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

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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.

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 swim-bladder 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 efficiency, 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 (Yokoyama 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 °C) 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*), alkaline phosphatase (*alp*), amylase (*amy*), pancreatic lipase (*pl*), bile salt-activated-lipases (*bal1* and *bal2*) and phospholipase A₂ (*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 °C, 37‰ salinity and continuous photoperiod, with a light intensity of 1000 lx. 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 × 10⁵ cells mL⁻¹ as green water. During the trial, photoperiod was maintained at 16 h/8 h light/dark (light intensity about 500 lx), temperature ranged between 24 and 26 °C 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).

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

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

2.2.1. Rotifer Brachionus rotundiformis culture and enrichment protocols

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

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

2.2.2. Cultivation of copepod Acartia tonsa

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

2.3. Sampling for biometrical, biochemical and molecular analysis

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

Triplicate samples of rotifers nutritionally boosted with the different enrichers and copepods (*Acartia*) were washed and filtered, excess water drained and blotted with filter paper, and immediately frozen in

liquid N_2 and stored at −80 °C prior to analysis. Two subsets of triplicate 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 °C for lipid analysis. All procedures were carried out according to the current national and EU legislation on the handling of experimental animals.

2.4. Biochemical analysis

2.4.1. 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 analyzed. 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).

2.4.2. 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 × 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 °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 °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 × 0.32 mm i.d. capillary column (CP Wax 52CB, Chrompak, London, U.K.) and on-column injection at 50 °C. Hydrogen was used as carrier gas and temperature programming was from 50 °C to 150 °C at 40 °C min^{-1} and then to 230 °C at 2.0 °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).

2.4.3. 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 × 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.

2.4.4. 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.

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

2.6. qPCR analysis

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

Primers for *fads2d6, elovl5, fabp2, 4* and *7, rxr, hmgcl, pparγ, aco, fas, lpl, myhc, anpep, amy, try* and *cat* were already available for ABT (Morais et al., 2011; Mazurais et al., 2015; Betancor et al., 2016). Primers for *alp* and *tropo* were designed on sequences of *Thunnus thynnus* (FM995226.1 and AB109447.1 respectively). Primers for *gpx1 and gpx4* were designed on the sequences of *Thunnus maccoyii* (EF452497.1 and EF452498.3 respectively; Thompson et al., 2010). Primers for *pl, bal1* and *bal2* were designed on the sequences of *Thunnus orientalis* (AB859991.1, AB859992.1 and AB859993.1, respectively). Primers for *pla2* and *sod* were designed on the sequence read archive (SAR) SRX2255758 by identifying and assembling the sequences.

Expression of genes of interest was determined by qPCR of all the RNA samples. *Elongation factor-1α* (*elf1α*) and *β-actin* were used as

reference genes. The cDNA was diluted 20-fold with milliQ water. The efficiency of the primers for each gene was previously evaluated by serial dilutions of cDNA pooled from the samples to guarantee it was > 85% for all primer pairs. qPCR was performed using a Biometra TOptical Thermocycler (Analytik Jena, Goettingen, 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 pretreatment 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

Growth performance of 15 dah ABT larvae fed on enriched rotifers *B. rotundiformis* (OG, MG, AG, RA) or 3 days post hatch nauplii of the copepod *A. tonsa* fed *R. baltica* (COP) are shown in Table 1. Highest total length, total dry mass and flexion index were shown in ABT larvae fed on nauplii of the copepod *Acartia* followed by larvae fed on rotifers enriched with AG. Lower performance parameters were observed in ABT larvae fed on rotifers enriched with MG > RA > OG. However, no significant differences were detected in final survival, which was around 8% in all the treatments.

