Title

Enriching *Artemia* nauplii with a high DHA-containing lipid emulsion: search for an optimal protocol

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Abstract

The present study aimed to investigate practical strategies to optimise the use of a high-DHA lipid emulsion (M70), a product with great potential in live prey enrichments for marine larviculture. Considering its particularly high content in docosahexaenoic acid (22:6n-3, DHA), the adequate utilisation of the emulsion for *Artemia* enrichments was evaluated in a series of six experiments. More specifically, the bioencapsulation efficiency of M70 into *Artemia* nauplii was tested under different experimental conditions of oxygen source, aeration flow, incubation temperature, concentration and dosage, as well as nauplial densities. Our results showed that an optimal utilisation of M70 is achieved with incubation temperatures of 28 ºC, moderate aeration flows and nauplial densities of 300 ind mL^{-1}. In addition, the emulsion can be dispensed in the enrichment medium in one single dose of 0.8 g L^{-1}, with no apparent detrimental effects on its oxidative stability and *Artemia* nauplii survival during enrichment.

1. Introduction

Marine fish, and particularly their larval stages, have high requirements for highly unsaturated fatty acids (HUFA), including the physiologically active arachidonic (20:4n-6, ARA), eicosapentaenoic (20:5n-3, EPA) and docosahexaenoic (22:6n-3, DHA) acids. Whereas the essential fatty acids (EFA) requirements of freshwater and salmonid species can be met by including the C18 polyunsaturated fatty acids (PUFA) α-linolenic acid (18:3n-3, LNA) and linoleic acid (18:2n-6, LA) (Buzzy *et al*. 1996; Bell *et al*. 2004), it is generally accepted that marine species have limited capability for the biosynthesis of HUFA from the C18 PUFA, although, exceptionally, some species have been proved to express genes encoding fatty acyl desaturases and elongases involved in these biochemical pathways (Yamamoto *et al*. 2010; Monroig *et al*. 2011; Morais *et al*. 2011). In consequence, adequate levels of pre-formed ARA, EPA and DHA need to be supplied through the diet to ensure normal growth and development (Bell *et al*. 1995, 2003; Estévez *et al*. 1998, 1999; Sargent *et al*. 2002; Tocher *et al*. 2003; Benitez-Santana *et al*. 2007).

Artemia nauplii are widely used as live prey in marine fish hatcheries worldwide, due to their availability and digestibility (Léger *et al*. 1986). The nutritional value of *Artemia* nauplii, however, is not adequate for marine fish larvae since they are largely deficient in essential HUFA, particularly DHA (Koven *et al*. 1990). In order to overcome the problem, *Artemia* nauplii are enriched through incubations in seawater containing HUFA-rich products that are passively incorporate by the nauplii (Léger *et al*. 1986). This procedure is called bioencapsulation (Navarro *et al*. 1999). Many different enrichment diets have been used to enhance *Artemia* EFA contents: microalgae (Watanabe *et al*. 1982; Aragão *et al*. 2004), microcapsules (Southgate & Lou 1995), liposomes (Hontoria *et al*. 1994; McEvoy *et al*. 1996; Monroig *et al*. 2003; 2006a, b, c),

and lipid emulsions (Léger *et al*. 1986; Evjemo *et al*. 1997; Han *et al*. 2000; 2001; 2005).

Lipid emulsions are arguably the most extended enrichment diets and a big variety of products are now commercially available. Composition of commercial lipid emulsions basically consists of fish oils, with other minor components including emulsifying agents, vitamins and fatty acid (FA) derivatives such as fatty acid ethyl esters (FAEE) added to compensate suboptimal natural profiles of fish oil (Monroig *et al*. 2007). Whereas the efficiency of enrichment protocols with emulsified diets is normally higher than that of other enrichment products (Coutteau & Sorgeloos 1997; Sorgeloos *et al*. 2001), their use has been related with detrimental side effects. Among them, the autoxidation of HUFA (McEvoy *et al*. 1995; Sargent *et al*. 1997) and the consequent bioaccumulation of potentially toxic lipid peroxides into larvae fed emulsion-enriched *Artemia* (Monroig *et al*. 2006) have been described. Furthermore, autooxidation of HUFA-rich products might deplete dissolved oxygen (DO) levels in the enrichment medium and thus can further compromise the overall performance of the enrichment process as well as favour *Artemia* mortalities (Léger *et al*. 1987; Figueiredo *et al*. 2009). Importantly, the most hindering and yet unresolved drawback from the use of emulsion-based enrichers involves specific difficulties in increasing the DHA content of nauplii.

The low efficiency of DHA enrichment in *Artemia* nauplii has been acknowledged as a major obstacle for their use as live prey for first-feeding larvae of marine fish (Bell *et al*. 2003; Haché *et al*. 2011). While the bioencapsulation of other HUFA including EPA and ARA has been readily achieved (Villalta *et al*. 2005a, b), specific factors determining the limited DHA incorporation into *Artemia* appear to exist. On one hand, the autooxidation mechanisms mentioned above might be particularly severe for DHA compared to other HUFA, due to its longer chain length and higher unsaturation degree

(Cosgrove *et al*., 1987). On the other hand, biological factors related to *Artemia* physiology also need to be considered. Naturally occurring trace levels of DHA found in *Artemia* lipids (Evjemo *et al*. 1997) suggest that *Artemia* DHA requirements are low, and excessive DHA input occurring during enrichment might be compensated through DHA retroconversion to EPA (Watanabe *et al*. 1983; Furuita *et al*. 1996; Barclay & Zeller 1996; Evjemo *et al*. 1997; Navarro *et al*. 1999). In order to overcome such adverse effects, novel enrichment strategies to enhance the DHA contents of *Artemia* nauplii need to be developed.

In the present study, we investigated the efficiency of a DHA-rich oil emulsion (M70) as enrichment diet for *Artemia* nauplii. Considering the particular aspects derived from its DHA-rich nature, a series of experiments were carried out to establish an optimal use of M70 under different oxygenation regimes, incubation temperatures, enrichment product concentrations and dosage modes, as well as different nauplial densities. All the investigated parameters were previously shown as relevant aspects during live prey enrichment procedures (Van Stappen 1996; Han *et al*. 2000, 2001; Monroig *et al*. 2006a, b; Sui *et al*. 2007; Hamre & Harboe 2008; Figueiredo *et al*. 2009).

2. Materials and methods

2.1 Emulsion formulation

The experimental lipid emulsion M70 consisted of a 1:1 (v/v) oil/water suspension, of which the synthetic oil DHA Algatrium (Brudy, Barcelona, Spain) contained approximately 70% DHA of total FA in the form of ethyl esters. Xanthan gum (0.36 %) and Tween 80 (0.10 %) were used as stabiliser and emulsifier, respectively (Giner 2005). M70 formulation was developed by Archivel Technologies S.L. (Barcelona,

Spain) and the The Physical and Sensory Properties Laboratory at the Instituto de Agroquímica y Tecnología de los Alimentos (IATA-CSIC, Valencia, Spain). The FA profiles of the M70 emulsion are reported in Table 1.

2.2 *Artemia* nauplii enrichments: general conditions

Low HUFA containing *Artemia* nauplii were obtained from the hatching of EG grade cysts (Inve, Ghent, Belgium). After an incubation period of 23 h at 28 ºC, nauplii were collected and rinsed with tap water to remove the hatching metabolites and debris. Newly hatched nauplii were thereafter placed in 1 L cylinder-conical glass vessels containing seawater for further enrichment with the experimental emulsion M70. Unless otherwise stated, *Artemia* enrichments were carried out at 28 ºC, aeration of 1 L min^{-1}, diffusion system consisting of a 25-cm long and 0.5-cm-diameter section glass tube applied from the bottom of the vessel, nauplial density of 300 nauplii mL^{-1}, and product concentrations of 0.8 g L^{-1} for M70 dispensed in a single dose at the beginning of the enrichment process. All the enrichment treatments were run in triplicates (n=3). After an enrichment period of 21 h, samples of *Artemia* nauplii were collected by filtering the enrichment medium through a 100 μm mesh carefully washed with tap water in order to eliminate remains of emulsion adhered to the nauplial shells, and subsequently rinsed with distilled water. *Artemia* samples were frozen at -20 ºC and freeze-dried previous to FA analysis.

