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1 **Assessment of stress and nutritional biomarkers in cultured *Octopus vulgaris* paralarvae:**  
2 **Effects of geographical origin and dietary regime**

3 D. Garrido<sup>a</sup>, I. Varó<sup>b</sup> (\*), A.E. Morales<sup>c</sup>, M.C. Hidalgo<sup>c</sup>, J.C. Navarro<sup>b</sup>, F. Hontoria<sup>b</sup>, O. Monroig<sup>d</sup>, J.  
4 Iglesias<sup>e</sup>, J.J. Otero<sup>e</sup>, A. Estévez<sup>f</sup>, J. Pérez<sup>f</sup>, M.V. Martín<sup>a</sup>, C. Rodríguez<sup>g</sup>, E. Almansa<sup>a</sup>, G.  
5 Cardenete<sup>c</sup>.

6  
7 <sup>a</sup>Centro Oceanográfico de Canarias (IEO), Vía Espaldón, Dársena Pesquera PCL 8, 38180 Santa  
8 Cruz de Tenerife, Spain.

9 <sup>b</sup>Instituto de Acuicultura Torre de la Sal. (IATS-CSIC), 12595 Ribera de Cabanes, Castellón,  
10 Spain.

11 <sup>c</sup>Departamento de Zoología, Universidad de Granada, Campus de Excelencia Internacional del  
12 Mar (CEI-MAR). Campus de Fuentenueva, 18071, Granada, Spain.

13 <sup>d</sup>Institute of Aquaculture, School of Natural Sciences, University of Stirling, Stirling FK9 4LA,  
14 Scotland, UK.

15 <sup>e</sup>Centro Oceanográfico de Vigo (IEO), Subida Radio Faro, 50, 36390, Vigo, Pontevedra, Spain.

16 <sup>f</sup>IRTA Sant Carles de la Ràpita, Ctra. Poble Nou, km. 5.5, 43540, Sant Carles de la Ràpita, Spain.

17 <sup>g</sup>Departamento de Biología Animal, Edafología y Geología, Universidad de La Laguna, Av.  
18 Astrofísico Francisco Sánchez, 38206, San Cristóbal de La Laguna, Santa Cruz de Tenerife Spain.

19

20 Corresponding author (\*): Inmaculada Varó. Instituto de Acuicultura Torre de la Sal.(IATS-CSIC),  
21 12595 Ribera de Cabanes, Castellón Spain. Email: inma@iats.csic.es.Tel: +34964319500

22

23 **Abbreviations**

24 **ARA**, Arachidonic acid; **CAT**, Catalase; **DHA**, docosahexaenoic acid; **EPA**, Eicosapentaenoic acid;  
25 **GPX**, Glutathione peroxidase; **GPX Se**, Selenium dependent glutathione peroxidase; **GPX T**,  
26 Total Glutathione peroxidase; **GR**, Glutathione reductase; **HSP70**, Heat shock proteins 70;  
27 **HUFA**, Highly unsaturated fatty acids; **LC60**, Marine Lecithin LC 60®; **MDA**, malondialdehyde;

28 **PUFAs**, Polyunsaturated fatty acids; **ROS**, Reactive oxygen species; **SGR**, Specific growth rate;  
29 **S**, Survival.

30

### 31 **Abstract**

32 The common octopus (*Octopus vulgaris*) is a promising species for aquaculture diversification,  
33 but massive mortality during the first life-cycle stages (paralarvae) is the main bottleneck for  
34 its commercial production in captivity. The aim of this study was to assess stress and  
35 nutritional condition biomarkers (HSP70, ROS enzymes and lipid peroxidation) (RNA/DNA,  
36 RNA/protein, protein/DNA and protein) in *O. vulgaris* paralarvae from different geographical  
37 origins and fed with *Artemia* enriched with marine phospholipids or microalgae (control  
38 group). To this end paralarvae were cultured for 30 days, in three different centres in Spain  
39 (Tarragona-Mediterranean area, Tenerife-Central Atlantic area and Vigo-North Atlantic area),  
40 under the same protocol, and fed on *Artemia* enriched with marine phospholipids (LC60  
41 (Marine Lecithin LC 60®, PhosphoTech Laboratoires) or microalgae (control group). Dry weight  
42 and most biomarkers analysed in hatchlings showed significant differences related to their  
43 origin (centre). Fifteen day old paralarvae presented significant differences in specific growth  
44 rate (SGR) associated with their dietary regime, and also showed differences in biomarkers  
45 associated both with their geographical origin and dietary regime. The results suggest that the  
46 SGR of paralarvae were positively influenced by LC60, promoting growth and in agreement  
47 with the results of nutritional condition biomarkers (nucleic acids ratios). The antioxidant  
48 defences against oxidative damage were also boosted in the LC60 paralarvae group, possibly  
49 as a result of the elevated content in highly polyunsaturated fatty acids. In addition, the partial  
50 correlations found between biomarkers varied according to diet. However, no positive effect  
51 of LC60 on survival was observed. The high variability found among geographical origins,  
52 despite the use of the same rearing protocol, highlights the need to clarify the sources of such  
53 variability.

54

55 **Keywords:** Antioxidant defences, Geographical origin, Heat shock protein, Marine  
56 phospholipids, Nucleic acid ratios, *Octopus vulgaris* paralarvae.

57

### 58 **1. Introduction**

59 *Octopus vulgaris* is a species of great interest for the diversification of marine aquaculture, due  
60 to its rapid growth rate and food conversion index, easy adaptation to captivity, and high

61 market price among other positive features (Iglesias and Fuentes, 2014; Iglesias et al., 2007).  
62 However, the high mortality rates of paralarvae occurring within the first 60 days of life in  
63 captivity have hindered its commercial production. In fact, the production of this species in  
64 captivity is restricted nowadays to on-growing of wild caught sub-adults until they reach a  
65 commercial size of 2-3 kg (García García et al., 2009; Rodríguez et al., 2006). With regards  
66 paralarval production, some authors have successfully reared a reduced number of paralarvae  
67 to juveniles when feeding them with crustacean zoeae in a co-feeding strategy with *Artemia*  
68 (Carrasco et al., 2006; Iglesias et al., 2004; Itami et al., 1963; Moxica et al., 2002; Villanueva,  
69 1994). Nonetheless, feeding paralarvae with crustacean zoeae is economically unfeasible due  
70 to availability reasons and concomitant high prices (Andrés et al., 2007, 2010).

71 *Artemia* is frequently used as live food in paralarvae cultures as a result of its easy availability,  
72 good acceptability and good handling/production logistics, making it a suitable live prey.  
73 However, *Artemia* in contrast to natural marine zooplankton, has an inadequate lipid  
74 composition, with low levels of polar lipids (PL) and highly unsaturated fatty acids (HUFA),  
75 especially docosahexaenoic acid (DHA) (Navarro et al., 1993), which is particularly relevant for  
76 octopus paralarvae development (Navarro and Villanueva, 2000, 2003). Recent studies  
77 (Monroig et al., 2013; Reis et al., 2014) point out that paralarvae have limited capacity to  
78 synthesize HUFA such as arachidonic acid (ARA), eicosapentaenoic acid (EPA) and DHA, from n-  
79 6 and n-3 precursors, confirming the essentiality of these fatty acids that must be supplied in  
80 the diet. Thus, in order to increase PL and HUFA levels in *Artemia metanauplii*, new efforts  
81 have been undertaken with the aim of developing novel enrichment protocols more suitable  
82 for *O. vulgaris* paralarvae (Guinot et al., 2013a, 2013b).

83 Although there is consensus about the nutritional factor being important (Navarro et al.,  
84 2014), and that many attempts have been made to develop optimum culture protocols, the  
85 reasons for high mortality during the culture of *O. vulgaris* paralarvae still remain unclear  
86 (Iglesias and Fuentes, 2014). In addition, many inconsistencies are found in the outcomes of  
87 feeding trials due to the great variability of the results obtained among studies in terms of  
88 survival and growth. This variability is reflected in differences in dry weight, number of suckers  
89 per arm, chromatophore patterns, etc..., found in the newly hatched paralarvae, probably  
90 related to their origin. In fact, *O. vulgaris* could be considered a species complex as has been  
91 suggested by Jereb et al. (2014). Recently, the species known until now as *O. vulgaris* in the  
92 East Asian and West Japan has been identified as *O. sinensis* by Gleadall (2016). Japanese  
93 hatchlings have a considerably higher dry weight and number of suckers per arm than  
94 European paralarvae (Arai et al., 2008; Iglesias and Fuentes, 2014; Kurihara et al., 2006;  
95 Okumura et al., 2005). Also differences in chromatophore patterns have been found between

96 European and Brazilian paralarvae (Vidal et al., 2010), although further research is still  
97 necessary in order to clarify the taxonomy as pointed out by Gleadall (2016). Moreover,  
98 differences in dry weight have been observed in Spanish paralarvae from different  
99 geographical origins (Moxica et al., 2002; Reis et al., 2015; Seixas et al., 2010a, 2010b; Viciano  
100 et al., 2011; Villanueva et al., 2004) although according to the study carried out by Cabranes et  
101 al. (2008) all belong to the same species. In addition to the geographical origin of paralarvae,  
102 other factors such as changes in spawning quality (female size, genetic and incubation  
103 temperature), prey nutritional profile (enrichment process and/or prey origin) or rearing  
104 conditions (tank volume, light intensity, density of paralarvae and/or preys) have also been  
105 pointed out as probable reasons for this variability, highlighting the need for a standardisation  
106 of octopus culture conditions (Iglesias and Fuentes, 2014; Iglesias et al., 2007; Villanueva and  
107 Norman, 2008). Therefore, a better understanding of the mechanisms underlying massive  
108 mortalities observed in early life-cycle stages of the common octopus paralarvae under the  
109 framework of standardized culture protocols is essential in order to advance in paralarval  
110 culture.

