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Comparative study on fatty acid metabolism of early stages of two crustacean species: *Artemia* sp. metanauplii and *Grapsus adscensionis* zoeae, as live prey for marine animals

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Abstract

The present study compared the lipid composition and *in vivo* capability of *Artemia* sp. metanauplii (the main live prey used in aquaculture) and *Grapsus adscensionis* zoeae (as a wild zooplankton model) to metabolise unsaturated fatty acids. The two species were incubated *in vivo* with 0.3 μM of individual [1-^{14}C]fatty acids (FA) including 18:1n-9, 18:2n-6, 18:3n-3, 20:4n-6 (ARA), 20:5n-3 (EPA) and 22:6n-3 (DHA) bound to bovine serum albumin (BSA). Compared to metanauplii, zoeae contained twice the content of polar lipids (PL) and eight-fold the content of long-chain polyunsaturated fatty acids (LC-PUFA). *Artemia* sp. metanauplii showed increased short chain fatty acid *de novo* synthesis from beta-oxidation of [1-^{14}C]LC-PUFA, preferentially DHA of the LC-PUFA, DHA showed the highest esterification rate into *Artemia* sp. triacylglycerols. In contrast, in *Grapsus* zoeae [1-^{14}C]DHA displayed the highest transformation rate into longer chain-length FAs and was preferentially esterified into PL. EPA and ARA, tend to be more easily incorporated and/or retained than DHA in *Artemia* sp.. Moreover, both EPA and ARA were preferentially esterified into *Artemia* PL, which theoretically would favour their bioavailability to the larvae. In addition to the inherent better nutritional value of *Grapsus* zoeae due to their lipid composition, the changes taking place after lipid enrichment of both prey points at two distinct models of lipid metabolism that indicate zoeae as a more suitable prey than *Artemia* sp. for marine animals.

Key words: *Artemia* sp. metanauplii; *Grapsus adscensionis* zoeae; Lipid metabolism; Marine larvae; Unsaturated fatty acids.

1. Introduction

In nature, marine animals feed on a wide spectrum of zooplankton and phytoplankton during their early life stage, which provides them with a complete and balanced diet. Nonetheless, mass production of zooplankton for large-scale aquaculture is still a challenge due to difficult logistics and cost-effective protocols (Støttrup and Norsker, 1997; Iglesias et al., 2007; Conceição et al., 2010). For that reason, more economical alternative protocols for marine larval production are required. Despite the recent progress in the development of inert diets (Hamre et al., 2013), the rearing of early life stages of marine animals still depends on the use of live feeds (Conceição et al., 2010).

Within live feeds, the larvae of the crustacean *Artemia* sp. is widely used in rearing of marine larvae because of its high availability and acceptance by a large number of species (Sorgeloos et al., 2001).

Artemia sp. is an Anostraca Branchiopod which has a wide geographical distribution, characterized by its adaptability to wide ranges of salinity (5-250 g/L) and temperature (6-35 ºC; Triantaphyllidis et al., 1998), and to varied nutrient resources as a non-selective filter feeder. Despite its adaptability to a wide range of environmental conditions, it is absent in most marine ecosystems, living in permanent salt lakes and coastal lagoons where bacteria, protozoa and algae are the base of the *Artemia* sp. diet (Amat, 1985). It is known that *Artemia* sp., among other nutritional issues, naturally possess high contents of neutral lipids (NL) and low content of long-chain polyunsaturated fatty acids (LC-PUFA), such as 20:5n-3 (EPA), and especially 22:6n-3 (DHA), which are essential fatty acids (EFA) for normal development of marine fish larvae (Sargent et al., 1999). In this respect, enrichment of *Artemia* sp. is used to tailor its lipid composition towards the nutritional needs of marine larvae (Van Stappen, 1996). A typical *Artemia* sp. enrichment protocol includes, for example, the incubation of newly hatched *Artemia* nauplii with lipid emulsions added every 12 h at 24-28 ºC during 24 h at densities around 2g cysts/L, dissolved oxygen near saturation and strong illumination (around 2000 Lux at water surface; Van Stappen, 1996; Sorgeloos et al., 2001). These lipid emulsions are basically formed by micelles (droplets) of triacylglycerols (TAG) from fish oils stabilised with emulsifiers, such as lecithin, which are filtered by *Artemia* sp. from seawater (Conceição et al., 2010). However, *Artemia* sp. naturally presents a low content of phospholipids (Navarro et al., 1991) and this enrichment protocol tends to promote an additional increase of NL.

Phospholipids are considered important for the development of marine species (Tocher et al., 2008; Cahu et al., 2009; Olsen et al., 2014; Li et al., 2015), not just to provide phosphorus or choline/inositol to larvae, but as a better way to provide EFA (see, Sargent et al., 1999 and Tocher et al., 2008). Therefore, in order to compensate the natural high amount of NL in *Artemia* sp., enrichment protocols based on increasing the polar lipid (PL) fraction of this organism have been attempted (McEvoy et al., 1996; Monroig et al., 2006; Guinot et al., 2013a). However, Navarro et al. (1999) showed retroconversion (partial beta-oxidation) of DHA, whereas Guinot et al. (2013b) verified that even after an enrichment period of only 4 h, DHA provided as PL to *Artemia* sp. metanauplii was actively metabolised and incorporated into NL classes, mainly TAG. It is thus important to consider this not merely as a passive carrier of fatty acids (FA), as these nutrients tend to be digested, incorporated and metabolised by *Artemia* sp. (Navarro et al., 1999), which may compromise the lipid enrichment of *Artemia* sp. required for rearing larval marine species.

Compared to *Artemia* sp., wild zooplankton species used in aquaculture, such as copepods and crustacean zoeae, naturally have lower total lipids and higher contents of phospholipids and EFA (McEvoy et al., 1998; Evjemo et al., 2003; Iglesias et al., 2014; Olsen et al., 2014; Reis et al., 2015), which could at least partly explain the better results obtained in the rearing of cultured marine species including, Atlantic halibut (Næss et al., 1995; Evjemo et al., 2003) and Atlantic cod (Imsland et al., 2006; Busch et al., 2010) larvae, or common octopus paralarvae (Iglesias et al., 2014; Reis et al., 2015). Nonetheless, the better growth and survival obtained when wild zooplankton is provided to larvae might go beyond the amount of EFA or PL, as the presence of EFA in specific lipid classes such as the PL, and the total or individual LC-PUFA contents and ratios are critical factors for marine larvae (Sargent et al., 1999; Olsen et al., 2014).

