, with high levels of n-3 PUFA,
577 aeration or oxygenation of the culture water, high temperature and bright light. Moreover, oxidative
578 stress associated with preroxisome proliferation is thought to be due, at least in part, to the
579 increased peroxisomal production of H_2O_2 via *aco* activity. In the present study, the expression of
580 *sod* and *cat* genes in ABT larvae fed different dietary treatments did not correlate with the *aco*
581 expression pattern but, in contrast, correlated with expression of *gpx1* (r = 0.88; P=0.024) and *gpx4*
582 (r=0.85; P=0.032) (Figs. 3 and 6). This may suggest that alternative antioxidant specific pathways
583 are activated (glutathione peroxidases) in addition to superoxide dismutase and catalase.

584 Therefore, it is important to supplement diets for larval marine fish with antioxidants such as
585 vitamin E. However, vitamin E at high levels in the absence of suficient vitamin C has been shown
586 to increase tissue lipid oxidation and mortality (Hamre et al., 2010; Betancor et al., 2012). It is
587 perhaps noteworthy that the Se content of live prey, either enriched rotifers or *Acartia* nauplii,
588 showed a negative correlation with final total length (r = -0.9; P = 0.0417), total weight (r = -0.94; P
589 = 0.008) and flexion index (r = -0.8; P = 0.053). Although Se is an essential micronutrient, it has the
590 narrowest window of any element between requirement and toxicity (Polatajko et al., 2006), with
591 reduced growth being one of the first symptons of toxicity (Jaramillo et al., 2009). Thus, it would
592 need to be established whether the Se levels used in enriched rotifers in the present study were
593 within safe limits for ABT larvae.

594 Apart from lipids there are other nutrients that, although outwith the scope of the present
595 trial, play a pivotal role in larvae performance such as taurine. Taurine is naturally found in high
596 levels in copepods compared to rotifers (Karlsen et al., 2015) and is involved in the production of
597 bile salts in fish (Vessey et al., 1990). Increased activity of bile salt-activated lipase has been found
598 in *Dentex dentex* juveniles fed diets supplemented with taurine (Chatzifotis et al., 2008), which is in

599 agreement with the highest expression of *bsl1* and *2* observed in COP-fed larvae in the present
600 study.

601 Several studies have dealt with the ontogenic development of the digestive system and the
602 functionality and expression of digestive enzymes during early stages of tuna development
603 (Miyashita et al., 1998; Murashita el al., 2014; Mazurais et al., 2015). In the present study,
604 pancreatic alkaline phosphatase (*alp*) trypsin (*tryp*), phospholipase A_2 (*pla2*) and two isoforms of
605 bile salt activated lipases (*bal1* and *bal2*) genes showed highest expression in 15 dah ABT larvae
606 that had been fed with *Acartia* nauplii and copepodites (Fig. 7). Similarly, up-regulation of *tryp* and
607 *lipase* expression was observed in red seabream (*Pagrus major*) fed a fish meal diet in contrast to
608 fish fed a soybean meal based diet, which could be due to the presence of stimulating factors such
609 as small peptides or free aminoacids in fish meal (Murashita et al., 2015). On the other hand, the
610 digestion of dietary polar lipids was investigated by analyzing the relative expression of *pla2* in
611 ABT larvae fed different dietary treatments. Results showed highest expression of *pla2* in larvae fed
612 copepods, which contained a higher proportion of polar lipids than enriched rotifers (Fig. 7). It is
613 commonly accepted that the major digestive lipase in teleosts, including larvae, appears to be bile
614 salt-dependent lipases (*bal*) (Rønnestad and Morais, 2008). It was also reported that bile salt-
615 dependent lipases were the main enzymes involved in lipid digestion in the larval stage of Pacific
616 bluefin tuna (Murashita et al., 2014). In the present study, the expression patterns of both isoforms
617 (*bal1* and *bal2*) were nutritionally regulated, showing a similar pattern. *Bal1* showed significantly
618 higher expression in larvae fed copepods (Fig. 7). In cod (*Gadus morhua*) larvae, *bal* expression
619 was correlated with the growth pattern of pyloric caeca, with a significant increase in expression
620 from 48 to 62 dah, which could be related to an increased demand for enzyme in the rapidly
621 increasing pyloric caeca or to an increase in pancreatic mass related to body size (Sæle et al., 2010).
622 Thus, the high *bal* expression found in ABT larvae fed COP in the present study could be reflecting
623 the larger size of COP-fed ABT larvae.

In conclusion, and in agreement with our previous trials, the present study showed that copepods were a superior live prey for first feeding ABT larvae compared to enriched rotifers, as indicated by the higher growth and flexion index achieved by COP-fed larvae. This may reflect the higher protein content of the copepods. Furthermore, some of the responses in lipid gene expression could be a consequence of dietary lipid and fatty acid content and composition, appearing to indicate that lipid levels provided by enriched rotifers exceeded ABT requirements. Although the effects of nutrient profiles of enriched rotifers on ABT larval production have been reported previously, no study has reported lipid requirements, including EPA and DHA requirements, during early larval stages (Buentello et al., 2016). Addition of organic selenium and α-tocopherol did not enhance larval performance, probably indicating that the levels contained in copepods (0.4 μg g^{-1} and 170 mg kg^{-1}, respectively) were sufficient to fulfill requirements. Indeed, it would be necesary to establish the safe inclusion limits of selenium for ABT larvae. Different expression patterns of digestive enzymes between ABT larvae fed copepods and enriched rotifers could be due to different lipid class/fatty acid compositions of the live prey or to differences in the size/development of the larvae. Therefore, further studies are required to investigate lipid requirements, lipid metabolism and accumulation during development of ABT larvae. Attention should be given to the analysis of expression of genes related to lipid metabolism and its regulation, combined with biochemical studies of tuna lipid metabolism in order to develop optimal feeds to facilitate the commercial culture of this iconic species.

Acknowledgements

This work was supported by the Consejería de Innovación, Ciencia y Empresa de la Junta de Andalucía, Proyecto de Excelencia de Promoción General del Conocimiento [Ref. RNM 733, 2012), and Programa Estatal de Investigación del Ministerio de Economía y Competitividad [Ref. AGL2014-52003-C2-1-R, 2014].

650 **References**

651 Ackman, R.G., 1980. Fish lipids. In: Connell, J. J. (Ed.), Advances in Fish Science and Technology:
652 Fishing News Books, Farnham, pp. 83-103.

653 Agawa, Y., Honryo, T., Ishii, A., Kobayashi, T., Oku, H., Sawada, Y., 2012. Molecular
654 identification and tissue distribution of peroxisome proliferators activated receptor gamma
655 transcript in cultured *Thunnus orientalis*. Aquacult. Res. 43, 1145-1158.

656 André, M., Ando, S., Ballagny, C., Durliat, M., Poupard, G., Briançon, C., Babin, P.J., 2000.
657 Intestinal fatty acid binding protein gene expression reveals the cephalocaudal patterning during
658 zebrafish gut morphogenesis. Int. J. Dev. Biol. 44, 249–252.

659 AOAC (2000) Official methods of analysis. Association of Official Analytical Chemists,
660 Washington, DC

661 Betancor, M.B., Caballero, M.J., Terova, G., Saleh, R., Atalah, E., Benítez-Santana, T., Bell, J.G.,
662 Izquierdo, M., 2012. Selenium inclusion decreases oxidative stress indicators and muscle injuries
663 in sea bass larvae fed high-DHA microdiets. Br. J. Nutr. 13, 1–14.

664 Betancor, M.B., Howarth, F.J.E., Glencross, B.D., Tocher, D.R., 2014. Influence of dietary
665 docosahexaenoic acid in combination with other long-chain polyunsaturated fatty acids on
666 expression of biosynthesis genes and phospholipid fatty acid compositions in tissues of post-
667 smolt Atlantic salmon (*Salmo salar*). Comp. Biochem. Physiol. 172–173B, 74–898.

668 Betancor, M.B., Sprague, M., Sayanova, O., Usher, S., Campbell, P.J., Napier, J.A., Caballero,
669 M.J., Tocher, D.R., 2015. Evaluation of a high-EPA oil from transgenic Camelina sativa in feeds
670 for Atlantic salmon (*Salmo salar* L.): Effects on tissue fatty acid composition, histology and
671 gene expression. Aquaculture 444, 1-12.

672 Betancor, M.B., Ortega, A., de la Gándara, F., Tocher, D.R., Mourente, G., 2016. Lipid
673 metabolism-related gene expression pattern of Atlantic bluefin tuna (*Thunnus thynnus* L.)
674 larvae fed on live prey. Fish Physiol. Biochem. doi:10.1007/s10695-016-0305-4

675 Boglino, A., Darias, M.J., Ortiz-Delgado, J.B., Özcam, F., Estévez, A., Andree, K.B., Hontoria, F.,
676 Sarasquete, C., Gisbert, E., 2012. Commercial products for Artemia enrichment with distinct
677 fatty acid profiles affect growth performance, digestive system maturation, ossification and
678 incidence of skeletal deformities in Senegalese sole (*Solea senegalensis*) larvae. Aquaculture
679 324-325, 290-302.

680 Bonacic, K., Campoverde, C., Sastre, M., Hachero-Cruzado, I., Ponce, M., Manchado, M., Estevez,
681 A., Gisbert, E., Morais, S., 2016. Mechanisms of lipid metabolism and transport underlying
682 superior performance of Senegalese sole (*Solea senegalensis*, Kaup 1858) larvae fed diets
683 containing n-3 polyunsaturated fatty acids. Aquaculture 450, 383-396.

684 Bower, N.I., Xuejun, L., Taylor, R., Johnston, I.A., 2008. Switching to fast growth: the insulin-like
685 growth factor (IGF) system in skeletal muscle of Atlantic salmon. J. Exp. Biol. 211, 3859-3870.

686 Buentello, A., Seoka, M., Suarez, J., 2016. Nutrition of cultured tuna species. In: Benetti, D.,
687 Partridge, G. J., Buentello, A. (eds.), Advances in Tuna Aquaculture: From Hatchery to Market.
688 Elsevier AP, New York, pp. 273-321.

689 Carmona-Antoñanzas, G., Tocher, D.R., Martinez-Rubio, L., Leaver, M.J., 2014. Conservation of
690 lipid metabolic gene transcriptional regulatory networks in fish and mammals. Gene 534, 1-9.

691 Chatzifotis, S., Polemitou, I., Divanach, P., Antonopoulo, E., 2008. Effect of dietary taurine
692 supplementation on growth performance and bile salt activated lipase activity of common
693 dentex, *Dentex dentex*, fed a fish meal/soy protein concentrate-based diet. Aquaculture 275, 201-
694 208.

695 Christie, W.W., 1993, Preparation of derivatives of fatty acids for chromatographic analysis. In:
696 Christie, W.W. (Ed.), Advances in Lipid Methodology-Two. The Oily Press, Dundee. pp. 69-
697 111.

