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

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21 **Abstract**

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

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

47 *Abbreviations:* ABT, Atlantic bluefin tuna; *aco*, acyl coA oxidase; *alp*, alkaline phosphatase; *amy*,
48 amylase; *anpep*, amino peptidase; ARA, arachidonic acid (20:4n-6); *ball*, bile salt activated lipase
49 1; *bal2*, bile salt activated lipase 2; C, free cholesterol; *cat*, catalase; *cpt1*, carnitine palmitoyl
50 transferase I; dah, days after hatch; DHA, docosahexaenoic acid (22:6n-3); EPA, eicosapentaenoic
51 acid (20:5n-3); *elovl5*, fatty acyl elongase 5; *fabp2*, fatty acid binding protein 2 (intestinal); *fabp4*,
52 fatty acid binding protein 4 (adipocyte); *fabp7*, fatty acid binding protein 7 (brain-type); *fads2d6*,
53 delta-6 fatty acyl desaturase; FAME, fatty acid methyl ester; *fas*, fatty acid synthase; *gpx1*,
54 glutathione peroxidase 1; *gpx4*, glutathione peroxidase 4; *hmgcl*, 3-hydroxy-3-methylglutaryl-CoA
55 lyase; HPLC, high-pressure liquid chromatography; HPTLC, high performance thin-layer
56 chromatography; LC-PUFA, long-chain polyunsaturated fatty acid; *lpl*, lipoprotein lipase; *lxr*, liver
57 X receptor; *myhc*, myosin heavy chain; PC, phosphatidylcholine; PE, phosphatidylethanolamine;
58 PI, phosphatidylinositol; *pl*, pancreatic lipase; *pla2*, phospholipase A₂; *ppara*, peroxisome
59 proliferator-activated receptor alpha; *ppary*, peroxisome proliferator-activated receptor gamma; PS,
60 phosphatidylserine; qPCR, quantitative real-time PCR; *rxr*, retinoid X receptor; SE, steryl ester;
61 *sod*, superoxide dismutase; *srebp1*, sterol regulatory element-binding protein 1; *srebp2*, sterol
62 regulatory element-binding protein 2; TAG, triacylglycerol; *tropo*, tropomyosin; *tryp*, trypsin.

63

64 **1. Introduction**

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

81 In fish, lipids and their constituent fatty acids (FA), play key roles in promoting and/or
82 maintaining optimum growth, survival, feed efficiency, health, neural and visual development, and
83 response to stressors in addition to generally being the main energy source (Sargent et al., 1989;
84 2002; Tocher, 2003; 2010). Among the lipids and their constituents, phospholipids and long-chain
85 polyunsaturated fatty acids (LC-PUFA) are particularly important due to their critical roles in the
86 physiological processes above mentioned. Appropriate uptake and accumulation of lipids improve
87 growth and survival of all fish but are particularly important in highly active migratory predator fish
88 species such as tuna (Mourente and Tocher, 2003; 2009). Additionally, omega 3 (n-3) LC-PUFA,
89 such as eicosapentaenoic acid (EPA; 20:5n-3) and docosahexaenoic acid (DHA; 22:6n-3), are

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

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

106 However, not only lipids and essential fatty acids, but other nutrients such as antioxidants
107 (vitamin E, vitamin C and Se) and taurine, have been proposed as essential components for fast
108 growing marine fish species, particularly in tuna which cannot synthesize these compounds
109 (Yokohama et al., 2001; Waagbø, 2010; NRC, 2011; Izquierdo and Betancor, 2015; Katagiri et al.,
110 2016). Indeed, growth in fish is primarily due to protein deposition with most marine fish larvae
111 having a high requirement for essential amino acids (Rønnestad et al., 2003). Additionally, fast
112 growth in larvae requires significant amounts of pro-oxidants such as n-3 LC-PUFA to be included
113 in live prey during larval stages and in feeds for juvenile fish. Moreover, high culture temperature
114 conditions (about 28 °C) and strong aeration/oxygenation in live food enrichment protocols and
115 rearing tanks, promotes high pro-oxidative conditions. As a consequence, during early

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

126 Our overarching aim is to gain knowledge to better understand the molecular basis of ABT
127 larval nutrition during first feeding. In order to achieve this goal, the effects of four enrichment
128 protocols for rotifer *B. rotundiformis* as well as copepod (*Acartia*) nauplii on survival, growth
129 performance and development, and expression of key genes of lipid metabolism, antioxidant
130 defence and digestive capabilities were investigated in early ABT larvae at the outset of exogenous
131 feeding period. Growth performance was evaluated by biometry (total length and total dry mass),
132 and development by notochord flexion index, as well as the expression of myosin heavy chain
133 (*myhc*) and tropomyosin (*tropo*) genes, both implicated in myogenesis (Johnston et al., 2011). Key
134 genes involved in major lipid pathways including fatty acid and LC-PUFA biosynthesis (*fas*, *fads2*
135 and *elovl5*), lipid transport (*fabp2*, 4 and 7), deposition (*lpl*) and β -oxidation (*cpt1* and *aco*) and
136 their control and regulation (transcription factors *ppara*, *ppar γ* , *lxr*, *rxr*, *srebl1* and 2), were
137 evaluated in ABT larvae fed different live prey. Additionally, ABT antioxidant protection status
138 was evaluated by determining the expression of genes encoding antioxidant enzymes superoxide
139 dismutase (*sod*), catalase (*cat*) and glutathione peroxidase (*gpx1* and 4). Whereas digestive
140 capabilities were assessed by the relative expression of the genes codifying for the enzymes trypsin
141 (*tryp*), aminopeptidase (*anpep*), alkaline phosphatase (*alp*), amylase (*amy*), pancreatic lipase (*pl*),

142 bile salt-activated-lipases (*ball* and *bal2*) and phospholipase A₂ (*pla2*).

143

144 **2. Materials and Methods**

145

146 *2.1 Atlantic bluefin tuna larvae rearing conditions*

147 The ABT eggs used in this study were obtained in June 2016 from ABT broodstock fish
148 maintained in captivity in a floating net cage located at El Gorguel, off the Cartagena coast, SE
149 Spain. Captive-reared ABT broodstock fish spawned naturally and spontaneously and floating eggs
150 were collected inside the cage by means of a net of 500 µm mesh screen size. A 1.5 m polyvinyl
151 sheet was also placed around the inside of the cage to avoid eggs drifting away from the cage by
152 means of currents and/or waves. Collected eggs were transported in a 500 L plastic tank supplied
153 with pure oxygen to the Spanish Institute of Oceanography (IEO) Planta Experimental de Cultivos
154 Marinos (Puerto de Mazarrón, Murcia, Spain) aquaculture facilities and placed in 100 L tanks with
155 gentle oxygenation and flow-through sterilized seawater. After 1 h, aeration and water flow were
156 stopped to separate buoyant (viable) from non-buoyant (non-viable) eggs. After washing and
157 counting, the fertilized eggs were incubated in 1500 L cylindrical tanks at a density of 10 eggs L⁻¹.
158 Incubation was carried out at 25 - 26 °C, 37 ‰ salinity and continuous photoperiod, with a light
159 intensity of 1000 lux. An upwelling flow-through with gentle aeration was employed in order to
160 maintain oxygen levels near to saturation. Larvae hatched approximately 32 h after fertilization,
161 with a hatching rate of almost 90 %, and were fed with enriched rotifers or copepod nauplii from 2
162 dah. A mixture of the microalgae *Isochrysis* sp. (T-Iso) and *Chlorella* (V12 DHA-enriched, Pacific
163 Trading Co., Japan) were added to tanks at a density of 2 - 3 x10⁵ cells mL⁻¹ as green water. During
164 the trial, photoperiod was maintained at 16 h / 8 h light/dark (light intensity about 500 lux),
165 temperature ranged between 24 - 26 °C and daily water renewal was 50 -70 %. Incoming seawater
166 was filtered at 10 µm and UV sterilized. An upwelling current was created to avoid larvae sinking
167 (mainly at night) and maintain oxygen level (Ortega, 2015; De la Gándara et al., 2016).

168

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

170

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

178

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

180

181 S-type rotifers *B. rotundiformis* were continuously cultured with commercial DHA enriched
182 algal paste (Chlorella V-12; Chlorella Industry, Kyushu, Japan), at a concentration of 3 mL
183 Chlorella paste per 10⁶ rotifers per day, in four 2000 L cylindro-conical tanks supplied with filtered
184 and sterilized sea water at 24 – 26 °C, 38 ‰ salinity, dissolved oxygen at saturation level and 24 h
185 continual illumination.

