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Title:

Oxidative stability and changes in the particle size of liposomes used in the *Artemia* enrichment

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Abstract

The oxidative stability and the particle size of several types of liposomes were assessed in order to characterize their behaviour when submitted to the aggressive conditions of the *Artemia* enrichments. Results show that all liposomes tested in this study were much more oxidatively stable than a commercial product based on fish oil emulsion. Whereas the initial thiobarbituric acid reactive substances (TBARS) concentration in the emulsion was only slightly higher than in liposomes, the

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1. Introduction

Deficiencies in the nutritional value of live preys used in the rearing of marine fish larvae has been solved through enrichment procedures with products containing high levels of essential nutrients. During this boosting process, live preys such as rotifers and *Artemia* nauplii are incubated in aqueous media where the enrichment diet is dispersed, thereby facilitating its incorporation through the passive filtering behaviour of these organisms (Léger et al., 1986). A broad variety of enrichment products are available and can be classified as those based on microalgae (Wickins, 1972; Watanabe et al., 1978; Watanabe et al., 1982), spray-dried cells of *Schizochytrium* sp. (Barclay and Zeller, 1996), microcapsules (Southgate and Lou, 1995) and oil emulsion products (Léger et al., 1986; Han et al., 2000; Copeman et al., 2002). All of them contain significant levels of highly polyunsaturated fatty acids (HUFA) as eicosapentaenoic

(EPA, 20:5n-3), docosahexaenoic (DHA, 22:6n-6) and arachidonic acids (ARA, 20:4n-6) which are essential for marine fish larvae (Sargent et al., 1997; Izquierdo et al., 2000). In addition to these compounds, several studies have focused their attention on the importance of other nutrients such as vitamins (Merchie et al., 1997; Halver, 2002) and free amino acids (Rønnestad et al., 1999; Tonheim et al., 2000; Rønnestad et al., 2003) on the early development of marine fish larvae. Although some effort has been made on the formulation of novel products which provide simultaneously all essential nutrients (Dhert et al., 2005), the different water or lipid solubility of such essential compounds has limited the success.

Since liposomes can include both hydrophilic and lipophilic substances, some authors have proposed their use as an alternative tool to deliver essential nutrients to marine fish larvae through their bioencapsulation in live preys (Ozkizilcik and Chu, 1994; Hontoria et al., 1994; McEvoy et al., 1996; Tonheim et al., 2000; Monroig et al., 2003; Monroig et al., 2006a, b). Simply stated, liposomes can be defined as phospholipid vesicles that enclose an aqueous internal compartment and with variable sizes ranging from some dozens of nanometres to several microns, (New, 1990). The previously described characteristics (i.e., the existence of an internal space, their phospholipid based composition and the small particle size) differentiate liposomes from other commercially available enrichment products, and hence determine their behaviour as enrichment diets.

As a preliminary attempt to study how liposomes behave under the *Artemia* enrichment conditions, Monroig et al. (2003) assessed the capacity of several types of vesicles to retain water soluble compounds dissolved in the internal aqueous space. It was concluded that lamellarity, the incubation time and the membrane formulation determine the leakage rate of water soluble compounds from the aqueous compartment

to the external space out of the membrane. However, other parameters are necessary to be studied for the proper assessment of the liposome capability for the live prey enrichment.

Lipid peroxidation, as an estimator of the oxidative stability, has been described to be an important degradation process of polyunsaturated fatty acids (Gurr and Harwood, 1991; Frankel, 1998). As aforementioned, most of the enrichment diets are basically composed of polyunsaturated fatty acids and hence are susceptible to peroxidize when exposed to the aggressive conditions of live prey enrichments i.e. illumination, high temperature and dissolved oxygen (McEvoy et al., 1995). Therefore, the oxidation process produces terminal toxic compounds such as aldehydes and ketones which can be accumulated in the live preys through their passive filtering behaviour. Finally, consumption of such harmful diets can lead to detrimental effects (Hipkiss et al., 1997; Tesoriere et al., 2002; Del Rio et al., 2005) on larvae. Alternatively, the phospholipid composition of liposomes can preserve the oxidative stability during enrichment since several studies have demonstrated the antioxidant properties of these compounds in comparison to other lipid classes such as triglycerides and fatty acid ethyl esters (King et al., 1992a, b; Song et al., 1997).

Particle size is an important property for the enrichment products as it can determine the bioencapsulation efficiency in the live prey (Gelabert-Fernández, R., 2002; Han et al., 2005). Generally speaking, commercial enrichment diets produce several micron-sized particles when dispersed in the enrichment medium (Léger et al., 1987; Southgate and Lou, 1995; Han et al., 2005). On the other hand, the unilamellar liposomes used in the present study are smaller with diameters varying around 150 nm. Likewise, incubation of liposomes in the enrichment medium itself can favour the occurrence of different phenomena such as fusion and aggregation, which can modify

100 the original size distribution (New, 1990), and therefore the bioencapsulation efficiency
101 in the nauplii.