111 Additionally, the capacity to assess paralarvae vulnerability under potential nutritional and  
112 physiological derived stress in rearing conditions seems of paramount importance in such an  
113 unpredictable environment. To this end, the selection of biomarkers capable of the early  
114 detection and quantification of stress appears as a support tool. Nucleic acids (DNA and RNA),  
115 as well as their ratios (RNA/DNA, RNA/protein, protein/DNA) have been used as classical  
116 biomarkers to estimate growth as well as physiological and nutritional conditions, in early life  
117 stages of fish and invertebrates including cephalopods (Buckley et al., 1999; Chícharo and  
118 Chícharo, 2008; Clemmesen, 1988; Houlian et al., 1990; Mathers et al., 1994; Moltschaniwskyj  
119 and Carter, 2010; Peragón et al., 1998; Pierce et al., 1999; Richard et al., 1991; Vidal et al.,  
120 2006). Their use is based on the assumption that total RNA, directly involved in protein  
121 synthesis, vary with age, life-stages, organism size, disease-state and under changeable  
122 environmental conditions (Gorokhova and Kyle, 2002), while the content of DNA is stable  
123 under changing environmental situations within the somatic cells of a species (Buckley et al.,  
124 1999). Therefore, the RNA/DNA ratio is used as an index of the cellular protein synthesis  
125 capacity that usually correlates with nutritional condition and growth. On the other hand,  
126 RNA/protein ratio provides information about the cellular protein synthesis capacity (Peragón  
127 et al., 1998), and some authors have also shown its direct link to growth rate (Houlian et al.,  
128 1990). Moreover, protein/DNA ratio is correlated with cell size and thus provides information  
129 about growth (Mathers et al., 1994).

130 Cells have different mechanisms directed at responding to environmental and intracellular  
131 stressful stimuli, one of them being the synthesis of so-called “heat shock proteins” (HSP). HSP  
132 are part of the cell’s strategy to protect itself from damage, and they are expressed  
133 constitutively. HSP70 is one of the major HSP families in molluscs, and is involved in a variety  
134 of physiological processes serving as molecular chaperones (Repolho et al., 2014), regulating  
135 apoptosis (Lyons et al., 2003), and also performing important roles in response to oxidative  
136 stress and/or to environmental stressors (Wang et al., 2013), to the point of becoming a  
137 common biomarker for assessing stress and health status in aquatic organisms, including  
138 cephalopods (Iwama et al., 1998, 1999; Repolho et al., 2014; Sanders, 1993).

139 Regarding antioxidant defences, aerobic organisms have a protection system charged with  
140 neutralising highly reactive oxygen species (ROS), including enzymatic and non-enzymatic  
141 antioxidants. Among the ROS scavenging enzymes, catalase (CAT), superoxide dismutase  
142 (SOD), and enzymes dependent on glutathione (glutathione peroxidase, GPX, and glutathione  
143 reductase, GR) can be found. Non-enzymatic antioxidants include low molecular weight  
144 antioxidants, such as glutathione and vitamin C (Zielinski and Pörtner, 2000). The use of  
145 polyunsaturated fatty acids (PUFA) rich diets in octopus paralarvae culture can induce  
146 oxidative stress, through lipid peroxidation, which can ultimately cause tissue damage through  
147 the formation of malondialdehyde acid (MDA) and other toxic substances, as has been  
148 reported in fish (Mourente et al., 2002; Rikans and Hornbrook, 1997; Zhang et al., 2009).  
149 Interestingly, significant increased levels of MDA have been associated with food deprivation  
150 in fish (Morales et al., 2004). Moreover, antioxidant defences as well as lipid peroxidation have  
151 been previously used to assess nutritional status in octopus paralarvae culture (Varó et al.,  
152 2013).

153 Within this context, the aim of this study was to assess stress and nutritional condition  
154 biomarkers (HSP70, ROS enzymes and lipid peroxidation, RNA/DNA, RNA/protein,  
155 protein/DNA and protein) in *O. vulgaris* paralarvae from different geographical origins, reared  
156 under the same culture protocol (Iglesias and Fuentes, 2014), and fed with standardised diets  
157 consisting of *Artemia* enriched with marine phospholipids or microalgae.

158

## 159 **2. Materials and methods**

160 All experimental work was performed according to Spanish law 6/2013 based on the European  
161 Union directive on animal welfare (Directive 2010/63/EU) on the protection of animals used  
162 for scientific purposes.

163 **2.1. Broodstock husbandry**

164 Broodstock rearing was carried out under common standard conditions in three Spanish  
165 research centres: IRTA (Research & Technology Food & Agriculture, Tarragona) and two  
166 centres belonging to the Spanish Institute of Oceanography (IEO), namely the Oceanographic  
167 Centre of the Canary Islands in Tenerife (TF) and the Oceanographic Centre of Vigo (VG). As a  
168 result, three different paralarval geographical origins are considered: Tarragona-  
169 Mediterranean area (40°37'N, 0°39'E), Tenerife-Central Atlantic area (28°30'N, 16°12'W,) and  
170 Vigo-North Atlantic area (42°11'N, 8°49'W). A total of 20 adult *O. vulgaris* per centre were  
171 captured from local fisheries using artisanal octopus traps. The adult specimens were kept in  
172 1000 L tanks (with a maximum density of 10 kg·tank<sup>-1</sup>) with water renovation (5L·min<sup>-1</sup>), under  
173 oxygen saturation conditions and low light intensity. The weight of the female broodstock and  
174 physicochemical parameters of water are presented in Table 1. The availability of food and  
175 centre-specific logistics conditioned the broodstock diet, but in all cases, considering the study  
176 carried out by Quintana et al. (2015), crabs and/or cephalopods (e.g. squid) were included in  
177 order to ensure an optimal spawning quality. IRTA and VG used a mixture of crabs and fish  
178 *Liocarcinus depurator/Boops boops* and *Carcinus maena/Merluccius merluccius*, respectively,  
179 whereas octopus broodstock were fed on squid (*Loligo gahi*) at TF.

180 **2.2. Paralarvae rearing conditions**

181 Culture of paralarvae was carried out under similar conditions in the three centres. Aspects  
182 such as density of paralarvae, volume and colour of tanks, light source, photoperiod, water  
183 flow and use of green water were all standardised among centres. Paralarvae were reared at a  
184 density of 6 individuals·L<sup>-1</sup>. Every trial was carried out in triplicate over 30 days in 500 L black  
185 fiberglass cylinder-conical tanks. Two fluorescent lights (OSRAM Dulux superstar 36W/840)  
186 were placed above each tank to attain 700 lx focused in the middle of the tank surface and 500  
187 lx at the tank edges. Temperature and oxygen were measured daily, and nitrite, ammonium  
188 and salinity once a week (see Table 1). A 12L:12D photoperiod was used. A water flow of  
189 1L·min<sup>-1</sup> (corresponding to over 1.5 renovations·day<sup>-1</sup>) was applied from 18:00 to 8:00. A flow-  
190 through seawater system equipped with 20, 5 and 1 µm filter cartridges and UV lamps were  
191 used in TF and VG, while in IRTA a recirculation unit (IRTAMar™) was used. The renovation flow  
192 allowed the unfed *Artemia* to go through a 500 µm outflow mesh located in the middle of the  
193 tanks. Moderated flux aeration stones were placed close to the mesh. A water surface  
194 skimmer was also applied at the tanks' edges. Finally, green-water was used by adding

195 *Nannochloropsis* sp ( $2.5 \cdot 10^5$  cell·mL<sup>-1</sup>), supplied by Monzon Biotech S.L, (Barcelona, Spain) in  
196 IRTA and by PhytobloomGreen Formula® (Olhão, Portugal) in VG and TF.

