Title

Enrichment of *Artemia* metanauplii in phospholipids and essential fatty acids as a diet for common octopus (*Octopus vulgaris*) paralarvae

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Running title

Enriched *Artemia* as a diet for octopus paralarvae

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Abstract

Highly unsaturated fatty acids (HUFA), like the eicosapentaenoic (EPA) and docosahexaenoic (DHA) acids and polar lipids (essentially phospholipids, PL) have been identified as essential nutrients for common octopus (*Octopus vulgaris*) paralarvae, but they are not available in sufficient amounts in live preys as *Artemia*, making necessary a supplementation of these nutrients previous use. A commercial emulsion, soya liposomes, and marine and soya lecithins were used to supply HUFA and PL to *Artemia* metanauplii, those being regarded as suitable size preys for octopus paralarvae. Our results prove that a simultaneous enrichment in HUFA and PL is possible using enrichment diets combining HUFA- and PL-rich products in short-term (4 h) incubations. Particularly interesting was the enrichment efficiency shown by the marine lecithin, which enabled the enhancement of the PL fraction of *Artemia* metanauplii and, importantly, also their HUFA with a remarkable 13% DHA of total fatty acids. Marine lecithin arises as a novel enrichment diet for *Artemia* and more effective than some commercial products currently used in hatcheries worldwide.

Introduction

The common octopus (*Octopus vulgaris*) is a promising candidate to diversify species in the Mediterranean aquaculture for its rapid growth, elevated food conversion index and its great commercial interest (Vaz-Pires *et al.*, 2004). Octopus culture has therefore become an attractive area of research, and extensive investigations devoted to understand diverse aspects of its biology have now made possible to on-grow wild-captured specimens in floating cages until commercial size (Iglesias *et al.*, 2006). However, the octopus life cycle in captivity has not been closed yet, as massive

mortalities during metamorphosis of early pelagic life stages, paralarvae, to benthic stages occur (Iglesias *et al.*, 2007; Villanueva & Norman, 2008).

Nutritional studies have highlighted the importance that some dietary components including proteins and amino acids (Villanueva *et al.*, 2004), essential and non-essential elements (Villanueva & Bustamante, 2006) and vitamins (Villanueva *et al.,* 2009) have to alleviate paralarval mortalities (Villanueva *et al.*, 2004). Moreover, Navarro & Villanueva (2000, 2003) studied the lipid requirements of early stages of cephalopods to conclude that suboptimal levels of polar lipids (essentially phospholipids, PL) and highly unsaturated fatty acids (HUFA) like eicosapentaenoic (EPA) and docosahexaenoic (DHA) acids in live preys such *Artemia* might be responsible for the high mortalities encountered in paralarvae.

Newly hatched *Artemia* nauplii are, arguably, the most commonly used live prey in marine finfish and crustacean larviculture (Sorgeloos *et al.*, 2001). However, their suitability as diet for marine larvae has been often questioned due to their relatively low PL and HUFA contents in comparison with natural preys (Navarro *et al.*, 1993). Beneficial aspects derived from dietary PL and HUFA have been reported on survival, growth and development of marine larvae (Sargent *et al.*, 1997; Tocher *et al.*, 2008, Tocher, 2010). In order to enhance the nutritional quality of *Artemia* nauplii, enrichment protocols have been optimised (Estévez & Kanazawa, 1995; Navarro *et al.*, 1999; Han *et al.*, 2001). Whereas enrichment of HUFA has been successfully achieved with the use of commercial products such as marine oil-based emulsions (Léger *et al.*, 1986; Han *et al.*, 2000; Copeman *et al.*, 2002) or spray-dried cells of *Schizochytrium* sp. (Barclay & Zeller, 1996), enhancement of PL contents in *Artemia* (McEvoy *et al.*, 1995; Monroig *et al.*, 2003; 2006) and also rotifers (Rainuzzo *et al.*, 1994) has been posed difficult due in

part to rapid metabolic conversions inside the living preys to other lipid classes such as triacylglicerides (TAG). Thus, liposomes, lipid vesicles with high PL contents, produced modest increases in the PL fraction when used as enrichment diets for *Artemia* nauplii (McEvoy *et al*., 1995; Monroig *et al*., 2003, 2006). Barr *et al*. (2005) also reported enhanced levels of PL in rotifers and *Artemia* nauplii that, after conventional HUFA enrichment procedures, were subsequently subjected to short-term incubations with liposomes formulated with soy phosphatidylcholine (PC). Whereas the alluded studies employed liposomes formulated with purified PL sources normally consisting of PC from either marine (McEvoy *et al*., 1995; Monroig *et al*., 2003, 2006) or terrestrial (Barr *et al*., 2005) origin, the so-called lecithins, often referred to coarse products containing a mixture of phospholipid types or even non-polar lipid classes such as TAG, appear as a cheap alternative to expensive highly purified PC-based PL sources that can be utilized for live prey enrichments at larger scale.

On-grown (metanaupliar) stages of *Artemia* have been regarded as more adequate preys for octopus paralarvae (Iglesias *et al*., 2006). Similarly to naupliar stages, *Artemia* metanauplii also have detrimental lipid profiles for octopus paralarvae, and experimental trials have partly solved the problem by on-growing *Artemia* in presence of marine phytoplankton (Iglesias *et al*., 2004; Viciano *et al*., 2011). While phytoplankton is not readily available and its use involved large-scale facilities, cheaper alternative protocols addressed to improve the nutritional value of *Artemia* metanauplii are required. We here report a series of experiments aiming at establishing optimized protocols for the simultaneous enrichment of PL and HUFA contents of *Artemia* metanauplii for their potential use as live preys in common octopus paralarvae rearing.