698 Chui, P.C., Guan, H.P., Lehrke, M., Lazar, M.A., 2005. PPAR gamma regulates adipocyte
699 cholesterol metabolism via oxidized LDL receptor 1. J. Clin. Invest. 115, 2244-2256.
700 Cowey, C.B., Adron, J.W., Walton, M.J., Murray, J., Youngson, A., Knox, D., 1981. Tissue
701 distribution, uptake and requirement for a-tocopherol of rainbow trout (Salmo gairdneri) fed
702 diets with a minimal content of unsaturated fatty acids. J. Nutr. 111, 1556–1567.
703 Cunha, I., Galante-Oliveira, S., Rocha, E., Planas, M., Urbatzka, R., Castro, L.F.C., 2013.
704 Dynamics of PPARs, fatty acid metabolism genes and lipid clases in eggs and early larvae of a
705 teleost. Comp. Biochem. Physiol. 164B, 247-258.
706 Darias, M.J., Boglino, A., Manchado, M., Ortiz-Delgado, J.B., Estévez, A., Andree, K.B., Gisbert,
707 E., 2012. Molecular regulation of both dietary vitamin A and fatty acid absorption and
708 metabolism associated with larval morphogenesis of Senegalese sole (*Solea senegalensis*).
709 Comp. Biochem. Physiol. 161A, 130-139.
710 De La Gandara F., Mylonas C., Coves D., Ortega A., Bridges C.R., Belmonte R.A., Vassallo-Agius
711 R., Papandroulakis N., Rosenfeld H., Tandler A., Medina A., Demetrio A., Corriero A., Fauvel
712 C., Falcon J., Sveinsvoll K., Ghysen A., Deguara S., Gordin H., 2010. Seedling production of
713 Atlantic bluefin tuna (ABFT) *Thunnus thynnus*. The selfdott project. In: S Miyashita, S.,
714 Sakamoto, W., Biswas, A. (Eds.), Joint International Symposium of Kinki University and
715 Setouchi Town on the 40th Anniversary of Pacific Bluefin Tuna Aquaculture, Towards the
716 Sustainable Aquaculture of Bluefin Tuna. University Press, Amami, Japan, pp. 45–52.
717 De La Gándara F., Ortega, A., Buentello, A., 2016. Tuna aquaculture in Europe. In: D. D. Benetti,
718 D.D., Partridge, G.J., Buentello, A. (Eds.), Advances in Tuna Aquaculture: From Hatchery to
719 Market. Elsevier AP, New York, pp. 273-321.
720 Fauconneau, B., Aguirre, P., Bergot, P., 1986. Protein synthesis in early life of coregonids:
721 influence of temperature and feeding. Arch. Hydrobiol. – Beihefte 22, 171-188.
722 Folch, J., Lees, M., Sloane-Stanley, G.H., 1957. A simple method for the isolation and purification
723 of total lipids from animal tissues. J. Biol. Chem. 226, 497-509.

724 Frohnert, B.I., Hui, T.Y., Bernlohr, D.A., 1999. Identification of a functional peroxisome
725 proliferator-responsive element in the murine fatty acid transport protein gene. J. Biol. Chem.
726 274, 3970-3977.
727 Glatz, J.F., van der Vusse, G.J., 1996. Cellular fatty acid-binding proteins: their function and
728 physiological significance. Prog. Lipid Res. 35, 243-282.
729 Gong, Y., Chen, W., Han, D., Zhu, X., Yang, Y., Jin, J., Liu, H., Xie, S., 2017. Effects of food
730 restriction on growth, body composition and gene expression related in regulation of lipid
731 metabolism and food intake in grass carp. Aquaculture 469, 28-35.
732 Gregory, M.K., See, V.H.L., Gibson, R.A., Schuller, K.A., 2010. Cloning and functional
733 characterisation of a fatty acyl elongase from southern bluefin tuna (*Thunnus maccoyii*). Comp.
734 Biochem. Physiol. 155B, 178-185.
735 Guan, H.P., Li, Y., Jensen, M.V., Newgard, C.B., Steppan, C.M., Lazar, M.A., 2002. A futile
736 metabolic cycle activated in adipocytes by antidiabetic agents. Nat. Med. 8, 1122-1128.
737 Hamre, K., 2006. Nutrition in cod (*Gadus morhua*) larvae and juveniles. ICES J. Mar. Sci. 63, 267-
738 274.

739 Hamre, K., Srivastava, A., Rønnestad, I., Mangor-Jensen, A., Stoss, J., 2008. Several micronutrients
740 in the rotifer *Brachionus sp*. may not fulfil the nutritional requirements of marine fish larvae.
741 Aquac. Nutr. 14, 51–60.

742 Hamre, K., Krossøy, C., Lock, E.J., Moren, M., 2010. Roles of lipid-soluble vitamins during
743 ontogeny of marine fish larvae. Aquac. Res. 41, 745–750.

744 Hamre, K., Yúfera, M., Rønnestad, I., Boglione, C., Conceição, L.E.C., Izquierdo, M., 2013. Fish
745 larval nutrition and feed formulation: Knowledge gaps and bottlenecks for advances in larval
746 rearing. Rev. Aquac. 5, S26-S58.

747 Hamre, K., 2016. Nutrient profiles of rotifers (*Brachionus sp*.) and rotifer diets from four different
748 marine fish hatcheries. Aquaculture 450, 136–142.

749 He, A.Y., Ning, L.J., Chen, L.Q., Chen, Y.L., Xing, Q., Li, J.M., Qiao, F., Li, D.L., Zhang, M.L.,
750 Du, Z.Y., 2015. Systemic adaptation of lipid metabolism in response to low- and high-fat diet
751 in Nile tilapia (*Oreochromis niloticus*). Physiol. Rep. 3, e12485.

752 Henderson, R.J., Tocher, D.R., 1992. Thin layer chromathography. In: Hamilton, R.J., Hamilton, S.,
753 (Eds.), Lipid analysis: a practical approach: IRL Press, Oxford, pp. 65–111.
754 Hibuse, T., Maeda, N., Funahashi, T., Yamamoto, K., Nagasawa, A., Mizunoya, W., Kishida, K.,
755 Inoue, K., Kuriyama, H., Nakamura, T., Fushiki, T., Kihara, S., Shimomura, I., 2005. Aquaporin
756 7 deficiency is associated with development of obesity through activation of adipose glycerol
757 kinase. Proc. Natl. Acad. Sci. U.S.A. 102, 10993-10998.
758 Hihi, A.K., Michalik, L., Wahli, W., 2002. PPARs: transcriptional effectors of fatty acids and their
759 derivatives. Cell. Mol. Life Sci. 59, 790-798.
760 Imsland, A.K., Le Francois, N.R., Lamarre, S.G., Ditlecadet, D., Sigurosson, S., Foss, A., 2006.
761 Myosin expression levels and enzyme activity in juvenile spotted wolffish (*Anarhichas minor*)
762 muscle: a method for monitoring growth rates. Can. J. Fish. Aquat. Sci. 63, 1959–1967.
763 Izquierdo, M.S., Betancor, M., 2015. Vitamin E. In: Lee, C.S., Lim, C., Gatlin, D., Webster, C.D.
764 (Eds.), Dietary Nutrients, Additives and Fish Health. Wiley-Backwell, New Jersey, pp. 173-194.
765 Jaramillo, F., Peng, L., Gatlin, D., 2009. Selenium nutrition of hybrid striped bass (*Morone*
766 *chrysops* x *M. saxatilis*) bioavailability, toxicity and interaction with vitamin E. Aquacult. Nutr.
767 15, 160-165.
768 Jeon, T.I., Osborne, T.F., 2012. SREBPs: metabolic integrators in physiology and metabolism.
769 Trends Endocrinol. Metab. 23, 65–72.

770 Ji, H., Liu, Y., Zhao, X., Zhang, M., 2011. N-acetyl-L-cysteine enhances the osteogenic
771 differentiations and inhibits the adipogenic differentiation through up regulation of Wnt 5a and
772 down regulation of PPARg in bone marrow stromal cells. Biomed. Pharmacother. 65, 369-374.

773 Johnston, I. A, Bower, N.I., Macqueen, D.J., 2011. Growth and the regulation of myotomal muscle
774 mass in teleost fish. J. Exp. Biol. 214, 1617–1628.

775 Karlsen, Ø., van der Meeren, T., Rønnestad, I., Mangor-Jensen, A., Galloway, T.F., Kjørsvik, E.,
776 Hamre, K., 2015. Copepods enhance nutritional status, growth and development in Atlantic cod
777 (*Gadus morhua* L.) larvae – can we identify the underlying factors? PeerJ 3:e902; DOI
778 10.7717/peerj.902.

779 Katagiri, R., Sasaki, T., Diaz, A., Ando, M., Margulies, D., Scholey, V.P., Sawada, Y., 2016. Effect
780 of taurine enrichment in rotifer (*Brachionus* sp.) on growth of larvae of Pacific bluefin tuna
781 *Thunnus orientalis* (Temminck & Schlegel) and yellowfin tuna *T. albacares* (Temminck &
782 Schlegel). Aquac. Res. 1–19.

783 Kersten, S., 2014. Physiological regulation of lipoprotein lipase. BBA- Mol. Cell Biol. Lipids.
784 1841, 919–933.

785 Kostopoulou, V., Miliou, H., Verriopoulos, G., 2015. Biochemical composition and fatty acid
786 profile in a strain of the lineage “Nevada”, belonging to the *Brachionus plicatilis* (Rotifera)
787 complex , fed different diets. Aquacult. Res. 46, 813–824.

788 Lehrke, M., Lazar, M.A., 2005. The many faces of PPARγ. Cell 123, 993-999.

789 Leng, X.J., Wu, X.F., Tian, J., Li, X.Q., Guan, K., Weng, D.C., 2012. Molecular cloning of fatty
790 acid synthase from grass carp (*Ctenopharyngodon idella*) and the regulation of its expression by
791 dietary fat level. Aquacult. Nutr. 18, 551-558.

792 Li, K., Østensen, M.A., Attramadal, K., Winge, P., Sparstad, T., Bones, A.M., Vadstein, O.,
793 Kjørsvik, E., Olsen, Y., 2015. Gene regulation of lipid and phospholipid metabolism in Atlantic
794 cod (*Gadus morhua*) larvae. Comp. Biochem. Physiol. Part B Biochem. Mol. Biol. 190, 16–26.

795 Mæhre, H.K., Hamre, K., Elvevoll, E.O., 2013. Nutrient evaluation of rotifers and zooplankton:
796 Feed for marine fish larvae. Aquac. Nutr. 19, 301–311.

797 Mazurais, D., Covès, D., Papandroulakis, N., Ortega, A., Desbruyeres, E., Huelvan, C., Le Gall,
798 M.M., De la Gándara, F., Cahu, C.L., 2015. Gene expression pattern of digestive and antioxidant
799 enzymes during the larval development of reared Atlantic bluefin tuna (ABFT), *Thunnus thynnus*
800 L. Aquacult. Res. 46, 2323-2331.

801 McMurray, C.H., Blanchflower, W.J., Rice, D.A., 1980. Influence of extraction techniques on
802 determination of α-tocopherol in animal feedstuffs. J AOAC 63, 1258–1261.