3.2. Macronutrient, vitamin E and Se content of enriched rotifers B. rotundiformis and Acartia nauplii

The contents of macronutrients, protein and total lipid (dry mass %), and vitamin E and Se in live feeds are shown in Table 2. Total protein content of *Acartia* nauplii was 63.8% of dry mass, and higher than in enriched rotifers at about 53% of dry mass. Conversely, total lipid content was higher in enriched rotifers at about 11% of dry mass, whereas *Acartia* nauplii had a lower lipid content of 5.6% of dry mass. Vitamin E content in enriched rotifers showed values ranging from 232 mg kg^{-1} in AG-enriched rotifers to 593 mg kg^{-1} in rotifers enriched with RA. *Acartia* nauplii had a lower level of vitamin E at only 170.1 mg kg^{-1}. Selenium content in rotifers ranged from 4.8 μg g^{-1} in those enriched with AG to 10.6 μg g^{-1} in those enriched with RA, while *Acartia* nauplii only contained selenium at 0.4 μg g^{-1}.

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*.

	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. Mean values bearing different superscript letter are significantly different (P < 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).

3.3. Lipid class compositions of enriched rotifers B. rotundiformis and Acartia nauplii

The lipid class composition of dietary live preys was predominantly neutral lipids with triacylglycerol (TAG) the predominant class (Table 3). However, class compositions reflected lipid content and, thus, copepods, with the lowest lipid content showed the lowest levels of neutral lipids and TAG, whereas all enriched rotifers had higher neutral lipids and TAG. Polar lipids were dominated by phosphatidylcholine (PC) and phosphatidylethanolamine (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.

3.4. 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.

3.5. 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).

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.

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 to 11%, and ARA from 1.4 to 2.5%. Total saturated fatty acids ranged from 34 to 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%.

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 ± 0.2[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 ± 0.30[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.
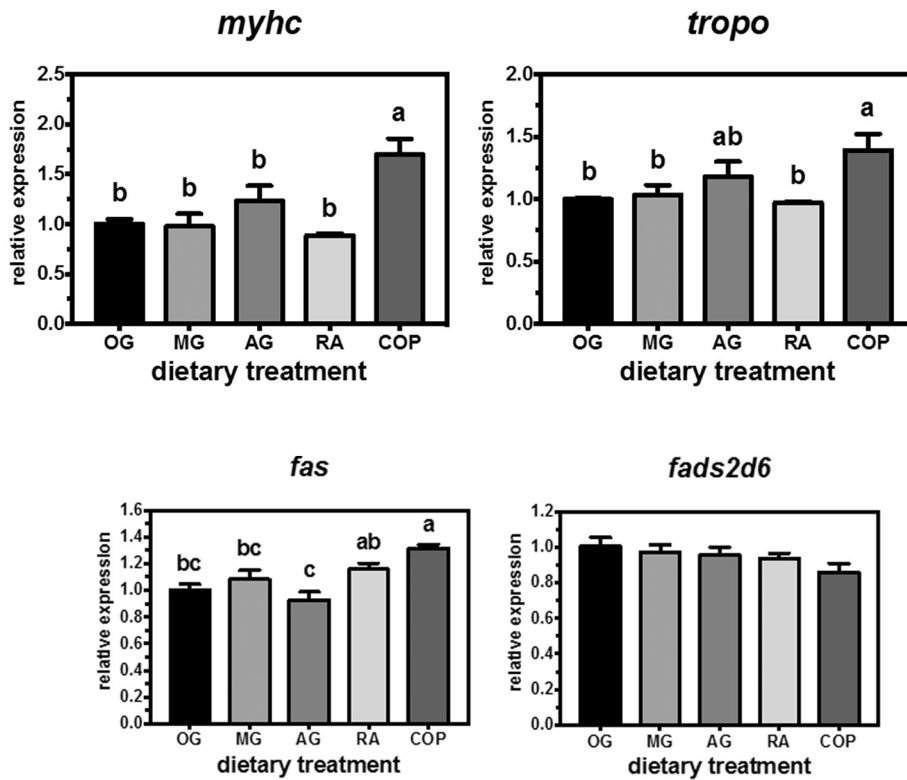
Fig. 1. Nutritional regulation of myosin heavy chain 2 (*myhc*) and tropomyosin *(tropo)* gene transcription in whole larvae of Atlantic bluefin tuna fed rotifers *Brachionus rotundiformis* enriched with four commercial enrichment products as described in Materials and Methods (OG, MG, AG and RA) or 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. 2. Nutritional regulation of fatty acid synthase (*fas*), delta-6 fatty acyl desaturase (*fads2d6*) and fatty acyl elongase 5 (*elovl5*) 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).
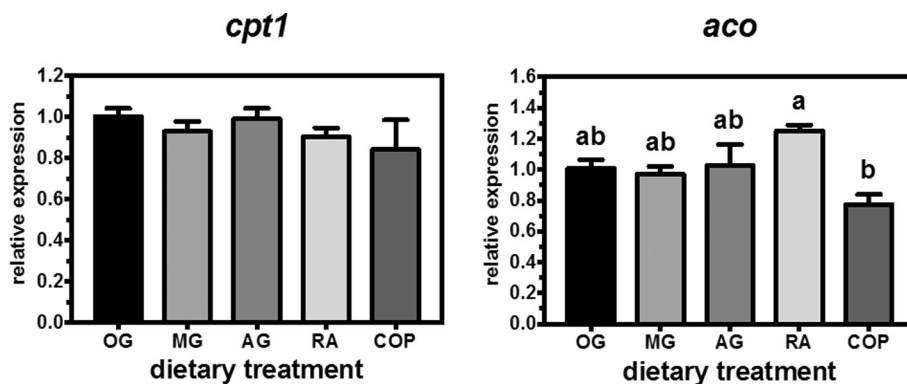
3.6. Expression of myogenic genes in 15 dah ABT larvae