2.3. *Artemia* nauplii enrichment with M70: protocol optimisation

Optimal conditions for the use of M70 in *Artemia* enrichments were assessed by analysing the FA composition of the nauplii obtained from a series of experiments (1-6) varying the type and source of aeration, temperature, dosage and concentration of

enrichment product and nauplial density. Conditions for each subsequent experiment were established based on the results from preceding experiments (Table 2).

2.3.1 Experiment 1: Effects of oxygen source

The effects of air quality on the enrichment efficiency of the emulsion M70 were tested. Three different treatments, all providing a fairly constant dissolved oxygen content above the recommended 4 mg L^{-1} (Van Stapen, 1996), were tested: "oxygen", with pure oxygen; "air", with compressed air; and "mixture", with both pure oxygen and aeration.

2.3.2. Experiment 2: Effects of aeration

In order to assess the effects of the aeration level in the *Artemia* enrichment with M70, three different air flows were tested: "1 lpm", "2 lpm" and "3 lpm" with air flows of 1.0, 2.0 and 3.0 L min^{-1}, respectively, being used. Air flows were individually controlled in each enrichment vessel by means of a rotameter (Key Instruments, Trevose, PA, USA).

2.3.3 Experiment 3: Effects of temperature

The effects of temperature in the enrichment process were assessed by incubating the nauplii in the presence of M70 at 24 ºC (treatment "24C") or 28 ºC (treatment "28C"). The temperature was kept (± 1 ºC) constant by placing the enrichment vessels in a thermostatic bath.

2.3.4 Experiment 4: Effects of enrichment product concentration

The effects of the M70 concentration on the enrichment efficiency were evaluated by enriching *Artemia* at two different concentrations: 0.8 g M70 L^{-1} (treatment "0.8"), this

concentration being equivalent to 0.6 g L^{-1} recommended for commercial emulsions after correction for the water content difference; and 0.6 g M70 L^{-1} (treatment "0.6").

2.3.5. Experiment 5: Effects of product dosage

In order to assess the effects of the enrichment product dosage on *Artemia* bioencapsulation, two experimental treatments were established: "2 doses", with M70 emulsion being splitted into two doses supplying 0.4 g L^{-1} at the beginning of the enrichment process and 0.4 g L^{-1} after 7 h; "1 dose", with the enrichment diet M70 being supplied in a single dose of 0.8 g L^{-1} at the beginning of the enrichment process.

2.3.6 Experiment 6: Effects of nauplial density

The effect of nauplial density in the *Artemia* enrichment with M70 was evaluated by incubating the nauplii at two different densities, 150 (treatment "low dens") and 300 nauplii mL^{-1} (treatment "high dens"). As the last of our experiments aiming at optimisation of M70 as enrichment diet, Experiment 6 was carried out under those experimental conditions (oxygen source, aeration flow, temperature, product concentration and product dosage) that had produced the optimal enrichment results in the preceding Experiments 1-5. Moreover, we compared the enrichment performance of M70 with that of DC Super Selco ("DCSS", Inve, Ghent, Belgium), a commercial product basically consisting of a fish oil emulsion. DCSS enrichment was carried out under the same conditions of M70, except for the product concentration, 0.8 g L^{-1} for M70, and 0.6 g L-1for DCSS**,** to compensate for the different water content of both products (50 % and 30 % for M70 and DCSS, respectively), as mentioned before.

2.4. Total lipids and FA analyses

Total lipids were extracted (Folch *et al*. 1957) from nauplii freeze-dried samples, measured gravimetrically (XS105 Mettler Toledo, Switzerland), and stored in chloroform: methanol (2:1; v/v) containing 0.01% butylated hydroxytoluene (BHT) at -20 ºC until further use. Total lipids were subjected to acid catalysed transmethylation for 16 h at 50 ºC using 1 mL toluene and 2 mL of 1% (v/v) sulphuric acid in methanol (Christie, 2003). Fatty acid methyl esters (FAME) were extracted with hexane:diethyl ether (1:1; v/v) containing 0.01% BHT and purified by thin-layer chromatography (Silica gel G60, 20 x 20 cm glass plates, Merck, Darmstadt, Germany) using hexane:diethyl ether:acetic acid (85:15:1.5; v/v/v) as a solvent system. FAME were then analysed with a Fisons Instruments GC 8000 Series (Rodano, Italy) gas chromatograph. Peaks were recorded using the Azur software package (version 4.0.2.0. Datalys, France). Individual FAME were identified by comparison with known standards. The relative amount of each FA was expressed as a percentage of the total amount of FA.

2.5. Statistical analysis

Analytical data were expressed as means ± standard deviations (n=3). Differences between treatments were analysed by one-way analysis of variance (ANOVA), followed by either Bonferroni's multiple comparison test or a Student's *t*-test when only two groups were compared (Sokal & Rohlf, 1981). If heterogeneity of variances existed, Welch test was used to detect differences, followed by Games-Howell test to assess the differences between groups. When significance was $P \leq 0.05$, means were considered statistically different. The FA analytical data from Experiment 6, for which a comparison with the commercial product DCSS was carried out, were further analysed by multivariate Principal Components Analysis (PCA), in order to highlight the effects of factors enrichment diet and nauplial density. Statistical analyses were performed using the SPSS statistical package (SPSS Inc., Chicago, Illinois, USA).

3. Results

3.1 Experiment 1: Effect of oxygen source

Whereas the DHA bioencapsulation into *Artemia* nauplii did not show statistical differences among the treatments in Experiment 1, the levels of EPA and ARA in nauplii from "air" treatment were significantly higher than those of treatments "oxygen" and "mixture" (Table 3). Thus, the DHA/EPA ratio also showed statistically significant differences and *Artemia* from "oxygen" treatment had higher DHA/EPA values (1.8 ± 0.0) in comparison with nauplii from treatments "air" and "mixture" (1.6 ± 0.1). As a preliminary conclusion from Experiment 1, DHA levels were similar in nauplii from all treatments and thus use of pure oxygen did not improve the enrichment efficiency of a more readily available source like compressed air.

3.2 Experiment 2: Effect of aeration

The FA profiles of the *Artemia* nauplii enriched under different air flows ("1 lpm", "2 lpm" and "3 lpm") are shown in Table 3. Our results suggested that M70 enrichments are more efficient under relatively low air flows. Thus, the DHA contents of *Artemia* from treatment "1 lpm" were significantly higher than those of *Artemia* from treatment "3 lpm". This was also reflected in the DHA/EPA ratio of both treatments, being 1.4 and 1.3 for "1 lpm" and "3 lpm" treatments, respectively. Experiment 2 results allowed us to conclude that enrichment procedures at 1 L min^{-1} improved M70 bioencapsulation efficiency.

3.3 Experiment 3: Effect of temperature

Table 3 shows the FA profiles of *Artemia* nauplii enriched with M70 at 24 and 28 ºC. Our results indicated that the contents of ARA, EPA and DHA, as well the ratio

DHA/EPA, were significantly higher in nauplii incubated at 28 ºC than at 24 ºC. Overall, 28 ºC was confirmed to be a more adequate incubation temperature for M70 enrichments compared to 24 ºC.