### 197 **2.3. Paralarvae feeding**

198 Two *Artemia* enrichments were tested as food in paralarval cultures: a control group (C)  
199 enriched with microalgae *Isochrysis galbana* (Iso) and *Nannochloropsis* sp, and an  
200 experimental group enriched with Marine Lecithin LC 60® (PhosphoTech Laboratoires, Saint  
201 Herblain, France), hereafter named LC60. *Artemia* cysts (Sep-Art EG) were supplied by INVE  
202 Aquaculture (Dendermonde, Belgium). The microalgae enrichments were performed according  
203 to Iglesias and Fuentes (2014) and the LC60 *Artemia* enrichment according to Guinot et al.  
204 (2013 a, 2013b). Two prey sizes were used throughout the experimental period as follows.

205 During the first 15 days of culture, paralarvae were fed with nauplii enriched either for 20h  
206 with Iso ( $10$  nauplii·mL<sup>-1</sup> and  $1 \cdot 10^6$  cell·mL<sup>-1</sup>), or for 8h with LC60 ( $250$  nauplii·mL<sup>-1</sup> and  $0.6$  g·L<sup>-1</sup>).  
207 Fresh Iso was used at IRTA and VG, and freeze-dried Iso (easy algae®, Cádiz, Spain) at TF.

208 From days 16 to 29 of culture, paralarvae were fed *Artemia* metanauplii. Enrichment was  
209 carried out after on-growing the metanauplii for three days with Iso ( $5$  metanauplii·mL<sup>-1</sup> and  
210  $4 \cdot 10^5$  cell·mL<sup>-1</sup>). Then, metanauplii for C diet were subsequently enriched with *Nannochloropsis*  
211 sp for 24h ( $5$  metanauplii·mL<sup>-1</sup> and  $1 \cdot 10^7$  cell·mL<sup>-1</sup>), whereas those for LC60 diet were enriched  
212 with marine phospholipids for 6h ( $50$  metanauplii·mL<sup>-1</sup> and  $0.6$  g·L<sup>-1</sup>). Paralarvae were fed three  
213 times per day (at 9:00, 12:00 and 15:00), at a density of  $0.3$  nauplii·mL<sup>-1</sup> from day 0 to 15 and  
214 at  $0.15$  metanauplii·mL<sup>-1</sup> from day 16 to 29. The enriched *Artemia* was kept in the dark at 4°C  
215 with gentle aeration until paralarval feeding.

### 216 **2.4. Growth and survival**

217 Individual dry weight (DW) was determined for each treatment and centre using 15 paralarvae  
218 at days 0 and 15 (5 paralarvae per tank and 3 tanks per treatment) (See Table 2). Paralarvae  
219 were killed in chilled seawater (-2°C), washed in distilled water, oven dried (110°C, 20h) and  
220 weighed as described by Fuentes et al. (2011). Specific growth rate (SGR, % DW·day<sup>-1</sup>) was  
221 calculated as:  $[(\ln DW_f - \ln DW_i) \cdot 100 / (t_f - t_i)]$ , where DW<sub>f</sub> and DW<sub>i</sub> are the dry weight at final  
222 time (t<sub>f</sub>) and initial time (t<sub>i</sub>) respectively. Survival (S, %) was calculated as  $S = 100 X_f / (X_i - X_s)$ ,  
223 where X<sub>f</sub> is the number of live individuals at the end of experiment, X<sub>i</sub> is the initial number of  
224 individuals and X<sub>s</sub> is the number of individuals sacrificed during the experiment.

### 225 **2.5. Biomarker assays**



226 Biomarkers were only determined in hatchlings (0 day old paralarvae) and 15 day old  
227 paralarvae due to problems of sample availability at 30 days of age (Table 2). For nucleic acids  
228 and HSP70 assays, at 0 days four pools of 7-8 paralarvae per centre were sampled, whereas at  
229 15 days one pool of 7-8 paralarvae per tank and per centre was collected. For antioxidant  
230 enzymes and lipid peroxidation, four samples of 300 mg wet weight per centre were taken at  
231 day 0, while for 15 day old paralarvae, the same quantity (300 mg) were obtained both per  
232 tank and per centre. Paralarvae were sacrificed in chilled seawater (-2°C), rinsed in distilled  
233 water and kept at -80°C until further analyses.

#### 234 **2.5.1. Nucleic acid determination**

235 Nucleic acids (RNA and DNA) were quantified following the procedure described in Varó et al.  
236 (2007), using RiboGreen™ RNA Quantitation Kit and PicoGreen™ DNA Quantitation Kit,  
237 respectively (Molecular Probes). Briefly, samples were homogenised in 1 mL ice-cold TE buffer  
238 (10 mM Tris–HCl buffer containing 1 mM EDTA, pH = 7.5). After centrifugation (10,000 g, 10  
239 min, 4°C) the supernatant was transferred to a clean tube for the analyses of nucleic acids and  
240 proteins. For RNA and DNA determinations 50 µL of diluted supernatant were transferred in  
241 triplicate into a 96-well black microplate containing 1 µL of DNase I or 10 µL of RNase A  
242 (diluted 1:400), respectively. After 1 h incubation at 37°C, the volume was adjusted to 100 µL  
243 with TE buffer and 100 µL of RiboGreen or PicoGreen was added for RNA and DNA  
244 quantification, respectively, and allowed to stain for 5 min in darkness before reading in a  
245 TECAN SPECTRA-FLUOR microplate reader, at 485 nm EX/535 nm EM (TECAN, Salzburg,  
246 Austria). Concentrations were calculated from high–range standard curves of RNA (20ng·mL<sup>-1</sup>-  
247 1µg·mL<sup>-1</sup>) or DNA (1ng·mL<sup>-1</sup>- 1µg·mL<sup>-1</sup>) prepared from standards supplied with Ribo-Green and  
248 Pico-Green reagent kits.

#### 249 **2.5.2. Heat shock protein (HSP70)**

250 Heat shock proteins 70 (HSP70) were determined according to the methodology described in  
251 Solé et al. (2015). Briefly, samples were homogenised in 1:10 (w:v) ice-cold calcium-  
252 magnesium free saline buffer (20 mM Hepes, 500 mM NaCl, 12.5 mM KCl, pH = 7.3), freshly  
253 complemented with 1mM dithiothreitol (DTT), 1mM phenylmethanesulfonyl fluoride (PMSF),  
254 Igepal (1%) and 1% protease inhibitor cocktail (Complete-Mini, EDTA-free, ROCHE). Samples  
255 were centrifuged at 15,000 g (4°C) for 20 min and the supernatant was kept at -80°C until  
256 further analyses. Protein samples (21 µg) were separated by 1D-SDS-PAGE using Bio-Rad Mini-  
257 Protean TGX Precast gels (4-20% resolving gel) in a Mini-Protean Tetra cell system (Bio-Rad),  
258 for 30 min at 300 V, and then, transferred onto PVDF membranes (0,2 µm, Trans-Blot® Turbo™

259 Mini PVDF Transfer Packs) at 1.3 A, 25 V for 10 min in a Trans-Blot® Turbo™ Blotting System  
260 (Bio-Rad). Blots were visualized on a VERSADOC Imaging system (Bio-Rad) using ELC-PRIME  
261 reagent (Amersham), and quantified by densitometry using the Quantity One software (Bio-  
262 Rad). Immunodetection was performed using HSP70 mouse monoclonal antibody (Sigma,  
263 H5147), and anti-mouse IgG secondary antibody conjugated with peroxidase (Sigma, A4416).  
264 The density of each band was normalized to the density of the HSP70 band of a commercial  
265 standard (Sigma, H 9776) in each blot (Varó et al., 2007), and HSP70 levels were expressed as  
266 arbitrary units HSP70·ng protein<sup>-1</sup>.

267

### 268 **2.5.3. Antioxidant enzymes and lipid peroxidation assays**

269 Samples were homogenised in 1:4 (w:v) ice-cold 100 mM Tris–HCl buffer containing 0.1 mM  
270 EDTA and 0.1% (v/v) Triton X-100 (pH 7.8). Homogenates were centrifuged at 30,000 g (4°C)  
271 for 30 min and the resultant supernatants were kept in aliquots and stored at –80°C for  
272 enzyme activity and lipid peroxidation assays. All enzyme assays were carried out at 25°C and  
273 changes in absorbance were monitored to determine the enzyme activity using a Power  
274 Wavex microplate scanning spectrophotometer (Bio-Tek Instruments, USA). The optimal  
275 substrate and protein concentrations for the measurement of maximal activity for each  
276 enzyme were established by preliminary assays. The extinction coefficients ( $\epsilon$ ) used for  
277 hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) and NADH/NADPH were 0.039 mM<sup>-1</sup> cm<sup>-1</sup> and 6.22 mM<sup>-1</sup> cm<sup>-1</sup>,  
278 respectively. The assay conditions were as follows:

279 Catalase (CAT; EC 1.11.1.6) activity was determined by measuring the decrease of H<sub>2</sub>O<sub>2</sub>  
280 concentration at 240 nm according to Aebi (1984). Reaction mixture contained 50 mM  
281 potassium phosphate buffer (pH 7.0) and 10 mM freshly added H<sub>2</sub>O<sub>2</sub>.