102 In order to better characterize the behaviour of liposomes during the *Artemia*
103 enrichment, we studied the oxidative stability of several liposome formulations when
104 submitted to standard enrichment conditions, together with the changes in the vesicle
105 size throughout the process. For this reason, the concentration of TBARS formed during
106 the incubation of liposomes was used as an estimator of oxidative stability. In a second
107 experiment, changes in the particle size of liposomes were monitored throughout the
108 incubation period by means of a light scattering laser technique.

109 **2. Materials and methods**

110 *2.1. Preparation of liposomes*

111 The oxidative stability and the changes in the vesicle size were assessed in five
112 liposome formulations submitted to standard *Artemia* enrichment conditions. Three
113 different phospholipid sources (see Table 1 for fatty acid and lipid class composition)
114 were used in the preparation of liposomes: krill phospholipid extract (KPE), dipalmitoyl
115 phosphatidylcholine (DPPC), and soybean phosphatidylcholine (SPC), all from Avanti
116 Polar Lipids Inc. (Alabaster, AL, USA).

117 All five liposomes were unilamellar vesicles and were prepared using two
118 different methodologies. Firstly, multilamellar liposome suspensions obtained by simple
119 hydration of a phospholipid film with seawater (Monroig et al., 2003) were extruded
120 using a LiposoFast engine (Avestin Inc., Ottawa, ON, Canada) equipped with 100 nm
121 polycarbonate membranes thereby yielding unilamellar vesicles (Lext). A second type
122 of unilamellar liposomes was prepared using a detergent solubilization methodology
123 (Ldet). Two solutions, a micelle suspension composed by a mixture of phospholipid and
124 the detergent sodium cholate (7:10, mol/mol), and a saline solution (0.9 % NaCl), were

mixed and dialyzed allowing the removal of detergent from the micelle solution. The continuous and very rapid detergent removal spontaneously leads to the formation of liposomes. More details about these liposome preparation methodologies can be found in New (1990). In both methodologies, original vesicle size was around 150 nm.

Membrane composition of all five liposomes used in the present study was as follows:

1. Ldet KPE: liposomes composed of krill phospholipid extract.
2. Ldet KPE:vitA: liposomes composed of krill phospholipid extract and retinyl palmitate (100:2, w/w).
3. Ldet SPC: liposomes composed of soybean phosphatidylcholine.
4. Lext SPC:CHO: liposomes composed of soybean phosphatidylcholine and cholesterol (4:1, w/w).
5. Lext DPPC:CHO: liposomes composed of dipalmitoyl phosphatidylcholine and cholesterol(4:1, w/w).

Cholesterol (CHO) was included as a membrane stabilizer (New, 1990, p. 22) in liposomes prepared by the extrusion methodology (Lext) since it has previously seen to reduce the leakage of water soluble substances in extrusion suspensions (Monroig et al., 2003). Another type of liposomes (Ldet KPE:vitA) was formulated by combining the antioxidant compound retinyl palmitate (Sigma, Alcobendas, Spain) with krill phospholipid (KPE) in order to check the effect on the oxidative stability of HUFA-rich KPE. Relevant features of the five liposome suspensions used in this study are gathered in Table 2.

2.2. Oxidative stability of liposomes during enrichment

This experiment assessed the oxidative stability of the five liposome formulations (Table 2) together with the self-emulsifying enrichment product Super

150 Selco (Inve, Ghent, Belgium) used as a reference product in previous studies (Monroig
151 et al., 2003, 2006a, b). The enrichment diets (liposomes or lipid emulsion) were
152 incubated in three replicate 1 litre cylinder-conical enrichment tubes filled with 700 ml
153 of filtered (0.45 µm) and UV light-sterilized sea water. During the incubation period (21
154 h), the enrichment tubes were placed in a thermostated bath at 28 ± 1ºC, illuminated
155 with fluorescent light tubes (1500-2000 lux), and aerated by means of 0.5 cm diameter
156 glass tubes giving an air flow of 1.0 litre per minute. Control of air flow was achieved
157 using rotameters (Key Instruments, Trevose, PA, USA). The experiment was carried out
158 in absence of nauplii to eliminate any effects of nauplii on the TBARS determination. In
159 this manner, the final lipid quantity would not be conditioned by the liposome type-
160 dependent filtratory efficiency of the nauplii. Furthermore, the inclusion of nauplii
161 implies the entry of microorganisms which could lead to false peroxide determinations
162 (McEvoy et al., 1995).

163 Sample collection was performed by taking an aliquot of the enrichment medium
164 at the beginning (t_o) and the end of the incubation period (t_o+21 h) under the standard
165 enrichment conditions detailed above. Samples of the enrichment medium were
166 immediately frozen and kept at -20 ºC until further analyses.

167 The oxidative stability was measured by determining the concentration of the
168 thiobarbituric acid reactive substances (TBARS) according to the method of Ohkawa et
169 al. (1979) with the following modifications. Enrichment medium aliquots (160 µl) were
170 reacted with 40 µl of 8.1% (w/v) sodium dodecyl sulphate containing 0.05% (w/v)
171 butylated hydroxytoluene (BHT), 300 µl of 20% acetic acid (pH=3.5) and 300 µl of
172 0.8% (w/v) thiobarbituric acid (TBA). A volume of sodium phosphate buffer was added
173 to complete a final volume in the test tubes of 800 µl. The reagents were mixed in
174 stoppered test tubes and incubated in a thermostated bath for 60 min at 95 ºC. After

175 cooling in an ice bath, 200 µl of distilled water and 1 ml of n-butanol:pyridine (1:1, v/v)
176 were added, and the mixture was shaken vigorously. After centrifugation at 10000 x g
177 for 3 min, the fluorescence of the organic layer was determined, setting excitation and
178 emission wavelengths at 515 nm and 553 nm, respectively. TBARS concentrations were
179 derived from a standard curve using 1,1,3,3-tetraethoxypropane (TEP) and values
180 expressed in terms of TEP µmolar equivalents per litre. All chemicals used in the
181 TBARS measurements were obtained from Sigma (Alcobendas, Spain).