Materials and methods

Artemia hatching and culture

Artemia franciscana metanauplii were obtained from the hatching of Great Salt Lake cysts purchased to INVE Aquaculture Nutrition (Dendermonde, Belgium). Cysts were incubated for 24 h in 1 L cylinder-conical tubes containing seawater at 28°C and 37 g L^{-1} salinity, vigorous bottom aeration and continuous light. After hatching, nauplii were placed in 90 L cylindrical methacrylate containers with seawater and maintained at room temperature and a density of 4000 individuals L^{-1}. Nauplii were fed microalgae *Tetraselmis suecica ad libitum* (around 200000 cells mL^{-1}). Fresh microalgae cultures were daily added to maintain cell density. *Artemia* metanauplii were grown for 5 d, attaining a mean length of 1.47 mm, and then used in the different enrichment experiments.

Preparation of enrichment diets

Multilamellar liposomes were formulated with soya PC from Avanti Polar Lipids Inc. (Alabaster, Alabama, USA), and cholesterol, purchased from Sigma-Aldrich Química S.A. (Alcobendas, Spain), included as a membrane stabilizer at a 1:4 w/w ratio (cholesterol:PC). Liposomes were prepared according to the method proposed by Bangham *et al.* (1965), but using filtered seawater as the aqueous phase as previously described (Monroig *et al*., 2003, 2006). Briefly, the lipid mixture was dried under nitrogen flux in a thin layer on the bottom of a flask and rehydrated with the aqueous phase for 1 h by vortexing frequently until a homogenous suspension was achieved.

The commercial emulsion Easy DHA Selco (INVE Aquaculture Nutrition) with 15 % DHA was self-dispersed in seawater to obtain a suspension following supplier's instructions. Marine lecithin LC60 (PhosphoTech Laboratories, St. Herblain, France),

containing 68 % of lipids, around 50 % PL, 13 % of total fatty acids (FA) as EPA, and 33% as DHA, was used after dispersion in seawater with a domestic blender. Soya lecithin (Korot SL, Alcoy, Spain) with 74 % of lipids, around 80 % PL, 48 % of total FA as linoleic acid (LOA), and lacking EPA and DHA, was prepared following the same procedure described for Marine lecithin LC60.

Artemia metanauplii enrichment experiments

A series of three experiments was conducted to explore practical strategies to enhance the contents of both HUFA and PL in *Artemia* metanauplii. Experiment 1 aimed to enrich metanauplii in HUFA or PL separately. The objective of Experiment 2 was to ascertain whether HUFA and PL could be supplied simultaneously. Experiment 3 was used to enrich metanauplii in HUFA and PL using the alternative cheaper products lecithins. All the enrichment experiments were carried out by placing ~30000 5 d old metanauplii in a 1 L cylinder-conical tube containing 0.5 L of filtered seawater at 28°C, strong aeration from the bottom, and continuous light.

Experiment 1

In Experiment 1, carried out to enhance either the PL or HUFA contents of *Artemia* metanauplii, two experimental treatments were established: "Selco", consisting of an enrichment with the commercial emulsion Easy DHA Selco and seeking to provide HUFA to metanauplii; and "Liposomes", consisting of liposomes formulated with soya PC and cholesterol, and used to improve PL contents of metanauplii. In order to establish the incorporation dynamics of both enrichment diets and therefore the optimal duration of the incubation, metanauplii samples were collected after 2, 4, 6 and

24 h, and immediately stored at -80 °C until further analysis. Both enrichment products were dispensed at concentrations of 0.6 g L^{-1}.

Experiment 2

The objective of Experiment 2 was to produce metanauplii simultaneously enriched in PL and HUFA. Three different enrichment treatments were assayed: "Selco", consisting of the same commercial emulsion (Easy DHA Selco) dispensed at a concentration of 0.6 g L^{-1} during 4 h; "Seq Selco+Lipos", consisting of a first incubation with Easy DHA Selco for 2 h dispensed at 0.6 g L^{-1}, followed by a medium (filtered seawater) renewal and a subsequent 2 h enrichment with soya liposomes at 0.6 g L^{-1}; and "Mix Selco+Lipos", consisting of a mixture of liposomes (0.3 g L^{-1}) and Easy DHA Selco (0.3 g L^{-1}), both dispensed simultaneously at the beginning of the incubation and maintained for 4 h. Metanauplii samples were collected after 4 h, and stored at -80 °C for further analyses.

Experiment 3

This experiment, while aiming at the simultaneous provision of PL and HUFA to *Artemia* metanauplii like Experiment 2, assessed the use of lecithins, readily available coarse materials with potential use beyond experimental scale. Three enrichment treatments were established: "ML", consisting of a dispersion of Marine lecithin LC60 at 0.6 g L^{-1}; "Mix ML+Selco", being a mixture of dispersed marine lecithin LC60 (0.3 g L^{-1}) and Easy DHA Selco (0.3 g L^{-1}) dispensed at the beginning of the incubation; and "Mix SL+Selco", consisting of a mixture of dispersed soya lecithin (Korot SL) (0.3 g L^{-1}) and Easy DHA Selco (0.3 g L^{-1}) dispensed at the beginning of the incubation.

Metanauplii samples were collected after 4 h and immediately stored at -80 °C for further analyses.

