Title

Antioxidant activity and lipid peroxidation in *Artemia* nauplii enriched with a DHA-rich oil emulsion and the effect of adding an external antioxidant based on hydroxytyrosol

Running head

Effect of hydroxytyrosol during *Artemia* enrichment

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Antioxidant enzymes, *Artemia*, docosahexaenoic acid, enrichment, hydroxytyrosol, oxidation.

Abstract

Artemia nauplii catabolise polyunsaturated fatty acids (PUFA); in particular, they retroconvert docosahexaenoic acid (DHA, 22:6n-3), so enrichment is a continuous quest towards increasing PUFA through the use of PUFA-rich enrichment products. However, optimal conditions during enrichment (aeration, illumination and temperatures around 28 ºC) tend to accelerate autoxidation of PUFA, and the formation of potentially toxic oxidation products. Water-soluble antioxidants like the polyphenolic compound hydroxytyrosol (3,4-dihydroxyphenylethanol), a polar molecule found in the water fraction resulting after the milling process of olives, arise as promising compounds to prevent oxidation during *Artemia* enrichments. We investigated the antioxidant activity and lipid peroxidation in *Artemia* nauplii during enrichment, and the effect of adding an external antioxidant based on hydroxytyrosol during the enrichment with a PUFA-rich emulsion (M70). For this purpose, the activity of antioxidant enzymes (catalase, superoxide dismutase, glutathione-S-transferase, glutathione peroxidase), as well as lipid peroxidation, were determined in enriched and unenriched *Artemia* nauplii. To validate antioxidant activity and lipid peroxidation, in a first experiment, nauplii were enriched with microalgae (*Tetraselmis suecica*), yeast (*Saccharomyces cerevisiae*) and M70 emulsion. In a second experiment, enrichment with a commercial emulsion (DC Super Selco), M70, and a combination of M70 and hydroxytyrosol (Hytolive, HYT) added as an external antioxidant was performed. The combination of M70 with HYT produced the best results, in terms of activity of antioxidant enzymes. The analysis of the fatty acids from total lipids showed that the addition of hydroxytyrosol preserved the DHA percentage of enriched nauplii.

52 **1. Introduction**

53 Adequate provision of polyunsaturated fatty acids (PUFA) is important in the diet of
54 marine organisms, in particular during early life stages, where these compounds
55 accumulate in rapidly developing tissues such as brain and eye (Bell et al., 1995, 2003;
56 Benitez-Santana et al., 2007; Tocher, 2010). Thus, enrichment of live preys like
57 *Artemia*, which are naturally deficient of essential PUFA such as docosahexaenoic acid
58 (22:6n-3, DHA), is critical for the survival and normal development of larval stages of
59 marine organisms, and thus the production of good quality fingerlings (Hamre &
60 Harboe, 2008; Tocher, 2010).

61 McEvoy et al. (1995) investigated the stability of PUFA in lipid emulsions throughout
62 the enrichment process since optimal enrichment conditions such as vigorous aeration,
63 illumination and temperature of 28 ºC, tend to accelerate autoxidation of PUFA and the
64 formation of potentially toxic oxidation products. It was found that, at later stages of the
65 enrichment process, all the emulsions tested showed autoxidation associated to notable
66 decreases in the concentration of PUFA in the lipids of *Artemia* nauplii, particularly for
67 eicosapentaenoic acid (EPA, 20:5n-3) and DHA. Interestingly, it was reported that
68 autoxidation of enrichment diets occurred even in commercial emulsions that are
69 formulated with antioxidants. Beyond the decrease in the enrichment efficiency
70 described above, the study of autoxidation in enrichment diets is also important because
71 it implies that *Artemia* nauplii can accumulate potentially toxic oxidation products that
72 can compromise the health and survival of fish and crustacean larvae. In fact, McEvoy
73 et al. (1995) recommended that *Artemia* enrichments, particularly those with DHA-rich
74 emulsions, should be shortened so that the alluded to accumulation of potentially toxic
75 compounds in nauplii could be reduced.

We have recently investigated the efficiency of a DHA-rich oil emulsion (M70) as enrichment diet for *Artemia* nauplii (Viciano et al., 2015). M70 consists of a 1:1 (v/v) oil/water suspension, based on the synthetic oil DHA Algatrium (Brudy Technology, Barcelona, Spain) that contains 70% of total fatty acids as DHA in the form of ethyl esters (Viciano et al., 2015). Additionally, the emulsion M70 is also formulated with Tocobiol Plus (BTSA Biotecnologías Aplicadas, Madrid, Spain), a liposoluble antioxidant. Considering the particular aspects derived from its DHA-rich nature, a series of experiments were carried out to optimise the use of M70 under different enrichment conditions including oxygenation regime, incubation temperature, nauplial density and enrichment product concentration and dosage mode, which have been regarded as important factors determining the enrichment efficiency (Van Stappen, 1996; Han et al., 2000, 2001; Monroig et al., 2006a, b; Sui et al., 2007; Hamre & Harboe, 2008; Figueiredo et al., 2009). Importantly, our results suggested that, despite the likely preservative action of the liposoluble antioxidant Tocobiol Plus, oxidation of DHA contained in M70 could not be ruled out as suggested by relatively low DHA incorporation in *Artemia* nauplii (Viciano et al., 2015).

Pro-oxidative conditions during enrichment could partly explain massive mortalities events reported in the literature during enrichments (Monroig et al., 2006b), since they can alter the capacity of live preys for catalyzing oxidative reactions, leading to the production of reactive oxygen species (ROS) as a general pathway of toxicity causing oxidative stress (Livingstone, 2001; Barata et al., 2005b). ROS include superoxide anion radical (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radical (OH^-) (Correia et al., 2003), compounds that are involved in cellular damage and with the capacity to promote morphological and physiological failures and peroxidation of PUFA, all this leading to pathologies and compromising normal development (Tovar-Ramirez et al.,

2010). To minimize oxidative damage to cellular components, organisms have developed key antioxidant enzyme defences that can be used as biomarkers (Barata et al., 2005a, b).

Water-soluble antioxidants appear to have potential for preventing DHA oxidation during *Artemia* enrichments. In a number of studies, the polyphenolic compound hydroxytyrosol (3,4-dihydroxyphenylethanol) (Hao et al., 2010), has been shown to be more efficient than antioxidant vitamins and synthetic antioxidants (Gordon et al., 2001; González, 2005; Deiana et al., 2008; Fernández-Bolaños et al., 2008). Hydroxytyrosol is a polar molecule found in the water fraction resulting after the milling process of olives (Schaffer et al., 2007). Several studies have investigated the potent activity of hydroxytyrosol to neutralize free radicals and slow oxidative stress (Chimi et al., 1991; Visioli et al., 1998, 2000). To the best of our knowledge, there are no data available concerning its potential role as protective agent of oil emulsions used for live prey enrichments.

The global aim of the present study was to investigate the protective effects of the addition of an external water-soluble natural olive fruit extract, rich in the polyphenol hydroxytyrosol (HYT, Hytolive Protect, Genosa I+D S.I., Malaga, Spain), to the enrichment of *Artemia* nauplii with a DHA-rich oil emulsion (M70). For this purpose, key enzymes (superoxide dismutase, catalase, glutathione peroxidase, glutathione-S-transferase) as biomarkers of antioxidant activity, and the lipid peroxidation of enriched *Artemia* nauplii were determined. To the best of our knowledge, such an approach has never been used before to study the effects of oxidation during enrichment. Two experiments were performed. Experiment 1 aimed at establishing the baseline of lipid oxidation in the enrichment of *Artemia* nauplii. We analyzed the antioxidant activity and lipid peroxidation of *Artemia* nauplii enriched with M70, and compared them with

126 those of nauplii enriched with yeast *Saccharomyces cerevisiae* or the green microalgae
127 *Tetraselmis suecica*, as well as those of non-enriched nauplii. Yeast and green
128 microalgae are known to have antioxidant properties (Greetham & Grant, 2009; Lee et
129 al., 2009). Experiment 2, aimed at studying the effect of the external antioxidant HYT
130 during enrichment with high PUFA emulsions. For this purpose we determined the
131 antioxidant activity, lipid peroxidation and fatty acid composition of: a) non-enriched
132 nauplii, used as a control; b) nauplii enriched with a commercial emulsion (DC Super
133 Selco, DCSS, Inve, Dendermonde, Belgium), as reference of conventional enrichment;
134 c) nauplii enriched with M70; and d) nauplii enriched with M70 plus the water-soluble
135 antioxidant HYT.

