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Corresponding Author: Dr. Francisco Hontoria,

Corresponding Author's Institution: Instituto de Acuicultura de Torre de la Sal (CSIC)

First Author: Óscar Monroig, Dr.

Order of Authors: Óscar Monroig, Dr.; Juan Carlos Navarro, Dr.; Francisco Amat, Dr.; Francisco Hontoria

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Title:

Enrichment of *Artemia* nauplii in vitamin A, vitamin C and methionine using liposomes

Authors:

Óscar Monroig, Juan Carlos Navarro, Francisco Amat, Francisco Hontoria

Affiliation:

Instituto de Acuicultura de Torre de la Sal (CSIC). 12595 Torre de la Sal. Castellón, Spain

Correspondence address:

Francisco Hontoria. Instituto de Acuicultura de Torre de la Sal (CSIC). 12595 Torre de la Sal. Castellón. Spain. Telephone +34 964 319500. Fax +34 964 319509.

E-mail: hontoria@iats.csic.es

Abstract

Several types of liposomes were used to enrich *Artemia* nauplii in vitamin A, vitamin C and free methionine. In a first experiment, unilamellar liposomes formulated with krill phospholipid extract and retinyl palmitate demonstrated their capability to enhance the retinol content of *Artemia* nauplii. Furthermore, the increase in retinol was related to the amount of retinyl palmitate included in the liposomes as vitamin A source. These findings yield the possibility of using such vesicles to bioencapsulate simultaneously both vitamin A and essential fatty acids present in the krill phospholipid extract. A second enrichment was carried out with unilamellar liposomes composed of soybean phosphatidylcholine and loaded with sodium ascorbate as vitamin C source.

Our results did not show that vitamin C content in the nauplii could be increased using unilamellar liposomes. This was most likely due to the degradation of the vitamin C during enrichment as well as the ascorbate leakage. Finally, a third experiment assessed enrichment in free methionine using liposomes of different lamellarity (unilamellar or multilamellar) and composed of either soybean phosphatidylcholine or dipalmitoyl phosphatidylcholine, both combined with cholesterol as a membrane stabilizer. Results indicated that multilamellar liposomes represent a useful tool to deliver methionine to *Artemia* nauplii. Enhanced protection given by their multiple bilayers in comparison to unilamellar liposomes could account for the higher ability displayed by multilamellar vesicles for free methionine bioencapsulation.

Keywords: *Artemia* enrichment; liposomes; methionine; vitamin A; vitamin C

1. Introduction

Most of the studies on nutrition of marine fish larvae focus their attention on the importance of essential fatty acids (Izquierdo, 1996; Sargent et al., 1997). However, vitamins and free amino acids are also considered as essential nutrients, and therefore they have to be included in the diet for satisfying the requirements to maintain the different biological functions working correctly, and guarantee the normal growth (Rønnestad et al., 1999; Halver, 2002).

Vitamins are a heterogeneous group of compounds with a wide range of biological activities. Vitamins are classified according to their solubility, as either lipid or water soluble compounds (Gouillou-Coustans and Guillaume, 2001). Among liposoluble vitamins, vitamin A represents an essential nutrient for fish since they cannot synthesize this compound *de novo*. Vitamin A has been widely studied in fish because it is involved in vision and most of the fish reared species are visual feeders

(Hunter, 1981). Consequently, dietary deficiencies in vitamin A can alter normal function of vision in larvae, thereby reducing their predator ability. Moreover, dietary vitamin A deficiencies have been related to the occurrence of pseudoalbinism in flatfish such as halibut (*Hippoglossus hippoglossus*), turbot (*Psetta maxima*) and Japanese flounder (*Paralichthys olivaceus*). This syndrome is characterized by the lack of pigmentation in the body skin of the individual (Seikai et al., 1987; Estévez and Kanazawa, 1995). Furthermore, low vitamin A content in the live preys as *Artemia* can lead to an incomplete migration of the eye during flat fish metamorphosis (Saele et al., 2003). On the contrary, hypervitaminosis of vitamin A has been related to the backbone disease in Japanese flounder (Dedi et al., 1995, 1997), together with the jaw deformation (Haga et al., 2003).

In regard to hydrosoluble vitamins, vitamin C or ascorbic acid is a dietary essential nutrient for fish since these animals lack the enzyme gulonolactone oxidase required in its synthesis (Dabrowski, 1990). In addition to its essentiality, vitamin C also offers antioxidant (Hwang and Lin, 2002) and immunostimulant (Cuesta et al., 2002) properties, and protection against stress (Henrique et al., 2002). For these reasons, most diets used in aquaculture contain megadoses of vitamin C as a strategy to prevent diseases in cultured fish.

Amino acids are believed to be important dietary nutrients for early larval stages because it has been observed to be present in high amounts in fish eggs and in marine zooplankton, the food of the larvae (Rønnestad, 1992, Fyhn et al., 1993; Rønnestad et al., 1999). The main properties of free amino acids include their contribution to the formation of new tissues during body growth, their role as energy source supply (Rønnestad et al., 1999), and as effectors of chemical attraction (Kolkovski et al., 1997; Hara, 2006). Among essential amino acids, methionine can be considered to be a

limiting amino acid since its content in *Artemia* nauplii is only at trace or low levels (Helland et al., 2000).