Among zooplanktonic marine organisms, zoeae from decapod crustacean have recently been shown to improve octopus paralarvae nutrition (Reis et al., 2015). *Grapsus adscensionis* is a marine Malacostraca decapod (Shcherbakova et al., 2011) with widespread distribution living in rocky shorelines and beaches (Henderson 2002), also adapted to a wide range of salinities (Evans 2009). It is an omnivorous and opportunistic feeder including cirripids, mytilids, other invertebrates, food remains and grazed algae in its feeding habits (Shcherbakova et al., 2011) On the basis of their superiority to *Artemia* sp. in the larval culture of commercially valuable species (Carro, 2004; Reis et al., 2015),

and their abundance in the Canary Islands, *G. adscensionis* zoeae were selected as a model of wild zooplankton in the present study.

The aim of the present study was to gain new insight into lipid metabolism of *Artemia* sp. nauplii and *G. adscensionis* zoeae. Therefore, we determined the *in vivo* capability of *Artemia* sp. metanauplii and *G. adscensionis* zoeae to assimilate, esterify into different lipid classes, and transform unsaturated FA, to determine differences between live prey, and to improve the design of *Artemi*a sp. enrichment protocols for marine larvae production.

2. Materials and Methods

2.1. Experimental animals

Artemia sp. nauplii were obtained by hatching EG *Artemia* cysts (INVE Aquaculture, Belgium). Following the protocol of Sorgeloos et al. (2001), 2 g of *Artemia* sp. cysts were decapsulated with bleach, followed by deactivation with $Na_2S_2O_3$ dissolved in filtered seawater (0.02% w/v). Incubation of cysts was performed over 24 h in a 3 L cylindro-conical fiberglass tank containing filtered seawater (36‰) at 28 ºC, with continuous light and vigorous aeration. After hatching, nauplii were separated from hatching wastes and placed in similar tanks with fresh filtered seawater at 24 ºC for 8 h until instar II stage (metanauplii stage - mouth and anus opening) was reached. Prior to incubation with radiolabelled FA substrates, metanauplii were filtered and concentrated in 400 mL of filtered seawater and metanauplii density was determined.

G. adscensionis broodstock (40 adult individuals) were caught off the N and NE coasts of Tenerife (Canary Islands, Spain) and reared in 3,000 L cylindro-conical fibreglass tanks in a flow-through system, under natural photoperiod (13L:11D) with a natural water temperature of 21 °C and salinity of 36‰. The tank water column was ~10 cm in height and the water flow was 6 L/min. Crabs were fed daily *ad libitum* on a diet consisting of 50% (w/w) frozen mackerel (*Scomber scombrus*) and squid (*Loligo opalescens*). Newly hatched crab zoeae were collected with a 500 µm mesh placed at out-flow system. Prior to incubation with radiolabelled fatty acid substrates, zoeae were thoroughly sorted from algae and other organisms found in the broodstock rearing tank, and placed into filtered seawater at similar salinity.

2.2. In vivo incubation of prey with labelled [1- ^{14}*C] fatty acids*

Artemia sp. metanauplii and *G. adscensionis* zoeae were incubated in 6-well flat-bottom tissue culture plates (Sarstedt AG & Co., Nümbrecht, Germany) in 10 mL of filtered seawater using a protocol adapted from Reis et al. (2014). Incubations were performed for 5 h at a density of 10,000 metanauplii or 1,000 zoeae per incubation well, with gentle stirring at 24 ºC and 21 ºC, respectively, with 0.2 µCi (0.3 µM) of [1-^{14}C]FA including 18:1n-9, 18:2n-6, 18:3n-3, ARA, EPA or DHA (n = 4). The [1-^{14}C]FA were added individually to separate wells, as their potassium salts bound to bovine serum albumin (BSA), as described by Ghioni et al. (1997). Control treatments of metanauplii and zoeae without addition of [1-^{14}C]FA were also assessed. A survival rate of 92 ± 4% was obtained over all incubations.

After incubation, *Artemia* sp. metanauplii and *Grapsus* zoeae were filtered with a 100 µm mesh and washed thoroughly with filtered seawater to remove excess radiolabelled FA. Extraction of total lipids (TL) was performed with chloroform/methanol (2:1, v/v) essentially according to the Folch method as modified by Christie (2003). The organic solvent was evaporated under a stream of nitrogen and lipid content determined gravimetrically. The TL extracts were stored until analysis at a concentration of 10 mg/mL in chloroform/methanol (2:1, v/v) with 0.01% butylated hydroxytoluene (BHT) as antioxidant at -20 ºC under an inert atmosphere of nitrogen.

2.3. *Lipid class and fatty acid composition* Artemia *sp. metanauplii and* G. adscensionis *zoeae*

Aliquots of 20 µg of TL extract of metanauplii and zoeae control groups were used to determine lipid class (LC) compositions. LC were separated by one-dimensional double-development high-performance thin-layer chromatography (HPTLC; Olsen and Henderson, 1989) on 10 cm x 10 cm plates using 1-propanol/chloroform/methyl acetate/methanol/0.25% KCL (5:5:5:2:1.8, v/v) for polar lipid class separation and hexane/diethyl ether/acetic acid (22.5:2.5:0.25, v/v) for neutral lipid class separation, and analysed by charring followed by calibrated densitometry using a dual-wavelength flying spot scanner CS-90001PC (Shimadzu Co., Japan; Tocher and Harvie, 1988). LC identification was performed by running known LC standards (cod roe lipid extract) on the same plates.

