Evaluation of different feeding protocols for larvae of Atlantic bluefin tuna (*Thunnus thynnus*, L.)

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Accepted refereed manuscript of:
Betancor M, Ortega A, de la Gándara F, Varela JL, Tocher DR & Mourente G (2019) Evaluation of different feeding protocols for larvae of Atlantic bluefin tuna (Thunnus thynnus, L.). *Aquaculture*, 505, pp. 523-538.
DOI: https://doi.org/10.1016/j.aquaculture.2019.02.063

Abstract

Mass mortality is still one of the main constraints in larval rearing of Atlantic bluefin tuna (*Thunnus thynnus* L.; ABT). Early data related to the feeding sequence of ABT larvae suggested that mortality observed during the first stages of life could be partly due to nutritional deficiencies. Previous studies demonstrated that copepods appeared to be a superior live prey compared to rotifers during the first two weeks of life. Our overarching aim was to evaluate different feeding strategies during first feeding of ABT larvae from a performance, compositional and molecular perspective. In order to do so, two groups of ABT larvae were fed with either copepod (*Acartia tonsa;* C) nauplii or rotifers (*Brachionus rotundiformis*; R) enriched with Algamac 3050® from mouth opening to 13 days after hatching (dah). After this, the group C-larvae was fed either *Artemia* enriched with Algamac 3050® (CA), *Acartia* nauplii and copepodites (CC) or sea bream (*Sparus aurata*) yolk-sac larvae (CY), while the R group were fed on *Artemia* enriched with Algamac 3050® (RA) up to 18 dah. At 13 dah, larvae fed copepods (C) had grown better than those fed enriched rotifers (R) although there were no significant differences in survival. ABT larvae fed R accumulated highest eicosapentaenoate (EPA) but lowest docosahexaenoate (DHA) and total n-3 long-chain polyunsaturated fatty acids (LC-PUFA) than C-fed larvae, reflecting the dietary contents. There was no activation in the expression of the enzymes involved in EPA and DHA biosynthesis. However, the different live prey showed regulation of transcription factor, digestive enzyme, lipid metabolism and oxidative stress genes. At 18 dah, larvae fed CY and CA treatments were largest in size, with larvae fed RA displaying the lowest growth, with no significant differences in survival among the dietary treatments. The highest DHA contents were found in ABT larvae fed CC and CY, whereas the lowest contents were found in RA-fed larvae. Indeed, larvae fed RA showed the highest level of the intermediate product n-3 docosapentaenoate, which could reflect increased activity of the biosynthetic pathway although this was not supported by gene expression data.

Keywords: Bluefin tuna, larvae, rotifers, copepods, Artemia, yolk sac larvae, lipid metabolism,

digestibility, antioxidant status, 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; *cat*, catalase; *cpt1*, carnitine palmitoyl transferase I; dah, days after hatch; DHA, docosahexaenoic acid (22:6n-3); DPA, docosapentaenoic acid (n-3 or n-6); 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; *fas*, fatty acid synthase; *gpx1*, glutathione peroxidase 1; *gpx4*, glutathione peroxidase 4; *hmgcl*, 3-hydroxy-3-methylglutaryl-CoA lyase; LA, linoleic acid (18:2n-6); LNA, α-linolenic acid (18:3n-3); LC-PUFA, long-chain polyunsaturated fatty acid; LPC, lyso phosphatidylcholine; *lpl*, lipoprotein lipase; *lxr*, liver X receptor; *myhc*, myosin heavy chain; PBT, Pacific bluefin tuna; *pl*, pancreatic lipase; *pla2*, phospholipase A_2; *pparα,* peroxisome proliferator-activated receptor alpha; *pparγ,* peroxisome proliferator-activated receptor gamma; *rxr*, retinoid X receptor; *sod*, superoxide dismutase; *srebp1*, sterol regulatory element-binding protein 1; *srebp2*, sterol regulatory element-binding protein 2; TF, transcription factor; *tropo*, tropomyosin; *tryp*, trypsin.

1. Introduction

Regardless of the efforts and progress that have been made in the Mediterranean area in the larval rearing of Atlantic bluefin tuna (ABT; *Thunnus thynnus*), a number of challenges and issues continue to restrict the hatchery production of ABT fingerlings in commercial quantities with early "mass mortality" during first feeding being a common occurence. (De la Gándara et al., 2016; Van Beijnen, 2017). Aside from improvement of zootechnical aspects of the culture system focussed on broodstock management and egg and larval production and quality, the refinement of the live food trophic chain (prey size, sequence and nutritional quality) is necessary until a reliable artificial commercial diet is fully developed. Currently, ABT larval first feeding is attained using either enriched rotifers (preferably *Brachionus rotundiformis*) and/or copepod nauplii (*Acartia tonsa*) as initial live prey (De la Gandara et al., 2010; 2016; Betancor et al., 2017a,b). Previous studies have shown that copepods were better live prey for first feeding ABT based on growth and survival data (Betancor et al., 2017a). In addition, broodstock nutrition was also identified as a possible factor explaining differences in growth performance and lipid metabolism observed between larvae from different year classes (Betancor et al., 2018).

The metabolism and deposition of lipids is a complex process in fish, involving multiple pathways such as lipogenesis, β–oxidation and biosynthesis of long–chain polyunaturated fatty acids (LC-PUFA) (Ayisi et al., 2018). In a recent trial where ABT larvae were fed on rotifers enriched with five commercial products or copepods nauplii, responses in lipid gene expression, which are a consequence of differences in dietary lipid contents and fatty acid compositions, appeared to indicate that lipid levels provided by enriched rotifers exceeded ABT requirements (Betancor et al., 2017b). This study corroborated that copepods were a superior live prey when compared to rotifers in first feeding ABT larvae, as indicated by the higher growth achieved by copepod-fed larvae, possibly reflecting the higher protein content of the copepods (Betancor et al., 2017b). However, the mass production of *Acartia* copepods its labourious and costly in comparison to the production of enriched

rotifers or *Artemia*, which explains why copepods are not widely used as a live prey in hatcheries for any species of marine fish.

In the present study, further feeding trials were performed to expand our knowledge of early nutrition and nutritional requirements of first feeding ABT larvae. Our overarching aim was to evaluate different strategies during first feeding (up to weaning onto an artificial, formulated diet) of ABT larvae from a performance, compositional and molecular perspective. In order to do so, two groups of ABT larvae were fed with either copepod (*Acartia tonsa;* C) nauplii, or rotifers (*Brachionus rotundiformis*; R) enriched with Algamac 3050® from mouth opening to 13 dah. After this, the C-larvae group was fed either *Artemia* enriched with Algamac 3050® (CA), *Acartia* nauplii and copepodites (CC) or sea bream (*Sparus aurata*) yolk-sac larvae (CY), while the R-larvae group were fed on *Artermia* enriched with Algamac 3050® (RA) up to 18 dah. Growth performance, developmental indices and survival were determined. Additionally, the expression of genes related to lipid metabolism (transcription factors, fatty acid metabolism and lipid homeostasis), antioxidant enzymes, myogenesis and digestive enzymes was carried out in order to assess the impacts of the different feeding protocols on larval metabolism and physiology.

2. Materials and Methods

2.1 Atlantic bluefin tuna larvae rearing conditions

The ABT eggs used in this study were obtained in June 2017 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 (or into 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 1400 L cylindrical tanks at a density of 8.5 eggs.L^{-1}. Incubation was carried out at a water temperature 23 - 25 ºC, 37 ‰ salinity, dissolved oxygen 6.5 mg.L^{-1} 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 (R-larvae) or copepod nauplii (C-larvae) from 2 dah to 13 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 feeding trial, photoperiod was maintained at 14 h / 10 h light/dark (light intensity about 500 lux), temperature ranged between 23 - 25 ºC and daily water renewal was 100 - 200 % tank volume.day^{-1}. Incoming seawater was filtered at 10 µm and UV sterilized. An upwelling current was created to avoid larvae sinking (mainly at night) and to maintain oxygen level (Ortega, 2015; De la Gándara et al., 2016; Betancor et al., 2017a,b). The rearing conditions for feeding trials are summarized in Supplementary Table 1.

2.2 Dietary trial 1 with live prey, rotifers and copepod nauplii, from first feeding up to 13 dah

In the first trial, ABT larvae at a density 8.5 larvae.L^{-1} were fed the two different live prey in quadruplicate (4 tank replicates/treatment) from mouth opening (2 dah) up to 13 dah (Supplementary Table 1). Larvae were fed either with rotifer *Brachionus rotundiformis* enriched with Algamac 3050® (treatment R) or *Acartia tonsa* copepod nauplii (treatment C). To maintain constant live prey concentration of 10 rotifer.mL^{-1} or copepod nauplii/copepodite.mL^{-1} within each experimental tank, three water samples (10 mL) from each tank were counted twice per day before supplying new feed (Ortega, 2015; De la Gándara et al., 2016; Betancor et al., 2017a,b).

139

140 *2.3 Dietary trial 2 from 13 to 18 dah*

141 After sampling ABT larvae at 13 dah, the remaining larvae were redistributed into four 1,400
142 L tanks at a density of 0.43 larvae.L^{-1} (Supplementary Table 1). The dietary treatments in single
143 replicate tanks were established as follows: group C-larvae (ABT larvae that had been previously fed
144 on *A. tonsa* nauplii) were given three different feeds, being either *Artemia* enriched with Algamac
145 3050® at 5 metanauplii.mL^{-1} (treatment CA), *Acartia* nauplii and copepodites at 10
146 nauplii/copepodites.mL^{-1} (treatment CC) or gilthead sea bream (*Sparus aurata*) yolk-sac larvae at 5
147 larvae.mL^{-1} (treatment CY). The R group (ABT larvae that had been fed on rotifer *Brachionus*
148 *rotundiformis* enriched with Algamac 3050®) were passed on to being fed only *Artermia* enriched
149 with Algamac 3050® (treatment RA) at 5 metanauplii.mL^{-1} to represent a reference treatment
150 reflecting the common commercial protocol for marine fish larvae. All four groups were fed these
151 treatments from 13 dah to 18 dah when larvae were again sampled.

152

153 *2.4 Rotifer culture and enrichment protocol*

154 S-type rotifers *B. rotundiformis* were continuously cultured with comercial DHA-enriched
155 algal paste (Chlorella V-12; Chlorella Industry, Kyushu, Japan), at a concentration of 3 mL Chlorella
156 paste per 10^6 rotifers per day, in four 2,000 L cylindro-conical tanks supplied with filtered and
157 sterilized sea water at 24 – 26 ºC, 38 ‰ salinity, dissolved oxygen at saturation level and 24 h
158 continual illumination. Enrichment treatment consisted of the commercial product, Algamac 3050®.
159 Additionally, rotifers were supplemented with taurine (0.5 g per 10^6 rotifers), organic Se (Selplex®
160 Alltech Spain SL; 3.0 mg per 10^6 rotifers), and vitamin E, as dl-α tocopheryl acetate (Lutavit E50;
161 (BASF; 0.9 mg per 10^6 rotifers), 18 h before the enrichment treatment. The enrichment protocols
162 were performed in 100 L cylindro-conical tanks at a density of 1000 rotifers.mL^{-1}, adding a dose of
163 the enrichment product of 0.3 g plus 3 mg Selplex® and 0.9 mg vitamin E per 10^6 rotifers over a
164 period of 6 h for Algamac 3050® according to manufacturer's recommendations.

165

166 *2.5 Cultivation of the copepod Acartia tonsa*

167 The copepods (*A. tonsa*) were cultivated in 4,000 L cylindrical tanks with seawater of 34 ‰
168 salinity at 20 ºC and were continuously fed with algae *Rhodomonas baltica* at a concentration not
169 below 3 x 10^4 cells.mL^{-1}. *Acartia* eggs were obtained every day with a harvesting arm to collect the
170 eggs deposited on the flat bottom of the tanks. The eggs were washed thoroughly and stored in flasks
171 at 2 ºC. Egg harvest started 3 months in advance of the ABT feeding trial and continued until the end
172 of the trial. The water in the flasks was renewed every 2 weeks and the number of eggs counted. The
173 copepod eggs were incubated in 100 L tanks at a maximum density of 150 eggs.mL^{-1}. From 2 days
174 after hatch the nauplii and copepodites were fed *ad libitum* with a mixture of *R. baltica* and *Isochrysis*
175 *galbana* clone T-Iso and, before harvesting, the nauplii/copepodite density in the tanks was estimated,
176 harvested with a siphon, concentrated in a 60 µm sieve and then transferred to the ABT larval tanks.
177

178 *2.6 Artemia metanauplii enrichment and gilthead sea bream yolk sac larvae*

179 *Artemia* cysts EG type were decapsulated and enriched with Algamac 3050® over 12 h before
180 being fed to ABT larvae. Recently hatched nauplii were disinfected with bronopol (100 ppm;
181 (Pyceze®; Novartis). Enriched *Artemia* metanauplii were deposited in the tanks at a density of 0.1-
182 0.2 metanauplii.mL^{-1}. Before reproduction, gilthead sea bream broodstock were fed Vitalis CAL and
183 Vitalis REPRO (Skretting®) at a rate of 0.6 – 0.8 % of broodstock biomass. Gilthead sea bream 1 dah
184 yolk sac larvae were added to tanks at a density of 5-10 larvae.L^{-1}.
185

186 *2.7 Sampling for biometrical, biochemical and molecular analysis*

187 Thirty and twenty randomly caught ABT larvae per replicate treatment were anaesthetized
188 (0.02 % 2-phenoxyethanol, Sigma, Spain) in Trial 1 and 2, respectively, total length measured and
189 individual larvae photographed. The tank was considered the experimental unit in Trial 1 (n = 4),
190 where individual fish were utilized in Trial 2 (n = 20). The developmental stage was assessed by

counting the number of ABT larva which had attained full flexion of the notochord by the end of the feeding trials (13 and 18 dah) in each replicate set of samples. Individual larvae dry mass was determined on a precision balance after maintaining samples at 110 °C for 24 h and cooling *in vacuo* for 1 h before weighing. Final survival (%) was calculated by counting individual live larvae at the beginning and end of the trial.

In trial 1, three samples of 15 larvae per sample of 13 dah larvae were collected per tank: i) two samples/tank were placed in 1 ml of RNA*later*® (Sigma, Madrid, Spain) for RNA extraction and molecular analysis (n = 8), and ii) one sample/tank was frozen in liquid N_2 and stored at -80 ºC for biochemical analysis (n = 4). In trial 2, nine samples of 15 larvae of 18 dah larvae were collected per tank with 6 samples/tank used for molecular analysis (n = 6) and 3 samples/tank used for biochemical analyses, respectively (n = 3). Triplicate samples of enriched rotifers, copepods (*Acartia*), enriched *Artemia* metanauplii, and 1 dah sea bream yolk sac larvae were filtered and washed, excess water drained and blotted with filter paper, immediately frozen in liquid N_2 and stored at -80 ºC prior to analysis. All procedures were carried out according to the current national and EU legislation on the handling of experimental animals.