186 In addition to the enrichment products, rotifers were supplemented with taurine (Andrés
187 Pintaluba S.A., 0.5 g per 10⁶ rotifers) 18 h before the enrichment treatment, and organic Se (Sel-
188 Plex[®] Alltech Spain SL; 3.0 mg per 10⁶ rotifers), and vitamin E as dl- α tocopheryl acetate Lutavit
189 E50 (BASF; 0.9 mg per 10⁶ rotifers) were added with the enrichment treatment. The enrichment
190 protocols were performed in 100 L cylindro-conical tanks at a density of 1000 rotifers mL⁻¹ with the
191 dose of enricher added over a period of 3 h for OG and MG, and 6 h for AG and RA according to
192 manufacturer's recommendations.

193

194 2.2.2 Cultivation of copepod *Acartia tonsa*

195

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

206

207 2.2 Sampling for biometrical, biochemical and molecular analysis.

208

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

216 Triplicate samples of rotifers nutritionally boosted with the different enrichers and copepods
217 (*Acartia*) were washed and filtered, excess water drained and blotted with filter paper, and
218 immediately frozen in liquid N₂ and stored at -80 °C prior to analysis. Two subsets of triplicate
219 samples (15 larvae per sample) of 15 dah ABT larvae fed the different live prey treatments were

220 collected: i) one subset of samples in 1 mL of RNAlater[®] (Ambion, Madrid, Spain) for RNA
221 extraction and molecular analysis, and ii) a second subset frozen in liquid N₂ and stored at -80 °C
222 for lipid analysis. All procedures were carried out according to the current national and EU
223 legislation on the handling of experimental animals.

224

225 *2.3 Biochemical analysis*

226 *2.3.1 Proximate composition*

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

235

236 *2.3.2. Total lipid, lipid class composition and fatty acid analysis*

237 Total lipid of live feeds (enriched rotifers and copepods) and ABT larvae fed the different
238 dietary regimes was extracted from triplicate pooled samples according to the method of Folch et al.
239 (1957). Approximately 200 mg of ABT larvae was placed in 10 mL of ice-cold
240 chloroform/methanol (2:1, by vol) and homogenized with an Ultra-Turrax tissue disrupter (Fisher
241 Scientific, Loughborough, U.K.). The non-lipid and lipid layers were separated by addition of 5 mL
242 of 0.88 % (w/v) KCl and allowed to separate on ice for 1 h. The upper aqueous layer was aspirated

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

245 Lipid class composition was determined by high-performance thin-layer chromatography
246 (HPTLC) using 10 x 10 cm plates (VWR, Lutterworth, England). Approximately 1 µg of total lipid
247 was applied as a single spot and the plates developed in methyl acetate/isopropanol/
248 chloroform/methanol/0.25 % aqueous KCl (25:25:25:10:9, by vol.) to two-thirds up the plate. After
249 drying for 20 min, the plate was fully developed with isohexane/diethyl ether/acetic acid (85:15:1,
250 by vol.). The lipid classes were visualized by charring at 160 °C for 15 min after spraying with 3 %
251 (w/v) aqueous cupric acetate containing 8 % (v/v) phosphoric acid and quantified by densitometry
252 using a CAMAG-3 TLC scanner (version Firmware 1.14.16). Scanned images were recorded
253 automatically and analyzed using winCATS Planar Chromatography Manager software (version
254 1.2.0) (Henderson and Tocher, 1992).

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

263

264 2.3.3. *Determination of alpha-tocopherol (vitamin E) content*

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

269 5 μ m C18 column (Phenomenex, CA, USA). The mobile phase was 98 % methanol pumped at 1.0
270 mL min⁻¹. The effluent from the column was monitored at a wavelength of 293 nm and
271 quantification achieved by comparison with alpha-tocopherol (Sigma-Aldrich) as external standard.

272

273 2.3.4. Selenium determination

274 Total selenium concentration was measured in feeds according to the method established in
275 Betancor et al. (2012). Dried samples were weighed in three replicates of between 0.04 and 0.1 g
276 and digested in a microwave digester (MarsXpress, CEM, USA) with 5 % of 69 % pure nitric acid
277 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
278 finally a 30 min cooling period. The digested solution was poured into a 10 mL volumetric flask
279 and made up to volume with distilled water. A total of 0.4 mL of this solution was added to 10 mL
280 tubes, 10 μ L of internal standard (Gallium and Scandium, 10 ppm, BDH, UK) included and 0.2 mL
281 of methanol added. The tube was made up to volume with distilled water and total selenium was
282 measured in a reaction cell by Inductively Coupled Plasma Mass Spectrometry (Thermo Scientific,
283 XSeries2 ICP-MS, USA) using argon and hydrogen as carrier gas.

284

285 2.4. Tissue RNA extraction and cDNA synthesis

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

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

296

297 2.5. qPCR analysis

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

304 Primers for *fads2d6*, *elovl5*, *fabp2*, 4 and 7, *rxr*, *hmgcl*, *ppary*, *aco*, *fas*, *lpl*, *myhc*, *anpep*,
305 *amy*, *try* and *cat* were already available for ABT (Morais et al., 2011; Mazurais et al., 2015;
306 Betancor et al., 2016). Primers for *alp* and *tropo* were designed on sequences of *Thunnus thynnus*
307 (FM995226.1 and AB109447.1 respectively). Primers for *gpx1* and *gpx4* were designed on the
308 sequences of *Thunnus maccoyii* (EF452497.1 and EF452498.3 respectively; Thompson et al.,
309 2010). Primers for *pl*, *ball* and *bal2* were designed on the sequences of *Thunnus orientalis*
310 (AB859991.1, AB859992.1 and AB859993.1, respectively). Primers for *pla2* and *sod* were
311 designed on the sequence read archive (SAR) SRX2255758 by identifying and assembling the
312 sequences.

313 Expression of genes of interest was determined by qPCR of all the RNA samples.
314 *Elongation factor-1 α* (*elf1a*) and *β -actin* were used as reference genes. The cDNA was diluted 20-
315 fold with milliQ water. The efficiency of the primers for each gene was previously evaluated by
316 serial dilutions of cDNA pooled from the samples to guarantee it was > 85 % for all primer pairs.
317 qPCR was performed using a Biometra TOptical Thermocycler (Analytik Jena, Goettingen,
318 Germany) in 96-well plates in duplicate 20 μ L reaction volumes containing 10 μ L of Luminaris

319 Color HiGreen qPCR Master Mix (Thermo Scientific, Hemel Hempstead, UK), 1 μ L of the primer
320 corresponding to the analyzed gene (10 pmol), 3 μ L of molecular biology grade water and 5 μ L of
321 cDNA (1/20 diluted). In addition, amplifications were carried out with a systematic negative control
322 (NTC, no template control) containing no cDNA. Standard amplification parameters contained an
323 UDG pre-treatment at 50 °C for 2 min, an initial denaturation step at 95 °C for 10 min, followed by
324 35 cycles: 15 s at 95 °C, 30 s at the annealing T_m and 30 s at 72 °C.

325

326 *2.7 Statistical analysis*

327 Results for biometry, lipid class and fatty acid compositions are presented as means \pm SD (n =
328 20 for biometry, and n = 3 for survival, lipid class and fatty acid compositions with tank being the
329 experimental unit). The data were checked for homogeneity of the variances by the Bartlett test
330 and, where necessary, arc-sin transformed before further statistical analysis. Differences between
331 mean values were analyzed by t-test and one-way analysis of variance (ANOVA), followed when
332 pertinent by a multiple comparison test (Tukey). Differences were reported as statistically
333 significant when $P < 0.05$ (Zar, 1999). All statistical analyses were performed using SPSS
334 software (IBM SPSS Statistics 19; SPSS Inc., Chicago, IL, USA).

335 Gene expression results were analyzed using the relative expression software tool (REST
336 2009), which employs a pairwise fixed reallocation randomization test (10,000 randomizations)
337 with efficiency correction (Pfaffl et al., 2002) to determine the statistical significance of
338 expression ratios (gene expression fold changes) between two treatments.

339

340 **3. Results**

341

342 *3.1 ABT larvae biometry and survival at 15 dah*

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

350

351 3.2. Macronutrient, vitamin E and Se content of enriched rotifers *B. rotundiformis* and *Acartia* 352 nauplii

353 The contents of macronutrients, protein and total lipid (dry mass %), and vitamin E and Se
354 in live feeds are shown in Table 2. Total protein content of *Acartia* nauplii was 63.8 % of dry mass,
355 and higher than in enriched rotifers at about 53 % of dry mass. Conversely, total lipid content was
356 higher in enriched rotifers at about 11 % of dry mass, whereas *Acartia* nauplii had a lower lipid
357 content of 5.6 % of dry mass. Vitamin E content in enriched rotifers showed values ranging from
358 232 mg kg⁻¹ in AG-enriched rotifers to 593 mg kg⁻¹ in rotifers enriched with RA. *Acartia* nauplii
359 had a lower level of vitamin E at only 170.1 mg kg⁻¹. Selenium content in rotifers ranged from 4.8
360 µg g⁻¹ in those enriched with AG to 10.6 µg g⁻¹ in those enriched with RA, while *Acartia* nauplii
361 only contained selenium at 0.4 µg g⁻¹.