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).

3.7. 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 expression than those fed COP, with larvae fed the other enriched rotifers showing intermediate values (Fig. 3). The expression of *fabp*2 showed a similar pattern to *aco*, with larvae fed on RA-rotifers showing higher expression than those fed COP, with larvae fed the other enriched rotifers showing intermediate values (Fig. 4). Expression of *fabp4* was also lowest in larvae fed COP, significantly so compared to rotifers enriched with MG and AG, although expression of *fabp7* showed no significant differences. In contrast, larvae fed AG-rotifers showed lower *lpl* expression than larvae fed OG-rotifers, with larvae fed the other live feeds showing intermediate values (Fig. 4). No differences among treatments were found in the expression of *hmgcl*.

Concerning transcription factors, larvae fed COP displayed lower expression of *ppar*γ than larvae fed OG- and MG-rotifers with larvae fed the other live feeds showing intermediate expression levels (Fig. 5).

Fig. 3. Nutritional regulation of carnitine palmitoyl transferase I (*cptI*) and acyl coA oxidase (*aco*) 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).
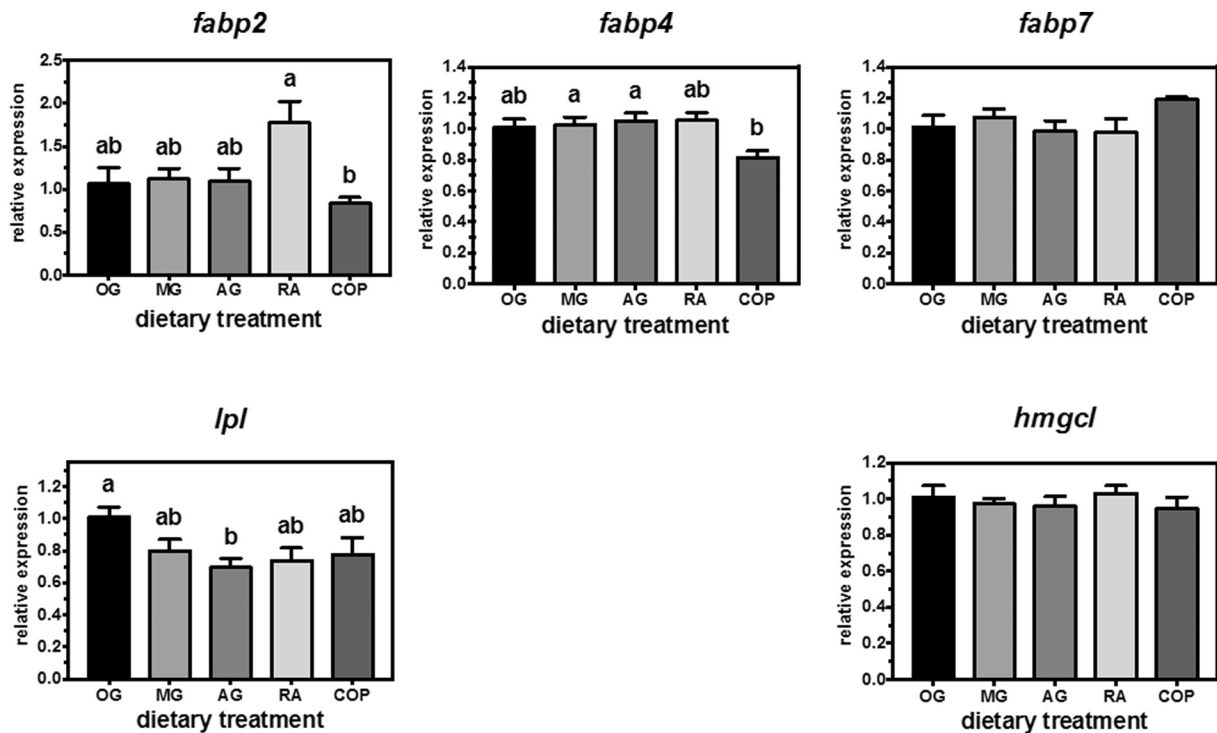