3.4 Experiment 4: Effect of product concentration

Experiment 4 compared the HUFA bioencapsulation performance of M70 dispensed to *Artemia* nauplii at product concentrations of 0.6 or 0.8 g L^{-1}. Generally, no differences among any of each individual FA analysed were detected between both treatments ($P>0.05$), possibly due to a remarkably high variability in the FA contents of "0.6" nauplii. Despite no significantly different, average DHA contents in nauplii from treatment "0.8" (8.3 ± 0.2) were still higher than those of "0.6" nauplii (6.6 ± 2.0) (Table 3). Moreover, statistically significant increases of the DHA/EPA ratio were observed for M70 concentrations of 0.8 g L^{-1}. We could therefore conclude that an M70 concentration of 0.8 g L^{-1} produced better bioencapsulation results than 0.6 g L^{-1}.

3.5 Experiment 5: Effect of product dosage

The results from Experiment 5 indicated that dispensation of the enrichment product M70 in one or two doses did not produce differences in the FA profiles from *Artemia* lipids (Table 3). It was concluded that dispensing the product M70 in one single dose at the beginning of the enrichment process was a more practical and simpler strategy.

3.6 Effects of nauplial density

No effect of the nauplial density (150 and 300 nauplii mL^{-1}) on the enrichment performance with M70 was observed. Thus, no significant differences among any of the FA analysed could be established. Interestingly, a notable impact on *Artemia* FA profiles was observed when comparing the enrichment products M70 and the

commercial emulsion DCSS, the control treatment in Experiment 6. The enrichment comparisons with DCSS showed that differences were mainly attributable to the effect of enrichment product (Table 3). This was further corroborated by the PCA, with the first component (PC1) explaining 92.5 % and the second 6.5 % (Fig. 2A), and subsequent score plot (Fig. 2B) that revealed two groups separated in the first component on the basis of the enrichment product used, whereas no separation based on the nauplial density was achieved. The association of the two groups to their respective variables allowed to identify 18:1n-9, LA and EPA with DCSS and 16:0, 18:0, 18:1n-7, LNA, ARA and DHA with M70 in the first component.

4. Discussion

It has been often reported in the literature that bioencapsulation of HUFA into *Artemia* nauplii is particularly difficult to achieve (Navarro *et al*.1999; Bell *et al*. 2003). Among the factors believed to account for such limited efficiency for HUFA delivery, some have a particular relevance when DHA-rich enrichment products are used. Here, we investigated diverse practical strategies to optimise the use of the emulsion M70, a product with great potential for its use in *Artemia* enrichments.

Dissolved oxygen (DO) availability in the culture medium is critical for *Artemia* survival during enrichment, and DO concentrations above 4 mg L^{-1} have been proposed (Van Stappen 1996). While standard enrichment procedures involve the use of compressed air to provide adequate levels DO, pure oxygen supply is particularly recommended when DO levels can be potentially compromised. That might be the case of live prey enrichments with high DHA contents, as oxygen depleting processes such as lipid peroxidation are especially favoured (McEvoy *et al*. 1995; Ries 2009). We therefore compared the performance of pure oxygen *vs*. compressed air when using M70 as enrichment diet, and conclude that no difference in either the HUFA

bioencapsulation into *Artemia* nor nauplial mortality were observed. Altogether, Experiment 1 allowed us to conclude that oxygen supplied with compressed air is an effective diffusion system to meet the DO demands in enrichment procedures with M70, and that other more expensive alternatives such as pure oxygen are not required.

Other factors like air flow and temperature during the enrichment procedure also need to be considered when a compromise between lipid peroxidation, oxygen supply and enrichment efficiency is pursued. In addition to oxygen solubility and its potential consequences in lipid peroxidation, the air flow during enrichment also determines the overall turbulence in the medium. It has been suggested that highly turbulent conditions during enrichment might impede the normal swimming behaviour of nauplii and damage the nauplial filtratory structures, thus ultimately hindering the incorporation of the enrichment product particles (Navarro *et al*. 1999; Monroig *et al*. 2006b). In agreement with those findings, the emulsion M70 exhibited a higher HUFA bioencapsulation efficiency when utilised at moderate air flows ("1 lpm") than that at highly turbulent conditions ("3 lpm"). In contrast with the results reported by Monroig *et al*. (2006b), who investigated the enrichment efficiency of HUFA-rich liposomes under the same air flow conditions set for Experiment 2, the enrichment with M70 did not produce an excessive foam in the medium and thus the *Artemia* mortality caused by such an effect did not occur for any the air flow studied.

Temperature of the enrichment medium is a key factor for efficient bioencapsulation of HUFA into *Artemia*. Beyond controlling the oxygen solubility and lipid peroxidation mechanisms, temperature also determines the metabolic capability of *Artemia* nauplii and therefore important development landmarks such as the mouth opening and vitelum absortion, as well as the onset of filtering/natatory appendixes (Hochanchka & Somero 1984; Anger 2001). Our results indicated that enhanced HUFA enrichment was

303 achieved at 28 ºC compared with 24 ºC, this being consistent with the vast majority of
304 studies in which 28 ºC has been established as preferred enrichment temperature (Harel
305 *et al*. 1999; Han *et al*. 2001; Sui *et al*. 2007; Boglino *et al*. 2012). In contrast, relatively
306 low temperatures (21-22 ºC) were established in other studies (Garcia *et al*. 2008;
307 Figueiredo *et al*. 2009), but unfortunately no specific reasons for the choice of these low
308 temperatures, and the causes of their effects, were given. Since the enrichment
309 efficiency of M70 did not appear to be compromised by potentially more pro-oxidant
310 temperatures ("28C" *vs*. "24C"), we continued our investigations evaluating other
311 experimental parameters potentially affecting the oxidative stability of M70 and
312 consequently its HUFA bioencapsulation efficacy.

313 Another strategy to preserve DHA-rich enrichment diets from oxidation involves an
314 adequate dosage along the enrichment protocol. While one single dose at the beginning
315 of the enrichment process appears to be the most extended dosage mode (Estevez *et al*.
316 1999; Villalta *et al*. 2005a, b), two doses, one at the beginning of the enrichment
317 process and another one at mid-period (Evjemo *et al*. 1997, 2001; Estévez *et al*. 1998;
318 Han *et al*. 2000, 2001; Sui *et al*. 2007; Hamre & Harboe, 2008) might contribute to
319 reduce lipid peroxidation as the time of exposure to pro-oxidant conditions (continuous
320 light, high temperature and limited dissolved oxygen) is minimised. The results from
321 the present study confirmed that no differences in HUFA bioencapsulation existed when
322 M70 was dispensed in one or two doses, and thus the oxidative stability of M70 enables
323 this enrichment diet to be dispensed in a unique dose without any evident detrimental
324 effect in terms of HUFA incorporation over 21 h of incubation.