362

363 3.2 Lipid class compositions of enriched rotifers *B. rotundiformis* and *Acartia* nauplii

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

369 rotifers than in copepods. Cholesterol did not show this tendency and values ranged between 0.66
370 and 1.04 $\mu\text{g mg}^{-1}$ in rotifers enriched with OG and AG, respectively.

371

372 3.3 Total lipid fatty acid compositions of enriched rotifers *B. rotundiformis* and *Acartia nauplii*

373 Total lipid of enriched rotifers contained higher levels of saturated fatty acids than copepods
374 (Table 4). Absolute values of monoenes in rotifers varied between 4.4 and 8.9 $\mu\text{g mg}^{-1}$ of total fatty
375 acids with the highest value in OG-rotifers, with COP showing the lowest value (2.8 $\mu\text{g mg}^{-1}$). Total
376 n-3 PUFA was the predominant fatty acid group ranging from 11.1 to 34.3 $\mu\text{g mg}^{-1}$ of total fatty
377 acids with DHA being predominant, ranging from 23.1 $\mu\text{g mg}^{-1}$ in AG-enriched rotifers to 5.1 μg
378 mg^{-1} in COP, which also showed the lowest DHA content (Table 4). The EPA levels ranged from
379 1.8 to 2.8 $\mu\text{g mg}^{-1}$ of total fatty acids, with the lowest value in MG-enriched rotifers. However,
380 when the data were expressed as percentage of total fatty acid, COP had the highest EPA content
381 (10.4 %), double the amount found in any enriched rotifers (Supplementary Table 2). The
382 DHA/EPA ratio was lowest in COP at 1.9, and ranged from 2.8 (OG) to 8.3 (AG) in enriched
383 rotifers. Total n-6 PUFA (primarily 18:2n-6) were higher in all enriched rotifer treatments (ranging
384 from 6.5 to 10.3 $\mu\text{g mg}^{-1}$) and much higher than in COP at around (1.4 $\mu\text{g mg}^{-1}$). Arachidonic acid
385 (ARA; 20:4n-6) was highest in RA-enriched rotifers (1.1 $\mu\text{g mg}^{-1}$) with COP displaying only 0.6 μg
386 mg^{-1} of this fatty acid.

387

388 3.4. Total lipid fatty acid compositions of 15 dah ABT larvae.

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

393 was more similar between all groups of larvae with highest levels in those fed OG-rotifers and
394 copepods 3.5-3.6 %, and lowest in larvae fed RA-rotifers (2.7 %). The DHA/EPA ratio varied
395 between 4.0 and 6.9 % and was highest in ABT larvae fed copepods and lowest in larvae fed OG-
396 rotifers. Larvae fed copepods had the lowest percentages of 18:2n-6, ARA and total n-6 PUFA at
397 3.9, 1.2 and 12.7 %, respectively. In contrast total n-6 PUFA ranged from 16 to 21 % in larvae fed
398 enriched rotifers with 18:2n-6 ranging from 8-11 %, and ARA from 1.4 to 2.5 %. Total saturated
399 fatty acids ranged from 34 – 41 % in larvae fed enriched rotifers with larvae fed copepods showing
400 an intermediate value of around 38 %. Similarly, total monoene content of ABT larvae ranged from
401 around 9 – 14 % in larvae fed enriched rotifers with larvae fed copepods showing an intermediate
402 value of around 12 %.

403 3.5. Expression of myogenic genes in 15 dah ABT larvae

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

407 3.6. Expression of lipid metabolism and transcription factor genes

408 Regarding fatty acid synthesis, the expression of *fas* showed differences among treatments,
409 that likely reflected the lipid content of the live feeds. Thus, relative expression of *fas* was higher in
410 larvae fed COP, which had the lowest lipid content, than in larvae fed enriched rotifers,
411 significantly so in all cases other than RA-rotifers, while larvae fed AG-rotifers, which had highest
412 lipid content, presented the lowest value (Fig.2). No significant differences were observed in the
413 expression levels of *fads2d6* and *elovl5* among 15 dah ABT larvae fed the different live preys (Fig.
414 2).

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

418 expression than those fed COP, with larvae fed the other enriched rotifers showing intermediate
419 values (Fig. 3). The expression of *fabp2* showed a similar pattern to *aco*, with larvae fed on RA-
420 rotifers showing higher expression than those fed COP, with larvae fed the other enriched rotifers
421 showing intermediate values (Fig. 4). Expression of *fabp4* was also lowest in larvae fed COP,
422 significantly so compared to rotifers enriched with MG and AG, although expression of *fabp7*
423 showed no significant differences. In contrast, larvae fed AG-rotifers showed lower *lpl* expression
424 than larvae fed OG-rotifers, with larvae fed the other live feeds showing intermediate values (Fig.
425 4). No differences among treatments were found in the expression of *hmgcl*.

426 Concerning transcription factors, larvae fed COP displayed lower expression of *ppary* than
427 larvae fed OG- and MG-rotifers with larvae fed the other live feeds showing intermediate
428 expression levels (Fig. 5). Similarly, larvae fed COP displayed lower expression of *sreb2* than
429 larvae fed OG- and RA-rotifers with larvae fed the other live feeds showing intermediate expression
430 levels. No differences in expression levels were observed among larvae fed the different dietary
431 treatments for *ppara*, *sreb1*, *rxr* or *lxr* (Fig. 5).

432

433 3.7. Expression of antioxidant defence enzyme genes.

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

441

442 3.8. Expression of digestive enzyme genes.

443 Trypsin (*tryp*) showed highest expression levels in ABT larvae fed COP, whereas

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

454

455 **4. Discussion**

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

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

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

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

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

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

496 (Worgall et al., 1998; Hihi et al., 2002; Cunha et al., 2013). Although the transcription factor genes
497 did not show regulation in the present trial, *fas*, a direct target of *srebp1*, was strongly regulated by
498 the different dietary treatments and followed a similar pattern as *srebp1*. Indeed, *fas* expression
499 levels were lowest in ABT larvae fed AG enriched rotifers, which contained the highest lipid level,
500 and highest in larvae fed COP, which contained the lowest total lipid content (Fig. 2). Previous
501 studies in teleosts have described an inhibition in hepatic *fas* expression when fish were fed in a
502 restricted manner (Tian et al., 2013; He et al., 2015; Gong et al., 2017) or with increasing dietary fat
503 levels (Leng et al., 2012), which is consistent with our findings. Another transcription factor
504 involved in lipid storage and lipogenesis, as well as osteogenesis (Nedergaard et al., 2005; Ji et al.,
505 2011; Agawa et al., 2012), *ppary*, was regulated in the present trial, showing a pattern opposite to
506 *fas*, with COP-fed ABT larvae displaying the lowest expression level (Fig. 5). An up-regulation in
507 *ppary* expression was also observed in rotifer-fed ABT in a previous trial (Betancor et al., 2016)
508 and this was apparently associated with dietary PC content, which was in agreement with results
509 found in blunt snout bream (*Megalobrama amblycephala*) where *ppary* expression in liver was
510 significantly affected by dietary phospholipid supplementation (Li et al., 2015).

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

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

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

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

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

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

573 and glutathione-S transferase, associated with antioxidant vitamins, such as alpha tocopherol
574 (vitamin E), retinoic acid (vitamin A), ascorbic acid (vitamin C) and Se as cofactor of glutathione
575 peroxidase (Mourente et al., 2007; Izquierdo and Betancor, 2015). Live feed production and
576 enrichment is performed under highly pro-oxidative conditions, with high levels of n-3 PUFA,
577 aeration or oxygenation of the culture water, high temperature and bright light. Moreover, oxidative
578 stress associated with peroxisome proliferation is thought to be due, at least in part, to the
579 increased peroxisomal production of H₂O₂ via *aco* activity. In the present study, the expression of
580 *sod* and *cat* genes in ABT larvae fed different dietary treatments did not correlate with the *aco*
581 expression pattern but, in contrast, correlated with expression of *gpx1* ($r = 0.88$; $P=0.024$) and *gpx4*
582 ($r=0.85$; $P=0.032$) (Figs. 3 and 6). This may suggest that alternative antioxidant specific pathways
583 are activated (glutathione peroxidases) in addition to superoxide dismutase and catalase.