Despite the fact that *Artemia* nauplii contain vitamin A, vitamin C and free amino acids (Merchie et al., 1997; Takeuchi et al., 1998; Helland et al., 2000), enrichment products normally include these compounds to enhance natural content of nauplii. However, inclusion of these compounds in the enrichment diet is somewhat difficult because of their different polarity. In the case of fish oil emulsions, one of the most habitual products employed in *Artemia* enrichment worldwide, lipid micelles formed when the product is dispersed in water posses a lipophilic core which impede the incorporation of hydrosoluble molecules. In the case of vitamin C, this handicap has been overcome using lipophilic derivatives such as ascorbyl palmitate which is stable in the emulsion micelles (Merchie et al., 1995a, b). Finally, amino acids are water soluble compounds and, consequently, their inclusion in lipid emulsions seems to be difficult.

Liposomes represent a potential alternative for the enrichment of live preys in both hydrophilic and lipophilic nutrients (Hontoria et al., 1994). Simply stated, liposomes are lipid vesicles enclosing an aqueous space (New, 1990). According to this structure, liposomes can be formulated with water-soluble compounds dissolved in the aqueous space and lipophilic nutrients imbibed in the apolar moiety of phospholipid fatty acid chains. Previous studies have assessed the capacity of liposomes to deliver different compounds to live preys used in marine larviculture (Hontoria et al., 1994; Ozkizilcik and Chu, 1994; Touraki et al., 1995; Tonheim et al., 2000; Monroig et al., 2006).

The present study assesses several types of liposomes in the enrichment of *Artemia* nauplii in vitamin A, vitamin C and methionine as models for lipophilic vitamins, hydrophilic vitamins and amino acids, respectively.

2. Materials and methods

2.1. Preparation of liposomes

Liposomes prepared by means of three different methodologies were employed to deliver vitamin A, vitamin C and methionine. Firstly, multilamellar vesicles (MLV) were prepared by the method proposed by Bangham et al. (1965), but using filtered seawater as the aqueous phase. Secondly, large unilamellar vesicles (LUV) were obtained by extrusion (LUVext) of MLV suspensions using an extruder LiposoFast equipped with 100 nm polycarbonate membranes (Avestin Inc., Ottawa, ON, Canada). Finally, a second type of unilamellar liposomes was prepared by a detergent solubilization methodology (LUVdet). In this latter typology, the aqueous phase consisted of a saline solution (0.9% NaCl). More details about these methodologies are found in Monroig et al. (2006).

Membranes of all three liposome typologies (MLV, LUVext and LUVdet) were formulated with three different phospholipid sources purchased from Avanti Polar Lipids (Alabaster, AL, USA): krill phospholipid extract (KPE), soybean phosphatidylcholine (SPC), and dipalmitoyl phosphatidylcholine (DPPC). Lipid class and fatty acid composition of phospholipids are presented in Table 1. Selection of the membrane components and liposome typology was adopted according to the lipophilic (retinyl palmitate) or hydrophilic (ascorbate and methionine) nature of the delivered nutrient. Hence, liposomes chosen to enrich *Artemia* in vitamin A were composed of KPE since this combination, although potentially leaky, would permit the simultaneous enrichment in both essential fatty acids and vitamin A. Contrarily, vitamin C and methionine were encapsulated in SPC and DPPC vesicles, which had demonstrated to efficiently retain water-soluble substances when submitted to *Artemia* enrichment conditions (Monroig et al., 2003). Occasionally, cholesterol (CHO) from Sigma

126 (Alcobendas, Spain) was included as a membrane stabilizer (New, 1990, p. 22).
127 Relevant features of the liposome suspensions used in this study are gathered in Table 2.
128 *2.2. Enrichment of* Artemia *nauplii in vitamin A*
129 Two types of unilamellar liposomes were used to enhance vitamin A of *Artemia*
130 nauplii, together with the commercial emulsion Super Selco (Inve, Ghent, Belgium) as a
131 reference product containing retinyl palmitate (Table 2). Both liposome suspensions
132 were formulated with KPE and retinyl palmitate in a ratio of 100:1 (w/w) in LUVext
133 (LUVext A), and 100:2 (w/w) in LUVdet (LUVdet A).
134 Commercial cysts (EG grade Inve, Ghent, Belgium) were incubated in aerated
135 seawater at a temperature of 28 ± 1ºC with an illumination of 1500-2000 lux. The cyst
136 incubation lasted for 23 h. During the following hour, the nauplii were separated from
137 the cyst shells and stocked at approximate densities of 300 nauplii ml^{-1} in 1 l cylinder-
138 conical enrichment tubes. At this point the nauplii were ready to begin the enrichment
139 process (t_0). Afterwards, the enrichment products were dispensed in a single dose
140 yielding retinyl palmitate concentrations of 1.3, 5.0 and 10.0 mg l^{-1} corresponding to
141 Emulsion, LUVext A and LUVdet A, respectively. Similarly to the cyst incubation, the
142 tubes were placed in a thermostated bath at a temperature of 28 ± 1ºC and illuminated
143 between 1500-2000 lux. Aeration was applied in each enrichment tube with 0.5
144 diameter section tubes, thereby yielding an approximate air flow of 1.0 litre per minute.
145 Samples of enriched nauplii were collected in a 100 µm plankton mesh and
146 gently washed with tap water, after 18 or 21 h of incubation in the enrichment medium.
147 Part of the nauplii were kept in clean seawater at the same conditions of nauplii
148 enrichment incubation stated above for 3 h more (24 h since the start of the incubation),
149 and then sampled. This sample point reflects the nutritional value of nauplii after some

150 hours in the culture tank, and emulates food quality in normal hatchery procedures.
151 Enriched nauplii samples were immediately frozen until nutrient analyses performed.