136 **2. Materials and methods**

137 2.1 Experimental design

138 *Artemia* nauplii were obtained from the hatching of EG grade cysts (Inve,
139 Dendermonde, Belgium). After an incubation period of 23 h at 28 ºC, nauplii were
140 collected and rinsed with tap water to remove the hatching debris and metabolites.
141 Newly hatched nauplii were thereafter placed in 1 L cylinder-conical vessels containing
142 seawater.

143 The *Artemia* enrichments were performed for 21 h with incubation temperatures of 28 ±
144 1 ºC, low/moderate aeration (1 L min^{-1}), the air diffusion system consisting of a 25 cm
145 long and 0.5 cm-diameter section glass tube applied from the bottom of the vessel.
146 Nauplial densities were 300 nauplii mL^{-1} (Monroig et al., 2006a, b), and illumination
147 was 1500 lux (Viciano et al., 2015). The temperature was kept (±1 ºC) constant by
148 placing the hatching and enrichment vessels in a thermostatic bath. The enrichment
149 product was dispensed in a single dose at the beginning of the incubation.

All the enrichment treatments were run in triplicates (n=3). After an enrichment period, samples of *Artemia* nauplii were collected by filtering the enrichment medium through a 100 µm mesh carefully washed with tap water and subsequently rinsed with distilled water.

Experiment 1: Baseline of M70 oxidation

Antioxidant activity and lipid peroxidation levels were determined in *Artemia* nauplii enriched with: a) 0.6 g L^{-1} *Saccharomyces cerevisiae* (fresh yeast (Levital, Lesaffre Ibérica S.A., Valladolid, Spain) dispersed in sea water using a mixer for 2 minutes), b) *Tetraselmis suecica* (algal culture stock from IATS facilities, at 300000 ± 20000 cells mL^{-1}), c) 0.8 g L^{-1} M70 emulsion (dispersed in sea water using a mixer for 2 minutes in a single dose at the beginning of the enrichment period), and d) a control group consisting of unenriched *Artemia* nauplii kept in the same conditions.

Experiment 2: External antioxidant

Antioxidant activity, fatty acid profile and lipid peroxidation of *Artemia* nauplii enriched with 0.6 g L^{-1} commercial emulsion DCSS, 0.8 g L^{-1} M70 emulsion, 0.8 g L^{-1} M70 in combination with 0.04 g L^{-1} of exogenously added water-soluble antioxidant HYT (hereafter named M70+HYT treatment) and a control group of unenriched *Artemia* nauplii were compared. All enrichment media were dispersed in sea water using a domestic blender for 2 min.

2.2 Sample preparation for antioxidant enzymes

After enrichment, nauplii were filtered through a 100 µm mesh, washed with distilled water and kept at -80 ºC until analysis. Samples were processed as soon as possible and always within 3 h of the end of the enrichment period to avoid possible oxidation during

173 the storage. *Artemia* samples were homogenized in a 1:4 wet weight: buffer volume
174 ratio in 100 mM phosphate buffer, pH 7.4 at 4 ºC, containing 100 mM KCl and 1 mM
175 ethylenediaminetetrasacetic acid (EDTA). Homogenates were further centrifuged at
176 10000 g for 10 min, and supernatants were immediately used as enzyme sources.
177 Supernatant proteins were measured by the Bradford method (Bradford, 1976) using
178 bovine serum albumin as standard.

179 2.3 Enzymatic activities

180 The following enzymatic activities were measured in *Artemia* homogenates: Superoxide
181 dismutase, SOD (EC 1.15.1.1, converts O_2^- to H_2O_2); catalase, CAT (EC 1.11.1.6,
182 reduces H_2O_2 to water); glutathione peroxidise, GPx (EC 1.11.1.9, detoxifies H_2O_2 or
183 organic hydroperoxides produced e.g. by lipid peroxidation); and Glutathione-S-
184 transferase, GST (EC 2.5.1.18, plays a role preventing oxidative damage by conjugating
185 breakdown products of lipid peroxides to glutathione (GSH)) (Barata et al., 2005a, b;
186 Tovar-Ramirez et al., 2010).

187 All the enzymatic assays were measured as described by Barata et al. (2005a, b). The
188 final results for enzymatic activities were normalized by protein content. Assays were
189 run at least in duplicate.

190 2.4 Lipid peroxides

191 Another factor affecting the potential oxidative damage is the level of target molecules
192 like PUFA, which are easily oxidized by ROS in lipid peroxides (Di Giulio et al., 1995).
193 Lipid peroxidation was evaluated analysing the lipid peroxidation levels (LPO).
194 Malondialdehyde (MDA) is a common substance produced in lipid peroxidation. LPO
195 method is based in the reaction of MDA with N-methyl-2-phenylindole (NMPI) to form
196 a coloured complex with known absorbance (586 nm) (Esterbauer et al., 1991). By this

197 method the interference from other lipid peroxidation products (such as 4-
198 hydroxyalkenals) is minimized.

199 2.5 Fatty acid analysis

200 Fatty acid analyses were performed to check any protective effect of the external
201 antioxidant (mainly on PUFA). Thus, in Experiment 2, two samples of each replicate of
202 every treatment (*Artemia* enriched with the different diets) were obtained, one was
203 assigned to the analysis of enzymatic activity (processed as specified above), and the
204 other was freeze-dried to proceed with the analysis of total lipids and fatty acids. Total
205 lipids were extracted following the method of Folch et al. (1957), and analyses of fatty
206 acids were carried out following the methods described in Viciano et al. (2015).

207 2.6 Statistical analysis

208 Analytical data were expressed as means ± standard deviation (SD) (n=3). When more
209 than two means were compared, differences with control group were analysed by
210 Dunnett test (Dunnett, 1964). Dunnett test is a multiple comparison procedure and is
211 performed by computing a Student t-statistic for each experimental or treatment group,
212 where the statistic compares the treatment group to a single control group. When
213 significance was $P \leq 0.05$, means were considered statistically different. For pair-wise
214 comparisons a Student t test was used.

215 Enzyme activities analytical data, and highly unsaturated fatty acids (HUFA), fatty
216 acids of 20 or more atoms of carbon and two or more double bonds, which are
217 considered to have the highest oxidative potential (Barata et al., 2005c), were included
218 as variables in multivariate Principal Component Analyses (PCA), to highlight the
219 effects of the different treatments on the patterns of enzyme activities and on the fatty
220 acid profiles composition. With such a parsimonious approach, the dataset of variables

221 is reduced into a smaller set of factors or components. Parsimony is achieved by
222 explaining the maximum amount of common variance in a correlation matrix using the
223 smallest number of explanatory concepts. Factors are statistical entities that can be
224 visualized as classification axes along which measurement variables can be plotted,
225 providing an idea of their correlation with the corresponding factor (loading). Score
226 plots are a graphical representation of individual (treatment groups) scores in the new
227 subset of measurement variables (factors). They illustrate the relationship among
228 individual cases (treatment groups), and the variables, and help in the analysis of data
229 by showing graphical display. Statistical analyses were performed using the SPSS
230 statistical package (SPSS Inc., Chicago, IL, USA).

231 **3. Results**

232 *Experiment 1: Baseline of M70 oxidation*

233 Figure 1 illustrates the levels of enzymatic activities and LPO measured in the control
234 group and in the *Artemia* nauplii enriched with different treatments. The *Tetraselmis*
235 group was not significantly different from control group in any of the enzymatic
236 activities or LPO measured. Yeast treatment presented lower activities of SOD, GPx-
237 Tot and GPx-Se, but a high GST activity. M70 group showed a decreased activity of
238 SOD and GPx-Tot and a significant ($P<0.05$) increase, near two-fold relatively to
239 control animals, in LPO.