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

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

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

601 Several studies have dealt with the ontogenic development of the digestive system and the
602 functionality and expression of digestive enzymes during early stages of tuna development
603 (Miyashita et al., 1998; Murashita et al., 2014; Mazurais et al., 2015). In the present study,
604 pancreatic alkaline phosphatase (*alp*) trypsin (*tryp*), phospholipase A₂ (*pla2*) and two isoforms of
605 bile salt activated lipases (*bal1* and *bal2*) genes showed highest expression in 15 dah ABT larvae
606 that had been fed with *Acartia* nauplii and copepodites (Fig. 7). Similarly, up-regulation of *tryp* and
607 *lipase* expression was observed in red seabream (*Pagrus major*) fed a fish meal diet in contrast to
608 fish fed a soybean meal based diet, which could be due to the presence of stimulating factors such
609 as small peptides or free aminoacids in fish meal (Murashita et al., 2015). On the other hand, the
610 digestion of dietary polar lipids was investigated by analyzing the relative expression of *pla2* in
611 ABT larvae fed different dietary treatments. Results showed highest expression of *pla2* in larvae fed
612 copepods, which contained a higher proportion of polar lipids than enriched rotifers (Fig. 7). It is
613 commonly accepted that the major digestive lipase in teleosts, including larvae, appears to be bile
614 salt-dependent lipases (*bal*) (Rønnestad and Morais, 2008). It was also reported that bile salt-
615 dependent lipases were the main enzymes involved in lipid digestion in the larval stage of Pacific
616 bluefin tuna (Murashita et al., 2014). In the present study, the expression patterns of both isoforms
617 (*bal1* and *bal2*) were nutritionally regulated, showing a similar pattern. *Ball* showed significantly
618 higher expression in larvae fed copepods (Fig. 7). In cod (*Gadus morhua*) larvae, *bal* expression
619 was correlated with the growth pattern of pyloric caeca, with a significant increase in expression
620 from 48 to 62 dah, which could be related to an increased demand for enzyme in the rapidly
621 increasing pyloric caeca or to an increase in pancreatic mass related to body size (Sæle et al., 2010).
622 Thus, the high *bal* expression found in ABT larvae fed COP in the present study could be reflecting
623 the larger size of COP-fed ABT larvae.

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

643

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649

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912 **Figure legends**

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

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921

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

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930

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

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939

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

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

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959

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

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

967

968 **Fig.7.** Nutritional regulation of trypsin (*tryp*), amino peptidase (*anpep*), amylase (*amy*), pancreatic
969 lipase (*pl*), phospholipase A₂ (*pla2*) and bile salt activated lipase 1 and 2 (*ball* and *bal2*
970 respectively) gene transcription in whole larvae of Atlantic bluefin tuna fed rotifers *Brachionus*
971 *rotundiformis* enriched with four commercial enrichment products as described in Materials
972 and Methods (OG, MG, AG and RA) or 3 days post hatch nauplii of the copepod *Acartia tonsa*
973 fed *Rhodomonas*. Values are normalized expression ratios, corresponding to an average of 6
974 pools of larvae (n = 6) with standard errors (SEM). Different superscript letters denote
975 differences among the dietary treatments (p < 0.05).

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

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

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

997 **Table 2.** Macronutrient (dry mass %), vitamin E (mg kg⁻¹) and selenium (µg g⁻¹) contents of rotifers
 998 *Brachionus rotundiformis* enriched with four commercial enrichment products as described in
 999 Materials and Methods (OG, MG, AG and RA) or 3 days post hatch nauplii of the copepod *Acartia*
 1000 *tonsa* fed *Rhodomonas baltica* (COP).

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

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

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

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

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

1052 **Table 4.** Total lipid fatty acid content (μg fatty acid/mg dry mass) of rotifers *Brachionus*
 1053 *rotundiformis* enriched with four commercial enrichment products as described in Materials and
 1054 Methods (OG, MG, AG and RA) or 3 days post hatch nauplii of the copepod *Acartia tonsa* fed
 1055 *Rhodomonas baltica* (COP).
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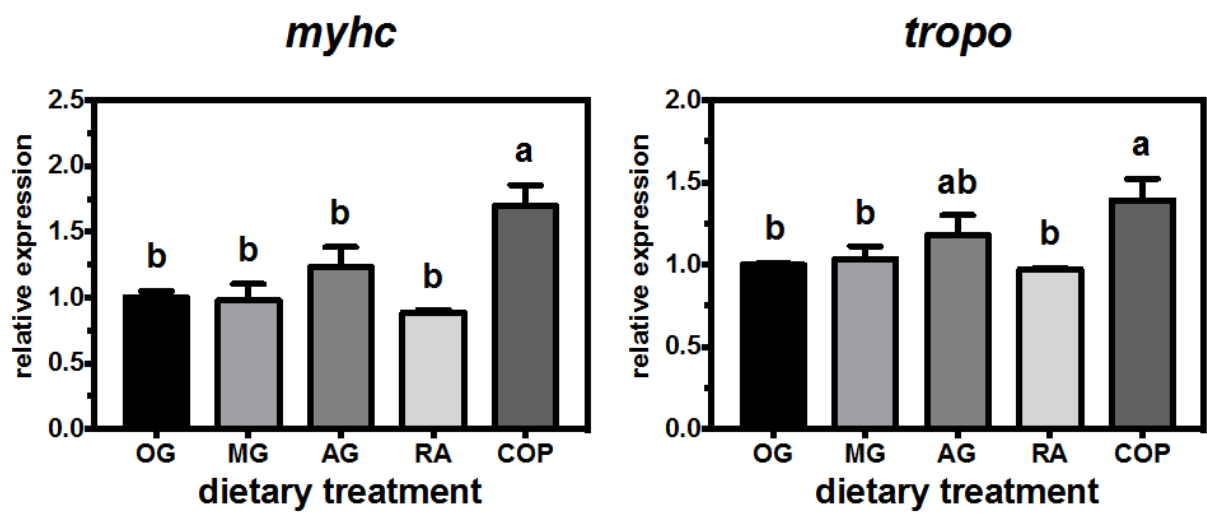
1057		OG	MG	AG	RA	COP
1058						
1059	Fatty acid					
1060						
1061	14:0	0.7 \pm 0.0 ^c	1.0 \pm 0.2 ^c	2.8 \pm 0.1 ^a	1.7 \pm 0.3 ^b	0.5 \pm 0.0 ^c
1062	16:0	9.8 \pm 0.4 ^c	10.4 \pm 0.6 ^{bc}	14.0 \pm 0.4 ^a	11.2 \pm 0.2 ^b	3.2 \pm 0.2 ^e
1063	18:0	3.1 \pm 0.1 ^a	2.1 \pm 0.1 ^b	2.9 \pm 0.2 ^a	2.4 \pm 0.1 ^b	1.3 \pm 0.1 ^c
1064	Total saturated ¹	15.3 \pm 0.4 ^{bc}	14.7 \pm 1.0 ^c	21.5 \pm 0.6 ^a	16.8 \pm 0.5 ^b	6.2 \pm 0.2 ^d
1065						
1066	16:1n-7	1.0 \pm 0.1 ^a	0.4 \pm 0.0 ^b	0.5 \pm 0.0 ^b	0.5 \pm 0.0 ^b	0.4 \pm 0.0 ^b
1067	18:1n-9	4.0 \pm 0.2 ^a	1.2 \pm 0.1 ^b	0.8 \pm 0.1 ^b	0.8 \pm 0.1 ^b	0.3 \pm 0.0 ^c
1068	18:1n-7	1.0 \pm 0.1 ^a	0.6 \pm 0.1 ^b	0.6 \pm 0.0 ^b	0.7 \pm 0.0 ^b	1.1 \pm 0.1 ^a
1069	20:1n-9	0.8 \pm 0.1 ^a	0.4 \pm 0.0 ^b	0.4 \pm 0.0 ^b	0.5 \pm 0.0 ^b	0.1 \pm 0.0 ^c
1070	Total monoenes ²	8.9 \pm 0.2 ^a	4.4 \pm 0.1 ^b	4.5 \pm 0.1 ^b	4.6 \pm 0.1 ^b	2.8 \pm 0.0 ^c
1071						
1072	18:2n-6	10.3 \pm 0.4 ^a	6.5 \pm 0.1 ^c	7.8 \pm 0.2 ^b	7.0 \pm 0.1 ^c	1.4 \pm 0.1 ^d
1073	20:4n-6	0.5 \pm 0.0 ^b	0.5 \pm 0.0 ^b	1.0 \pm 0.0 ^a	1.1 \pm 0.3 ^a	0.6 \pm 0.0 ^b
1074	22:5n-6	0.6 \pm 0.0 ^d	2.8 \pm 0.1 ^c	7.1 \pm 0.3 ^a	5.0 \pm 0.1 ^b	0.3 \pm 0.1 ^d
1075	Total n-6PUFA ³	13.8 \pm 0.6 ^c	11.9 \pm 0.4 ^d	18.6 \pm 0.8 ^a	15.3 \pm 0.4 ^b	3.7 \pm 0.1 ^e
1076						
1077	18:3n-3	2.6 \pm 0.1 ^a	1.6 \pm 0.0 ^b	2.4 \pm 0.1 ^a	1.7 \pm 0.0 ^b	1.2 \pm 0.1 ^c
1078	18:4n-3	0.1 \pm 0.0 ^c	0.1 \pm 0.0 ^c	0.3 \pm 0.0 ^b	0.2 \pm 0.1 ^{bc}	0.7 \pm 0.1 ^a
1079	20:4n-3	0.5 \pm 0.0 ^b	0.6 \pm 0.0 ^b	0.9 \pm 0.0 ^a	0.8 \pm 0.0 ^a	0.1 \pm 0.0 ^c
1080	20:5n-3	2.8 \pm 0.1 ^a	1.8 \pm 0.1 ^b	2.8 \pm 0.1 ^a	2.6 \pm 0.0 ^a	2.8 \pm 0.2 ^a
1081	22:5n-3	2.3 \pm 0.1 ^a	1.4 \pm 0.1 ^c	1.8 \pm 0.1 ^b	1.7 \pm 0.0 ^b	0.1 \pm 0.0 ^d
1082	22:6n-3	7.8 \pm 0.4 ^d	10.3 \pm 0.5 ^c	23.1 \pm 0.9 ^a	16.1 \pm 0.3 ^b	5.1 \pm 0.3 ^e
1083	Total n-3PUFA ⁴	18.1 \pm 0.9 ^c	17.2 \pm 0.7 ^c	34.3 \pm 1.3 ^a	25.2 \pm 0.5 ^b	11.1 \pm 0.5 ^d
1084						
1085	C16 PUFA	2.4 \pm 0.0 ^b	1.6 \pm 0.0 ^d	2.9 \pm 0.1 ^a	1.8 \pm 0.0 ^c	1.4 \pm 0.0 ^c
1086						
1087	Total PUFA	31.8 \pm 1.5 ^c	29.1 \pm 1.0 ^c	52.8 \pm 2.1 ^a	40.6 \pm 0.5 ^b	14.9 \pm 0.4 ^d
1088	n-3/n-6	1.3 \pm 0.1 ^c	1.4 \pm 0.1 ^c	1.8 \pm 0.1 ^b	1.6 \pm 0.1 ^{bc}	3.0 \pm 0.2 ^a
1089	DHA/EPA	2.8 \pm 0.1 ^d	5.7 \pm 0.1 ^c	8.3 \pm 0.1 ^a	6.2 \pm 0.1 ^b	1.9 \pm 0.2 ^e

