Effects of algae-enriched rotifers on winter flounder (*Pseudopleuronectes americanus*) gene expression during metamorphosis

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Abstract The aim of this study was to evaluate the effect of a dietary highly unsaturated fatty acid (HUFA) deficiency on winter flounder *Pseudopleuronectes americanus* metamorphosis by examining growth and the expression of genes involved in some key metabolic processes: lipid digestion, oxidative stress, and growth. Three groups of fish were fed rotifers enriched with different blends of microalgae providing different HUFA profiles: (1) a diet comprising a mixture of three microalgae, *Nannochloropsis oculata*, *Isochrysis galbana*, and *Pavlova lutheri* (Cocktail diet), which contained a balanced combination of ecosapentaenoic acid (EPA), docosahexaenoic acid (DHA), and arachidonic acid (AA); (2) the *N. occulata* diet (Nanno diet), with a low level of DHA; and (3) the *I. galbana* diet (Tiso diet), characterized by low levels of EPA and AA. The results indicate that the need for DHA increased from settlement and for EPA and AA from 15 days after settlement. The lower HUFA content in the Tiso and Nanno diets did not affect larval development or lipid reserve accumulation. The *superoxide dismutase* gene expression suggests a reduced oxidative stress in the Cocktail group, and overall results indicate that *gh* gene expression could be a valuable indicator of development at the molecular level in response to dietary HUFA quality during metamorphosis in winter flounder.

Introduction

In many marine fish species, metamorphosis is a critical step during which animals undergo profound physiological and morphological modifications that are controlled by a coordinated change in gene expression (Bao et al. 2005; Hildahl et al. 2007; Wang et al. 2011). Metamorphosis processes differ between flatfish and pelagic fish species: flatfish metamorphosis is characterized by striking anatomical transformations that involve a 90° rotation in body position, asymmetrical pigmentation, and the migration of one eye towards the other on the upper

side of the fish. This process is correlated with a transition from the pelagic to the benthic habitat (Fuiman 1997; Gibson 1997; Geffen et al. 2007) that involves modifications in feeding habits and digestive physiology (Tanaka et al. 1996; Lagardère et al. 1999; Cañavate et al. 2006). In marine fish production, metamorphosis is a crucial phase, and its success is strongly related to survival rate, growth, and pigmentation development (Geffen et al. 2007).

It is well known that metamorphosis is affected by environmental factors such as temperature and photoperiod (Policansky 1982; Solbakken and Pittman 2004) as well as the nutritional environment (Tocher 2010; Pinto et al. 2010; Olivotto et al. 2011). The nutritional environment is of particular importance during marine fish metamorphosis because it must provide the energy required for cellular, tissular, and functional remodelling (Sargent 1999; Tocher 2010). Nutritional deficiencies have been shown to be the cause of abnormal pigmentation and bone deformities commonly encountered during the culture of larval fishes (Miki et al. 1990; Kanazawa 1993; Bolker and Hill 2000; Hamre et al. 2005; Mazurais et al. 2009). In particular, lipid deficiencies may impair larval health, growth, and feeding efficiency and may also cause anaemia and high larval mortality (Sargent 1999; Copeman et al. 2002; Cahu 2003; Olivotto et al. 2011). Among lipids, 20- and 22-carbon highly unsaturated fatty acids from the n-3 and n-6 series (n-3 and n-6 HUFAs), such as ecosapentaenoic acid (EPA, 20:5n-3), docosahexaenoic acid (DHA, 22:6n-3), and arachidonic acid (AA, 20:4n-6), perform a variety of important physiological functions in all vertebrates (Sargent et al. 2002). In fish larvae, they are preferentially incorporated into membrane phospholipids (Linares and Henderson 1991) and have been shown to play key roles in ontogenesis, growth, survival, pigmentation, and resistance to stress and disease as well as in the development and functionality of the brain, vision, and the nervous system (for reviews, see Sargent et al. 2002; Glencross 2009; Tocher 2010). While they are essential for several vital functions, HUFAs from the n-3 and n-6 series are generally only

76 minimally synthesized *de novo* in marine fishes and must therefore be supplied by food (Teshima
77 et al. 1992).

78 Lipid digestion is a key metabolic process that develops during metamorphosis: dietary
79 lipids play an important role as energy sources to achieve metamorphosis in carnivorous fishes,
80 which have few carbohydrates available for energy (Watanabe 1982). Lipid digestion is
81 facilitated by the activation of lipases (Iijima et al. 1998), the most important of which in teleosts
82 is bile salt-activated lipase (Bal) (Patton et al. 1977; Gjellesvik 1992; Murray et al. 2003; Darias
83 et al. 2007; Sæle et al. 2010). Bal hydrolyzes the ester bonds of triacylglycerols (TAGs), and the
84 digestion products are absorbed by the enterocytes located on the gut epithelial wall.

85 The development of digestive pathways and of all other metabolic pathways occurring
86 during metamorphosis creates a high metabolic demand. To meet this demand, fishes increase
87 their exogenous oxygen consumption (Fernández-Díaz et al. 2001) which can increase the
88 production of reactive oxygen species (ROS). ROS are waste products from mitochondrial
89 oxidation and may cause damage to lipids, proteins, and DNA in fish tissues (Fridovich 2004;
90 Mourente et al. 2007). ROS are continually detoxified and removed from cells by antioxidant
91 enzymes. The study of the mechanisms behind oxidative stress in fish is an emerging field in
92 aquaculture, and enzymatic activities as well as mRNA transcription levels have been
93 characterized in several species (Mourente 1999; Fontagné et al. 2008; Todorcevic et al. 2009;
94 Tovar-Ramírez et al. 2010; Ji et al. 2011; Zuo et al. 2012). Among antioxidant enzymes,
95 superoxide dismutase (Sod) catalyzes the dismutation of superoxide radicals to hydrogen
96 peroxide and oxygen (Halliwell 2006). Studies on turbot (Peters and Livingstone 1996), common
97 dentex *Dentex dentex* (Mourente 1999), and rainbow trout *Oncorhynchus mykiss* (Fontagné et al.
98 2008) have shown that Sod is required at very early developmental stages to reduce elevated
99 tissue concentrations of oxygen. Moreover, in rainbow trout, Sod was the only antioxidant

enzyme with readily measurable activity in embryos, contrary to catalase and glutathione peroxidase. Sod activity during metamorphosis has recently been shown to depend on the n-3 HUFA dietary content in Atlantic salmon *Salmo salar* (Todorcevic et al. 2009), juvenile grass carp *Ctenopharyngodon idellus* (Ji et al. 2011), and yellow croaker *Larimichthys crocea* (Zuo et al. 2012). The mRNA levels of antioxidant enzymes are known to be valid biomarkers of oxidative stress (Olsvik et al. 2005). However, to our knowledge, there are no reports concerning the effect of dietary HUFA content on the expressions of genes coding for antioxidant enzymes during early developmental stages of marine fish larvae.

While several studies have focussed on flatfish metamorphosis and the effects of HUFA dietary content on key process during flatfish development at the enzymatic level, very little is known about the genetic mechanisms underlying metamorphosis and about the role of HUFA in these mechanisms. In this context, our aim was to study the effect of a dietary HUFA deficiency on flatfish metamorphosis through the measure of growth and the expression of genes involved in some key metabolic processes occurring during metamorphosis: lipid digestion, oxidative stress, and growth. The model we used was winter flounder, a common inshore flatfish geographically distributed from Labrador (Atlantic Canada, 53° N) to Georgia (southeast United States, 33° N) (Scott and Scott 1988). Since the 1970s, this species has been identified as a promising candidate for coldwater marine aquaculture due to its tolerance to a wide range of temperatures (from -1.9 to 25°C; Duman and Devries 1974; Fletcher and Smith 1980) and salinities (from 3 to 40; McCracken 1963), its good response to gamete stripping as well as the possibility of cryopreserving sperm (Rideout et al. 2003), and its high commercial value (Fairchild et al. 2007).

Starting at mouth opening, three groups of winter flounder larvae were fed rotifers enriched with different blends of microalgae providing different HUFA profiles. The expressions of genes involved in growth (*growth hormone gh*), lipid digestion (*bile salt-activated lipase bal*,

and *triacylglycerol lipase tag*), and oxidative stress (*superoxide dismutase sod*) were surveyed for 30 d starting at settlement; these were compared to gene expressions in pelagic larvae just prior to settlement.

Materials and methods

Fish rearing conditions

All experiments were conducted at the Station aquicole de Pointe-au-Père (ISMER / UQAR, 48° 27' N; 68° 32' W, QC, Canada), and all fish manipulations were done according to the Canadian Council of Animal Protection recommendations and protocols approved by the University's Animal Care Committee.