282 Glutathione peroxidase (GPX T and GPX Se; EC 1.11.1.9) activity was measured following the  
283 method of Flohé and Günzler (1984). The glutathione disulfide (GSSG) generated by GPX was  
284 reduced by GR, and NADPH oxidation was monitored at 340 nm. The reaction mixture  
285 consisted of 50 mM potassium phosphate buffer (pH 7.1), 1 mM EDTA, 3.9 mM GSH, 3.9 mM  
286 sodium azide, 1 IU mL<sup>-1</sup> glutathione reductase, 0.2mM NADPH, and 0.05 mM H<sub>2</sub>O<sub>2</sub> for Total-  
287 GPX, or cumene hydroperoxide for GPX Se-dependent.

288 Glutathione reductase (GR; EC 1.6.4.2) activity was assayed as described by Morales et al.  
289 (2004), measuring the oxidation of NADPH at 340 nm. Reaction mixture consisted of 0.1 M  
290 sodium phosphate buffer (pH 7.5), 1 mM EDTA, 0.63mM NADPH, and 0.16 mM GSSG.

291 Enzyme activity was expressed as units (CAT) or milliunits (GPX and GR) per mg of soluble  
292 protein. One unit of enzyme activity was defined as the amount of enzyme required to  
293 transform 1  $\mu\text{mol}$  of substrate per min under the above assay conditions.

294 For lipid peroxidation, the concentration of thiobarbituric acid reacting substances (TBARS)  
295 was determined according to the method of Buege and Aust (1978). An aliquot of the  
296 supernatant from the homogenate (100  $\mu\text{L}$ ) was mixed with 500  $\mu\text{L}$  of a previously prepared  
297 solution containing 15% (w/v) trichloroacetic acid (TCA), 0.375% (w/v) thiobarbituric acid  
298 (TBA), 80% (v/v) hydrochloric acid 0.25 N and 0.01% (w/v) butylated hydroxytoluene (BHT).  
299 The mixture was heated to 100°C for 15 min and after cooling at room temperature,  
300 centrifuged at 1,500 g for 10 min. Absorbance in the supernatant was measured at 535 nm  
301 compared with blank. Concentration was expressed as nanomoles of malondialdehyde (MDA)  
302 per gram of tissue ( $\text{nmol MDA}\cdot\text{g}^{-1}$ ), calculated from a calibration curve.

#### 303 **2.5.4. Total protein content**

304 Total soluble protein contents of samples for nucleic acid and HSP70 were determined using  
305 Lowry Bio Rad DC-Protein Assay kit, and the absorbance was read at 750nm. Moreover,  
306 protein contents antioxidant enzymes activities were determined by the Bradford method  
307 (1976), and the absorbance read at 595nm. BSA (bovine serum albumin) was used as standard.  
308 All protein determinations were performed in triplicate for each sample and the absorbance  
309 read in a microplate reader.

#### 310 **2.6. Statistical analysis**

311 Results are presented as means  $\pm$  standard deviation (SD). Data were checked for normal  
312 distribution with the one-sample Kolmogorov-Smirnoff test, as well as for homogeneity of the  
313 variances with the Levene's test (Zar, 1999) and transformed (ln) when needed (Fowler et al.,  
314 1998).

315 Differences among geographical origins (centres) in dry weight and biomarkers for each  
316 condition (age or diet) were assessed by one-way ANOVA followed by a Bonferroni's *post hoc*  
317 test (Zar 1999). When normal distribution and/or homoscedasticity were not achieved, data  
318 were subjected to Kruskal–Wallis non-parametric test, followed by Games-Howell non-  
319 parametric multiple comparison test (Zar, 1999).

320 A two-way ANOVA (Zar, 1999) model was used to analyse Specific Growth Rate (SGR), survival,  
321 RNA/DNA, RNA/protein, protein/DNA, protein, CAT, GPX T, GPX Se, GR and MDA using the

322 effect of diet (C and LC60) and centre (IRTA, TF and VG) as main factors. Bonferroni's *post hoc*  
323 test was used for pairwise comparisons for SGR and biomarkers in order to assess the effect of  
324 diet in each centre. When normality and/or homoscedasticity assumptions were not achieved,  
325 data were subjected to a non-parametric two-way ANOVA test based on rank transformation  
326 (Zar, 1999).

327 A Principal Components Analysis (PCA) was used to integrate the information of the analyses  
328 carried out with 15 days old paralarvae. The values from the biomarkers, RNA/DNA,  
329 RNA/protein, protein/DNA, protein, CAT, GPX T, GPX Se, GR and MDA, were used as variables.  
330 Subsequently, the scores obtained for the two first components were plotted and identified by  
331 origin (centre) and diet in order to establish potential graphical patterns of identity, and  
332 further submitted to one-way ANOVA to analyse significant differences.

333 Partial correlation analysis was used to explore associations between SGR and biomarkers for  
334 each dietary group. A summary with the experimental design of the paralarvae cultures as well  
335 as the statistical tests used is shown in Table 2. Statistical significance was established at  
336  $P < 0.05$ . Statistical analyses were performed using the SPSS package version 15.0 (SPSS Inc,  
337 Chicago, USA)

338

### 339 **3. Results and Discussion**

#### 340 **3.1. Growth and survival**

341 The dry weight of the hatchlings ( $0.22 \pm 0.03$  mg) from the experiment carried out in TF was  
342 significantly lower ( $P < 0.05$ ) than that obtained in IRTA ( $0.32 \pm 0.03$  mg) and VG ( $0.32 \pm 0.02$  mg)  
343 (Fig. 1). These values are within previously reported results for *O. vulgaris* hatchlings in each  
344 one of the geographical areas considered in this study. In this sense, the weight of hatchlings  
345 from the Central Atlantic area (TF) range from 0.17 to 0.25 mg (Franco-Santos et al., 2016; Reis  
346 et al., 2015; Roo et al., 2015), from the Mediterranean area (IRTA) from 0.27 to 0.34 mg  
347 (Navarro and Villanueva, 2000; Villanueva et al., 2002, 2004), and from the North Atlantic area  
348 (VG) from 0.29 to 0.36 mg (Carrasco et al., 2006; Moxica et al., 2002; Viciano et al., 2011).  
349 These differences could be associated with a possible counter-gradient growth adaptation in  
350 octopus species as pointed out by Noyola et al. (2013), and/or genetic differences among  
351 Spanish *O. vulgaris* populations (Cabranes et al., 2008). Despite that, the hatchlings size could  
352 be affected by other factors such as the incubation temperature of eggs (Vidal et al., 2002) and

353 broodstock diet (Caamal-Monsreal et al., 2015; Quintana et al., 2015). In our study,  
354 temperature does not seem to be the cause of the differences observed in dry weight, since  
355 the incubation temperatures were in the same range among centres (19-21°C). On the other  
356 hand, broodstocks' diet might be a source of variation since there were differences at the  
357 species level of the foodstuff. For instance, IRTA and VG based their dietary regimes on a  
358 combination of crabs and fish whereas in TF the diet included squid only. However, it must be  
359 taken into account that all diets used in this study included crabs or squid, which have been  
360 successfully used to feed *O. vulgaris* broodstock obtaining high spawning quality (Quintana et  
361 al., 2015).

362 Specific growth rate (SGR) was used to estimate growth in order to standardise and compare  
363 the results between the three centres. Regardless of the diet used, in this study SGR ranged  
364 between 3.99 and 6.36% DW·day<sup>-1</sup> at 15 days of culture (Table 3). This growth rate was higher  
365 than previously reported for *O. vulgaris* paralarvae by Seixas et al. (2010b) (2.5-3.3% DW·day<sup>-1</sup>)  
366 and Villanueva et al. (2004) (2.59-3.90% DW·day<sup>-1</sup>), using HUFA enriched *Artemia* as food. On  
367 the other hand, our study showed lower values than those reported by Villanueva (1995)  
368 (7.07% DW·day<sup>-1</sup> at 20 days), Iglesias et al. (2004) (7.76% DW·day<sup>-1</sup> at 15 days) and Carrasco et  
369 al. (2006) (7.90-8.90% DW·day<sup>-1</sup>, at 20 days), where paralarvae were fed decapod zoeae in co-  
370 feeding with *Artemia*.

371 The SGR values in 15 day old paralarvae only showed significant differences between diets  
372 (Two-way ANOVA,  $P < 0.05$ , Table 3), with paralarvae from VG fed with the LC60 diet having the  
373 highest (albeit non-significant) SGR. Differences in terms of growth (SGR) could not be  
374 assessed in 30 day old paralarvae due to the absence of a sample caused by high mortalities.  
375 These results suggest that the initial differences in size and/or dry weight of the paralarvae  
376 from different geographical origins were not a relevant factor in the growth rate. As opposed  
377 to the results reported by Leporati et al. (2007) who point out that differences in the initial size  
378 of cephalopods could lead to significant differences in SGR. Regarding the effect of diet, LC60  
379 enriched *Artemia* seemed to improve paralarvae growth, probably due to an increase in the  
380 levels of DHA and polar lipids (Garrido et al., 2016; Guinot et al., 2013a, 2013b), both lipid  
381 components are considered to have pivotal roles during the early development of cephalopods  
382 (Monroig et al., 2013; Navarro and Villanueva, 2003; Reis et al., 2014).