1090
 1091 Results are means \pm SD (n = 3). An SD of 0.0 implies an SD of < 0.05. Mean values bearing
 1092 different superscript letter are significantly different (P<0.05). ¹, Totals include 15:0, 20:0, 22:0 and
 1093 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,
 1094 20:2n-6 and 22:4n-6; ⁴, Totals include 20:3n-3 and 22:3n-3; DHA, docosahexaenoic acid; EPA,
 1095 eicosapentaenoic acid; PUFA, polyunsaturated fatty acid.
 1096
 1097

1098 **Table 5.** Fatty acid composition (weight %) of total lipid of 15 days post hatch ABT larvae fed
 1099 rotifers *B. rotundiformis* enriched with four commercial enrichment products as described in
 1100 Materials and Methods (OG, MG, AG and RA) or 3 days post hatch nauplii of the copepod *Acartia*
 1101 *tonsa* fed *Rhodomonas baltica* (COP).
 1102
 1103

1104		OG	MG	AG	RA	COP
1106						
1107	14:0	1.6 ± 0.1 ^b	0.9 ± 0.1 ^c	1.1 ± 0.1 ^c	1.0 ± 0.0 ^c	2.7 ± 0.1 ^a
1108	16:0	18.2 ± 0.4 ^b	19.9 ± 1.4 ^{ab}	21.0 ± 1.2 ^a	21.7 ± 1.3 ^a	20.0 ± 0.3 ^{ab}
1109	18:0	10.8 ± 0.3 ^b	11.6 ± 0.9 ^{ab}	11.7 ± 0.6 ^{ab}	13.0 ± 0.9 ^a	10.6 ± 0.2 ^b
1110	Total saturated ¹	34.5 ± 0.9 ^b	37.3 ± 2.6 ^{ab}	40.9 ± 2.3 ^a	40.1 ± 1.1 ^a	37.7 ± 0.6 ^a
1111						
1112	16:1n-7	2.4 ± 0.1 ^a	2.6 ± 0.2 ^a	0.8 ± 0.1 ^b	0.9 ± 0.1 ^b	2.6 ± 0.2 ^a
1113	18:1n-9	5.5 ± 0.1 ^a	3.7 ± 0.3 ^b	3.3 ± 0.2 ^b	3.5 ± 0.1 ^b	5.1 ± 0.1 ^a
1114	18:1n-7	1.9 ± 0.1 ^{ab}	1.4 ± 0.1 ^c	1.4 ± 0.1 ^c	1.7 ± 0.1 ^b	2.1 ± 0.1 ^a
1115	20:1n-9	0.7 ± 0.0 ^a	0.5 ± 0.1 ^a	0.5 ± 0.2 ^a	0.5 ± 0.1 ^a	0.2 ± 0.0 ^b
1116	Total monoenes ²	14.4 ± 0.3 ^a	11.5 ± 0.8 ^b	9.1 ± 0.4 ^c	9.5 ± 0.1 ^c	11.9 ± 0.2 ^b
1117						
1118	18:2n-6	10.8 ± 0.2 ^a	8.5 ± 0.6 ^b	8.4 ± 0.4 ^b	7.9 ± 0.3 ^b	3.9 ± 0.1 ^c
1119	20:4n-6	1.4 ± 0.1 ^c	1.9 ± 0.2 ^b	2.4 ± 0.1 ^a	2.5 ± 0.2 ^a	1.2 ± 0.0 ^c
1120	22:5n-6	1.3 ± 0.1 ^d	3.4 ± 0.3 ^b	4.8 ± 0.3 ^a	4.7 ± 0.1 ^a	2.7 ± 0.0 ^c
1121	Total n-6PUFA ³	16.6 ± 0.4 ^b	16.7 ± 1.2 ^b	20.7 ± 0.8 ^a	20.1 ± 0.3 ^a	12.7 ± 0.2 ^c
1122						
1123	18:3n-3	1.9 ± 0.0 ^a	1.1 ± 0.1 ^b	1.3 ± 0.1 ^b	1.1 ± 0.1 ^b	1.9 ± 0.0 ^a
1124	18:4n-3	1.0 ± 0.0 ^b	0.4 ± 0.0 ^c	0.3 ± 0.0 ^c	0.4 ± 0.1 ^c	2.1 ± 0.1 ^a
1125	20:4n-3	0.7 ± 0.0 ^b	0.7 ± 0.1 ^b	0.7 ± 0.0 ^b	0.9 ± 0.1 ^a	0.7 ± 0.0 ^b
1126	20:5n-3	3.6 ± 0.1 ^a	2.9 ± 0.2 ^b	2.9 ± 0.1 ^b	2.7 ± 0.0 ^b	3.5 ± 0.1 ^a
1127	22:5n-3	3.1 ± 0.1 ^a	2.5 ± 0.2 ^b	2.4 ± 0.1 ^b	2.5 ± 0.0 ^b	0.4 ± 0.0 ^c
1128	22:6n-3	14.3 ± 0.3 ^c	15.4 ± 1.2 ^c	19.5 ± 1.3 ^b	17.8 ± 0.2 ^b	24.3 ± 0.3 ^a
1129	Total n-3PUFA ⁴	27.2 ± 0.6 ^b	25.8 ± 1.9 ^b	28.8 ± 1.5 ^b	28.0 ± 0.4 ^b	36.4 ± 0.6 ^a
1130						
1131	C16 PUFA	2.4 ± 0.3 ^b	2.4 ± 0.1 ^b	4.4 ± 0.2 ^a	4.2 ± 0.1 ^a	4.8 ± 0.3 ^a
1132						
1133	Total PUFA	43.9 ± 1.0 ^b	42.6 ± 3.2 ^b	50.5 ± 2.2 ^a	48.2 ± 0.7 ^{ab}	49.1 ± 0.8 ^a
1134	n-3/n-6	1.6 ± 0.3 ^b	1.5 ± 0.1 ^b	1.4 ± 0.1 ^b	1.4 ± 0.1 ^b	2.9 ± 0.2 ^a
1135	DHA/EPA	4.0 ± 0.2 ^c	5.7 ± 0.1 ^b	6.7 ± 0.3 ^a	6.6 ± 0.2 ^a	6.9 ± 0.3 ^a