803 Minghetti, M., Leaver, M.J., Tocher, D.R., 2011. Transcriptional control mechanisms of genes of
804 lipid and fatty acid metabolism in the Atlantic salmon (*Salmo salar* L.) established cell line,
805 SHK-1. BBA- Mol. Cell Biol. Lipids. 1811, 194-202.

806 Miyashita, S., Kato, Y., Sawada, Y., Murata, O., Ishitani, Y., Shimizy, K., Yamamoto, S., Kumai,
807 H., 1998. Development of digestive system and digestive enzyme activities of larval and juvenile
808 bluefin tuna, *Thunnus thynnus*, reared in the laboratory. Suisanzoshoku 46, 111–120.

809 Morais, S., Mourente, G., Ortega, A., Tocher, J.A., Tocher, D.R., 2011. Expression of fatty acyl
810 desaturase and elongase genes, and evolution of DHA/EPA ratio during development of unfed
811 larvae of Atlantic bluefin tuna (*Thunnus thynnus* L.). Aquaculture 313, 129-139.

812 Morais, S., Castanheira, F., Martinez-Rubio, L., Conceição, L.E.C., Tocher, D.R., 2012. Long chain
813 polyunsaturated fatty acid synthesis in a marine vertebrate: Ontogenetic and nutritional
814 regulation of a fatty acyl desaturase with Δ4 activity. Biochim. Biophys. Acta - Mol. Cell Biol.
815 Lipids 1821, 660–671.

816 Mourente, G., Tocher, D.R., 2003. An approach to study the nutritional requirements of the bluefin
817 tuna (*Thunnus thynnus thynnus* L.). Cahiers Options Méditerranéennes 60, 143-150.
818 Mourente, G., Tocher, D.R., 2009. Tuna nutrition and feeds: current status and future perspectives.
819 Rev. Fisheries Sci. 17, 374-391.
820 Mourente, G., Bell, J.G., Tocher, D.R., 2007. Does dietary tocopherol affect fatty acid metabolism
821 in fish? Fish Physiol. Biochem. 33, 269-280.

822 Murashita, K., Matsunari, H., Kumon, K., Tanaka, Y., Shiozawa, S., Furuita, H., Oku, H.,
823 Yamamoto, T., 2014. Characterization and ontogenetic development of digestive enzymes in
824 Pacific bluefin tuna *Thunnus orientalis* larvae. Fish Physiol. Biochem. 40, 1741–1755.

825 Murashita, K., Fukasa, H., Takahashi, N., Hosomi, N., Matsunari, H., Furuita, H., Oku, H.,
826 Yamamoto, T., 2015. Effect of feed ingredients on digestive enzyme secretion in fish. Bull. Fish.
827 Res. Agen. 40, 69-74.

828 Nedergaard, J., Ricquier, D., Kozak, L.P., 2005. Uncoupling proteins: curren status and
829 therapeutic prospects. EMBO Rep., 6, 917–921.
830 Nilsson-Ehle, P., Grafinkel, A.S., Schotz, M.C., 1980. Lipolytic enzymes and plasma lipoprotein
831 metabolism. Annu. Rev. Biochem. 49, 667-693.
832 NRC, 2011. Nutrient requirements of fish and shrimp. National Research Council, Academic Press,
833 Washington DC.
834 Ortega, A., 2015. Cultivo Integral de dos especies de escómbridos: Atún rojo del Atlántico
835 (*Thunnus thynnus*, L. 1758) y Bonito Atlántico (*Sarda sarda*, Bloch 1793). PhD Thesis,
836 Universidad de Murcia, Murcia (Spain).
837 Partridge, G.J., 2013. Closed-cycle hatchery production of tuna. In: Allan, G., Burnell, G. (Eds.),
838 Advances in Aquaculture Hatchery Technology. Woodhead Publishing Series in Food Science,
839 Technology and Nutrition: Number 242, Woodhead Publishing Limited, Oxford, pp. 457-497.

840 Pfaffl, M.W., Morgan, G.W., Dempfle, L., 2002. Relative expression software tool (REST) for
841 group-wise comparison and statistical analysis of relative expression results in real-time PCR.
842 Nucleic Acids Res. 30, e36.
843 Polatajko, A., Jakubowski, N., Szpunar, J., 2006. State of the art report of selenium speciation in
844 biological samples. J. Anal At. Spectrom. 21, 639-654.
845 Rajkumar, M., Kumaraguru vasagam, K.P., 2006. Suitability of the copepod, *Acartia clausi* as a
846 live feed for Seabass larvae (*Lates calcarifer* Bloch): Compared to traditional live-food
847 organisms with special emphasis on the nutritional value. Aquaculture 261, 649-658.
848 Rønnestad, I., Morais, S., 2008. Digestion. In: Finn, R.N., Kapoor, B.G. (Eds.), Fish Larval
849 Physiology, Science Publishers, Enfield, NH, USA, pp. 201-262.
850 Sargent, J.R., Henderson, R.J., Tocher, D.R., 1989. The lipids. In: Halver, J.E. (Ed.), Fish Nutrition,
851 2nd Edition: Academic Press, Inc., San Diego, pp. 154-218.
852 Sargent, J.R., Tocher, D.R., Bell, J.G., 2002. The lipids. In: Halver J.E., Hardy, R.W. (Eds), Fish
853 Nutrition, 3rd Edition: Academic Press, Inc., San Diego, pp. 182–246.
854 Sawada, Y., Okada, T., Miyashita, S., Murata, O., Kumai, H., 2005. Completion of the Pacific
855 bluefin tuna *Thunnus orientalis* (Temminck et Schlegel) life cycle. Aquac. Res. 36, 413–421.
856 Sæle, Ø., Nordgreen, A., Olsvik, P.A., Hamre, K., 2010. Characterization and expression of
857 digestive neutral lipases during ontogeny of Atlantic cod (*Gadus morhua*). Comp. Biochem.
858 Physiol. 157A, 252-259.
859 Scholefield, A.M., Tocher, D.R., Schuller, K.A., 2015. Dynamics of fatty acid metabolism in a cell
860 line from southern bluefin tuna (*Thunnus maccoyii*). Aquaculture 449, 58-68.
861 Schoonjans, K., Peinado-Onsurbe, J., Lefebvre, A.M., Heyman, R.A., Briggs, M., Deeb, S., Staels,
862 B., Auwerx, J., 1996. PPAR alpha and PPAR gamma activators direct a distinct tissue-specific
863 transcriptional response via a PPRE in the lipoprotein lipase gene. EMBO J. 15, 5336-5348.

864 Takeuchi, T., 2014. Progress on larval and juvenile nutrition to improve the quality and health of
865 seawater fish: A review. Fish. Sci. 80, 389–403.

866 Tian, J., Wen, H., Zeng, L.B., Jiang, M., Wu, F., Liu, W., Yang, G.C., 2013. Changes in the
867 activities and mRNA expression levels of lipoprotein lipase (LPL), hormone-sensitive lipase
868 (HSL) and fatty acid synthetase (FAS) of Nile tilapia (*Oreochromis niloticus*) during fasting
869 and re-feeding. Aquaculture 400-401, 29-35.

870 Thompson, J.L., See, V.H., Thomas, P.M., Schuller, K.A., 2010. Cloning and functional
871 characterization of two glutathione peroxidase cDNAs from souther bluefin tuna (*Thunnus*
872 *maccoyii*). Comp. Biochem. Physiol. 156B, 287-297.

873 Tocher, D.R., Harvie, D.G., 1988. Fatty acid composition of the major phosphoglycerides from fish
874 neutral tissues: (n-3) and (n-6) polyunsaturated fatty acids in rainbow trout (*Salmo gairdneri* L.)
875 and cod (*Gadus morhua* L.) brains and retinas. Fish Physiol. Biochem. 5, 229-239.
876 Tocher, D.R., 2003. Metabolism and functions of lipids and fatty acids in teleost fish. Rev.
877 Fisheries Sci. 11, 107-184.
878 Tocher, D.R., 2010. Fatty acid requirements in ontogeny of marine and freshwater fish. Aquacult.
879 Res. 41, 717-732.
880 Toledo, J.D., Golez, M.S., Ohna, A., 1999. Use of copepod nauplii during early feeding stage of
881 grouper *Epinephelus coioides*. Fish. Sci. 65, 390-397.
882 Uotani, I., Saito, T., Hiranuma, K., Nishikawa, Y., 1990. Feeding habit of bluefin tuna *Thunnus*
883 *thynnus* larvae in the western North Pacific Ocean. Nippon Suisan Gakkaishi 56, 713-717.

884 van der Meeren, T., Olsen, R.E., Hamre, K., Fyhn, H.J., 2008. Biochemical composition of
885 copepods for evaluation of feed quality in production of juvenile marine fish. Aquaculture 274,
886 375–397.

887 Vessey, D.A., Benfatto, A.M., Zerweck, E., Vestweber, C., 1990. Purification and cgaracterization
888 of the enzymes of bile acid conjugation from fish liver. Comp Biochem Physiol 95B, 647-652.

889 Waagbø, R., 2010. Water-soluble vitamins in fish ontogeny. Aquac. Res. 41, 733–744.

890 Witt, U., Quantz, G., Kuhlmann, D., Kattner, G., 1984. Survival and growth of turbot larvae
891 (*Scophtalmus maximus*) reared on different food organisms with special regard to long-chain
892 polyunsaturated fatty acids. Aquacult. Eng. 3, 177-190.
893 Worgall, T.S., Sturley, S.L., Seo, T., Osborne, T.F., Deckelbaum, R.J., 1998. Polyunsaturated fatty
894 acids decrease expression of promoters with sterol regulatory elements by decreasing levels of
895 mature sterol regulatory element-binding protein. J. Biol. Chem. 273, 25537–25540.
896 Yasunori, I., 2012. Fingerling production II. In Kumai, H., Miyashita, S., Sakamoto, W., Ono, S.,
897 (Eds.), Full-Life Cycle Aquaculture of the Pacific Bluefin Tuna. Agriculture and Forestry
898 Statistics Publishing Inc., Tokyo, Japan.
899 Yuan, X., Liang, X.F., Liu, L., Fang, J., Li, J., LI. A., Cai, W., Xue, M., Wang, J., Wang, Q., 2016.
900 Fat deposition pattern and mechanism in response to dietary lipid levels in grass carp,
901 *Ctenopharyngodon idellus*. Fish Physiol. Biochem. 42, 1557-1569.

902 Yokoyama, M., Takeuchi, T., Park, G.S., Nakazoe, J., 2001. Hepatic cysteinesulphinate
903 decarboxylase activity in fish. Aquac. Res. 32, 216–220.