Fig. 4. Nutritional regulation of fatty acid binding protein 2, 4 and 6 (*fabp2*, *fabp4* and *fabp7* respectively), lipoprotein lipase (*lpl*) and 3-hydroxy-3-methylglutaryl-CoA lyase (*hmgcl*) gene transcription in whole larvae of Atlantic bluefin tuna fed rotifers *Brachionus rotundiformis* enriched 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).
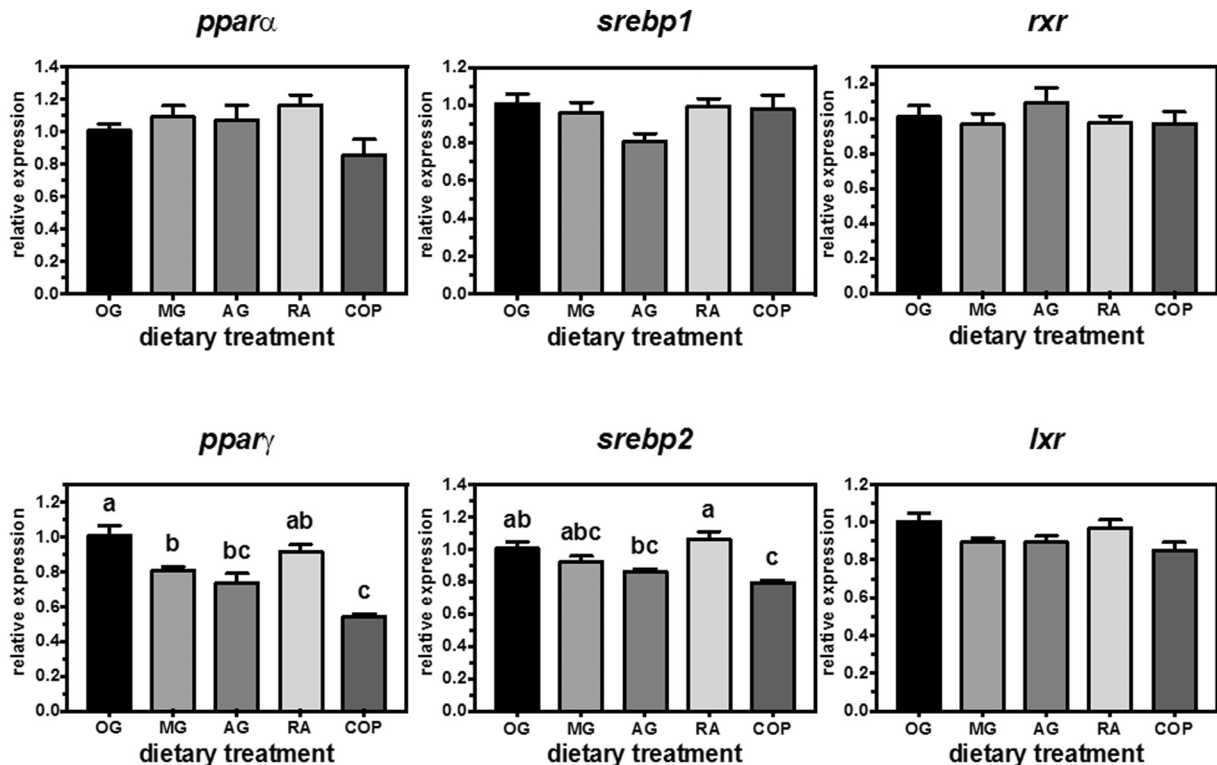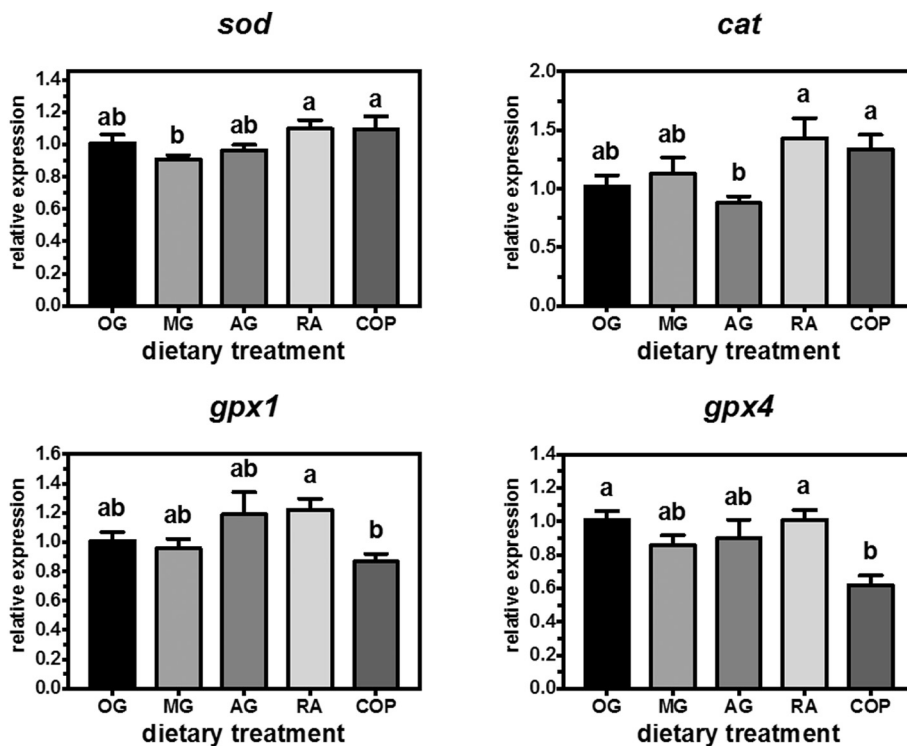
Fig. 6. Nutritional regulation of superoxide dismutase (*sod*), catalase (*cat*) and glutathione peroxidase 1 and 4 (*gpx1* and *gpx4* respectively) gene transcription in whole larvae of Atlantic bluefin tuna fed rotifers *Brachionus rotundiformis* enriched with four commercial enrichment products as 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).