325 Our experiments investigating the effects of the M70 concentration and the nauplial
326 density on *Artemia* bioencapsulation efficiency evidenced that M70 behaved similarly
327 to other enrichment products, and no special specification derived from its particularly

high-DHA content needs to be considered. Thus, a dose of 0.8 g L^{-1} of M70, equivalent in dry weight basis to the recommended 0.6 g L^{-1} for commercial products (McEvoy *et al*. 1995, 1996, 1997; Smith *et al*. 2002; Villalta *et al*. 2005a, b; Sui *et al*. 2007), resulted in increased DHA bioencapsulation into *Artemia* compared to lower concentration enrichments. At first glance, no significant differences in DHA contents between *Artemia* enriched at M70 concentrations of 0.6 and 0.8 g L^{-1} would have indicated that a concentration of 0.6 g M70 L^{-1} was sufficient to produce as much DHA enrichment as 0.8 g L^{-1}. However, the high variability observed in the FA profiles from *Artemia* enriched at concentration of 0.6 g L^{-1} could have certainly masked a lowered bioencapsulation efficiency in comparison with that obtained at 0.8 g L^{-1}. Indeed we did observe significant higher DHA/EPA ratio, as well as higher average contents of individual DHA for *Artemia* enriched at 0.8 g M70 L^{-1}. These results enabled us to choose 0.8 g L^{-1} of the M70 emulsion as a more adequate enricher concentration than 0.6 g L^{-1}. Additionally, our experiment assessing the effect of nauplial density on HUFA enrichment revealed that M70 can be used at nauplial densities of 300 nauplii mL^{-1}, with the potential limitation of DO not causing any apparent effect on nauplial motility (Southgate & Lou 1995). Qualitative (FA profile) enrichment efficiency of M70 did not vary between 150 and 300 nauplii mL^{-1}. However, from a quantitative point of view (lipid %), a higher uptake of enrichment product was achieved at 150 nauplii mL^{-1}. Thus, any positive cost-effectiveness aspects related, among others, to a more restricted utilisation of enrichment product, pointing at 300 nauplii mL^{-1} as the recommended nauplial density for M70 enrichments, has to be counterbalanced by the higher uptake achieved at lower nauplial density.

The DHA/EPA ratio is a common biochemical parameter used to evaluate the nutritional suitability of diets for marine finfish larviculture (Reitan *et al*. 1994; Evjemo

353 *et al*. 1997). For instance, the yolk of marine fish eggs and the polar lipids of
354 copepodites, natural preys of marine fish larvae in the wild, have DHA/EPA ratios
355 around 2.0 (Fraser *et al*. 1989; McEvoy *et al*. 1997; Sargent *et al*. 1997; Sorgeloos *et al*.
356 2001; Evjemo *et al*. 2003; Van der Meeren *et al*. 2008). Generally the results from the
357 present study showed that the emulsion M70 allowed us to obtain in most cases *Artemia*
358 nauplii with DHA/EPA ratios above 1.3 and only suboptimal experimental conditions
359 (i.e., excessive aeration or relatively low temperature) resulted in lower values. It is
360 worth mentioning that the DHA/EPA ratios of *Artemia* nauplii were particularly high
361 (1.8-1.9) for Experiment 6, supporting that the final (optimised) experimental conditions
362 developed for a more efficient use of M70 as enrichment diet had been adequately
363 established. Moreover, M70-enriched nauplii had higher DHA individual contents and
364 DHA/EPA ratios than nauplii enriched with commercial enrichment products like the
365 one utilised in this study (DC Super Selco) and previous studies (Léger *et al*. 1986;
366 Woods 2003; Lund *et al*. 2007; Naz 2008). Interestingly, Haché *et al*. (2011) recently
367 reported DHA/EPA ratios of 3.6 in *Artemia* nauplii enriched with Algamac 3050, a
368 commercial product based on spray-dried cells of the marine protist *Schizochytrium* sp.
369 (Barclay & Zeller 1996). Since DHA concentration of Algamac 3050 (~40 % of total
370 FA) is lower than that of M70 (~70 %), factors possibly related to the physical and
371 biochemical nature of Algamac 3050 might account for such unexpectedly high HUFA
372 bioencapsulation. Nevertheless, the results obtained from the present study clearly show
373 that M70 emulsion was able to consistently produce DHA/EPA ratios above 1 in
374 *Artemia* lipids, with particularly high values when optimised conditions are used.

375 In summary, the results from the present study indicated that an efficient utilisation of
376 the emulsion M70 as enrichment diet for *Artemia* nauplii is achieved with incubation
377 temperatures of 28ºC, low/moderate aeration (1 L min^{-1}) and nauplial densities of 150

ind mL^{-1}. Moreover, the emulsion M70 can be administered to newly hatched nauplii through a single dose of 0.8 g L^{-1}, with no detrimental effects such as DHA autooxidation and nauplial mortalities becoming apparent.

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6. References

Anger, K., 2001. The biology of decapod crustacean larvae. In: Vonk, R. (Ed.), Crustacean Issues, vol. 14. A. A. Balkema Publishers, Lisse, 419 p.

Aragão, C., Conceição, L.E.C., Dinis, M.T., Hans-Jørgen, F., 2004. Amino acid pools of rotifers and *Artemia* under different conditions: nutritional implications for fish larvae. Aquaculture 234, 429-445.

Barclay, W., Zeller, S., 1996. Nutritional enhancement of n-3 and n-6 fatty acids in rotifers and *Artemia* nauplii by feeding spray-dried *Schizochytrium* sp. J. World Aquac. Soc. 27, 214-322.

Bell, M.V., Batty, R.S., Dick, J.R., Fretwell, K., Navarro, J.C., Sargent, J.R., 1995. Dietary deficiency of docosahexaenoic acid impairs vision at low light intensities in juvenile herring (*Clupea harengus* L.). Lipids 30, 443-449.

Bell, J.G., McEvoy, L.A., Estévez, A., Shields, R.J., Sargent, J.R., 2003. Optimising lipid nutrition in first-feeding flatfish larvae. Aquaculture 227, 211-220.

Bell. M.V., Dick, J.R., 2004. Changes in capacity to synthesise 22:6n-3 during early development in rainbow trout (*Oncorhynchus mykiss*). Aquaculture 235, 393-409.

Boglino, A., Darias, M.J., Ortiz-Delgado, J.B., Özcan, F., Estévez, A., Andree, K.B., Hontoria, F., Sarasquete, C., Gisbert, E., 2012. Commercial products for *Artemia* enrichment affect growth performance, digestive system maturation, ossification and incidence of skeletal deformities in Senegalese sole (*Solea senegalens*is) larvae. Aquaculture 324, 290-302

414 Buzzy, M., Henderson, R.J., Sargent, J.R., 1996. The desaturation and elongation of
415 linolenic acid and eicosapentaenoic acid by hepatocytes and liver microsomes
416 from rainbow trout (*Oncorhynchus mykiss*) fed diets containing fish oil or olive
417 oil. Biochim. Biophys. Acta 1299, 235-244.

418 Christie, W.W., 2003. Lipid analysis, third ed. Oily Press, Bridgwater.

419 Cosgrove, J.P., Church, D.F., Pryor, W.A., 1987. The kinetics of the autoxidation of
420 polyunsaturated fatty acids. Lipids 22, 299–304.

421 Coutteau, P., Sorgeloos, P., 1997. Manipulation of dietary lipids, fatty acids and
422 vitamins in zooplankton cultures. Freshwater biology 38, 501-512.

423 Estévez, A., McEvoy, L.A., Bell, J. G., Sargent, J. R., 1998. Effects of temperature and
424 starvation time on the pattern and rate of loss of essential fatty acids in *Artemia*
425 nauplii previously enriched using archidonic acid and eicosapentaenoic acid-rich
426 emulsions. Aquaculture 165, 295-311.

427 Estévez, A., McEvoy, L.A., Bell, J.G., Sargent, J.R., 1999. Growth, survival, lipid
428 composition and pigmentation of turbot (*Scophthalmus maximus*) larvae fed
429 live-prey enriched in Arachidonic and Eicosapentaenoic acids. Aquaculture 180,
430 321-343.

431 Evjemo, J.O., Coutteau, P., Olsen, Y., Sorgeloos, P., 1997. The stability of
432 docosahexaenoic acid in two *Artemia* species following enrichment and
433 subsequent starvation. Aquaculture 155, 135-148.

434 Evjemo, J.O., Danielsen, T.L., Olsen, Y., 2001. Losses of lipid, protein and n-3 fatty
435 acids in enriched *Artemia franciscana* starved at different temperatures.
436 Aquaculture 193, 65-80.

437 Evjemo, J.O., Reitan, K.I., Olsen, Y., 2003. Copepods as live food organisms in the
438 larval rearing of halibut larvae (*Hippoglossus hippoglossus* L.) with special
439 emphasis on the nutritional value. Aquaculture 227, 191–210.