904 Yufera, M., Ortiz-Delgado, J., Hoffman, T., Siguero, I., Urup, B., Sarasquete, C., 2014.
905 Organogenesis of digestive system, visual system and other structures in Atlantic bluefin tuna
906 (*Thunnus thynnus*) larvae reared with copepods in mesocosm system. Aquaculture 426, 126-137.
907 Zar, J.H., 1999. Biostatistical Analysis 4th Edition Prentice-Hall, New Jersey.
908 Zheng, K., Zhu, X., Han, D., Yang, Y., Lei, W., Xie, S., 2010. Effects of dietary lipid levels on
909 growth, survival and lipid metabolism during early ontogeny of *Pelteobagrus vachelli* larvae.
910 Aquaculture 299, 121-127.
911

912 **Figure legends**

913 **Fig. 1.** Nutritional regulation of myosin heavy chain 2 (*myhc*) and tropomyosin *(tropo*) gene
914 transcription in whole larvae of Atlantic bluefin tuna fed rotifers *Brachionus rotundiformis*
915 enriched with four commercial enrichment products as described in Materials and Methods
916 (OG, MG, AG and RA) or 3 days post hatch nauplii of the copepod *Acartia tonsa* fed
917 *Rhodomonas.* Values are normalized expression ratios, corresponding to an average of 6 pools
918 of larvae (n = 6) with standard errors (SEM). Different superscript letters denote differences
919 among the dietary treatments ($p < 0.05$).

920

921

922 **Fig. 2.** Nutritional regulation of fatty acid synthase (*fas*), delta-6 fatty acyl desaturase (*fads2d6*) and
923 fatty acyl elongase 5 (*elovl5*) gene transcription in whole larvae of Atlantic bluefin tuna fed
924 rotifers *Brachionus rotundiformis* enriched with four commercial enrichment products as
925 described in Materials and Methods (OG, MG, AG and RA) or 3 days post hatch nauplii of the
926 copepod *Acartia tonsa* fed *Rhodomonas.* Values are normalized expression ratios,
927 corresponding to an average of 6 pools of larvae (n = 6) with standard errors (SEM). Different
928 superscript letters denote differences among the dietary treatments ($p < 0.05$).

929

930

931 **Fig.3.** Nutritional regulation of carnitine palmitoyl transferase I (*cptI*) and acyl coA oxidase (*aco*)
932 gene transcription in whole larvae of Atlantic bluefin tuna fed rotifers *Brachionus*
933 *rotundiformis* enriched with four commercial enrichment products as described in Materials
934 and Methods (OG, MG, AG and RA) or 3 days post hatch nauplii of the copepod *Acartia tonsa*
935 fed *Rhodomonas.* Values are normalized expression ratios, corresponding to an average of 6
936 pools of larvae (n = 6) with standard errors (SEM). Different superscript letters denote
937 differences among the dietary treatments ($p < 0.05$).

Fig.4. Nutritional regulation of fatty acid binding protein 2, 4 and 6 (*fabp2, fabp4* and *fabp7* respectively), lipoprotein lipase (*lpl*) and 3-hydroxy-3-methylglutaryl-CoA lyase (*hmgcl*) gene transcription in whole larvae of Atlantic bluefin tuna fed rotifers *Brachionus rotundiformis* enriched with four commercial enrichment products as described in Materials and Methods (OG, MG, AG and RA) or 3 days post hatch nauplii of the copepod *Acartia tonsa* fed *Rhodomonas.*. Values are normalized expression ratios, corresponding to an average of 6 pools of larvae (n = 6) with standard errors (SEM). Different superscript letters denote differences among the dietary treatments ($p < 0.05$).

Fig.5. Nutritional regulation of peroxisome proliferator-activated receptor alpha (*pparα*), gamma (*pparγ*), sterol regulatory element-binding protein 1 and 2 (*srebp1*and *srebp2* respectively), retinoid X receptor (*rxr*) and liver X receptor (*lxr*) gene transcription in whole larvae of Atlantic bluefin tuna fed rotifers *Brachionus rotundiformis* enriched with four commercial enrichment products as described in Materials and Methods (OG, MG, AG and RA) or 3 days post hatch nauplii of the copepod *Acartia tonsa* fed *Rhodomonas.* Values are normalized expression ratios, corresponding to an average of 6 pools of larvae (n = 6) with standard errors (SEM). Different superscript letters denote differences among the dietary treatments ($p < 0.05$).

Fig.6. Nutritional regulation of superoxide dismutase (*sod*), catalase (*cat*) and glutathione peroxidase 1 and 4 (*gpx1* and *gpx4* respectively) gene transcription in whole larvae of Atlantic bluefin tuna fed rotifers *Brachionus rotundiformis* enriched with four commercial enrichment products as described in Materials and Methods (OG, MG, AG and RA) or 3 days post hatch

nauplii of the copepod *Acartia tonsa* fed *Rhodomonas.* Values are normalized expression ratios, corresponding to an average of 6 pools of larvae (n = 6) with standard errors (SEM). Different superscript letters denote differences among the dietary treatments ($p < 0.05$).

Fig.7. Nutritional regulation of trypsin (*tryp*), amino peptidase (*anpep*), amylase (*amy)*, pancreatic lipase (*pl*), phospholipase A_2 (*pla2*) and bile salt activated lipase 1 and 2 (*bal1* and *bal2* respectively) gene transcription in whole larvae of Atlantic bluefin tuna fed rotifers *Brachionus rotundiformis* enriched with four commercial enrichment products as described in Materials and Methods (OG, MG, AG and RA) or 3 days post hatch nauplii of the copepod *Acartia tonsa* fed *Rhodomonas.* Values are normalized expression ratios, corresponding to an average of 6 pools of larvae (n = 6) with standard errors (SEM). Different superscript letters denote differences among the dietary treatments ($p < 0.05$).

Table 1. Growth performance of 15 days after hatch ABT larvae fed rotifers *Brachionus rotundiformis* enriched with four commercial enrichment products as described in Materials and Methods (OG, MG, AG and RA) or 3 days post hatch nauplii of the copepod *Acartia tonsa* fed *Rhodomonas baltica.*

	OG	MG	AG	RA	COP
Length (mm)	6.4 ± 0.2 [b]	6.6 ± 0.2 [b]	6.7 ± 0.2 [ab]	6.5 ± 0.3 [b]	7.4 ± 0.1 [a]
Dry mass (mg)	2.7 ± 1.2 [c]	3.1 ± 1.3 [b]	3.2 ± 1.3 [b]	2.5 ± 1.1 [c]	4.3 ± 1.3 [a]
Flexion Index	42.7 ± 6.6 [b]	43.7 ± 0.9 [b]	50.7 ± 7.4 [ab]	45.0 ± 4.9 [b]	63.0 ± 1.2 [a]
Survival (%)	9.3 ± 3.7	5.6 ± 1.6	8.9 ± 4.5	7.7 ± 5.3	7.4 ± 0.3

Results are means ± SD (n = 15 for total length, n = 30 for dry mass and flexion index, n = 3 for survival). An SD of 0.0 implies an SD of < 0.05.

Table 2. Macronutrient (dry mass %), vitamin E (mg kg^{-1}) and selenium (µg g^{-1}) contents of rotifers *Brachionus rotundiformis* enriched with four commercial enrichment products as described in Materials and Methods (OG, MG, AG and RA) or 3 days post hatch nauplii of the copepod *Acartia tonsa* fed *Rhodomonas baltica* (COP).

	OG	MG	AG	RA	COP
Protein	55.2 ± 0.1^{b}	53.8 ± 0.4^{b}	51.6 ± 0.6^{b}	52.7 ± 0.1^{b}	63.8 ± 3.0^{a}
Total lipid	10.1 ± 0.3^{b}	9.8 ± 0.4^{b}	12.9 ± 1.0^{a}	11.4 ± 0.2^{b}	5.6 ± 0.0^{c}
Vitamin E	310.3 ± 15.5^{b}	294.6 ± 5.9^{b}	232.3 ± 1.7^{bc}	593.2 ± 4.4^{a}	170.1 ± 6.5^{c}
Selenium	6.4 ± 0.1^{b}	5.4 ± 0.1^{c}	4.8 ± 0.2^{d}	10.6 ± 0.2^{a}	0.4 ± 0.0^{e}

Results are means ± SD (n = 3). An SD of 0.0 implies an SD of < 0.05. Mean values bearing different superscript letter are significantly different ($P<0.05$).

Table 3. Total lipid lipid class content (µg lipid class/mg dry mass) of rotifers *Brachionus rotundiformis* enriched with four commercial enrichment products as described in Materials and Methods (OG, MG, AG and RA) or 3 days post hatch nauplii of the copepod *Acartia tonsa* fed *Rhodomonas baltica* (COP).

	OG	MG	AG	RA	COP
Lipid Class (µg lipid class/mg dry mass)					
PC	1.05 ± 0.05 ^{ab}	0.94 ± 0.01 ^{b}	1.07 ± 0.03 ^{ab}	1.08 ± 0.06 ^{a}	0.76 ± 0.07 ^{c}
PE	1.15 ± 0.03 ^{ab}	1.03 ± 0.10 ^{b}	1.22 ± 0.03 ^{a}	1.15 ± 0.03 ^{ab}	0.58 ± 0.01 ^{c}
PS	0.35 ± 0.01 ^{a}	0.20 ± 0.01 ^{b}	0.25 ± 0.01 ^{b}	0.21 ± 0.07 ^{b}	0.07 ± 0.04 ^{c}
PI	0.12 ± 0.00	0.12 ± 0.02	0.11 ± 0.01	0.10 ± 0.02	0.09 ± 0.04
Sphingomyelin	0.04 ± 0.00	0.04 ± 0.00	0.05 ± 0.00	0.05 ± 0.00	0.02 ± 0.00
LPC	0.19 ± 0.01 ^{b}	0.19 ± 0.01 ^{b}	0.22 ± 0.02 ^{b}	0.19 ± 0.01 ^{b}	0.24 ± 0.03 ^{a}
Sulphatides	0.05 ± 0.00 ^{c}	0.04 ± 0.02 ^{c}	0.15 ± 0.00 ^{a}	0.10 ± 0.02 ^{b}	0.05 ± 0.02 ^{c}
Pigments	0.86 ± 0.01 ^{b}	0.80 ± 0.02 ^{c}	0.95 ± 0.02 ^{a}	0.77 ± 0.03 ^{c}	0.64 ± 0.01 ^{d}
Total Polar	**3.82 ± 0.02 ^{a}**	**3.37 ± 0.13 ^{b}**	**4.03 ± 0.09 ^{a}**	**3.66 ± 0.21 ^{ab}**	**2.47 ± 0.18 ^{c}**
Triacylglycerol	3.31 ± 0.03 ^{d}	4.02 ± 0.16 ^{c}	5.86 ± 0.11 ^{a}	5.47 ± 0.22 ^{b}	1.60 ± 0.05 ^{e}
Cholesterol	0.66 ± 0.04 ^{b}	0.71 ± 0.04 ^{b}	1.04 ± 0.01 ^{a}	0.69 ± 0.03 ^{b}	0.71 ± 0.03 ^{b}
SE/WE	0.99 ± 0.04 ^{ab}	0.89 ± 0.08 ^{b}	1.03 ± 0.01 ^{a}	0.61 ± 0.07 ^{c}	0.32 ± 0.02 ^{d}
Free fatty acid	1.31 ± 0.03 ^{a}	0.81 ± 0.02 ^{c}	0.94 ± 0.02 ^{b}	0.97 ± 0.03 ^{b}	0.49 ± 0.08 ^{d}
Total Neutral	6.28 ± 0.02 ^{c}	6.43 ± 0.13 ^{c}	8.87 ± 0.09 ^{a}	7.74 ± 0.21 ^{b}	3.12 ± 0.18 ^{d}

Results are means ± SD (n = 3). An SD of 0.0 implies an SD of < 0.05. Mean values bearing different superscript letter are significantly different ($P < 0.05$). LPC, lyso-phosphatidylcholine; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; SE/WE, steryl/wax ester.