Total lipid and fatty acid determination

Total lipids and FA were determined as described by Monroig *et al.* (2006). Briefly, total lipids from liophylized metanauplii samples were extracted with chloroform:methanol (2:1 v/v) according to the method of Folch *et al.* (1957). FA determinations were carried out according to the methodology described by Christie (1982). FA methyl esters (FAME) were extracted with hexane:diethyl ether (1 :1, v/v), and purified by thin layer chromatography (Silica gel G 60, Merck, Darmstadt, Germany) using a mix of hexane:diethyl ether:acetic acid (85 :15: 1.5, v/v/v) as solvent system. The analyses of FAME were performed with a Thermo (Thermo Trace GC Ultra, Thermo Electron Corporation, Waltham, MA, USA) gas chromatograph equipped with a fused silica 30 m x 0.25 mm open tubular column (Tracer, TR-WAX, film thickness: 0.25 µm, Teknokroma, Barcelona, Spain) and a cold on-column injection system. Helium was used as carrier, and a 50 to 220 ºC thermal gradient was established during the running of samples. A personal computer system equipped with Azur Datalys (St Martin d'Heres, France) software was used in the recording and processing the data proceeding from the flame-ionization detector. Peaks were determined by comparison with known standards.

Phospholipid determination

Phospholipids were estimated through the quantification of the inorganic phosphorous (Pi) of the total lipid fraction according to Zhou & Arthur (1992) and with the following modifications. Total lipid aliquots (50 µg) in duplicates were placed into

assay glass tubes and the solvents were evaporated off under nitrogen flux. After addition of 0.2 mL of perchloric acid (37% purity), samples were heated at 180 ºC for 1 h. After cooling, 0.2 mL of distilled water and 2 ml of working solution containing malachite green were added. Absorbance at 660 nm was then measured in a U-2001 Spectrophotometer (Hitachi, Tokio, Japan). Pi concentrations (µg g dw^{-1}) were calculated according to calibration curves constructed with KH_2PO_4 standard solutions of 1, 2.5, 5, 7.5 and 10 µg mL^{-1}.

Statistical analysis

Statistical analyses were performed with the SPSS for Windows 15.0 statistical package (SPSS Inc., Chicago, IL, USA). Data are expressed as means ± standard deviations. One-way ANOVAs were used to assess differences between treatments. *A posteriori* mean comparison Tukey's tests were utilized when appropriate. Differences in FA and Pi contents of the two treatments in Experiment 1 were compared with a Student's *t*-test. Comparisons of the means with P values less or equal than 0.05 were considered significantly different.

Results

Experiment 1

FA profiles of metanauplii collected from Experiment 1 are shown in Table 1. Differences between the treatments assayed in every FA at the same time of exposure were significant (*t*-test, $P \leq 0.05$) in all cases, except for linolenic acid (18:3n-3) at 24 h of enrichment. Results of *t*-tests are not shown in Table 1. Interestingly, the EPA contents of "Selco" metanauplii were significantly higher (*t*-test, $P \leq 0.001$) than those of

"Liposomes" metanauplii after only 2 h of exposure. Compared to EPA contents at 2 h, no significant increases in "Selco" metanauplii were detected despite longer exposure times (4, 6 and 24 h) (Table 1). Similarly, DHA was rapidly incorporated and only 2 h were required to achieve maximum contents in "Selco" *Artemia* (Table 1). DHA/EPA ratios, calculated only for "Selco" treatment as soya liposomes lack DHA, were significantly higher (ANOVA, $P \leq 0.001$) at 2, 4 and 6 h than at 24 h (Table 1), consistent with a steady decrease in the DHA contents of metanauplii from 4 to 24 h. These results indicate that 2 h of exposure is sufficient for delivering EPA and DHA through conventional commercial emulsion into *Artemia* metanauplii.

PL enrichment of *Artemia* metanauplii was also shown to be a rapid process when using liposomes. Thus, "Liposomes" metanauplii contained significantly more (*t*-test, $P \leq 0.05$) Pi than "Selco" treatment after 2 h, with also significant higher levels at 6 and 24 h of treatment (Fig. 1).

Experiment 2

The results obtained from Experiment 2 showed that it is possible to simultaneously enrich *Artemia* metanauplii in PL and HUFA, but increases in one of these components occur in detriment of the other. Whereas "Selco" metanauplii, treated exclusively with the commercial emulsion Easy DHA Selco, showed the highest EPA and DHA among treatments in Experiment 2 (Table 2), their Pi content was significantly lower than those of liposome-based treatments ("Seq Selco+Lipos" and "Mix Selco+Lipos") (Fig. 2). However, the increase of Pi in metanauplii treated with liposomes was achieved in detriment of HUFA incorporation, with an obvious dilution effect derived from the incorporation of LOA (18:2n-6) present in liposome soya PC

(Table 2). DHA/EPA ratios reflected the enrichment product FA composition, and significantly higher values (0.8) were obtained in "Selco" compared to liposome-treated nauplii (0.5-0.6). Comparison of liposome-enriched metanauplii (sequential or mixed treatments) showed that "Seq Selco+Lipos" metanauplii contained more PL (Fig. 2), although no differences (ANOVA, $P \leq 0.05$) in the contents of EPA and DHA were detected (Table 2).

Experiment 3

The use of lecithins (soya and marine origins) for enriching *Artemia* metanauplii was explored in Experiment 3. Thus, metanauplii treated with Marine lecithin LC60 (treatments "ML" and "Mix ML+Selco") exhibited notable EPA and DHA levels, particularly in "ML" metanauplii with 12.6 % (EPA) and 13.1 % (DHA) (Table 3). Consequently, DHA/EPA ratio also reached significant values, with the highest (1.0) corresponding to that of "ML" metanauplii. Moreover, "ML" metanauplii showed the highest Pi contents, significantly different (ANOVA, $P \leq 0.001$) compared to the levels of "Mix SL+Selco" metanauplii, despite they were treated with soya lecithin (Fig. 3).