Egg stripping and fertilization were done according to Ben Khemis et al. (2000). Once hatched (day 0), larvae were transferred into nine 55 L cylindro-conical tanks (density: 250 larvae L^{-1}) placed in a temperature-controlled room (10°C) and exposed to a 12:12 (light:dark) photoperiod cycle. These tanks were supplied with flowing filtered ambient sea water except during the feeding period (09:00–17:00), when flow was stopped. A permanent up-welling was maintained in each tank by the aeration system placed at the bottom of a vertical strainer. From mouth opening (4 days post-hatching, dph) until the end of the experiment, larvae were fed the rotifer *Brachionus plicatilis* (5 ind. ml^{-1}) enriched with one of three different microalgal diets to modify their fatty acid profiles (see Seychelles et al. 2009 for the enrichment protocol): 1) the Cocktail diet (*Nannochloropsis oculata*, *Isochrysis galbana*, and *Pavlova lutheri*), containing a balanced combination of EPA, DHA, and AA (EPA/DHA/AA = 3.8/2.9/1), 2) the Nanno diet (*N.*

147 *oculata*), with a low level of DHA (EPA/DHA/AA = 3.4/0.5/1), and 3) the Tiso diet (*I. galbana*),
148 with low levels of EPA and AA (EPA/DHA/AA = 1.3/6.3/1). The fatty acid composition of each
149 diet is reported in Table 1. Three larval tanks were used for each experimental diet (N = 3 per
150 diet).

151 When settlement occurred (~ 45 dph), newly settled larvae were collected every three
152 days and transferred into rectangular tanks (35.5 × 65 × 6.5 cm). Each replicate tank contained
153 300 individuals. Post-settled larvae were reared according to Fraboulet et al. (2010), using
154 flowing filtered seawater (50 µm, 2 L min^{-1}) under natural conditions of temperature (10.4 ±
155 1°C), salinity (28.8 ± 1.3), and photoperiod (artificial light 400 lux). Each day, post-settled larvae
156 were fed the same diet as during pelagic larval stage (*i.e.*, Nanno, Tiso, or Cocktail; 5 rotifers ml^{-1}
157 at 11:00, 13:00, and 16:00), supplemented with 10 microdiet meals (Gemma wean;
158 www.skretting.com) every 30 min between 09:00 and 11:00 and between 13:30 and 15:30.
159 Seawater flow was stopped between 09:00 and 12:00 and between 13:00 and 17:00 to avoid
160 rotifer loss. Water was renewed between 12:00 and 13:00 and overnight. Dead individuals and
161 excess microdiet were removed every day and tanks were cleaned every two weeks.

162

163 Rotifer culture and sampling

164

165 Rotifers were cultured in triplicate in 18 L tanks and enriched with fresh microalgae produced in
166 a semi-continuous system in a closed photobioreactor (Seychelles et al. 2009). Microalgae were
167 added once a day, with the total amount of cells provided being adjusted based on rotifer numbers
168 in the culture tanks (10^6 rotifers L^{-1}). Microalga concentration was 10×10^9 cells L^{-1} for *I.*
169 *galbana* (Tiso diet), 20×10^9 cells L^{-1} for *N. oculata* (Nanno diet), and 12×10^9 cells L^{-1} for the

Cocktail diet (1:1:1 *N. oculata, I. galbana*, and *P. lutheri*). After 72 h of enrichment, two aliquots of 20 000 rotifers from each replicate were taken for lipid analysis (N = 3 tanks per diet). Rotifers were rinsed with filtered sea water (0.2 µm) on a 50 µm filter and stored at -80°C in 1 ml dichloromethane in amber glass vials with Teflon-lined caps until lipid extraction.

Larval sampling

Larvae were sampled in the morning before their first meal (12h fast prior to sampling). At the peak of settlement, early settled larvae (S0) and pelagic larvae (PL) were sampled. Larvae were also sampled 15 (S15) and 30 (S30) days after settlement. At each sampling period, 10 individuals per tank were collected and anaesthetized (MS-222, 0.05 g L^{-1}) for growth measurements, three subsamples of five or six larvae per tank were frozen at -80°C for lipid analysis, and four subsamples of six larvae per tank were preserved in five volumes of RNA*later*® (Applied Biosystems, CA, USA) for 24h before being frozen at -80°C for gene expression measurements.

Lipid analysis

For each subsample, whole frozen larvae and enriched rotifers were weighed and homogenized (Dounce homogenizer) at 4°C in dichloromethane/methanol (2:1 v/v). Total lipids were extracted (Folch et al. 1957) with chloroform replaced by dichloromethane. Lipid classes (triacylglycerols TAG, free sterols ST, phospholipids PLP, acetone-mobile polar lipids AMPL, free fatty acids FFA, and ketones KET) were determined on 4 µl of total lipids by thin-layer chromatography with flame ionization detection (TLC-FID) using an Iatroscan MK6 (Shell USA, VA, USA;

194 Parrish 1987). Extracts were spotted onto chromarods coated with silica gel (SIII, Shell USA),
195 and a three-stage development system was used. Chromatograms were recorded using
196 PeakSimple software (v3.21, SRI Inc., CA, USA), and peak areas were quantified using
197 calibration curves obtained from scans of standards (Sigma Chemicals, Inc., MO, USA). Lipid
198 classes were calculated in μg of lipids per mg of dry mass, summed, and expressed as percentages
199 of total lipids.

200 Total lipid extracts were dried and fatty acid methyl esters (FAMEs) were prepared
201 (Lepage and Roy 1984) and analyzed in mass spectrometry scan mode (ionic range: 60–650 m/z)
202 on a Polaris Q ion trap coupled to a trace gas chromatography GC (Thermo Finnigan,
203 Mississauga, ON, Canada) equipped with a Valcobond VB-5 capillary column (VICI Valco
204 Instruments Co. Inc., Broakville, ON, Canada); data were treated using Xcalibur v.1.3 software
205 (Thermo Scientific, Mississauga, ON, Canada). FAMEs were identified by comparing retention
206 times with known standards (Supelco 37 Component FAME Mix and menhaden oil; Supleco
207 Inc., Belfonte, PA, USA). Data acquisition and processing were performed using the Excalibur
208 2.1 software (ThermoScientific, Fisher, ON, Canada).

209

210 Growth measurements

211

212 Total length, standard length (*i.e.*, notochord length), and maximum width were measured using a
213 micrometer (± 0.001 mm) on ten larvae per tank (N = 3 tanks per treatment) at PL, S0, S15, and
214 S30 stages.

215

216 Primer design for superoxide dismutase *sod,* growth hormone *gh*, and glyceraldehyde-3
217 phosphate dehydrogenase *gapdh* cloning and sequencing

Primers were designed from mRNA sequences to obtain PCR products ranging from 90 to 150 bp using Primer Express® software v.3.0 (Applied Biosystems, CA, USA). While the mRNA sequences for the *tag* and *bal* genes were available for *P. americanus* (Benson et al. 2005), those for *sod*, *gh*, and *gapdh* were not. Primers for *sod*, *gh*, and *gapdh* were designed from sequences available for *Platichtys flesus* and *Paralichtys olivaceus*. Sequences of primers used for each gene, the percentages of similarity between the sequences obtained, the source sequences and the length of the amplicon obtained are presented in Table 2.

PCR reactions using the newly designed primers were carried out on a Mastercycler® epGradient S (Eppendorf) in a total volume of 25 µl containing 5 µl of cDNA (initial concentration: 500 ng μl^{-1}), 2.25 µl of each forward and reverse primers (10 µmol L^{-1}), 2.5 µl of buffer (Expand High Fidelity PCR Buffer 10X with $MgCl_2$; Roche diagnostics, QC, Canada), 0.1 µl of DNA polymerase (Expand High Fidelity 3.5 U/µl, Roche diagnostics, QC, Canada), 1 µl dNTP (2.5 mM Mix, Roche diagnostics, QC, Canada), and 11.9 µl of sterile water. Thermal cycling was initiated with 10 min at 95°C then 2 min at 50°C, followed by 40 cycles consisting of 15 sec at 95°C and 1 min at 60°C. A last cycle of 10 min at 60°C was performed to obtain poly-A tails for future cloning.