Fatty acid methyl esters (FAME) were obtained by acid-catalysed transmethylation of 1 mg of TL extract for 16 h at 50 ºC. FAME were purified by thin-layer chromatography (TLC; Christie, 2003) using hexane/diethyl ether/acetic acid (90:10:1, v/v) and then

separated and analysed using a TRACE-GC Ultra gas chromatograph (Thermo Fisher Scientific Inc., Waltham, Massachusetts, USA). The column temperature was programmed for four different ramps of temperature: 1st ramp was programmed for a linear increase of 40 ºC per minute from 50 to 150 ºC; the 2nd ramp for a linear increase of 2 ºC per minute until 200 ºC; the 3rd ramp for a linear increase of 1 ºC per minute until 214 ºC; and the 4th ramp for a linear increase of 40 ºC per minute until 230 ºC and hold at that temperature for 5 minutes. FAME were identify by comparison with retention times of a standard mixture containing F.A.M.E Mix C4-C24 (Supelco 18919-1AMP) diluted to 2 mg/mL in hexane, PUFA Nº 3 from menhaden oil (Supelco 47085-U) diluted to 2 mg/mL in hexane; and cod roe FAME. When necessary, identification of individual FAME was confirmed by GC-MS chromatography (DSQ II, Thermo Fisher Scientific Inc. Waltham, Massachusetts, USA).

2.4. Incorporation of radiolabelled fatty acids into total lipids

An aliquot of 0.1 mg of *Artemia* sp. metanauplii and *G. adscensionis* zoeae TL extract was taken to determine total radioactivity incorporated. Extracts were transferred to scintillation vials and radioactivity determined on a LKB Wallac 1214 Rackbeta liquid scintillation β-counter (PerkinElmer Inc., Waltham, Massachusetts, USA). Results in disintegration per minute (dpm) were converted into pmoles per mg protein per h of incubation (pmol/mg pp/h), considering efficiency of counting (including quenching), specific activity of each substrate, and metanauplii and zoeae total lipid and protein contents. Protein was determined in both metanauplii and zoeae according to Lowry et al. (1951).

2.5. Esterification of radiolabelled fatty acids into lipid classes

An aliquot of 0.1 mg of TL extract from radioactive samples was applied to HPTLC plates to determine the esterification of [1-^{14}C]FA into the different LC. Lipid classes were separated as described on Section 2.3. Esterification pattern of each [1-^{14}C]FA into LC was determined by image analysis following Reis et al. (2014).

2.6. Transformation of radiolabelled fatty acids

An aliquot of 0.9 to 1.1 mg of TL extract from radioactive samples was subjected to acid-catalysed transmethylation to obtain FAME as detailed above (Christie, 2003). FAME were separated by TLC using plates impregnated with a solution of 2 g silver nitrate in

20 mL acetonitrile followed by activation at 110 ºC for 30 min. TLC plates were fully developed in toluene/acetonitrile (95:5, v/v), which resolved FAME into discrete bands based on both degree of unsaturation and chain length (Wilson and Sargent, 1992). FAME identification and quantification was performed by image analysis following the method described in Reis et al. (2014).

2.7. Materials

Organic solvents used were of reagent grade and were purchased from Merck KGaA (Düsseldorf, Germany), Sigma-Aldrich Co. (St. Louis, Missouri, USA) and Panreac Química S.L.U. (Barcelona, Spain). [1-^{14}C]C18 FAs (18:1n-9, 18:2n-6 and 18:3n-3) were purchased from PerkinElmer, Inc. (Waltham, Massachusetts, USA) and [1-^{14}C] LC-PUFA (ARA, EPA, and DHA) were purchased from American Radiolabelled Chemicals, Inc. (St. Louis, Missouri, USA). BSA was purchase from Sigma-Aldrich Co. (St. Louis, Missouri, USA). TLC plates (20 × 20 cm × 0.25 mm) were purchased from Macherey-Nagel GmbH & Co. KG (Düren, Germany). HPTLC plates, (10 × 10 cm × 0.15 mm) pre-coated with silica gel 60 (without fluorescent indicator), were purchased from Merck KGaA (Düsseldorf, Germany). OptiPhase "HiSafe" 2 scintillant liquid was purchased from PerkinElmer, Inc. (Waltham, Massachusetts, USA).

2.8. Data analysis

Results are presented as means ± SD (n = 4). For all statistical tests, $p < 0.05$ was considered significantly different. Data were checked for normal distribution with the one-sample Shapiro-Wilk test, as well as for homogeneity of variances with the Levene test (Zar, 1999). Arcsine square root transformation was applied to all data expressed as percentage (Fowler et al., 1998). Comparisons between the six FA means and within [1-^{14}C]C18 FAs (18:1n-9, 18:2n-6, 18:3n-3) and [1-^{14}C] LC-PUFA (ARA, EPA, DHA) were analysed by one-way analysis of variance (ANOVA) followed by a Tukey's post hoc test (Zar, 1999). When normal distribution and/or homogeneity of the variances were not achieved, data were subjected to the Welch robust test, followed by a Games-Howell non-parametric multiple comparison test (Zar, 1999). Differences between LC and FA compositions of *Artemia* sp. metanauplii and *G. adscensionis* zoeae control groups as well as comparisons of [1-^{14}C]FAs individual incorporation into TL and its transformation rate between both species, were tested using Student´s *t*-test (Zar, 1999). The statistical analysis was performed using IBM SPSS statistics 22.0 (IBM Co., USA).

256

257 **3. Results**

258 3.1. *Lipid composition of* Artemia sp. *metanauplii and* G. adscensionis *zoeae*

259 *Artemia* sp. metanauplii TL was particularly rich in NL, with TAG being the main lipid
260 component (51.9 ± 2.3%), followed by cholesterol (14.4 ± 0.5%; Table 1). *G.*
261 *adscensionis* zoeae also presented a high proportion of NL, although lower than that of
262 metanauplii ($p < 0.05$), and TAG and cholesterol were the most abundant lipid classes.
263 Zoeae possess twice the amount of PL than metanauplii ($p < 0.05$). Within the PL fraction,
264 zoeae contained 17.9 ± 0.7% phosphatidylcholine (PC) and 12.0 ± 1.1%
265 phosphatidylethanolamine (PE) whereas metanauplii contained 7.8 ± 1.1% and 6.8 ±
266 0.8% PC and PE, respectively (Table 1).