440 Figueiredo, J., van Woesik, R., Lin, J., Narciso, L., 2009. *Artemia franciscana*
441 enrichment model – How to keep them small, rich and alive? Aquaculture 294,
442 212-220.

443 Folch, J., Lees, M., Sloane Stanley, H., 1957. A simple method for the isolation and
444 purification of total lipides from animal tissues. J. Biol. Chem.. 226, 497-509.

445 Fraser, A.J., Sargent, J.R., Gamble, J.C., 1989. Lipid class and fatty acid composition of
446 *Calanus finmarchicus* (Gunnerus), *Pseudocalanus* sp. and *Temora longicornis*
447 Muller from a nutrient-enriched seawater enclosure. J. Exp. Mar. Biol. Ecol.
448 130, 81-92.

449 Furuita, H., Takeuchi, T., Toyota, M., Watanabe, T., 1996. EPA and DHA requirements
450 in early juvenile sea bream using HUFA enriched *Artemia* nauplii. Fish. Sci. 62,
451 246-251.

452 Garcia, A.S., Parrish, C.C., Brown, J.A., 2008. Growth and lipid composition of
453 Atlantic cod (*Gadus morhua*) larvae in response to differently enriched *Artemia*
454 *franciscana*. Fish. Physiol. Biochem. 34, 77-94.

455 Giner, E., 2005. Desarrollo de emulsiones de alto contenido en ácidos grasos
456 poliinsaturados para complementar el valor nutritivo de presas vivas en
457 acuicultura. Universidad Politécnica de Valencia (Valencia). pp 75.

458 Hamre, K., Harboe, T., 2008. *Artemia* enriched high n-3 HUFA may give a large
459 improvement in perfomance of Atlantic halibut (*Hippoglossus* L.) larvae.
460 Aquaculture 277, 239-243.

461 Haché, R., Plante, S., 2011. The relationship between enrichment, fatty acid profiles and
462 bacterial load in cultured rotifers (*Brachionus plicatillis* L-strain) and *Artemia*
463 (*Artemia salina* strain *franciscana*). Aquaculture 311, 201-208.

464 Han, K., Geurden, I., Sorgeloos, P., 2000. Enrichment strategies for *Artemia* using
465 emulsions providing differents levels of n-3 highly unsaturated fatty acids.
466 Aquaculture 183, 335-347.

467 Han, K., Geurden, I., Sorgeloos, P., 2001. Fatty acid changes in enriched and
468 subsequently starved *Artemia franciscana* nauplii enriched with different
469 essential fatty acids. Aquaculture 199, 93-105.

470 Han, K., Geurden, I., Van der Meeren, P., Bai, S.C., Sorgeloos, P., 2005. Particle size
471 distribution in two lipid emulsion used for the enrichment of *Artemia* nauplii as
472 a function of their preparation method and storage time. J. World Aquac. Soc.
473 36, 196-202.

474 Harel, M., Ozkizilcik, S., Lund, E., Behrens, P., Place, A.R., 1999. Enhanced absorption
475 of docosahexaenoic acid (DHA, 22:6n3) in *Artemia* nauplii using a dietary
476 combination of DHA-rich phospholipids and DHA-sodium salts. Comp.
477 Biochem. Physiol. B 124, 169-176.

478 Hontoria, F., Crowe, J.H., Crowe, L.M., Amat, F., 1994. Potential use of liposomes in
479 larviculture as a delivery system through Artemia nauplii. Aquaculture 127, 255-
480 264.

481 Koven, W.M., Tandler, A., Kissil, G.W., Sklan, D., Friezlander, O., Harel, M.,
482 1990.The effect of dietary (*n*-3) polyunsaturated fatty acids on growth, survival
483 and swim bladder development in *Sparus aurata* larvae. Aquaculture 91, 131-
484 141.

485 Léger, P., Bengtson, D.A., Simpson, K.L., Sorgeloos, P., 1986. The use and nutritional
486 value of *Artemia* as a food source. Oceanogr. Mar. Biol. Annu. Rev. 24, 521-
487 623.

488 Léger, P., Bengtson, D.A., Sorgeloos, P., Simpson, K.L., Beck, A.D., 1987. The
489 nutritional value of *Artemia*: a review: p.357-372. In: Sorgeloos, P., Bengtson,
490 D.A., Declier, W. and Jaspers, E. (Eds.), *Artemia* Research and its applications.
491 Vol. 3. Universa Press, Wetteren, Belgium, 556 p.

492 Lund, I., Steenfeldt, S.J., Hanses, B.W., 2007. Effect of dietary arachidonic acid,
493 eicosapentaenoic acid and docosahexaenoic acid on survival, growth and
494 pigmentation in larvae of common sole (*Solea solea* L.). Aquaculture 273, 532-
495 544.

496 McEvoy, L.A., Navarro, J.C., Bell, J.G., Sargent, J.R., 1995. Autoxidation of oil
497 emulsions during the *Artemia* enrichment process. Aquaculture 134, 101-112.

498 McEvoy, L.A., Navarro, J.C., Hontoria, F., Amat, F., Sargent, J.R., 1996. Two novel
499 *Artemia* enrichment diets containing polar lipid. Aquaculture 144, 339-352.

500 McEvoy, L.A., Navarro, J.C., Amat, F., Sargent, J.R., 1997. Application of soya
501 phosphatidylcholine in tuna orbital oil enrichment emulsions for *Artemia*.
502 Aquaculture international 5, 517-526.

503 Monroig, O., Navarro, J.C., Amat, I., González, P., Amat, F., Hontoria, F., 2003.
504 Enrichment of *Artemia* nauplii in PUFA, phospholipids, and water-soluble
505 nutrients using liposomes. Aquaculture international 11, 151-161.

506 Monroig. O., 2006. Diseño y optimización de liposomas para su uso como sistema de
507 suministro de nutrientes a larvas de peces marinos. PhD thesis. University of
508 Valencia, Spain, 239 p.

509 Monroig, O., Navarro, J.C., Amat, F., González, P., Bermejo, A., Hontoria, F., 2006a.
510 Enrichment of *Artemia* nauplii in essential fatty acids with different types of
511 liposomes and their use in the rearing of gilthead sea bream (*Sparus aurata*)
512 larvae. Aquaculture 251, 491-508.

513 Monroig, O., Navarro, J.C., Amat, F., Gonzalez, P., Hontoria, F., 2006b. Effect of
514 aeration on the efficiency of *Artemia* enrichment with EFA-rich emulsion and
515 liposomes. Aquaculture 257, 382-392.

516 Monroig, O., Navarro, J.C., Amat, F., Gonzalez, P., Hontoria, F., 2006c. Effects of
517 nauplial density, product concentration and product dosage on the survival of the
518 nauplii and EFA incorporation during *Artemia* enrichment with liposomes.
519 Aquaculture 261, 659-669.

520 Monroig, O., Navarro, J.C., Amat, F., Hontoria, F., 2007. Enrichment of *Artemia*
521 nauplii in vitamin A, vitamin C and methionine using liposomes. Aquaculture
522 269, 504-513.

523 Monroig, O., Webb, K., Ibarra-Castro, L., Holt, G.J., Tocher, D.R., 2011. Biosynthesis
524 of long-chain polyinsaturated fatty acids in marine fish: Characterization of an

525 Elovl4-like elongase from cobia *Rachycentron canadum* and activation of the
526 pathway during early life stages. Aquaculture 312, 145-153.

527 Morais, S., Mourente, G., Ortega, A., Tocher, J.A., Tocher, D.R., 2011. Expression of
528 fatty acyl desaturase and elongase genes, and evolution of DHA:EPA ratio
529 during development of unfed larvae of Atlantic bluefin tuna (*Thunnus Thynnus*
530 L.). Aquaculture 313, 129-139.

