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

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Abbreviations

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

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

Abstract

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

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

1. Introduction

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

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

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

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

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

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

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

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

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

2. Materials and methods

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

2.1. Broodstock husbandry

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

2.2. Paralarvae rearing conditions

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

195 *Nannochloropsis sp* (2.5·10^5 cell·mL^{-1}), 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^{-1} and 1·10^6 cell·mL^{-1}), or for 8h with LC60 (250 nauplii·mL^{-1} and 0.6 g·L^{-1}).
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^{-1} and
210 4·10^5 cell·mL^{-1}). Then, metanauplii for C diet were subsequently enriched with *Nannochloropsis*
211 sp for 24h (5 metanauplii·mL^{-1} and 1·10^7 cell·mL^{-1}), whereas those for LC60 diet were enriched
212 with marine phospholipids for 6h (50 metanauplii·mL^{-1} and 0.6 g·L^{-1}). Paralarvae were fed three
213 times per day (at 9:00, 12:00 and 15:00), at a density of 0.3 nauplii·mL^{-1} from day 0 to 15 and
214 at 0.15 metanauplii·mL^{-1} 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^{-1}) was
221 calculated as: [(Ln DWf- Ln DWi)·100/(tf-ti)], where DWf and DWi are the dry weight at final
222 time (tf) and initial time (ti) respectively. Survival (S, %) was calculated as S = 100 Xf/(Xi-Xs),
223 where Xf is the number of live individuals at the end of experiment, Xi is the initial number of
224 individuals and Xs is the number of individuals sacrificed during the experiment.

225 **2.5. Biomarker assays**

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

2.5.1. Nucleic acid determination

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

2.5.2. Heat shock protein (HSP70)

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

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

2.5.3. Antioxidant enzymes and lipid peroxidation assays

Samples were homogenised in 1:4 (w:v) ice-cold 100 mM Tris–HCl buffer containing 0.1 mM EDTA and 0.1% (v/v) Triton X-100 (pH 7.8). Homogenates were centrifuged at 30,000 g (4°C) for 30 min and the resultant supernatants were kept in aliquots and stored at −80°C for enzyme activity and lipid peroxidation assays. All enzyme assays were carried out at 25°C and changes in absorbance were monitored to determine the enzyme activity using a Power Wavex microplate scanning spectrophotometer (Bio-Tek Instruments, USA). The optimal substrate and protein concentrations for the measurement of maximal activity for each enzyme were established by preliminary assays. The extinction coefficients (ε) used for hydrogen peroxide (H_2O_2) and NADH/NADPH were 0.039 mM^{-1} cm^{-1} and 6.22 mM^{-1} cm^{-1}, respectively. The assay conditions were as follows:

Catalase (CAT; EC 1.11.1.6) activity was determined by measuring the decrease of H_2O_2 concentration at 240 nm according to Aebi (1984). Reaction mixture contained 50 mM potassium phosphate buffer (pH 7.0) and 10 mM freshly added H_2O_2.

Glutathione peroxidase (GPX T and GPX Se; EC 1.11.1.9) activity was measured following the method of Flohé and Günzler (1984). The glutathione disulfide (GSSG) generated by GPX was reduced by GR, and NADPH oxidation was monitored at 340 nm. The reaction mixture consisted of 50 mM potassium phosphate buffer (pH 7.1), 1 mM EDTA, 3.9 mM GSH, 3.9 mM sodium azide, 1 IU mL^{-1} glutathione reductase, 0.2mM NADPH, and 0.05 mM H_2O_2 for Total-GPX, or cumene hydroperoxide for GPX Se-dependent.

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

Enzyme activity was expressed as units (CAT) or milliunits (GPX and GR) per mg of soluble protein. One unit of enzyme activity was defined as the amount of enzyme required to transform 1 µmol of substrate per min under the above assay conditions.