2.8 Biochemical analysis.

2.8.1 Proximate gross 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 prior to analyses. 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). Carbohydrate content was calculated as 100 – (percentages of protein + lipid

based on dry weight). The gross energy content was calculated from gross composition data and using values of 5.65, 9.45 and 4.20 kcal.g^{-1} for protein, lipid and carbohydrates, respectively (Henken *et al.* 1986).

2.8.2. Preparation of hydrolysates, derivatisation, UPLC analysis of taurine and total amino acid content of live prey and ABT larvae, and calculation of amino acid sufficiency index

A Waters AccQ-Tag Ultra Method® was used in determining taurine and amino acids contents in samples of enriched rotifers *B. rotundiformis* and 13 dah ABT larvae. Hydrolysis and derivatization were carried out according to manufacturer's instructions, and amino acid analysis (including taurine) was performed using a Waters H-Class UPLC fitted with an ACQUITY BEH Phenyl 1.7µ UPLC column (AAA for H-Class System Guide, Waters Corporation 2012). An essential amino acid sufficiency index (SIa.a.) was calculated by assuming the total amino acid profile/content of 1 dah ABT yolk sac larvae as an indicator of ABT larvae amino acid requirements. The index was calculated by dividing the content of a determined amino acid in a live prey by the content of the same amino acid in 1 dah ABT larvae and multiplying the result by 100.

SIa.a. = ([a.a. prey]/ [a.a. 1 dah ABT larvae]) x 100

Values of a SIa.a. equal or above 100 will indicate sufficient dietary amount of that amino acid in the live prey, whereas values below 100 show potential insufficiency for that amino acid (Oser, 1959).

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

Total lipid of live feeds (enriched rotifers, copepods, enriched *Artemia* and gilthead sea bream yolk sac larvae) 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 samples of feeds/ABT

larvae were 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) from the extracted total lipids were prepared by acid-catalyzed transesterification at 50 ^{o}C for 16 h according to the method of Christie (1993). Methyl esters were separated and quantified by gas-liquid chromatography (Agilent Technologies 7890B GC System) using a 30 m x 0.32 mm i.d. fused silica capillary column (SUPELCOWAX^{TM}-10, Supelco Inc., Bellefonte, USA) 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 per min and then to 230 ^{o}C at 2.0 ^{o}C per min. 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 Agilent Technologies Openlab CDS Chemstation for Windows (version A.02.05.21).

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

Alpha-tocopherol concentrations in enriched rotifers and Artemia as well as 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 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.

2.8.5. Selenium determination

Total selenium concentration was measured in feeds according to the method established by 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.

2.8.6. Stable isotope analysis

Triplicate samples of rotifer *B. rotundiformis* enriched with Algamac 3050®, *A. tonsa* copepod nauplii and 13 dah ABT larvae fed on rotifers and/or copepods were frozen in liquid nitrogen and subsequently lyophilized. Then, samples were ground to powder by pestle and mortar, packed into tin capsules to be analyzed for isotopic relative abundance (δ) of ^{15}N and ^{13}C, carbon (%) and nitrogen

290 (%). Prior to δ^{13}C analysis, samples with high lipid content (C:N ratio > 3.5; see Post et al., 2007)
291 were subjected to total lipid extraction by chloroform/methanol (2:1, by volume) (Varela et al., 2012;
292 2013). The relative abundances of ^{13}C and ^{15}N (δ^{13}C and δ^{15}N, repectively) were measured by a
293 continuous gas flow system using a Thermo Finnigan Elementary Analyzer Flash EA1112 coupled
294 to a Finnigan MAT Delta Plus mass spectrometer. All carbon and nitrogen isotope data are reported
295 in δ notation according to the following equation: $\delta X = [(R_{sample}/R_{standard}) - 1] \times 1000$, where
296 X is ^{13}C or ^{15}N and R is the ratio ^{13}C/^{12}C or ^{15}N/^{14}N (Peterson and Fry, 1987). Standard materials are
297 Vienna Pee Dee belemnite for carbon and atmospheric N_2 for nitrogen and expressed as parts per
298 thousand (‰) relative to standards (Peterson and Fry, 1987). The isotopic enrichmen (Δ) of the
299 predator in relation to its prey was calculated as: $\Delta^{15}N = \delta^{15}N_{predator} - \delta^{15}N_{prey}$ and $\Delta^{13}C = \delta^{13}C_{predator} -$
300 $\delta^{13}C_{prey}$, respectively.

301

302 *2.9. Tissue RNA extraction and cDNA synthesis*

303 Samples of pooled larvae (approximately 100 mg) [2 samples per tank in Trial 1 (n = 8) and
304 6 samples per tank in trial 2 (n = 6)] were placed in RNA*later*® (Sigma–Aldrich, Dorset, UK) and
305 frozen at -20 °C prior to total RNA extraction. Samples were homogenized in 1 mL of TriReagent®
306 (Sigma-Aldrich) RNA extraction buffer using a bead tissue disruptor (Bio Spec, Bartlesville,
307 Oklahoma, USA). Total RNA was isolated following manufacturer's instructions and quantity and
308 quality determined by spectrophotometry using a Nanodrop ND-1000 (Labtech Int., East Sussex,
309 UK) and electrophoresis using 200 ng of total RNA in a 1 % agarose gel. cDNA was synthesized
310 using 2 µg of total RNA and random primers in 20 µL reactions and the high capacity reverse
311 transcription kit without RNase inhibitor according to the manufacturer's protocol (Applied
312 Biosystems, Warrington, UK).

313

314 *2.10. qPCR analysis*

315 Several genes related to lipid and fatty acid metabolism, antioxidant and digestive enzymes,
316 as well as growth markers were evaluated in the present study. Quantitative real-time PCR (qPCR)
317 was carried out on transcription factors peroxisome proliferator-activated receptor alpha (*pparα*),
318 peroxisome proliferator-activated receptor gamma (*pparγ*), liver X receptor (*lxr*), retinoid X receptor
319 (*rxr*), sterol regulatory element-binding protein 1 (*srebp1*) and sterol regulatory element-binding
320 protein 2 (*srebp2*); LC-PUFA biosynthesis genes delta-6 fatty acyl desaturase (*fads2d6*) and fatty
321 acyl elongase 5 (*elovl5*), and fatty acid metabolism genes fatty acid synthase (*fas*), carnitine palmitoyl
322 transferase I (*cpt1*), acyl coA oxidase (*aco*), fatty acid binding protein 2 (intestinal; *fabp2*), fatty acid
323 binding protein 4 (adipocyte; *fabp4*), fatty acid binding protein 7 (brain; *fabp7*), lipoprotein lipase
324 (*lpl*) and 3-hydroxy-3-methylglutaryl-CoA lyase (*hmgcl*); the antioxidant enzymes superoxide
325 dismutase (*sod*), catalase (*cat*), glutathione peroxidase 1 (*gpx1*) and glutathione peroxidase 4 (*gpx4*);
326 myogenesis growth indicators myosin heavy chain (*myhc*) and tropomyosin (*tropo*); and digestive
327 genes trypsin (*tryp*), amino peptidase (*anpep*), alkaline phosphatase (*alp*), amylase (*amy*), pancreatic
328 lipase (*pl*), phospholipase A2 (*pla2*), bile salt activated lipase 1 (*bal1*) and bile salt activated lipase 2
329 (*bal2*) (see Supplementary Table 2).

330 Expression of genes of interest was determined by qPCR of all the RNA samples with
331 *Elongation factor-1α* (*elf1α*) and *β-actin* used as reference genes. The cDNA was diluted 20-fold with
332 milliQ water. The efficiency of the primers for each gene was previously evaluated by serial dilutions
333 of cDNA pooled from the samples to guarantee it was > 85 % for all primer pairs. qPCR was
334 performed using a Biometra TOptical Thermocycler (Analytik Jena, Goettingen, Germany) in 96-
335 well plates in duplicate 20 µL reaction volumes containing 10 µL of Luminaris Color HiGreen qPCR
336 Master Mix (Thermo Scientific, Hemel Hempstead, UK), 1 µL of the primer corresponding to the
337 analyzed gene (10 pmol), 3 µL of molecular biology grade water and 5 µL of cDNA (1/20 diluted).
338 In addition, amplifications were carried out with a systematic negative control (NTC, no template
339 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.

2.11 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). The data were checked for homogeneity of the variances by the Bartlett test and, where necessary, arc-sin transformed before further statistical analysis. Relations between dietary components and the different variables measured were surveyed by correlation and linear regression analysis. Differences between mean values were analyzed by t-test and one-way ANOVA followed by Tukey's multiple comparisons test performed using GraphPad Prism version 7.00 for Mac OSX (GraphPad Software, La Jolla California USA, www.graphpad.com). Differences were reported as statistically significant when $P < 0.05$ (Zar, 1999). 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 Analyzed composition of the different dietary treatments (rotifer, Artemia, copepods and gilthead sea bream yolk sac larvae)

Gross composition (% dry mass), energy content (kcal.g^{-1} dry mass), taurine (mg.g^{-1} dry mass), vitamin E and selenium contents (µg.g^{-1} dry mass) and lipid class composition (total lipid %) of the different live preys used in the present study are shown in Table 1. Total protein content was significantly highest in *Acartia* copepod (63.2 %) followed by sea bream yolk sac larvae (58.7 %)

and then enriched rotifer and *Artemia* (about 52 %). In contrast, total lipid content was highest in sea bream yolk sac larvae and enriched *Artemia* (about 18 %), followed by enriched rotifer (12 %) and copepod (8.8 %). Carbohydrates (generally mostly chitin) was highest in enriched rotifers, followed by *Acartia* and *Artemia* and, as expected, lowest in sea bream yolk sac larvae. Total caloric content was significantly highest in sea bream yolk sac larvae and enriched *Artemia* (about 56 $kcal.g^{-1}$), due to the highest lipid caloric contribution, followed by enriched rotifers and *Acartia* (about 52 $kcal.g^{-1}$). The significantly highest total polar lipid contents and polar/neutral lipid ratios were found in *Acartia* nauplii and sea bream yolk sac larvae, mainly due to presenting highest levels of PC and PE. Enriched *Artemia* and rotifer had the significantly lowest polar/neutral lipid ratios, due to their high triacylglycerol (TAG) contents. Free chlolesterol was highest sea bream yolk sac larvae >*Acartia* nauplii >enriched *Artemia* metanuplii > rotifers. Vitamin E content was highest in sea bream yolk sac larvae, followed by enriched rotifers, *Acartia* nauplii and lastly enriched *Artemia*. Se content was highest in enriched rotifers followed by enriched *Artemia* and yolk sac sea bream larvae and then *Acartia* copepods. Highest taurine content was found in gilthead sea bream yolk sac larvae (11 $mg.g^{-1}$ dry mass), followed by enriched *Artemia* (6.1 $mg.g^{-1}$ dry mass), *Acartia* nauplii (4.1 $mg.g^{-1}$ dry mass) and finally enriched rotifer (2.5 $mg.g^{-1}$ dry mass).

3.2 Amino acid contents in live preys and ABT larvae

Highest total amino acid content was presented by *Acartia* nauplii (571.5 $mg.g^{-1}$ dry mass) > gilthead sea bream yolk sac larvae (528.3 $mg.g^{-1}$ dry mass) > enriched *Artemia* metanauplii (411.6 $mg.g^{-1}$ dry mass) > enriched rotifer (334.3 $mg.g^{-1}$ dry mass) (Table 1 and Supplementary Table 3). However, the highest total essential amino acid content was shown by sea bream yolk sac larvae (280.4 $mg.g^{-1}$ dry mass) > *Acartia* nauplii (267.1 $mg.g^{-1}$ dry mass) > enriched *Artemia* metanauplii (214.6 $mg.g^{-1}$ dry mass) > enriched rotifer (157.5 $mg.g^{-1}$ dry mass). The most deficient content of essential amino acids, according to the sufficiency indices, was presented by enriched rotifers (Table 2). Enriched *Artemia* showed some insufficiencies with regards to valine, leucine, phenylalanine,

391 threonine and methionine. *Acartia* nauplii showed shortfall of histidine, and methionine, whereas sea
392 bream yolk sac larvae only showed a slight shortage of valine.
393
394 *3.3 Total lipid fatty acid compositions and contents of the live preys used to feed ABT larvae in Trials*
395 *1 and 2*

396 Total lipid fatty acid composition (percentage of weight) of rotifer *B. rotundiformis* enriched
397 with Algamac 3050®, nauplii of the copepod *A. tonsa* fed with the microalgae *R. baltica*, *Artemia*
398 metanauplii enriched with Algamac 3050® and gilthead sea bream (*S. aurata* L.) yolk sac larvae used
399 to feed ABT larvae are presented in Table 3. The highest values of total saturated fatty acids were
400 shown by *Acartia* nauplii and gilthead sea bream yolk sac larvae (27.8 % and 26.0 %, respectively),
401 followed by enriched rotifer (23.9 %) and *Artemia* (22.4 %). The highest values for total monoenes
402 were presented by enriched *Artemia* and yolk sac larvae (27.5 % and 26.3 %, respectively), mainly
403 due to the large proportions of 18:1n-9 (14.9 % and 15.9 %, respectively). Copepods and enriched
404 rotifers showed monoene values significantly lower (11.5 % and 12.6 %, respectively). Total n-6
405 PUFA were highest in enriched rotifers, followed by *Acartia* nauplii, enriched *Artemia* and yolk sac
406 larvae, largely due to their contents of linoleic acid (18:2n-6, LA). Yolk-sac larvae showed a very
407 low level of arachidonic acid (20:4n-3, ARA) compared to the other live preys. Total n-3 PUFA were
408 highest in copepod nauplii (43.9 %) > enriched *Artemia* and yolk sac larvae (39.3 % and 37.2 %,
409 respectively) > enriched rotifers (32.9 %). Enriched *Artemia* presented the significantly highest value
410 (23.0 %) of linolenic acid (18:3n-3; LNA) characteristic of EG grade *Artemia*. The proportion of
411 eicosapentaenoic acid (20:5n-3, EPA) was significantly highest (7.2 %) and that of docosahexaenoic
412 acid (22:6n-3, DHA) second highest (25.1 %) in yolk sac larvae, whereas the proportion of
413 docosahexaenoic acid was highest in *Acartia* (27.0 %). The DHA/EPA ratio was highest in *Acartia*
414 (6.1), followed by yolk sac larvae (3.5), enriched rotifers (2.8) and finally enriched *Artemia* (1.9),
415 respectively (Table 3). Quantitative results as total lipid fatty acid content (µg fatty acid.mg dry mass^{-1})
416 of the live preys used to feed ABT larvae in Trials 1 and 2 are shown in Supplementary Table 4.