267 The FA compositions of *Artemia* sp. metanauplii and *G. adscensionis* zoeae were also
268 substantially different (Table 2). *Artemia* sp. metanauplii were particularly rich in 18:3n-
269 3, followed by 18:1n-9 and 16:0, while *G. adscensionis* zoeae were rich in 18:1n-9, 16:0,
270 ARA, EPA and DHA. The total polyunsaturated fatty acids (PUFA) content was higher
271 in *Artemia* sp. metanauplii ($p < 0.05$), but LC-PUFA represented only 3.0 ± 0.4% of total
272 FA in metanauplii, while *G. adscensionis* zoeae contained 25.8 ± 0.7% LC-PUFA (Table
273 2).

274

275 *3.2. Incorporation of radiolabelled fatty acids into total lipids*

276 Table 3 shows the incorporation of radiolabelled FA into TL of *Artemia* sp. metanauplii
277 and *G. adscensionis* zoeae. Most notably, the incorporation of [1-^{14}C]DHA into
278 metanauplii TL was approximately only 50% of the incorporation of all other
279 radiolabelled FA substrates. All [1-^{14}C]C18 FAs were incorporated into metanauplii TL
280 at similar levels. In contrast, within LC-PUFA, [1-^{14}C]ARA showed highest
281 incorporation, whereas DHA was the lowest incorporated one ($p < 0.05$). Compared to
282 *Artemia* sp. metanauplii, the incorporation of [1-^{14}C]FA into zoeae TL was generally
283 lower, although only statistically different for 18:1n-9, 18:2n-6 and ARA (Table 3). [1-
284 ^{14}C]18:1n-9 and [1-^{14}C]DHA were the FA least incorporated into zoeae TL ($p < 0.05$).

285

286 *3.3. Esterification of radiolabelled fatty acids into lipid classes*

287 The distribution of incorporated radioactivity into lipid classes of *Artemia* sp. metanauplii
288 is presented in Table 4. All radiolabelled FA were extensively esterified by metanauplii,
289 with less than 10% of the incorporated radioactivity being recovered as free fatty acids

(FFA). After 5 h, the majority of the radiolabelled substrates were esterified into PL, mainly in PE and PC, except for [1-^{14}C]ARA that showed much higher esterification into phosphatidylinositol (PI; 26.3 ± 2.6% of radioactivity incorporated). Some differences were detected between [1-^{14}C]C18 FA and [1-^{14}C]LC-PUFA substrates, with higher (almost two-fold) esterification of [1-^{14}C]C18 FA into TAG, and of [1-^{14}C]LC-PUFA into PI and phosphatidylserine (PS). The esterification patterns of [1-^{14}C]18:1n-9 and [1-^{14}C]18:2n-6 were similar (PE > TAG > PC > sterol esters (SE) > FFA ≥ partial acylglycerols (PAG) > PI > PS). In contrast, the esterification pattern of [1-^{14}C]18:3n-3, was different to the other [1-^{14}C]C18 FA, being predominantly esterified into PE, PC and TAG. The esterification pattern of [1-^{14}C]LC-PUFA into metanauplii LC also varied between FA ($p < 0.05$; Table 4). [1-^{14}C]ARA was esterified into PI > PC > PE, [1-^{14}C]EPA into PC > PE > PI and [1-^{14}C]DHA into PC ≥ PE > TAG.

Table 5 shows the esterification pattern of FA into the TL of *G. adscensionis* zoeae. A higher percentage of [1-^{14}C] FA were recovered as FFA compared with *Artemia* sp.. [1-^{14}C]DHA was the most esterified substrate, with only 8.4 ± 1.3% of radioactivity recovered as FFA ($p < 0.05$), and it was predominantly esterified into PL (78.5 ± 3.9% of incorporated radioactivity; $p < 0.05$). While [1-^{14}C]C18 FA showed a tendency for similar esterification into PL and NL (approximately 50:50), LC-PUFA were mostly esterified into PL. [1-^{14}C]C18 FA were mainly esterified into TAG, PE and PC, while [1-^{14}C]LC-PUFA were predominantly esterified into PC and PE. Within [1-^{14}C]C18 FA, [1-^{14}C]18:3n-3 presented a slightly different pattern from [1-^{14}C]18:1n-9 and [1-^{14}C]18:2n-6 with higher esterification into PE and lower esterification into TAG ($p < 0.05$). The esterification patterns of the LC-PUFA were generally similar with the only difference found in the esterification of [1-^{14}C]DHA into PC, which was reflected in differences regarding esterification into total PL and NL, compared to the other LC-PUFA ($p < 0.05$; Table 5).

3.4. *Transformation of radiolabelled fatty acids*

The majority of radioactivity incorporated into *Artemia* sp. metanauplii and *G. adscencionis* zoeae TL was present as the unmodified FA substrate (Table 6). Nonetheless, with the exception of [1-^{14}C]18:1n-9 metabolism that was similar for both species, higher percentages of radioactivity from incubated FA were recovered in shorter chain FAs in *Artemia* sp. metanauplii ($p < 0.05$). Likely derived from recycling of labelled acetyl-CoA produced from oxidation of the [1-^{14}C]-labelled FA, recovery of radioactivity

in FA with shorter chain-lengths (fatty acids with a chain-length of 14, 16 and 18 carbons) was higher in *Artemia* sp. metanauplii for all the FA substrates incubated, although it was also evident for C18 FAs in *Grapsus* zoeae. The higher catabolism of DHA (almost 30% of incorporated radioactivity) by metanauplii was also noteworthy. Interestingly, in metanauplii this was the only fate of the FA incubated, since no elongated or desaturated FA products were detected for any of the FA substrates. In contrast, some elongation of LC-PUFA incorporated into zoeae was observed, although no desaturation products were detected from any FA substrate.

4. Discussion

The present study demonstrated the feasibility of the developed methodology to investigate the *in vivo* fate of incorporated [1-^{14}C]FA, bound to BSA and added to seawater, not only by determining transformation through elongation and desaturation, but also esterification into different lipid classes, enabling basal FA metabolism in different zooplankton species to be determined. *In vitro* incubations with radiolabelled FAs are normally performed over 2 to 3 h (Bell et al., 2001; Díaz-López et al., 2010; Rodríguez et al., 2002). Nonetheless, an incubation period of 5 h provided a higher incorporation rate, which led to enhanced visualization of the radiolabelled bands and increased validation of the data. Considering the suitability of the methodology for determining *in vivo* FA metabolism of different zooplankton species, the method may also be a useful tool to determine, not only endogenous FA metabolism, but also the effect of rearing conditions (e.g. diet, temperature, salinity) on *in vivo* FA metabolism of marine and freshwater zooplankton species. Moreover, this method may also be applied in metabolic studies on the physiology, ecology and ecotoxicology of marine organisms also applied in the frame of global change (Beaugrand, 2005; Nunes et al., 2006).