152 *2.3. Enrichment of* Artemia *nauplii in vitamin C*

153 Newly obtained *Artemia* nauplii were incubated with four enrichment diets
154 (Table 2). In addition to the commercial emulsion Super Selco (Emulsion) that contains
155 ascorbyl palmitate as vitamin C source, *Artemia* nauplii were incubated with LUVdet
156 liposomes composed of SPC as a unique membrane component and prepared with
157 sodium ascorbate (Sigma, Alcobendas, Spain) dissolved in the aqueous phase at a
158 concentration of 100 mg ml^{-1}. Liposomes were administered in a single dose at the
159 beginning of the incubation (t_0) (LUVdet C 1) or in two separate doses at t_0 and t_0+7 h
160 (LUVdet C 2). Finally, a fourth treatment consisted of sodium ascorbate directly
161 dissolved in the enrichment medium (Solution C). Both liposomes and solution
162 treatments yielded a sodium ascorbate concentration of 0.5 g l^{-1}, whereas the ascorbyl
163 palmitate content of the commercial emulsion was not specified by the manufacturer.
164 Sampling was carried out as described in the vitamin A enrichment.

165 *2.4. Enrichment of* Artemia *nauplii in methionine*

166 Four liposome-based treatments were used to enhance the free methionine
167 content of *Artemia* nauplii (Table 2). LUVext formulated with SPC and CHO (4:1, w/w)
168 as membrane components and methionine dissolved in the aqueous phase (40 mg ml^{-1})
169 were administered in a single dose at t_0 (LUVext M 1) or in two separate doses at t_0 and
170 t_0+8 h (LUVext M 2). A third enrichment product consisted of LUVext composed of
171 DPPC and CHO (4:1, w/w) and methionine dissolved in the aqueous phase (40 mg ml^{-1})
172 (LUVext M 3) administered in a single dose at the start of the nauplii incubation (t_0).
173 Liposome treatments were completed with multilamellar vesicles (MLV M) with an
174 equal composition than LUVext M 1 and LUV M 2, and administered in a single dose at

175 the beginning of incubation. As performed in the vitamin C experiment, liposomes were
176 compared with the efficiency displayed by methionine directly dissolved in the
177 enrichment medium and dispensed at t_0 (Solution M). In all five treatments,
178 concentration of methionine in the enrichment medium was 0.2 g l^{-1} after administration
179 of the total amount of product.

180 *2.5. Determination of vitamin A*

181 Analysis of retinol was carried out following the method proposed by Takeuchi
182 et al. (1998) with the following modifications. Total lipids were extracted from
183 biological samples (around 10 mg DW) according to the method described by Folch et
184 al. (1957), and then transferred to amber vials to avoid photodegradation. Lipid extracts
185 were dissolved in a methanol:acetone solution (1:1. v/v) containing 3.0 µg ml^{-1} of
186 retinyl acetate (Sigma, Alcobendas, Spain) used as an internal standard (Aust et al.,
187 2001).

188 Lipid extracts (20 µl) were injected in a high performance liquid
189 chromatography (HPLC) system (Thermo Liquid Chromatography, San Jose, CA,
190 USA) equipped with a quaternary pump P4000 employed at isocratic conditions.
191 Detection was performed using a UV-VIS UV6000LP detector set at a wavelength of
192 325 nm. An oven Croco-Cil (Cluzeau, Sainte-Foy-la-Grande, France) was used to keep
193 a C-18 Lichrospher (Merck, Darmstadt, Germany) at 30ºC during the elution. The
194 mobile phase consisted of 98% methanol containing 0.5% ammonium acetate (w/v) and
195 chloroform 85:15 (v/v) which was pumped at a flow rate of 1.5 ml min^{-1}

196 Peak identification was performed by comparing with commercial standards
197 obtained from Sigma (Alcobendas, Spain) and quantification of each peak was carried
198 out by comparing its relative area with that of the internal standard retinyl acetate.

ChromQuest Software (Thermo Quest, San Jose, CA, USA) was used in peak integration.

2.6. Determination of vitamin C

Analyses of ascorbate (vitamin C) were performed following the method described by Shiau and Hsu (1994). A weighed portion of enriched nauplii (around 10 mg DW) was suspended in 2 ml of ice cold 5% (w/v) metaphosphoric acid (Sigma, Alcobendas, Spain) and homogenized with a tissue disrupter (Ika Laborteknik, Staufen, Germany) for 30 s in an ice bath. Homogenates were centrifuged at 3000 g for 5 min, and supernatants were filtered through a 0.45 μm-pore-size glass fiber-nylon syringe filter (Whatman, Clifton, NJ, USA).

Ascorbate extracts (20 μl) were injected in the same HPLC system as described in the vitamin A analysis. In this case, mobile phase consisting of an aqueous solution of 0.05 M KH_2PO_4 (pH 2.8) was pumped at a flow rate of 0.9 ml min^{-1}. Elution was carried out at room temperature and the relative peak area was compared with ascorbic acid (Sigma, Alcobendas, Spain) as an external standard using a UV–VIS detector set at 254 nm. ChromQuest Software (Thermo Quest, San Jose, CA, USA) was used in peak integration.

2.7. Determination of free methionine

A modification of the method described by Koop et al. (1982) was used in the free amino acid determination. This method is based on a pre-column derivatization with phenylisothiocyanate (PITC, Sigma, Alcobendas, Spain). Samples of enriched nauplii (around 10 mg DW) were extracted in 1 ml of 6% (w/v) trichloracetic acid (Panreac, Castellar del Vallés, Spain) using a tissue disrupter (Ika Laborteknik, Staufen, Germany). After maintaining homogenates for 24 h at 4ºC, they were centrifuged

223 (10000 g) for 20 min, and supernatants were filtered through a 0.45 µm-pore-size glass
224 fiber-nylon syringe filter (Whatman, Clifton, NJ, USA).