383 Finally, no significant differences were found for the survival rates (S%) that ranged between  
384 0.14-3.77% at 30 days, either between diets or among geographical origins (Table 3), certainly  
385 due to the elevated mortality and the high intra-group variability observed, although generally,

386 survival was similar to that reported previously by other authors, especially when fed *Artemia*  
387 based diets. Villanueva et al. (2002) reported paralarval survivals from 0.8 to 4.6%, after 30  
388 days of feeding with *Artemia* nauplii enriched with DC Super Selco in co-feeding with different  
389 inert diets, while Viciano et al. (2011) obtained a survival of 3% using 4 days *I. galbana* on-  
390 grown *Artemia* further enriched for 24h with a DHA-rich oil emulsion. These results are far  
391 from the those obtained by Fuentes et al. (2011) at 30 days (27.2%) or by Iglesias et al. (2004)  
392 at 40 days (31.5%), using juvenile *Artemia* complemented with sand eel flakes or crab zoeae  
393 (*Maja squinado*), respectively.

### 394 **3.2. Total protein and nucleic acid ratios**

395 Changes in RNA, DNA and protein ratios are frequently used as efficient biomarkers to  
396 estimate growth and nutritional condition in early life stages of marine species. In this study,  
397 RNA/DNA, RNA/protein and protein/DNA ratios were determined both in hatchlings and 15  
398 day old paralarvae from the three different geographical origins fed either C or LC60 diets (Fig.  
399 2). Hatchlings from TF showed the lowest RNA/DNA and protein/DNA ratios, whereas those  
400 from IRTA showed the lowest RNA/protein ratio (One-way ANOVA  $P < 0.05$ , Fig. 2, capital  
401 letters). Generally, organisms in good condition tend to have higher RNA/DNA ratios than  
402 those in poor condition (Chícharo and Chícharo, 2008), whereas low RNA/DNA ratios have  
403 been associated with starvation in larval stages and juveniles of fish (Clemmesen, 1987; Gwak  
404 et al., 2003; Mathers et al., 1994; Raae et al., 1988; Richard et al., 1991), and cephalopods  
405 (Sykes et al., 2004; Vidal et al., 2006).

406 Overall, the hatchlings from each geographical origin showed specific cell growth patterns  
407 according to the results obtained from the nucleic acid analysis. This fact, along with the  
408 differences found in dry weight, highlighted the paralarva-origin associated variability.  
409 Therefore, more research is required in order to elucidate the possible relation between  
410 nucleic acid ratios and dry weight, and the effect of the geographical/genetic origin on newly  
411 hatched paralarvae.

412 The combined effects of diet and geographical origin (centres), as well as their interactions,  
413 were studied in 15 day old paralarvae (Table 4). Significant effects of geographical origin and  
414 diet were observed for the RNA/protein values, whereas the origin of the paralarvae was the  
415 only factor affecting RNA/DNA and protein/DNA ratios. On the contrary, protein content did  
416 not seem to be influenced by any of these factors, either geographical origin or diet, and no  
417 interaction between factors was observed for any of the biomarkers studied.

418 Furthermore, changes among hatchlings and 15 day old paralarvae were assessed within each  
419 geographical origin (Fig. 2, lower letters). Generally speaking, almost all ratios studied (protein,  
420 RNA/DNA, RNA/protein and protein/DNA) increased with age (development), with protein  
421 content being the only parameter that significantly increased in all centres regardless of diet  
422 ( $P<0.05$ ) and thus reflecting growth. In addition, the results suggest that paralarvae from each  
423 geographical origin presented a different pattern of nucleic acid ratios throughout  
424 development. Firstly, paralarvae from IRTA displayed an increment in RNA/DNA and  
425 RNA/protein ratios ( $P<0.05$ ) from hatchlings to 15 days old up to the highest values among  
426 geographical origins (Fig. 2, capital letters,  $P<0.05$ ). RNA concentration, expressed as a ratio of  
427 RNA/protein, has been proposed in octopus as a measure of the capacity or potential for  
428 protein synthesis (Houlihan et al., 1990), suggesting therefore that IRTA paralarvae growth was  
429 due indeed to an increased protein synthesis. Secondly, paralarvae from TF showed slightly  
430 increased levels of RNA/DNA and protein/DNA ratios ( $P<0.05$ ) from hatchlings to 15 days, but  
431 despite these increments, this group maintained the lowest RNA/DNA and protein/DNA ratios  
432 at 15 days ( $P<0.05$ ), which suggested a suboptimal nutritional condition. Thirdly, at 15 days,  
433 paralarvae from VG showed a significant increase in protein/DNA ratio ( $P<0.05$ ), which seems  
434 to indicate a growth pattern based on an increment of cell size rather than cell number  
435 (Mathers et al., 1994; Peragón et al., 1998).

436 When considering the effect of diet on paralarval growth, those fed LC60 at TF showed lower  
437 RNA/protein ratio (protein synthesis capacity) than those fed the control diet ( $P<0.05$ ). Also in  
438 VG, RNA/protein was lower in the LC60 dietary group ( $P<0.05$ ). Taking into account that this  
439 group showed the highest growth rate (Table 3) the results suggest that growth promoted by  
440 LC60 was not solely due to an increase in protein synthesis, and that the phospholipid content  
441 of the diet may have played a role as an energy source in stages of high cell proliferation, as  
442 has been proposed by Takii et al. (1994). Given the scarcity of in-depth research into the  
443 patterns and correlations of the selected biomarkers and growth in common octopus, further  
444 studies are needed in order to ascertain the mechanisms underlying the growth processes  
445 related with age (development) and diet.

### 446 **3.3. Heat shock proteins (HSP70)**

447 HSP70 levels from hatchlings and 15 day old paralarvae from the three different geographical  
448 origins (centres) fed with C or LC60 diets are shown in Fig. 3. In the hatchlings, differences in  
449 HSP70 were found among geographical origins, with IRTA showing significantly higher values  
450 than TF and VG ( $P<0.05$ ) (Fig. 3, capital letters). HSP are constitutive proteins, which take part

451 in protein folding and transport, and apoptosis, whose expression can be altered by several  
452 stressors (Deng et al., 2009; Solé et al., 2004). Some studies on early life stages of fish and  
453 cephalopods have shown variability in the HSP70 response to starvation or nutritional stress.  
454 In fact, in fish larvae, increased (Cara et al., 2005), decreased (Deng et al., 2009) or unchanged  
455 (Han et al., 2012) HSP70 levels have been found related to food restriction periods. In *O.*  
456 *vulgaris*, decreased HSP70 levels were detected in 5 day old starved paralarvae, whereas  
457 increased HSP70 levels were associated with fed paralarvae (Varó et al., 2013). Although  
458 HSP70 is considered highly conserved, Wang et al. (2013) reported that the N-terminal  
459 domains of the molluscs *Ostrea edulis*, *Crassostrea gigas* and *Argopecten irradians* from  
460 different geographical populations showed some variations. From this point of view, we can  
461 only hypothesise about the different origin of hatchlings being related to the HSP70 inter-  
462 centres differences, and further research should be carried out to test the HSP70 structural  
463 variability in different populations of *O. vulgaris*, and if that variability affects the constitutive  
464 and/or induced levels of these proteins.

465 The results in 15 day old paralarvae parallel those obtained in hatchlings in that significant  
466 differences in HSP70 values were only found among geographical origins (Fig. 3), whereas diet  
467 or the interaction of both diet and origin had no effect (Two-way ANOVA, Table 4). On the one  
468 hand, the differences observed at the hatchling stage among geographical origins may suggest  
469 that the initial variability on HSP70 expression is probably decisive throughout *O. vulgaris*  
470 paralarval culture. On the other hand, Hamza et al. (2010) working with pikeperch larvae  
471 (*Sander lucioperca*), have shown that HSPs expression is modulated, for example, in response  
472 to dietary phospholipids. Consequently, the results of this study seem more supportive of the  
473 first hypothesis since no modulatory stress sensitivity effect of the phospholipid content of  
474 LC60 diet was found, although other unknown stressful factors equally affecting both dietary  
475 groups cannot be ruled out.

#### 476 **3.4. Antioxidant enzymes and lipid peroxidation assays**

477 In hatchlings, no significant differences were found in CAT and GR activities among centres,  
478 while GPX T, GPX Se activities and lipid peroxidation (MDA) were significantly different (One-  
479 way ANOVA,  $P < 0.05$ ) (Fig. 4, capital letters). Hatchlings from VG showed higher GPX T and GPX  
480 Se activities than those from TF, with those from IRTA showing values in-between. MDA values  
481 moved in the opposite trend, TF hatchlings displayed the highest values and significantly  
482 different ( $P < 0.05$ ) from those observed in VG, with IRTA showing values in-between. These  
483 significant origin-related differences could indicate the differential ability of hatchlings to cope



484 with oxidative stress. It is important to note that apart from geographical origin, maternal  
485 effect could also have influenced the variability found in hatchlings. In fish it has been reported  
486 that population origin as well as maternal effect affect larvae condition (Bunnell et al., 2005).

