Effects of supplementation of decapods zoea to *Artemia* basal diet on fatty acid composition and digestive gland histology in common octopus (*Octopus vulgaris*) paralarvae.

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Abstract

The present study aimed to evaluate the effect of the supplementation of different crab zoeas to enriched *Artemia* basal diet for *O. vulgaris* paralarvae during the first month of life. Paralarvae were fed using: enriched *Artemia* nauplii alone and *Artemia* co-fed with either first zoea stages of *Grapsus adscensionis* or *Plagusia depressa*. The experiment was carried out over a period of 28 days, in 0.12 m^3 tanks with a flow-through rearing system. Growth in dry weightas well as mantle length and width were assessed weekly. Additionally, prey and paralarvae fatty acid composition and digestive gland (DG) histology were evaluated. Addition of low amounts of crab zoeas (approx.100 indv. L^{-1} day^{-1}) provided during critical life stages of *O.vulgaris* proved to be good enough to improve paralarvae growth and survival in comparison to those fed exclusively on enriched *Artemia.* These results were supported by the finding of a higher number of glycoprotein absorption vacuoles in the DG from paralarvae co-fed with crab zoeas, suggesting a higher feeding activity. In addition, the fatty acid analysis of crab zoea showed that these are good sources of dietary arachidonic and eicosapentaenoic acids during the octopus planktonic life stage, whereas the low docosahexaenoic (DHA) content, suggests the use of additional DHA sources or higher zoeas densities to meet paralarvae nutritional demand to carry out a successful metamorphosis to benthic life.

Introduction

Among the different cephalopods, the common octopus, *Octopus vulgaris*, is considered one of the most economically interesting species for aquaculture diversification, largely due to its high growth rates, high fecundity and wide market demand (Iglesias, Sánchez, Bersano, Carrasco, Dhont, Fuentes, Linares, Okumura, Van der Meeren, Muñoz, Roo, Vidal & Villanueva 2007; Iglesias, Fuentes & Villanueva 2014). The ongrowing phase under experimental and pilot scale conditions has shown promising results in both tanks and sea cages, either floating or in benthic systems (García-García, & Cerezo Valverde 2006; Rodríguez, Carrasco, Arronte & Rodríguez 2006; García-García, Cerezo-Valverde, Aguado-Giménez, García-García, & Hernández 2009; Domingues, Garcia & Garrido 2010; Estefanell, Roo, Guirao, Fernández-Palacios, Izquierdo & Socorro 2012a; Estefanell, Roo, Guirao, Izquierdo & Socorro 2012b; Iglesias *et al*. 2014). However, to date the ongrowing experiences with *O. vulgaris* were exclusively based on the capture of sub adult octopus (less than 1 kg) from the wild.

Although the first studies on *O. vulgaris* paralarvae culture were conducted more than 30 years ago, the development of the farming industry is still mainly constrained by the lack of a sustainable supply of reared juveniles; with high paralarvae mortality during the early stages its main bottleneck (Iglesias *et al.* 2007). Therefore, determining the factors affecting paralarvae mortality in *O. vulgaris* is still one of the main goals to be researched. During the last twenty years, different zootechnical parameters such as tank volume (Sánchez, Fuentes, Otero, Lago, Linares, Pazos & Iglesias 2013), light intensity (Fernández-López, Roo, Socorro, Hernandez-Cruz, Fernández-Palacios & Izquierdo

2005) or water quality (Feyjoo, Riera, Felipe, Skalli & Almansa 2011), were studied to improve paralarvae survival. Nevertheless, the most important effort has been focused on nutritional studies. Thus, some experimental studies to try to determine the nutritional requirements for essential fatty acids (EFA), amino acids, vitamins or minerals have been carried out (Navarro & Villanueva 2000; Navarro & Villanueva 2003; Seixas, Rey-Méndez, Valente & Otero 2010; Villanueva, Riba, Ruíz-Capillas, González, & Baeta 2004; Villanueva & Bustamante 2006; Villanueva, Escudero, Deulofeu, Bozzano & Casoliva 2009). In addition, histological studies on the digestive gland (DG), with important roles in several digestive processes in cephalopods (Martínez, López-Ripoll, Avila-Poveda, Santos-Ricalde, Mascaró & Rosas 2011) described the apparition of feeding related vacuoles at early stages (Roo, Socorro, Alonso & Izquierdo 2003). Thus, recognizing the morphological changes that occur throughout *O. vulgaris* paralarvae DG development may improve our understanding of the absorption and assimilation mechanism of nutrients.