240 The component plot (Figure 2a) obtained from PCA shows variables that were
241 responsible for separation along the two principal components. The first two
242 components of the PCA explained more than 68% of total variance (43.9% and 24.2%
243 for the first and second component, PC1 and PC2, respectively). The factor score plot
244 (Figure 2b) showed groups separated in the second component on the basis of lipid

peroxidation. M70 treatment appeared to be related with high loads of LPO and clearly separated from the other treatments. Control and *Tetraselmis* treatments appeared very close to each other and on the opposite side of M70 treatment, indicating less lipid peroxidation, and higher activities of GPx-Tot, GPx-Se and SOD than M70. The Yeast treatment was related to high GST activity. This graph allowed us to establish the baseline of antioxidant activity for the M70 emulsion, and to investigate the effect of including an external antioxidant into the enrichment media.

Experiment 2: External antioxidant

Figure 3 shows the enzyme activities and levels of LPO of the control and enriched *Artemia* nauplii with and without the antioxidant HYT. Observed patterns of antioxidant enzyme activities and LPO levels for M70 versus controls were quite similar to those reported in Figure 1, except for GST. *Artemia* nauplii incubated with the two tested enriched media (DCSS and M70) showed similar enzymatic activity and LPO patterns. The addition of the antioxidant HYT decreased the activities of CAT and GST, and increased those of GPx-Tot of *Artemia* nauplii enriched with M70.

LPO were significantly ($P<0.05$) higher in nauplii enriched with DCSS and M70 treatments. Adding an external antioxidant (HYT) dramatically reduced LPO.

Table 1 lists the main fatty acids (% of total fatty acids) from total lipids of *Artemia* nauplii enriched with different treatments. EPA values were significantly ($P<0.05$) higher in DCSS treatment (12.2 ± 2.6) compared to all other treatments. The n-3 HUFA increased in all treatments when compared with the control group, but n-6 HUFA were only significantly ($P<0.05$) higher in M70+HYT treatment. DHA levels were significantly ($P<0.05$) higher in M70+HYT treatment compared to other treatments, and this result is reflected in the highest DHA/EPA ratio among all treatments.

Figure 4a illustrates the component plot for the enzymatic activity and HUFA data. The two first components of PCA accounted for the 66.9 % of variation, PC1 explaining 45.0% and PC2 explaining 21.9% of the variation of the data set. Figure 4b shows the factor score plot based on enrichment and antioxidant scores from the constituent variables. All treatments were clearly separated from each other, with control treatment to the left, DCSS and M70+HYT in the middle, and M70 to the right. The PCA grouping indicates that M70 treatment has higher LPO than the other treatments. Figure 4 also shows that the scores of M70+HYT treatment were associate to both n-3 and n-6 HUFA variables pointing at the protective role of HYT during enrichment.

Analysing the PCA results of both experiments, the M70 group is always linked to lipid peroxidation as evidenced by the association of the scores to the location of the LPO variable in the components graph. The association is lost by adding HYT to the enrichment. The separation of the two groups (M70 and M70+HYT) was achieved, with the LPO values of M70+HYT treatment approaching those of the commercial emulsion DCSS and control group.

Discussion

High levels of DHA are important in the development of nervous and sensory system during early development of vertebrates, including fish (Hamre & Harboe, 2008; Tocher, 2010). For that reason, enrichment of live preys including *Artemia* that naturally lack DHA is critical for the successful rearing of fish larvae, particularly those from marine species (Tocher, 2010). DHA levels achieved in *Artemia* nauplii with the M70 and M70+HYT treatments were different. The DHA/EPA ratio is a common biochemical parameter used to evaluate the nutritional suitability of diets for marine finfish larviculture (Evjemo et al., 1997), and high DHA/EPA ratios were also reported as important to promote growth, stress resistance and pigmentation (Mourente et al.,

294 1993; Reitan et al., 1994; Sorgeloos et al., 2001). Rodríguez et al. (1997) observed
295 significantly higher growth rates in sea bream larvae fed rotifers with a DHA/EPA ratio
296 of 1.5 compared to those fed DHA/EPA ratio < 0.6. In natural preys such as copepods,
297 this ratio is around 2.0 (Fraser et al., 1989; Sorgeloos et al., 2001; Van der Meeren et
298 al., 2011). Currently satisfactory results in an enrichment process with *Artemia* nauplii
299 is achieved with DHA/EPA ratios of 2 and higher (Dhert et al., 1993; Sorgeloos et al.,
300 2001) but, on the other hand, this has been acknowledged as a major challenge due to an
301 apparent inability of *Artemia* nauplii to retain DHA (Evjemo et al., 1997). In fact,
302 *Artemia* nauplii actively retroconvert DHA to EPA during and after enrichment
303 (Navarro et al., 1999). Thus, enrichment of *Artemia* nauplii is a continuous quest
304 towards increasing PUFA and DHA, and despite the use of high DHA emulsions like
305 M70 which consistently produces *Artemia* DHA/EPA ratios higher than 1 (Viciano et
306 al., 2015), the fact that it only produces moderate levels of DHA in the enriched nauplii
307 (5-8 % of total fatty acids) illustrates the problems of DHA retroconversion in *Artemia*.

308 *Artemia* nauplii possess antioxidant enzymes required to metabolize O_2^- (SOD), H_2O_2
309 (CAT and GPx) and organic hydroperoxides activity (GPx-Se) (Nunes et al., 2006).
310 These enzymes allow the organism to prevent, intercept and repair the damage caused
311 by the free radicals (Olsen et al., 2013). However, during live feed enrichments, it is
312 possible that these endogenous protective mechanisms are not sufficient to prevent
313 oxidative damage as HUFA contained in the enrichment diet are very prone to
314 oxidation, particularly under physical-chemical conditions set up during the enrichment
315 procedures (McEvoy et al., 1995; Monroig et al., 2007), thus contributing to the low
316 efficiency in HUFA enrichment in general and DHA in particular.

317 SOD is the first defence mechanism in the detoxification process and, along with CAT,
318 is regarded as playing an important antioxidant role in aquatic invertebrates

(Livingstone, 1991; Barata et al., 2005b). In Experiment 1, SOD activity was inhibited in the nauplii enriched with yeast and M70, in comparison with those enriched with *Tetraselmis*, and the control group (unenriched nauplii). These differences can be explained by SOD *modus operandi*, with SOD activity increasing in early stages of oxidative stress when ROS abundance increases. If the oxidative stress persists, SOD becomes depleted and unable to cope with free radicals (Lukaszewicz-Hussan & Moniuszko-Jakoniuk, 2004). This is consistent with increased LPO levels achieved in *Artemia* nauplii enriched with M70 and yeast in Experiment 1. In Experiment 2 differences in SOD activity were not observed, although lipid peroxidation in M70 treatment was very high. Inter experiment difference in SOD supports the previous argument that antioxidant enzyme activities are transient (Livingstone, 1991; Barata et al., 2005b).