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

2.5.4. Total protein content

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

2.6. Statistical analysis

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

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

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

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

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

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

3. Results and Discussion

3.1. Growth and survival

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

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

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

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

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

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

3.2. Total protein and nucleic acid ratios

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

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

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

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

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

3.3. Heat shock proteins (HSP70)

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

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

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

3.4. Antioxidant enzymes and lipid peroxidation assays

In hatchlings, no significant differences were found in CAT and GR activities among centres, while GPX T, GPX Se activities and lipid peroxidation (MDA) were significantly different (One-way ANOVA, $P<0.05$) (Fig. 4, capital letters). Hatchlings from VG showed higher GPX T and GPX Se activities than those from TF, with those from IRTA showing values in-between. MDA values moved in the opposite trend, TF hatchlings displayed the highest values and significantly different ($P<0.05$) from those observed in VG, with IRTA showing values in-between. These 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**

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

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

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

Partial correlations between some biomarkers were found in both dietary groups. Thus RNA/DNA was positively correlated with GPX T (C, $r = 0.817$, $P<0.05$; LC60, $r = 0.937$, $P<0.01$) and GPX Se (C, $r = 0.780$, $P<0.05$; LC60, $r = 0.905$, $P<0.01$), biomarkers involved in stress response. However, several partial correlations found among biomarkers varied depending on the diet supplied. For C fed paralarvae, RNA/DNA was positively correlated to GR ($r = 0.740$, $P<0.05$). Protein level was also correlated to HSP70 ($r = 0.923$, $P<0.01$), SGR ($r = 0.741$, $P<0.05$) and MDA ($r = -0.757$, $P<0.05$). These correlations have also been observed in individuals of *O. vulgaris* by Houlihan et al. (1990) confirming protein as a good growth indicator. The lack of 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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Table 1. Female weight and physicochemical parameters for broodstock and paralarvae reared in three Spanish research centres.

	IRTA	**TF**	**VG**
BROODSTOCK			
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_3/NH_4^+(mg/L)	0	0	0
NO_2^-(mg/L)	<0.3	<0.3	<0.3
PARALARVAE			
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_3/NH_4^+(mg/L)	0	0	0
NO_2^-(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^{-1}) and Survival (S, %) of *O. vulgaris* 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		
	Control	LC60	Control	LC60	Control	LC60	Centres	Diets	Interaction
SGR	4.27 ± 1.49	5.01 ± 0.91	3.99 ± 1.59	5.30 ± 2.03	5.05 ± 1.76	6.36 ± 1.01		*	
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).

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

(*) 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 ± 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®, n=9). Data are shown as mean ± 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®, n=9). See Fig 1 and Fig 2 for details. Data are shown as mean ± 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®, n=9). Data are shown as mean ± 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®, n=9). (B) Factor Score plot: for abbreviations see Fig. 1.

Figure(s)