For each gene, the amplicon obtained with the newly designed primers was sequenced to verify whether its sequence corresponded to the targeted gene sequence. The amplicon was first cloned using the TOPO TA Cloning Kit for Sequencing® (Invitrogen Inc., ON, Canada). Plasmid cDNA was extracted using the EZNA Plasmid Mini Kit I® (Omega Bio-Tek, GA, USA) and sequenced in forward and reverse directions using the Big Dye Terminator v3.1 Cycle sequencing kit (Applied Biosystems, CA, USA). The sequencing reactions were performed with a PerkinElmer DNA Thermal Cycler 480 in a total volume of 10 µl containing 3 µl of cDNA, 1 µl

of 5 X buffer (Applied Biosystems, CA, USA), 2 µl of Big Dye Terminator v3 chemistry® (Applied Biosystems, CA, USA), 2 µl of forward and reverse primers (1.0 µM) for plasmid (T3 or T7; Applied Biosystems, CA, USA), and 2 µl of sterile water. The sequencing parameters were as follows: one minute at 95°C, 35 cycles of 10 sec at 96°C, 5 sec at 50°C, and 4 min at 60°C. Unincorporated nucleotides were removed using Ultra-Step Dye Terminator Removal Kit® (EaZy Nucleic Isolation, Ezna, Omega Bio-Tek, GA, USA). Electrophoresis was carried out using an ABI 3130 Genetic Analyzer (Applied Biosystems, CA, USA). For each gene, the sequence specificity was verified using BLAST software (Altschul et al. 1990).

Gene expression measurements

Relative expressions of *sod*, *bal*, *tag*, and *gh* were determined as in Vagner et al. (2013). Briefly, total RNA was extracted from 30 mg of larvae in three tanks per treatments using an RNeasy Plus Mini Kit® (Qiagen, Inc., ON, Canada) according to the manufacturer's instructions. Total RNA purity and concentrations were determined using the 260 nm / 280 nm absorbance ratio measured with a Nanodrop ND-1000 Spectrophotometer v3.3.0 (NanoDrop Technologies, Inc., DE, USA). RNA purity was also assessed by running an aliquot of all RNA samples on 1.2% agarose gel stained with ethidium bromide. The 260nm / 280nm ratio for all samples ranged from 1.6 to 2.0, and the intensity ratio of the 28s and 18s rRNA bands was always approximately 2:1.

Duplicate measures of cDNAs were immediately obtained by reverse transcription on 1 µg of total RNA for each sample using a Quantitect Reverse Transcription kit® with integrated removal of genomic DNA contamination (Qiagen, Inc., ON, Canada). cDNA concentrations were determined using a Nanodrop spectrophotometer. cDNA duplicates were pooled for each sample and stored at -20°C until analyses. For each gene, qPCR analyses were performed in duplicate

(Biorad MyiQ I cycler, Bio-Rad Laboratories, Inc., ON, Canada) on each pool of cDNA in a total volume of 15 µl containing 5 µl of cDNA (mean initial concentration 20.0 ± 2.4 µg ml^{-1}), 0.5 µl of primers (10 µmol l^{-1}), 1.5 µl of sterile water, and 7.5 µl of 2X iQ SYBR Green Supermix® (Bio-Rad Laboratories, Inc., ON, Canada). Thermal cycling of real-time PCR was initiated with an incubation at 95°C for 13.5 min for activation of the hot-start enzyme, iTaq™ DNA polymerase. After this initial step, 45 cycles of PCR were performed. Each PCR cycle consisted of 30 s at 95°C for denaturing, 60 s at 60°C for annealing, and 30 s at 72°C for extension. To test the amplification specificity, the PCR product was subjected to a melting curve analysis during qPCR assays: the 45 cycles for cDNA amplification were followed by one minute at 95°C, 60 s at 55°C, and 80 cycles consisting of 0.5°C increments from 55°C to 90°C for 10 s each.

Cycle threshold values (CT) correspond to the number of cycles during which the fluorescence emission monitored in real time exceeds the threshold limit. CT values were automatically calculated on the log curve for each gene.

To determine the relative quantity of target-gene–specific transcripts present in each subsample, CTs were averaged for each duplicate and then for each tank, and relative expression was calculated according to equation 1 (Livak and Schmittgen 2001):

$$2^{-\Delta\Delta\text{^CT}} = 2\text{^}{-(\Delta CTe - \Delta CTc)} \qquad 1$$

$CTe = CT_{\textit{target gene}} - CT_{\textit{reference gene}}$ for sample x,

$CTc = CT_{\textit{target gene}} - CT_{\textit{reference gene}}$ for the calibrator.

In our study, the calibrator was the pelagic larval stage fed the Cocktail diet (PL-Cocktail group) and the reference gene was *gapdh,* which was already used for this species by Vagner et al. (2013). To test the stability of *gapdh* gene expression between samples and developmental stages, standard curves were established for each developmental stage in triplicate by plotting the CT

289 values against the $\log_{10}$ of five different dilutions (in triplicate) of a pool of a representative
290 cDNA sample solutions.
291
292 Statistical analyses
293
294 All statistical tests were performed with Statistica 6 for Windows (Statsoft v.6.1, Tulsa, OK,
295 USA). Normality and homoscedasticity of data were tested using Kolmogorov-Smirnov and
296 Levene tests, respectively. In order to meet these conditions, gene expression values were log (x
297 + 1) transformed and fatty acids in percentages were arcsine–square-root transformed. The effect
298 of microalgal treatments on rotifer fatty acid content was tested using one-way ANOVA. The
299 effects of developmental stage and diet on fish growth (total length, standard length, and width),
300 gene expression, and fatty acid content were tested using two-way ANOVA. A test of slope
301 homogeneity followed by an ANCOVA was performed on *gapdh* gene expression to show its
302 stability among developmental stages. When significant effects were found, the unequal Tukey
303 test was applied if ANOVA assumptions were met; the Fisher LSD test was applied on rank-
304 transformed data if homoscedasticity was violated (Quinn and Keough 2002). Differences were
305 considered significant at $P < 0.05$.
306
307 **Results**
308
309 Lipid composition of diets
310
311 No significant difference was found among diets with respect to lipid classes, but the fatty acid
312 proportions were significantly different (Table 1). The Tiso diet was characterized by (i) the

313 highest content of 18:3*n*-3 (3.7 ± 1.4% of total FA) and by an EPA (20:5*n*-3) content (1.9 ± 0.0%
314 of total FA) roughly six- and five-fold lower than in the Nanno and Cocktail diets, respectively.
315 The 14:0, 18:2*n*-6c, and 18:4*n*-3 FA contents were respectively two-, two-, and four-fold higher
316 in the Tiso diet than in the Nanno diet, while the 16:0, 16:1, and AA (20:4*n*-6) FA contents were
317 respectively two-, six-, and two-fold higher in the Nanno diet compared to the Tiso diet. DHA
318 (22:6*n*-3) was approximately five- to six-fold lower in the Nanno diet (1.7 ± 0.2% of total FA)
319 than in the Cocktail and Tiso diets, respectively (7.7 ± 1.9 and 9.5 ± 0.5% of total FA). No
320 significant difference was found between diets with respect to the content of other FA ($P > 0.05$).
321
322 Fatty acid and lipid composition of pelagic larvae, early-settled larvae, and post-settled larvae
323
324 Diet greatly influenced the FA composition in early developmental stages (Table 3). The 14:0
325 and MUFA contents were significantly lower in the groups fed the Nanno diet than in the other
326 two for all developmental stages (14:0: $F_2 = 13.96$, $P < 0.001$; MUFA: $F_2 = 14.34$, $P < 0.001$). At
327 S30, the accumulation of 14:0 and MUFA was 69% and 59% lower in post-settled larvae fed with
328 Nanno-enriched rotifers than in the groups fed Cocktail- and Tiso-enriched rotifers, respectively.
329 Larvae fed the Nanno diet had a 70% lower 17:0 content than those fed the Cocktail diet (F_2=
330 4.6, $P < 0.05$). They had 74% less 18:1*n*-7 ($F_2 = 6.87$, $P < 0.01$), 36% less 22:1*n*-9 ($F_2 = 4.74$, P
331 < 0.05), 32% less 20:5*n*-3 ($F_2 = 4.1$, $P < 0.05$), and 81% less PUFA ($F_2 = 3.69$, $P < 0.05$) than
332 larvae fed the Tiso diet but 20% higher SFA ($F_2 = 10.6$, $P < 0.01$) compared to larvae fed the
333 other two diets.
334 FA composition varied during winter flounder development (Table 3). MUFA content (F_3
335 = 7.40, $P < 0.01$) was 19% higher at S0 than at S15. Moreover, EPA ($F_3 = 5.60$, $P < 0.01$), DHA
336 ($F_3 = 7.40$, $P < 0.01$), and EFA ($F_3 = 5.93$, $P < 0.01$) contents were 71, 68, and 69%, respectively,

337 higher at S30 than at S0. DHA content also increased significantly (two-fold higher) between S15
338 and S30 ($F_3 = 7.40$, $P < 0.01$).