531 Navarro, J.C., Henderson, R.J., McEvoy, L.A., Bell, M.V., Amat, F., 1999. Lipid
532 conversions during enrichment of *Artemia* . Aquaculture 174, 155-166.

533 Naz, M., 2008. The changes in the biochemical composition and enzymatic activities of
534 rotifer (*Brachionus plicatillis*, Müller) and *Artemia* during the enrichment and
535 starvation periods. Fish Phys. and Biochem. 34, 391-404.

536 Reitan, K.I., Rainuzzo, J.R., Olsen, Y., 1994. Influence of lipid composition of live feed
537 on growth, survival and pigmentation of turbot larvae. Aquaculture international
538 2, 22-48.

539 Ries, D., 2009. Studies on the antioxidant activity of milk proteins in model oil-in-water
540 emulsions. PhD thesis. Riddet Institute (Massey University) Palmerston North,
541 New Zealand, 256 pp.

542 Sargent, J.R., McEvoy, L.A., Bell, J.G., 1997. Requirements, presentation and sources
543 of polyunsaturated fatty acids in marine fish larval feeds. Aquaculture 155, 117-
544 127.

545 Sargent, J.R., Tocher, D.R., Bell, J.G., 2002. The lipids. In: Halver, J.E., Hardly, R.W.
546 (Eds.). Fish Nutrition Academic Press, San Diego, CA, USA. Pp: 181-257.

547 Smith, G.G., Ritar, A.J., Phleger, C.F., Nelson, M.M., Mooney, B., Nichols, P.D., Hart,
548 P.R., 2002. Changes in gut content and composition of juvenile *Artemia* after oil
549 enrichment and during starvation. Aquaculture 208, 137-158.

550 Sokal, R.R. and Rohlf, F.J., 1981. Biometry. New York. 859 pp.

551 Sorgeloos, P., Dhert, P., Candreva, P., 2001. Use of the brine shrimp, *Artemia* spp., in
552 marine fish larviculture. Aquaculture 200, 147-159.

553 Southgate, P.C. and Lou, D.C., 1995. Improving the *n* - 3 HUFA composition of
554 *Artemia* using microcapsules containing marine oils. Aquaculture 134, 91-99.

555 Sui, L., Wille, M., Cheng, Y., Sorgeloos, P., 2007. The effect of dietary n-3 HUFA
556 levels and DHA/EPA ratios on growth, survival and osmotic stress tolerance of
557 Chinese mitten crab *Eriocheir sinensis* larvae. Aquaculture 273, 139-150.

558 Tocher, D.R., 2003. Metabolism and functions of lipids and fatty acids in teleost fish.
559 Rev. Fish. Sci. 11, 107-184.

560 Van der Meeren, T., Olsen, R.E., Hamre, K.,Fyhn, H., 2008. Biochemical composition
561 of copepods for evaluation of feed quality in production of juvenile marine fish.
562 Aquaculture 274, 375-397.

563 Van Stappen, G., 1996. Introduction , biology and ecology of *Artemia*. In: Lavens, P.,
564 Sorgeloos, P., (Eds.), Manual on the production and use of live food for
565 aquaculture. FAO Fisheries Technical Paper (361), Roma.

566 Villalta, M., Estévez, A., Bransden, M.P., 2005a. Arachidonic acid enriched live prey
567 induces albinism in Senegal sole (*Solea senegalensis*) larvae. Aquaculture 245,
568 193-209.

569 Villalta, M., Estévez, A., Bransden, M.P., Bell, J.G., 2005b. The effect of graded
570 concentrations of dietary DHA on growth, survival and tissue fatty acid profile
571 os Senegal Sole (*Solea senegalensis*) larvae during the *Artemia* feeding period.
572 Aquaculture 249, 353-365.

573 Watanabe, T., Ohta, M., Kitajima, C., Fujita, S., 1982. Improvement of dietary value of
574 brine shrimp *Artemia salina* for fish larvae by feeding them on w3 highly
575 unsaturated fatty acids. Bull. Jpn. Soc. Sci. Fish. 48, 1775-1782.

576 Watanabe, T., Kitajima, C, Fujita, S., 1983. Nutritional values of live organisms used in
577 Japan for mass propagation of fish: A review. Aquaculture 34, 115-143.

578 Woods, C.M.C., 2003. Effects of varying *Artemia* enrichment on growth and survival of
579 juvenile seahorses, *Hippocampus abdominalis*. Aquaculture 220, 537-548.

580 Yamamoto, Y., Kabeya, N., Takeuchi, Y., Alimuddin, Haga, Y., Satoh, S., Takeuchi,
581 T., Yoshizaki, G., 2010. Cloning and nutritional regulation of polyunsaturated
582 fatty acid desaturase and elongase of a marine teleost, the nibe croaker *Nibea*
583 *mitsukurii*. Fisheries science 76, 463-472.

584 **Figure Legends**

585 Figure 1. Component plot (A) and factor score plot (B) of the multivariate Principal
586 Components Analysis of selected FA from total lipids of enriched *Artemia* nauplii from
587 Experiment 6.

588

589 Table 1. Fatty acid composition (percent of total fatty acids) of the enrichment products
590 M70 and DC Super Selco (DCSS, Inve, Ghent, Belgium) utilised in the present study

Fatty acid	M70	DCSS
14:0	0.1	1.3
16:0	0.9	5.2
16:1n-7	0.4	1.9
18:0	0.4	2.7
18:1n-9	3.7	9.2
18:1n-7	0.5	1.6
18:2n-6	0.7	4.8
18:3n-3	0.6	1.0
18:4n-3	0.4	1.8
20:0	N.D.	0.4
20:1n-9	0.3	2.3
20:3n-6	0.1	0.1
20:4n-6	2.4	1.6
20:3n-3	0.1	0.2
20:4n-3	0.5	1.1
20:5n-3	7.7	31.6
22:0	0.1	0.4
22:1n-11	0.1	0.2
22:5n-3	2.7	2.2
22:6n-3	70.3	20.6
Saturated	1.8	10.3
Monounsaturated	5.2	18.5
Polyunsaturated	91.1	66.2
HUFA n-3	81.3	55.6
HUFA n-6	8.0	2.6

DHA/EPA ratio	9.1	0.6

N.D.: not detected; HUFA n-3: ≥20:3n-3; HUFA n-6: ≥20:2n-6; DHA/EPA: docosahexaenoic and eicosapentanoic fatty acid ratio.

Table 2. Experimental conditions set up in the experiments carried out in the present study. DC Super Selco (DCSS, Inve, Ghent, Belgium) was used as control treatment in Experiment 6 as indicated in Materials and Methods

	Treatment	Oxygen source	Air flow (L min^{-1})	Temperature (ºC)	Product concentration (g L^{-1})	Doses	Nauplial density (nauplii mL^{-1})
Exp. 1	oxygen	Oxygen	1	28	0.8	1	300
	air	Air	1	28	0.8	1	300
	mixture	Oxygen+Air	1	28	0.8	1	300
Exp. 2	1 lpm	Air	1	28	0.8	1	300
	2 lpm	Air	2	28	0.8	1	300
	3 lpm	Air	3	28	0.8	1	300
Exp. 3	24C	Air	1	24	0.8	1	300
	28C	Air	1	28	0.8	1	300
Exp. 4	0.6	Air	1	28	0.6	1	300
	0.8	Air	1	28	0.8	1	300
Exp. 5	2 doses	Air	1	28	0.8	2	300
	1 dose	Air	1	28	0.8	1	300
Exp. 6	low dens	Air	1	28	0.8	1	150
	high dens	Air	1	28	0.8	1	300
	DCSS	Air	1	28	0.6	1	300

Table 3. Fatty acids (percentage of total fatty acids) from enriched *Artemia* nauplii collected from Experiment 1-6 (see Materials and Methods for treatment details). Data represent means ± standard deviations (n=3).Treatments in each Experiment with different letter are significantly different ($P \leq 0.05$). If no superscript appears, values are not different.