Discussion

On-grown stages of *Artemia* (metanauplii) have been regarded as adequate live preys of suitable size for common octopus paralarvae, since eating bigger size preys has several advantages as it leads to a higher food intake per hunting effort, improving growth and survival (Villanueva *et al.,* 2002; Okumura *et al.,* 2005; Iglesias *et al*., 2006). Like naupliar stages, however, *Artemia* metanauplii appear to have suboptimal dietary lipid profiles as live preys for feeding marine organism larvae. The present study aimed to

develop adequate enrichment protocols, yet barely unexplored, for the enrichment of *Artemia* metanauplii with key lipid nutrients for octopus paralarvae, namely PL and HUFA (Navarro and Villanueva, 2000). Our results clearly show that it is possible to obtain *Artemia* metanauplii enriched in PL and HUFA, if appropriate materials and procedures are considered.

Results of Experiment 1 demonstrate that individual supply of HUFA or PL into *Artemia* metanauplii is possible. Thus, remarkable contents of HUFA, including EPA and DHA, were obtained by treating *Artemia* metanauplii with a commercial emulsion like Easy DHA Selco. Fish oil-based emulsions are widely used as enrichment diets for *Artemia* nauplii (Estévez & Kanazawa, 1995; Han *et al*., 2001), and the herein reported results clearly show that these products can be efficiently utilized as well for metanauplii enrichments. The use of emulsion-based products, however, appears inefficient for delivering PL into *Artemia* metanauplii, consistently with their formulation based on neutral lipids, in particular TAG (Monroig *et al*., 2006). Liposomes, in contrast, are an efficient means for delivering PL into metanauplii, and *Artemia* enriched with soya PC liposomes clearly exhibited enhanced PL levels compared to the emulsion-enriched metanauplii. Rather than emulsion-treated *Artemia*, a more adequate control treatment reflecting the basal PL levels of *Artemia* was unfortunately not available, as non-enriched on-grown metanauplii still retained phytoplankton cells, thus altering *Artemia* natural PL composition (Ruiz *et al*., 2008). Similarly, early studies pointed out that liposomes can enhance the PL fraction of *Artemia* naupliar stages (McEvoy *et al.*, 1996; Monroig *et al*. 2003, 2006), despite their inherent metabolic activity addressed to conserve the homeostasis phospholipid fraction in live preys as previously hypothesized (Rainuzzo *et al.*, 1994; Coutteau *et al.*, 1997).

Additionally to the provision of PL, it is noteworthy to mention that liposomes can facilitate the delivery of cholesterol that, while added as membrane stabilizer in our liposome formulations, can also exert beneficial effects for the octopus paralarvae as suggested by Navarro & Villanueva (2000). Further investigations are underway in our laboratories to ascertain the efficiency of liposomes as a tool for the cholesterol enrichment of *Artemia*.

The incorporation of HUFA or PL into *Artemia* metanauplii, which have a better developed filter-feeding apparatus (Dhont *et al*., 1991), appears to be a faster process. In contrast to longer (18-24 h) incubations required in the enrichment procedures of newly hatched *Artemia* nauplii (Monroig *et al*., 2006), enhanced HUFA or PL levels in metanauplii were achieved within only 2 h, with unremarkable increases observed thereafter. These results suggest that short-term incubations of 2 h for delivering each lipid ingredient (HUFA or PL) are sufficient and that longer incubations can be therefore avoided to prevent autoxidation of enrichment diets (McEvoy *et al*., 1995; Monroig *et al*., 2007) or undesired metabolic conversions of lipid classes (Rainuzzo *et al*., 1994; McEvoy *et al*., 1996;) and fatty acids (Navarro *et al*., 1999).

Combined use of emulsions and liposomes was investigated in order to achieve a simultaneous enhancement of HUFA and PL in metanauplii, and two different approaches were assessed. One strategy consisted of a sequential supply of commercial emulsion and a subsequent replacement by soya liposomes (treatment "Seq Selco+Lipos", Experiment 2). The other strategy consisted of a mixture of emulsion and liposomes that was dispensed at the beginning of the incubation (treatment "Mix Selco+Lipos", Experiment 2). While the two strategies resulted in an increase of PL fractions compared to emulsion-enriched *Artemia*, PL content was significantly higher

in metanauplii enriched through the sequential emulsion/liposome treatment compared to the mixture emulsion/liposome approach. Interestingly, no differences in HUFA contents including EPA and DHA were encountered in metanauplii enriched through the two distinct strategies. These results enlighten a novel strategy for simultaneously delivering HUFA and PL into *Artemia*, and more importantly, simpler and cheaper than the sequential emulsion/liposome treatment proposed by Barr *et al*. (2005) for naupliar stages that involved renewal and larger total amounts of enrichment products.

Despite liposome-based treatments in Experiment 2 demonstrated their ability to increase the HUFA contents of *Artemia* metanauplii, the significance of such increases might still be insufficient, if it is compared to natural preys (Navarro & Villanueva 2000; Iglesias *et al*., 2004) or to metanauplii treated exclusively with commercial emulsions. Thus, in Experiment 2, the DHA/EPA ratios, regarded as a good indicator of nutritional quality in live preys for marine larvae (Rodríguez *et al*., 1998; Sui *et al*., 2007), were significantly lower in "Seq Selco+Lipos" (0.5) and "Mix Selco+Lipos" (0.6) than in "Selco" treatment (0.8). These results indicated a dilution effect caused by the soya PC fatty acid composition with which the liposomes were formulated. This was further supported by the increased levels of LOA (18:2n-6) accounting for over 24 % of total FA in liposome-treated metanauplii. In order to increase the PL contents of *Artemia* metanauplii while preserving their HUFA, a marine lecithin, a coarse material containing HUFA-rich PL, was examined in Experiment 3.