339 AA, EPA, and DHA available from the Cocktail diet seemed to be sufficient for larval and
340 post-settled larval development, since the ratios between organism FA content and dietary FA
341 content were always below one for that treatment (Fig. 1). However, we observed strong selective
342 retention for DHA from settlement to S30 with the Nanno diet and for EPA (from S15) and AA
343 (from S30) with the Tiso diet, with organism/diet ratios above one (Copeman et al. 2002). This
344 suggests that the availability of essential fatty acids from the Nanno and Tiso diets were below
345 the physiological needs of early settled (S0) and post-settled (S15 and S30) larvae.

346 No significant differences were found between developmental groups for the main lipid
347 classes associated with structural lipids or energy reserves (PLP, TAG, ST, and AMPL; $P > 0.05$;
348 Table 3). A significant interaction between diet and developmental stage was observed for KET
349 content ($F_{16} = 5.40$, $P < 0.01$), but no clear pattern emerged.

350

351 Growth performance

352

353 Total length varied from an average of 6.60 ± 0.08 mm in PL, S0, and S15 to 7.48 ± 0.17 mm in
354 S30 ($F_3 = 9.96$, $P < 0.001$; Fig. 2a). Between PL and S0, body width increased significantly by a
355 factor of 1.6, remained unchanged between S0 and S15, and increased again from S15 to S30 (F_3
356 = 30.70, $P < 0.001$; Fig. 2b). No significant effect of diet or interaction between developmental
357 stage and diet was observed for growth in total length, standard length (data not shown), or
358 maximum width ($P > 0.05$; Fig. 2).

359

360 Gene expression

A test of slope homogeneity ($F = 0.00$, $N = 4$, $P > 0.05$) followed by an ANCOVA ($F = 0.11$, $N = 4$, $P > 0.05$) did not reveal any effect of developmental stage on *gapdh* expression, allowing its use as a housekeeping gene.

While *bal* gene expression was not influenced by diet ($P > 0.05$), it differed significantly among development stages (Fig. 3a). It was 2.5 times higher at S30 than at PL and S0 for all dietary groups ($F_3 = 4.74$, $P < 0.05$). Relative gene expression was intermediate at S15, indicating that the expression activation had already begun at this stage. The *tag* gene expression was not affected by diet or by developmental stage ($P > 0.05$; Fig. 3b).

The *gh* and *sod* gene expressions differed according to both developmental stage and diet ($F_6 = 2.66$, $P < 0.05$; Fig. 3c and $F_6 = 2.85$, $P < 0.05$; Fig. 3d). In young winter flounder fed the Cocktail diet, *gh* gene expression increased significantly and continuously from PL to S30: it was about 5-fold higher at S15 than PL and about 16-fold higher at S30 than PL (Fig. 3c). In those fed the Nanno diet, it increased only by about 4-fold from PL to S30, while it increased with the Tiso diet by about 7-fold from PL to settlement before stabilizing. The *sod* gene expression decreased significantly by 2-fold from PL to S15 in the Cocktail group and remained stable during this period with the two other diets. It increased significantly by about 2- to 3-fold from S15 to S30 in all treatments (Fig. 3d).

Discussion

Effect of dietary HUFA levels on the FA profile of pelagic, early settled, and post-settled larvae

384 The low amounts of essential fatty acids (EFA) present in the Tiso and Nanno diets were not
385 reflected in the fatty acid composition of pelagic larvae. However, in larvae that had initiated
386 metamorphosis, a selective retention of DHA, indicating potential deficiency, appeared at
387 settlement in the group fed the Nanno diet, and selective EPA and AA retention appeared from
388 S15 in the group fed the Tiso diet. These results suggest that EFA levels in the Nanno and Tiso
389 diets were below the physiological needs of early settled and post-settled larvae, and that the
390 larvae had to retain the low levels of EPA, AA, and DHA in tissues to support growth and
391 development during metamorphosis. Such a process was suggested for yellow tail flounder
392 (Copeman et al. 2002) and sea scallop *Placopecten magellanicus* larvae (Pernet and Tremblay
393 2004). Pelagic and settled larvae that were fed rotifers enriched with the microalgal mix (Cocktail
394 diet) did not show any selective retention of DHA, EPA, or AA, indicating that this enrichment
395 seemed adequate to support the physiological needs in EFA during metamorphosis (Copeman et
396 al. 2002; Pernet and Tremblay 2004).

397 The low levels of EFA in the groups fed the Nanno and Tiso diets could indicate that,
398 before S0 (for the Nanno group) or S15 (for the Tiso group), (1) the dietary EFA content satisfied
399 larvae needs, or (2) the larvae were able to produce EFAs from FA precursors (18:2*n*-6 or 18:3*n*-
400 3) using desaturation and elongation processes. The first hypothesis is the most likely, since it is
401 generally considered that elongation/desaturation processes are of minor importance in marine
402 fish that require preformed HUFA, contrary to freshwater fish (see Glencross 2009 and Tocher
403 2010 for reviews). From settlement, it is possible that the DHA content in the Nanno diet is no
404 longer sufficient to sustain the high metabolic demand during this developmental stage. Our
405 results suggest that the requirement for DHA increases at settlement while those for EPA and AA
406 increase 15 days later. These results could be related to winter flounder lifestyle in the natural
407 environment. Indeed, prior to settlement, pelagic carnivorous larval fish, such as flatfish larvae,

have access to abundant DHA sources in the pelagic food chain (Drake and Arias 1993; Kainz et al. 2004) through copepods and bivalve veligers that are known to be rich in DHA (Holland 1978; Sargent and Falk-Peterson 1988; Morehead et al. 2005). Following settlement, benthic-dwelling flatfish are exposed mainly to an abundance of EPA via diatoms and polychaetes, which are particularly rich in this HUFA (Kates and Volcani 1966; Graeve et al. 1997; Cabral 2000; Copeman and Parrish 2003). It is important to note that the period of settlement coincides with the introduction of the commercial diet to supplement the enriched rotifer diet in our experiment. However, this commercial diet was quantitatively and qualitatively similar for all dietary groups. We thus assume that the fatty acid compositions of all groups were similarly impacted by this inert diet, and consequently that the differences in fatty acid composition observed between groups are only related to rotifer enrichments.

Effect of dietary HUFA levels on growth performance

Despite the selective retention measured in the Nanno and Tiso groups, which indicates a potential EFA deficiency (Copeman et al. 2002; Pernet and Tremblay 2004), growth performance in width and total length were similar in all three groups, indicating that DHA, EPA, and AA were sufficient in all three diets to sustain normal growth in winter flounder. The similar growth rates among groups despite EFA deficiencies could also be explained by the presence of MUFA and SFA in fish tissues. MUFA and SFA are considered as the fuel for fish growth and can easily be synthesized by fish (Sargent et al. 2002). While we found $\sum$ MUFA and $\sum$ SFA to be lower in the Nanno group than in the other two dietary groups, these low levels did not represent deficiencies since the ratios between fish content and dietary content were always below one in the three groups (results not presented; Copeman et al. 2002; Pernet and Tremblay 2004).

Moreover, similar growth rates could also be explained by a good balance between HUFA, which is required for the functional integrity of cell membranes, and the less unsaturated FA required for energy (Sargent 1995). For instance, Villalta et al. (2005) observed a lower growth rate in Senegalese sole fed a high DHA dietary content compared to those fed a DHA-deficient diet once fish became benthic. These authors explained their results by the reduction of dietary MUFA in the DHA-enriched diet.

Several studies have highlighted the importance of dietary EPA/AA and DHA/EPA ratios rather than the individual dietary FA contents in sustaining higher growth rates since each of these FA plays a specific physiological role (Sargent 1999; Sargent et al. 2002; Bell et al. 2003; Zuo et al. 2012). A higher growth rate was reported at 19°C in sea bream *Sparus aurata* fed rotifers with a DHA/EPA ratio of 1.5 compared to those fed rotifers with a DHA/EPA ratio < 0.6 (Rodriguez et al. 1997). Moreover, Zuo et al. (2012) reported a higher growth rate for a DHA/EPA ratio between 2.17 and 3.04 in yellow croacker reared between 21.5 and 30°C. In the present study, the DHA/EPA ratio varied widely among the three diets, from 0.15 ± 0.02 for the Nanno diet to 5.04 ± 0.02 for the Cocktail diet, but these differences did not affect growth performance. Sargent (1999) found growth to be markedly impaired in several larval fish species when the EPA/AA ratio was below 1.5. Such impairment was not observed in our study, where the dietary EPA/AA content was not significantly different among groups and varied between 1.26 ± 0.02 and 3.93 ± 1.19. Thus our results indicate that the DHA/EPA and EPA/AA ratios used were not limiting growth performance in winter flounder reared at 10°C, as opposed to what has been observed for other fish species. Such differences may be explained by the different temperatures used in the different studies, since growth has already been shown to be affected by both temperature and dietary HUFA content in marine fishes (Person-Le Ruyet et al. 2004; Skalli et al. 2006). It is known that elevated temperature increases cellular turnover (Hagar and Hazel

1985), which should increase the need for HUFA to make new cell membranes. The EPA/DHA/AA ratios should then be more critical at high temperatures because of higher cell turnover.