1136
 1137 Results are means ± SD (n = 3). An SD of 0.0 implies an SD of < 0.05. Mean values bearing
 1138 different superscript letter are significantly different (P < 0.05). ¹, Totals include 15:0, 20:0, 22:0
 1139 and 24:0; ², Totals include 16:1n-9, 18:1n-11, 20:1n-7, 22:1 isomers and 24:1; ³, Totals include
 1140 18:3n-6, 20:2n-6 and 22:4n-6; ⁴, Totals include 20:3n-3 and 22:3n-3; DHA, docosahexaenoic acid;
 1141 EPA, eicosapentaenoic acid; PUFA, polyunsaturated fatty acid.
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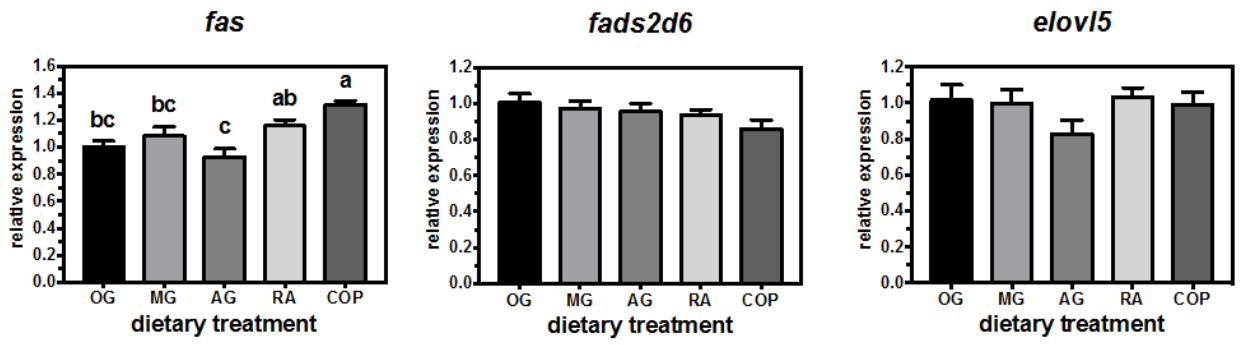
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1146 Figure 1

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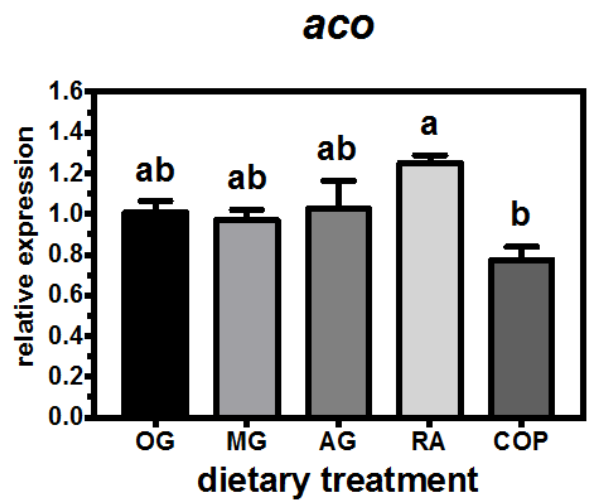
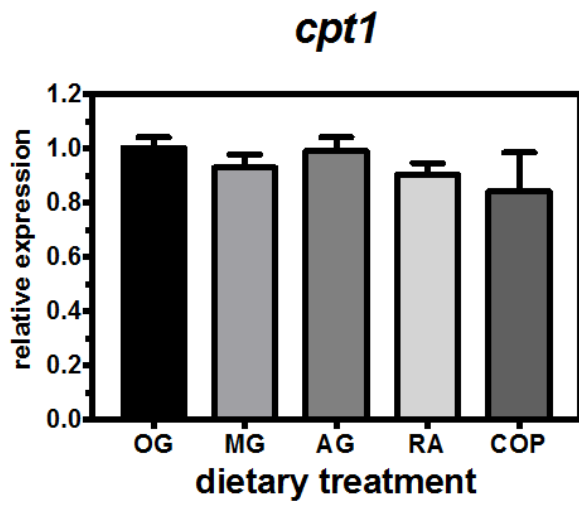


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1155 Figure 3

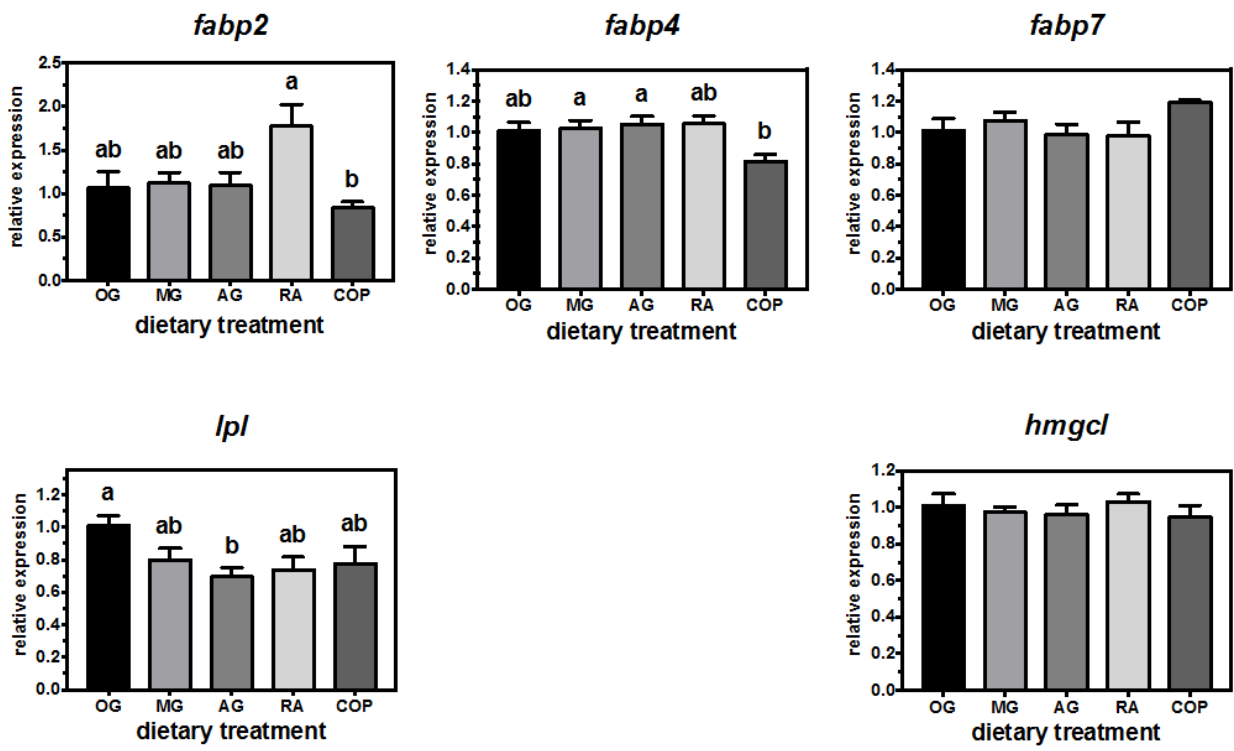
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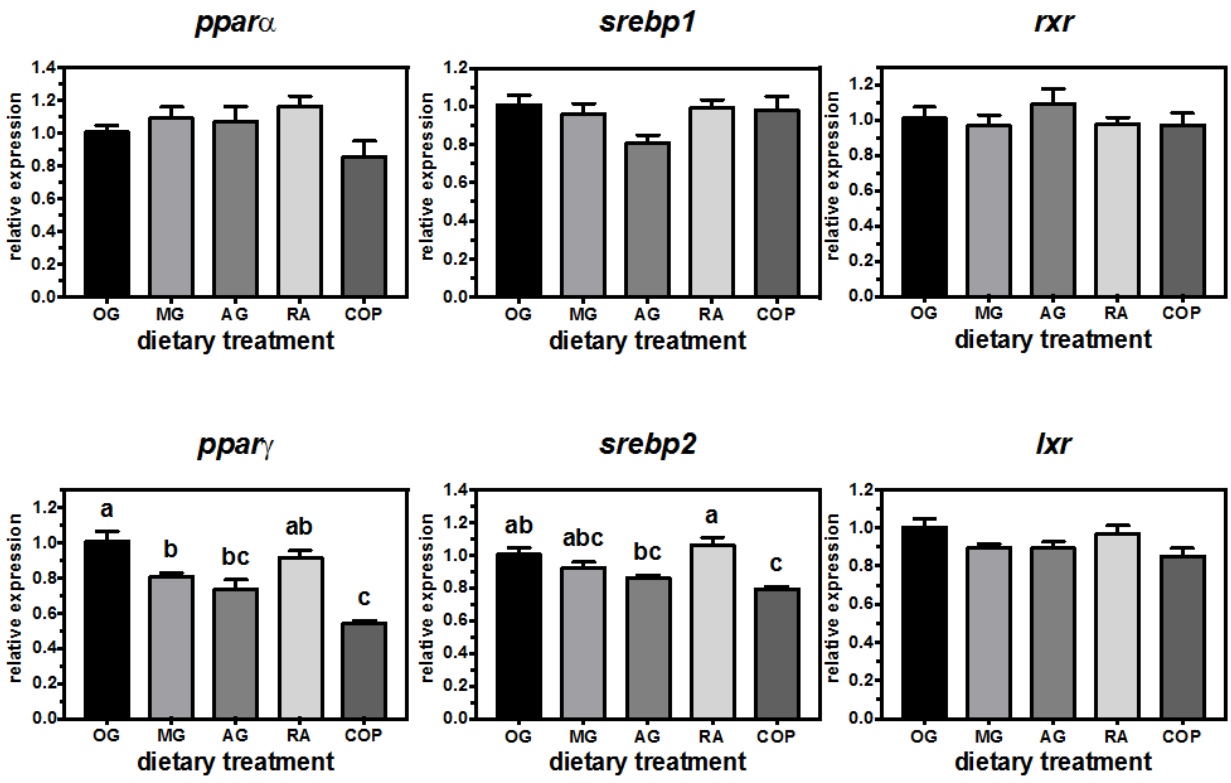
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1163 Figure 4