Table 4. Total lipid fatty acid content (µg fatty acid/mg dry mass) of rotifers *Brachionus rotundiformis* enriched with four commercial enrichment products as described in Materials and Methods (OG, MG, AG and RA) or 3 days post hatch nauplii of the copepod *Acartia tonsa* fed *Rhodomonas baltica* (COP).

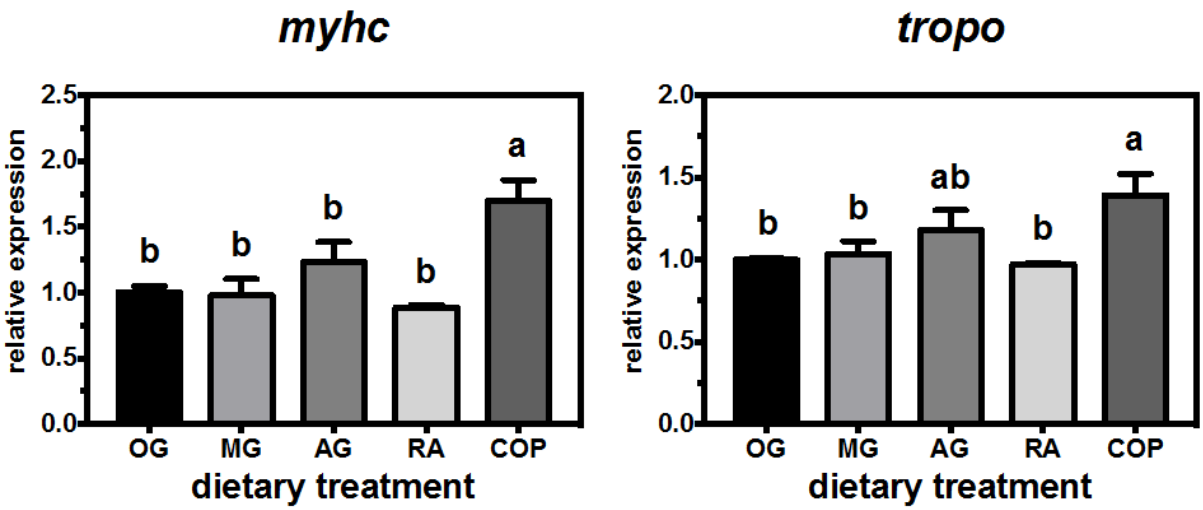
	OG	MG	AG	RA	COP
Fatty acid					
14:0	0.7 ± 0.0 ^{c}	1.0 ± 0.2 ^{c}	2.8 ± 0.1 ^{a}	1.7 ± 0.3 ^{b}	0.5 ± 0.0 ^{c}
16:0	9.8 ± 0.4 ^{c}	10.4 ± 0.6 ^{bc}	14.0 ± 0.4 ^{a}	11.2 ± 0.2 ^{b}	3.2 ± 0.2 ^{e}
18:0	3.1 ± 0.1 ^{a}	2.1 ± 0.1 ^{b}	2.9 ± 0.2 ^{a}	2.4 ± 0.1 ^{b}	1.3 ± 0.1 ^{c}
Total saturated[1]	15.3 ± 0.4 ^{bc}	14.7 ± 1.0 ^{c}	21.5 ± 0.6 ^{a}	16.8 ± 0.5 ^{b}	6.2 ± 0.2 ^{d}
16:1n-7	1.0 ± 0.1 ^{a}	0.4 ± 0.0 ^{b}	0.5 ± 0.0 ^{b}	0.5 ± 0.0 ^{b}	0.4 ± 0.0 ^{b}
18:1n-9	4.0 ± 0.2 ^{a}	1.2 ± 0.1 ^{b}	0.8 ± 0.1 ^{b}	0.8 ± 0.1 ^{b}	0.3 ± 0.0 ^{c}
18:1n-7	1.0 ± 0.1 ^{a}	0.6 ± 0.1 ^{b}	0.6 ± 0.0 ^{b}	0.7 ± 0.0 ^{b}	1.1 ± 0.1 ^{a}
20:1n-9	0.8 ± 0.1 ^{a}	0.4 ± 0.0 ^{b}	0.4 ± 0.0 ^{b}	0.5 ± 0.0 ^{b}	0.1 ± 0.0 ^{c}
Total monoenes[2]	8.9 ± 0.2 ^{a}	4.4 ± 0.1 ^{b}	4.5 ± 0.1 ^{b}	4.6 ± 0.1 ^{b}	2.8 ± 0.0 ^{c}
18:2n-6	10.3 ± 0.4 ^{a}	6.5 ± 0.1 ^{c}	7.8 ± 0.2 ^{b}	7.0 ± 0.1 ^{c}	1.4 ± 0.1 ^{d}
20:4n-6	0.5 ± 0.0 ^{b}	0.5 ± 0.0 ^{b}	1.0 ± 0.0 ^{a}	1.1 ± 0.3 ^{a}	0.6 ± 0.0 ^{b}
22:5n-6	0.6 ± 0.0 ^{d}	2.8 ± 0.1 ^{c}	7.1 ± 0.3 ^{a}	5.0 ± 0.1 ^{b}	0.3 ± 0.1 ^{d}
Total n-6PUFA[3]	13.8 ± 0.6 ^{c}	11.9 ± 0.4 ^{d}	18.6 ± 0.8 ^{a}	15.3 ± 0.4 ^{b}	3.7 ± 0.1 ^{e}
18:3n-3	2.6 ± 0.1 ^{a}	1.6 ± 0.0 ^{b}	2.4 ± 0.1 ^{a}	1.7 ± 0.0 ^{b}	1.2 ± 0.1 ^{c}
18:4n-3	0.1 ± 0.0 ^{c}	0.1 ± 0.0 ^{c}	0.3 ± 0.0 ^{b}	0.2 ± 0.1 ^{bc}	0.7 ± 0.1 ^{a}
20:4n-3	0.5 ± 0.0 ^{b}	0.6 ± 0.0 ^{b}	0.9 ± 0.0 ^{a}	0.8 ± 0.0 ^{a}	0.1 ± 0.0 ^{c}
20:5n-3	2.8 ± 0.1 ^{a}	1.8 ± 0.1 ^{b}	2.8 ± 0.1 ^{a}	2.6 ± 0.0 ^{a}	2.8 ± 0.2 ^{a}
22:5n-3	2.3 ± 0.1 ^{a}	1.4 ± 0.1 ^{c}	1.8 ± 0.1 ^{b}	1.7 ± 0.0 ^{b}	0.1 ± 0.0 ^{d}
22:6n-3	7.8 ± 0.4 ^{d}	10.3 ± 0.5 ^{c}	23.1 ± 0.9 ^{a}	16.1 ± 0.3 ^{b}	5.1 ± 0.3 ^{e}
Total n-3PUFA[4]	18.1 ± 0.9 ^{c}	17.2 ± 0.7 ^{c}	34.3 ± 1.3 ^{a}	25.2 ± 0.5 ^{b}	11.1 ± 0.5 ^{d}
C16 PUFA	2.4 ± 0.0 ^{b}	1.6 ± 0.0 ^{d}	2.9 ± 0.1 ^{a}	1.8 ± 0.0 ^{c}	1.4 ± 0.0 ^{e}
Total PUFA	31.8 ± 1.5 ^{c}	29.1 ± 1.0 ^{c}	52.8 ± 2.1 ^{a}	40.6 ± 0.5 ^{b}	14.9 ± 0.4 ^{d}
n-3/n-6	1.3 ± 0.1 ^{c}	1.4 ± 0.1 ^{c}	1.8 ± 0.1 ^{b}	1.6 ± 0.1 ^{bc}	3.0 ± 0.2 ^{a}
DHA/EPA	2.8 ± 0.1 ^{d}	5.7 ± 0.1 ^{c}	8.3 ± 0.1 ^{a}	6.2 ± 0.1 ^{b}	1.9 ± 0.2 ^{e}

Results are means ± SD (n = 3). An SD of 0.0 implies an SD of < 0.05. Mean values bearing different superscript letter are significantly different ($P<0.05$). [1], Totals include 15:0, 20:0, 22:0 and 24:0; [2], Totals include 16:1n-9, 18:1n-11, 20:1n-7, 22:1 isomers and 24:1; [3], Totals include 18:3n-6, 20:2n-6 and 22:4n-6; [4], Totals include 20:3n-3 and 22:3n-3; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; PUFA, polyunsaturated fatty acid.

Table 5. Fatty acid composition (weight %) of total lipid of 15 days post hatch ABT larvae fed rotifers *B. rotundiformis* enriched with four commercial enrichment products as described in Materials and Methods (OG, MG, AG and RA) or 3 days post hatch nauplii of the copepod *Acartia tonsa* fed *Rhodomonas baltica* (COP).