The high availability and simple hatching protocol makes *Artemia* sp. the most convenient live prey available for aquaculture (Lavens and Sorgeloos 2000; Sorgeloos et al., 2001). However, this prey was selected mainly due to its convenience of use, rather than for its nutritional value for marine animals (Conceição et al., 2010). Compared to *G. adscensionis* zoeae (a model of wild zooplankton prey), *Artemia* sp. have a higher TL content but a lower proportion of phospholipids and LC-PUFA. These differences could be related with species origin, with the *Artemia* sp. strain used having a fresh-water origin, while *G. adscensionis* is a marine species (Henderson 2002). In order to tailor *Artemia* sp. lipid composition towards the nutritional needs of marine larvae, this live

prey is normally enriched with lipid emulsions (Van Stappen, 1996; Conceição et al., 2010). Despite this, better rearing results are obtained when wild zooplankton is provided (Næss et al., 1995; Evjemo et al., 2003; Imsland et al., 2006; Busch et al., 2010; Iglesias et al., 2014; Reis et al., 2015). This might go beyond dietary LC-PUFA or phospholipid contents, as the presence of EFA in specific lipid classes and the total or individual LC-PUFA and ratios, may also influence marine larvae performance (Sargent et al., 1999; Olsen et al., 2014).

The FA profiles of marine fish phospholipids are characterised by a high pro portion of 16:0 and a relatively lower LC-PUFA content in PC; an intermediate level of saturated FA and monounsaturated FA and high levels of C20 and C22 PUFA in PE; PS is characterised by high 18:0 and C22 LC-PUFA; and PI also present a high 18:0 and relatively lower LC-PUFA but with a particularly high content of ARA (Tocher, 1995). The preservation of this characteristic FA esterification pattern among lipid classes is not only highly influenced by the endogenous capability of organisms to complete the de-acylation/re-acylation turnover processes, but also by the FA profile of diets (Tocher, 2003; Olsen et al., 2014). It is assumed that phospholipid digestion in fish occurs by the action of phospholipase A_2 (PLA_2) at the sn-2 position of phospholipids, which results in the production of 1-acyl lyso-phospholipids and FFAs (Tocher et al., 2008). In contrast, TAG digestion is performed by 1,3 lipases that cleave the FAs from sn-1 and sn-3 positions leaving 2- monoacylglycerols (MAG). LC-PUFA are generally esterified at the sn-2 position of phospholipids and TAG molecules (Sargent et al., 1999). Therefore, the action of these enzymes cleave the LC-PUFA from phospholipids molecules, but these FAs would be retained in 2-MAG obtained from TAG digestion (Tocher, 2003; Olsen et al., 2014). After dietary lipid de-acylation the pool of FFAs available for re-acylation would contain LC-PUFA from phospholipids, and saturated, monosaturated and PUFAs from TAG. As observed in the present study, *Artemia* sp. normally has high levels of TAG and PUFA, but lower levels of phospholipids and LC-PUFA (Seixas et al., 2010a, 2010b; Fuentes et al., 2011; Viciano et al., 2011; Reis et al., 2015). Consequently, the dietary FFA pool when *Artemia* sp. is used, would contain high C18 FAs and low LC-PUFA. In this sense, the probability for re-acylation of C18 FAs into phospholipids would be higher than that for LC-PUFA, influencing the characteristic FA esterification pattern of lipid classes. It is important to note, however, that after enrichment, *Artemia* nauplii incorporate preferentially exogenous fatty acids into sn-1 and sn-3 positions of TAG, especially DHA into sn-3 (Ando et al., 2002, 2004), which would be readily available for

392 the FA pool after lipase action. On the other hand, *G. adscensionis* zoeae show a high
393 content of phospholipids and LC-PUFA, which may promote a more suitable/natural
394 phospholipid FA profile.
395 As previously mention, the main features that favour the use of *Artemia* sp. in aquaculture
396 are its convenient handling and storage possibilities, as well as its availability, and the
397 possibility of enrichment with nutrients such as LC-PUFA (Van Stappen, 1996). *Artemia*
398 sp. are defined as continuous non-selective filter feeders (Reeve, 1963), where the amount
399 of incorporated FA is directly related to its abundance in the enrichment medium (Navarro
400 et al., 1999). Nonetheless, the results of the present study showed a lower incorporation
401 of DHA into metanauplii TL compared to other substrates. A substantial reduction in
402 DHA incorporation by *Artemia* sp. has been reported to occur due to a preferential (at
403 least partial) oxidation of this FA (Estévez et al., 1998; Navarro et al., 1999), which was
404 consistent with the almost 30% of shorter chain labelled FAs obtained when using DHA
405 in the present study. Moreover, it is known that *Artemia* sp. has the capacity to
406 retroconvert DHA into EPA (Navarro et al., 1999). In the present study [1-^{14}C]DHA was
407 labelled only at the C1 position, so any chain shortening of this FA would remove the
408 labelled carbon (acetyl-CoA) and the shorter FA obtained from the original labelled DHA
409 would be undetectable. *De novo* synthesis of shorter chain FAs was also evident from all
410 incubated FA substrates. Likewise, a *de novo* synthesis of shorter chain-length FAs was
411 also observed in *G. adscensionis* zoeae metabolism. Nonetheless, the catabolism of
412 labelled FAs was significantly higher in *Artemia* sp. metanauplii and for [1-^{14}C]C18 FA
413 substrates incorporated into zoeae total lipid content.
414 Despite the preferential incorporation of all FA substrates into *Artemia* sp. PL, C18 FAs
415 and DHA presented a high esterification rate into metanauplii TAG. Navarro et al. (1999)
416 and Guinot et al. (2013b) also observed a high proportion of DHA incorporated in *Artemia*
417 sp. TAG. It has been previously reported that the most appropriate form to present DHA
418 to marine fish larvae is through phospholipids (Gisbert et al., 2005; Wold et al., 2009;
419 Olsen et al., 2014). Consequently, several attempts to increase the amount of DHA in
420 *Artemia* sp. PL have been made (Rainuzzo et al., 1994; McEvoy et al., 1996; Harel et al.,
421 1999; Monroig et al., 2006, 2007; Seixas et al., 2008, 2010b; Guinot et al., 2013a).
422 Nonetheless, the high presence in *Artemia* of 18:3n-3 which may compete with DHA for
423 PC and other sn-2 phospholipid positions and the inherent redistribution of DHA from
424 dietary phospholipids to the NL fraction of *Artemia* sp., observed even during the first 4
425 h of enrichment, represent major handicaps for its enrichment with essential lipid