487 Within each centre, the biomarkers of antioxidant defences were compared among hatchlings  
488 and 15 day old paralarvae from both dietary groups (Fig. 4, lower letters) and, in general  
489 terms, few differences were observed. CAT is the only biomarker showing a significant increase  
490 with age in all centres ( $P < 0.05$ ) regardless of the diet provided. This result is in agreement with  
491 those reported for paralarvae of the same species by Varó et al. (2013) and by other authors in  
492 fish larvae (Fernández-Díaz et al., 2006; Mourente et al., 2002; Peters and Livingstone, 1996;  
493 Zhang et al., 2009). Centre-specific developmental differences ( $P < 0.05$ ) were found for lipid  
494 peroxidation (MDA) in IRTA paralarvae (Fig. 4), supporting the results obtained by Zielinski and  
495 Pöter (2000) for *Sepia officinalis* and by Fernández-Díaz et al. (2006) for *Solea senegalensis* and  
496 Mourente et al. (2002) for *Dentex dentex* larvae. On the contrary, a decreased GR activity  
497 related to age was only observed in 15 day old control paralarvae with respect to hatchlings  
498 from VG, as has also been shown in 30 day old octopus paralarvae fed with *Artemia* from the  
499 same origin (Varó et al., 2013). Overall, the antioxidant system of *O. vulgaris* paralarvae  
500 changes with age, and paralarvae origin (centre) does influence the oxidative stress response.

501 On the other hand, the two factors studied (geographical origin and diet) showed significant  
502 effects (two-ways ANOVA,  $P < 0.05$ , Table 4) in GPX Se and GR antioxidant activities. However,  
503 lipid peroxidation (MDA) that was significantly different among centres remained unaffected  
504 by diet. Lipids and particularly polyunsaturated fatty acids (PUFA) are highly susceptible of  
505 oxidation due to their conjugated double bond structure (Lesser et al., 2006; Mourente et al.,  
506 1999; Zielinski and Pörtner, 2000), leading to lipid peroxides and secondary cytotoxic  
507 compounds, in an autocatalytic process that can be detoxified by GPX/GR activities through  
508 glutathione sulfhydryl (GSH)/GSSG dependent mechanism. GPX Se-dependent acts as a  
509 phospholipid hydroperoxidase, and GR together with NADPH allow oxidative glutathione  
510 sulfhydryl to recover its reduced state and in consequence its availability to join GPX Se in  
511 order to avoid the adverse effect of oxidative stress (Ferrari et al., 2008; Regoli et al., 2011).  
512 Consequently, an increment in MDA should have been expected in paralarvae fed the PUFA  
513 rich diet. However, no significant dietary differences were evident for lipid peroxidation  
514 suggesting that the enhanced antioxidant activity found in paralarvae fed LC60 evidences an  
515 efficient defence mechanism against oxidative damage (Mourente et al., 1999).

### 516 **3.5. Integrated assessment of the biomarkers in 15 day old paralarvae**

517 Figure 5 shows the results of the PCA analysis. The two principal components accounted for  
518 72.17 % of total variance. Variables related to oxidation-defence (GPX T, GPX Se), and  
519 Prot/DNA, heavily loaded on the positive side of component 1 (43.76 % of variance), apart  
520 from MDA. Component 2 (28.26 % of variance) associated with HSP70 and GR on the positive  
521 side and CAT on the negative side (Fig. 5A). The score plot (Fig. 5 B) allowed a clear and  
522 significant (ANOVA,  $P<0.05$ ) separation of the scores according to geographical origin in  
523 component 1 (TF, IRTA, VG), and a trend in the segregation of the two dietary groups, with the  
524 LC60 scores tending to distribute towards the positive side (with the antioxidant defence  
525 variables). The second component significantly separated ( $P<0.05$ ) the IRTA scores from the  
526 rest, thus associating them with HSP70. Globally these graphs depict the main trends of the  
527 results presented above and underline MDA, GPX T, GPX Se, HSP70, GR and CAT as the main  
528 explicative variables.

529 The distribution and association of these variables also offer a prospective tool for assessing  
530 trends and sources of variability to support the choice of the most explicative biomarkers. For  
531 example, in component 1, Prot/DNA, reporting on cell size (Mathers et al., 1994; Peragón et  
532 al., 1998) associated with VG paralarvae (see Fig. 2). Also the almost exactly equal contribution  
533 and proximity of GPX T and GPX Se could indicate that there were very little differences in total  
534 GPX activity aside from that of GPX Se, a fact that has been reported by Mourente et al. (1999)  
535 during early development in *Dentex dentex*. A gradient in the activation of the antioxidant  
536 defences from TF to VG with IRTA in between can also be identified, with LC60 treatment also  
537 associating with antioxidant activity. In addition, GPX Se as mentioned above acts as a co-  
538 factor against MDA (Ferrari et al., 2008), for this reason GPX T and GPX Se appeared opposite  
539 MDA driving the distribution of TF scores.

### 540 **3.6 Partial correlation among biomarkers and growth rate (SGR) in 15 day old paralarvae**

541 Partial correlations between some biomarkers were found in both dietary groups. Thus  
542 RNA/DNA was positively correlated with GPX T (C,  $r = 0.817$ ,  $P<0.05$ ; LC60,  $r = 0.937$ ,  $P<0.01$ )  
543 and GPX Se (C,  $r = 0.780$ ,  $P<0.05$ ; LC60,  $r = 0.905$ ,  $P<0.01$ ), biomarkers involved in stress  
544 response. However, several partial correlations found among biomarkers varied depending on  
545 the diet supplied. For C fed paralarvae, RNA/DNA was positively correlated to GR ( $r = 0.740$ ,  
546  $P<0.05$ ). Protein level was also correlated to HSP70 ( $r = 0.923$ ,  $P<0.01$ ), SGR ( $r = 0.741$ ,  $P<0.05$ )  
547 and MDA ( $r = -0.757$ ,  $P<0.05$ ). These correlations have also been observed in individuals of *O.*  
548 *vulgaris* by Houlihan et al. (1990) confirming protein as a good growth indicator. The lack of  
549 correlation for these biomarkers in paralarvae fed LC60 may be linked to the fact that, the

550 predictive capability of RNA and protein contents as growth indicators may be limited (Gwak et  
551 al., 2003). As for the negative relationships of protein and HSP70 with MDA ( $r = -0.757$ ,  $P < 0.05$   
552 and  $r = -0.768$ ,  $P < 0.05$ , respectively) in the paralarvae fed with control diet, it is interesting to  
553 point out that protein content and HSP70 could be modulated by the products of lipid  
554 peroxidation or by other associated substances, since upregulation of HSP70 by free radicals  
555 has been reported in mammals before (Zhou et al., 2001). However, this correlation is not  
556 apparent in paralarvae fed the LC60 diet. Further studies are needed to establish the  
557 relationship between these two biomarkers and to elucidate the role of lipid peroxidation in  
558 HSP70 expression patterns.

559 Conversely, protein content was positively correlated with antioxidant enzymes (GPX T ( $r =$   
560  $0.884$ ,  $P < 0.01$ ), GPX Se ( $r = 0.761$ ,  $P < 0.05$ ) and GR ( $r = 0.776$ ,  $P < 0.05$ ) in paralarvae fed LC60,  
561 suggesting that the variations in the activity of these enzymes could be associated with  
562 fluctuations in their amount in the cells (Boudjema et al., 2014). In this dietary group higher  
563 levels of lipids and LC-PUFA are present (Guinot et al., 2013a), and the expression/synthesis of  
564 these antioxidant enzymes could be upregulated to counteract potential injury derived from  
565 lipid peroxidation (Fernández-Díaz et al., 2006; Mourente et al., 1999).

566

#### 567 **4. Conclusion**

568 Overall, this study reveals that initial size (dry weight) and biomarkers of hatchlings were  
569 related to the geographical origin (research centre) of the broodstock. The SGR of 15 day old  
570 paralarvae seemed positively linked to their diet. Moreover, antioxidant defences against the  
571 oxidative damage were boosted in the LC60 paralarvae group, probably as a result of the high  
572 content in PUFA of this diet. The partial correlations found between biomarkers were  
573 differentially dependent on the diet supplied to paralarvae. Despite these results no clear  
574 positive effect of LC60 diet was observed on survival, still indicating potential overriding  
575 effects of unknown factors other than the quality and quantity of the lipid composition. The  
576 high variability found among the results obtained in paralarvae from different geographical  
577 origins, despite the use of similar protocols, highlights the need to clarify the sources of this  
578 variability. Further studies are necessary to better understand the mechanisms underlying *O.*  
579 *vulgaris* paralarvae metabolism and stress in response to their diet and geographical origin.