225 Derivatization of free amino acids was accomplished after mixing 125 µl of the
226 filtrate and 25 µl of 2 mM of norleucine used as an internal standard (Sigma,
227 Alcobendas, Spain) with the following procedure. After desiccation of the mixture in a
228 speed vacuum, 20 µl of methanol:distilled water:trimethilamine (2:2:1, v/v/v) were
229 added to facilitate complete desiccation, and again introduced in the speed vacuum.
230 Finally, 15 µl of freshly prepared derivatizing reagent composed of methanol:distilled
231 water:trimethilamine:PITC (7:1:1:1, v/v/v/v) were added and incubated for 20 min at
232 room temperature. After this period, derivatized samples were completely desiccated in
233 the speed vacuum. At this point, samples were preserved at -20ºC until HPLC injection.

234 Sample injection (20 µl) was carried out after dissolving derivatized samples
235 with 200 µl of 5 mM Na_2HPO_4 (pH 7.4) containing 5% (v/v) acetonitrile. A C-18
236 Lichrospher (Merck, Darmstadt, Germany) column maintained at 52ºC during the
237 elution was employed. Injected samples were eluted through a gradient mixture of two
238 mobile phases: mobile phase A consisted of an aqueous solution of 70 nM sodium
239 acetate (pH 6.55) containing 2.5% (v/v) acetonitrile; and mobile phase B was composed
240 of acetronitrile:distilled water:methanol (45:40:15, v/v/v). All solvents were HPLC
241 grade (Merck, Darmstadt, Germany). The flow rate of the mobile phase was 1 ml min^{-1},
242 and followed the gradient program detailed in Table 3. The same UV-VIS detector as
243 described above was employed at 254 nm. Peaks were identified by comparison with
244 the amino acid commercial standard AA-S-18 (Sigma, Alcobendas, Spain), whereas
245 quantification was obtained by comparing its relative area with that of the internal
246 standard norleucine. ChromQuest Software (Thermo Quest, San Jose, CA, USA) was
247 used in peak integration.

248 *2.8. Statistical analyses*

249 Analytical data are expressed as means ± standard deviations. Homogeneity of
250 variances was checked by Barlett's test. In cases of homoscedasticity, differences in the
251 nutrient content among treatments in each sampling point were analyzed by one-way
252 analysis of variance (ANOVA) followed, where appropriate, by Tukey's multiple
253 comparison test (Sokal and Rohlf, 1981). If heterogeneity of variances existed, robust
254 tests were applied. Welch's test was used to check differences between treatments and
255 Game-Howell's test to establish differences among groups. Comparisons of the means
256 with *P* values equal or less than 0.05 were considered significantly different. All the
257 statistical analyses were carried out using the SPSS statistical package (SPSS Inc.,
258 Chicago, IL, USA).

259 **3. Results**

260 *3.1. Enrichment of* Artemia *nauplii in vitamin A*

261 Fig. 1 shows the retinol content of nauplii enriched with two different liposome
262 preparations and the commercial emulsion. A relationship between the amount of
263 retinyl palmitate present in the enrichment diet and the retinol measured in the treated
264 nauplii can be observed. Thus, nauplii enriched with LUVdet A displayed higher retinol
265 levels than the other two treatments in all three sampling points ($P \leq 0.05$). At the same
266 time, LUVext A nauplii show higher retinol concentration than nauplii enriched with
267 the commercial emulsion after 18 and 21 h of incubation, although no differences can
268 be seen at the 24 h sampling point ($P \leq 0.05$). It is noteworthy to mention that nauplii
269 enriched with the emulsion, that includes retinyl palmitate in its composition, exhibit a
270 retinol contents below the detection threshold. In regards to retinol incorporation
271 throughout enrichment, both liposome-based treatments did not exhibit important

changes from 18 to 21 h. Nonetheless, the subsequent starvation period (24 h sampling point) produced a drop in the retinol content of liposome enriched nauplii to the extent that retinol from LUVext A nauplii was below detection threshold at the 24 h sampling point (Fig. 1).

3.2. Enrichment of Artemia *nauplii in vitamin C*

Ascorbate analyses from nauplii enriched with the different products are shown in Fig. 2. In general, nauplii enriched with the commercial emulsion contained lower ascorbate levels in comparison to the other treatments. This difference became significant ($P \leq 0.05$) at 18 h of incubation. Another remarkable result is the lack of significant differences at any sampling point ($P \leq 0.05$) between the ascorbate content of nauplii enriched with liposomes administered in a single dose (LUVdet C 1) and two doses (LUVdet C 2). Nauplii incubated with dissolved ascorbate (Solution C) showed not only statistically equivalent levels than the other treatments, but also their ascorbate content was even higher ($P \leq 0.05$) than the other's after the starvation period. Concerning the temporal incorporation, ascorbate levels from nauplii did not undergo conclusive patterns, except for the increase observed during the starvation period in nauplii treated with dissolved ascorbate (Fig. 2).