426 compounds and consequently for their use as live prey in larviculture (Guinot et al.,
427 2013b). When provided to *Grapsus* zoeae, [1-^{14}C]DHA was the LC-PUFA least
428 incorporated, but the FA most esterified, as well as the [1-^{14}C]LC-PUFA with the highest
429 transformation rate into larger chain-length FAs. In addition, there was a preferential
430 esterification of this FA into PL, which would favour the use of zoeae as prey for marine
431 organisms.
432 The enrichment of *Artemia* sp. with EPA or ARA appears to be less problematic than that
433 of DHA since during the enrichment process these FAs tend to be greater incorporated
434 than DHA (Dhert et al., 1993; McEvoy et al., 1996; Estévez et al., 1998). Similar to those
435 studies, the present results showed a higher incorporation rate of [1-^{14}C]ARA and [1-
436 ^{14}C]EPA into *Artemia* sp. metanauplii lipids compared to DHA. Moreover, both EPA and
437 ARA were preferentially esterified into PL, which theoretically would favour the
438 bioavailability of these FAs to the larvae. EPA and ARA are precursors of eicosanoids,
439 which are hormone-like compounds known to regulate many physiological processes,
440 including immune and inflammatory responses, cardiovascular tone, renal and neural
441 function including that related to camouflage behaviour and reproduction (Sargent et al.,
442 2002). While EPA produces eicosanoids of lower biological activity, ARA is the
443 preferred substrate and produces eicosanoids of higher biological activity in fish (Bell et
444 al., 1994). In addition, increased dietary ARA appears to have a positive effect on
445 development of fish larvae (Atalah et al., 2011a). Nonetheless, dietary ARA levels must
446 be controlled and balanced, as it can influence EPA incorporation (Villalta et al., 2005;
447 Atalah et al., 2011a, 2011b; Reis et al., 2014, 2015) and bioconversion (Sargent et al.,
448 2002; Furuita et al., 2003). Furthermore, competition between these LC-PUFA for
449 eicosanoid production can also influence fish development (Sargent et al., 2002).
450 Interestingly, *Grapsus* zoeae differed from the esterification pattern of ARA observed in
451 *Artemia* sp. (this study) and fish larvae (Bell and Sargent, 2003) as this FA was not
452 preferentially esterified into PI, but into PE and PC.
453 In summary, there are several features that favour the use of decapod crustacean zoeae as
454 live prey for marine larvae rearing compared to *Artemia* sp. which are directly related to
455 their endogenous lipid and fatty acid composition. In addition, the changes occurring after
456 lipid enrichment of both live prey suggest two distinct models of lipid metabolism that
457 could be related with species origin. *Artemia* sp. metanauplii show lower contents of PL
458 and LC-PUFA, higher FA catabolism rates, and a preferential esterification of EFA into
459 TAG. The opposite is true for *Grapsus* zoeae illustrated, for example, in preferential

esterification of EFA into PL classes. The present results not just illustrate zoeae inherent better nutritional value due to their lipid composition that may possibly make them as a more suitable live prey than *Artemia* sp., but essentially further highlight the difficulties of efficient EFA enrichment of *Artemia* as food for marine organisms.

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Table 1 – Total lipid (TL) content and lipid class composition of *Artemia* sp. metanauplii and *G. adscensionis* zoeae

	Artemia sp.	*G. adscensionis*
TL content (μg lipid/mg protein)	624.6 ± 77.8	453.0 ± 150.4
Lipid classes (%)		
Sphingomyelin	0.0 ± 0.0	0.5 ± 0.3*
Phosphatidylcholine	7.8 ± 1.1	17.9 ± 0.7*
Phosphatidylserine	1.2 ± 0.3	5.2 ± 0.1*
Phosphatidylinositol	1.4 ± 0.4	2.3 ± 0.2*
Phosphatidylglycerol	2.2 ± 0.2	3.5 ± 0.7*
Phosphatidylethanolamine	6.8 ± 0.8	12.0 ± 1.1*
∑ Polar lipids	19.4 ± 1.7	41.4 ± 2.1*
Diacylglycerols	1.5 ± 0.6	0.0 ± 0.0*
Cholesterol	14.4 ± 0.5	18.5 ± 1.9*
Free Fatty Acids	5.3 ± 2.3	2.6 ± 0.3
Triacylglycerols	51.9 ± 2.3	31.3 ± 1.0*
Sterol Esters	7.6 ± 0.9	6.2 ± 1.0
∑ Neutral lipids	80.6 ± 1.7	58.6 ± 2.1*

Results represent means ± SD; n = 4. Lipid classes are presented in percentage of total lipid content. Significance was evaluated by Student´s t-test. * Represents significant differences between *Artemia* sp. and *G. adscensionis* ($p < 0.05$).