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588

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**Table 1.** Female weight and physicochemical parameters for broodstock and paralarvae reared in three Spanish research centres.

	<b>IRTA</b>	<b>TF</b>	<b>VG</b>
<b>BROODSTOCK</b>			
Female weight (kg)	2.5	4.5	1.5
Temperature (°C)	19-21	19-21	19-21
Salinity (PSU)	35.5	36.8	35.0
Oxygen (mg/L)	7-7.5	6.8-7.4	7-7.8
NH <sub>3</sub> /NH <sub>4</sub> <sup>+</sup> (mg/L)	0	0	0
NO <sub>2</sub> <sup>-</sup> (mg/L)	<0.3	<0.3	<0.3
<b>PARALARVAE</b>			
Temperature (°C)	21-22	21.3-23.4	21-22
Salinity (PSU)	35.5	36.8	35.0
Oxygen (mg/L)	7-7.5	6.8-7.3	7.0-8.0
NH <sub>3</sub> /NH <sub>4</sub> <sup>+</sup> (mg/L)	0	0	0
NO <sub>2</sub> <sup>-</sup> (mg/L)	<0.3	<0.3	<0.3

IRTA: Research & Technology Food & Agriculture; TF: Oceanographic Centre of the Canary Islands; VG: Oceanographic Centre of Vigo.

**Table 2.** Experimental design of the paralarvae cultures.

Dependent variables	Independent variables	Age	N	Statistical test	Observations
DW	Centres	0	15	One-way ANOVA	15 paralarvae per centre
NA and HSP70	"	"	4	"	4 pools of 7-8 paralarvae per centre
AE and MDA	"	"	4	"	4 pools of 300 mg wet weight per centre
SGR	Centres and diets	15	6 and 9	Two-way ANOVA	5 paralarvae per tank and per centre
NA and HSP70	"	"	"	"	1 pool of 7-8 paralarvae per tank and per centre
AE and MDA	"	"	"	"	1 pool of 300 mg wet weight per tank and per centre
S	"	30	"	"	
NA and HSP70	Diets	15	9	One-way ANOVA	Each dietary treatment was studied considering all centres
AE and MDA	"	"	"	"	"
NA and HSP70	Age	0/15	10	"	0 and 15 day old paralarvae were analysed for each centre
AE and MDA	"	"	"	"	"
All biomarkers	Centres and diets	15	6 and 9	PCA	
"	"	"	"	ANOVA	
SGR and all biomarkers	Diets	15	9	Partial correlation	

Abbreviations: DW, dry weight; NA, Nucleic acid and protein; HSP70, Heat shock protein 70; AE, Antioxidant enzymes; MDA, lipid peroxidation; SGR, Specific growth rate; S, survival; Centres (IRTA: Research & Technology Food & Agriculture; TF: Oceanographic Centre of the Canary Islands; VG: Oceanographic Centre of Vigo); Diets (Control and LC60); Age (days); PCA: Principal components analysis.

**Table 3.** Results of two-way ANOVA for specific growth rate (SGR, % dry weight·day<sup>-1</sup>) and Survival (S, %) of *O. vulgarris* paralarvae cultured from different geographical origins (centres) and fed with *Artemia* enriched with microalgae (Control diet) or Marine Lecithin LC 60® (LC60 diet).

	IRTA		TF		VG		Two-way ANOVA Centres Diets Interaction
	Control	LC60	Control	LC60	Control	LC60	
	SGR	4.27 ± 1.49	5.01 ± 0.91	3.99 ± 1.59	5.30 ± 2.03	5.05 ± 1.76	
S (%)	2.18 ± 3.36	0.78 ± 0.94	0.14 ± 0.07	0.31 ± 0.37	3.77 ± 6.52	2.55 ± 4.42	

Data are presented as means ± SD (standard deviation).

SGR was obtained in 15 day old paralarvae (Centre n=6 and diets n=9).

S was obtained in 30 day old paralarvae (Centre n=6 and diets n=9).

IRTA: Research & Technology Food & Agriculture; TF: Oceanographic Centre of the Canary Islands; VG: Oceanographic Centre of Vigo.

(\*) Indicate significant differences in the studied variables ( $P < 0.05$ ).



**Table 4.** Results of two-way ANOVA, for total protein content (Prot) and RNA/DNA, RNA/protein and protein/DNA ratios, heat shock protein, (HSP70), antioxidant enzymes activity (CAT, GPX T, GPX Se and GR) and lipid peroxidation (MDA) of 15 day old *O. vulgaris* paralarvae cultured from different geographical origins (centres, n=6) and fed with *Artemia* enriched with microalgae (Control diet, n=9) or Marine Lecithin LC 60® (LC60 diet, n=9).

<b>Two-way ANOVA</b>		
	<b>Centres</b>	<b>Diets</b>
<b>Prot</b>		
<b>RNA/DNA</b>	*	
<b>RNA/Prot</b>	*	*
<b>Prot/DNA</b>	*	
<b>HSP70</b>	*	
<b>CAT</b>		
<b>GPX T</b>	*	
<b>GPX Se</b>	*	*
<b>GR</b>	*	*
<b>MDA</b>	*	

(\*) Indicate significant differences ( $P < 0.05$ ). CAT: catalase; GPX T and GPX Se: total and selenium dependent glutathione peroxidases, respectively; GR: glutathione reductase; MDA: malondialdehyde.

**Fig. 1.** Dry weight (mg) of hatchlings from different geographical origins (centres): Research & Technology Food & Agriculture (IRTA), Oceanographic Centre of the Canary Islands (TF) and Oceanographic Centre of Vigo (VG). Data are shown as mean  $\pm$  SD (standard deviation); n=15. Different letters show significant differences among geographical origins after one-way ANOVA, followed by Bonferroni's post hoc test ( $P < 0.05$ ).

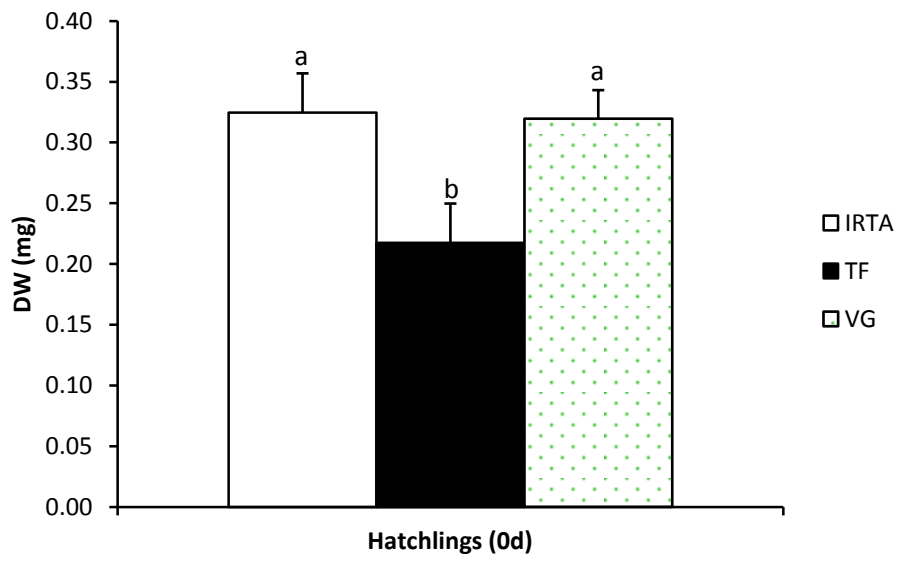
**Fig. 2.** Total protein and nucleic acid ratios of hatchlings (n=4) and 15 day old paralarvae cultured from different geographical origins (centres, n=6; see Fig. 1 for details) and fed with control diet (C, *Artemia* enriched with microalgae, n=9) or LC60 diet (*Artemia* enriched with Marine Lecithin LC 60<sup>®</sup>, n=9). Data are shown as mean  $\pm$  SD (standard deviation). Different capital letters show significant differences among geographical origins for both hatchlings and 15 day old paralarvae fed with C diet or LC60 diet; and different lower letters display significant differences between hatchlings and 15 day old paralarvae in each centre, after one-way ANOVA, followed by Bonferroni's *post hoc* test, or kruskall-Wallis non parametric test, followed by Games-Howell *post hoc* test ( $P < 0.05$ ).

**Fig. 3.** HSP70 (AU/ng protein) levels for hatchlings (n=4) and 15 day old paralarvae cultured from different geographical origins (centres, n=6) and fed with control diet (C, *Artemia* enriched with microalgae, n=9) or LC60 diet (Marine Lecithin LC 60<sup>®</sup>, n=9). See Fig 1 and Fig 2 for details. Data are shown as mean  $\pm$  SD (standard deviation). Different capital letters show significant differences among geographical origins for both hatchlings and 15 day old paralarvae fed with C diet or LC60 diet, after one-way ANOVA, followed by Bonferroni's *post hoc* test ( $P < 0.05$ ).