3.3. Enrichment of Artemia *nauplii in free methionine*

Levels of free methionine measured in *Artemia* nauplii incubated with different enrichment products are represented in Fig. 3. After 18 h of incubation, nauplii enriched with MLV liposomes (MLV M) registered significantly higher contents of free methionine in comparison to the other treatments ($P \leq 0.05$). Such a difference was maintained for up to 21 h, although LUVext M 2 (2 doses) did not differ from MLV nauplii ($P \leq 0.05$) at that sampling point. Although this tendency is also observed at 24 h,

296 free methionine of nauplii enriched with MLV liposomes only showed significant
297 higher contents if compared to LUVext M 1 and Solution M nauplii. As seen in the
298 retinol and ascorbate experiments, temporal evolution of the free methionine levels of
299 nauplii showed minor changes from 18 to 21 h, except for LUVext M 1 nauplii, which
300 seemed to exhibit an increase during this period. On the contrary, the starvation period
301 was accompanied by a general decrease in the free methionine visible in all five
302 treatments.

303 **4. Discussion**

304 The ability of liposomes to deliver both hydrophilic and lipophilic substances
305 has been employed to improve the nutritional value of live preys used for feeding
306 marine fish larvae (Ozkizilcik and Chu, 1994; McEvoy et al., 1996; Tonheim et al.,
307 2000). With the exception of the pioneer study undertaken by Hontoria et al. (1997) on
308 vitamin encapsulation with liposomes, the present study represents the first report of
309 vitamin A bioencapsulation in live preys by means of these lipid vesicles. Some
310 nutritional studies on flat fish larvae have assessed the efficiency of several compounds
311 with vitamin A activity (i.e., retinol, retinyl esters, etc.) included in oil emulsions and
312 bioencapsulated in *Artemia* nauplii (Dedi et al., 1995; Estévez and Kanazawa, 1995;
313 Takeuchi et al., 1998; Haga et al., 2004). Results obtained in the present study illustrate
314 the efficiency of liposomes formulated with krill phospholipids and retinyl palmitate to
315 enhance the retinol content in *Artemia* nauplii. Additionally, the amount of retinol in the
316 nauplii can be directly related to the retinyl ester included in the liposome membrane,
317 thereby pointing at a metabolic transformation by nauplial enzymatic system as
318 previously described by Takeuchi et al. (1998). Retinyl palmitate incorporated in
319 liposomes would be hydrolyzed to free retinol before its absorption in the intestinal cells
320 (Furr and McGrane, 2003). Metabolic activity of nauplii may also explain the decrease

321 in the retinol levels observed throughout the starvation period in liposome enriched
322 nauplii, probably due to a resterification of retinol (Furr and McGrane, 2003) within
323 digestive tract cells. A shocking result is the low retinol level obtained in nauplii
324 incubated with the commercial emulsion despite containing retinyl palmitate. This poor
325 efficiency of the commercial emulsion may, in part, be due to a partial degradation
326 during the incubation of the enrichment product under aggressive conditions of light,
327 oxygen and temperature (Woollard and Indyk, 2003). Indeed, Moren et al. (2005) have
328 recently proposed this degradation mechanism to explain why no vitamin A was
329 detected when another enrichment product (DC-DHA Selco) was submitted to
330 simulated enrichment conditions during 12 h. Despite the fact that liposomes could
331 undergo the same process, the higher amount of retinyl palmitate present in liposomes
332 in comparison to the commercial emulsion would preserve enough quantity to be
333 bioencapsulated in the *Artemia* nauplii before degradation.

334 Regarding the vitamin C enrichment, our results revealed that enhancement in
335 the ascorbate content of *Artemia* nauplii was more efficient when ascorbate was
336 encapsulated in liposomes or simply dissolved in the water in comparison to the oil
337 emulsion formulated with ascorbyl palmitate. The importance of such a result is
338 difficult to evaluate since the amount of vitamin C available in all treatments was not
339 the same. Whereas nauplii treated with liposomes and the aqueous solution were
340 incubated at the same initial ascorbate concentration in the medium (0.5 g l^{-1}), the
341 amount of vitamin C available for nauplii enriched with the commercial emulsion
342 depends on the concentration of ascorbyl palmitate present in the product (not specified
343 by the manufacturer), along with the efficiency of the nauplii to hydrolyze the ester
344 derivative to free ascorbate (Merchie et al., 1995a). However, results do not prove the
345 presumable advantage of using liposomes instead of the aqueous solution, since

346 differences between liposomes and the aqueous solution did not exist. Firstly, the low
347 efficiency of liposomes to bioencapsulate ascorbate in *Artemia* can be partly explained
348 through the susceptibility of ascorbate to degrade (Halver, 2002) as occurred in vitamin
349 A. Preservation of vitamin C by means of other antioxidant compounds such as vitamin
350 E included in the membrane (Urano et al., 1987; Fukuzawa et al., 1993a, b) or the
351 utilization of more resistant derivatives (Schüep et al., 1989; Shiau and Hsu, 1994;
352 Yoshitomi, 2004) would improve the efficiency of liposomes as an ascorbate carrier to
353 *Artemia* nauplii. Secondly, the turbulent regime of enrichment would produce the
354 leakage of water-soluble compounds encapsulated in liposomes. This phenomenon,
355 which is expected to affect other hydrosoluble substances such as methionine, is
356 discussed later on.