Table 2 – Fatty acid composition (% total FA) of *Artemia* sp. metanauplii and *G. adscensionis* zoeae

	Artemia sp.	*G. adscensionis*
C16:0	13.5 ± 0.6	19.1 ± 0.2*
C18:0	7.6 ± 0.5	7.2 ± 0.2
Total Saturated[a]	24.6 ± 0.8	31.3 ± 0.2*
C16:1n-9	0.8 ± 0.1	0.4 ± 0.0*
C16:1n-7	2.8 ± 0.0	4.1 ± 0.1*
C18:1n-9	20.5 ± 0.5	24.0 ± 0.4*
C18:1n-7	7.3 ± 0.3	5.5 ± 0.1*
C20:1n-9	0.6 ± 0.1	1.1 ± 0.1*
Total Monoenes[a]	35.5 ± 0.5	38.3 ± 0.9*
C18:2n-6	5.9 ± 0.4	2.0 ± 0.1*
C20:2n-6	0.2 ± 0.0	1.5 ± 0.0*
C20:4n-6	0.3 ± 0.0	7.1 ± 0.2*
Total n-6 FA[a]	6.8 ± 0.4	11.1 ± 0.2*
C18:3n-3	25.4 ± 0.5	0.3 ± 0.0*
C18:4n-3	3.9 ± 0.1	0.0 ± 0.0*
C20:3n-3	0.8 ± 0.0	0.3 ± 0.0*
C20:4n-3	0.6 ± 0.0	0.3 ± 0.0*
C20:5n-3	1.0 ± 0.1	7.4 ± 0.3*
C22:6n-3	0.1 ± 0.0	10.0 ± 0.3*
Total n-3 FA[a]	32.8 ± 0.5	18.6 ± 0.6*
UK[b]	0.0 ± 0.0	0.8 ± 0.2*
Total PUFA[c]	39.9 ± 0.3	29.7 ± 0.7*
Total LC-PUFA [d]	3.0 ± 0.4	25.8 ± 0.7*
DHA/EPA[e]	0.1 ± 0.0	1.4 ± 0.0*
EPA/ARA[e]	2.9 ± 0.1	1.0 ± 0.0*
DHA/ARA	0.2 ± 0.0	1.4 ± 0.0*

Results represent means ± SD; n = 4. Data are presented in percentage of total fatty acids content. Significance was evaluated by Student´s t-test. * Represents significant differences between groups ($p<0.05$). [a] Totals include some minor components not shown. [b] UK – unknown. [c] PUFA – polyunsaturated fatty acids. [d] LC-PUFA – long-chain polyunsaturated fatty acids (≥ 20C and ≥ 3 double bonds)[e] ARA – 20:4n-6; EPA – 20:5n-3; DHA – 22:6n-3.

Table 3 - Incorporation of radioactivity into total lipid (pmoles/mg pp/h) of *Artemia* sp. metanauplii and *G. adscensionis* zoeae

	18:1n-9	18:2n-6	18:3n-3	20:4n-6	20:5n-3	22:6n-3
Artemia sp.	15.6 ± 2.8^{a}	18.3 ± 2.5^{a}	13.6 ± 3.9^{ab}	$19.4 \pm 2.6^{\Delta a}$	$13.4 \pm 2.7^{\circ ab}$	$6.8 \pm 0.8^{\square b}$
G. adscensionis	$3.8 \pm 1.8^{\bullet b*}$	$8.6 \pm 1.4^{\blacktriangle a*}$	$10.1 \pm 2.7^{\blacktriangle a}$	$10.1 \pm 0.9^{\Delta a*}$	$10.5 \pm 2.2^{\Delta a}$	$4.5 \pm 1.5^{\circ b}$

Results represent means ± SD; n = 4. Data are presented in pmoles of ^{14}C fatty acid incorporated/mg of protein per hour of incubation. Significance within species was evaluated by one-way ANOVA. Significance between species was evaluated by Student´s t-test. Different letters in superscript within the same row represent significant differences among all fatty acids ($p < 0.05$). Different full symbols in superscript ($^{\blacktriangle\bullet\blacksquare}$) within the same row represent significant differences between C18 fatty acids ($p < 0.05$). Different hollow symbols in superscript ($^{\Delta\circ\square}$) within the same row represent significant differences between LC-PUFA ($p < 0.05$). * Within the same column represent significant differences for a specific fatty acid between *Artemia* sp. metanauplii and *G. adscensionis* zoeae ($p < 0.05$).

Table 4 – Esterification (%) of [1-^{14}C]FA substrates into *Artemia* sp. metanauplii lipid classes

	18:1n-9	18:2n-6	18:3n-3	20:4n-6	20:5n-3	22:6n-3
Phosphatidylcholine	19.2 ± 0.9 $^{▲c}$	19.3 ± 1.2 $^{▲c}$	26.1 ± 0.8 $^{•b}$	24.1 ± 0.4 ^{b}	31.5 ± 3.6 ^{a}	24.7 ± 1.6 ^{b}
Phosphatidylserine	3.8 ± 1.0 ^{b}	3.5 ± 0.5 ^{b}	2.4 ± 1.0 ^{b}	6.7 ± 0.4 ^{Δa}	8.4 ± 0.2 $^{○a}$	6.1 ± 0.6 ^{Δa}
Phosphatidylinositol	4.1 ± 0.9 $^{▲c}$	5.5 ± 0.5 $^{•c}$	4.9 ± 0.5 $^{▲•c}$	26.3 ± 2.6 ^{Δa}	11.2 ± 1.0 $^{○b}$	9.3 ± 0.7 $^{○b}$
Phosphatidylethanolamine	29.8 ± 4.8 ^{a}	29.0 ± 5.5 ^{a}	28.0 ± 4.9 ^{a}	20.3 ± 1.3 ^{b}	23.6 ± 5.2 ^{ab}	24.0 ± 2.0 ^{ab}
∑ Polar Lipids	56.9 ± 2.5 ^{a}	57.2 ± 4.7 ^{a}	61.4 ± 3.3 ^{a}	77.5 ± 3.7 ^{c}	74.7 ± 8.8 ^{bc}	64.1 ± 2.5 ^{ab}
Partial Acylglycerols	5.2 ± 3.9	7.4 ± 2.2	4.6 ± 1.1	5.7 ± 0.9	6.0 ± 2.8	7.3 ± 0.8
Free Fatty Acids	7.4 ± 0.9 $^{▲ab}$	6.7 ± 1.0 $^{▲ab}$	4.7 ± 0.3 $^{•b}$	4.8 ± 1.4 ^{ab}	5.9 ± 2.6 ^{ab}	8.6 ± 0.6 ^{a}
Triacylglycerols	21.7 ± 2.2 $^{▲ab}$	20.7 ± 1.9 $^{▲b}$	25.5 ± 0.8 $^{•a}$	9.1 ± 0.5 ^{Δd}	9.2 ± 1.5 ^{Δd}	14.4 ± 0.6 $^{○c}$
Sterol Esters	8.8 ± 0.9 $^{▲a}$	8.1 ± 1.2 $^{▲a}$	3.8 ± 1.6 $^{•b}$	2.9 ± 0.9 ^{b}	4.2 ± 2.0 ^{b}	5.6 ± 0.9 ^{ab}
∑ Neutral Lipids	43.1 ± 2.5 ^{a}	42.8 ± 4.7 ^{a}	38.6 ± 3.3 ^{a}	22.5 ± 3.7 ^{c}	25.3 ± 8.8 ^{bc}	35.9 ± 2.5 ^{ab}