The response to oxidative stress has been related to increased CAT activities (Tovar-Ramirez et al., 2010) and therefore, the activity of this enzyme is expected to increase with the presence of substrates that enhance or produce ROS (Barata et al., 2005a; Tovar-Ramirez et al., 2010). In this study, CAT activity in *Artemia* nauplii from the control group remained at low levels, increasing in those nauplii enriched with yeast, *Tetraselmis*, DCSS or M70 emulsion. Nauplii enriched with a combination of the DHA-rich emulsion M70 and the antioxidant Hytolive (HYT) treatment showed significantly lower CAT activities than those enriched with the same emulsion (M70) without antioxidant, and a commercial emulsion (DCSS). Clearly, these results strongly suggest the protective effect of Hytolive added to the enrichment diet. CAT and GPx have complementary roles in the detoxification of hydrogen peroxides, but have different subcellular localization (peroxisomal and cytosolic, respectively) and affinity for H_2O_2 levels. Thus, high levels of H_2O_2 produce an increased affinity for CAT, whereas low

344 levels of H_2O_2 produce an increased affinity for GPx (Orbea et al., 2000; Barata et al.,
345 2005b). Interestingly, GPx activity (GPx-Tot and GPx-Se) reached the highest values in
346 unenriched nauplii (control). On the contrary, *Artemia* nauplii enriched with M70
347 showed low activity values for both GPx enzymes, suggesting high levels of H_2O_2 and
348 oxidative stress. Addition of the antioxidant HYT increased the GPx activity in nauplii
349 enriched with M70 and, together with the decreased CAT activity mentioned above,
350 made the nauplii enriched with M70+HYT exhibit the best antioxidant status among all
351 enrichment diets tested and most closely resembled the control *Artemia*. These results
352 clearly indicate that the Hytolive exerts an effective antioxidant effect during *Artemia*
353 enrichment and, in addition to lipophilic antioxidants that are commonly used in the
354 formulation of enrichment diets, inclusion of water-soluble antioxidants may also help
355 live preys to cope with exposure to toxic oxidized compounds.

356 GSTs are a family of Phase II detoxification enzymes that catalyze the conjugation of
357 glutathione (GSH) to a wide variety of endogenous and exogenous electrophilic
358 compounds (Townsend & Tew, 2003). GST activity detoxifies endogenous compounds
359 such as peroxidized lipids and enables the breakdown of xenobiotics (Sherrat & Hayes,
360 2001). GST activity was higher than the experimental control in nauplii enriched with
361 yeast, DCSS, M70 (Expt. 2) and M70+HYT treatment. This is consistent with previous
362 reports showing that GST activity increases in response to exogenous chemical sources
363 including those promoting ROS (Barata et al., 2005b). Increased GST activity indicates
364 indirect conjugation to its endogenous substrate during Phase II detoxification, which
365 would help eliminate toxic metabolites (Townsend & Tew, 2003). Phenolic compounds
366 from olive oil can restore redox balance by free radical quenching (El-Azem, 2013). By
367 neutralizing the free radicals formed, the amount of antioxidant enzymes such as GSH
368 is preserved in the body (Masella et al., 2004; El-Azem, 2013). The previous effect may

369 explain the slight but significant reduction of GST in *Artemia* nauplii co-exposed to
370 M70 and HYT.

371 Because ROS have extremely short half-lives, they are difficult to measure directly and
372 therefore oxidative stress can be inferred through measurement of several products of
373 the damage produced (Pryor, 1991). Lipid peroxidation (LPO) can be considered an
374 index of oxidative damage to lipids produced by toxic compounds (Fernández et al.,
375 2012). Results from this study showed that *Artemia* nauplii enriched with M70 and
376 DCSS emulsions presented significantly higher LPO levels than those supplemented
377 with HYT and the control group.

378 It is reasonable to assume that lipid peroxides and other potentially toxic oxidation
379 products are filtered by *Artemia* nauplii during the enrichment procedure (McEvoy et
380 al., 1995). *Artemia* enrichment is largely regarded as a "bioencapsulation" process
381 whereby the *Artemia* ingest enrichment diet particles until the gut is full (Figueiredo et
382 al., 2009). The supplementation of M70 enrichments with HYT was hypothesised to
383 decrease the lipid peroxidation and therefore the presence of oxidized lipid available in
384 the enrichment media. Consequently, the PCA results strongly suggest that such a
385 protective action from HYT took place, as the HYT-supplemented treatment clearly
386 separated from the M70 group, the latter containing higher levels of the oxidation
387 biomarker LPO. There are no previous studies using hydroxytyrosol on live prey
388 enrichment. In humans, hydroxytyrosol is rapidly absorbed in a dose-dependent manner
389 in the small intestine and colon by bidirectional passive diffusion (El-Azem, 2013), and
390 then is mostly conjugated as glucuronoconjugates, the most abundant form found in
391 plasma. From this conjugated form, it displays its antioxidant potential. Hydroxytyrosol
392 is rapidly metabolized and, after five minutes of administration, it is possible to find
393 derivatives in plasma (Miro-Casas et al., 2003). McEvoy et al. (1995) demonstrated

394 autoxidation in three enrichment emulsions when *Artemia* were present, however the oil
395 emulsions in the control media without *Artemia* did not show autoxidation within the
396 experimental period. It is expected that once introduced into the enrichment media, with
397 the brine shrimp present, the protective effect of hydroxytyrosol begins due to its water
398 soluble nature, protecting against oxidation of the fatty acids. Thereby oxidation may be
399 reduced before the fatty acids entrance in the digestive tract of *Artemia*, ensuring that
400 the enzymatic system is not oversaturated and remains active. This is shown in the
401 improvement of the enzymatic activity when hydroxytyrosol was added in the
402 enrichment medium.

403 In conclusion, the addition of a natural water-soluble olive fruit extract with a high
404 amount of hydroxytyrosol to the lipid emulsion enrichment improves the antioxidant
405 defence of *Artemia* nauplii and decreases the levels of the oxidative biomarker LPO.
406 Moreover, the addition of this olive fruit extract makes the activity of all antioxidant
407 enzymes in *Artemia* enriched with a DHA-rich oil emulsion resemble those of
408 homeostasis as defined by unenriched nauplii. Such an antioxidant protective role of
409 hydroxytyrosol is reflected in the lipid profile of *Artemia*, since higher levels of DHA
410 and a higher DHA/EPA ratio were found in nauplii enriched in the presence of
411 hydroxytyrosol. As a first approach, here, only a concentration of 0.04 g L^{-1} was tested
412 and, although the benefits prove significant, probably higher concentrations would
413 produce even better results, since there is evidence of an inhibitory effect of HYT in
414 LPO at increasing concentrations (Gargouri et al., 2011). Thus further dose dependent
415 studies are advisable in order to establish if the antioxidant potential can be further
416 enhanced and to verify how this improvement may be applicable in large-scale
417 enrichments.

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424

425 **5. Bibliography**

426 Barata, C., Lekumberri, I., Vila-Escalé, M., Prat, N., Porte, C., 2005a. Trace metal
427 concentration, antioxidant enzyme activities and susceptibility to oxidative stress
428 in the tricoptera larvae *Hydropsyche exocellata* from the Llobregat river basis
429 (NE Spain). Aquatic Toxicology 74, 3-19.

430 Barata, C., Varo, I., Navarro, J.C., Arun, S., Porte, C., 2005b. Antioxidant enzyme
431 activities and lipid peroxidation in the freshwater cladoceran *Daphnia magna*
432 exposed to redox cycling compounds. Comparative Biochemistry and
433 physiology Part C: Toxicology & pharmacology 140, 175-186.

434 Barata, C., Navarro, J.C., Varo, I., Riva, M.C., Arun, S., Porte, C., 2005c. Changes in
435 antioxidant enzyme activities, fatty acid composition and lipid peroxidation in
436 *Daphnia magna* during the aging process. Comparative Biochemistry and
437 Physiology Part B 140, 81-90.

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

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

443 Benítez-Santana, T., Masuda, R., Juárez Carrillo, E., Ganuza, E., Valencia, A.,
444 Hernández-Cruz, C.M, Izquierdo, M.S., 2007. Dietary n-3 HUFA deficiency
445 induces a reduced visual response in gilthead seabream *Sparus aurata* larvae.
446 Aquaculture 264, 408-417.

447 Bradford, M., 1976. A rapid and sensitive method for the quantification of microgram
448 quantities of protein utilizing the principle of protein-dye binding. Analytical
449 biochemistry 72, 248-254.

450 Chimi, H., Cillard, J., Cillard, P., Rahmani, M., 1991. Peroxyl and hydroxyl radical
451 scavenging activity of some natural phenolic antioxidants. JAOCS 68, 307-312.