417

418 *3.4 Growth performance and survival rates of ABT larvae in Trial 1 (13 dah) and Trial 2 (18 dah).*

419 Growth performance and survival of ABT larvae in Trial 1 (13 dah) and Trial 2 (18 dah) are
420 presented in Table 4. In Trial 1, 13 dah ABT larvae fed on *Acartia* copepods showed significantly
421 higher values for total length, total weight and flexion index than larvae fed on enriched rotifers.
422 However, no significant differences were observed in survival rates. In Trial 2, 18 dah ABT larvae,
423 previously fed on copepods in Trial 1 and then on sea bream yolk sac larvae (treatment CY), showed
424 the significantly highest values for total length, total weight and survival (32 %), and second lowest
425 for dry mass %. The next best set of values for growth and survival were obtained for ABT larvae fed
426 on treatment CA, followed by those of larvae fed on treatment CC, with poorest performance obtained
427 with ABT larvae fed treatment RA.

428

429 *3.5 Total lipid content and lipid class composition of 13 dah and 18 dah ABT larvae under different*
430 *live prey feeding protocols in Trials 1 and 2.*

431 Total lipid content (% of live and dry mass) and lipid class composition (total lipid %) of ABT
432 larvae 13 dah fed on treatments R and C in Trial 1, and ABT larvae 18 dah that had been fed on
433 treatments RA, CA, CC and CY in Trial 2, are presented in Table 5. In Trial 1, ABT 13 dah larvae
434 fed either on treatment R or C did not show significant differences in total lipid content. Neither
435 showed significant differences in total polar or total neutral lipids. Nevertheless, total polar lipids
436 predominated (56.5 and 58.5 %, respectively) over total neutral lipids (43.5 and 41.5 %, respectively)
437 in ABT larvae fed on treatments R or C, respectively. Moreover, ABT larvae fed on treatment R
438 showed significant higher level of TAG than larvae fed on treatment C (14.5 % vs 10.7 %). In Trial
439 2, ABT 18 dah fed on treatments CY and RA showed significant highest lipid contents followed by
440 those ABT larvae that were fed on treatments CC and CA. Total polar lipid showed highest (59.4 %)
441 in ABT larvae from treatment CC, followed by ABT larvae from treatments CA and RA (56.4 % and

54.6 %, respectively) and lowest value for larvae from CY treatment, due to their highest level of total neutral lipid (51.8 %) primarily TAG (22.4 %) (Table 5).

3.6 Total lipid fatty acid composition of 13 dah and 18 dah ABT larvae under different live prey feeding protocols in Trials 1 and 2.

In Trial 1, 13 dah ABT larvae fed treatments R or C showed no significant differences between total saturated fatty acids, although larvae from treatment R showed significantly higher 16:0 contents than larvae from treatment C (Table 6). Total monoenes were significantly higher in larvae from treatment R, mainly due to higher levels of 16:1n-7 and 18:1n-7 in total lipids compared to larvae fed treatment C. The same trend was observed for total n-6 PUFA, which were significantly higher in ABT larvae fed treatment R, due to higher values of LA, ARA and n-6 docosapentaenoic acid (22:5n-6, DPA) compaed to larvae fed treatment C. In contrast, total n-3 PUFA content was highest in ABT larvae fed treatment C (34.2 % *vs* 19.5 %), mainly due to contain higher levels of LNA, SDA, EPA and DHA. The DHA/EPA ratio was almost 14-fold higher in ABT larvae fed treatment C.

In Trial 2, larvae fed treatments CC and CY showed significantly higher total saturated fatty acids, mainly due to higher levels of 16:0, followed by ABT larvae of the RA and CA treatments. Similarly, high levels of total monoenoic fatty acids (about 20 % of total fatty acids, with half represented by 18:1n-9) were found in larvae from treatments RA, CA and CY, with larvae from treatment CC showing lower levels. Total n-6 PUFA were highest in larvae from treatment RA (14.9 %) > larvae fed treatments CC and CA (12.5 % and 12.1 %, respectively), and were lowest in larvae fed treatment CY (9.8 %). These values mainly reflected the level of 18:2n-6 that followed the same order per treatment. ARA was significantly highest in larvae fed treatment CA (1.9 %), with no significant differences among the other treatments. Total n-3 PUFA was significantly higher in ABT larvae fed treatment CC (33.2 %) > CA, CY and RA (30.9 %, 30.7 % and 28.2 %, respectively). These values reflected the major contribution of DHA, which was highest in larvae fed treatments

CC and CY (24.1 % and 22.2 %, respectively), followed by larvae fed treatments CA and RA (15.8 % and 10 %). Levels of EPA in ABT larvae showed, in decreasing order of abundance, treatments RA and CY (4.7 % and 4.4 %, respectively, and not significantly different) followed by treatments CA and CC (3.4 % and 2.1 %, respectively). The DHA/EPA ratio was significantly highest in 18 dah ABT larvae fed treatment CC (11.3), followed bylarvae fed treatments CY (5.1), CA (4.6) and RA (2.1).

3.7 Relative abundance (δ) of stable isotopes ^{15}N and ^{13}C and C:N ratios of enriched rotifer B. rotundiformis, A. tonsa nauplii and 13 dah ABT larvae

The relative abundance (δ) of ^{15}N and ^{13}C (‰) and C:N ratio of rotifer *B. rotundiformis* enriched with Algamac 3050®, nauplii of the copepod *A. tonsa* fed on the microalgae *R. baltica* and ABT larvae 13 dah fed on *B. rotundiformis* and *A. tonsa* nauplii, as well as isotopic enrichment (Δ) of ABT larvae in relation to its preys are presented in Table 7. The relative abundance of ^{15}N was significantly higher in *A. tonsa* nauplii than in enriched rotifers, as was the case for 13 dah ABT larvae fed *Acartia* compared to larvae fed enriched rotifer. In contrast, the relative abundance of ^{13}C showed significantly higher values in enriched rotifers than in *Acartia*. The same was observed for ABT larvae, with ABT larvae fed enriched rotifers presenting significantly higher values than larvae fed copepod nauplii. On the other hand, the C:N ratio was significantly higher in enriched rotifers and ABT larvae fed rotifers than in copepod nauplii and ABT larvae fed copepods. The isotopic enrichments $\Delta^{15}N$ and $\Delta^{13}C$ of 13 dah ABT larvae in relation to their preys were also significantly higher in larvae fed copepods than in larvae fed rotifers.

3.8. Gene expression

3.8.1. Myogenic genes

The expression of both *myhc* and *tropo* was significantly higher in ABT larvae fed *A. tonsa* nauplii in Trial 1 (Fig. 1). On the other hand, in Trial 2, the expression of *myhc* in 18 dah ABT larvae

was significantly up-regulated in larvae from treatment CY whereas no significant differences were observed among the expression of this gene in larvae from the others treatments (RA, CA and CC). With regard to the expression of *tropo* in 18 dah ABT larvae, no differences were observed among larvae fed the different treatments.

3.8.2. Fatty acid synthesis genes.

No significant nutritional regulation was detected at 13 dah between larvae fed on treatments R and C with regard to the relative expression of the 3 fatty acid synthesis genes *fas*, *fad2d6* and *elovl5* (Fig. 2). The expression values for the same 3 genes followed a similar pattern at 18 dah, with ABT larvae fed CA displaying the highest expression levels, albeit not significantly different to expression in larvae fed RA and CC in the case of *fad2d6*. The lowest transcript copy numbers of *fas* and *elovl5* were found in larvae fed CY, whereas the expression of *fad2d6* did not differ between this group and larvae fed RA or CC.

3.8.3. Fatty acid catabolism genes

In relation to fatty acid catabolism, the expression of *cpt1* did not show dietary regulation at 13 dah among larvae fed R or C in Trial 1 (Fig. 3), whereas at 18 dah the highest mRNA copy numbers were observed in larvae fed CY > RA > CA = CC. In contrast, relative expression of *aco* was significantly lower in 13 dah ABT larvae fed treatment C and, in Trial 2, larvae fed CY.

3.8.4. Fatty acid and lipid transport genes

In Trial 1, the relative transcript abundance of *fabp2*, *fabp7* and *lpl* was not significantly different between larvae fed treatments R and C (Fig. 4). In addition, the expression of *fabp4* and *hmgcl* were significantly lower in larvae fed treatment C. In Trial 2, the lowest copy numbers for *fabp2*, *lpl* and *hmgcl* were observed in larvae fed CY, whereas these larvae showed the highest expression levels of *fabp4* and fabp7, albeit not different to those of larvae fed CA in the case of *fabp7* (Fig. 4).

519

520 *3.8.5. Transcription factors genes regulating lipid metabolism*

521 In Trial 1, the expression levels of *pparα*, *pparγ*, *srebp1* and *srebp2* were significantly lower
522 in larvae fed treatment C (Fig. 5). In contrast, the expression of *rxr* was significantly higher in larvae
523 fed treatment C, whereas no significant differences were observed for expression of *lxr*. In Trial 2,
524 lower expression of *pparα*, *srebp1*, *pparγ* and *lxr* was observed in CY-fed larvae. The relative
525 expression value of *srebp2* was also lowest for larvae fed treatment CY, albeit not different to the
526 level in larvae fed CC. No regulation was observed in the expression levels of *rxr* at 18 dah (Fig. 5).

527

528 *3.8.6. Antioxidant defence enzyme genes*

529 In Trial 1, the expressions of *sod*, *gpx1* and *gpx4* were significantly lower in larvae fed *Acartia*
530 nauplii (C), whereas the expression of *cat* was not regulated between larvae fed treatments R or C
531 (Fig. 6). In Trial 2, *sod* expression was significantly higher in ABT larvae fed treatment CY, whereas
532 abundance of *cat* and *gpx4* was lower in the same larvae. The highest *gpx1* mRNA copy number was
533 found in larvae fed RA, albeit not different to that of larvae fed CY (Fig. 6).

534

535 *3.8.7. Digestive enzyme genes*

536 In Trial 1, the expression values of *tryp*, *alp*, and *bal1* showed no regulation between 13 dah
537 ABT larvae fed treatments R or C (Fig.7). In contrast, the expression levels of *anpep*, *amy*, *pl*, *pla2*
538 and *bal2* were significantly lower in ABT larvae fed treatment C. In contrast, in Trial 2, larvae fed
539 CY displayed the lowest expression levels for all the digestive genes, excepting for *alp*, where no
540 differences were found among larvae fed the different dietary treatments. ABT larvae fed treatment
541 CC displayed the highest expression levels of *tryp*, *pla2 bal1* and *bal2* (Fig. 7).

542

543 **4. Discussion**

544 Previous studies had shown that copepods supported better growth performance than other

545 live prey for larvae of ABT at first feeding (Yufera et al., 2014; Betancor et al., 2017a,b). The present
546 study was consistent with these data, as larvae fed this live prey displayed larger biometric data,
547 advanced stage of development (highest flexion index), and highest expression of the myogenic
548 genes, *myhc* and *tropo* than ABT larvae fed on enriched rotifer at 13 dah. Additionally, in the present
549 study we evaluated the effect that different live prey can have on later stages (18 dah) of ABT larvae
550 performance in combination with the earlier use of either rotifers or copepods. A higher C:N ratio in
551 the rotifer coincides with a significantly higher total lipid content in this prey than in the copepod, as
552 was the case for rotifer-fed 13 dah ABT larvae, whose average total lipid content was higher than
553 ABT larvae fed copepods, albeit not statistically significant. The $\delta^{15}N$ has been used to examine the
554 trophic position of organisms in food webs (Owens 1987; Hobson and Welch, 1992; Michener and
555 Kaufman, 2007), with ^{15}N values of consumers generally increasing with trophic level, with an
556 average 3.2 ‰ of enrichment per trophic level (Michener and Kaufman, 2007). In the present study,
557 ABT larvae fed *A. tonsa* nauplii showed higher values of enrichment of N ($\Delta^{15}N$) and C ($\Delta^{13}C$) than
558 ABT larvae fed on enriched *B. rotundiformis* (2.79 vs. 1.46 for $\Delta^{15}N$, and 3.06 vs. 0.48 for $\Delta^{13}C$,
559 respectively). This is also consistent with *Acartia* nauplii having a significantly higher content of total
560 protein (63.2 % vs. 51.3 % on a dry matter basis) and total amino acids (571.5 vs. 334.3 $mg.g^{-1}$ dry
561 mass) than rotifers. Gilthead sea bream yolk sac larvae, as a live prey showed the highest content of
562 essential amino acids with indication of a slight deficiency only for valine. Thus, the stable isotope
563 data support growth performance data, where ABT larvae fed on copepods grew and developed faster
564 than ABT larvae fed on enriched rotifers as they were prey of a supposedly "higher trophic position",
565 and had better nutritional value, in consequence fixing more N and C.

566 Fast growth in ABT larvae during early life stages, under standard rearing conditions, occurs
567 after notochord flexion, when piscivory is attained (Uriarte et al., 2016). The results of the present
568 study showed that ABT larvae fed *Acartia* nauplii attained 54.5 % of flexioned larvae *versus* only
569 35.8 % for ABT larvae fed enriched rotifers at 13 dah. This implies an advantage to reach the faster
570 growth phase after flexion with larvae that had been fed copepods attaining higher growth rates than

571 those fed rotifers at 18 dah. Indeed, highest growth was obtained in ABT larvae fed copepods first
572 (to 13 dah) and then on gilthead sea bream yolk sac larvae (to 18 dah), reflecting the contribution of
573 piscivory in the diet. Once ABT larvae attained piscivory, a very high growth performance was
574 obtained, in agreement with previous reports with Pacific Bluefin tuna (PBT; *Thunnus orientalis*)
575 larvae (Tanaka et al., 2007, 2010, 2014, 2015). Moreover, results on PBT larvae suggested that fast-
576 growing larvae at the onset of piscivory could survive in the mass culture tank and were characterized
577 by growth-selective mortality based on direct evidence by comparing feeding (nitrogen stable isotope
578 ratios) and growth between live and dead fish (Tanaka et al., 2017).

579 In the present trial, survival was not different among treatments at 13 dah, since the variability
580 of average survival rate among replicates was relatively large. Such a difference in survival is
581 currently the norm in the tuna hatchery phase at the present time and limited the number of fish
582 available for the second phase of the trial, 13-18 dph, which was cosequently carried out in single
583 replicate tanks to provide fish density to promote adequate feeding response. Great differences in
584 survival/growth have been apparent among different cohorts of fish produced since the first eggs were
585 hatched in 2009. These differences appear related to broodstock, as presently eggs are collected from
586 floating cages and therefore there is no accurate control of each broodstock contribution. However,
587 the mortality of ABT larvae at first feeding should not be quantitatively related to the nutritional
588 quality of the live prey. Survival of tuna larvae in artificial rearing has been reported to be relatively
589 low compared to other fish species (Miyashita, 2002). Multiple factors are thought to contribute to
590 mortality of larval tuna in rearing systems during the early larval stages, such as suboptimal physical
591 condition, including increased bacterial loads, surface adhesion and, of course, malnutrition as an
592 added negative effect (Margulies et al., 2016; Honryo et al., 2017). At these early stages, the vitality
593 and strength of the larvae also depends to a great extent on broodstock nutrition and the quality of the
594 eggs and yolk sac larvae produced, and this may vary with and within every spawned batch. Some
595 other neglected zootechnical factors dealing with rearing conditions in individual replicates/tanks
596 may affect swim bladder inflation and larval survival. Besides, mortality and poor development

during larval stages has also been attributed to antioxidant status (Mourente et al., 1999; Fernandez-Díaz et al., 2006; Mazurais el al., 2015; Penglase et al., 2015), as discussed below. In any case, the provision of a high-quality nutritional enhancement of planktonic prey during ABT larvae first feeding, a period of high metabolic demand required to support exponential growth, is of paramount importance.