	Experiment 1			Experiment 2			Experiment 3		Experiment 4		Experiment 5		Experiment 6		
% Fatty acid	oxygen	air	mixture	1 lpm	2 lpm	3 lpm	24C	28C	0.6	0.8	2 doses	1 dose	low dens	high dens	DCSS
14:0	0.5±0.0	0.5±0.0	0.6±0.3	0.6±0.0	0.7±0.0	0.6±0.0	0.5±0.0	0.5±0.0	1.0±0.7	0.6±0.0	0.6±0.0	0.6±0.0	0.6±0.0^{b}	0.6±0.0^{b}	0.8±0.0^{a}
15:0	0.5±0.0	0.6±0.0	0.5±0.0	0.9±0.0	0.9±0.1	0.9±0.0	0.7±0.1	0.7±0.0	0.6±0.0	0.6±0.0	0.9±0.0	0.9±0.0	0.4±0.3	0.6±0.0	0.3±0.3
16:0	9.5±0.1	9.3±0.0	9.6±0.7	10.1±0.1	10.5±0.4	10.4±0.1	10.0±0.0	9.7±0.2	11.1±1.1	10.4±0.1	10.0±0.1	9.8±0.1	9.9±0.2	10.0±0.1	10.0±0.4
16:1n-9	0.5±0.0	0.5±0.0	0.5±0.0	0.4±0.0^{a}	0.6±0.2^{b}	0.7±0.0^{c}	0.6±0.0	0.6±0.0	0.5±0.1	0.6±0.0	0.6±0.2	0.6±0.2	0.6±0.0^{a}	0.5±0.0^{b}	0.5±0.0^{b}
16:1n-7	1.9±0.0	1.9±0.0	2.1±0.3	4.7±0.0^{a}	4.7±0.2^{ab}	4.5±0.0^{b}	2.9±0.1	2.9±0.1	2.7±0.9	2.1±0.0	4.4±0.2	4.4. ±0.1	2.1±0.1^{b}	2.1±0.0^{b}	2.3±0.0^{a}
16:2	0.0±0.0	0.0±0.0	0.0±0.0	0.6±0.0	0.7±0.0	0.7±0.1	0.5±0.0	0.5±0.0	0.5±0.0	0.5±0.0	0.6±0.0	0.7±0.0	0.5±0.0^{a}	0.5±0.0^{a}	0.4±0.0^{b}
16:3	0.5±0.0	0.5±0.0	0.5±0.0	0.7±0.2	0.9±0.1	0.8±0.1	0.6±0.0	0.6±0.0	0.2±0.3	0.0±0.0	0.9±0.1	0.7±0.2	0.0±0.0	0.0±0.0	0.1±0.0
18:0	4.7±0.2	4.6±0.1	4.5±0.1	4.1±0.0	4.3±0.2	4.3±0.1	4.2±0.0	4.3±0.1	4.4±0.2	4.7±0.0	4.1±0.0	4.20±0.1	4.5±0.1^{a}	4.4±0.0^{a}	4.1±0.2^{b}
18:1n-9	16.1±0.2	16.0±0.2	16.0±0.1	22.4±0.2	23.2±1.1	23.3±0.1	19.2±0.1^{a}	18.7±0.1^{b}	16.8±0.6	16.8±0.1	22.2±0.3	21.7±0.2	16.4±0.2^{b}	16.4±0.1^{b}	17.0±0.2^{a}
18:1n-7	6.0±0.1	6.1±0.1	6.1±0.1	8.2±0.1	8.6±0.5	8.8±0.1	7.5±0.2	7.4±0.1	6.3±0.4	6.3±0.0	8.3±0.1	8.4±0.3	6.3±0.0^{a}	6.3±0.0^{a}	5.5±0.1^{b}
18:2n-6	4.9±0.1	4.7±0.0	4.7±0.1	4.3±0.0	4.4±0.2	4.4±0.0	4.8±0.0^{a}	4.6±0.0^{b}	4.9±0.5	5.0±0.0	4.3±0.0	4.3±0.0	4.8±0.1^{b}	4.9±0.0^{b}	6.3±0.0^{a}
18:3n-3	24.2±0.9	24.6±0.3	24.4±0.9	19.1±0.3	19.9±1.0	19.8±0.2	25.6±0.1^{a}	23.9±0.4^{b}	25.2±3.2	25.4±0.1	19.1±0.2	19.3±0.1	24.8±0.6^{a}	24.6±0.1^{a}	19.8±0.0^{b}
18:4n-3	3.0±0.1	2.9±0.1	3.0±0.1	1.8±0.0	1.9±0.1	1.7±0.0	3.0±0.0	2.6±0.1	3.1±0.2	3.2±0.1	1.8±0.0	1.9±0.0	3.1±0.1^{a}	3.1±0.0^{s}	2.8±0.1^{b}
20:0	0.1±0.0	0.1±0.0	0.1±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.1±0.0	0.1±0.0	0.0±0.0	0.0±0.0	0.1±0.0	0.1±0.0	0.1±0.0
20:1n-9	0.6±0.1	0.6±0.0	0.6±0.1	0.5±0.0	0.5±0.0	0.5±0.0	0.4±0.0	0.5±0.0	0.5±0.1	0.5±0.0	0.5±0.0	0.5±0.0	0.5±0.0^{b}	0.5±0.0^{b}	0.9±0.0^{a}
20:2n-6	0.2±0.0	0.2±0.0	0.1±0.0	0.1±0.0	0.1±0.0	0.0±0.0	0.1±0.0	0.1±0.1	0.2±0.0	0.2±0.0	0.0±0.0	0.1±0.1	0.2±0.0	0.2±0.0	0.2±0.0
20:3n-6	0.1±0.0	0.1±0.0	0.1±0.0	0.1±0.0	0.0±0.0	0.0±0.0	0.1±0.0	0.0±0.0	0.1±0.0	0.1±0.0	0.0±0.0	0.0±0.0	0.1±0.0	0.1±0.0	0.1±0.0
20:4n-6	1.3±0.0^{b}	1.5±0.0^{a}	1.4±0.0^{ab}	1.5±0.0	1.4±0.1	1.5±0.0	1.2±0.0^{b}	1.5±0.0^{a}	1.0±0.1	1.1±0.0	1.6±0.0	1.7±0.0	1.2±0.1^{a}	1.2±0.0^{a}	1.0±0.0^{b}
20:3n-3	0.7±0.0	0.7±0.0	0.7±0.0	0.2±0.0	0.2±0.1	0.1±0.1	0.5±0.0	0.5±0.0	0.6±0.1	0.6±0.0	0.1±0.1	0.1±0.1	0.6±0.0^{a}	0.6±0.0^{a}	0.5±0.0^{b}
20:4n-3	0.6±0.0	0.6±0.0	0.6±0.0	0.2±0.0	0.2±0.0	0.2±0.0	0.5±0.0	0.4±0.0	0.6±0.0	0.6±0.0	0.2±0.0	0.1±0.1	0.6±0.0^{b}	0.6±0.0^{b}	0.8±0.0^{a}
20:5n-3	5.7±0.2^{b}	6.2±0.1^{a}	6.4±0.2^{a}	6.2±0.1	5.6±0.6	5.7±0.0	5.2±0.0^{b}	6.2±0.2^{a}	5.0±1.3	4.6±0.0	6.2±0.1	6.5±0.0	5.2±0.2^{b}	5.1±0.1^{b}	13.8±0.5^{a}
22:0	0.3±0.1	0.2±0.0	0.2±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.1±0.2	0.0±0.0	0.2±0.0	0.2±0.0	0.1±0.1	0.0±0.0	0.2±0.0^{b}	0.2±0.0^{b}	0.3±0.0
22:5n-3	0.7±0.0	0.6±0.0	0.6±0.0	0.3±0.0	0.2±0.1	0.1±0.0	0.2±0.2	0.1±0.0	0.4±0.1	0.4±0.0	0.1±0.1	0.1±0.2	0.5±0.0	0.5±0.0	0.6±0.0^{a}
22:6n-3	10.4±0.4	10.2±0.1	10.3±0.5	8.2±0.2^{a}	6.2±2.8^{b}	7.3±0.1^{b}	6.9±0.1^{b}	9.1±0.3^{a}	6.6±2.0	8.3±0.2	9.0±0.6	9.4±0.1	9.2±1.0^{a}	9.6±0.3^{a}	4.9±0.2^{b}
Saturated	15.7±0.4	15.5±0.1	15.8±1.1	15.8±0.2	16.6±0.7	16.2±0.0	15.6±0.3	15.3±0.3	17.5±1.7	16.7±0.1	15.7±0.2	15.4±0.2	15.8±0.5	16.0±0.1	15.2±0.6
Monounsat.	25.8±0.2	25.7±0.2	25.8±0.5	36.3±0.3	37.7±1.8	37.8±0.2	30.6±0.2^{a}	30.0±0.2^{b}	27.0±0.6	26.5±0.2	35.9±0.4	35.5±0.6	26.0±0.2^{b}	26.0±0.1^{b}	27.1±0.3^{a}
Polyunsat.	53.3±0.5	53.7±0.1	53.6±1.3	43.4±0.4	41.7±2.4	42.4±0.2	49.2±0.2	50.1±0.6	49.3±1.8	50.6±0.4	44.1±0.6	44.9±0.4	51.0±0.7	51.7±0.4	51.8±0.9
HUFA n-3	18.1±0.6	18.3±0.2	18.5±0.4	15.2±0.3	12.4±3.6	13.5±0.1	13.2±0.1^{b}	16.3±0.5^{a}	13.2±3.0	14.6±0.2	15.7±0.7	16.3±0.4	16.2±1.2^{b}	16.5±0.4^{b}	20.6±0.7^{a}
HUFA n-6	2.3±0.1	2.3±0.1	2.2±0.1	1.7±0.0	1.5±0.2	1.5±0.0	1.4±1.1	1.6±0.1	1.7±0.2	1.9±0.0	1.7±0.1	1.8±0.1	2.0±0.1^{a}	2.1±0.0^{a}	1.6±0.0^{b}
DHA/EPA	1.8±0.0^{a}	1.6±0.1^{b}	1.6±0.1^{b}	1.4±0.0^{a}	1.1±0.4	1.3±0.0	1.3±0.0^{b}	1.5±0.0^{a}	1.4±0.4^{b}	1.8±0.0^{a}	1.4±0.1	1.4±0.0	1.8±0.1^{a}	1.9±0.0^{a}	0.3±0.0^{b}
% lipids (DW)	31.4±0.8^{a}	27.6±0.5^{b}	27.5±1.0^{b}	25.7±1.6	24.0±2.0	26.4±0.6	22.7±0.2	22.9±0.4	25.2±0.4	26.6±1.8	31.6±0.7	31.3±0.8	25.7±1.0^{b}	22.4±1.2^{c}	28.8±0.6^{a}