	OG	MG	AG	RA	COP
14:0	1.6 ± 0.1 ^{b}	0.9 ± 0.1 ^{c}	1.1 ± 0.1 ^{c}	1.0 ± 0.0 ^{c}	2.7 ± 0.1 ^{a}
16:0	18.2 ± 0.4 ^{b}	19.9 ± 1.4 ^{ab}	21.0 ± 1.2 ^{a}	21.7 ± 1.3 ^{a}	20.0 ± 0.3 ^{ab}
18:0	10.8 ± 0.3 ^{b}	11.6 ± 0.9 ^{ab}	11.7 ± 0.6 ^{ab}	13.0 ± 0.9 ^{a}	10.6 ± 0.2 ^{b}
Total saturated[1]	34.5 ± 0.9 ^{b}	37.3 ± 2.6 ^{ab}	40.9 ± 2.3 ^{a}	40.1 ± 1.1 ^{a}	37.7 ± 0.6 ^{a}
16:1n-7	2.4 ± 0.1 ^{a}	2.6 ± 0.2 ^{a}	0.8 ± 0.1 ^{b}	0.9 ± 0.1 ^{b}	2.6 ± 0.2 ^{a}
18:1n-9	5.5 ± 0.1 ^{a}	3.7 ± 0.3 ^{b}	3.3 ± 0.2 ^{b}	3.5 ± 0.1 ^{b}	5.1 ± 0.1 ^{a}
18:1n-7	1.9 ± 0.1 ^{ab}	1.4 ± 0.1 ^{c}	1.4 ± 0.1 ^{c}	1.7 ± 0.1 ^{b}	2.1 ± 0.1 ^{a}
20:1n-9	0.7 ± 0.0 ^{a}	0.5 ± 0.1 ^{a}	0.5 ± 0.2 ^{a}	0.5 ± 0.1 ^{a}	0.2 ± 0.0 ^{b}
Total monoenes[2]	14.4 ± 0.3 ^{a}	11.5 ± 0.8 ^{b}	9.1 ± 0.4 ^{c}	9.5 ± 0.1 ^{c}	11.9 ± 0.2 ^{b}
18:2n-6	10.8 ± 0.2 ^{a}	8.5 ± 0.6 ^{b}	8.4 ± 0.4 ^{b}	7.9 ± 0.3 ^{b}	3.9 ± 0.1 ^{c}
20:4n-6	1.4 ± 0.1 ^{c}	1.9 ± 0.2 ^{b}	2.4 ± 0.1 ^{a}	2.5 ± 0.2 ^{a}	1.2 ± 0.0 ^{c}
22:5n-6	1.3 ± 0.1 ^{d}	3.4 ± 0.3 ^{b}	4.8 ± 0.3 ^{a}	4.7 ± 0.1 ^{a}	2.7 ± 0.0 ^{c}
Total n-6PUFA[3]	16.6 ± 0.4 ^{b}	16.7 ± 1.2 ^{b}	20.7 ± 0.8 ^{a}	20.1 ± 0.3 ^{a}	12.7 ± 0.2 ^{c}
18:3n-3	1.9 ± 0.0 ^{a}	1.1 ± 0.1 ^{b}	1.3 ± 0.1 ^{b}	1.1 ± 0.1 ^{b}	1.9 ± 0.0 ^{a}
18:4n-3	1.0 ± 0.0 ^{b}	0.4 ± 0.0 ^{c}	0.3 ± 0.0 ^{c}	0.4 ± 0.1 ^{c}	2.1 ± 0.1 ^{a}
20:4n-3	0.7 ± 0.0 ^{b}	0.7 ± 0.1 ^{b}	0.7 ± 0.0 ^{b}	0.9 ± 0.1 ^{a}	0.7 ± 0.0 ^{b}
20:5n-3	3.6 ± 0.1 ^{a}	2.9 ± 0.2 ^{b}	2.9 ± 0.1 ^{b}	2.7 ± 0.0 ^{b}	3.5 ± 0.1 ^{a}
22:5n-3	3.1 ± 0.1 ^{a}	2.5 ± 0.2 ^{b}	2.4 ± 0.1 ^{b}	2.5 ± 0.0 ^{b}	0.4 ± 0.0 ^{c}
22:6n-3	14.3 ± 0.3 ^{c}	15.4 ± 1.2 ^{c}	19.5 ± 1.3 ^{b}	17.8 ± 0.2 ^{b}	24.3 ± 0.3 ^{a}
Total n-3PUFA[4]	27.2 ± 0.6 ^{b}	25.8 ± 1.9 ^{b}	28.8 ± 1.5 ^{b}	28.0 ± 0.4 ^{b}	36.4 ± 0.6 ^{a}
C16 PUFA	2.4 ± 0.3 ^{b}	2.4 ± 0.1 ^{b}	4.4 ± 0.2 ^{a}	4.2 ± 0.1 ^{a}	4.8 ± 0.3 ^{a}
Total PUFA	43.9 ± 1.0 ^{b}	42.6 ± 3.2 ^{b}	50.5 ± 2.2 ^{a}	48.2 ± 0.7 ^{ab}	49.1 ± 0.8 ^{a}
n-3/n-6	1.6 ± 0.3 ^{b}	1.5 ± 0.1 ^{b}	1.4 ± 0.1 ^{b}	1.4 ± 0.1 ^{b}	2.9 ± 0.2 ^{a}
DHA/EPA	4.0 ± 0.2 ^{c}	5.7 ± 0.1 ^{b}	6.7 ± 0.3 ^{a}	6.6 ± 0.2 ^{a}	6.9 ± 0.3 ^{a}

Results are means ± SD (n = 3). An SD of 0.0 implies an SD of < 0.05. Mean values bearing different superscript letter are significantly different ($P < 0.05$). [1], Totals include 15:0, 20:0, 22:0 and 24:0; [2], Totals include 16:1n-9, 18:1n-11, 20:1n-7, 22:1 isomers and 24:1; [3], Totals include 18:3n-6, 20:2n-6 and 22:4n-6; [4], Totals include 20:3n-3 and 22:3n-3; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; PUFA, polyunsaturated fatty acid.



1144

1145

1146 Figure 1

1147

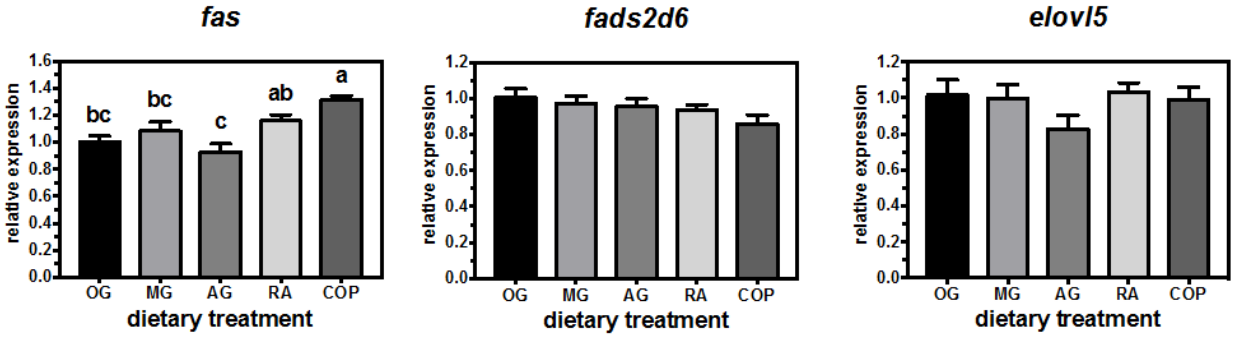

fas
fads2d6
elovl5
relative expression
dietary treatment
OG
MG
AG
RA
COP
bc
bc
c
ab
a

Figure 2

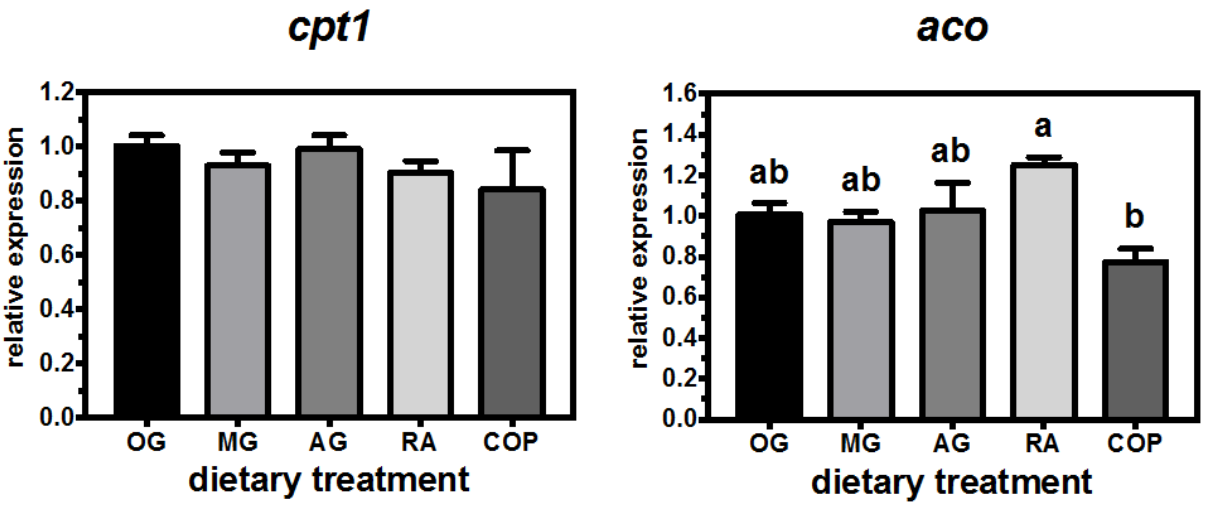

cpt1
relative expression
1.2
1.0
0.8
0.6
0.4
0.2
0.0
OG
MG
AG
RA
COP
dietary treatment
aco
relative expression
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
ab
ab
ab
a
b
OG
MG
AG
RA
COP
dietary treatment

Figure 3

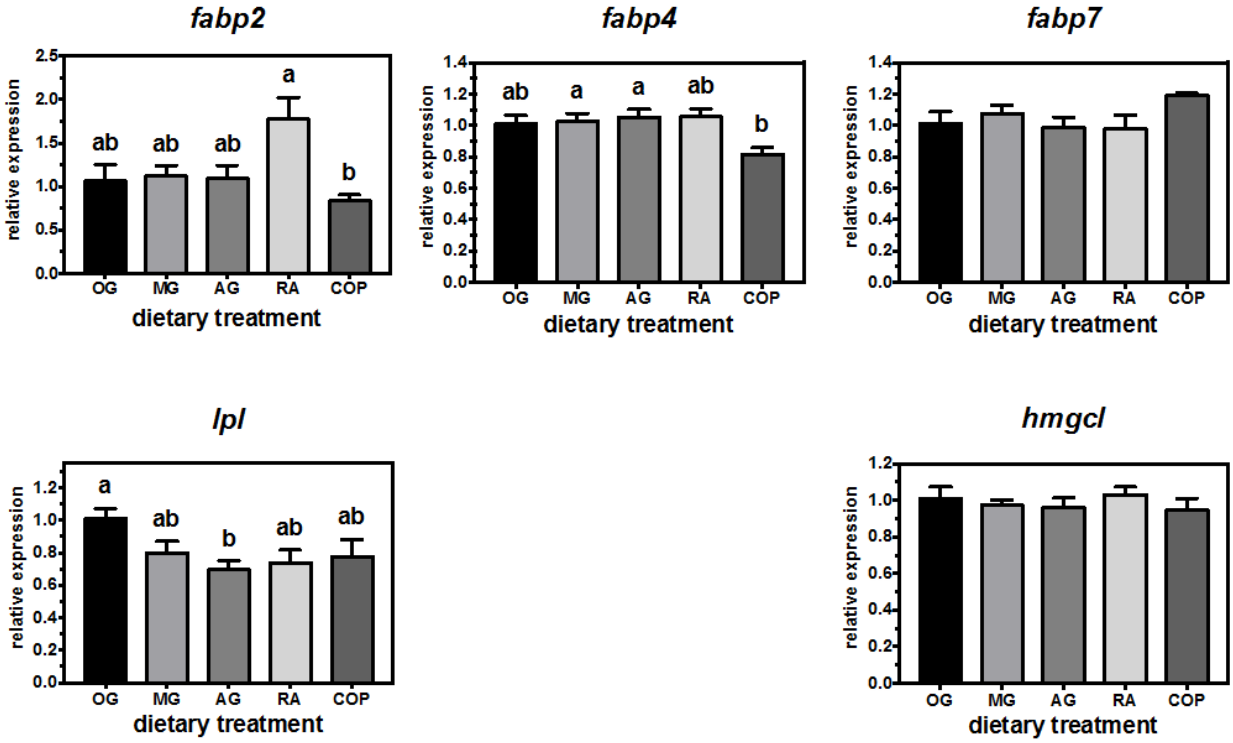

fabp2
relative expression
2.5
2.0
1.5
1.0
0.5
0.0
ab
ab
ab
a
b
OG
MG
AG
RA
COP
dietary treatment
fabp4
relative expression
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
ab
a
a
ab
b
OG
MG
AG
RA
COP
dietary treatment
fabp7
relative expression
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
OG
MG
AG
RA
COP
dietary treatment
lpl
relative expression
1.2
1.0
0.8
0.6
0.4
0.2
0.0
a
ab
b
ab
ab
OG
MG
AG
RA
COP
dietary treatment
hmgcl
relative expression
1.2
1.0
0.8
0.6
0.4
0.2
0.0
OG
MG
AG
RA
COP
dietary treatment