Dietary fatty acid composition is known to have a modulatory effect on enzyme activities controlling fatty acid biosynthesis and bioconversion pathways in cultured teleost species (Zheng et al., 2004; Thanuthong et al., 2011; Ayisi et al., 2018). In fish, fatty acids can arise from two sources; either synthesized *de novo* from non-lipid carbon sources, or directly from dietary lipid. Fatty acid synthase (Fas) plays a key role in the process of *de novo* lipogenesis, and the rate of fatty acid *de novo* synthesis has been shown to be negatively correlated to the level of dietary lipids (Henderson, 1996). In trial 2 in the present study, there was a strong negative correlation between dietary total lipid content and *fas* expression ($r = -0.63$; $P = 0.05$), indicating and inhibition of *fas* expression by increased dietary lipid levels, as stated above. In contrast, in Trial 1, *fas* expression showed no differences in larvae fed on either rotifers or copepods, possibly due to sufficient lipid content in these preys. On the other hand, *fas* is a direct target gene of the transcription factor *srebp1*, and both genes followed a similar pattern of expression ($r = 1.0$; $P = 0.08$). These data were in contrast to the results of our previous studies with ABT larvae (Betancor et al., 2017a,b).

As a general consideration, increased expression of *fads2d6* has been observed previously in fish fed low levels of dietary n-3 LC-PUFA, whereas high dietary levels of these fatty acids has been associated with reduced relative transcript abundance (Morais et al., 2012; Betancor et al., 2015). In the present study, in Trial 1, no dietary regulation was detected at 13 dah among ABT larvae fed treatments R or C with regard to the relative expression of the 3 fatty acid synthesis genes *fas*, *fas2d6* and *elovl5*. These results could be interpreted in the following manner: i) both prey (enriched rotifers and copepod nauplii) supplied sufficient lipid to maintain *fas* expression at a baseline level and ii) both prey supplied sufficient n-3 and n-6 PUFA to keep *fad2d6* and *elovl5* expression at a baseline

623 level. This may also mean that prey lipid and essential fatty acid contents in Trial 1 satisfied ABT
624 larvae requirements for these nutrients at first feeding and did not significantly affect fatty acid or
625 LC-PUFA synthesis gene expression. In contrast, in Trial 2, the expression of *fad2d6* in 18 dah ABT
626 larvae was higher in larvae fed enriched *Artemia* for 5 days after feeding on copepod nauplii diet
627 (treatment CA), and those fed treatments RA and CC were also higher than larvae fed CY. This seems
628 logical taking into account that the *Artemia* diet presented the lowest level of total n-3 PUFA and
629 DHA. In contrast, ABT larvae fed treatment CY showed lower expression of *fads2d6*, possibly due
630 to higher levels of DHA and total n-3 PUFA in both copepod nauplii and sea bream yolk sac larvae
631 than other live prey. A similar pattern of expression to dietary treatments was shown by *elolv5* in 18
632 dah ABT larvae, which further supports up-regulation of the LC-PUFA pathway in response to the
633 low dietary LC-PUFA in treatment CA. This suggests that n-3 LC-PUFA requirement of 18 dah ABT
634 larvae could be above the level supplied by treatment CA (mainly *Artemia*) and below or similar to
635 that of CY. In agreement, a previous study showed that an increased DHA/EPA ratio in ABT larvae
636 in parallel with increased expression of *fad2d6* and *elovl5* during development of unfed ABT larvae
637 was associated with elongation and desaturation of EPA in order to maintain adequate DHA levels
638 (Morais et al., 2011).

639 High dietary levels of n-3 LC-PUFA, particularly DHA, can act as ligands for transcription
640 factors such as *pparα* and *srebp1*, down-regulating the biosynthesis of LC-PUFA (Worgall et al.,
641 1998; Hihi et al., 2002; Cunha et al., 2013; Peng et al., 2014), and regulating the expression of their
642 target genes such as *fas*, *cptI*, *aco* or *lpl*. In the present study, *srebp1* was strongly positively correlated
643 with the expression of *fas* ($r = 1.0$; $p = 0.08$) and *elovl5* ($r = 1.0$; $p = 0.08$) in Trial 2. However, no
644 correlation was observed in Trial 2 between dietary DHA levels and the expression values of *pparα*
645 and *srebp1*. Previous studies in teleosts have described an inhibition in hepatic *fas* expression when
646 fish were fed in a restricted manner (Tian et al., 2013; He et al., 2015; Gong et al., 2017) or with
647 increased dietary lipid (Leng et al., 2012), including our previous results with ABT larvae at first
648 feeding (Betancor et al., 2017b). Although the transcription factor genes *pparα* and *srebp1* did not

649 show nutritional regulation in Trials 1 and 2, *fas*, a direct target of *srebp1*, was strongly regulated by
650 the different dietary treatments in Trial 2 and followed a similar pattern of expression as *srebp1* in
651 response to diet.

652 *Pparγ*, a transcription factor involved in adipocyte function and differentiation and lipid
653 storage by adipocytes (Nedergaard et al., 2005; Ji et al., 2011; Agawa et al., 2012; Ayisi et al., 2018),
654 showed lower expression in ABT larvae fed copepods in Trial 1 and 2, with its expression negatively
655 related to lipid content. Thus, taking into account that copepods are among the natural live prey of
656 ABT larvae in the wild (Uotani et al., 1990; Catalán et al., 2011; Tilley et al., 2016; Kodama et al.,
657 2017), it is feasible to suggest that the high lipid content of enriched rotifers (treatment R in Trial 1
658 and treatment RA in Trial 2) triggered a response in ABT larvae that modulated lipogenetic/lipolytic
659 mechanisms in order to adapt to an energy-dense ration. In this sense, lower *pparγ* expression was
660 also observed in copepod-fed ABT larvae in previous trials (Betancor et al., 2017a,b). In mammals,
661 targets directly regulated by *pparγ* include genes that favour uptake of circulating fatty acids by
662 adipocytes (Schoonjans et al., 1996; Frohnert et al., 1999; Chui et al., 2005) and others that promote
663 recycling rather than export of intracellular fatty acids (Guan et al., 2002; Hibuse et al., 2005). These
664 paradoxical effects on adipocyte biology means that, apart from enhancing fatty acid deposition
665 similar to *pparα*, *pparγ* can lead to increased fatty acid oxidation (Lehrke and Lazar, 2005). The β-
666 oxidation of fatty acids takes place in both mitochondria and peroxisomes, but mitochondrial β-
667 oxidation is quantitatively more important and can utilize a wider range of fatty acids as substrate
668 (Henderson, 1996). In this respect, *pparγ* expression was down-regulated by dietary treatments with
669 higher n-3 LC-PUFA (CA, CC and CY) and the same trend was followed by the expression of *aco* in
670 Trial 2. Nevertheless, the *cpt1* expression pattern for larvae fed this treatment was the opposite, being
671 higher in ABT larvae fed yolk sac larvae. CPT-1 is considered to be the main regulatory enzyme in
672 long-chain fatty acid β-oxidation and the higher expression observed in larvae fed yolk sac larvae
673 might be explained by the superior content of LC-PUFA in this live prey.

SREBP1 preferentially regulates fatty acid and LC-PUFA synthesis, whereas 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 an inverse correlation between cholesterol levels in enriched rotifers and copepods and *srebp2* expression in ABT larvae in Trial 1. Moreover, in Trial 2, the expression of *srebp2* was also negatively correlated ($r = -0.95$; $P = 0.17$) to dietary cholesterol levels.

LXR is important in controlling intermediary metabolism mediating cross-regulation between sterol and fatty acid metabolism (Ayisi et al., 2018), and its ligands can be antagonized by fatty acids (Cruz-Garcia et al., 2012). Thus, high levels of unsaturated fatty acids activate *lxr,* whereas activated *lxr* induces cholesterol catabolism and *de novo* fatty acid biosynthesis in liver, which has led to the suggestion that *lxr*s are sensors of the balance between cholesterol and fatty acid metabolism (Ayisi et al., 2018). In Trial 2, a weak negative correlation ($r = -0.32$; $P = 0.67$) was observed between dietary cholesterol levels and *lxr* expression. Although there was no significant correlation between dietary cholesterol levels and *lxr* expression in Trial 2, *lxr* expression was positively correlated with the expression of the bile acid activated lipases *bal1* and *bal2,* ($r = 0.80$; $p = 0.33$). The lack of a clear relation between dietary cholesterol and *lxr* expression may be due to the fact 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 addition to the role of *lxr* in regulating cholesterol catabolism, storage, absorption and transport through the transcriptional regulation of key target genes involved in these processes, it may also act in fatty acid metabolism by increasing the expression of the transcription factor *srebp1* or genes such as *fas* or *lpl*. In this sense, the mRNA levels of *lxr* and *lpl* showed a strong positive correlation ($r = 1.0$; $p = 0.08$) and the same pattern of expression in 18 dah ABT larvae. Lpl is a lipase highly expressed in muscle and liver of ABT (Betancor et al., 2017a) 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; Ayisi et al., 2018). In agreement, high levels of *lpl* expression and activity have been associated with increased lipid utilization in darkbarbel catfish (*Pelteobagrus vachelli*) larvae fed high-lipid diets (Zheng et al., 2010).

Fatty acid binding proteins (Fabp) in general are noted for the intracellular transport of fatty acids and play an intermediary role in orchestrating gene transcription involved in lipid homeostasis (Tocher 2003; Ayisi et al., 2018). In agreement with our previous studies (Betancor et al., 2017a,b), *fabp4*, a carrier protein involved in fatty acid uptake, transport and metabolism in adipocytes (Glatz and van der Vusse, 1996), was down-regulated in copepod-fed ABT larvae in Trial 1, possibly reflecting increased uptake and accumulation of lipid into larval tissues. On the other hand, *fabp2* (intestinal) and *fabp7* (brain) showed no differences in their expression between 13 dah ABT larvae fed treatments R or C. In agreement, a recent study in Senegalese sole (*Solea senegalensis*) 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 Trial 2 in the present study, different patterns of regulation were presented by different *fabp* genes in response to dietary treatments. However, the positive correlation ($r = 0.80$; $p = 0.33$) between *fas* expression and *fabp2* (intestinal) expression in ABT larvae fed the different dietary treatments in Trial 2 was noteworthy.

Rotifers and *Artemia* have low levels of several nutrients, including antioxidants, compared to copepods (Hamre et al., 2013). Nutrient intake plays a role in regulating the redox system in fish larvae, suggesting that nutrient-induced changes in the redox system may contribute to differences in larval fish growth and development (Mourente et al., 2007; Izquierdo and Betancor, 2015; Penglase et al., 2015). In Trial 1, C-fed larvae showed lower expression of *sod*, *gpx1* and *gpx4*, possibly in response to lower contents of antioxidant nutrients such as Se or vitamin E. It was more complicated in Trial 2 as the pattern of expression was variable among the different dietary treatments. Differences

726 could not only be attributed to the feed, but also to the stage of development of the larvae as it is
727 known that lipid peroxidation levels in larvae show diet and age/growth dependence in their responses
728 (Fernandez-Diaz et al., 2006). Therefore, the differences in growth among the larvae could influence
729 lipid oxidation levels. It is perhaps noteworthy that the Se content of live prey, either enriched rotifers
730 or *Acartia* nauplii, showed a significant negative correlation with final total length ($r = -0.9$; $P = 0.0417$), total weight ($r = -0.9$; $P = 0.008$) and flexion index ($r = -0.8$; $P = 0.053$). Although Se is an
731
732 essential micronutrient, it has the narrowest window of any element between requirement and toxicity
733 (Polatajko et al., 2006), with reduced growth being one of the first symptons of toxicity (Jaramillo et
734 al., 2009). Thus, it would need to be established whether the Se levels used in enriched rotifers in the
735 present study were within safe limits for ABT larvae.
736 Molecular methodologies could contribute to the understanding of the real digestive capacities
737 of developing larvae under different dietary protocols. In the present study, expression of trypsin
738 (*tryp*), pancreatic alkaline protease (*alp*) and bile acid activated lipase 1 (*bal1*) was not regulated by
739 dietary treatments in Trial 1. In contrast, in Trial 2, all digestive anzyme genes studied were
740 significantly up-regulated by treatment CC and down-regulated by treatment CY, which were the
741 largest larvae, which could indeed indicate that the expression levels are related to the developmental
742 stage. It is commonly accepted that the major digestive lipase in teleosts, including larvae, appears to
743 be bile salt-dependent lipases (*bal*) (Rønnestad and Morais, 2008). It was also reported that *Bal* were
744 the main enzymes involved in lipid digestion in the larval stage of PBT (Murashita et al., 2014). In
745 the present study, the expression patterns of both isoforms (*bal1* and *bal2*) were nutritionally
746 regulated, showing a similar pattern.
747 In conclusion, and in agreement with our previous trials, the present study showed that
748 copepods (*Acartia*) were a superior live prey for first feeding ABT larvae compared to enriched
749 rotifers, as indicated by higher growth performance. This rapid growth would enable rapid attainment
750 of the piscivory feeding stage, which would also enable earlier weaning to inert formulated diet. This
751 may reflect the higher protein and essential amino acids, polar lipid and n-3 LC PUFA and other

752 nutrient contents of copepods as first prey. When the start of piscivory is delayed (larvae fed Artemia
753 or copepods) larvae previously fed copepods do not show any advantage in growth compared to larvae
754 fed with rotifers. The highest DHA contents were found in ABT larvae fed CC and CY, whereas the
755 lowest contents were found in RA-fed larvae. Although, RA-fed larvae showed the highest level of
756 the intermediate product n-3 DPA, this was not supported by gene expression data. Different
757 expression patterns of digestive enzymes between ABT larvae fed copepods and enriched rotifers
758 could be due to different lipid class/fatty acid compositions of the live prey or to differences in the
759 size/development of the larvae.
760

761 **Acknowledgements**

762 We wish to thank the technical staff at Laboratory of Marine Aquaculture (IEO), Puerto de Mazarrón
763 (Murcia, Spain) and Nutritional Analytical Services (NAS; Institute of Aquaculture, University of
764 Stirling, UK) that contributed to this work. This work was supported by the Consejería de Innovación,
765 Ciencia y Empresa de la Junta de Andalucía, Proyecto de Excelencia de Promoción General del
766 Conocimiento [Ref. RNM 733, 2012), and Programa Estatal de Investigación del Ministerio de
767 Economía y Competitividad [Ref. AGL2014-52003-C2-1-R, 2014 and Ref. RTC-2016-5835-2].
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1009