Results represent means ± SD; n = 4. Data of esterification are given in percentage. Significance was evaluated by one-way ANOVA. Different letters in superscript within the same row represent significant differences between all fatty acids ($p < 0.05$). Different full symbols in superscript ($^{▲•■}$) within the same row represent significant differences among C18 FA ($p < 0.05$). Different hollow symbols in superscript ($^{Δ○□}$) within the same row represent significant differences between LC-PUFA ($p < 0.05$).

Table 5 – Esterification (%) of [1-^{14}C]FA substrates into *G. adscensioni*s zoeae lipid classes

	18:1n-9	18:2n-6	18:3n-3	20:4n-6	20:5n-3	22:6n-3
Phosphatidylcholine	16.1 ± 2.7 ^{c}	16.6 ± 2.1 ^{c}	18.3 ± 0.5 ^{c}	28.9 ± 6.2 $^{\circ b}$	28.5 ± 1.2 $^{\circ b}$	37.4 ± 0.7 $^{\Delta a}$
Phosphatidylserine	3.7 ± 2.1	5.0 ± 0.8	4.2 ± 0.3	4.3 ± 1.3	3.8 ± 0.4	4.6 ± 0.7
Phosphatidylinositol	3.8 ± 2.2	5.3 ± 1.3	5.1 ± 1.1	5.3 ± 1.5	7.1 ± 1.1	4.8 ± 0.4
Phosphatidylethanolamine	18.2 ± 2.6 $^{\bullet d}$	21.0 ± 2.2 $^{\bullet cd}$	25.5 ± 1.3 $^{\blacktriangle bc}$	29.9 ± 1.1 ^{ab}	26.9 ± 0.9 ^{ab}	31.8 ± 4.6 ^{a}
∑ Polar Lipids	41.8 ± 5.9 $^{\bullet d}$	47.8 ± 3.2 $^{\blacktriangle\bullet cd}$	53.1 ± 2.2 $^{\blacktriangle c}$	66.1 ± 7.3 $^{\Delta\circ b}$	64.8 ± 3.0 $^{\circ b}$	78.5 ± 3.9 $^{\Delta a}$
Partial Acylglycerols	9.1 ± 1.5 $^{\blacktriangle a}$	8.7 ± 2.1 $^{\blacktriangle a}$	5.2 ± 1.2 $^{\bullet b}$	5.7 ± 1.2 ^{ab}	7.9 ± 0.6 ^{ab}	5.7 ± 0.9 ^{ab}
Free Fatty Acids	19.1 ± 1.6 ^{a}	20.3 ± 5.2 ^{a}	19.8 ± 0.7 ^{a}	14.1 ± 4.6 $^{\Delta ab}$	14.5 ± 0.2 $^{\Delta a}$	8.4 ± 1.3 $^{\circ b}$
Triacylglycerols	30.1 ± 5.2 $^{\blacktriangle a}$	23.1 ± 3.8 $^{\blacktriangle\bullet a}$	21.9 ± 2.6 $^{\bullet a}$	11.7 ± 3.7 ^{b}	11.2 ± 0.9 ^{b}	7.4 ± 2.0 ^{b}
∑ Neutral Lipids	58.2 ± 5.9 $^{\blacktriangle a}$	52.2 ± 3.2 $^{\blacktriangle\bullet ab}$	46.9 ± 2.2 $^{\bullet b}$	33.9 ± 7.3 $^{\Delta\circ c}$	35.2 ± 3.0 $^{\Delta c}$	21.4 ± 3.9 $^{\circ d}$

Results represent means ± SD; n = 4. Data of esterification are given in percentage. Significance was evaluated by one-way ANOVA. Different letters in superscript within the same row represent significant differences between all fatty acids ($p < 0.05$). Different full symbols in superscript ($^{\blacktriangle\bullet\blacksquare}$) within the same row represent significant differences among C18 FA ($p < 0.05$). Different hollow symbols in superscript ($^{\Delta\circ\square}$) within the same row represent significant differences between LC-PUFA ($p < 0.05$).

Table 6 – Recovery of radioactivity (%) from [1-^{14}C]FA substrates in FA metabolites

Substrates	Products	*Artemia* sp.	*G. adscensionis*
[1-^{14}C]18:1n-9			
	18:1n-9	89.1 ± 3.3	91.3 ± 3.0
	de novo[a]	10.9 ± 3.3	8.7 ± 3.0
[1-^{14}C]18:2n-6			
	18:2n-6	84.0 ± 3.6	94.0 ± 1.2*
	de novo	16.0 ± 3.6	6.0 ± 1.2*
[1-^{14}C]18:3n-3			
	18:3n-3	79.9 ± 6.6	91.7 ± 0.9*
	de novo	20.1 ± 6.6	8.3 ± 0.9*
[1-^{14}C]20:4n-6			
	20:4n-6	92.8 ± 3.8	97.4 ± 1.8
	22:4n-6	-	2.4 ± 2.1*
	de novo	7.2 ± 3.8	0.2 ± 0.3*
[1-^{14}C]20:5n-3			
	20:5n-3	88.3 ± 2.4	96.7 ± 1.2*
	22:5n-3	-	1.7 ± 1.5*
	de novo	11.7 ± 2.4	1.6 ± 0.3*
[1-^{14}C]22:6n-3			
	22:6n-3	70.6 ± 6.8	93.4 ± 6.9*
	24:6n-3	-	3.4 ± 5.9*
	de novo	29.4 ± 6.8	3.1 ± 3.0*

Results represent means ± SD; n=4. Data of transformation are given in percentage. Significance was evaluated by Student´s t-test. * Represents significant differences between groups ($p < 0.05$).
[a] *De novo* synthesis of fatty acids with shorter chain-length (less than 18 carbons).