182 *2.3. Changes in the liposome size during enrichment*

183 The size (diameter) of liposome vesicles was estimated throughout a 22 h period
184 in three replicate cylinder-conical tubes. In comparison to the oxidative stability
185 experiment, smaller enrichment tubes were used (200 ml) filled with 100 ml of filtered
186 sea water (0.45 µm). Because of the smaller capacity of the tubes, the air flow was
187 decreased to give a similar hydrodynamic regime in both experiments. Lipid
188 concentration, illumination and temperature conditions were the same described in the
189 peroxide formation experience. In this case, changes in the liposome size distribution
190 were evaluated both in the presence and absence of nauplii in the enrichment medium to
191 precisely imitate the real enrichment conditions. For treatments containing *Artemia*
192 nauplii, newly hatched nauplii (Inve, Ghent, Belgium) were stocked at the beginning of
193 the trial (t_o) at naupliar densities of 300 individuals ml^{-1}

194 Medium samples were collected at the beginning (t_o) and after 12, 17 and 22 h of
195 incubation. When nauplii were present, the enrichment medium aliquot was filtered
196 through a 100 µm pore plankton mesh to eliminate the *Artemia* nauplii. About 4 ml of
197 deionized water and a portion of the enrichment medium were transferred into a 4.5 ml
198 polymethyl methacrylate cuvette, in order to obtain an intensity between 10^4 and 10^6
199 particles counted per second. Measurements of vesicle diameter were performed with a

Nanosizer Coulter N4 Plus instrument (Coulter Coultronics, Margency, France), which uses the principle of light scattering applied to the Brownian motion of the particles. Essentially, a laser light source is emitted through a sample producing a scattering pattern which is then analysed by a personal computer system equipped with N4 Plus software. Taking into account the parameters of temperature (20ºC), viscosity and refractive index of the suspending medium (1.33 for deionized water), the mixture was left to achieve thermostatic equilibrium for 5 min. After this period, three measures were carried out using the following parameters: acquisition angle 90º and analysis time of 180 s. The final diameter is calculated as the mean of the three measurements taken during 180 s in every sample. The particle size of the lipid globules formed by the commercial emulsion was not analysed because they are above the upper threshold of the Nanosizer Coulter N4 Plus instrument.

2.4. Lipid classes and fatty acid determinations

The lipid class and fatty acid analyses of the phospholipid sources used in the liposome preparation were analysed after a lipid extraction procedure carried out by the method described by Folch et al. (1957). Lipid classes were determined using a high-performance thin-layer chromatography (HPTLC) method as proposed Olsen and Henderson (1989). Analyses of fatty acids were carried by gas chromatography as described in Monroig et al. (2006a).

2.5. Statistical analyses

Analytical data are expressed as means ± standard deviations. Homogeneity of variances was checked by Barlett's test. In cases of homoscedasticity, a Student's *t*-test (Sokal and Rohlf, 1981) determined the differences in the TBARS concentration at t_o and $t_o + 21$ h in all the enrichment products (liposomes or the commercial emulsion). In the particle size experiment, a Student's *t*-test evaluated differences between the

presence and absence of nauplii in each sample point. If heterogeneity of variances existed, a Welch's test was used to check differences between treatments. Comparisons of the means with *P* values less or equal than 0.05 were considered significantly different. All the statistical analyses were carried out using the SPSS statistical package (SPSS Inc., Chicago, IL, USA).

3. Results

3.1. Oxidative stability of liposomes during enrichment

Fig. 1 illustrates the concentration of TBARS measured in the enrichment media of all five liposomes and the commercial emulsion. The results indicate a significant increase in the concentration of lipid peroxides in three liposome formulations incubated during the 21 h period (Ldet KPE, Ldet KPE:vitA, and Lext SPC:CHO). On the contrary, both Ldet SPC and Lext DPPC:CHO did not display significant increase in the peroxide concentration after their incubation under the experimental conditions. In spite of the formation of lipid peroxides in some formulations, the liposome treatments showed a higher oxidative stability in comparison to the commercial emulsion whose peroxide levels increased up to three orders of magnitude beyond liposome oxidation rates. In the case of liposomes, little differences were observed among formulations and final TBARS concentration registered values of the same order of magnitude in all treatments.