1010 **Figure legends**

1011 **Fig. 1.** Nutritional regulation of myosin heavy chain 2 (*myhc*) and tropomyosin *(tropo*) gene
1012 transcription in Atlantic bluefin tuna (ABT) larvae 13 days after hatch (Trial 1) fed with rotifer
1013 *B. rotundiformis* enriched with Algamac 3050® (R), *A. tonsa* copepod nauplii (C), and ABT
1014 larvae 18 days after hatch (Trial 2) fed with rotifer *B. rotundiformis* and then *Artemia* metanauplii
1015 enriched with Algamac 3050® (RA), *A. tonsa* copepod nauplii and then *Artemia* enriched with
1016 Algamac 3050® (CA), *A. tonsa* copepod nauplii and then followed with *A. tonsa* nauplii and
1017 copepodites (CC), and *A. tonsa* nauplii and then gilthead sea bream (*S. aurata*) yolk sac larvae
1018 (CY). Values are normalized expression ratios, corresponding to an average of 8 pooled samples
1019 in Trial 1 (n = 8) and 6 pooled samples in Trial 2 (n = 6) with standard errors (SEM). Different
1020 superscript letters denote differences among the dietary treatments.
1021
1022 **Fig. 2.** Nutritional regulation of fatty acid synthase (*fas*), delta-6 fatty acyl desaturase (*fads2d6*) and
1023 fatty acyl elongase 5 (*elovl5*) gene transcription in Atlantic bluefin tuna (ABT) larvae 13 days
1024 after hatch (Trial 1) fed with rotifer *B. rotundiformis* enriched with Algamac 3050® (R), *A. tonsa*
1025 copepod nauplii (C), and ABT larvae 18 days after hatch (Trial 2) fed with rotifer *B.*
1026 *rotundiformis* and then *Artemia* metanauplii enriched with Algamac 3050® (RA), *A. tonsa*
1027 copepod nauplii and then *Artemia* enriched with Algamac 3050® (CA), *A. tonsa* copepod nauplii
1028 and then followed with *A. tonsa* nauplii and copepodites (CC), and *A. tonsa* nauplii and then
1029 gilthead sea bream (*S. aurata*) yolk sac larvae (CY). Values are normalized expression ratios,
1030 corresponding to an average of 8 pooled samples in Trial 1 (n = 8) and 6 pooled samples in Trial
1031 2 (n = 6) with standard errors (SEM). Different superscript letters denote differences among the
1032 dietary treatments.
1033
1034 **Fig.3.** Nutritional regulation of carnitine palmitoyl transferase I (*cptI*) and acyl coA oxidase (*aco*)
1035 gene transcription in Atlantic bluefin tuna ((ABT) larvae 13 days after hatch (Trial 1) fed with

rotifer *B. rotundiformis* enriched with Algamac 3050® (R), *A. tonsa* copepod nauplii (C), and ABT larvae 18 days after hatch (Trial 2) fed with rotifer *B. rotundiformis* and then *Artemia* metanauplii enriched with Algamac 3050® (RA), *A. tonsa* copepod nauplii and then *Artemia* enriched with Algamac 3050® (CA), *A. tonsa* copepod nauplii and then followed with *A. tonsa* nauplii and copepodites (CC), and *A. tonsa* nauplii and then gilthead sea bream (*S. aurata*) yolk sac larvae (CY). Values are normalized expression ratios, corresponding to an average of 8 pooled samples in Trial 1 (n = 8) and 6 pooled samples in Trial 2 (n = 6) with standard errors (SEM). Different superscript letters denote differences among the dietary treatments.

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 Atlantic bluefin tuna (ABT) larvae 13 days after hatch (Trial 1) fed with rotifer *B. rotundiformis* enriched with Algamac 3050® (R), *A. tonsa* copepod nauplii (C), and ABT larvae 18 days after hatch (Trial 2) fed with rotifer *B. rotundiformis* and then *Artemia* metanauplii enriched with Algamac 3050® (RA), *A. tonsa* copepod nauplii and then *Artemia* enriched with Algamac 3050® (CA), *A. tonsa* copepod nauplii and then followed with *A. tonsa* nauplii and copepodites (CC), and *A. tonsa* nauplii and then gilthead sea bream (*S. aurata*) yolk sac larvae (CY). Values are normalized expression ratios, corresponding to an average of 8 pooled samples in Trial 1 (n = 8) and 6 pooled samples in Trial 2 (n = 6) with standard errors (SEM). Different superscript letters denote differences among the dietary treatments.

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 Atlantic bluefin tuna (ABT) larvae 13 days after hatch (Trial 1) fed with rotifer *B. rotundiformis* enriched with Algamac 3050® (R), *A. tonsa* copepod nauplii (C), and ABT larvae 18 days after hatch (Trial 2)

fed with rotifer *B. rotundiformis* and then *Artemia* metanauplii enriched with Algamac 3050® (RA), *A. tonsa* copepod nauplii and then *Artemia* enriched with Algamac 3050® (CA), *A. tonsa* copepod nauplii and then followed with *A. tonsa* nauplii and copepodites (CC), and *A. tonsa* nauplii and then gilthead sea bream (*S. aurata*) yolk sac larvae (CY). Values are normalized expression ratios, corresponding to an average of 8 pooled samples in Trial 1 (n = 8) and 6 pooled samples in Trial 2 (n = 6) with standard errors (SEM). Different superscript letters denote differences among the dietary treatments.

Fig.6. Nutritional regulation of superoxide dismutase (*sod*), catalase (*cat*) and glutathione peroxidase 1 and 4 (*gpx1* and *gpx4* respectively) gene transcription in Atlantic bluefin tuna (ABT) larvae 13 days after hatch (Trial 1) fed with rotifer *B. rotundiformis* enriched with Algamac 3050® (R), *A. tonsa* copepod nauplii (C), and ABT larvae 18 days after hatch (Trial 2) fed with rotifer *B. rotundiformis* and then *Artemia* metanauplii enriched with Algamac 3050® (RA), *A. tonsa* copepod nauplii and then *Artemia* enriched with Algamac 3050® (CA), *A. tonsa* copepod nauplii and then followed with *A. tonsa* nauplii and copepodites (CC), and *A. tonsa* nauplii and then gilthead sea bream (*S. aurata*) yolk sac larvae (CY). Values are normalized expression ratios, corresponding to an average of 8 pooled samples in Trial 1 (n = 8) and 6 pooled samples in Trial 2 (n = 6) with standard errors (SEM). Different superscript letters denote differences among the dietary treatments.

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 Atlantic bluefin tuna (ABT) larvae 13 days after hatch (Trial 1) fed with rotifer *B. rotundiformis* enriched with Algamac 3050® (R), *A. tonsa* copepod nauplii (C), and ABT larvae 18 days after hatch (Trial 2) fed with rotifer *B. rotundiformis* and then *Artemia* metanauplii enriched with Algamac 3050® (RA), *A. tonsa* copepod nauplii and then *Artemia* enriched with Algamac 3050® (CA), *A. tonsa* copepod nauplii and then followed with

1089 *A. tonsa* nauplii and copepodites (CC), and *A. tonsa* nauplii and then gilthead sea bream (*S.*
1090 *aurata*) yolk sac larvae (CY). Values are normalized expression ratios, corresponding to an
1091 average of 8 pooled samples in Trial 1 (n = 8) and 6 pooled samples in Trial 2 (n = 6) with
1092 standard errors (SEM). Different superscript letters denote differences among the dietary
1093 treatments.
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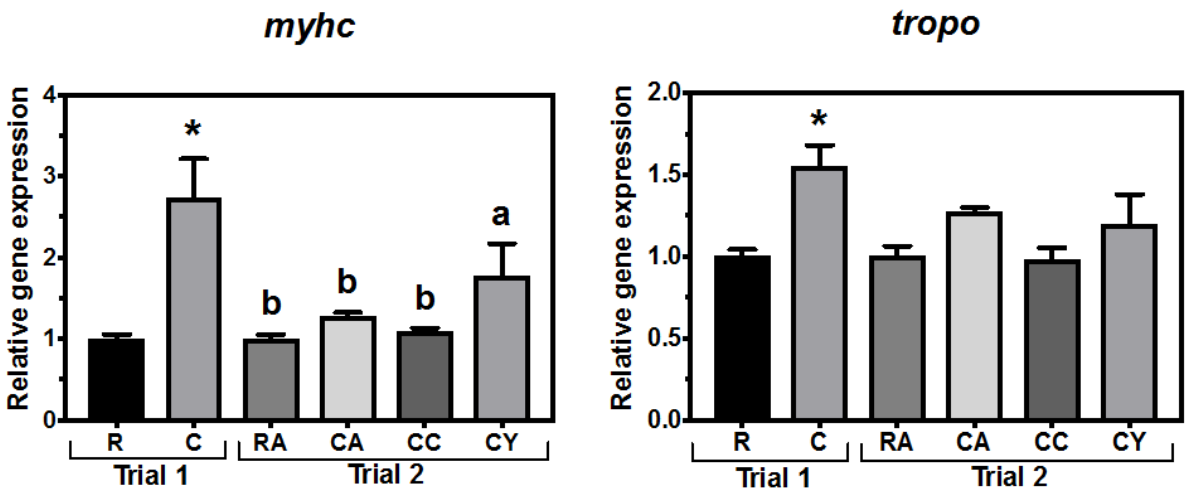

Fig.1

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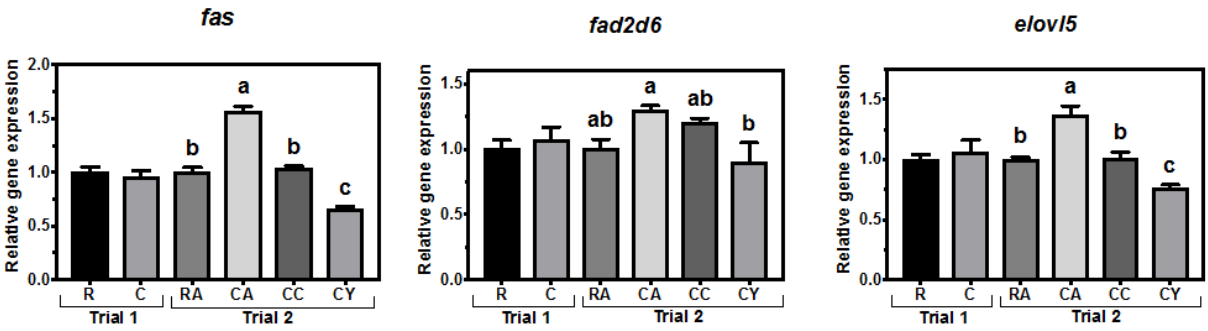

Fig.2

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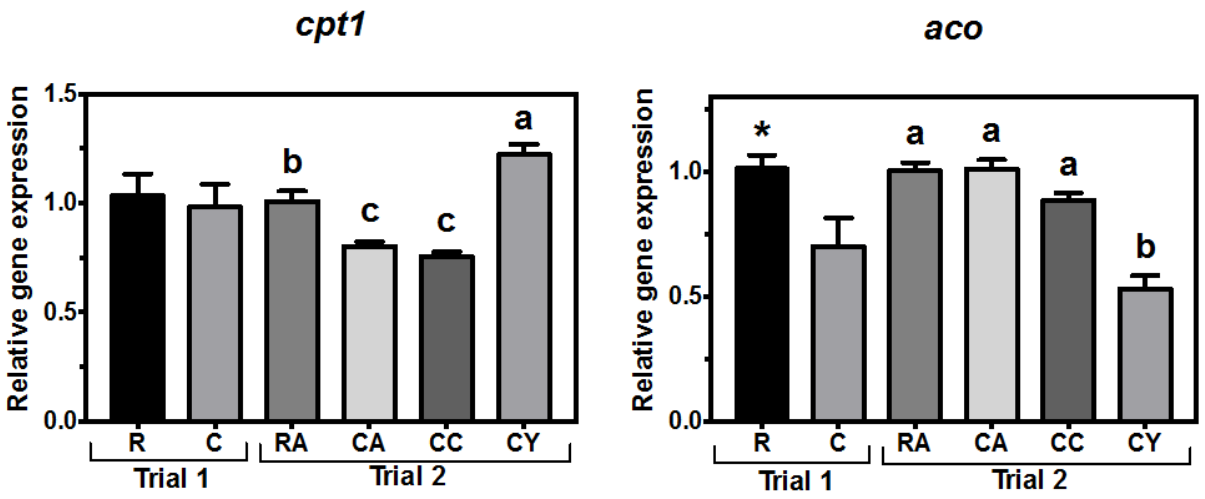
cpt1
aco
Relative gene expression
1.5
1.0
0.5
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a
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R
C
RA
CA
CC
CY
Trial 1
Trial 2

Fig.3

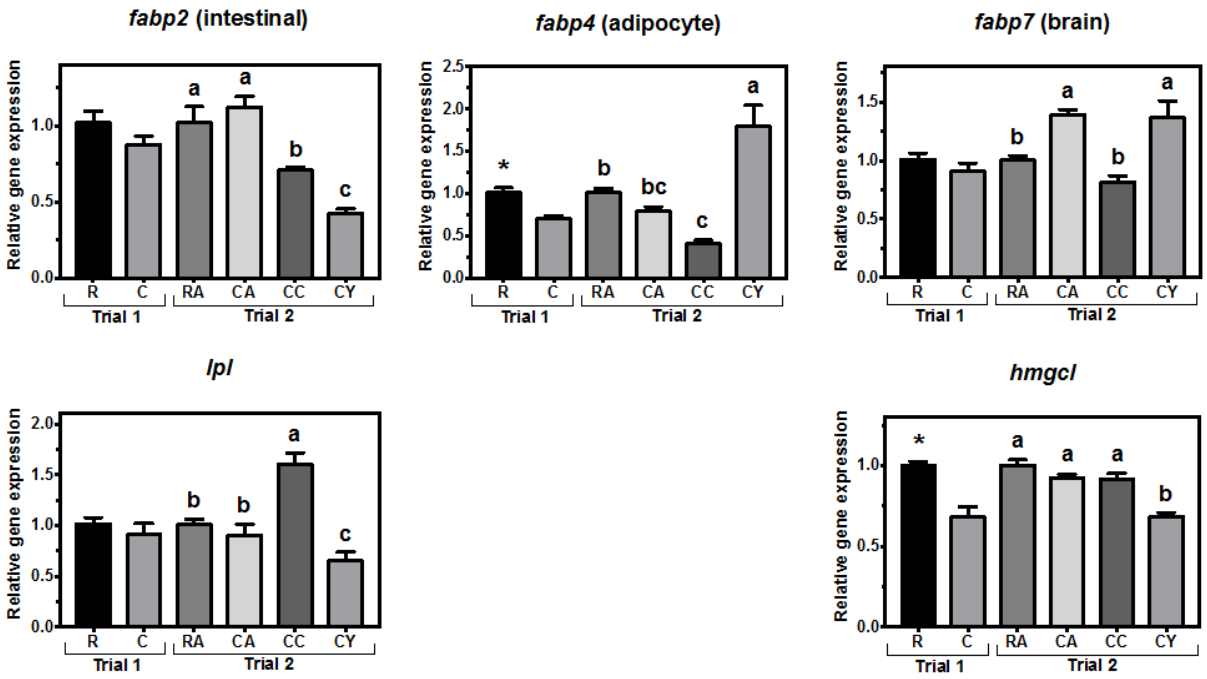
fabp2 (intestinal)
fabp4 (adipocyte)
fabp7 (brain)
lpl
hmgcl
Relative gene expression
R
C
RA
CA
CC
CY
Trial 1
Trial 2