357 Results from the methionine enrichment indicate the possibility of using
358 liposomes to enhance the free amino acid pool in *Artemia* nauplii. Nauplii enriched with
359 multilamellar liposomes (MLV) presented higher levels of free methionine than
360 unilamellar liposomes (LUV), despite the fact that LUV are considered to have the most
361 efficient typology to maximize encapsulation of water-soluble compounds because of
362 the high internal volume:membrane lipid ratio (New, 1990, p. 28). Nevertheless, under
363 high turbulence conditions of enrichment procedures, MLV formulations seem to be
364 more suitable than LUV possibly due to their protective multiple bilayer structure
365 (between 5 and 20 layers). Therefore, methionine dissolved in the more internal
366 intermembrane spaces of MLV would remain encapsulated, whereas the turbulence
367 during incubation would cause the leakage of water-soluble compounds from the
368 aqueous compartments located between the more outer membranes, as would also occur
369 in the unique aqueous space of LUV. This low performance of LUV could neither be
370 improved through the dosage of liposomes in two separate doses, despite the fact that

371 this strategy would increase the time during which vesicles are still loaded with the
372 water-soluble nutrient (Monroig et al., 2003). Consequently, bioencapsulation of
373 methionine loaded particles (MLV liposomes) would be favoured in comparison to
374 methionine simply dissolved in the enrichment medium (case of original methionine
375 solution and empty unilamellar liposomes) since filtering appendages of nauplii are
376 adapted to retain discrete particles of a certain size present in the water column.

377 The direct incorporation of methionine through ingestion of enrichment medium
378 by nauplii should also be considered, as *Artemia* nauplii drink medium for
379 osmoregulatory balance (Navarro et al., 1993). Considering the content of free
380 methionine from newly hatched nauplii (88.4 µg g DW^{-1}) as the basal level, free
381 methionine measured in nauplii enriched with the methionine solution (Solution M)
382 (between 300 and 400 µg g DW^{-1}) possibly indicate a net incorporation of the amino
383 acid through ingestion of the medium. The existence of this direct pathway was
384 demonstrated in *Artemia* enrichments with radioactive methionine (Tonheim et al.,
385 2000). In regards to temporal incorporation, analyses did not indicate an important
386 increase in the free methionine content during the period between 18 and 21 h. Despite
387 the supply of methionine during this period, the free methionine already
388 bioencapsulated in the nauplii could not be detected since nauplii can assimilate it into
389 the protein structure, transform it into other amino acids (cysteine or taurine), and
390 oxidize it to sulphate products (Tonheim et al., 2000). Moreover, these mechanisms
391 could still occur during the starvation period (from 21 to 24 h), thus explaining the drop
392 of free methionine levels in all treatments. Indeed, total absorbed methionine was
393 estimated to be two fold compared to that present in a free state (Tonheim et al., 2000).

394 In light of these results, unilamellar vesicles cannot be considered the best
395 alternative to encapsulate hydrophilic nutrients in liposomes employed in *Artemia*

396 enrichments, contrarily to the results obtained from another study (Monroig et al., 2003)
397 where LUV suspensions retained a fluorescent marker during a longer period than MLV
398 liposomes. This apparent discrepancy can be related to several reasons. Firstly, MLV
399 suspensions tested by Monroig et al. (2003) were formulated with krill phospholipids
400 which would form leaky vesicles due to the unsaturation degree of their fatty acid
401 chains (Alberts et al., 1996, p.512-513). On the contrary, formulation of multilamellar
402 liposomes with purified soybean phosphatidylcholine in the present study would
403 improve the retention of water-soluble substances included in these vesicles. Secondly,
404 differences in the hydrodynamic conditions set in both studies could also explain the
405 different behaviour of multilamellar liposomes. Incubation of nauplii in the present
406 study was carried out in 1 l tubes with an aeration flow around 1.0 lpm, whereas we
407 previously used smaller tubes (200 ml) where aeration was reduced to simulate normal
408 turbulence applied in standard 1 l enrichment tubes (Monroig et al., 2003). While
409 attempting to standardize turbulence in the 200 ml tubes, the degree of agitation in the
410 enrichment medium in this study could have resulted to be higher than the previous
411 study by Monroig et al. (2003). Finally, differences between the solubility of the
412 fluorescent marker carboxyfluorescein used in our preliminary experiment (Monroig et
413 al., 2003) and the water-soluble substances used here, such as methionine, could affect
414 their leakage rate and, consequently, make results from both studies not fully
415 comparable.

416 In summary, liposomes can be presented as an efficient system for the
417 enrichment of *Artemia* nauplii in retinol. Furthermore, retinol incorporation was related
418 to the amount of retinyl palmitate included in the liposome formulation. These findings
419 demonstrate the potential of these vesicles to bioencapsulate simultaneously both
420 vitamin A and essential fatty acids present in the krill phospholipid extract. Regarding

water-soluble nutrients, the present study could not demonstrate the efficiency of unilamellar liposomes to increase the vitamin C content of nauplii probably due to degradation during the enrichment process. The use of more stable derivatives, together with the combination with other antioxidant compounds could improve liposome capacity for vitamin C enrichment. In addition, leakage of vitamin C could be another factor limiting the liposome efficiency. As shown in the methionine enrichment, multilamellar liposomes could be a good candidate to deliver hydrosoluble compounds to nauplii. Enhanced protection given by their multiple bilayers could account for a high ability of water-soluble retention inside the liposomes. In these circumstances, nauplii would incorporate particulate liposomes more efficiently than dissolved molecules by means of their adapted filtering-natatory appendages.

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578 Tables

579 Table 1. Selected fatty acid (percent of total fatty acids) and lipid class composition
580 (percent of total lipids) of phospholipid sources used in the liposome formulation.