452 Correia, A.D., Costa, M.H., Luis, O.J., Livingstone, D.R., 2003. Age-related changes in
453 antioxidant enzyme activities, fatty acid composition and lipid peroxidation in
454 whole body *Gammarus locusta* (Crustacea: Amphipoda). J. Exp. Mar. Biol. and
455 Ecol. 289, 83-101.

456 Deiana, M., Incani, A., Rosa, A.,Corona, G., Atzeri, A., Loru, D., Melis, M.P., Dessi,
457 A., 2008. Protective effect of hydroxytyrosol and its metabolite homovanillic
458 alcohol on H_2O_2 induced lipid peroxidation in renal tubular epitelial cells. Food
459 and Chem. Toxixology 46, 2984-2990.

460 Dhert, P., Sorgeloos, P., Devresse, B., 1993. Contributions towards a specific DHA
461 enrichment in the live food *Brachionus plicatilis* and *Artemia* sp. In: Reinertsen,
462 H., Dahle, L.A., Jorgensen, L., Tvinnereim, K. (Eds.), Fish Farming
463 Technology. Balkema, Rotterdam, Netherlands, pp. 109-115.

464 Di Giulio, R.T., Benson, W.H., Sanders, B.M., Van Veld, P.A., 1995. Biochemical
465 mechanisms: metabolism, adaptation and toxicity. In: Rand, G. (Ed.),
466 Fundamentals of aquatic toxicology, effects, environmental fate and risk
467 assessment, Taylor & Francis, London, pp. 523-561.

468 Dunnett, C.W., 1964. New tables for multiple comparisons with a control. Biometrics
469 20, 482-491.

El-azem, N., 2013. Efecto del hidroxitirosol sobre la cardiotoxicidad producida por doxorubicina. PhD. Universidad de Granada. Pp. 202.

Esterbauer, H., Schaur, R.J., Zollner, H., 1991. Chemistry and biochemistry of 4-hydroxynonenal malonaldehyde and related aldehydes. Free radic. Biol. Med. 11, 81-128.

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

Fernández, B., Campillo, J.A., Martínez-Gómez, C., Benedicto, J., 2012. Assessment of the mechanisms of detoxification of chemical compounds and antioxidant enzymes in the digestive gland of mussels, *Mytilus galloprovincialis*, from Mediterranean coastal sites. Chemosphere 87, 1235-1245.

Fernández-Bolaños, J.G., López, Ó., Fernández-Bolaños, J., Rodríguez-Guitiérrez, G., 2008. Hydroxytyrosol and derivates: Isolation, synthesis, and biological properties. Curr. Org. Chem. 12, 442-463

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

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

Fraser, A.J., Sargent, J.R., Gamble, J.C., 1989. Lipid class and fatty acid composition of *Calanus finmarchicus* (Gunnerus), *Pseudicalanus* sp. and *Temora longicornis*

492 Muller from a nutrient-enriched seawater enclosure. J. Exp. Mar. Biol. Ecol.
493 130, 81-92.

494 Gargouri, B., ben Mansour, R., Jmal, M.M., Boujelben, R.N., Allouch, N., Lassoued, S.,
495 Hamden, K. 2011. Hydroxytyrosol supplementation inhibits oxidative DNA
496 damage, suppresses protein-lipid oxidation and modulates antioxidant enzymes
497 in human peripheral blood lymphocytes. J. Medicinal Plants Research 5, 4863-
498 4869.

499 González, M.P., 2005. Estudio de los efectos cardiovasculares y la absorción oral del
500 hidroxitirosol en modelos animales y humanos. Ph D. University of Granada,
501 Spain. 201 pp.

502 Gordon, M.H., Paiva-Martins, F., Almeida, M., 2001. Antioxidant activity of
503 hydroxytyrosol acetate compared with that of other olive oil polyphenols. J.
504 Agric. Food Chem. 49, 2480-2485.

505 Greetham, D. & Grant, C.M., 2009. Antioxidant activity of the yeast mitochondrial one-
506 cys peroxidation is depedent on thioredoxin reductase and glutathione in vivo.
507 Mol. Cell. Boil. 29: 3229-40

508 Hamre, K. & Harboe, T., 2008. *Artemia* enriched with high n-3 HUFA may give a large
509 improvement in performance of Atlantic halibut (*Hippoglossus hippoblossus* L.)
510 larvae. Aquaculture 277, 239-243

511 Han, K., Geurden, I., Sorgeloos, P., 2000. Enrichment strategies for *Artemia* using
512 emulsions providing different levels of n-3 highly unsaturated fatty acids.
513 Aquaculture 183, 335-347.

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

517 Hao, J., Shen, W., Yu, G., Jia, H., Li, X., Feng, Z., Wang, Y., Weber, P., Wertz, Z.,
518 Sharman, E, Liu, J., 2010. Hydroxytyrosol promotes mitochondrial biogenesis
519 and mitochondrial function in 3T3-L1 adypocites. J. Nutr. Biochem. 21: 634-44.

520 Lee, S., Chang, D., Lee, B., Jeon, Y., 2009. Antioxidant activity of solubilized
521 *Tetraselmis suecica* and *Chlorella ellipsoidea* by enzymatic digest. J. Food Sci.
522 and Nutrition 14, 21-28.

523 Livingstone, D.R., 1991. Organic xenobiotics metabolism in marine invertebrates. Adv.
524 Comp. Environ. Physiol. 7, 45-185.

525 Livingstone, D.R., 2001. Contaminant-stimulated reactive oxygen species production
526 and oxidative damage in aquatic organisms. Marine pollution bulletin 42, 656-
527 666.

528 Lukaszewicz-Hussain, A. & Moniuszko-Jakoniuk, J., 2004. Liver catalase, glutathione
529 peroxidase and reductase activity, reduced glutathione and hydrogen peroxide
530 levels in acute intoxication with chlorfenviphos, an organophosphate insecticide.
531 Pol. J. Environ. Studies 13, 303-309.

532 Masella, R., Varí, R., D'Archivio, M., Di Benedetto, R., Matarrese, P., Malorni, W.,
533 Scazzocchio, B., Giovannini, C., 2004. Extra virgin olive oil biophenols inhibit
534 cell-mediated oxidation of LDL by increasing the mRNA transcription of
535 glutathione-related enzymes. J. Nutr. 134, 785-791.

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

538 Miro-Casas, E., Covas, M.I., Farre, M., Fito, M., Ortuño, J., Weinbrenner, T., Roset, P.,
539 de la Torre, R., 2003. Hydroxytyrosol disposition in humans. Clin. Chem. 49,
540 945-952.

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

545 Monroig, Ó., Navarro, J.C., Amat, F., González, P., Hontoria, F., 2006b. Effect of
546 aeration on the efficiency of *Artemia* enrichment with EFA-rich emulsion and
547 liposomes. Aquaculture 257, 382-392.

548 Monroig, Ó, Navarro, J.C., Amat, F., González, P., Hontoria, F., 2007. Oxidative
549 stability and changes in the particle size of liposomes used in the *Artemia*
550 enrichment. Aquaculture 266, 200-210.

551 Mourente, G., Rodríguez, A., Sargent, J.R., 1993. Effects of dietary docosahexaenoic
552 acid (DHA; 22:6*n*-3) on lipid and fatty acid composition and growth in gilthead
553 sea bream (*Sparus aurata* L.) larvae during first feeding. Aquaculture 112, 79-
554 98.

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

557 Nunes, B., Carvalho, F., Guilhermino, L., 2006. Effects of widely used pharmaceuticals
558 and a detergent on oxidative stress biomarkers of the crustacean *Artemia*
559 *parthenogenetica*. Chemosphere 62, 581-594.

560 Olsen, E.K., Hansen, E., Isaksson, J., Andersen, J.H., 2013. Cellular antioxidant effect
561 of four bromophenols from the red algae, *Vertebrata lanosa*. Mar. Drugs 11,
562 2769-2784.