HUFA n-3: ≥20:3n-3; HUFA n-6: ≥20:2n-6; DHA/EPA: docosahexaenoic and eicosapentaenoic acid ratio; FAME: Fatty acid methyl ester.

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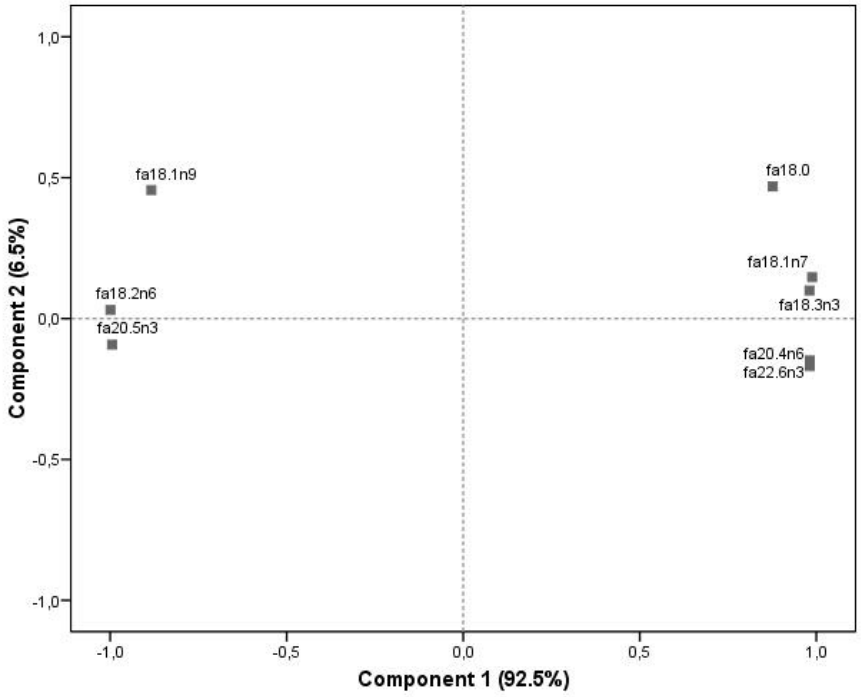
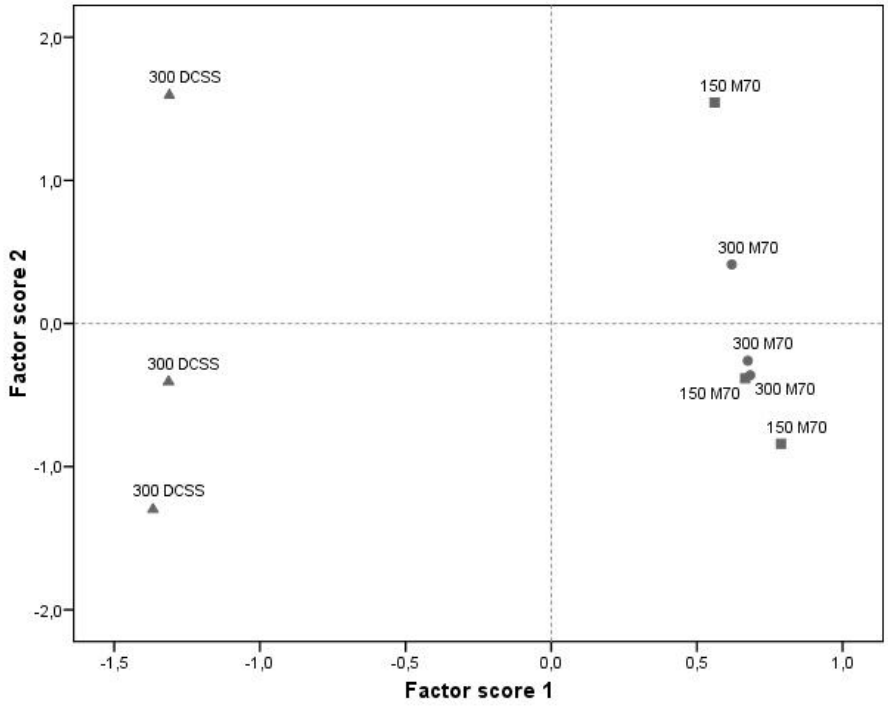
599 (A)



600

601 (B)



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