Similarly, larvae fed COP displayed lower expression of *srebp2* than larvae fed OG- and RA-rotifers with larvae fed the other live feeds showing intermediate expression levels. No differences in expression levels were observed among larvae fed the different dietary treatments for *ppara*, *srebp1*, *rxr* or *lxr* (Fig. 5).

3.8. Expression of antioxidant defence enzyme genes

Expression of superoxide dismutase (*sod*) was higher in ABT larvae fed COP and RA-rotifers than in MG-rotifers with larvae fed OG- and AG-rotifers displaying intermediate values (Fig. 6). A similar pattern was observed for catalase (*cat*) with higher expression in ABT larvae fed COP and RA-rotifers than larvae fed AG-rotifers with larvae fed the other live feeds showing intermediate values. In contrast, larve fed COP had low expression of both glutathione peroxidase genes (*gpx1* and *gpx4*), significantly lower than RA-rotifers in both cases with larve fed the other enriched rotifers generally showing intermediate expression levels (Fig. 6).

3.9. Expression of digestive enzyme genes

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 et 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, > 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
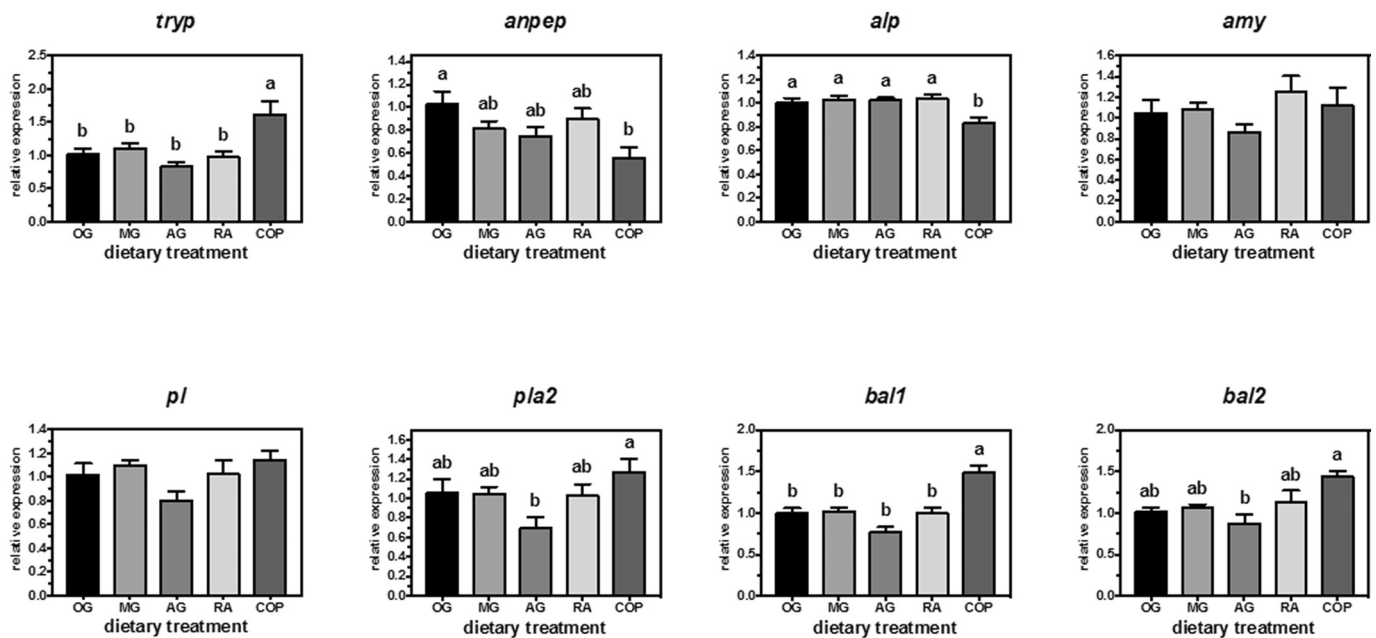
Fig. 7. Nutritional regulation of trypsin (*tryp*), amino peptidase (*anpep*), amylase (*amy*), pancreatic lipase (*pl*), phospholipase A₂ (*pla2*) and bile salt activated lipase 1 and 2 (*bal1* and *bal2* respectively) gene transcription in whole larvae of Atlantic bluefin tuna fed rotifers *Brachionus 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 ($p < 0.05$).