3.2. Changes in the liposome size during enrichment

Diameters of liposomes throughout the enrichment period are represented in Fig. 2. All five liposome formulations showed minor changes in their size during the incubation time when exposed to the experimental conditions in absence of *Artemia* nauplii. In these circumstances, all formulations maintained their original size of around

249 150 nm. However, the presence of *Artemia* nauplii in the medium gave rise to an
250 increase in the particle diameter of liposomes. Except for Ldet SPC and Ldet KPE, a
251 significant increase ($P \leq 0.05$) in the liposome size in the presence of *Artemia* was
252 observed at 17 h of incubation and onwards. Thus, Ldet KPE did not show differences
253 between the absence and presence of *Artemia* nauplii at any sampling point (Fig. 2a),
254 whereas Ldet SPC liposomes (Fig. 2c) exhibited a significant increase ($P \leq 0.05$) only at
255 the end of incubation ($t_o + 22$ h). The inclusion of retinyl palmitate in krill liposomes
256 (Ldet KPE:vitA) was associated to a slight increase in the liposome diameters in the
257 presence of *Artemia* nauplii (Fig. 2b).

258 The most obvious augment in the particle diameter was seen in Lext SPC:CHO
259 liposomes (Fig. 2d). In this formulation, the presence of nauplii caused a marked
260 increase in particle sizes, which was associated with high variability at 12 h and
261 onwards. Another remarkable result is the different behaviour showed by Lext
262 SPC:CHO (Fig. 2d) and Lext DPPC:CHO (Fig. 2e). Despite the fact that both
263 suspensions were formulated with purified phospholipid sources mixed with CHO at the
264 same phospholipid:CHO ratio, DPPC liposomes showed much lower increase in size in
265 the presence of nauplii compared to SPC vesicles (Fig. 2e).

266 **4. Discussion**

267 The use of liposomes as an enrichment diet implies their submission to
268 aggressive conditions which can modify their original properties. In this context,
269 Monroig et al. (2003) studied the leakage shown by several types of liposomes
270 submitted to enrichment conditions. It was concluded that liposomes become leaky
271 under the aggressive environment of enrichment, contrarily to their stable behaviour
272 when stored at room conditions (i.e., no agitation, room temperature, etc.) (Hontoria et

al., 1998). In a second approach, the study of the oxidative stability and the changes in the size distribution of liposomes during the *Artemia* enrichment process contribute to a better global assessment of the capacity of liposomes to be used in *Artemia* enrichment procedures.

McEvoy et al. (1995) reported the oxidative stability of several enrichment diets submitted to standard *Artemia* enrichment conditions. These authors found that all the enrichment products tested, including the commercial product Super Selco used as reference in the present study, showed autoxidation of polyunsaturated fatty acids after 23 h of incubation. The results of the present study partially confirm the occurrence of this degradation process when liposomes are employed as *Artemia* enrichment diets during a period of 21 h. However, the oxidation rates of liposomes were clearly lower than that of the lipid emulsion Super Selco despite the fact that this product contains antioxidant substances (McEvoy et al., 1995). The difference in the oxidative stability between liposomes and the commercial emulsion might be related to their lipid class composition. Whereas the commercial emulsion is essentially composed of triacylglycerides (TAG) and fatty acid ethyl esters (FAEE) (see Table 1), liposomes are vesicles mostly comprised of phospholipids. Consequently, fatty acids included in liposome phospholipids would be efficiently protected since these compounds have been described to possess antioxidant properties in comparison to other lipid classes such as TAG and FAEE (King et al., 1992a, b; Song et al., 1997). In order to explain the antioxidant mechanisms acting on phospholipids, King et al. (1992a) cited several postulates which included synergism between phospholipids and tocopherol, chelation of pro-oxidant metals by the phospholipid phosphate groups, formation of Maillard-type products between phospholipids and oxidation products, and, finally, their action as an oxygen barrier through the oil/air interfaces. More recently, Lyberg et al. (2005) studied

298 the oxidative stability of free DHA, DHA mixed with phosphatidylcholine (PC) and
299 DHA incorporated in PC, phosphatidylethanolamine (PE) and TAG. It was found that
300 DHA included in the structure of the phospholipid (PC and PE) was the most protected
301 alternative against oxidation, and proposed a key role of the polar group in the
302 protection mechanism. These authors hypothesized that the best protection was
303 achieved in those parts of the fatty acid chain that were close to the polar group. Thus,
304 in addition to carbons close to the carboxyl group, DHA terminal carbons are also
305 protected due to the curve exhibited by DHA as a result of its high degree of
306 unsaturation. Consequently, the oxidative stability of liposomes would reduce the
307 formation of lipid peroxides during enrichment, its subsequent accumulation in live
308 preys and, finally, the potential harmful effects (Hipkiss et al., 1997; Tesoriere et al.,
309 2002; Del Rio et al., 2005) on larvae fed with those nauplii. In a larval feeding trial,
310 Monroig et al. (2006a) obtained higher concentrations of lipid peroxides in larvae fed
311 *Artemia* nauplii enriched with the same commercial emulsion employed in the present
312 study when compared to larvae fed liposome enriched nauplii.