Effect of dietary HUFA levels on lipid reserves

The low EFA levels measured in the Nanno and Tiso groups did not affect lipid reserve accumulations during winter flounder metamorphosis as shown by the similar TAG contents as well as by the similar *tag* gene expression measured in all groups. Moreover, the TAG ratio between fish content and diet content measured in all groups from PL to S30 was below one (results not presented), revealing that TAG was not incorporated into fish tissues. The TAG enzyme is responsible for the degradation of triglycerides to FAs available for energy (Henderson and Tocher 1987). This lack of accumulated lipid reserves could be due to their immediate utilization during metamorphosis to support fast growth and metamorphosis. This hypothesis is in accordance with the significant increase in body width measured at S0 and S30 and in body length measured at S30. The absence of accumulated lipid reserves in fish could also be the consequence of increased β-oxidation due to a sufficiently high n-3 HUFA level in all groups, as has been observed in Atlantic salmon (Kjaer et al. 2008; Todorcevic et al. 2009). Increased β-oxidation would reduce FA availability (the substrate for TAG synthesis) and thus reduce TAG synthesis. The mechanisms underlying the reduction are not known in fish, nor have they been completely elucidated in mammals (see Shearer et al. 2012 for a review). Along with stable TAG reserves, we also measured stable *bal* gene expression in all groups from the pelagic larval stage (about 45 dph) to 30 days post settlement (about 75 dph), suggesting a stable capacity for lipid digestion throughout the studied developmental stages. Our results could also suggest that—

480 whatever the levels of DHA, EPA, and AA in the diet treatments—they were not limiting factors
481 for pancreas development since *bal* is mostly produced by the pancreas in winter flounder.
482
483 Effect of diet on the expression of genes coding for growth hormone and antioxidative defences
484
485 While low HUFA levels did not affect growth performance or lipid reserves, it reduced *gh* gene
486 expression in the Tiso and Nanno groups. Although *gh* expression was continuously stimulated in
487 the Cocktail group from PL to S30, it remained at the settlement level in the Tiso and Nanno
488 groups. This suggests that an essential combination of EPA, AA, and DHA—as in the Cocktail
489 group—is required to sustain the up-regulation of this gene expression throughout metamorphosis
490 in winter flounder. Thus *gh* gene expression could be an indicator of development at the
491 molecular level in response to the dietary HUFA quality during winter flounder metamorphosis.
492 The selective retention indicating potential EFA deficiency that was observed in the
493 Nanno and Tiso groups may have limited the reduction of antioxidative defences, while the
494 combination of n-3 and n-6 HUFA, as in the Cocktail group, would instead have reduced the
495 level of antioxidative defences through a lowering in the reactive oxygen species (ROS)
496 concentration in cells. Indeed, *sod* gene expression decreased in the Cocktail group at S15 while
497 it remained stable in the two others. It is known that the Sod activity correlates well with ROS
498 production (Mourente et al. 2007). The high levels of EPA and AA in the Nanno diet as well as
499 the high level of DHA in the Tiso diet may have promoted oxidative stress in cells, as suggested
500 for Atlantic salmon (Todorcevic et al. 2009). Moreover, a recent study reported that Sod activity
501 increased significantly in juvenile grass carp with increasing dietary HUFA content (Ji et al.
502 2011).

503 The higher *sod* gene expression measured at S30 in all dietary groups may be related to an
504 increased metabolic rate towards the end of metamorphosis due to the increase in growth rate
505 measured at the same time (*gh* gene expression, total length, and maximum width) (Aceto et al.
506 1994; Fernández-Díaz et al. 2001; Vagner et al. 2013). The increased growth rate would have led
507 to increased oxygen uptake, which may have the potential to increase ROS production in the
508 early life stages of fish. This higher *sod* gene expression could be a final response to strong
509 metabolic changes occurring throughout metamorphosis, as suggested in common dentex
510 (Mourente 1999) and Senegalese sole (Solé et al. 2004; Fernández-Díaz et al. 2006). Our results
511 are in accordance with previous studies on rainbow trout (Fontagné et al. 2008), *Salmo iridaeus*
512 (Aceto et al. 1994), and several other fish species (Rudneva 1999), all of which reported
513 increasing *sod* gene expression during larval development.
514
515 **Conclusion**
516
517 This study reveals the increased requirement of DHA from settlement in winter flounder while
518 the EPA and AA content seem critical starting 15 days later. The lower HUFA content in the Tiso
519 and Nanno diets had no effect on larval growth performance or lipid reserve accumulations. The
520 *gh* gene expression could be an indicator of development at the molecular level in response to the
521 dietary HUFA quality during metamorphosis in winter flounder. The results indicate that
522 potential EFA deficiencies may limit antioxidative defences, and a combination of n-3 and n-6
523 HUFA (as in the Cocktail group) may be necessary to reduce oxidative stress in winter flounder
524 during metamorphosis. Overall results also suggest that the *gh* gene expression could be a
525 valuable indicator of development in response to the dietary EFA quality during metamorphosis.
526

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785 *irritans*). Aquaculture 334-337: 101-109. doi:10.1016/j.aquaculture.2011.12.045.

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788 **Table 1** Lipid composition (lipid classes: % of total lipids, mean ± SD; fatty acids: % of total
789 fatty acids, mean ± SD) of rotifers enriched with the Tiso (*Isochrysis galbana*), Nanno
790 (*Nannochloropsis oculata*), and Cocktail (*Isochrysis galbana, Nannochloropsis oculata, Pavlova*
791 *lutheri*) diets (N = 3 tanks per diet). Only FAs with a content > 2% are presented. Diets not
792 sharing a common letter are significantly different ($P < 0.05$)

793

Lipid class	Cocktail diet		Tiso diet		Nanno diet		*p*	*F*	*df*
	Mean	SD	Mean	SD	Mean	SD			
KET	70.9	6.5	41.8	23.7	36.0	25.8	0.33	1.7	2
TAG	7.2	8.6	17.1	17.8	22.6	8.0	0.53	0.8	2
ST	0.0	0.0	7.5	0.3	1.1	1.5	0.06	7.8	2
AMPL	9.2	2.7	11.2	8.3	23.9	11.5	0.30	1.8	2
PLP	12.1	0.3	22.5	2.2	16.4	7.7	0.22	2.6	2
Fatty acid									
14:0	7.5^{ab}	0.7	11.3^{b}	0.9	4.8^{a}	1.2	0.03	16.0	2
16:0	18.3^{ab}	0.3	14.2^{a}	0.9	25.2^{b}	1.4	0.00	16.0	2
18:0	2.7	1.1	2.4	0.0	2.8	0.3	0.70	0.4	2
22:0	3.3	2.0	3.0	0.1	2.8	0.4	0.95	0.1	2
24:0	1.0	1.3	2.9	0.1	2.8	0.3	0.16	3.7	2
16:1	11.7^{ab}	2.0	2.6^{a}	0.2	16.6^{b}	2.4	0.02	16.0	2
18:1*n*-9c	7.2	0.9	12.8	0.5	6.9	0.2	0.13	4.3	2
18:1*n*-7	2.4	0.4	2.8	0.4	1.4	0.3	0.06	8.6	2
18:2*n*-6c	3.3^{ab}	0.4	5.0^{b}	0.0	2.4^{a}	0.0	0.03	16.0	2
18:3*n*-3	2.0^{b}	0.4	3.7^{c}	1.4	0.8^{a}	0.1	0.01	24.0	2
18:4*n*-3	4.8^{ab}	0.4	6.2^{b}	0.1	1.5^{a}	0.2	0.03	16.0	2
20:4*n*-6 (AA)	2.6^{ab}	0.6	1.5^{a}	0.1	3.4^{b}	0.0	0.03	16.0	2
20:5*n*-3 (EPA)	9.9^{b}	2.1	1.9^{a}	0.0	11.6^{b}	0.7	0.02	18.4	2
22:6*n*-3 (DHA)	7.7^{b}	1.9	9.5^{b}	0.5	1.7^{a}	0.2	0.03	15.5	2
Σ SFA	53.6	11.9	52.9	0.9	56.8	4.1	0.58	0.7	2