	KPE	SPC	DPPC
Fatty acid			
16:0	25.5	15.1	100.0
18:0	1.0	3.0	0.0
18:1n-9	5.4	13.6	0.0
18:2n-6	1.8	60.6	0.0
18:3n-3	1.4	3.6	0.0
20:4n-6	0.7	0.0	0.0
20:5n-3	29.7	0.0	0.0
22:5n-3	0.8	0.0	0.0
22:6n-3	18.4	0.0	0.0
Saturates	28.6	18.7	100.0
Monounsaturates	13.2	15.8	0.0
Polyunsaturates	55.8	64.5	0.0
Total n-3	52.5	3.8	0.0
Total n-6	3.2	60.7	0.0
HUFA n-3	49.3	0.2	0.0
HUFA n-6	1.4	0.1	0.0
DHA/EPA	0.6	0.0	0.0
Phospholipid class			
PC	67.0	95.0	100.0
PE	9.0	0.0	0.0
Other	24.0	5.0	0.0

581 HUFA n-3: ≥20:3n-3; HUFA n-6: ≥20:2n-6; DHA/EPA: docosahexaenoic to
582 eicosapentaenoic fatty acid ratio; PC: phosphatidylcholine; PE:
583 phosphatidylethanolamine.

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586

587 Table 2. Enrichment products assessed in the *Artemia* enrichment in vitamin A, vitamin
588 C and methionine.

Delivered nutrient	*Enrichment products*	*Lamellarity (liposomes)*	*Preparation method (liposomes)*	*Dosage*	*Membrane composition (w/w)*
Vitamin A	Emulsion	-	-	1	-
	LUVext A	Unilamellar	Extrusion	1	KPE:vitA (100:1)
	LUVdet A	Unilamellar	Detergent solubilization	1	KPE:vitA (100:2)
Vitamin C	Emulsion	-	-	1	-
	LUVdet C 1	Unilamellar	Detergent solubilization	1	SPC (100%)
	LUVdet C 2	Unilamellar	Detergent solubilization	2	SPC (100%)
	Solution C	-	-	1	-
Methionine	LUVext M 1	Unilamellar	Extrusion	1	SPC:CHO (4:1)
	LUVext M 2	Unilamellar	Extrusion	2	SPC:CHO (4:1)
	LUVext M 3	Unilamellar	Extrusion	1	DPPC:CHO (4:1)
	MLV M	Multilamellar	Simple lipid hydration	1	SPC:CHO (4:1)
	Solution M	-	-	1	-

589 LUVdet: large unilamellar vesicles prepared by the detergent solubilization
590 methodology; LUVext: large unilamellar vesicles prepared by extrusion through
591 polycarbonate membranes; MLV: multilamellar vesicles; KPE: krill phospholipid
592 extract; SPC: soybean phosphatidylcholine; DPPC: dipalmitoyl phosphatidylcholine;
593 CHO: cholesterol; vitA: retinyl palmitate.

594

595 Table 3. Gradient elution program followed in the analysis of free amino acids.

Time (min)	% phase A	% phase B
0.0	100.0	0.0
13.5	97.0	3.0
16.5	97.0	3.0
19.0	96.5	3.5
21.0	95.5	4.5
24.0	94.0	6.0
25.0	93.0	7.0
26.5	92.0	8.0
28.5	91.0	9.0
30.0	91.0	9.0
50.0	66.0	34.0
65.0	30.0	70.0

596 Mobile phase A, aqueous solution of 70 nM sodium acetate (pH 6.55) containing 2.5%
597 (v/v) acetonitrile; Mobile phase B, solution of acetonitrile:destilled water:methanol
598 (45:40:15, v/v/v).
599
600
601 Figure legends
602 Figure 1. Contents of retinol (µg per gram of dry weight) of *Artemia* nauplii enriched
603 with different products. Samples of 24 h belong to nauplii kept during 3 h in clean
604 seawater after 21 h of incubation with the enrichment product. Data are means of three
605 replicates. Error bars represent standard deviations. Treatments sharing the same
606 superscript letter in a sampling point are not significantly different from each other
607 ($P \leq 0.05$).
608 Figure 2. Contents of ascorbate (mg per gram of dry weight) of *Artemia* nauplii
609 enriched with different products. Samples of 24 h belong to nauplii kept during 3 h in
610 clean seawater after 21 h of incubation with the enrichment product. Data are means of
611 three replicates. Error bars represent standard deviations. Treatments sharing the same
612 superscript letter in a sampling point are not significantly different from each other
613 ($P \leq 0.05$).
614 Figure 3. Contents of free methionine (µg per gram of dry weight) of *Artemia* nauplii
615 enriched with different products. Samples of 24 h belong to nauplii kept during 3 h in
616 clean seawater after 21 h of incubation with the enrichment product. Data are means of
617 three replicates. Error bars represent standard deviations. Treatments sharing the same
618 superscript letter in a sampling point are not significantly different from each other
619 ($P \leq 0.05$).
620