Figure 4

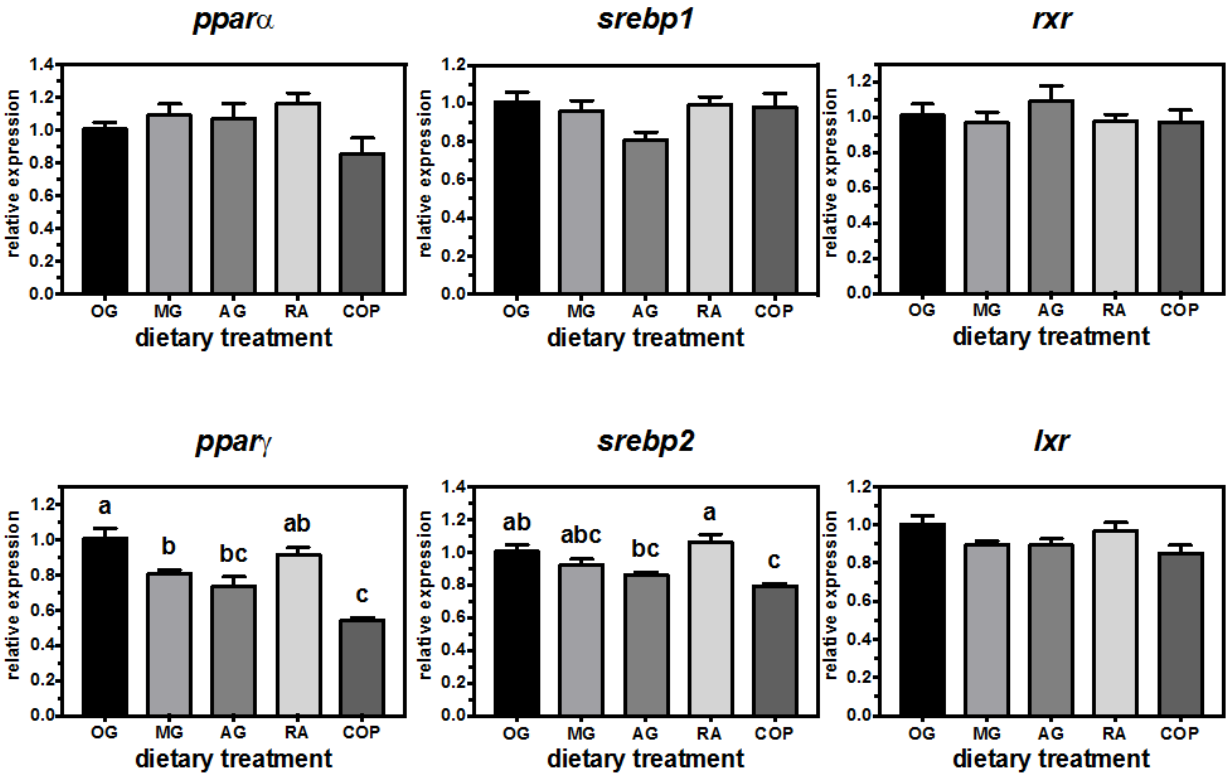
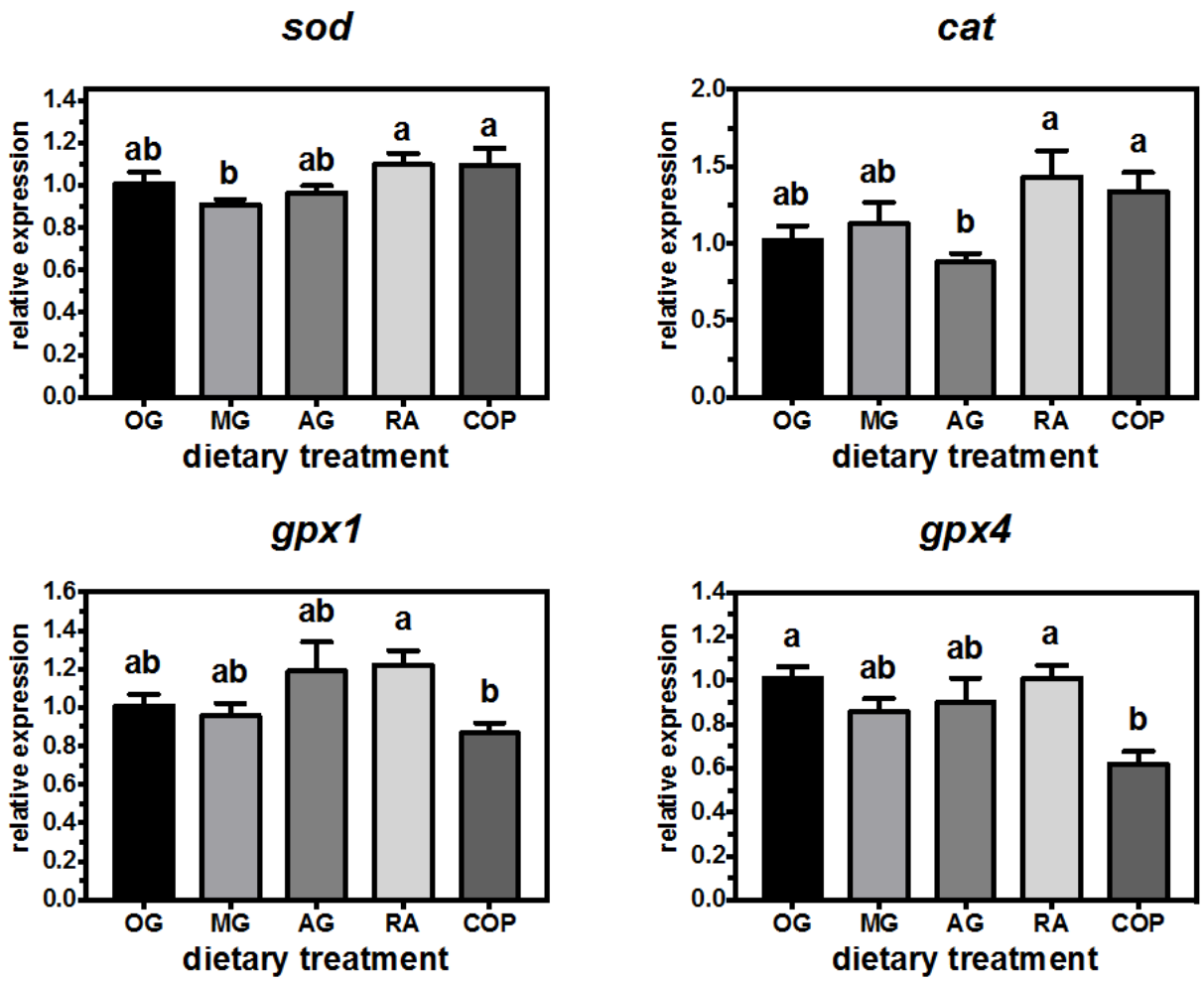

pparα
srebp1
rxr
pparγ
srebp2
lxr
relative expression
dietary treatment
OG
MG
AG
RA
COP
a
b
bc
ab
c
ab
abc
bc
a
c

Figure 5



1173

1174 Figure 6

1175

1176

1177

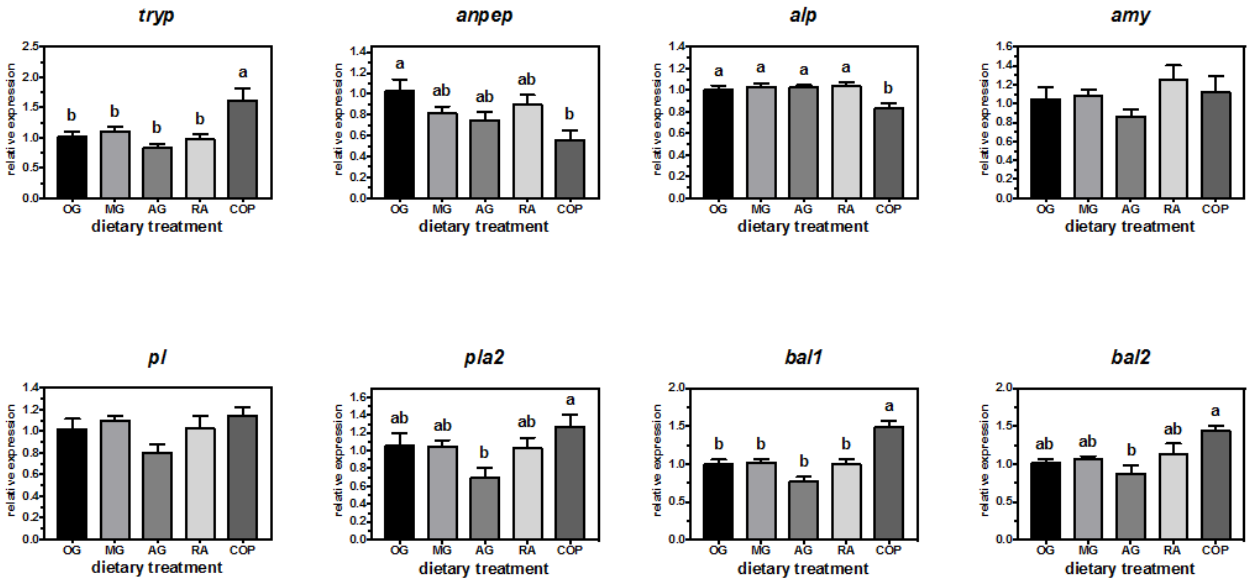

tryp
anpep
alp
amy
pl
pla2
bal1
bal2
relative expression
dietary treatment
OG
MG
AG
RA
COP

Figure 7

Supplementary Table 1. Sequence, annealing temperature (Tm) and size of the fragment produced by the primer pairs used for quantitative PCR (qPCR).