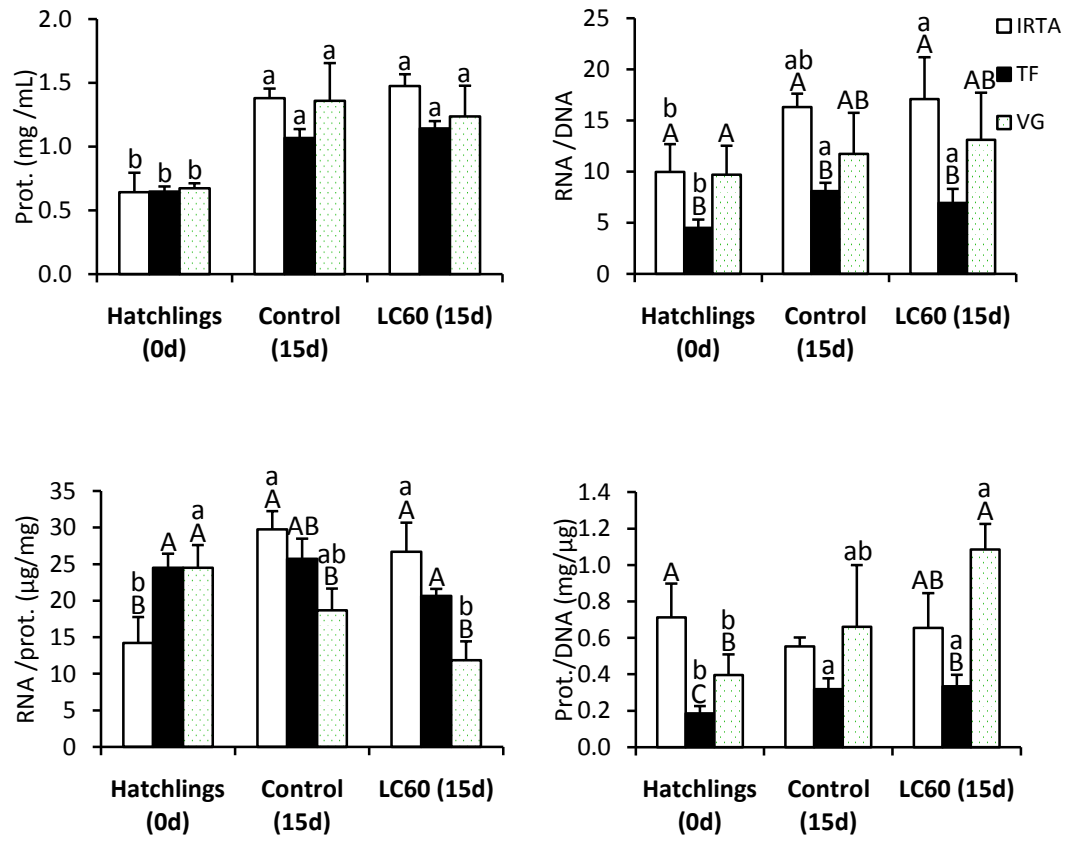
**Fig. 4.** Antioxidant enzymes activities and lipid peroxidation (MDA) of hatchlings (n=4) and 15 day old paralarvae cultured from different geographical origins (centres, n=6, see Fig 1) and fed with control diet (C, *Artemia* enriched with microalgae, n=9) or LC60 diet (Marine Lecithin LC 60<sup>®</sup>, n=9). Data are shown as mean  $\pm$  SD (standard deviation). See Fig 2 for details. CAT: catalase; GPX T and GPX Se: total and selenium dependent glutathione peroxidases, respectively; GR: glutathione reductase; MDA: malondialdehyde.

**Fig. 5.** (A) Principal Components Analysis (PCA) for total protein content (Prot) and RNA/DNA, RNA/protein and protein/DNA ratios, heat shock protein, (HSP70), antioxidant enzymes activity (CAT, GPX T, GPX Se and GR) and lipid peroxidation (MDA) of 15 day old paralarvae cultured from different geographical origins (centres, n=6) and fed with control diet (C, *Artemia* enriched with microalgae, n=9) or LC60 diet (Marine Lecithin LC60<sup>®</sup>, n=9). (B) Factor Score plot: for abbreviations see Fig. 1.

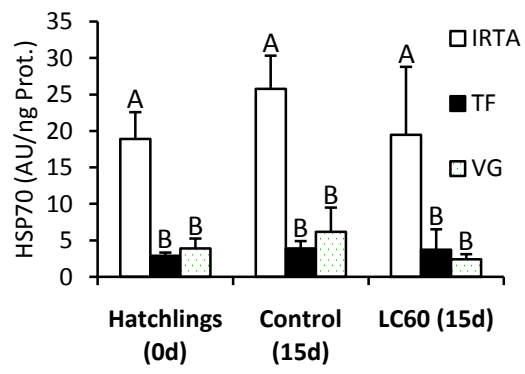
Fig. 1



**Fig. 2**



**Fig. 3.**



**Fig. 4.**

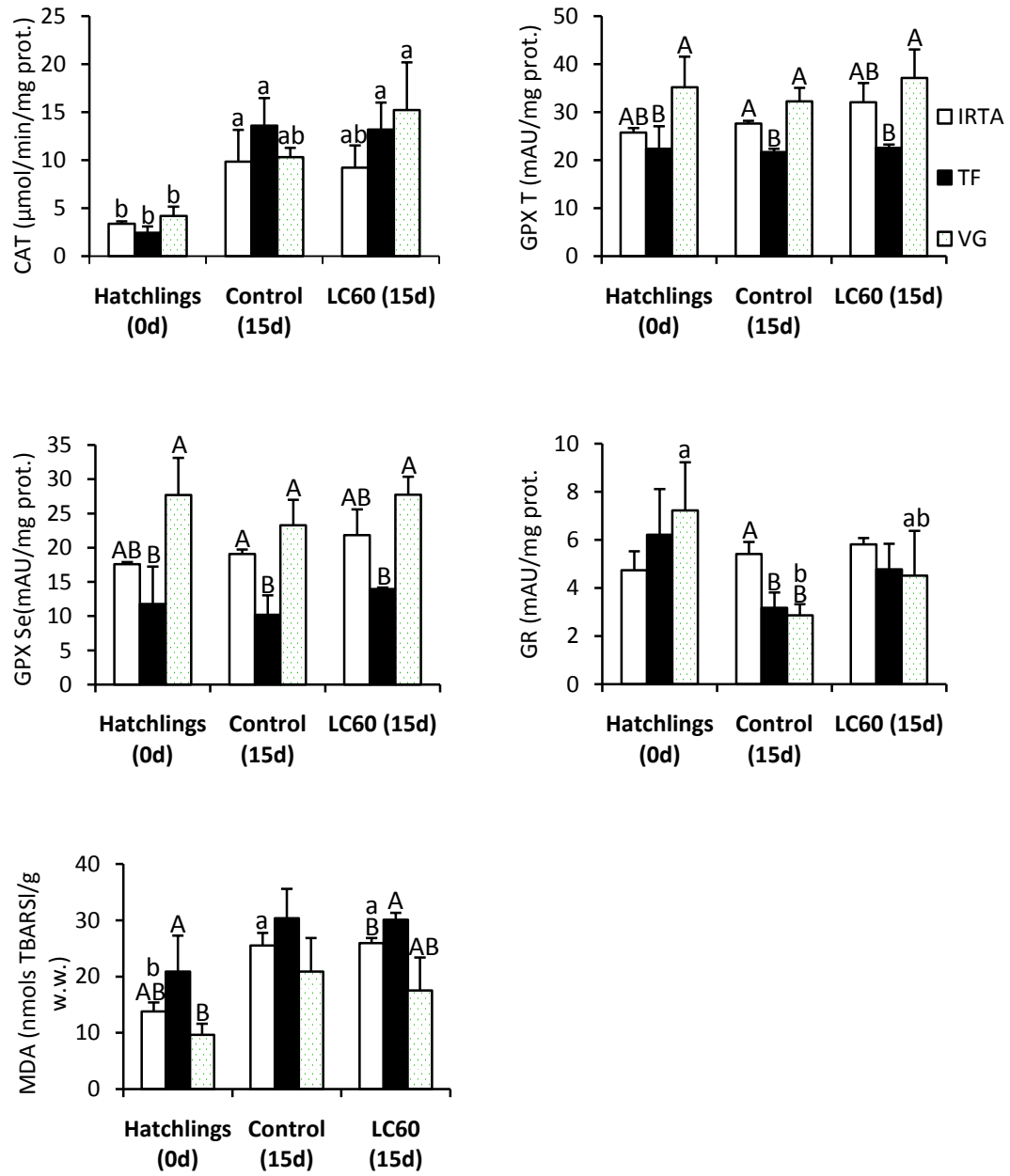


Fig. 5.