Fig.4

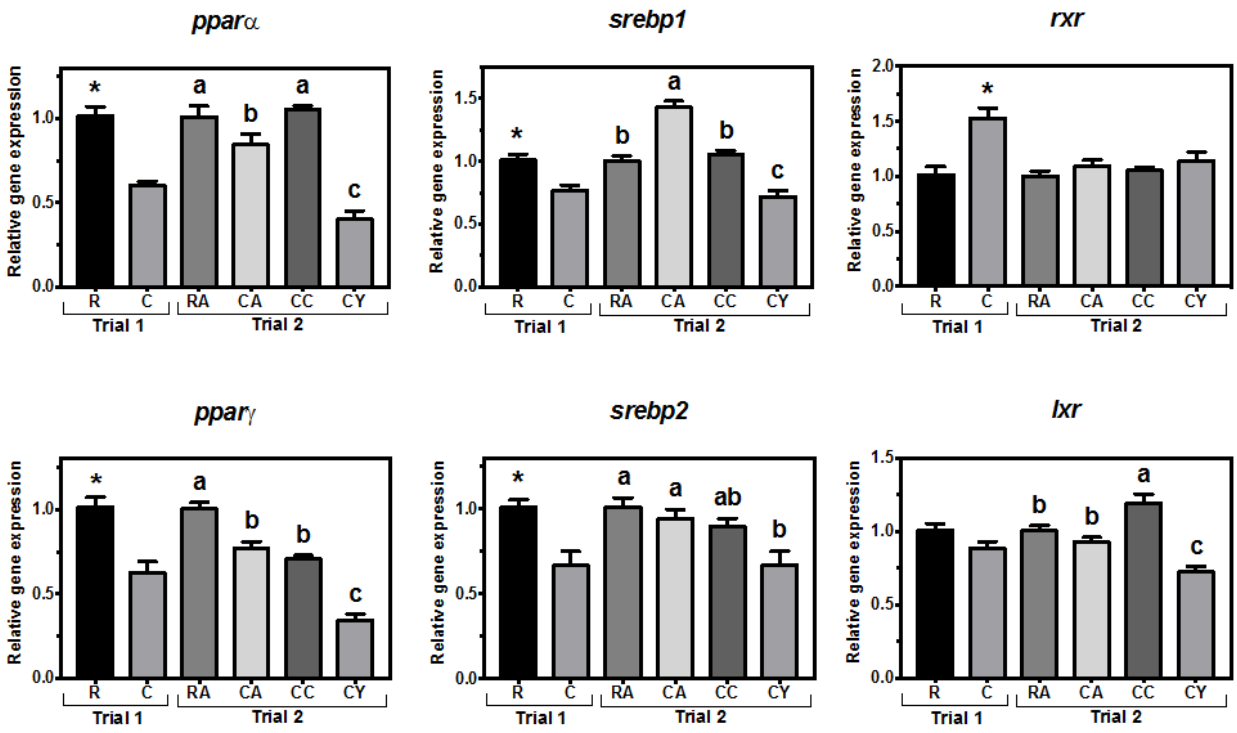

Fig.5

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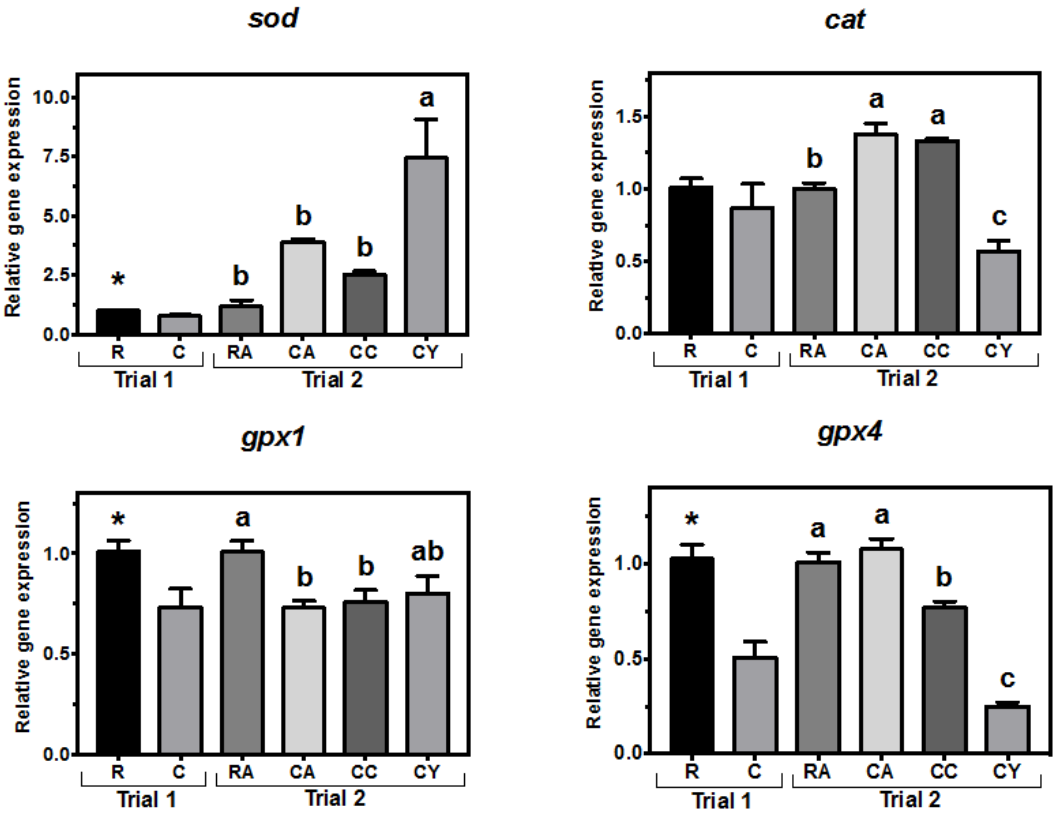

Fig.6

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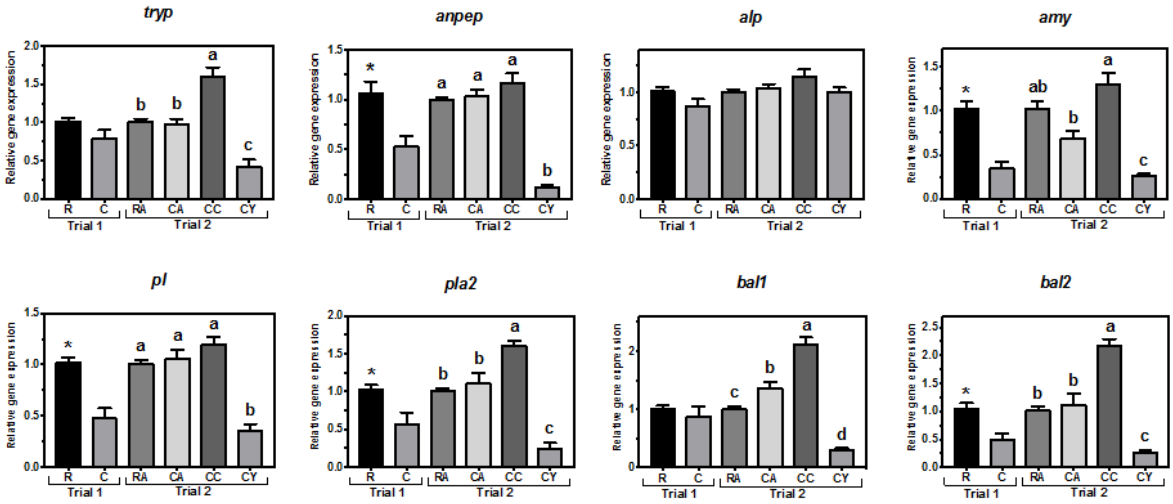

Fig.7

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Table 1. Total gross composition (% dry mass), energy content (kcal.g^{-1}), taurine (mg.g^{-1}), vitamin E (µg.g^{-1}dry mass), Se (µg.g^{-1}dry mass) and lipid class composition (total lipid %) of rotifer *Brachionus rotundiformis* (ROT) enriched with Algamac 3050®, nauplii of the copepod *Acartia tonsa* (COP) fed with the microalgae *Rhodomonas baltica, Artemia* metanauplii (ART) enriched with Algamac 3050®, and gilthead sea bream (*Sparus aurata* L.) yolk sac larvae (YSL) used to feed Atlantic bluefin tuna (*Thunnus thynnus* L.) larvae.

	ROT	COP	ART	YSL
Composition (% dry mass)				
Protein	51.3 ± 0.4^{c}	63.2 ± 2.1^{a}	53.8 ± 0.8^{c}	58,7 ± 0,4^{b}
Lipid	12.0 ± 0.5^{b}	8.8 ± 0.3^{c}	18.1 ± 0.9^{a}	18.9 ± 0.4^{a}
Carbohydrate	30.1 ± 0.6^{a}	20.4 ± 1.1^{b}	20.0 ± 0.7^{b}	13.7 ± 0.3^{c}
Ash	6.7 ± 0.3^{c}	7.6 ± 0.3^{b}	8.1 ± 0.1^{a}	8.7 ± 0.2^{a}
Energy (kcal.g^{-1})				
Protein	28.6 ± 0.7^{c}	35.3 ± 0.9^{a}	30.2 ± 0.6^{c}	33.0 ± 0.4^{b}
Lipid	11.3 ± 0.4^{b}	8.5 ± 0.3^{c}	16.9 ± 0.7^{a}	17.9 ± 0.6^{a}
Carbohydrate	12.6 ± 0.3^{a}	8.4 ± 0.3^{b}	8.4 ± 0.5^{b}	5.9 ± 0.3^{c}
Total Energy	52.5 ± 0.6^{b}	52.2 ± 0.7^{b}	55.5 ± 0.6^{a}	56.8 ± 0.5^{a}
Taurine (mg.g^{-1})	2.5 ± 0.2^{d}	4.1 ± 0.2^{c}	6.1 ± 0.2^{b}	11.0 ± 0.8^{a}
Vitamin E (µg.g^{-1})	232.3 ± 1.7^{b}	170.1 ± 6.5^{c}	78.9 ± 5.8^{d}	308.7 ± 4.6^{a}
Se (µg.g^{-1})	4.8 ± 0.2^{a}	0.4 ± 0.0^{c}	1.8 ± 0.5^{b}	1.4 ± 0.1^{b}
Lipid classes (% total lipid)				
PC	12.3 ± 0.3^{c}	16.2 ± 0.3^{b}	9.2 ± 0.2^{d}	18.7 ± 1.0^{a}
PE	13.1 ± 1.1^{b}	11.8 ± 0.2^{b}	7.7 ± 0.2^{c}	17.1 ± 0.4^{a}
TPL	41.0 ± 0.3^{c}	52.9 ± 0.7^{a}	28.6 ± 1.0^{d}	47.9 ± 0.5^{b}
Cholesterol	6.6 ± 0.3^{c}	11.9 ± 0.7^{b}	11.8 ± 0.4^{b}	21.7 ± 0.8^{a}
Triacylglycerol	40.2 ± 0.3^{b}	23.3 ± 0.3^{c}	46.3 ± 0.5^{a}	10.0 ± 0.7^{d}
TNL	59.0 ± 0.3^{b}	47.1 ± 0.7^{d}	71.4 ± 1.0^{a}	52.1 ± 0.5^{c}
TPL/TNL	0.7 ± 0.1^{ab}	1.1 ± 0.2^{a}	0.4 ± 0.1^{b}	0.9 ± 0.2^{a}

Results are means ± SD (n = 3). An SD of 0.0 implies an SD of < 0.05. Values bearing different superscript letter are significantly different (P < 0.05). TNL, total neutral lipids; PC, phosphatidylcholine; PE, phosphatidylethanolamine; TPL, total polar lipids.

Table 2. Sufficiency Index (SI) of essential amino acids (Oser, 1959) of rotifer *Brachionus rotundiformis* (ROT) enriched with Algamac 3050®, nauplii of the copepod *Acartia tonsa* (COP) fed with the microalgae *Rhodomonas baltica*, *Artemia* metenauplii (ART) enriched with Algamac 3050® and gilthead sea bream (*Sparus aurata* L.) yolk sac larvae (YSL) used to feed Atlantic bluefin tuna (*Thunnus thynnus* L.) larvae in Trials 1 and 2.

	ROT	COP	ART	YSL
Taurine	60.5	99.3	147.7	266.3
Valine	68.0	107.9	80.2	97.2
Isoleucine	78.4	970.1	835.8	947.8
Leucine	73.3	101.5	79.3	110.7
Phenylalanine	87.4	116.1	91.4	114.3
Histidine	54.1	93.7	74.6	121.9
Lysine	74.6	113.8	100.2	124.4
Arginine	79.4	127.6	110.4	127.6
Threonine	69.3	121.6	79.6	115.0
Methionine	48.5	95.9	63.5	101.1

Results are means (n = 3). Values equal or above 100 indicate sufficient amount of that amino acid in the live prey, whereas values below 100 show insufficiency for that amino acid.

Table 3. Total lipid fatty acid composition (weight %) of rotifer *Brachionus rotundiformis* (ROT) enriched with Algamac 3050®, nauplii of the copepod *Acartia tonsa* (COP) fed with the microalgae *Rhodomonas baltica*, *Artemia* metenauplii (ART) enriched with Algamac 3050 and gilthead sea bream (*Sparus aurata* L.) yolk sac larvae (YSL) used to feed Atlantic bluefin tuna (*Thunnus thynnus* L.) larvae.