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 were 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 *ppara* 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 ($y = -2.3296 \times + 0.8105$; $R^2 = 0.8594$), 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 that may activate *lxr* might be similar. In this sense, it was shown that oxysterols and not cholesterol activated salmon *lxr* in a heterologous *in vitro* system (Carmona-Antoñanzas et al., 2014).

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

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

peroxidases) in addition to superoxide dismutase and catalase.

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

Apart from lipids there are other nutrients that, although outwith the scope of the present trial, play a pivotal role in larvae performance such as taurine. Taurine is naturally found in high levels in copepods compared to rotifers (Karlsen et al., 2015) and is involved in the production of bile salts in fish (Vessey et al., 1990). Increased activity of bile salt-activated lipase has been found in *Dentex dentex* juveniles fed diets supplemented with taurine (Chatzifotis et al., 2008), which is in agreement with the highest expression of *bsl1* and *2* observed in COP-fed larvae in the present study.

Several studies have dealt with the ontogenic development of the digestive system and the functionality and expression of digestive enzymes during early stages of tuna development (Miyashita et al., 1998; Murashita et al., 2014; Mazurais et al., 2015). In the present study, pancreatic alkaline phosphatase (*alp*) trypsin (*tryp*), phospholipase A$_2$ (*pla2*) and two isoforms of bile salt activated lipases (*bal1* and *bal2*) genes showed highest expression in 15 dah ABT larvae that had been fed with *Acartia* nauplii and copepodites (Fig. 7). Similarly, up-regulation of *tryp* and *lipase* expression was observed in red seabream (*Pagrus major*) fed a fish meal diet in contrast to fish fed a soybean meal based diet, which could be due to the presence of stimulating factors such as small peptides or free aminoacids in fish meal (Murashita et al., 2015). On the other hand, the digestion of dietary polar lipids was investigated by analyzing the relative expression of *pla2* in ABT larvae fed different dietary treatments. Results showed highest expression of *pla2* in larvae fed copepods, which contained a higher proportion of polar lipids than enriched rotifers (Fig. 7). It is commonly accepted that the major digestive lipase in teleosts, including larvae, appears to be bile salt-dependent lipases (*bal*) (Rønnestad and Morais, 2008). It was also reported that bile salt-dependent lipases were the main enzymes involved in lipid digestion in the larval stage of Pacific bluefin tuna (Murashita et al., 2014). In the present study, the expression patterns of both isoforms (*bal1* and *bal2*) were nutritionally regulated, showing a similar pattern. *Bal1* showed significantly higher expression in larvae fed copepods (Fig. 7). In cod (*Gadus morhua*) larvae, *bal.* expression was correlated with the growth pattern of pyloric caeca, with a significant increase in expression from 48 to 62 dah, which could be related to an increased demand for enzyme in the rapidly increasing pyloric caeca or to an increase in pancreatic mass related to body size (Sæle et al., 2010). Thus, the high *bal.* expression found in ABT larvae fed COP in the present study could be reflecting 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 necessary 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.

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