The metanauplii treated with marine lecithin ("ML", Experiment 3) exhibited a DHA/EPA ratio of 1. More importantly, the individual HUFA content was notably high, with impressive percentages of EPA and DHA (~13% each). These results revealed a remarkable efficiency for the HUFA enrichment in *Artemia* metanauplii. Thus, if the

efficiency is estimated in an approximate way as [DHA incorporated in metanauplii/DHA present in the enrichment diet] x 100, it can be easily deduced that the marine lecithin enrichment is highly efficient with values of 40 % incorporation, whereas much lower efficiency (11%) was observed for metanauplii enriched with a prototype DHA-rich emulsion containing 70% of total FA that produced DHA contents of 8 % of total FA in nauplii (Viciano *et al*., 2011). In addition to HUFA delivery, the marine lecithin was also efficient in the provision of PL into metanauplii, showing the highest PL content among treatments in Experiment 3, even higher than PL levels of metanauplii enriched with soya lecithin ("Mix SL+Selco"). Overall our results demonstrate that Marine lecithin LC60 is a promising novel enrichment diet capable to enhance the HUFA and PL contents of *Artemia* metanauplii. Moreover, the marine lecithin can also be efficient when used for enrichment of other live preys employed in marine larviculture, including rotifers or *Artemia* naupliar stages.

In conclusion, the present study demonstrates that it is possible to increase simultaneously the contents of PL and essential HUFA of *Artemia* metanauplii treated with both fish oil-based emulsions and liposomes, as well as with marine-origin lecithins, resulting in a hypothetically more equilibrated living food for octopus paralarvae.

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Figure legends

Figure 1. Inorganic phosphorous content in the *Artemia* metanauplii lipid fraction collected during Experiment 1. Data represent mean ± SD (n=3). * Indicates significant differences between treatments in each experimental time (*t*-test, P≤0.05). Selco: Easy DHA Selco. Liposomes: multilamellar liposomes of soya phosphatidylcholine.

Figure 2. Inorganic phosphorous content in the *Artemia* metanauplii lipid fraction collected during Experiment 2. Data represent mean ± SD (n=3). Data that do not share the same letter differ significantly (ANOVA, P≤0.05). Selco: 4 h enrichment with Easy DHA Selco. Seq Selco+Lipos: 2 h enrichment with Easy DHA Selco + 2 h enrichment with soya phosphatidylcholine liposomes. Mix Selco+Lipos: 4 h enrichment with a mixture of Easy DHA Selco and soya phosphatidylcholine liposomes.

Figure 3. Inorganic phosphorous content in the *Artemia* metanauplii lipid fraction collected during Experiment 3. Data represent mean ± SD (n=3). Data that do not share the same letter differ significantly (ANOVA, P≤0.05). ML: 4 h enrichment with Marine lecithin LC60. Mix ML + Selco: 4 h enrichment with a mixture of Marine lecithin LC60 and Easy DHA Selco. Mix SL + Selco: 4 h enrichment with a mixture of soya lecithin and Easy DHA Selco.

Table 1. Selected fatty acid content (percentage of total fatty acids) in the *Artemia* metanauplii total lipid fraction collected during Experiment 1. Data represent mean ± SD (n=3). Data that do not share the same letter among incubation times in the same treatment differ significantly (ANOVA, $P \leq 0.05$)

	Selco				Liposomes			
Time (h)	2	4	6	24	2	4	6	24
16:0	12.9 ± 0.2^{a}	12.7 ± 0.5^{a}	12.7 ± 0.5^{a}	12.6 ± 0.0^{a}	11.0 ± 0.0^{a}	10.3 ± 0.1^{b}	10.1 ± 0.1^{b}	11.1 ± 0.3^{a}
18:0	8.3 ± 0.1^{a}	7.7 ± 0.1^{b}	7.7 ± 0.1^{b}	8.1 ± 0.1^{a}	7.4 ± 0.1^{a}	6.8 ± 0.2^{b}	6.5 ± 0.1^{b}	7.5 ± 0.2^{a}
18:1n-9	19.1 ± 0.2^{a}	19.9 ± 0.2^{b}	20.6 ± 0.2^{c}	23.5 ± 0.0^{d}	14.7 ± 0.0^{a}	14.3 ± 0.0^{ab}	14.0 ± 0.0^{b}	14.0 ± 0.4^{b}
18:1n-7	8.9 ± 0.1^{ab}	8.6 ± 0.2^{a}	8.6 ± 0.3^{a}	9.3 ± 0.1^{b}	7.6 ± 0.1^{a}	6.9 ± 0.0^{b}	6.68 ± 0.0^{bc}	6.5 ± 0.3^{c}
18:2n-6	4.4 ± 0.2^{a}	4.8 ± 0.1^{b}	5.1 ± 0.2^{b}	5.7 ± 0.0^{c}	31.9 ± 0.7^{a}	36.8 ± 0.4^{b}	39.0 ± 0.3^{bc}	40.0 ± 1.6^{c}
18:3n-3	9.8 ± 0.3^{a}	9.2 ± 0.3^{ab}	9.0 ± 0.3^{ab}	8.8 ± 0.2^{b}	10.9 ± 0.1^{a}	10.4 ± 0.1^{b}	10.0 ± 0.2^{c}	8.5 ± 0.2^{d}
20:4n-6	1.3 ± 0.0^{a}	1.3 ± 0.0^{ab}	1.4 ± 0.0^{b}	1.5 ± 0.0^{c}	0.6 ± 0.0^{a}	0.5 ± 0.0^{b}	0.5 ± 0.0^{b}	0.5 ± 0.0^{b}
20:5n-3	8.1 ± 0.1^{a}	8.4 ± 0.3^{a}	8.5 ± 0.3^{a}	8.3 ± 0.0^{a}	4.1 ± 0.1^{a}	3.7 ± 0.0^{ab}	3.4 ± 0.1^{b}	3.0 ± 0.2^{c}
22:6n-3	6.2 ± 0.1^{ab}	6.4 ± 0.1^{b}	6.1 ±0.1^{a}	2.6 ± 0.2^{c}	ND	ND	ND	ND
DHA:EPA Ratio	0.8 ± 0.0^{a}	0.8 ± 0.0^{a}	0.7 ± 0.0^{a}	0.3 ± 0.0^{b}	NA	NA	NA	NA