	ROT	COP	ART	YSL
Fatty acid				
14:0	2.3 ± 0.1^{b}	9.8 ± 0.3^{a}	1.5 ± 0.1^{c}	2.1 ± 0.2^{b}
16:0	15.1 ± 0.4^{b}	13.0 ± 0.3^{c}	14.9 ± 0.3^{b}	18.7 ± 0.3^{a}
18:0	4.8 ± 0.2^{a}	3.0 ± 0.1^{b}	5.1 ± 0.1^{a}	4.6 ± 0.4^{ab}
Total saturated[1]	23.9 ± 0.3^{b}	27.8 ± 0.8^{a}	22.4 ± 0.4^{b}	26.0 ± 0.4^{a}
16:1n-7	1.9 ± 0.2^{c}	3.3 ± 0.2^{b}	2.3 ± 0.1^{c}	4.8 ± 0.2^{a}
18:1n-9	2.2 ± 0.1^{c}	4.2 ± 0.1^{b}	17.2 ± 0.2^{a}	15.9 ± 0.4^{a}
18:1n-7	1.6 ± 0.2^{c}	2.0 ± 0.1^{bc}	6.5 ± 0.1^{a}	2.8 ± 0.2^{b}
20:1n-9	0.9 ± 0.2^{a}	0.3 ± 0.0^{c}	0.6 ± 0.1^{a}	0.6 ± 0.1^{a}
Total monoenes[2]	12.6 ± 0.4^{b}	11.5 ± 0.3^{b}	27.5 ± 0.6^{a}	26.3 ± 0.4^{a}
C16 PUFA	6.3 ± 0.3^{a}	3.1 ± 0.1^{b}	0.8 ± 0.2^{c}	0.9 ± 0.2^{c}
18:2n-6	16.3 ± 0.7^{a}	5.1 ± 0.3^{c}	4.3 ± 0.1^{d}	7.1 ± 0.3^{b}
20:4n-6	1.1 ± 0.1^{c}	1.4 ± 0.1^{b}	1.8 ± 0.2^{a}	1.6 ± 0.2^{ab}
22:5n-6	2.9 ± 0.4^{a}	3.3 ± 0.2^{a}	3.2 ± 0.2^{a}	0.3 ± 0.1^{b}
Total n-6PUFA[3]	24.8 ± 0.7^{a}	13.6 ± 0.3^{b}	10.0 ± 0.3^{bc}	9.7 ± 0.6^{c}
18:3n-3	3.9 ± 0.2^{b}	4.7 ± 0.2^{b}	23.0 ± 0.2^{a}	0.9 ± 0.1^{c}
18:4n-3	0.2 ± 0.0^{c}	4.8 ± 0.3^{a}	2.8 ± 0.1^{b}	0.6 ± 0.1^{c}
20:4n-3	1.0 ± 0.1^{a}	0.6 ± 0.1^{b}	0.6 ± 0.1^{b}	0.6 ± 0.1^{b}
20:5n-3	5.1 ± 0.3^{b}	4.4 ± 0.3^{bc}	4.1 ± 0.3^{c}	7.2 ± 0.2^{a}
22:5n-3	3.2 ± 0.4^{a}	0.2 ± 0.0^{b}	0.2 ± 0.0^{b}	2.6 ± 0.2^{a}
22:6n-3	14.1 ± 0.6^{b}	27.0 ± 0.9^{a}	8.0 ± 0.2^{c}	25.1 ± 0.9^{a}
Total n-3PUFA[4]	32.9 ± 0.9^{c}	43.9 ± 1.6^{a}	39.3 ± 0.3^{b}	37.2 ± 0.8^{b}
Total PUFA	57.7 ± 1.6^{a}	57.6 ± 1.4^{a}	50.1 ± 0.5^{b}	47.7 ± 0.9^{b}
n-3/n-6	1.3 ± 0.1^{c}	3.2 ± 0.2^{b}	3.9 ± 0.1^{a}	3.8 ± 0.2^{a}
DHA/EPA	2.8 ± 0.3^{c}	6.1 ± 0.7^{a}	1.9 ± 0.1^{d}	3.5 ± 0.2^{b}

Results are means ± SD (n = 3). An SD of 0.0 implies an SD of < 0.05. Values bearing different superscript letters 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 4. Growth performance and survival of ABT larvae in Trial 1 (13 dah) and Trial 2 (18 dah). Total length (mm), total weight as live mass or dry mass per larvae (mg), dry mass (%) and survival rate (%) of ABT larvae 13 days after hatch fed on rotifer *B. rotundiformis* enriched with Algamac 3050® (treatment R) or *A. tonsa* copepod nauplii (treatment C), and ABT larvae 18 days after hatch that were fed rotifer *B. rotundiformis* and then *Artemia* metanauplii enriched with Algamac 3050® (RA), *A. tonsa* copepod nauplii and then *Artemia* enriched with Algamac 3050® (treatment CA), *A. tonsa* copepod nauplii and then followed with *A. tonsa* nauplii and copepodites (treatment CC), and *A. tonsa* nauplii and then gilthead sea bream (*S. aurata*) yolk sac larvae (treatment CY).

	Trial 1(13 dah ABT larvae)		Trial 2 (18 dah ABT larvae)			
Dietary treatments	R	C	RA	CA	CC	CY
Total length (mm)	6.8 ± 0.3	7.6 ± 0.4 *	8.2 ± 0.4^{b}	9.7 ± 0.5^{ab}	8.9 ± 0.6^{b}	10.3 ± 0.5^{a}
Total weight (mg/larvae live mass)	4.3 ± 0.2	8.2 ± 0.5 *	9.7 ± 0.5^{c}	11.6 ± 0.5^{b}	11.4 ± 0.6^{b}	24.3 ± 0.9^{a}
Total weight (mg/larvae dry mass)	0.6 ± 0.1	1.1 ± 0.2 *	1.2 ± 0.3^{c}	2.5 ± 0.4^{b}	1.9 ± 0.3^{bc}	3.6 ± 0.4^{a}
Dry mass (%)	14.4 ± 0.8	12.8 ± 0.9	12.2 ± 0.7^{d}	21.2 ± 1.1^{a}	16.9 ± 0.9^{b}	14.9 ± 0.8^{c}
Flexion Index (%)	35.8 ± 3.9	54.5 ± 5.0 *	100	100	100	100
Survival at 13 dah (%)	13.9 ± 11.8	8.2 ± 3.8				
Survival at 18 dah (%)			2.4	2.1	2.3	3.6
Survival (13 -18 dah, 5 days) (%)			17.6	19.5	21.7	32.0

Results are means ± SD (n =4 in Trial 1 and n = 20 in Trial 2 for total length, total weight, dry mass and flexion index; n = 4 per treatment for survival in Trial A). An SD of 0.0 implies an SD of < 0.05. Values bearing an asterisk (*) between samples of 13 dah ABT larvae are significantly different ($P < 0.05$). Values of 18 dah ABT larvae bearing different superscript letters are significantly different ($P < 0.05$).

Table 5. Total lipid content (% of live and dry mass) and lipid class composition (total lipid %) of Atlantic bluefin tuna (*Thunnus thynnus* L.) larvae (ABT) 13 days after hatch fed with rotifer *Brachionus rotundiformis* enriched with Algamac 3050® (R), *Acartia tonsa* copepod nauplii (C), and ABT larvae 18 days after hatch that had been fed with rotifer *B. rotundiformis* and then *Artemia* metanauplii enriched with Algamac 3050® (RA), *Acartia* copepod nauplii and then *Artemia* enriched with Algamac 3050 ® (CA), *Acartia* copepod nauplii and then followed with *Acartia* nauplii and copepodites (CC), and *Acartia* nauplii and then gilthead sea bream (*Sparus aurata* L.) yolk sac larvae (CY).

	13 dah ABT larvae		18 dah ABT larvae			
Dietary treatments	R	C	RA	CA	CC	CY
Total lipid (% live mass)	1.3 ± 0.1	1.1 ± 0.1	1.3 ± 0.1	1.7 ± 0.2	1.5 ± 0.2	1.8 ± 0.3
Total lipid (% dry mass)	9.1 ± 0.6	8.0 ± 0.7	10.5 ± 0.9^{a}	7.4 ± 0.6^{b}	8.3 ± 0.5^{b}	12.1 ± 0.9^{a}
Lipid classes (total lipid %)						
Phosphatidylcholine	21.2 ± 1.1	22.2 ± 1.2	21.6 ± 1.0	21.6 ± 01.4	23.2 ± 0.7	20.6 ± 0.9
Phosphatidylethanolamine	14.5 ± 1.2	14.6 ± 0.8	14.7 ± 0.8^{a}	14.0 ± 0.5^{a}	14.6 ± 0.3^{a}	11.4 ± 0.3^{b}
Phosphatidylserine	6.6 ± 0.2	7.2 ± 0.7	5.8 ± 0.4^{b}	6.5 ± 0.3^{ab}	7.8 ± 0.4^{a}	4.8 ± 0.2^{c}
Phosphatidylinositol	4.6 ± 0.3	3.7 ± 0.2 *	4.2 ± 0.6	4.5 ± 0.5	4.6 ± 0.2	4.0 ± 0.5
Sphingomyelin	2.2 ± 0.1	3.8 ± 0.4 *	2.1 ± 0.1^{c}	3.3 ± 0.1^{b}	4.0 ± 0.2^{a}	2.5 ± 0.2^{bc}
Phosphatidic acid/cardiolipin	3.3 ± 0.2	3.3 ± 0.1	3.2 ± 0.3^{a}	2.9 ± 0.7^{ab}	2.5 ± 0.2^{b}	2.5 ± 0.3^{b}
Lysophosphatidylcholine	0.4 ± 0.1	0.6 ± 0.2	0.4 ± 0.1	0.5 ± 0.1	0.5 ± 0.1	0.4 ± 0.0
Pigmented material	3 .6 ± 0.8	3.1 ± 0.3	2.6 ± 0.2^{ab}	3.3 ± 0.5^{a}	2.5 ± 0.3^{ab}	2.0 ± 0.1^{b}
Total polar lipids	56.5 ± 1.7	58.5 ± 3.4	54.6 ± 2.1^{b}	56.4 ± 1.8^{b}	59.4 ± 0.6^{a}	$48.2 \pm 3.2b^{c}$
Cholesterol/sterols	18.1 ± 0.6	17.5 ± 1.4	16.4 ± 1.1^{ab}	16.1 ± 1.8^{ab}	18.5 ± 0.6^{a}	14.6 ± 2.1^{b}
Free fatty acids	4.9 ± 1.1	6.2 ± 0.1	5.6 ± 0.7	5.1 ± 1.1	6.0 ± 0.6	5.7 ± 0.8
Diacylglycerol	2.1 ± 0.1	1.8 ± 0.2	1.4 ± 0.1^{b}	1.8 ± 0.5^{ab}	2.0 ± 0.3^{a}	1.9 ± 0.2^{a}
Triacylglycerol	14.5 ± 0.7	10.7 ± 1.0 *	19.8 ± 1.2^{a}	15.4 ± 0.5^{b}	11.5 ± 0.3^{c}	22.4 ± 1.2^{a}
Wax/Sterol esters	3.9 ± 1.0	5.3 ± 0.7 *	2.2 ± 0.4^{c}	5.4 ± 1.3^{b}	2.7 ± 0.4^{c}	7.2 ± 0.9^{a}
Total neutral lipids	43.5 ± 1.7	41.5 ± 2.4	45.4 ± 1.1^{b}	43.7 ± 1.7^{bc}	40.6 ± 0.8^{c}	51.8 ± 2.3^{a}

Results are means ± SD (n = 4 in Trial 1 and n = 3 in Trial 2). An SD of 0.0 implies an SD of < 0.05. Values bearing an asterisk (*) between samples of 13 dah ABT larvae (R and C) are significantly different ($P < 0.05$). Values of 18 dah ABT larvae (samples RA, CA, CC and CY) bearing different superscript letters are significantly different ($P < 0.05$).

Table 6. Total lipid fatty acid composition (weight %) of Atlantic bluefin tuna (*T. thynnus* L.) larvae 13 days after hatch fed with rotifer *B. rotundiformis* enriched with Algamac 3050 ® (R), *A. tonsa* copepod nauplii (C), and bluefin tuna larvae 18 days after hatch fed with rotifer *B. rotundiformis* and then *Artemia* metanauplii enriched with Algamac 3050® (RA), *A. tonsa* copepod nauplii and then *Artemia* enriched with Algamac 3050® (CA), *A. tonsa* copepod nauplii and then followed with *A. tonsa* nauplii and copepodites (CC), and *A. tonsa* nauplii and then gilthead sea bream (*S. aurata*) yolk sac larvae (CY).

	13 dah ABT larvae		18 dah ABT larvae			
Dietary treatments	R	C	RA	CA	CC	CY
Fatty acid (weight %)						
14:0	1.1 ± 0.2	1.4 ± 0.2	0.4 ± 0.1^{c}	0.8 ± 0.2^{b}	1.6 ± 0.2^{a}	0.4 ± 0.1^{c}
16:0	24.9 ± 0.9	21.2 ± 0.6 *	17.7 ± 0.7^{b}	16.7 ± 0.5^{b}	20.5 ± 0.2^{a}	21.8 ± 0.3^{a}
18:0	11.4 ± 0.8	12.4 ± 0.4	12.9 ± 0.5^{a}	13.0 ± 0.4^{a}	11.4 ± 0.1^{b}	12.0 ± 0.2^{ab}
Total SFA[1]	39.5 ± 1.5	37.5 ± 1.2	33.7 ± 1.3^{b}	33.1 ± 0.3^{b}	36.9 ± 0.9^{a}	36.3 ± 0.5^{a}
16:1n-7	5.5 ± 0.2	2.2 ± 0.2 *	1.8 ± 0.1^{b}	1.8 ± 0.1^{b}	2.4 ± 0.3^{a}	2.1 ± 0.2^{ab}
18:1n-9	6.8 ± 0.2	6.9 ± 0.2	10.9 ± 0.6^{b}	10.8 ± 0.2^{b}	7.5 ± 0.5^{c}	13.2 ± 0.8^{a}
18:1n-7	3.1 ± 0.2	2.1 ± 0.1 *	4.8 ± 0.4^{a}	4.6 ± 0.5^{a}	2.0 ± 0.3^{c}	2.9 ± 0.3^{b}
20:1n-9	0.7 ± 0.2	1.1 ± 0.3	0.7 ± 0.1	0.4 ± 0.2	0.5 ± 0.1	0.6 ± 0.1
Total MUFA[2]	20.0 ± 0.6	14.0 ± 0.4 *	20.2 ± 0.8^{a}	19.3 ± 0.4^{a}	14.2 ± 0.5^{b}	20.7 ± 1.4^{a}
C16 PUFA	2.1 ± 0.1	3.5 ± 0.5 *	3.1 ± 0.2^{a}	3.0 ± 0.2^{a}	3.5 ± 0.3^{a}	2.1 ± 0.1^{b}
18:2n-6	4.5 ± 0.3	3.0 ± 0.1 *	5.6 ± 0.3^{a}	4.8 ± 0.3^{ab}	4.6 ± 0.7^{ab}	4.1 ± 0.5^{b}
20:4n-6	5.1 ± 0.2	1.0 ± 0.1 *	1.6 ± 0.1^{b}	1.9 ± 0.2^{a}	1.1 ± 0.0^{b}	1.2 ± 0.1^{b}
22:5n-6	4.3 ± 0.3	3.1 ± 0.2 *	2.7 ± 0.2^{b}	3.0 ± 0.2^{ab}	3.6 ± 0.4^{a}	1.1 ± 0.1^{c}
Total n-6 PUFA[3]	17.5 ± 0.6	10.1 ± 0.8 *	14.9 ± 0.6^{a}	12.1 ± 0.5^{b}	12.5 ± 0.3^{b}	9.8 ± 0.2^{c}
18:3n-3	0.3 ± 0.1	0.6 ± 0.1 *	6.9 ± 0.3^{a}	5.7 ± 0.4^{b}	1.8 ± 0.3^{c}	0.4 ± 0.1^{d}
18:4n-3	0.5 ± 0.1	1.5 ± 0.3 *	1.0 ± 0.2^{b}	1.8 ± 0.3^{a}	1.4 ± 0.3^{ab}	0.2 ± 0.0^{c}
20:4n-3	0.6 ± 0.1	0.6 ± 0.0	0.8 ± 0.2^{a}	0.8 ± 0.1^{a}	0.6 ± 0.1^{ab}	0.5 ± 0.1^{b}
20:5n-3	10.2 ± 0.8	3.1 ± 0.3 *	4.7 ± 0.2^{a}	3.4 ± 0.3^{b}	2.1 ± 0.2^{c}	4.4 ± 0.2^{a}
22:5n-3	0.3 ± 0.0	0.4 ± 0.1	2.3 ± 0.4^{a}	0.3 ± 0.0^{b}	0.4 ± 0.1^{b}	1.8 ± 0.3^{a}
22:6n-3	5.9 ± 0.4	25.3 ± 0.9 *	10.0 ± 0.4^{c}	15.8 ± 0.4^{b}	24.1 ± 1.0^{a}	22.2 ± 1.5^{a}
Total n-3 PUFA[4]	19.5 ± 0.6	34.2 ± 1.8 *	28.2 ± 1.1^{b}	30.9 ± 1.4^{ab}	33.2 ± 1.3^{a}	30.7 ± 1.6^{ab}
Total PUFA	39.1 ± 1.7	47.5 ± 1.6 *	43.1 ± 1.6^{ab}	43.0 ± 0.7^{ab}	45.7 ± 1.0^{a}	40.5 ± 1.6^{b}
DHA/EPA	0.6 ± 0.1	8.2 ± 0.4 *	2.1 ± 0.1^{b}	4.6 ± 0.3^{a}	11.3 ± 0.7^{b}	5.1 ± 0.2^{a}
n-3/n-6	1.1 ± 0.2	3.4 ± 0.4 *	1.9 ± 0.3^{c}	2.6 ± 0.2^{b}	2.6 ± 0.2^{b}	3.1 ± 0.2^{a}
Unknown	1.4 ± 0.2	1.0 ± 0.2	3.0 ± 0.3^{b}	4.6 ± 0.2^{a}	3.2 ± 0.4^{b}	2.5 ± 0.4^{c}