313 As aforesaid, McEvoy et al. (1995) assessed the autoxidation of several
314 enrichment products including the commercial product Super Selco also tested in the
315 present study. Assuming that the concentration of total lipid present in the enrichment
316 medium at the beginning and at the end of incubation was 0.5 g l^{-1}, our data can be
317 converted into the same units used by these authors (moles of TBARS per lipid weight
318 unit) and, hence, data from both studies can be compared. At the beginning of the trials,
319 the concentration of TBARS was 1420±300 nmol g $lipid^{-1}$ in our experiment (Fig. 1)
320 and 2300 nmol g $lipid^{-1}$ in study of McEvoy et al (1995) (data estimated from Fig. 6).
321 However, the incubation of Super Selco under the enrichment conditions and in the
322 absence of nauplii produced a much higher increase in the TBARS concentration in the

present study (196240±13060 nmol g lipid^{-1}) in comparison to data obtained by McEvoy et al. (1995) (approximately 3200 nmol g lipid^{-1}), even if the incubation period in this latter experiment was 2 h longer. Some hypotheses arise to explain the high lipid peroxide production in our assay. Firstly, McEvoy et al. (1995) administered the emulsion Super Selco in two separate doses. This strategy is recommended by the manufacturers to minimize the degradation of the enrichment product given that it is exposed to a damaging environment during a shorter period, and it has been used in numerous studies concerning live prey enrichment trials (McEvoy et al., 1995, 1996; Han et al., 2005). Another possibility explaining the high degradation of the commercial emulsion in our study may be due to the depletion of the antioxidant compounds present in our product stock. Although maintained at the manufacturer's recommended temperature of 4 ºC and stored under nitrogen, the antioxidant compounds of the stock could be depleted and, despite the fact the initial TBARS concentration was similar to that of McEvoy et al. (1995), antioxidant protection may not have acted once the product is exposed to the aggressive conditions of the enrichment process. One of the antioxidant substances included in the formulation of Super Selco is retynil palmitate. This compound, incorporated in the Ldet KPE:vitA liposomes, did not enhance the oxidative stability of krill pure liposomes (Ldet KPE). Pro-oxidant conditions of enrichment procedures (temperature, oxygenation, and illumination) could partially degrade retynil palmitate of liposomes thus compromising its protection role against autoxidation of unsaturated fatty acid chains of krill phospholipid (Woollard and Inkyk, 2003). Indeed, Moren et al. (2005) were not able to detect vitamin A after 12 h of exposure of a commercial product similar to Super Selco (DC-DHA Selco, Inve, Ghent, Belgium) in simulated enrichment conditions.

Additionally to the lipid class composition, autoxidation of lipids depends on the acyl chain length and the unsaturation degree of fatty acids (Holman and Elmer, 1947; Cosgrove et al., 1987). This could explain differences in the oxidative stability among liposome formulation assessed in this study. In fact, krill liposomes (Ldet KPE and Ldet KPE:vitA), which contain both the longest and the highest unsaturated fatty acids (note HUFA levels in Table 1), underwent autoxidation during the incubation period. Conversely, soybean liposomes (Lext SPC:CHO and Ldet SPC) showed lower lipid peroxide concentrations than krill vesicles, and even Ldet SPC did not suffer any significant increase in the peroxide concentration throughout the assay. This high oxidative stability was also observed in Lext DPPC:CHO whose fatty acids are totally saturated (palmitic acid, 16:0). All these results are in agreement with the general acceptance that the oxidative stability of fatty acids decreases with increasing degrees of unsaturation. However, Nara et al. (1998) and Araseki et al. (2002) found that PC liposomes containing HUFAs were more stable than liposomes formulated with more saturated PCs. These authors suggested that the degree of unsaturation was not the main factor affecting the oxidative stability of PC in liposomes and hypothesized that DHA in liposomes would adopt a tightly packed conformation. The higher reactivity of DHA derived from the higher degree of unsaturation may compensate for the difficulty of free radicals and/or oxygen to attack this tighter conformation of DHA in liposomes. Nevertheless, the results of these authors were obtained under very well-defined conditions (temperature of 37 ºC, darkness, no agitation, etc.) in which liposomes were formulated with pure PCs containing fatty acid chains in known positions of the glycerol moiety. All these differences could limit the extrapolation of the results to those obtained in the present study where pro-oxidative conditions are most likely to be drastic.

The changes in liposome size during the enrichment process showed different results depending on the absence or presence of *Artemia* nauplii in the enrichment medium. Despite the habitual occurrence of physical phenomena such as fusion and aggregation observed in liposome suspensions which originate a final increase in the particle size of vesicles (New, 1995, p. 17), no liposome formulation displayed visible changes in their vesicle diameter in the absence nauplii. Simply stated, fusion can be defined as the interaction of two separate membrane sheets such that they combine to form a single sheet in which membrane lipids are shared. Additionally, aggregation occurs when neutral planar phospholipid membranes establish weak interactions such as van der Waals forces. However, both mechanisms do not seem to occur in liposomes submitted to our experimental conditions according to the minor changes observed when liposomes were incubated in seawater without nauplii. This result would disagree with those obtained by Hontoria et al. (1994), who corroborated that extruded liposomes maintained under no agitation conditions aggregate after their preparation. In addition, Koven et al. (1999) point out the presence of divalent ions in seawater to explain aggregation of extruded liposomes to form particles of 20-30 microns. The apparent discrepancy can be explained by means of several mechanisms. Firstly, the turbulent hydrodynamic regime of the enrichment process might handicap the establishment of the above mentioned interactions between vesicles so that liposomes remain in the single original state. In the case of liposomes prepared by the detergent solubilization methodology (Ldet), a second antifusogenic mechanism may be acting. Given that the detergent removal during the preparation of Ldet is not complete (Allen et al., 1980), along with the negative charge of sodium cholate, Ldet would exhibit a decreased fusion rate because of the repulsion forces between charged vesicles. A similar mechanism was proposed by Lasic (1989) to explain the reduced size of Ldet prepared with sodium