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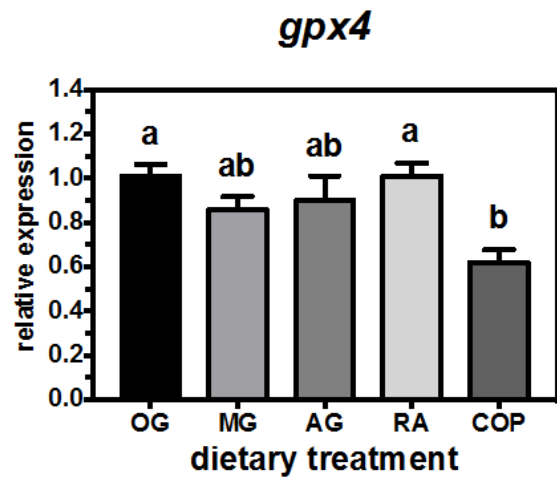
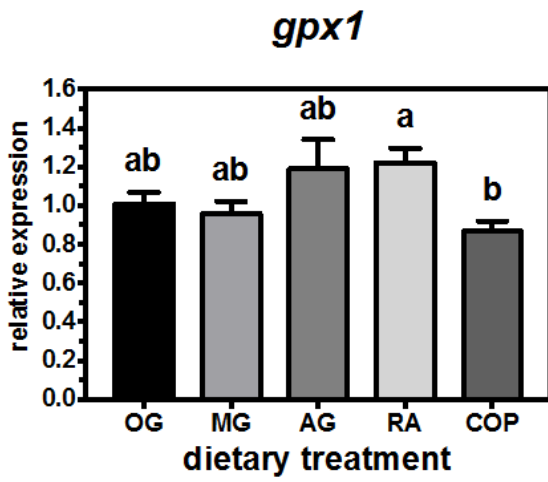
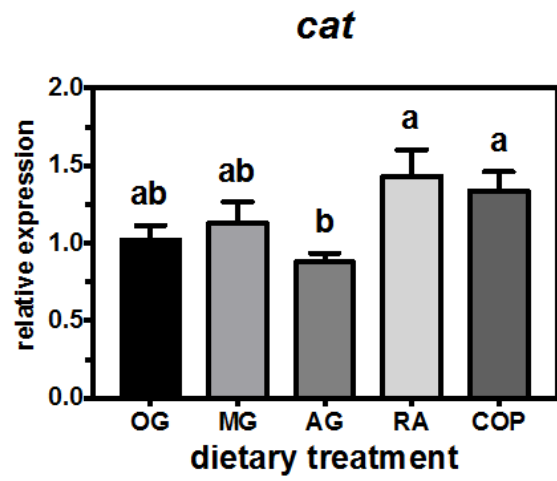
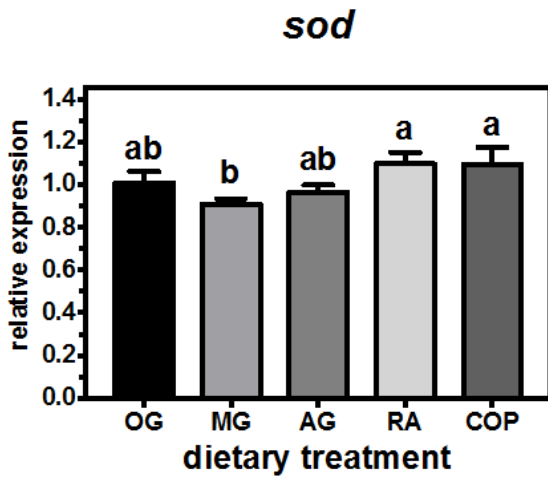
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Figure 5



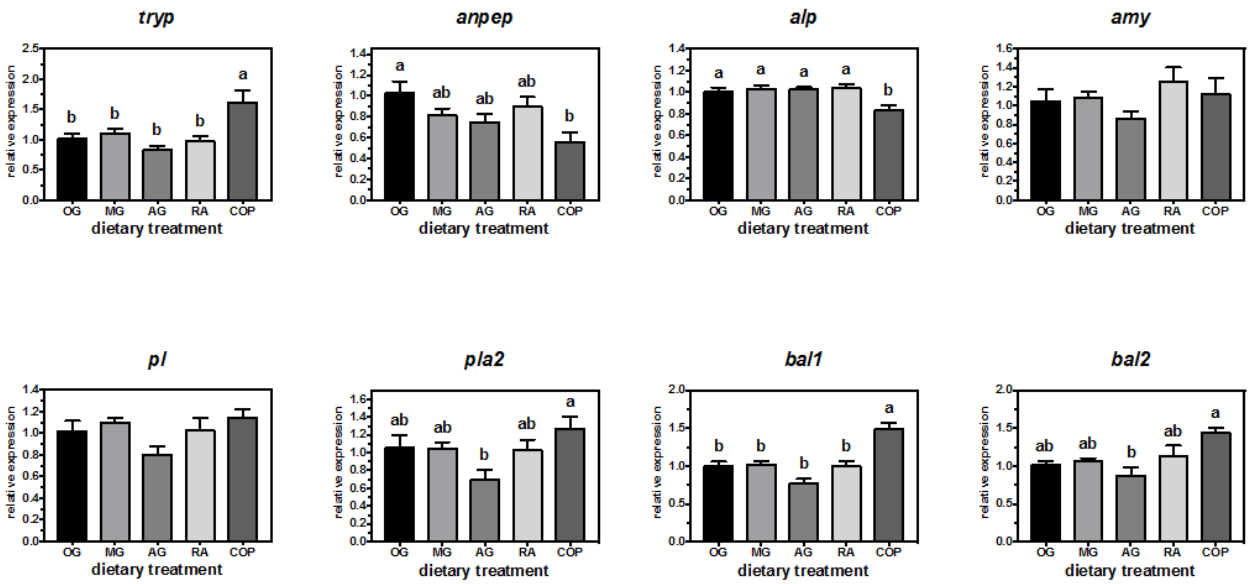
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1174 Figure 6

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1181 Figure 7

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1187 **Supplementary Table 1.** Sequence, annealing temperature (T_m) and size of the fragment produced
 1188 by the primer pairs used for quantitative PCR (qPCR).

Name	Sequence (5'-3')	Amplicon size (bp)	T _m °C
<i>elovl5</i>	F: CCACGCTAGCATGCTGAATA	236	60
	R: ATGGCCATATGACTGCACAC		
<i>fads2d6</i>	F: CCGTGCCTGTGTGAGAAAC	152	60
	R: CAGTGTAAGCGATAAAAATCAGCTG		
<i>ppara</i>	F: TGGTCATGGAGGTGGAAGAC	152	60
	R: ATGGATGACGAAAGGAGGGG		
<i>ppary</i>	F: ACCTGACCAACATGGACTAC	118	60
	R: GAGAAAACAGGACTGTCAGC		
<i>lxr</i>	F: CACACTGGATCCACAACAGC	192	58
	R: ATCTCCTGCACCGACATGAT		
<i>rxr</i>	F: TGAGGGAAAAGGTCTACGCA	212	59
	R: TGTGATCTGATGTGGTGCCT		
<i>srebp1</i>	F: CCAGCTACACATGACAGGGA	153	59
	R: GCTTTGACCCTTAGAGCTGC		
<i>srebp2</i>	F: AGATCCAGTGAGTCGTTGGT	212	60
	R: CTACAGCCCCTTCTCCCTTC		
<i>fabp2</i>	F: CGCAGCGAGAATTATGACAA	244	55
	R: AGCATGTCACCCTCCATCTC		
<i>fabp4</i>	F: ACTGCAATGACCGAAAGACC	175	55
	R: CCTCCTTTCCGTAGGTCCTC		
<i>fabp7</i>	F: CCTACACCTGATGACCGACA	212	55
	R: GCTGGGATGATTTGCTCATT		
<i>cpt1</i>	F: TGGAGGCTGTCCACCAGTCA	211	60
	R: TGCTGGAGATGTGGAAGTTG		
<i>lpl</i>	F: CCGAAAGAACCGCTGCAATA	212	59
	R: GATCCTCCTTCTCTCCGTGG		
<i>fas</i>	F: ATACCGTGGCAATGTAACGC	188	59
	R: GTGAGCTGTGGATGATGCTG		
<i>aco</i>	F: AGCGCTATGACCAGGCTATT	164	59
	R: GTACAGGGTTGGGAGGAACA		
<i>hmgcl</i>	F: CGTGCCAACAGAGACGAAA	173	59
	R: GGGTGAGGACTGGGTAAGAC		
<i>gpx1</i>	F: TGGAGAAAGTGGATGTGAACGG	309	55
	R: GTGCTGTGGAAGCTGTATGATGG		
<i>gpx4</i>	F: TGGGGAATAGCATCAAGTGG	206	55
	R: CGAGAAAGGAGGGAAACAGG		
<i>cat</i>	F: ATGGTGTGGGACTTCTGGAG		60
	R: ATGAAACGGTAGCCATCAGG		
<i>sod</i>	F: TCCCAGATCACCTACATGCC	182	59
	R: CTGCGGAGAGTTGCTTGATC		
<i>myhc</i>	F: GATTCAGCTGAGCCATGCCA	190	60
	R: TCTCAGCTCCTCAATCTCAG		
<i>anpep</i>	F: CCTGAGGTGGTGGAAATGACT		60
	R: GGGTTCAGCTTTGTCTGCTC		
<i>amy</i>	F: TCATGTGGAAGCTGGTTCAG		60
	R: AATATTGCCACTGCCAGTCC		