Results are means ± SD (n = 4 in Trial 1 and n = 3 in Trial 2). An SD of 0.0 implies an SD of < 0.05. Values bearing an asterisk (*) between samples of 13 dah ABT larvae (a and b) are significantly different ($P < 0.05$). Values of 18 dah ABT larvae (samples c to f) bearing different superscript letters 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; MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid; SFA, saturated fatty acid.

Table 7. Relative abundance (δ) of $^{15}N/^{14}N$ and $^{13}C/^{12}C$ (‰) and C:N ratio of rotifer *Brachionus rotundiformis* enriched with Algamac 3050® (R), nauplii of the copepod *Acartia tonsa* fed on the microalgae *Rhodomonas baltica* (C) and 13 dah Atlantic bluefin tuna (*Thunnus thynnus* L.) larvae from Trial 1 fed on *B. rotundiformis* (ABT R) and *A. tonsa* nauplii (ABT C), and isotopic enrichment (Δ) of ^{15}N and ^{13}C (‰) in ABT larvae in relation to its prey, rotifers and copepods.

C	R (e)	C	ABT R	ABT C
$\delta^{15}N$ (‰)	-0.81 ± 0.02	4.39 ± 0.04 *	0.65 ± 0.05	7.18 ± 0.01 *
$\delta^{13}C$ (‰)	-11.09 ± 0.02	-20.17 ± 0.06 *	-10.61 ± 0.17	-17.11 ± 0.01 *
C:N	4.64 ± 0.03	3.74 ± 0.02 *	3.92 ± 0.01	3.67 ± 0.03 *
$\Delta^{15}N$ (‰)			1.46 ± 0.03	2.79 ± 0.02 *
$\Delta^{13}C$ (‰)			0.48 ± 0.08	3.06 ± 0.04 *

Results are means ± SD ((n = 4 in Trial 1 and n = 3 in Trial 2). An SD of 0.0 implies an SD of < 0.05. Values between live prey, or ABT larvae fed on these live prey, bearing an asterisk (*), are significantly different ($P < 0.05$).

Supplementary Table 1. Rearing conditions for feeding trials of Atlantic bluefin tuna (*Thunnus thynnus* L.) larvae.

	Trial A (0-13dph)	Trial B (13-18dph)
Age of initial fish (days after hatching)	just hatched larvae	13 dph
Experimental period	13 days	5 days
Tank volume (m^3)	1.4	1.4
Number of tanks per treatment	4	1
Number of fish (ind./tank)	11.900 eggs	600
Initial ABT larval density (larvae L^{-1})	8.5	0.4
Live preys density (individuals mL^{-1})		
Rotifers (*Brachionus rotundiformis*)	10	-(5)
Copepods (*Acartia tonsa*)	10	2.5
Artemia	-	0.5
Yolk sac larvae (*Sparus aurata*)	-	0.1
Water temperature (ºC)	24.8±0.4ºC	22.8±0,6ºC
Dissolved oxygen (mg/l)	6.41±0.06	6.56±0.19
Photoperiod (hL:hD)	14:10	14:10
Exchange rate of sea water (% tank volume/day)	100-200%	100-200%

Supplementary Table 2. 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 3. Total amino acid content, including taurine (mg/g dry mass) of 1 day post hatch ABT yolk sac larvae (ABT), rotifers *B. rotundiformis* enriched with Algamac 3050 Bio Marine® (ROT), *Artemia* metanauplii enriched with Algamac 3050 Bio Marine® (ART), 3 days post hatch nauplii of the copepod *Acartia tonsa* fed *Rhodomonas baltica* (COP) and gilthead sea bream (*Sparus aurata* L.) yolk sac larvae (YSL).

	(ABT)	(ROT)	(ART)	(COP)	(YSL)
Taurine	4.1 ± 0.1^{c}	2.5 ± 0.2^{d}	6.1 ± 0.2^{b}	4.1 ± 0.2^{c}	11.0 ± 0.8^{a}
EAA					
Valine	33.5 ± 0.3^{ab}	22.8 ± 0.8^{c}	26.9 ± 0.6^{bc}	36.2 ± 5.6^{a}	32.6 ± 1.2^{ab}
Isoleucine	2.7 ± 0.2^{c}	2.1 ± 0.1^{c}	22.4 ± 2.3^{b}	26.0 ± 1.3^{a}	25.4 ± 0.6^{ab}
Leucine	41.4 ± 0.2^{b}	30.3 ± 0.6^{c}	32.8 ± 1.1^{c}	42.0 ± 2.5^{b}	45.8 ± 1.3^{a}
Phenylalanine	22.3 ± 0.7^{bc}	19.5 ± 0.5^{c}	20.4 ± 0.6^{b}	25.9 ± 1.0^{a}	25.5 ± 0.7^{a}
Histidine	13.1 ± 0.2^{b}	7.1 ± 0.2^{d}	9.8 ± 0.3^{c}	12.3 ± 1.2^{b}	16.1 ± 0.1^{a}
Lysine	37.5 ± 0.2^{b}	28.0 ± 0.6^{c}	37.6 ± 0.7^{b}	42.7 ± 5.3^{ab}	46.7 ± 0.8^{a}
Arginine	27.8 ± 0.2^{b}	22.1 ± 0.4^{c}	30.7 ± 1.2^{b}	35.5 ± 2.2^{a}	35.5 ± 1.3^{a}
Threonine	21.2 ± 0.4^{b}	14.7 ± 0.6^{c}	16.9 ± 0.4^{c}	25.8 ± 1.8^{a}	24.4 ± 0.8^{a}
Methionine	17.3 ± 0.3^{a}	8.4 ± 0.1^{c}	11.0 ± 0.6^{b}	16.6 ± 1.7^{a}	17.5 ± 0.9^{a}
Total EAA	221.1 ± 2.6^{c}	157.5 ± 4.1^{d}	214.6 ± 2.8^{c}	267.1 ± 2.4^{b}	280.4 ± 4.6^{a}
NEAA					
Aspartic acid	35.8 ± 0.6^{c}	38.1 ± 0.8^{c}	39.4 ± 0.7^{bc}	54.5 ± 3.6^{a}	44.1 ± 1.3^{b}
Glutamic acid	56.1 ± 0.9^{c}	49.0 ± 1.4^{d}	57.3 ± 2.8^{c}	76.5 ± 4.5^{a}	67.5 ± 1.7^{b}
Serine	22.4 ± 0.5^{a}	16.1 ± 0.4^{b}	15.3 ± 0.5^{b}	23.5 ± 0.6^{a}	22.3 ± 0.9^{a}
Proline	19.6 ± 0.5^{b}	19.7 ± 0.7^{b}	21.1 ± 0.8^{b}	34.2 ± 1.7^{a}	21.7 ± 0.8^{b}
Glycine	18.6 ± 0.4^{c}	17.2 ± 0.4^{c}	24.0 ± 0.9^{b}	38.6 ± 1.8^{a}	26.0 ± 0.8^{b}
Alanine	34.2 ± 0.5^{b}	17.1 ± 0.5^{d}	23.2 ± 0.8^{c}	36.9 ± 1.1^{a}	33.8 ± 0.9^{b}
Tyrosine	20.8 ± 0.8^{c}	15.6 ± 0.7^{d}	13.0 ± 0.6^{d}	30.6 ± 2.6^{a}	26.4 ± 0.9^{b}
Cysteine	2.7 ± 0.4^{d}	4.0 ± 0.1^{c}	3.7 ± 0.2^{cd}	9.6 ± 0.6^{a}	6.1 ± 0.7^{b}
Total NEAA	210.2 ± 2.3^{c}	176.8 ± 1.6^{e}	197.0 ± 2.4^{d}	304.4 ± 2.7^{a}	247.9 ± 2.9^{b}
Total AA	431.2 ± 6.8^{c}	334.3 ± 9.1^{e}	411.6 ± 4.5^{d}	571.5 ± 7.3^{a}	528.3 ± 5.7^{b}
EAA/NEAA	1.05	0.48	1.09	0.52	1.13

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 ($P<0.05$). AA, amino acid; EAA, essential amino acid; NEAA, non essential amino acid.

Supplementary Table 4. Total lipid fatty acid content (µg fatty acid/mg dry mass) of rotifer *Brachionus rotundiformis* enriched with Algamac 3050 ® (ROT), nauplii of the copepod *Acartia tonsa* fed with the microalgae *Rhodomonas baltica* (COP), *Artemia* metenauplii enriched with Algamac 3050 (ART) and sea bream (*Sparus aurata* L.) yolk sac larvae (YSL) used to feed Atlantic bluefin tuna (*Thunnus thynnus* L.) larvae.

Live prey	ROT (e)	COP	ART	YSL
Fatty acid				
14:0	1.8 ± 0.2^{c}	5.1 ± 0.3^{a}	1.4 ± 0.1^{c}	2.6 ± 0.2^{b}
16:0	11.8 ± 0.9^{c}	6.8 ± 0.3^{d}	13.5 ± 0.4^{b}	22.3 ± 0.6^{a}
18:0	3.7 ± 0.3^{c}	1.6 ± 0.1^{d}	4.6 ± 0.2^{b}	5.5 ± 0.2^{a}
Total saturated[1]	18.6 ± 0.6^{b}	14.4 ± 0.8^{c}	20.6 ± 0.5^{b}	31.0 ± 1.1^{a}
16:1n-7	1.5 ± 0.2^{b}	1.7 ± 0.1^{b}	2.1 ± 0.2^{b}	5.8 ± 0.2^{a}
18:1n-9	1.7 ± 0.3^{c}	2.2 ± 0.2^{c}	15.5 ± 0.5^{b}	19.0 ± 0.7^{a}
18:1n-7	1.3 ± 0.2^{c}	1.1 ± 0.1^{c}	5.9 ± 0.1^{a}	3.3 ± 0.1^{b}
20:1n-9	0.7 ± 0.1^{a}	0.2 ± 0.0^{c}	0.5 ± 0.1^{b}	0.7 ± 0.1^{a}
Total monoenes[2]	9.8 ± 0.7^{c}	5.9 ± 0.3^{d}	24.9 ± 0.6^{b}	31.4 ± 1.2^{a}
C16 PUFA	4.9 ± 0.7^{a}	1.6 ± 0.1^{b}	0.7 ± 0.1^{c}	1.0 ± 0.1^{c}
18:2n-6	12.7 ± 1.0^{a}	2.6 ± 0.3^{d}	3.9 ± 0.2^{c}	8.5 ± 0.3^{b}
20:4n-6	0.8 ± 0.1^{b}	0.7 ± 0.1^{b}	1.6 ± 0.2^{a}	1.9 ± 0.2^{a}
22:5n-6	2.3 ± 0.4^{a}	1.7 ± 0.2^{b}	2.9 ± 0.2^{a}	0.3 ± 0.1^{c}
Total n-6PUFA[3]	19.4 ± 0.9^{a}	7.1 ± 0.3^{d}	9.0 ± 0.3^{c}	11.6 ± 0.5^{b}
18:3n-3	3.0 ± 0.5^{b}	2.4 ± 0.2^{b}	20.8 ± 0.4^{a}	1.1 ± 0.1^{c}
18:4n-3	0.2 ± 0.0^{c}	2.5 ± 0.3^{a}	2.6 ± 0.1^{a}	0.7 ± 0.1^{b}
20:4n-3	0.8 ± 0.1^{a}	0.3 ± 0.1^{b}	0.6 ± 0.1^{a}	0.7 ± 0.1^{a}
20:5n-3	3.9 ± 0.6^{b}	2.3 ± 0.2^{c}	3.7 ± 0.3^{b}	8.6 ± 0.4^{a}
22:5n-3	2.5 ± 0.4^{b}	0.1 ± 0.0^{c}	0.2 ± 0.0^{c}	3.2 ± 0.1^{a}
22:6n-3	10.9 ± 0.8^{b}	14.0 ± 0.4^{b}	7.2 ± 0.4^{c}	30.0 ± 1.3^{a}
Total n-3PUFA[4]	25.7 ± 1.9^{c}	22.8 ± 0.5^{d}	35.6 ± 0.6^{b}	44.4 ± 2.0^{a}
Total PUFA	50.1 ± 1.9^{b}	31.5 ± 1.4^{d}	45.3 ± 0.7^{c}	57.0 ± 2.5^{a}
n-3/n-6	1.3 ± 0.1^{b}	3.2 ± 0.2^{a}	3.9 ± 0.1^{a}	3.8 ± 0.4^{a}
DHA/EPA	2.8 ± 0.3^{c}	6.1 ± 0.7^{a}	1.9 ± 0.1^{d}	3.5 ± 0.2^{b}

Results are means ± SD (n = 3). An SD of 0.0 implies an SD of < 0.05. 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.