Figure 1

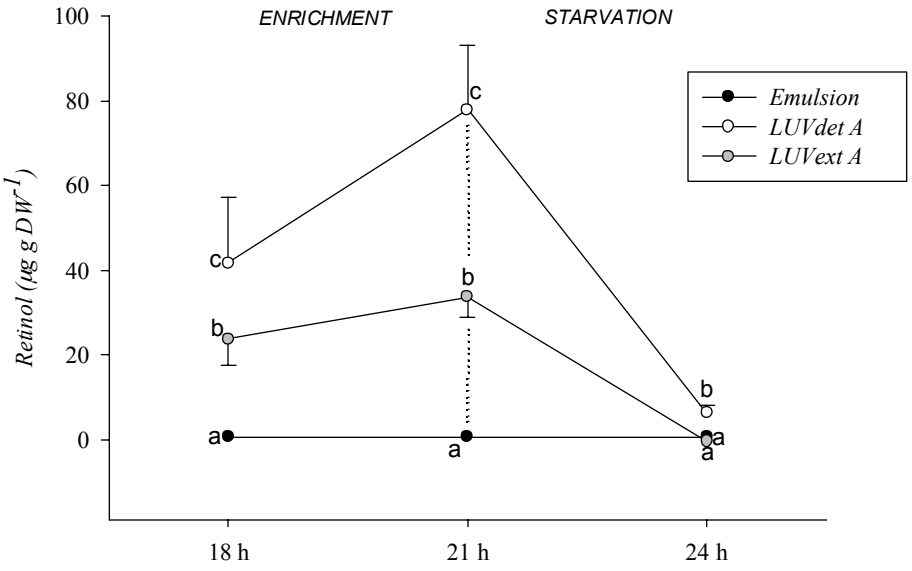

ENRICHMENT
STARVATION
Emulsion
LUVdet A
LUVext A
Retinol (µg g DW-1)
100
80
60
40
20
0
18 h
21 h
24 h
a
b
c

Figure 2

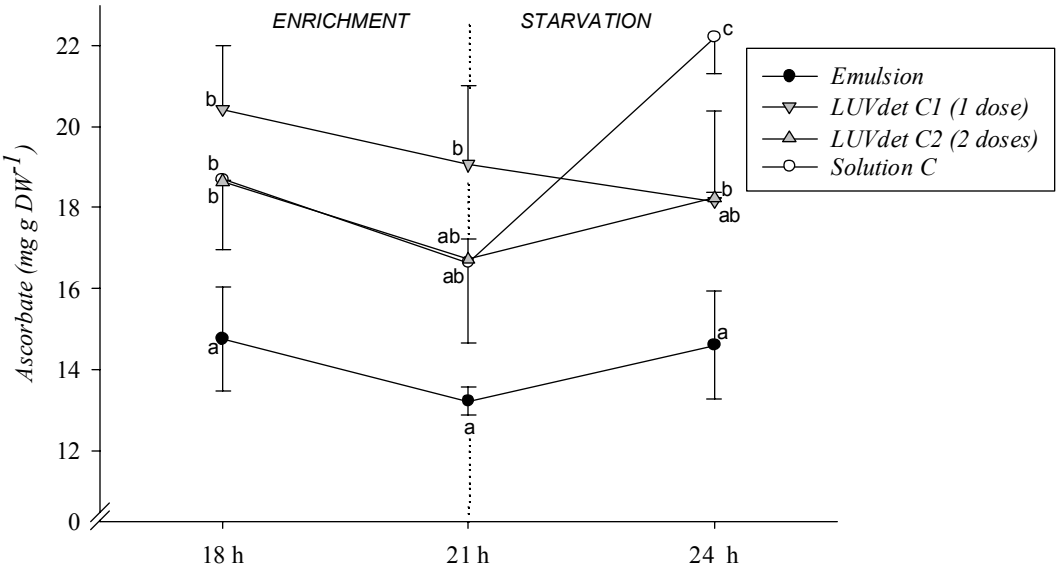
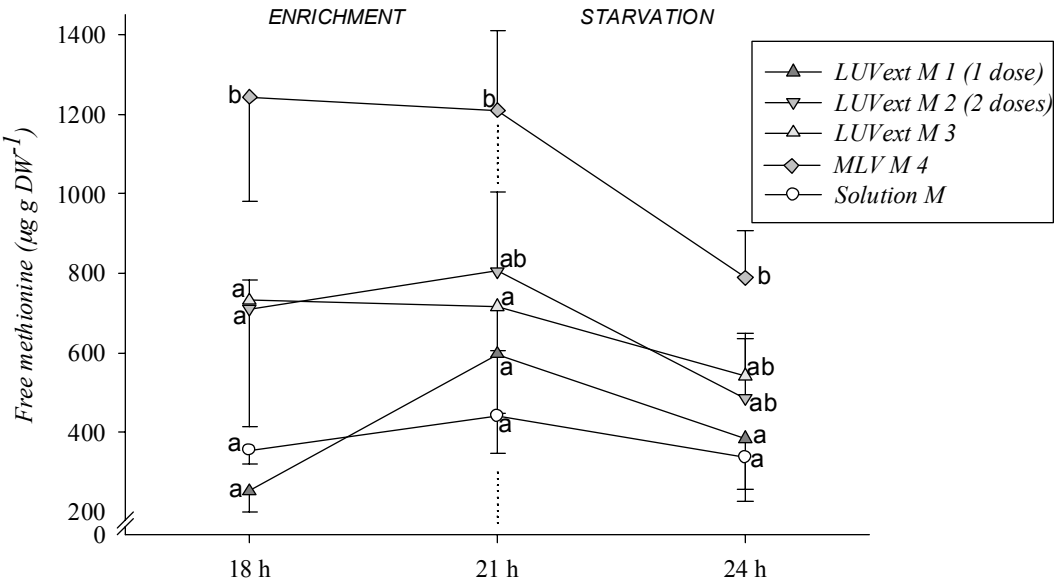

ENRICHMENT
STARVATION
Ascorbate (mg g DW-1)
22
20
18
16
14
12
0
18 h
21 h
24 h
Emulsion
LUVdet C1 (1 dose)
LUVdet C2 (2 doses)
Solution C
a
a
a
b
b
b
b
b
ab
ab
ab
c

Figure 3


ENRICHMENT
STARVATION
Free methionine (µg g DW-1)
1400
1200
1000
800
600
400
200
0
18 h
21 h
24 h
LUVext M 1 (1 dose)
LUVext M 2 (2 doses)
LUVext M 3
MLV M 4
Solution M
b
b
b
ab
a
a
a
ab
ab
a
a
a
a
a