1189

1190

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

1191 *elovl5*, fatty acyl elongase 5; *fads2d6*, delta-6 fatty acyl desaturase; *ppara*, peroxisome proliferator-
1192 activated receptor alpha; *ppary*, peroxisome proliferator-activated receptor gamma; *lxr*, liver X
1193 receptor; *rxr*, retinoid X receptor; *srebp1*, sterol regulatory element-binding protein 1; *srebp2*; sterol
1194 regulatory element-binding protein 2; *fabp2*, fatty acid binding protein 2 (intestinal); *fabp4*, fatty
1195 acid binding protein 4 (adipocyte); *fabp7*, fatty acid binding protein 7 (brain-type); *cptI*, carnitine
1196 palmitoyl transferase I; *lpl*, lipoprotein lipase; *fas*; fatty acid synthase; *aco*, acyl coA oxidase;
1197 *hmgcl*; 3-hydroxy-3-methylglutaryl-CoA lyase; *gpx1*, glutathione peroxidase 1; *gpx4*, glutathione
1198 peroxidase 4; *cat*, catalase; *sod*, superoxide dismutase; *myhc*, myosin heavy chain; *anpep*, amino
1199 peptidase; *amy*, amylase; *tryp*, trypsin; *alp*, alkaline phosphatase; *pl*, pancreatic lipase; *ball*, bile
1200 salt activated lipase 1; *bal2*, bile salt activated lipase 2; *pla2*, phospholipase A2; *tropo*,
1201 tropomyosin; *ef1a*, elongation factor 1 alpha; *bactin*, beta actin.

1202

1203

1204 **Supplementary Table 2.** Total lipid fatty acid composition (weight %) of rotifers *B. rotundiformis*
 1205 enriched with four commercial enrichment products as described in Materials and Methods (OG,
 1206 MG, AG and RA) or 3 days post hatch nauplii of the copepod *Acartia tonsa* fed *Rhodomonas*
 1207 *baltica* (COP).
 1208

1209		OG	MG	AG	RA	COP
1210						
1211	Fatty acid					
1212						
1213	14:0	1.2 ± 0.1 ^b	1.9 ± 0.4 ^b	3.4 ± 0.1 ^a	2.7 ± 0.5 ^a	1.9 ± 0.1 ^b
1214	16:0	16.9 ± 0.3 ^b	20.9 ± 1.1 ^a	17.2 ± 0.5 ^b	17.3 ± 0.1 ^b	11.9 ± 0.8 ^c
1215	18:0	5.3 ± 0.1	4.2 ± 0.1	3.5 ± 0.2	3.7 ± 0.1	4.8 ± 0.1
1216	Total saturated ¹	26.5 ± 0.2 ^b	29.7 ± 1.8 ^a	26.4 ± 0.9 ^b	26.2 ± 0.4 ^b	23.5 ± 0.7 ^c
1217						
1218	16:1n-7	1.8 ± 0.1 ^a	0.9 ± 0.1 ^b	0.7 ± 0.1 ^b	0.8 ± 0.1 ^b	1.7 ± 0.2 ^a
1219	18:1n-9	7.0 ± 0.1 ^a	2.5 ± 0.1 ^b	1.0 ± 0.1 ^c	1.2 ± 0.1 ^{cd}	1.3 ± 0.1 ^d
1220	18:1n-7	1.7 ± 0.1 ^b	1.1 ± 0.1 ^c	0.8 ± 0.1 ^c	1.1 ± 0.1 ^c	4.0 ± 0.2 ^a
1221	20:1n-9	1.3 ± 0.1 ^a	0.8 ± 0.1 ^b	0.5 ± 0.1 ^{ce}	0.7 ± 0.1 ^{bc}	0.3 ± 0.1 ^e
1222	Total monoenes ²	15.4 ± 0.2 ^a	9.0 ± 0.2 ^c	5.5 ± 0.1 ^e	7.1 ± 0.2 ^d	10.6 ± 0.1 ^b
1223						
1224	18:2n-6	17.7 ± 0.4 ^a	13.2 ± 0.4 ^b	9.5 ± 0.1 ^d	10.8 ± 0.1 ^c	5.3 ± 0.3 ^e
1225	20:4n-6	0.9 ± 0.0 ^c	1.1 ± 0.1 ^c	1.2 ± 0.1 ^c	1.8 ± 0.4 ^b	2.4 ± 0.1 ^a
1226	22:5n-6	1.0 ± 0.1 ^d	5.6 ± 0.2 ^c	8.7 ± 0.1 ^a	7.9 ± 0.1 ^b	1.1 ± 0.3 ^d
1227	Total n-6PUFA ³	23.8 ± 0.5 ^a	24.1 ± 0.9 ^a	22.8 ± 0.5 ^a	23.9 ± 0.8 ^a	14.2 ± 0.2 ^b
1228						
1229	18:3n-3	4.6 ± 0.1 ^a	3.2 ± 0.1 ^b	2.9 ± 0.1 ^b	2.7 ± 0.1 ^c	4.7 ± 0.3 ^a
1230	18:4n-3	0.2 ± 0.1 ^b	0.2 ± 0.1 ^b	0.4 ± 0.1 ^b	0.4 ± 0.1 ^b	2.7 ± 0.1 ^a
1231	20:4n-3	0.9 ± 0.1 ^b	1.2 ± 0.1 ^a	1.2 ± 0.1 ^a	1.2 ± 0.1 ^a	0.5 ± 0.1 ^c
1232	20:5n-3	4.9 ± 0.2 ^b	3.7 ± 0.2 ^c	3.4 ± 0.1 ^c	4.1 ± 0.1 ^b	10.4 ± 0.6 ^a
1233	22:5n-3	4.0 ± 0.2 ^a	2.8 ± 0.2 ^b	2.2 ± 0.1 ^c	2.5 ± 0.1 ^{bc}	0.5 ± 0.1 ^d
1234	22:6n-3	13.5 ± 0.5 ^d	20.9 ± 1.1 ^c	28.3 ± 0.4 ^a	25.0 ± 0.2 ^b	19.4 ± 0.9 ^c
1235	Total n-3PUFA ⁴	31.3 ± 0.9 ^c	34.8 ± 1.5 ^b	42.1 ± 0.5 ^a	39.3 ± 0.2 ^a	42.2 ± 1.8 ^a
1236						
1237	C16 PUFA	4.1 ± 0.3 ^b	3.3 ± 0.1 ^c	3.5 ± 0.2 ^c	3.0 ± 0.1 ^c	5.3 ± 0.3 ^a
1238						
1239	Total PUFA	55.1 ± 1.5 ^b	58.8 ± 2.4 ^b	64.9 ± 1.0 ^a	63.1 ± 0.7 ^a	56.4 ± 1.6 ^b
1240	n-3/n-6	1.3 ± 0.1 ^c	1.4 ± 0.1 ^c	1.8 ± 0.1 ^b	1.6 ± 0.1 ^{bc}	3.0 ± 0.2 ^a
1241	DHA/EPA	2.8 ± 0.1 ^d	5.7 ± 0.1 ^c	8.3 ± 0.1 ^a	6.2 ± 0.1 ^b	1.9 ± 0.2 ^e

1242
 1243 Results are means ± SD (n = 3). An SD of 0.0 implies an SD of < 0.05. Mean values bearing
 1244 different superscript letter are significantly different (P<0.05). ¹, Totals include 15:0, 20:0, 22:0 and
 1245 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,
 1246 20:2n-6 and 22:4n-6; ⁴, Totals include 20:3n-3 and 22:3n-3; DHA, docosahexaenoic acid; EPA,
 1247 eicosapentaenoic acid; PUFA, polyunsaturated fatty acid.
 1248
 1249