Σ MUFA	25.7	0.9	25.1	0.4	26.8	3.0	0.72	0.4	2
DHA/EPA	0.8	0.0	5.0	0.1	0.2	0.0	0.00	1658	2
EPA/AA	3.9	1.2	1.3	0.0	3.4	0.1	0.13	4.2	2
Σ PUFA	37.0	2.3	36.3	1.0	29	1.4	0.17	3.5	2
Σ EFA	20.2	3.5	12.9	0.6	16.7	0.5	0.13	4.3	2
Total FA (mg g^{-1})	14.3	6.2	13.2	0.5	13.6	1.3	0.98	0.02	2

794 KET: ketone, TAG: triglyceride, ST: sterols, AMPL: acetone-mobile polar lipids, PLP:
795 phospholipids, AA: arachidonic acid, EPA: ecosapentaenoic acid, DHA: docosahexaenoic acid,
796 SFA: saturated fatty acids, MUFA: monounsaturated fatty acids, PUFA: polyunsaturated fatty
797 acids, EFA: essential fatty acids, FA: fatty acids.
798

Table 2 Primers used for *Pseudopleuronectes americanus* in qPCR analysis. The GenBank accession number identifies the sequence of the species used for primer design. The size of the PCR amplicon (bp) as well as the percentage of similarity obtained between the sequence of the amplicon and that of the GenBank species are provided

Target	Primer sequence (5' → 3')	Sequence used for primer design (GenBank accession number)	Sequence similarity (%)	Amplicon size (bp)
tag	F: GTGGCTTCGACGAGAAAAAC R: AAGTCAAACGCTGCCAGTCT	*P. americanus* (AF512562)	99	138
bal	F: GGACAACGCCTACTCCACAT R: GCCTGTGTAGGAACCAGGAA	*P. americanus* (AF512561)	98	116
sod	F: TGGAGACAACACAAACGGG R: CATTGAGGGTGAGCATCTTG	*Platichthys flesus* (AJ291980)	95	138
gh	F: CCTGAAGCTGATAGAGGCCAAT R: GGAGCACCGAACTCTCAGAGA	*Paralichtys olivaceus* (M23439)	96	76
gapdh	F: CAACGGCGACACTCACTCCTC R: TCGCAGACACGGTTGCTGTAG	*P. olivaceus* (AB029337)	85	87

tag: *triacylglycerol lipase*, *bal*: *bile salt-activated lipase*, *sod*: *superoxide dismutase*, *gh*: *growth hormone*, *gapdh*: *glyceraldehyde-3 phosphate dehydrogenase*.

Table 3 Fatty acid composition of early developmental stages of winter flounder fed Cocktail (C; *Isochrysis galbana*, *Nannochloropsis oculata*, *Pavlova lutheri*), Tiso (T; *Isochrysis galbana*), and Nanno (N; *Nannochloropsis oculata*) enriched diets at the pelagic larval stage (PL), at settlement (S0), and 15 (S15) and 30 (S30) days after settlement. Results are expressed in % of total lipids (TL) ± SD, and TL (first line) is expressed in mg of fatty acid per g of dry matter. Only FA with content > 2% of TL are presented. *: $P < 0.05$; **: $P < 0.01$; ***: $P < 0.001$. The letters d, s, and d × s indicate respectively an effect of diet, developmental stage, and their interaction. NS indicates that no significant effect was observed. Groups not sharing a common letter are statistically different ($P < 0.05$)

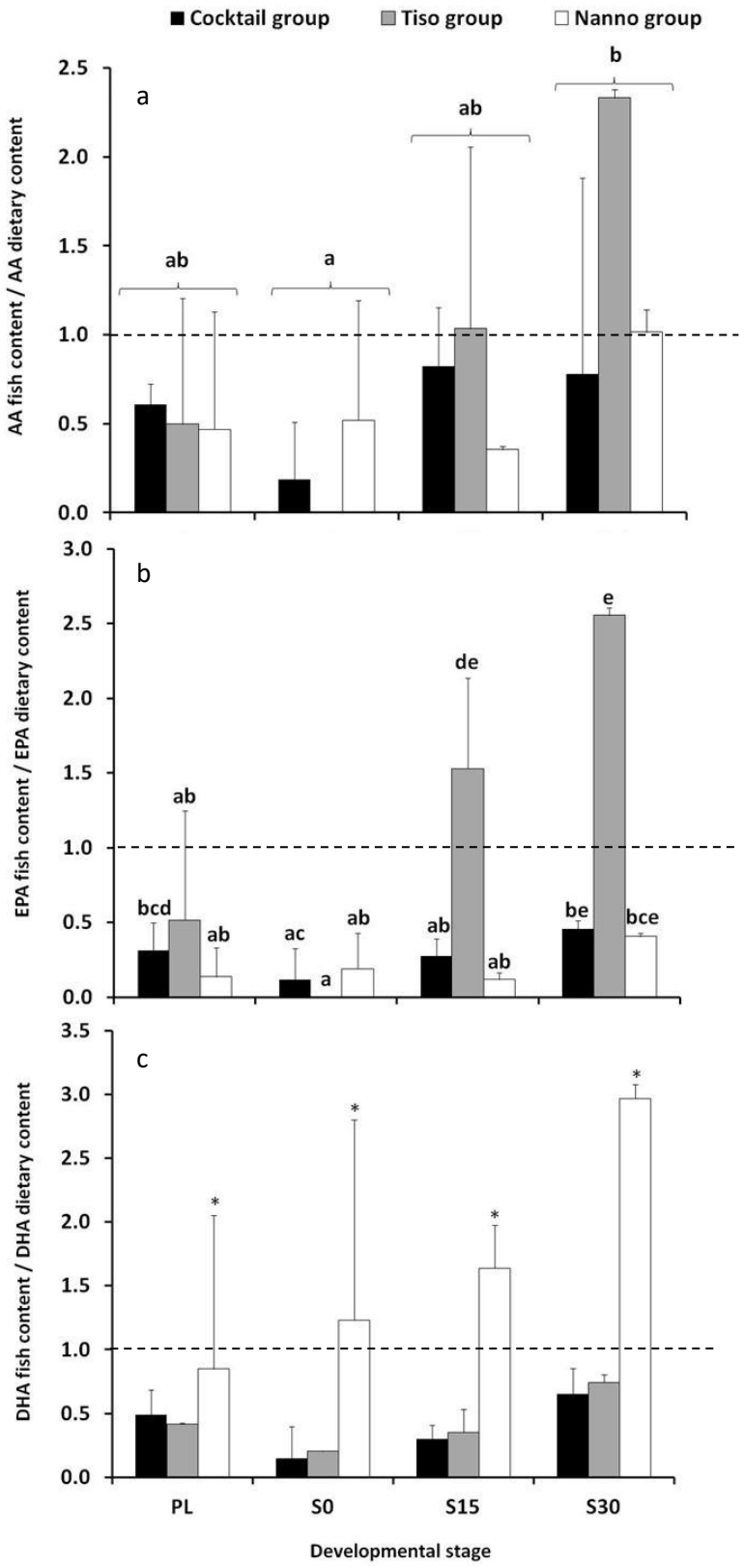
	PL			S0			S15			S30		
	C	T	N	C	T	N	C	T	N	C	T	N
TL NS	64.0±56.5	50.2±1.9	31.3±1.9	20.1±11.4	63.9±33.3	45.9±36.2	66.4±36.7	49.1±14.1	63.1±16.5	84.9±45.6	34.2±0.3	155.2±174.4
PLP NS	55.8±9.4	52.5±9.6	69.5±9.5	68.2±2.9	63.5±0.4	59.7±12.5	61.4±4.5	62.3±8.3	56.5±4.3	62.7±0.1	61.5±5.6	60.5±8.9
TAG NS	14.0±7.2	11.3±1.1	0.0±0.0	5.1±5.8	2.8±1.0	5.7±2.5	5.2±7.3	1.1±1.9	2.7±2.1	0.0±0.0	6.7±9.5	0.0±0.0
ST NS	14.8±3.8	21.3±5.5	12.4±4.7	11.8±6.5	18.7±4.8	19.1±4.7	26.3±6.7	23.3±2.8	15.3±1.1	19.5±4.1	18.2±12.9	22.1±5.6
KET d × s **	5.05±2.7^{c}	4.08±1.6^{bc}	1.1±1.6^{ab}	1.6±1.4^{ab}	2.8±0.5^{abc}	0.7±1.2^{a}	0.0±0.0^{a}	2.2±3.9^{ab}	9.5±3.6^{c}	5.6±2.5^{c}	1.3±1.9^{ab}	5.6±2.2^{c}
AMPL NS	10.3±6.8	10.8±4.6	14.4±10.3	13.4±12.2	12.2±5.0	14.6±12.4	7.1±3.9	11.0±7.1	15.9±0.3	11.9±6.4	11.7±8.0	11.7±12.4
14:0 d **	3.1±0.3 N < C; N < T; C = T	1.3±1.9	1.2±1.7	2.5±0.6	3.3±0.2	2.2±0.8	1.5±2.1	2.7±0.6	0.0±0.0	3.7±1.0	2.2±0.1	0.0±0.0
16:0 NS	10.7±0.3	11.2±1.4	12.8±4.1	13.9±1.6	12.1±0.7	16.7±3.0	10.7±1.4	12.6±2.4	10.5±4.0	15.2±4.8	10.3±1.7	13.3±4.7
17:0 d*	2.5±0.4 N < C; C = T; C = N	1.1±1.5	2.2±3.2	2.7±0.9	2.7±0.2	1.4±1.3	1.3±1.8	2.2±0.5	0.0±0.0	3.0±1.0	1.8±0.1	0.0±0.0
19:0	36.7±8.3	35.9±7.1	55.1±2.8	36.8±12.4	39.5±4.8	36.4±10.6	46.0±22.6	30.8±5.4	69.8±11.5	25.1±4.6	23.5±3.6	64.0±8.1