cholate instead of the zwitterionic detergent CHAPS. In these latter liposomes, the simultaneous co-existence of positive and negative charges in the CHAPS molecule would facilitate fusion of vesicles and thus their bigger size in comparison to charged sodium cholate liposomes. On the other hand, liposomes prepared by extrusion do not contain detergent and, consequently, would be more prone to aggregate.

The presence of *Artemia* nauplii in the enrichment medium would affect in a different way liposomes prepared by the extrusion (Lext) or detergent solubilization (Ldet) methodologies. In the case of Ldet, the above mechanisms seem to remain avoiding vesicle condensation. On the contrary, a growth in size was observed in Lext which was especially visible in Lext containing SPC. Although not presented in this study, other liposomes prepared by extrusion and composed of CHO and other phospholipid sources (krill phospholipid extract and egg PC) have been observed to undergo a similar increase in their size in the presence of nauplii (unpublished data). The inclusion of *Artemia* could bring some shedding metabolites, degradation by-products, etc., which may facilitate the aggregation of the Lext formulations contrarily to Ldet. On the other hand, Lext DPPC:CHO liposomes would represent the exception among other Lext formulations since they did not display important changes in their size, as seen in Ldet . The result is probably due to the fact that DPPC molecules would remain in a tightly ordered solid phase at 28 ºC (New, 1990, p. 8), whereas more unsaturated phospholipids (SPC and KPE) would be in a liquid-crystalline phase. Consequently, the membrane fluidity of DPPC liposomes would be reduced and phenomena such as fusion hindered. This hypothesis, valid for pure DPPC liposomes, can also be applicable to the formulation DPPC:CHO because the presence of CHO at molar ratio DPPC:CHO of 2:1 would only displace the phase transition temperature

421 from 41 ºC to 44 ºC (New, 1990, p. 20). Consequently, Lext DPPC:CHO would remain
422 in a solid stable phase at the *Artemia* enrichment temperature of 28 ºC.

423 Despite the fact that some liposomes can exhibit an increase in size during the
424 enrichment process, this cannot be considered as a disadvantage for their use as live
425 prey enrichment diets. Indeed, we studied the efficiency of different types of krill
426 liposomes to enhance the HUFA content in *Artemia* nauplii and found that Ldet
427 enriched nauplii showed the maximal HUFA contents among liposome treatments
428 (Monroig et al., 2006a). These latter results confirmed the usefulness of these small
429 vesicles despite the fact that other studies had proposed that the optimal size of
430 enrichment products has to be higher than that of liposomes assessed in this study
431 (Caers et al., 2000; Gelabert-Fernández, 2002; Han et al., 2005).

432 In summary, the results indicate that liposomes are more stable against oxidation
433 than other commercial products based on fish oil emulsion. Whereas the initial lipid
434 peroxide levels of liposomes and emulsion were similar, their incubation under standard
435 *Artemia* enrichment conditions produced an increase in TBARS in the emulsion up to
436 values three orders of magnitude above those registered by liposomes after 21 h of
437 incubation. Differences of oxidation rates among liposomes are related to the chain
438 length and the unsaturation degree of their fatty acids. Thus, liposomes composed of
439 phospholipids containing long-chain highly unsaturated fatty acids (krill phospholipid
440 extract) were normally less stable than those composed of shorter length-chain and more
441 saturated liposomes.

442 The study of the vesicle size throughout enrichment showed minor changes in
443 the liposome diameter when the incubations were carried out without nauplii. In the
444 presence of nauplii, liposomes do not exhibit notable changes in their size and only SPC
445 extruded liposomes displayed an evident size increase.

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596 Tables

597

598 Table 1. Selected fatty acid (percent of total fatty acids) and lipid class composition

599 (percent of total lipids) of the enrichment products.

	Commercial emulsion	KPE	SPC	DPPC
Fatty acid				
16:0	3.3	25.5	15.1	100.0
18:0	3.0	1.0	3.0	0.0
18:1n-9	10.8	5.4	13.6	0.0
18:2n-6	6.1	1.8	60.6	0.0
18:3n-3	1.4	1.4	3.6	0.0
20:4n-6	1.6	0.7	0.0	0.0
20:5n-3	19.8	29.7	0.0	0.0
22:5n-3	4.8	0.8	0.0	0.0
22:6n-3	25.4	18.4	0.0	0.0
Saturates	8.0	28.6	18.7	100.0
Monounsaturates	25.4	13.2	15.8	0.0
Polyunsaturates	63.0	55.8	64.5	0.0
Total n-3	53.8	52.5	3.8	0.0
Total n-6	9.7	3.2	60.7	0.0
HUFA n-3	51.4	49.3	0.2	0.0
HUFA n-6	2.8	1.4	0.1	0.0
DHA/EPA	1.3	0.6	0.0	0.0
Lipid class				
FAEE/TAG	91.1	0.0	0.0	0.0
PC	0.4	67.0	95.0	100.0
PE	0.0	9.0	0.0	0.0
Other	8.4	24.0	5.0	0.0