Fig. 1

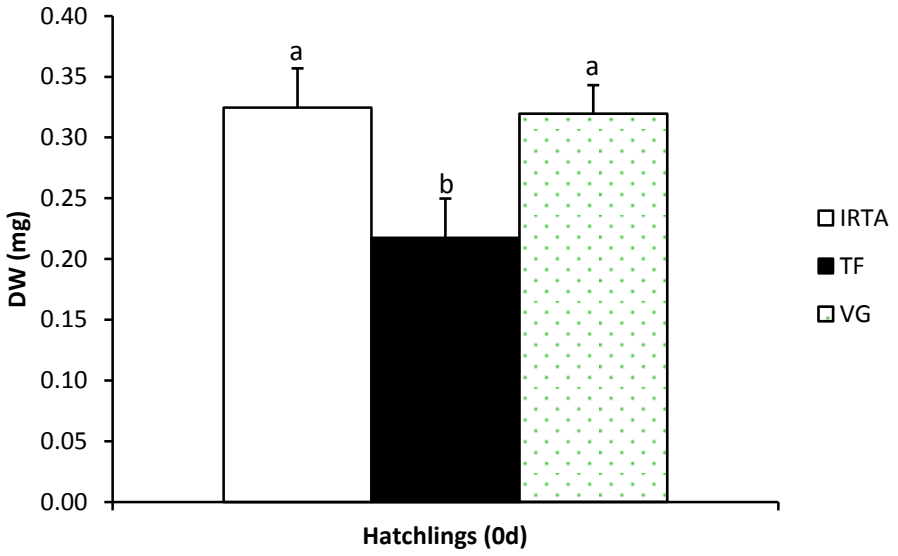


Fig. 2

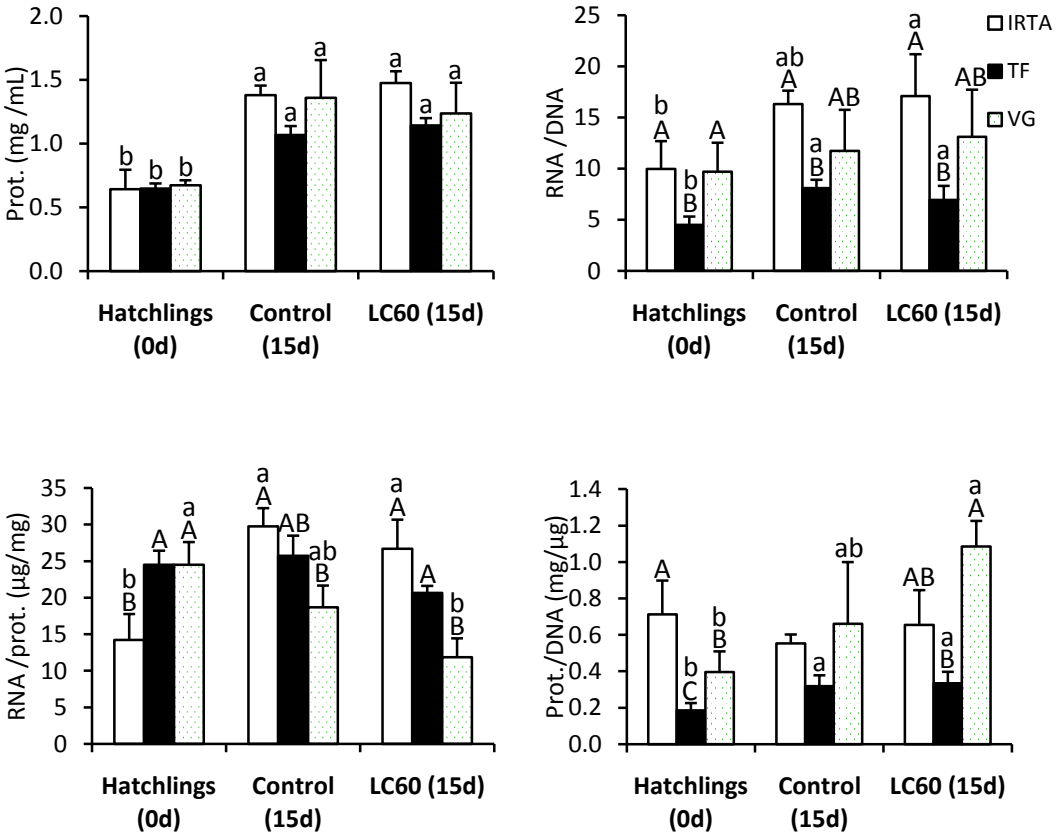

Prot. (mg /mL)
RNA /DNA
RNA /prot. (µg/mg)
Prot./DNA (mg/µg)
IRTA
TF
VG
Hatchlings (0d)
Control (15d)
LC60 (15d)

Fig. 3.