d** N < C; N < T; C = T												
20:0 NS	3.1±1.5	2.8±1.0	0.6±0.9	2.3±2.0	3.2±1.6	2.6±1.4	2.1±3.0	3.1±1.8	1.1±0.2	5.1±1.7	2.9±0.2	0.7±0.9
22 :0 NS	2.9±2.8	2.9±4.1	0.0±0.0	3.0±3.0	3.2±0.1	2.6±2.3	3.4±4.8	5.0±2.9	0.8±1.2	3.8±0.3	3.9±0.9	0.0±0.0
23 :0 d** N < C; N < T; C = T	2.2±0.8	2.6±0.8	0.0±0.0	1.8±1.6	1.1±1.6	0.0±0.0	1.8±2.6	1.2±1.1	0.0±0.0	1.6±0.5	1.7±1.0	0.0±0.0
24 :0 NS	2.2±2.2	0.0±0.0	1.9±2.7	3.4±3.3	1.8±2.5	1.0±0.9	1.3±1.8	1.6±1.4	3.7±5.2	0.0±0.0	1.0±1.4	1.4±2.0
18:1n-9c NS	6.3±0.4	6.2±0.4	6.0±5.4	6.3±1.7	6.5±0.2	6.6±0.7	5.8±1.9	6.8±0.6	2.4±3.4	7.6±1.5	6.1±0.5	5.1±0.5
18:1n-7 d** N < T; C = N; C = T	2.9±0.4	2.8±0.4	0.0±0.0	2.1±0.5	2.9±0.2	2.2±1.9	0.7±1.0	2.5±0.5	0.0±0.0	2.3±1.3	2.6±0.3	0.0±0.0
20:1 NS	1.8±1.2	2.1±0.8	0.8±1.1	1.7±1.5	2.3±1.2	2.0±0.5	1.4±0.2	1.1±1.1	0.0±0.0	1.4±0.4	2.4±0.2	1.7±0.5
22:1n-9 d* N < T; C = N; C = T	1.2±1.2	1.3±1.8	0.4±0.6	1.2±1.3	2.6±1.2	1.6±1.4	0.5±0.7	1.0±0.9	0.0±0.0	0.0±0.0	1.0±1.4	0.0±0.0
24:1 s* S0 > S15; PL = S0; PL = S15; PL = S30; S0 = S30; S15 = S30	2.0±1.8	1.4±0.3	0.6±0.9	3.5±1.3	2.5±1.3	1.7±0.5	0.6±0.8	0.7±1.2	0.0±0.0	0.6±0.8	1.1±1.6	1.2±0.3
18:2n-6c NS	1.5±0.2	0.8±1.1	2.3±3.2	0.5±0.8	0.0±0.0	2.7±3.8	2.8±1.6	1.6±1.7	2.2±0.7	1.8±2.6	3.3±0.4	3.7±0.7
AA NS	1.4±0.3	0.7±0.9	1.4±2.0	0.4±0.7	0.0±0.0	1.3±2.2	1.9±0.7	1.4±1.3	1.1±0.1	1.8±2.5	3.1±0.1	3.1±0.4
EPA s* S0 < S30; PL = S0; PL = S15; PL = S30; S0 = S15; S15 = S30	2.7±1.6	0.9±1.2	1.4±2.0	1.0±1.8	0.0±0.0	2.0±2.5	2.4±1.0	2.5±1.0	0.8±1.1	3.9±0.5	4.2±0.1	4.2±0.2
DHA s** S30 > S0; S30 > S15; PL = S0; PL = S15; PL = S30; S0 = S15	3.3±1.3	3.5±0.1	1.3±1.8	1.0±1.7	1.7±0.0	1.9±2.4	2.0±0.7	2.9±1.5	2.5±0.5	4.4±1.3	6.2±0.5	4.5±0.2
22:5n-3 d* N < C; N < T; C = T	1.0±0.9	0.7±1.0	0.0±0.0	0.5±0.8	0.0±0.0	0.0±0.0	0.7±1.0	1.5±1.4	0.0±0.0	0.8±1.1	2.5±1.3	0.0±0.0
Σ SFA d** N < C; N < T; C = T	72.2±2.9	71.7±4.2	84.4±8.3	78.7±7.6	79.6±1.8	76.9±5.6	76.4±3.4	70.2±14.5	92.7±0. 1	71.6±10.8	55.8±7.2	87.6±1.8
Σ MUFA d*** : N < C; N < T; C = T; s** : S15 < S0; PL = S0; PL = S15; PL = S30; S0 = S30; S15 = S30	16.0±2.6	17.7±0.6	7.8±2.8	16.6±1.0	18.7±1.9	16.8±3.4	11.2±4.6	14.6±3.3	5.4±5.5	13.4±0.6	18.0±1.7	10.3±0.4
Σ PUFA d* N < T; C = N; C = T	11.8±2.7	10.6±4.9	7.8±11.1	4.7±8.1	1.7±0.0	6.3±4.1	12.5±1.2	15.3±11.2	2.8±4.0	15.1±11.4	26.3±5.5	2.2±1.4
Σ EFA s** S30 > S0; PL = S0; PL = S15; PL = S30; S0 = S15; S15 = S30	7.3±2.5	5.0±2.1	4.1±5.8	2.4±4.2	1.7±0.0	5.4±6.9	6.2±2.4	6.8±3.6	4.8±1.1	10.0±4.3	13.4±0.5	11.7±0.3

Abbreviations: AA: arachidonic acid 20:4n-6, AMPL: acetone-mobile polar lipids, d: diet, DHA docosahexaenoic acid 22:6n-3, EFA essential fatty acids, EPA: ecosapentaenoic acid 20:5n-3, i: interaction between diet and developmental stage; KET: ketones, MUFA:

816 mono-unsaturated fatty acids, NMI: non-methylene-interrupted, NS: not significant; PLP: phospholipids, PUFA: poly-unsaturated fatty
817 acids, s: developmental stage, SFA: saturated fatty acids, ST: sterols, TAG: triglycerides

Fig. 1


Cocktail group
Tiso group
Nanno group
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ab
a
ab
b
b
EPA fish content / EPA dietary content
bcd
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ab
ac
a
ab
ab
de
ab
be
e
bce
c
DHA fish content / DHA dietary content
*
*
*
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PL
S0
S15
S30
Developmental stage