600 KPE: krill phospholipid extract; SPC: soybean phosphatidylcholine; DPPC: dipalmitoyl

601 phosphatidylcholine; HUFA n-3: ≥20:3n-3; HUFA n-6: ≥20:2n-6; DHA/EPA:

602 docosahexaenoic and eicosapentaenoic fatty acid ratio; FAEE/TAG: combined fraction

603 of fatty acid ethyl esters and triacylglycerols; PC: phosphatidylcholine; PE:

604 phosphatidylethanolamine.

605

606 Table 2. Characteristics of liposomes used in the present study.

Name	*Lamellarity*	*Preparation method*	*Composition (w/w)*
Ldet KPE	Unilamellar	Detergent solubilization	KPE 100%
Ldet KPE:vit A	Unilamellar	Detergent solubilization	KPE:retinyl palmitate (100:2)
Ldet SPC	Unilamellar	Detergent solubilization	SPC 100%
Lext SPC:CHO	Unilamellar	Extrusion	SPC:CHO (4:1)
Lext DPPC:CHO	Unilamellar	Extrusion	DPPC:CHO (4:1)

607 KPE: krill phospholipid extract; SPC: soybean phosphatidylcholine; DPPC: dipalmitoyl
608 phosphatidylcholine; CHO: cholesterol; vitA: retinyl palmitate; Ldet: unilamellar
609 vesicles prepared by a detergent solubilization methodology; Lext: unilamellar vesicles
610 prepared by extrusion through a polycarbonate membranes.

611

612 Figure legends

613

614 Figure 1

615 Concentration of TBARS in the *Artemia* enrichment media containing different
616 products. Data correspond to three replicate samples measured at the beginning (t_o) and
617 after 21 h of incubation ($t_o + 21$). Error bars are standard deviations. Significant
618 differences in the TBARS concentration at t_o and $t_o + 21$ h in all the enrichment
619 products are indicated with the symbol "*" ($P \leq 0.05$).
620 Ldet: unilamellar vesicles prepared by a detergent solubilization methodology; Lext:
621 unilamellar vesicles prepared by extrusion through a polycarbonate membranes; KPE:
622 krill phospholipid extract; SPC: soybean phosphatidylcholine; DPPC: dipalmitoyl
623 phosphatidylcholine; CHO: cholesterol; vitA: retinyl plamitate.

624

625 Figure 2

626 Particle size from several liposome formulations Ldet (a, b, and c) and Lext (d and e)
627 incubated under enrichment conditions in the presence and absence of *Artemia* nauplii.

628 Data represent means and error bars are standard deviations (n=3). Treatments with
629 different letters in a sampling point are significantly different from each other ($P \leq 0.05$).
630 Ldet: unilamellar vesicles prepared by a detergent solubilization methodology; Lext:
631 unilamellar vesicles prepared by extrusion through a polycarbonate membranes; KPE:
632 krill phospholipid extract; SPC: soybean phosphatidylcholine; DPPC: dipalmitoyl
633 phosphatidylcholine; CHO: cholesterol; vitA: retinyl plamitate.
634