Σ % FAMES	95.0 ± 0.2[a]	95.3 ± 0.4[a]	95.5 ± 0.3[a]	94.3 ± 0.1[a]	96.1 ± 0.2[a]	96.7 ± 0.0[b]	96.8 ± 0.1[bc]	97.2 ± 0.1[c]
mg of total lipids/g dw	15.2 ± 0.2[a]	14.4 ± 0.3[a]	17.3 ± 0.6[a]	7.0 ± 2.5[b]	16.4 ± 1.1[a]	18.1 ± 0.4[a]	18.5 ± 0.3[a]	18.2 ± 1.5[a]

Selco: Easy DHA Selco.

Liposomes: multilamellar liposomes of soya phosphatidylcholine.

Tabla 2. Selected fatty acid content (percentage of total fatty acids) in the *Artemia* metanauplii total lipid fraction collected during Experiment 2. Data represent mean ± SD (n=3). Data that do not share the same letter differ significantly (ANOVA, P≤0.05)

	DHA Selco	Seq Selco+Lipos	Mix Selco+Lipos
16:0	13.7 ± 0.5^{a}	12.4 ± 0.0^{b}	12.9 ± 0.0^{b}
18:0	6.4 ± 0.1^{a}	5.9 ± 0.2^{b}	6.2 ± 0.2^{ab}
18:1n-9	18.7 ± 0.5^{a}	15.6 ± 0.4^{b}	14.9 ± 0.3^{b}
18:1n-7	6.6 ± 0.2^{a}	5.8 ± 0.2^{b}	6.0 ± 0.2^{b}
18:2n-6	8.5 ± 0.4^{a}	27.2 ± 0.2^{b}	24.9 ± 1.4^{c}
18:3n-3	11.6 ± 1.1^{a}	11.3 ± 1.2^{a}	11.9 ± 1.3^{a}
20:4n-6	2.2 ± 0.1^{a}	1.7 ± 0.1^{b}	1.6 ± 0.0^{b}
20:5n-3	7.5 ± 0.3^{a}	5.1 ± 0.3^{b}	4.8 ± 0.1^{b}
22:6n-3	6.0 ± 0.4^{a}	2.6 ± 0.4^{b}	2.7 ± 0.3^{b}
DHA:EPA Ratio	0.8 ± 0.0^{a}	0.5 ± 0.1^{b}	0.6 ± 0.0^{b}
Σ % FAMES	94.7 ± 0.2^{a}	95.6 ± 1.3^{a}	94.0 ± 0.5^{a}
mg of Total lipids/g dw	18.3 ± 0.6^{a}	19.6 ± 1.3^{a}	18.6 ± 0.8^{a}

Selco: 4 h enrichment with Easy DHA Selco.

Seq Selco+Lipos: 2 h enrichment with Easy DHA Selco + 2 h enrichment with soya phosphatidylcholine liposomes.

Mix Selco+Lipos: 4 h enrichment with a mixture of Easy DHA Selco and soya phosphatidylcholine liposomes.

Tabla 3. Selected fatty acid content (percentage of total fatty acids) in the *Artemia* metanauplii total lipid fraction collected during Experiment 3. Data represent mean ± SD (n=3). Data that do not share the same letter differ significantly (ANOVA, P≤0.05)

	ML	Mix ML+Selco	Mix SL+Selco
16:0	19.4 ± 0.4^{a}	14.2 ± 0.3^{b}	14.2 ± 0.2^{a}
18:0	7.6 ± 0.1^{a}	7.0 ± 0.1^{b}	7.2 ± 0.1^{c}
18:1n-9	11.7 ± 0.0^{a}	18.4 ± 0.2^{b}	19.6 ± 0.2^{c}
18:1n-7	6.1 ± 0.0^{a}	6.5 ± 0.2^{a}	6.6 ± 0.3^{a}
18:2n-6	2.8 ± 0.1^{a}	4.9 ± 0.1^{b}	9.1 ± 0.9^{c}
18:3n-3	11.2 ± 0.5^{a}	11.4 ± 0.4^{a}	11.2 ± 0.5^{a}
20:4n-6	1.8 ± 0.1^{a}	1.8 ± 0.0^{a}	1.5 ± 0.1^{b}
20:5n-3	12.6 ± 0.4^{a}	9.4 ± 0.2^{b}	7.6 ± 0.3^{c}
22:6n-3	13.1 ± 0.3^{a}	8.6 ± 0.2^{b}	5.8 ± 0.3^{c}
DHA:EPA Ratio	1.0 ± 0.0^{a}	0.9 ± 0.0^{b}	0.8 ± 0.0^{c}
Σ % FAMES	95.9 ± 0.2^{a}	96.6 ± 0.1^{b}	96.8 ± 0.3^{b}
mg of Total lipids/g dw	17.6 ± 0.1^{a}	17.3 ± 0.1^{b}	16.5 ± 1.0^{b}

ML: 4 h enrichment with marine lecithin LC60.