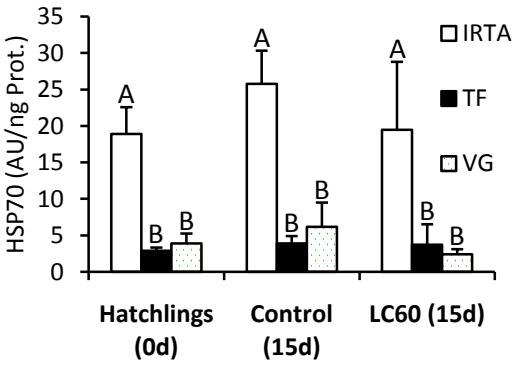

HSP70 (AU/ng Prot.)
35
30
25
20
15
10
5
0
A
B
B
A
B
B
A
B
B
IRTA
TF
VG
Hatchlings (0d)
Control (15d)
LC60 (15d)

Fig. 4.

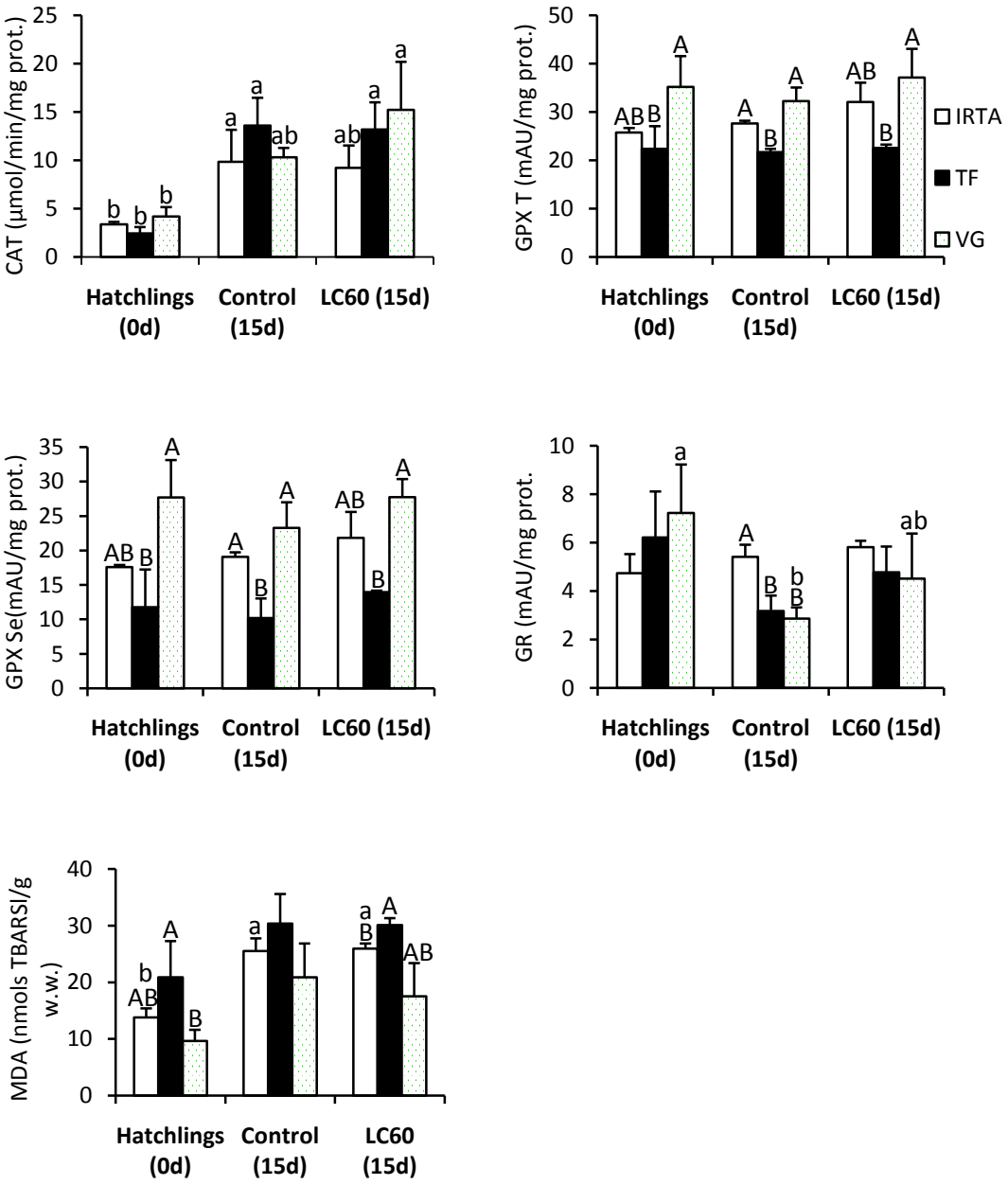