Name	Sequence (5'-3')	Amplicon size (bp)	Tm°C
elovl5	F: CCACGCTAGCATGCTGAATA	236	60
	R: ATGGCCATATGACTGCACAC		
fads2d6	F: CCGTGCACTGTGTGAGAAAC	152	60
	R: CAGTGTAAGCGATAAAATCAGCTG		
pparα	F: TGGTCATGGAGGTGGAAGAC	152	60
	R: ATGGATGACGAAAGGAGGGG		
pparγ	F: ACCTGACCAACATGGACTAC	118	60
	R: GAGAAAACAGGACTGTCAGC		
lxr	F: CACACTGGATCCACAACAGC	192	58
	R: ATCTCCTGCACCGACATGAT		
rxr	F: TGAGGGAAAAGGTCTACGCA	212	59
	R: TGTGATCTGATGTGGTGCCT		
srebp1	F: CCAGCTACACATGACAGGGA	153	59
	R: GCTTTGACCCTTAGAGCTGC		
srebp2	F: AGATCCAGTGAGTCGTTGGT	212	60
	R: CTACAGCCCCTTCTCCCTTC		
fabp2	F: CGCAGCGAGAATTATGACAA	244	55
	R: AGCATGTCACCCTCCATCTC		
fabp4	F: ACTGCAATGACCGAAAGACC	175	55
	R: CCTCCTTTCCGTAGGTCCTC		
fabp7	F: CCTACACCTGATGACCGACA	212	55
	R: GCTGGGATGATTTGCTCATT		
cptI	F: TGGAGGCTGTCCACCAGTCA	211	60
	R: TGCTGGAGATGTGGAAGTTG		
lpl	F: CCGAAAGAACCGCTGCAATA	212	59
	R: GATCCTCCTTCTCTCCGTGG		
fas	F: ATACCGTGGCAATGTAACGC	188	59
	R: GTGAGCTGTGGATGATGCTG		
aco	F: AGCGCTATGACCAGGCTATT	164	59
	R: GTACAGGGTTGGGAGGAACA		
hmgcl	F: CGTGCCAACAGAGACGAAAA	173	59
	R: GGGTGAGGACTGGGTAAGAC		
gpx1	F: TGGAGAAAGTGGATGTGAACGG	309	55
	R: GTGCTGTGGAAGCTGTATGATGG		
gpx4	F: TGGGGAATAGCATCAAGTGG	206	55
	R: CGAGAAAGGAGGGAAACAGG		
cat	F: ATGGTGTGGGACTTCTGGAG		60
	R: ATGAAACGGTAGCCATCAGG		
sod	F: TCCCAGATCACCTACATGCC	182	59
	R: CTGCGGAGAGTTGCTTGATC		
myhc	F: GATTCAGCTGAGCCATGCCA	190	60
	R: TCTCAGCTCCTCAATCTCAG		
anpep	F: CCTGAGGTGGTGGAATGACT		60
	R: GGGTTCAGCTTTGTCTGCTC		
amy	F: TCATGTGGAAGCTGGTTCAG		60
	R: AATATTGCCACTGCCAGTCC		

tryp	F: CCCCAACTACAACCCCTACA		60
	R: CCAGCCAGAGACAAGACACA		
alp	F: ACTCTGACAACGAGATGCCA	189	60
	R: TTCCGTCTTTTCTTGTGCCG		
pl	F: TTCCAGGACACTCCTGTTTCTGTGC	107	59
	R: ATCCCCAGACCAAGTTTGGAGTTGA		
bal1	F: CATGGATGGACACCTCTTTACTGGT	126	59
	R: AAACCAGCCTGGCCCTTCTCTTTAG		
bal2	F:GGATGGGCACCTCTTCACATCACAG	120	59
	R: CCAGCTTGGCCCTTCTCTTTGGTAT		
pla2	F: GGATGATCTGGACAGGTGCT	217	59
	R: TCTGGCAAAACACTCAACGG		
tropo	F: AGAATGCCTTGGACAGAGCT	227	60
	R: ACGTCTGTTAAGGGAAGCGA		
ef1a	F: CCCCTGGACACAGAGACTTC	119	60
	R: GCCGTTCTTGGAGATACCAG		
bactin	F: ACCCACACAGTGCCCATCTA	155	61
	R: TCACGCACGATTTCCCTCT		

elovl5, fatty acyl elongase 5; *fads2d6*, delta-6 fatty acyl desaturase; *pparα*, peroxisome proliferator-activated receptor alpha; *pparγ*, peroxisome proliferator-activated receptor gamma; *lxr*, liver X receptor; *rxr*, retinoid X receptor; *srebp1*, sterol regulatory element-binding protein 1; *srebp2*; sterol regulatory element-binding protein 2; *fabp2*, fatty acid binding protein 2 (intestinal); *fabp4*, fatty acid binding protein 4 (adipocyte); *fabp7*, fatty acid binding protein 7 (brain-type); *cptI*, carnitine palmitoyl transferase I; *lpl*, lipoprotein lipase; *fas*; fatty acid synthase; *aco*, acyl coA oxidase; *hmgcl*; 3-hydroxy-3-methylglutaryl-CoA lyase; *gpx1*, glutathione peroxidase 1: *gpx4*, glutathione peroxidase 4; *cat*, catalase; *sod*, superoxide dismutase; *myhc*, myosin heavy chain; *anpep*, amino peptidase; *amy*, amylase; *tryp*, trypsin; *alp*, alkaline phosphatase; *pl*, pancreatic lipase; *bal1*, bile salt activated lipase 1; *bal2*, bile salt activated lipase 2; *pla2*, phospholipase A2; *tropo*, tropomyosin; *ef1α*, elongation factor 1 alpha; *bactin*, beta actin.

Supplementary Table 2. Total lipid fatty acid composition (weight %) of rotifers *B. rotundiformis* enriched with four commercial enrichment products as described in Materials and Methods (OG, MG, AG and RA) or 3 days post hatch nauplii of the copepod *Acartia tonsa* fed *Rhodomonas baltica* (COP).

	OG	MG	AG	RA	COP
Fatty acid					
14:0	1.2 ± 0.1 ^{b}	1.9 ± 0.4 ^{b}	3.4 ± 0.1 ^{a}	2.7 ± 0.5 ^{a}	1.9 ± 0.1 ^{b}
16:0	16.9 ± 0.3 ^{b}	20.9 ± 1.1 ^{a}	17.2 ± 0.5 ^{b}	17.3 ± 0.1 ^{b}	11.9 ± 0.8 ^{c}
18:0	5.3 ± 0.1	4.2 ± 0.1	3.5 ± 0.2	3.7 ± 0.1	4.8 ± 0.1
Total saturated[1]	26.5 ± 0.2 ^{b}	29.7 ± 1.8 ^{a}	26.4 ± 0.9 ^{b}	26.2 ± 0.4 ^{b}	23.5 ± 0.7 ^{c}
16:1n-7	1.8 ± 0.1 ^{a}	0.9 ± 0.1 ^{b}	0.7 ± 0.1 ^{b}	0.8 ± 0.1 ^{b}	1.7 ± 0.2 ^{a}
18:1n-9	7.0 ± 0.1 ^{a}	2.5 ± 0.1 ^{b}	1.0 ± 0.1 ^{c}	1.2 ± 0.1 ^{cd}	1.3 ± 0.1 ^{d}
18:1n-7	1.7 ± 0.1 ^{b}	1.1 ± 0.1 ^{c}	0.8 ± 0.1 ^{c}	1.1 ± 0.1 ^{c}	4.0 ± 0.2 ^{a}
20:1n-9	1.3 ± 0.1 ^{a}	0.8 ± 0.1 ^{b}	0.5 ± 0.1 ^{ce}	0.7 ± 0.1 ^{bc}	0.3 ± 0.1 ^{e}
Total monoenes[2]	15.4 ± 0.2 ^{a}	9.0 ± 0.2 ^{c}	5.5 ± 0.1 ^{e}	7.1 ± 0.2 ^{d}	10.6 ± 0.1 ^{b}
18:2n-6	17.7 ± 0.4 ^{a}	13.2 ± 0.4 ^{b}	9.5 ± 0.1 ^{d}	10.8 ± 0.1 ^{c}	5.3 ± 0.3 ^{e}
20:4n-6	0.9 ± 0.0 ^{c}	1.1 ± 0.1 ^{c}	1.2 ± 0.1 ^{c}	1.8 ± 0.4 ^{b}	2.4 ± 0.1 ^{a}
22:5n-6	1.0 ± 0.1 ^{d}	5.6 ± 0.2 ^{c}	8.7 ± 0.1 ^{a}	7.9 ± 0.1 ^{b}	1.1 ± 0.3 ^{d}
Total n-6PUFA[3]	23.8 ± 0.5 ^{a}	24.1 ± 0.9 ^{a}	22.8 ± 0.5 ^{a}	23.9 ± 0.8 ^{a}	14.2 ± 0.2 ^{b}
18:3n-3	4.6 ± 0.1 ^{a}	3.2 ± 0.1 ^{b}	2.9 ± 0.1 ^{b}	2.7 ± 0.1 ^{c}	4.7 ± 0.3 ^{a}
18:4n-3	0.2 ± 0.1 ^{b}	0.2 ± 0.1 ^{b}	0.4 ± 0.1 ^{b}	0.4 ± 0.1 ^{b}	2.7 ± 0.1 ^{a}
20:4n-3	0.9 ± 0.1 ^{b}	1.2 ± 0.1 ^{a}	1.2 ± 0.1 ^{a}	1.2 ± 0.1 ^{a}	0.5 ± 0.1 ^{c}
20:5n-3	4.9 ± 0.2 ^{b}	3.7 ± 0.2 ^{c}	3.4 ± 0.1 ^{c}	4.1 ± 0.1 ^{b}	10.4 ± 0.6 ^{a}
22:5n-3	4.0 ± 0.2 ^{a}	2.8 ± 0.2 ^{b}	2.2 ± 0.1 ^{c}	2.5 ± 0.1 ^{bc}	0.5 ± 0.1 ^{d}
22:6n-3	13.5 ± 0.5 ^{d}	20.9 ± 1.1 ^{c}	28.3 ± 0.4 ^{a}	25.0 ± 0.2 ^{b}	19.4 ± 0.9 ^{c}
Total n-3PUFA[4]	31.3 ± 0.9 ^{c}	34.8 ± 1.5 ^{b}	42.1 ± 0.5 ^{a}	39.3 ± 0.2 ^{a}	42.2 ± 1.8 ^{a}
C16 PUFA	4.1 ± 0.3 ^{b}	3.3 ± 0.1 ^{c}	3.5 ± 0.2 ^{c}	3.0 ± 0.1 ^{c}	5.3 ± 0.3 ^{a}
Total PUFA	55.1 ± 1.5 ^{b}	58.8 ± 2.4 ^{b}	64.9 ± 1.0 ^{a}	63.1 ± 0.7 ^{a}	56.4 ± 1.6 ^{b}
n-3/n-6	1.3 ± 0.1 ^{c}	1.4 ± 0.1 ^{c}	1.8 ± 0.1 ^{b}	1.6 ± 0.1 ^{bc}	3.0 ± 0.2 ^{a}
DHA/EPA	2.8 ± 0.1 ^{d}	5.7 ± 0.1 ^{c}	8.3 ± 0.1 ^{a}	6.2 ± 0.1 ^{b}	1.9 ± 0.2 ^{e}

Results are means ± SD (n = 3). An SD of 0.0 implies an SD of < 0.05. Mean values bearing different superscript letter are significantly different ($P<0.05$). [1], Totals include 15:0, 20:0, 22:0 and 24:0; [2], Totals include 16:1n-9, 18:1n-11, 20:1n-7, 22:1 isomers and 24:1; [3], Totals include 18:3n-6, 20:2n-6 and 22:4n-6; [4], Totals include 20:3n-3 and 22:3n-3; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; PUFA, polyunsaturated fatty acid.