Mix ML+Selco: 4 h enrichment with a mixture of marine lecithin LC60 and Easy DHA Selco.

Mix SL+Selco: 4 h enrichment with a mixture of soya lecithin and Easy DHA Selco.

Figure 1

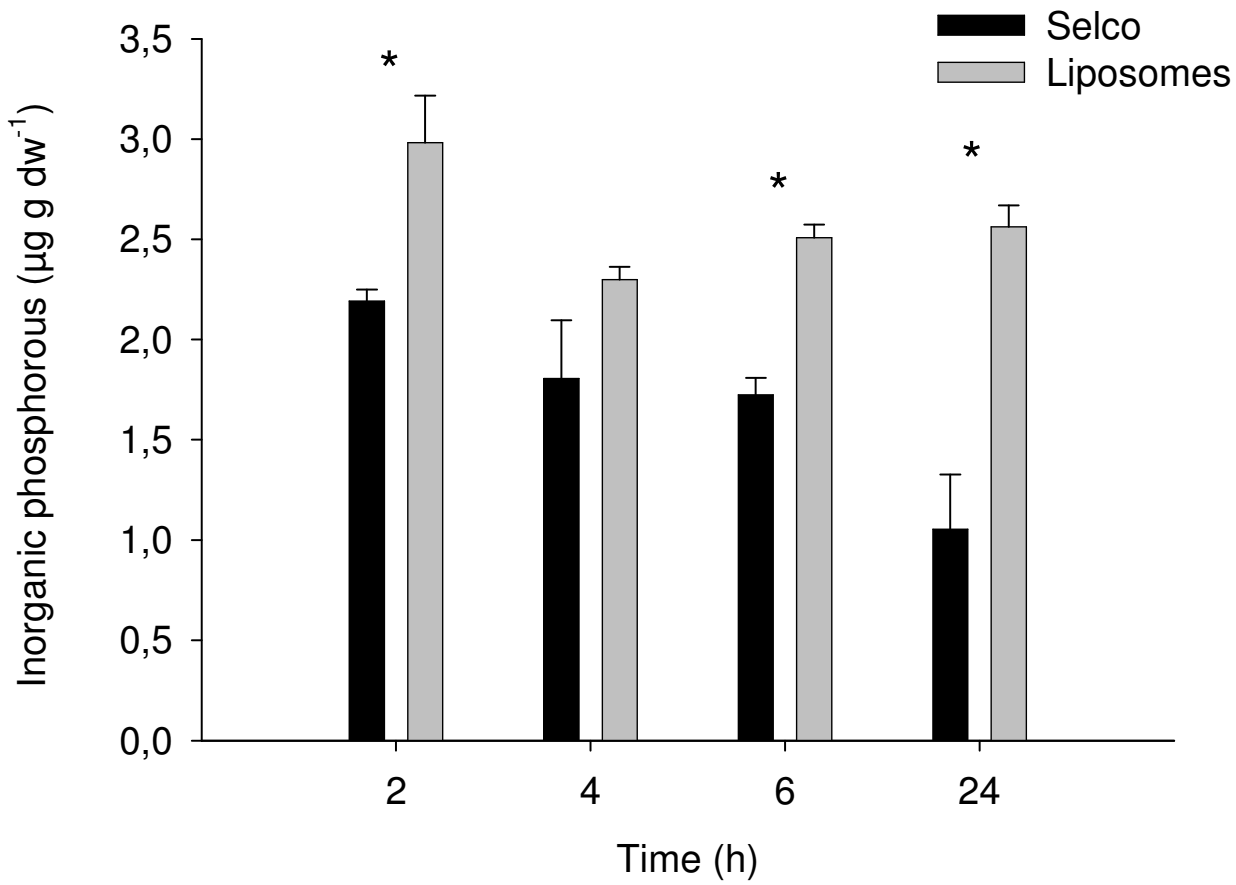

Selco
Liposomes
Inorganic phosphorous (µg g dw-1)
3,5
3,0
2,5
2,0
1,5
1,0
0,5
0,0
*
*
*
2
4
6
24
Time (h)