563 Orbea, A., Fahimi, H.D., Cajaraville, M.P., 2000. Immunolocalization of four
564 antioxidant enzymes in digestive glands of mollusks and crustaceans and fish
565 liver. Histochem. Cell. Biol. 114, 393-404.

566 Pryor, W., 1991. The antioxidant nutrients and disease prevention – What do we know
567 and what do we need to find out?. Am. J. Clin. Nutr 53, 391S-393S.

568 Reitan, K.I., Rainuzzo, J.R., Olsen, Y., 1994. Influence of lipid composition of live feed
569 on growth, survival and pigmentation of turbot larvae. Aquacult. Int. 2, 33-48.

570 Rodríguez, C., Perez, J.A., Diaz, M., Izquierdo, M.S, Fernández-Palacios, H., Lorenzo,
571 A., 1997. Influence of EPA/DHA ratio in rotifers on gilthead seabream (*Sparus*
572 *aurata*) larval development. Aquaculture 150, 77-89.

573 Schaffer, S., Podstawa, M., Visioli, F., Bogani, P., Müller, W.E., Eckert, G.P., 2007.
574 Hydroxytyrosol-rich olive mill wastewater extract protects brain cells in vitro
575 and ex vivo. J. Agric. Food Chem. 55, 5043-5049.

576 Sherrat, P.J & Hayes, J.D., 2001. Glutathione-S-transferases. In: Ioannides, C. (Ed.),
577 Enzyme system that metabolise drugs and other xenobiotics. John Wiley & Sons
578 Ltd. pp. 319-352.

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

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

584 Tocher, D., 2010. Fatty acid requirements in ontogeny of marine and freshwater fish.
585 Aquacult. Res. 41, 717-732.

586 Tovar-Ramirez, D., Mazurais, D., Gatesoupe, J.F., Quazuguel, P., Cahu, C.L.,
587 Zambonino-Infante, J.L., 2010. Dietary probiotic live yeast modulates
588 antioxidant enzyme activities and gene expression of sea bass (*Dicentrarchus*
589 *labrax*) larvae. Aquaculture 300, 142-147.

590 Townsend, D.M. & Tew, K.D., 2003. The role of glutathione-S-transferase in anti-
591 cancer drug resistance. Oncogene 22, 7369-7375.

592 Van der Meeren, T., Olsen, R.E., Hamre, K., Fyhn, H., 2011. Biochemical composition
593 of copepods for evaluation of feed quality in production of juvenile marine fish.
594 Aquaculture 274, 375-397.

595 Van Stappen, G., 1996. Introduction, biology and ecology of *Artemia*. In: Manual on the
596 production and use of live food for Aquaculture, (ed. by P. Lavens & P.
597 Sorgeloos), FAO Fisheries Technical Paper (361), Roma.

598 Viciano, E., Monroig, Ó., Salvador, A., Amat, J., Fiszman, S, Navarro; J.C., 2015.
599 Enriching *Artemia* nauplii with a high DHA-containing lipid emulsion: search
600 for an optimal protocol. Aquaculture Research 46, 1066-1077.

601 Visioli, F., Bellomo, G., Galli, C., 1998. Free radical-scavenging properties of olive oil
602 polyphenols. Biochem. Biophys. Res. Commun. 247, 60-64.

603 Visioli, F., Caruso, D., Galli, C, Viappiani, S, Galli, G., Sala, A., 2000. Olive oils rich in
604 natural catecholic phenols decrease isoprostane excretion in humans. Biochem.
605 Biophys. Res. Commun. 278, 797-799.

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Figures

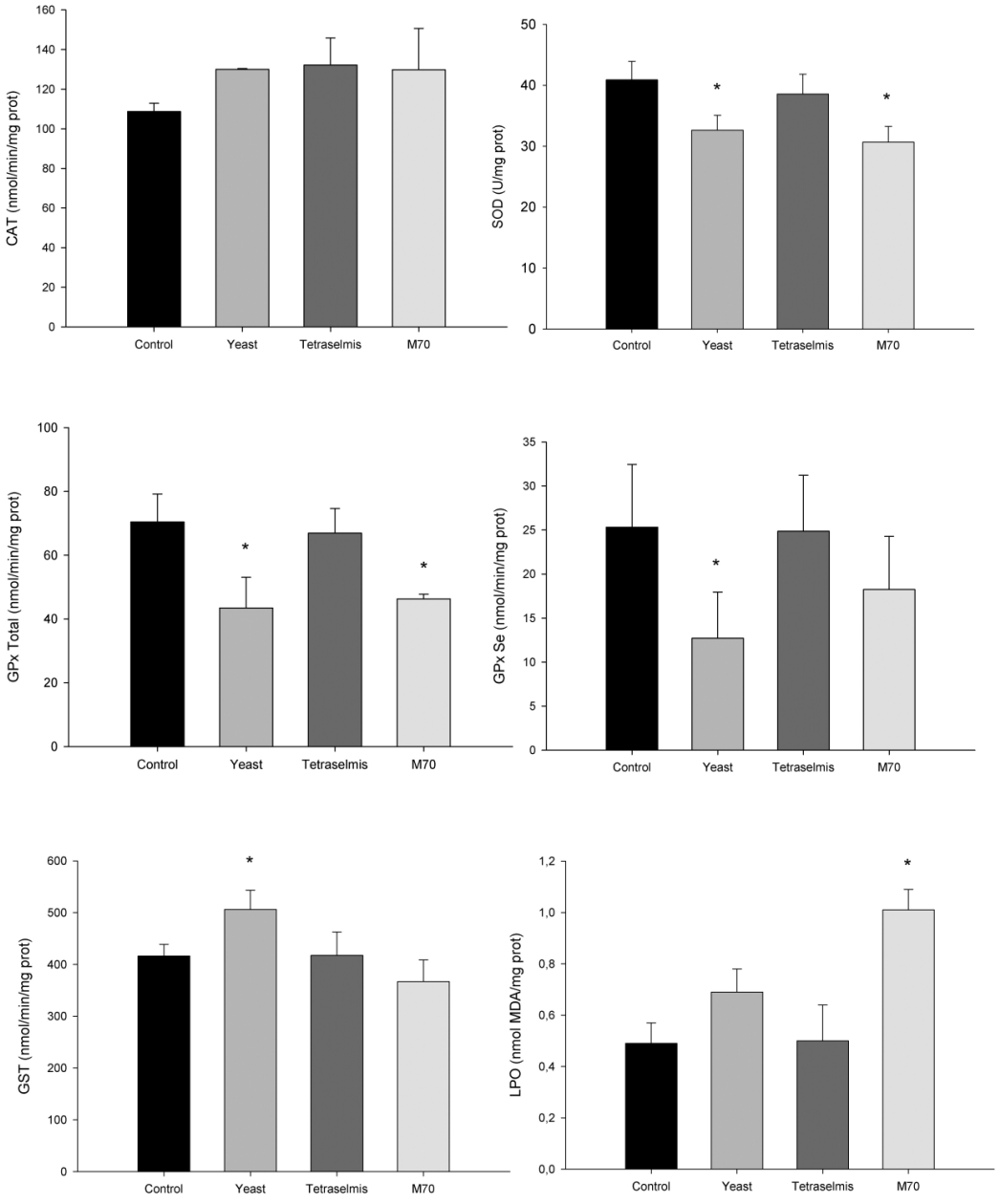

Figure 1. Activity of Catalase (CAT), superoxide dismutase (SOD), total glutathione peroxidase (GPx Total), Selenium dependent glutathione peroxidase (GPx Se-dep), glutathione-s-transferase (GST) and lipid peroxidation (LPO) in whole body of *Artemia* nauplii enriched with M70 emulsion, *Tetraselmis suecica* algae, yeast (*Saccharomyces cerevisiae*) and non-enriched *Artemia*. Asterisk shows statistical differences between the different treatments and the control group (Dunnett test).