821 **Fig. 2**

822

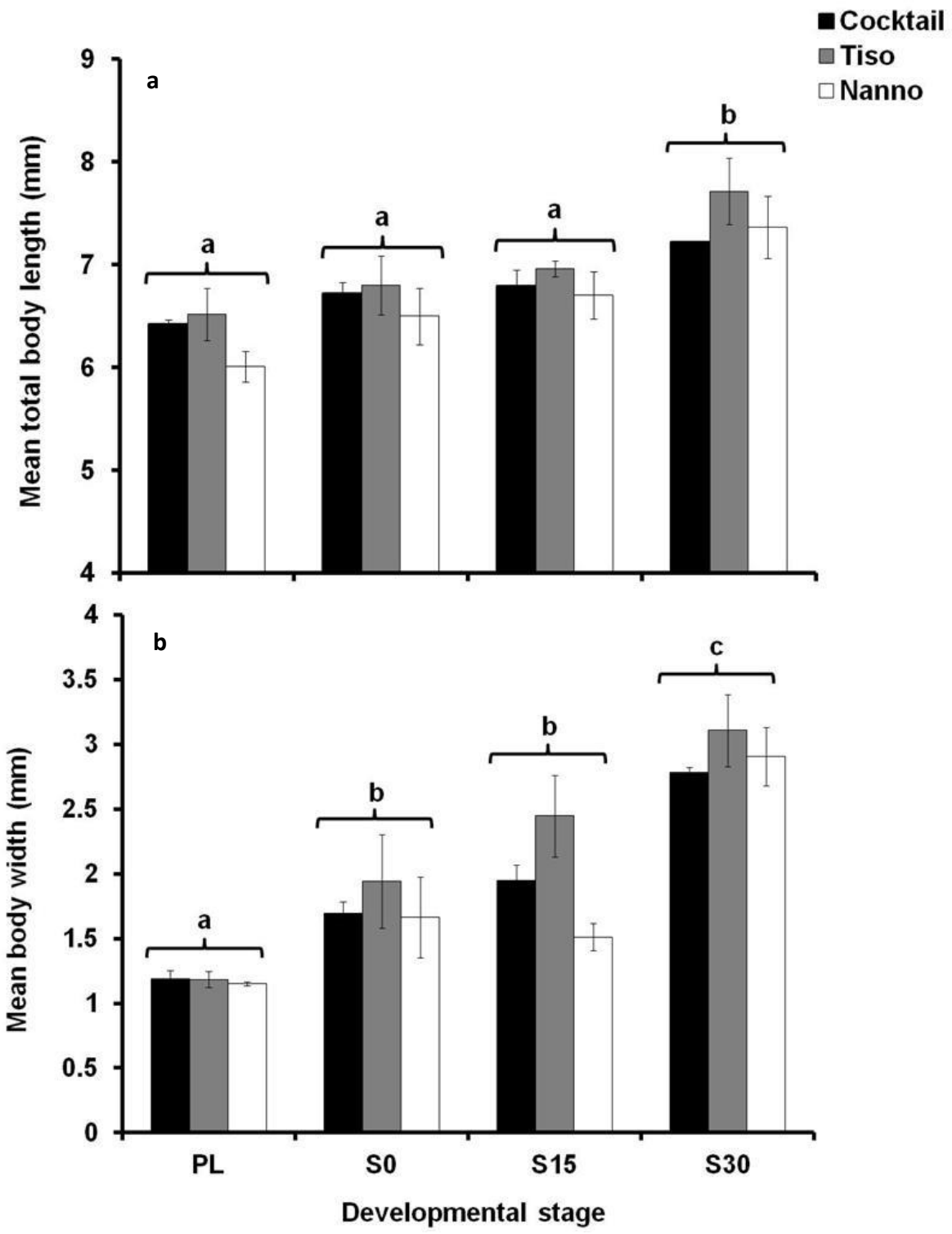

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Fig. 3

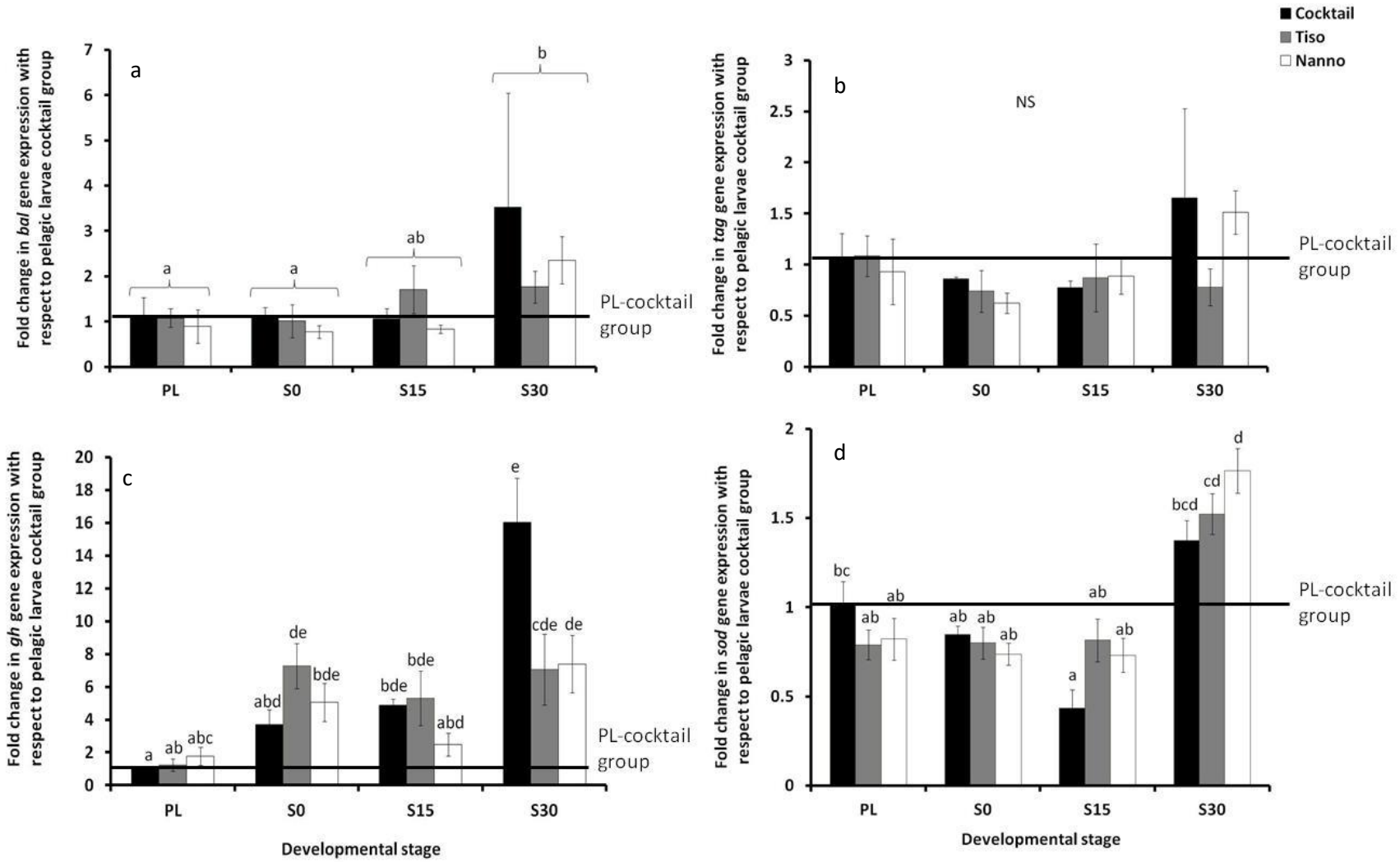
Cocktail
Tiso
Nanno
a
Fold change in bal gene expression with respect to pelagic larvae cocktail group
PL-cocktail group
PL
S0
S15
S30
b
NS
Fold change in tag gene expression with respect to pelagic larvae cocktail group
c
Fold change in gh gene expression with respect to pelagic larvae cocktail group
Developmental stage
d
Fold change in sod gene expression with respect to pelagic larvae cocktail group

Figure captions

Fig. 1 Change in fatty acid (FA) ratios between fish FA content and dietary FA content for the three main polyunsaturated fatty acids found in fish membranes by developmental stage: (a) arachidonic acid (AA; 20:4*n*-6), (b) ecosapentaenoic acid (EPA; 20:5*n*-3), and (c) docosahexaenoic acid (DHA; 22:6*n*-3). Results are expressed as mean ± SD. PL: pelagic larvae; S0: at settlement; S15: 15 days after settlement; S30: 30 days after settlement. The dotted lines indicate the 1:1 ratio. For each graph, bars or developmental groups not sharing a common letter are significantly different at $P < 0.05$; Dietary groups sharing an asterisk are significantly different from the others at $P < 0.05$

Fig. 2 Growth performance of *Pseudopleuronectes americanus* during development and according to diet: (a) total body length and (b) maximum body width in mm (mean ± SE) by developmental stage (PL: pelagic larvae; S0: at settlement; S15: 15 days after settlement; and S30: 30 days after settlement). Developmental groups not sharing a common letter are significantly different (two-way ANOVA; $P < 0.05$)

Fig. 3 Fold-change in (a) *bile salt-activated lipase* (*bal*), (b) *triacylglycerol lipase* (*tag*), (c) *growth hormone* (*gh*), and (d) *superoxide dismutase* (*sod*) gene expression in *Pseudopleuronectes americanus* with respect to pelagic larvae of the Cocktail group (mean ± SE; $N = 4$ subsamples of 6 larvae per tank) and according to developmental stage (PL: pelagic larvae; S0: settlement; S15: 15 days after settlement; and S30: 30 days after settlement). Bars not sharing a common letter are significantly different ($P < 0.05$). The black horizontal line indicates the level of gene expression in the reference group (PL-Cocktail group) from which the gene expression of other groups was calculated (two-way ANOVA; $P < 0.05$)