Figure 2

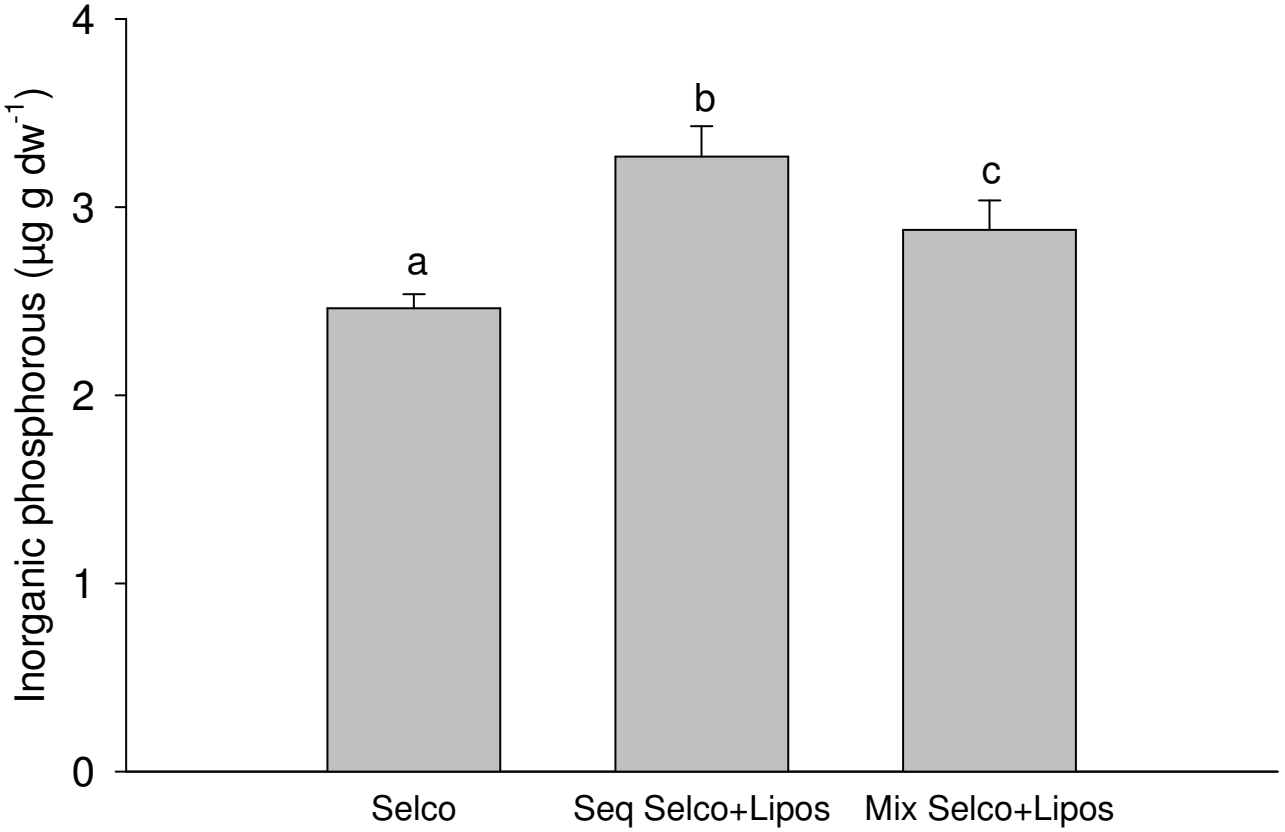
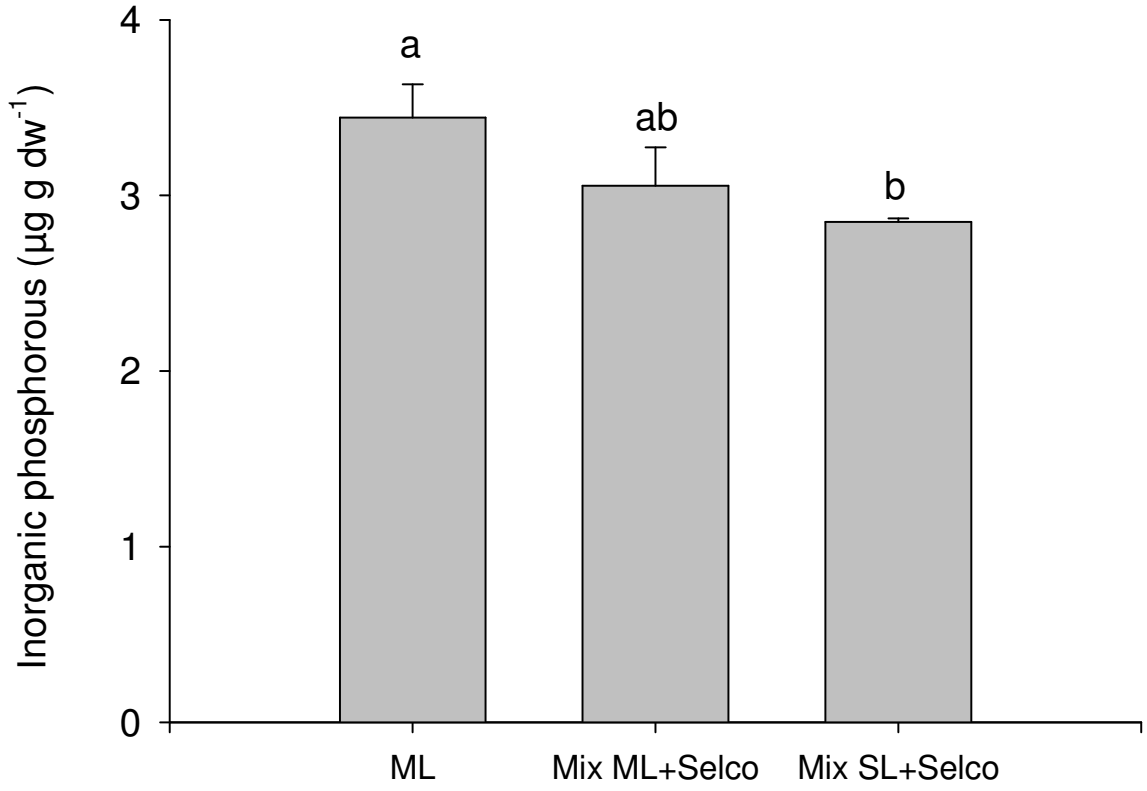

Inorganic phosphorous (µg g dw-1)
0
1
2
3
4
a
b
c
Selco
Seq Selco+Lipos
Mix Selco+Lipos

Figure 3


Inorganic phosphorous (µg g dw-1)
4
3
2
1
0
a
ab
b
ML
Mix ML+Selco
Mix SL+Selco