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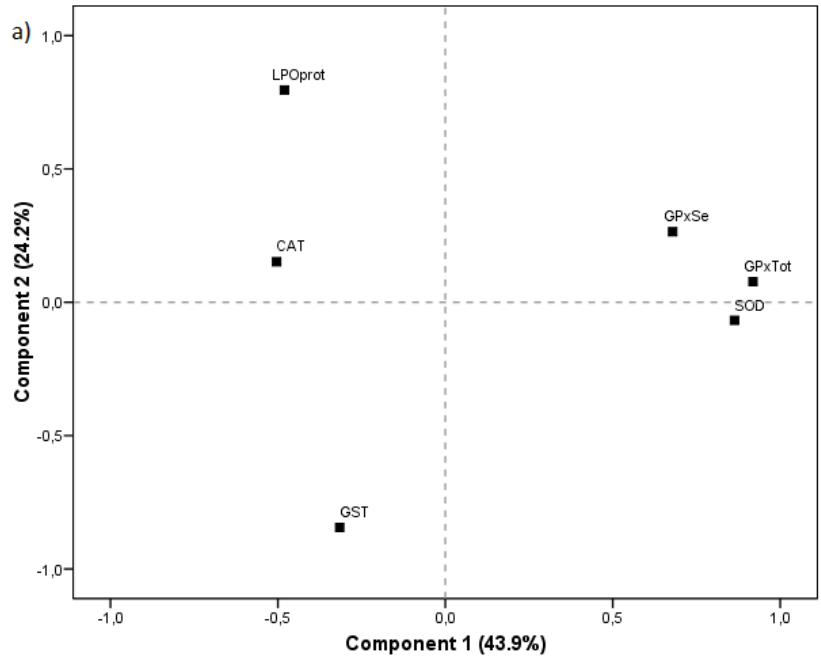

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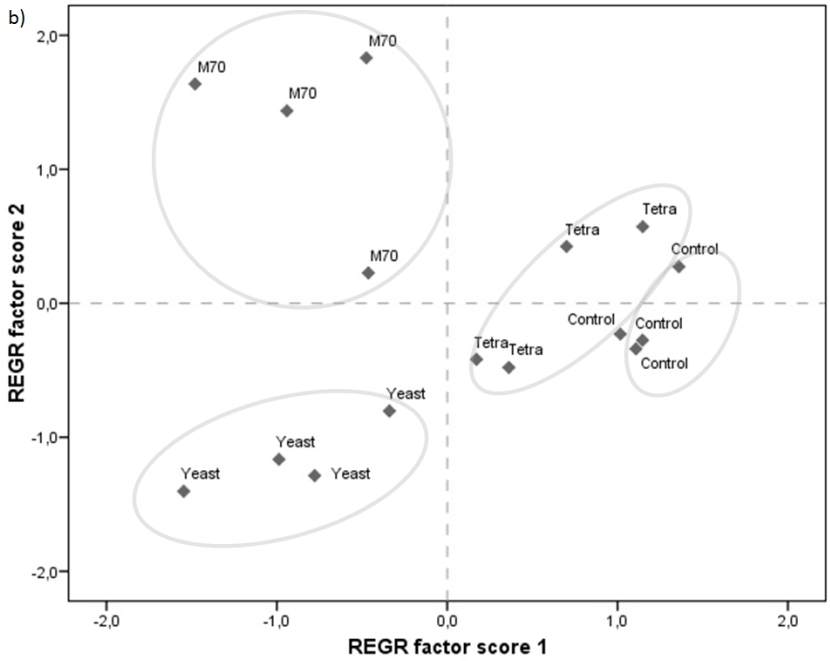

617 Figure 2. Component plot (a) and factor score plot (b) of the multivariate Principal
618 Components Analysis of lipid peroxidation (LPOprot) and antioxidant enzymes
619 (Catalase (CAT), glutathione-s-transferase (GST), superoxide dismutase (SOD), total
620 glutathione peroxidase (GPxTot), Selenium dependent glutathione peroxidase (GPxSe))

621 of enriched *Artemia* nauplii with M70 emulsion, *Tetraselmis suecica* algae (Tetra),
622 yeast (*Saccharomyces cerevisiae*) and non-enriched *Artemia* from Experiment 1.

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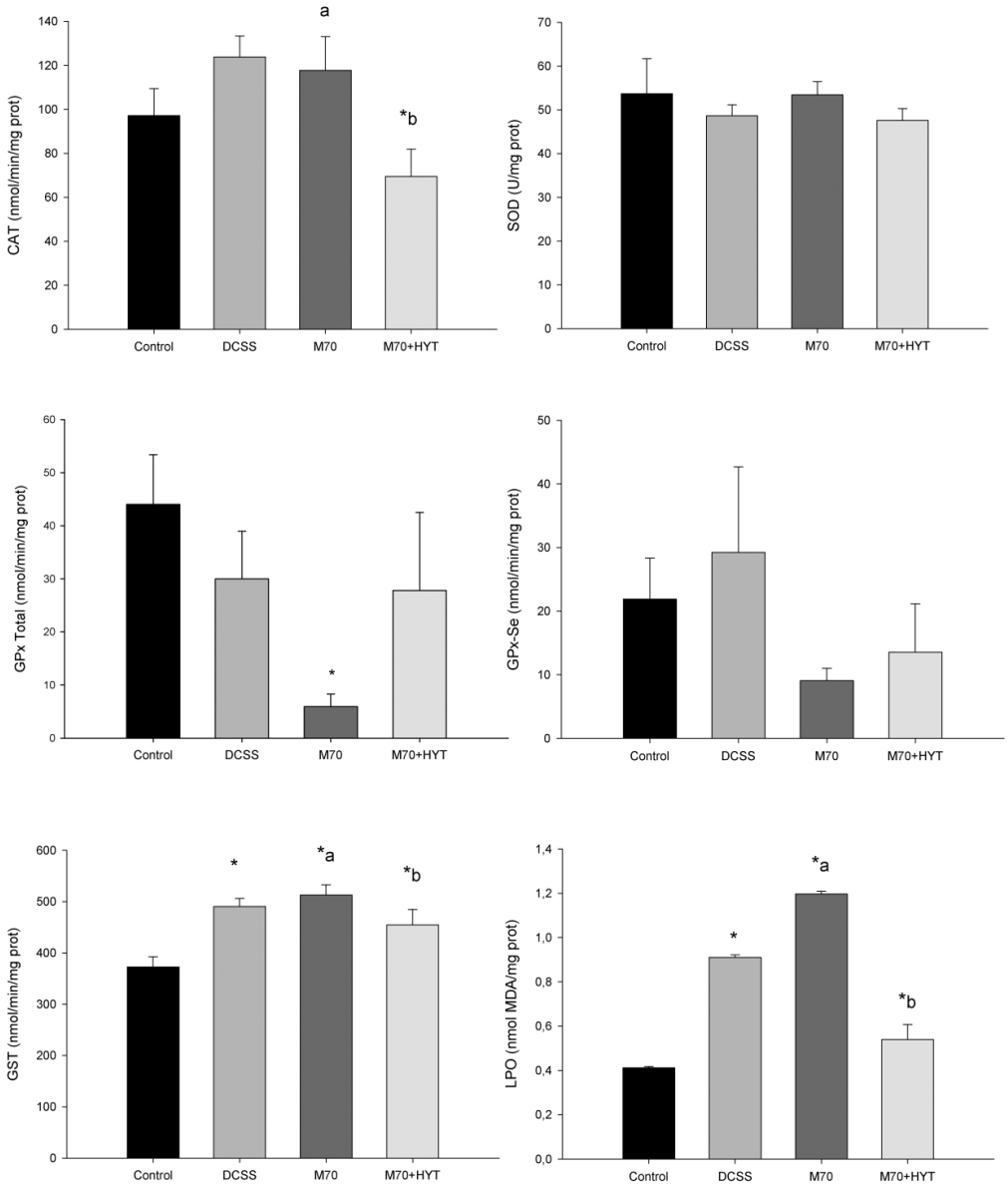

Figure 3. Activity of Catalase (CAT), superoxide dismutase (SOD), total glutathione peroxidase (GPx Total), Selenium dependent glutathione peroxidase (GPx Se-dep), glutathione-s-transferase (GST) and lipid peroxidation (LPO) in whole body of non-enriched *Artemia* nauplii and *Artemia* nauplii enriched with commercial emulsion DCSS, M70 emulsion and M70 emulsion in combination with an external antioxidant HYT, M70+HYT treatment. Asterisk shows statistical differences between the treatment and the control group (Dunnett test) and lowercase letter superscript shows statistical differences between M70 and M70+HYT treatment (Student t-test).