Figure 1

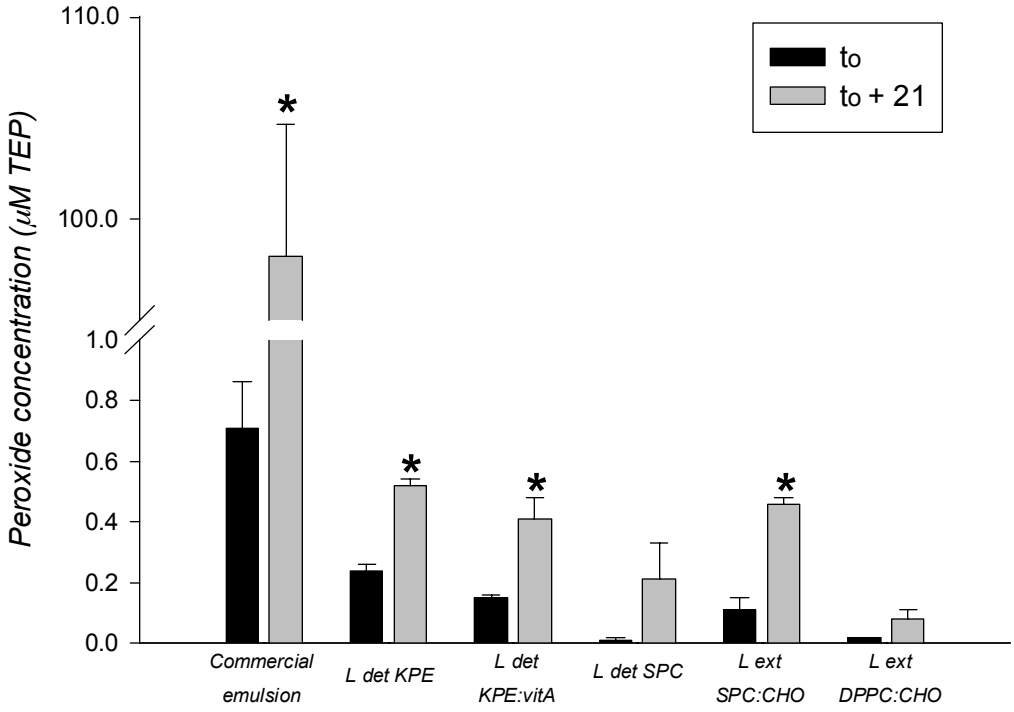
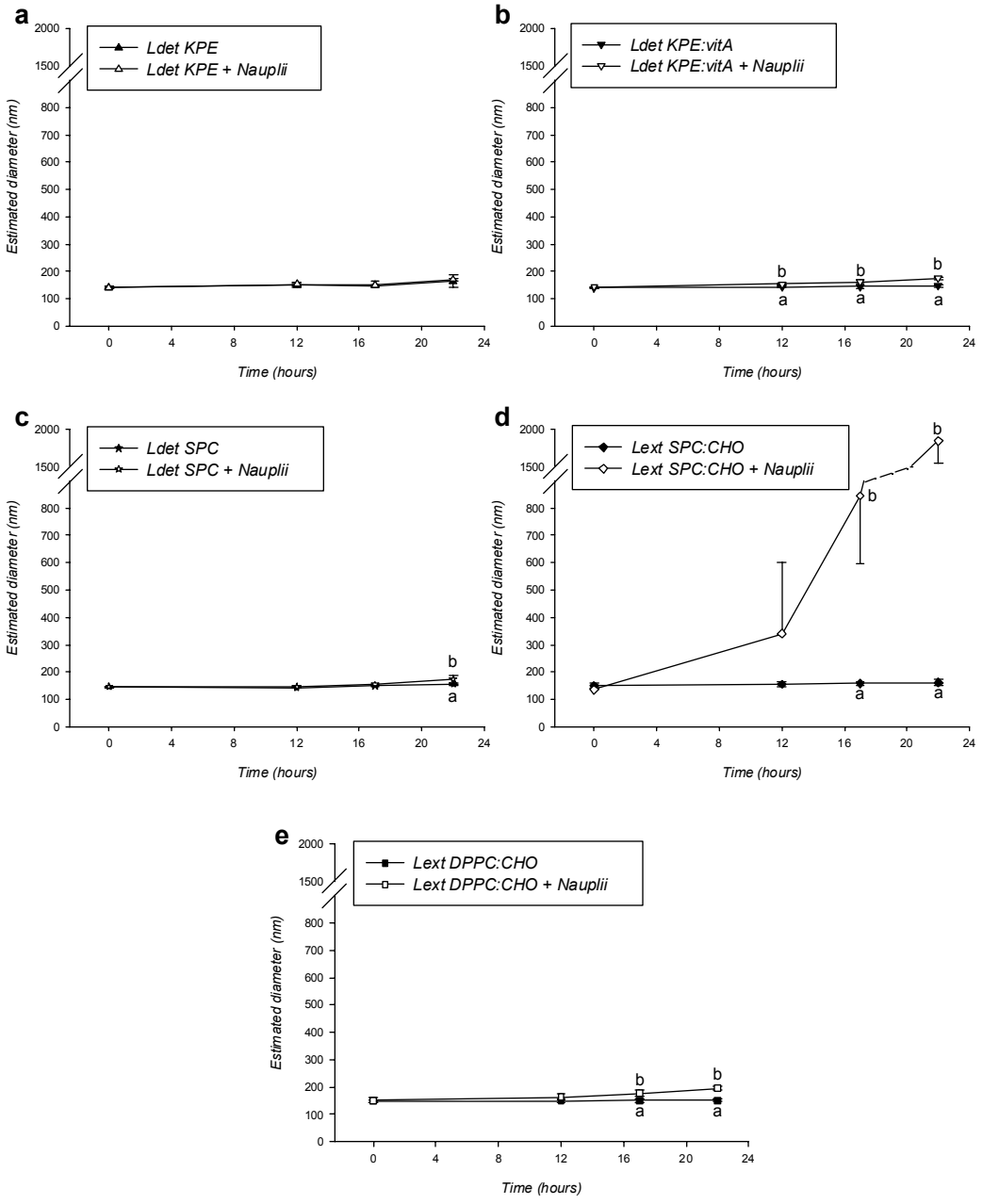

Peroxide concentration (μM TEP)
110.0
100.0
1.0
0.8
0.6
0.4
0.2
0.0
to
to + 21
*
*
*
*
Commercial emulsion
L det KPE
L det KPE:vitA
L det SPC
L ext SPC:CHO
L ext DPPC:CHO

Figure 2


a
Ldet KPE
Ldet KPE + Nauplii
b
Ldet KPE:vitA
Ldet KPE:vitA + Nauplii
c
Ldet SPC
Ldet SPC + Nauplii
d
Lext SPC:CHO
Lext SPC:CHO + Nauplii
e
Lext DPPC:CHO
Lext DPPC:CHO + Nauplii
Estimated diameter (nm)
Time (hours)