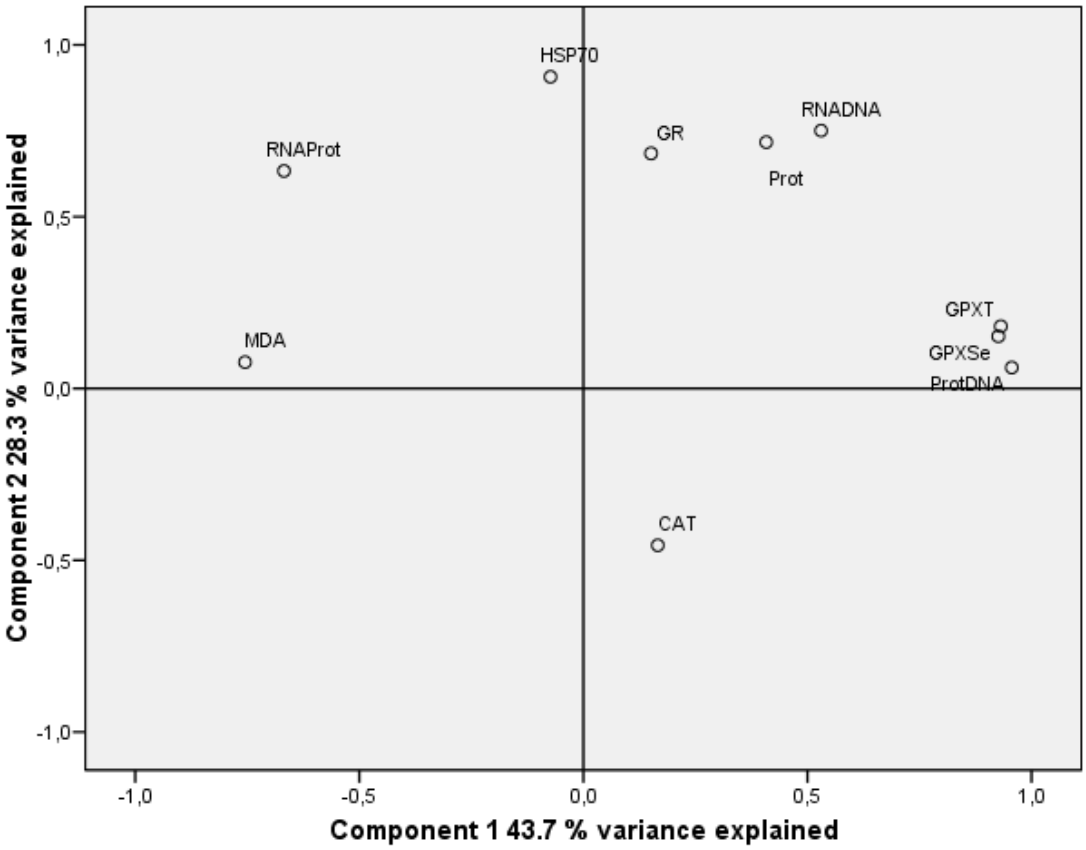

CAT (µmol/min/mg prot.)
GPX T (mAU/mg prot.)
GPX Se(mAU/mg prot.)
GR (mAU/mg prot.
MDA (nmols TBARSl/g w.w.)
Hatchlings (0d)
Control (15d)
LC60 (15d)
LC60
IRTA
TF
VG

Fig. 5.