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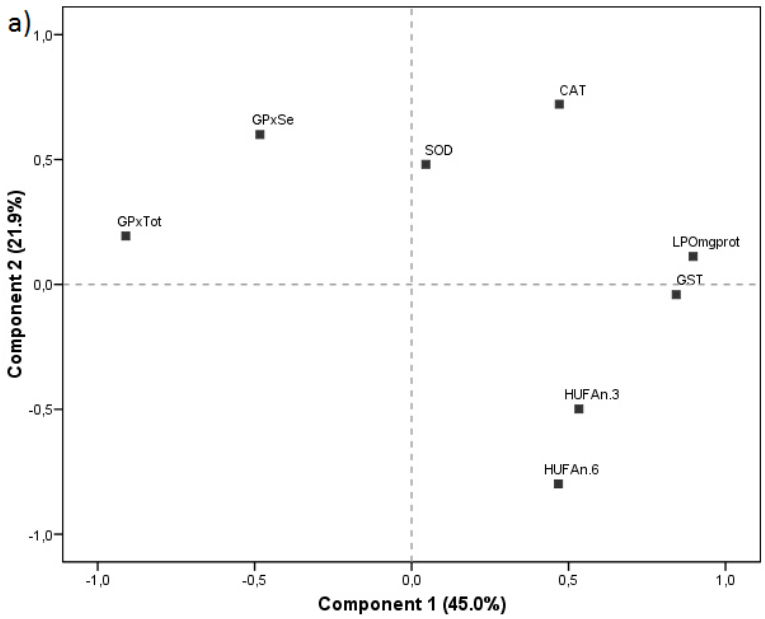

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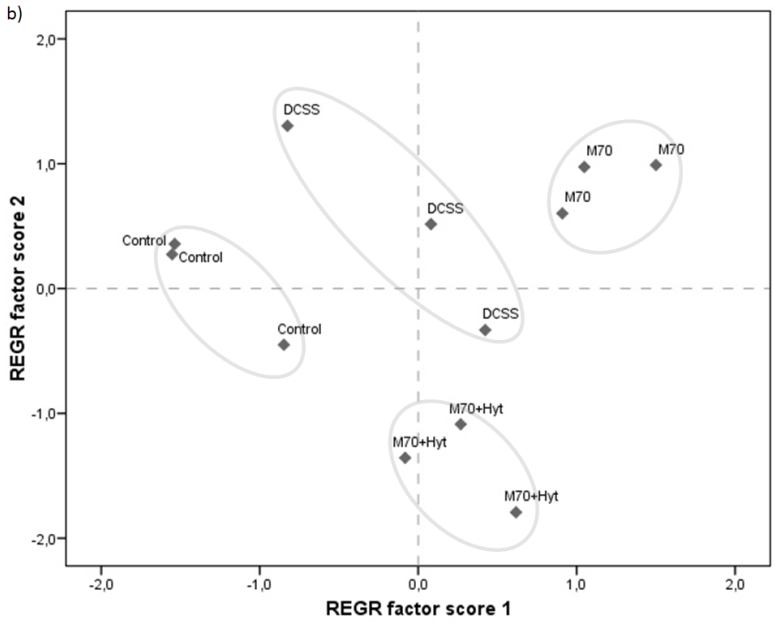

635 Figure 4. Component plot (a) and factor score plot (b) of the multivariate Principal
636 Components Analysis of lipid peroxidation (LPOprot), antioxidant enzymes (Catalase
637 (CAT), glutathione-s-transferase (GST), superoxide dismutase (SOD), total glutathione
638 peroxidase (GPxTot), Selenium dependent glutathione peroxidase (GPxSe)) and total
639 HUFA (n-3 and n-6 series) of enriched *Artemia* nauplii with commercial emulsion
640 DCSS, M70 emulsion, M70 emulsion in combination with an external antioxidant, HYT
641 (M70+HYT treatment) and non-enriched *Artemia* from Experiment 2.

642 **Tables**

643 Table 1. Selected fatty acids (percentage of total fatty acids) from enriched *Artemia*
644 nauplii from Experiment 2. Data represent means ± SD (n=3). Treatments with asterisk
645 are significantly different ($P \leq 0.05$) from control treatment (Dunnett test). If no
646 superscript appears, values are not different. T-Student test was performed between
647 M70 and M70+HYT treatments, and statistical differences were marked with a capital
648 letter superscript (A, B)

% Fatty acids	Control	DCSS	M70	M70+HYT
14:0	1.1±0.1	1.0±0.1	1.2±0.2	1.1±0.1
16:0	12.2±0.7	10.0±0.5*	10.8±0.3*	10.8±0.4*
16:1n-9	0.6±0.1	0.5±0.1	0.7±0.1	0.7±0.2
16:1n-7	2.4±0.1	2.3±0.2	2.4±0.1	2.3±0.1
18:0	6.7±0.0	5.0±0.1*	5.0±0.1*	5.0±0.1*
18:1n-9	17.5±0.9	16.4±0.8	15.7±0.7*	15.5±0.3*
18:1n-7	8.6±0.4	6.7±0.5*	7.1±0.5*	6.8±0.2*
18:2n-6	5.4±0.1	5.9±0.4	5.1±0.3	4.9±0.1
18:3n-3	25.1±0.6	22.0±0.8*	25.5±0.7A	23.8±0.4B
18:4n-3	3.2±0.1	2.8±0.2	3.1±0.3	3.0±0.0
20:0	0.2±0.0	0.2±0.0	0.2±0.0	0.2±0.0
20:2n-6	0.2±0.0	0.3±0.0	0.2±0.0	0.2±0.0
20:3n-6	0.2±0.0	0.2±0.0	0.2±0.0	0.2±0.0
20:4n-6	0.8±0.1	1.0±0.2	1.1±0.1*	1.2±0.0*
20:3n-3	0.7±0.0	0.6±0.0	0.7±0.1	0.6±0.0
20:4n-3	0.6±0.0	0.8±0.0*	0.6±0.0	0.6±0.0
20:5n-3	2.2±0.4	12.0±2.6*	4.3±0.7	4.6±0.2
22:0	0.4±0.0	0.3±0.0*	0.3±0.0*	0.3±0.0*
22:5n-3	0.0±0.0	0.4±0.1*	0.4±0.1*	0.5±0.0*
22:6n-3	0.1±0.0	2.4±0.3*	5.1±0.8*A	8.4±0.5*B
Saturated	22.8±0.8	18.1±0.8*	19.5±0.4*	19.0±0.6*
Monounsaturated	31.0±0.9	28.6±1.0*	27.4±1.2*	26.7±0.5*
Polyunsaturated	40.0±1.4	48.0±2.5*	47.9±0.9*	49.1±1.0*
HUFA n-3	3.6±0.5	15.5±5.2*	12.0±3.0*	14.3±1.3*
HUFA n-6	1.4±0.3	1.6±0.2	1.9±0.2	2.2±0.2*
DHA/EPA ratio	0.0±0.0	0.2±0.1*	1.2±0.1*A	1.8±0.0*B
Total FAME (mg g^{-1})	43.7±7.9	138.3±12.1*	105.7±17.1*	121.3±9.2*
% Lipid (DW)	20.9±0.4	27.8±1.3	27.4±0.5	26.2±0.4

649 HUFA n-3: ≥20:3n-3; HUFA n-6: ≥20:2n-6; DHA/EPA:
650 docosahexaenoic/eicosapentaenoic fatty acid ratio.