A


HSP70
RNADNA
GR
RNAProt
Prot
GPXT
MDA
GPXSe
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CAT
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Component 1 43.7 % variance explained
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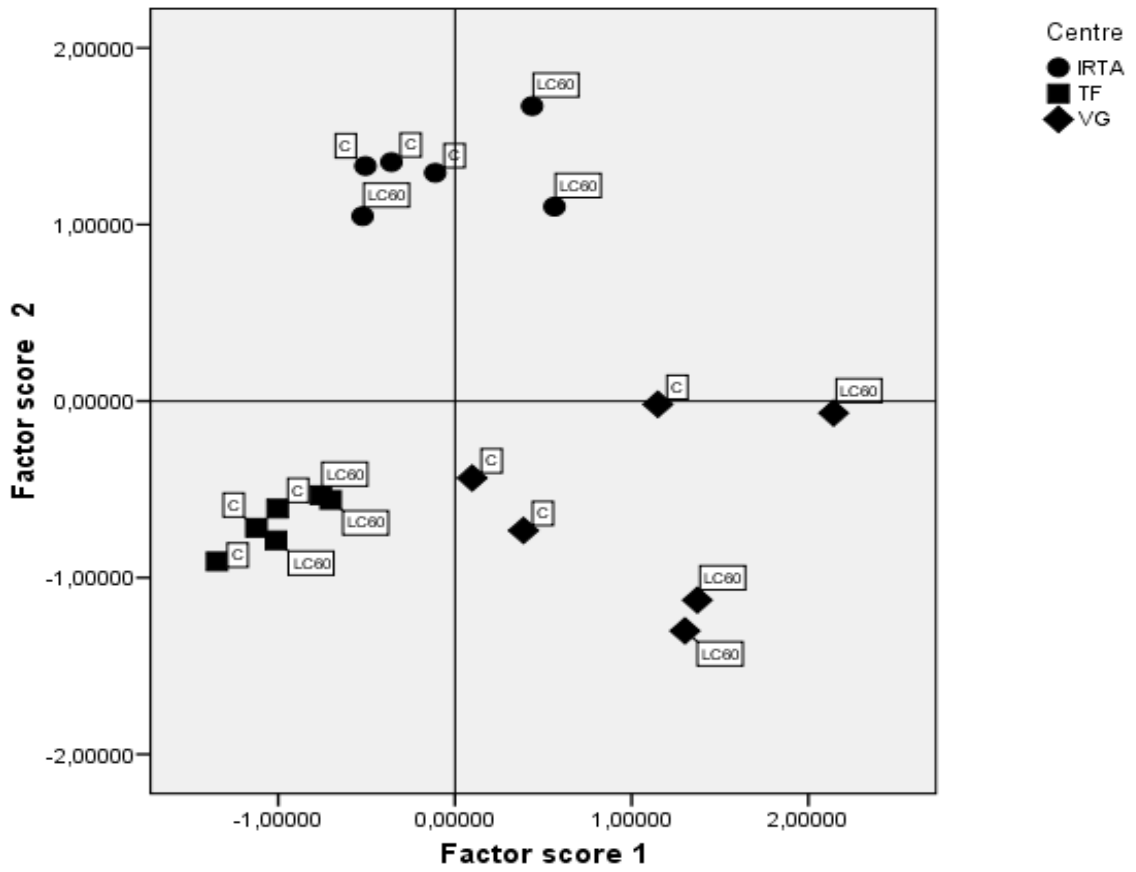
0,5
0,0
-0,5
-1,0

B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