The effect of different feeding regimes with a variety of live prey combinations, such as crustacean zoeas (Villanueva 1994, 1995; Moxica, Linares, Otero, Iglesias & Sánchez 2002; Roo *et al.* 2003; Carrasco, Arronte & Rodríguez 2006; Reis, García-Herrero, Riera, Felipe, Rodríguez, Sykes, Martín, Andrade & Almansa 2014; Iglesias *et al.* 2014), enriched *Artemia* at diverse live stages (Seixas, Rey-Méndez, Valente & Otero 2008; Solorzano, Viana, López, Correa, True & Rosas 2009; Fuentes, Sánchez, Lago, Iglesias, Pazos & Linares 2011) or copepods (Vidal, Di Marco, Wormuth & Lee 2002) were assessed. Those studies which fed different crab zoea during the paralarvae pelagic stages obtained better survival results than those using *Artemia* or microdiets.

However, in most of these studies, the benthic life stage was not achieved, with only a few octopuses (1-10) surviving to reach the juvenile stage. Subsequently, recent studies using PCR based methods with wild octopus paralarvae have shown that early hatchlings of *O. vulgaris* prey upon a reduced number of species (around 20), belonging to the Decapoda order (Roura, González, Red & Guerra 2012). These authors reported that Brachyura (crabs) and Caridea (shrimps) zoea are the most preyed upon species, followed by Anomura (hermit crabs) and Thalassinidea (mud shrimps) zoea, whereas early hatchlings of *O. vulgaris* do not seem to prey upon copepods. These results suggest that *O. vulgaris* paralarvae should be considered as specialist predator during their first days of life, probably throughout their planktonic phase, where not only zoea morphology but also type of movement, escape velocity and/or zoea dimensions might affect prey selectivity. This specialized way of feeding might explain the high mortality of *O. vulgaris* hatchlings under culture conditions even when different species were used as preys in experimental trials. All of these studies, provided considerable amount of basic and applied information, but reliable standard methodologies for both, research studies and/or commercial production are still not available.

In the Canary Islands, different ecological studies have shown that there is a close relationship between the most frequent reproduction periods for *O. vulgaris* (March-April; August-September) and the presence of decapods zoea of rock crab, *Grapsus adscensionis,* and Atlantic grapsic crab, *Plagusia depressa,* (Carro, Socorro, Roo, Montero & Izquierdo 2004; Landeira 2010). Both species are widely distributed in the western Atlantic coast and share similar morpho anatomical characteristics to those reported as natural live preys for *O. vulgaris* paralarvae by Roura and co-workers

(2012). Moreover, the high fecundity rate (>50.000 eggs/female) and the presence of healthy populations in the wild with no or low commercial interest in the Canaries (Carro *et al.* 2004; Ramirez & Haroun 2014), makes this species an interesting candidate to be utilized as preys for *O. vulgaris* paralarvae rearing.

Thus, the present study aimed to evaluate the effects of supplementation of crab zoeas to *Artemia* basal diet on the biological performance and nutritional status of octopus paralarvae during the critical time window of specialized prey selection at the first month of life. To achieve so, *O. vulgaris* paralarvae were fed three diets containing solely enriched *Artemia* (control) or enriched *Artemia* in combination with first zoea stages of either *Grapsus adscensionis* or *Plagusia depressa* for 28 days. The paralarvae performance, fatty acid profile as well as histological studies were performed. The successful implementation of the use of proposed crabs zoeas in paralarval diet might stablish new opportunities for both hatchery operations of *O. vulgaris* as well as the utilization of underexploited crab species as a marine resource.

Material and methods

Experimental conditions

The trial was carried out at the experimental facilities of the Scientific and Technological Park from the University of Las Palmas de Gran Canaria (Canary Islands, Spain). Twelve wild captured adult octopuses, (2000 ± 750 g average weight) with a male: Female ratios of 1:2 were kept in four rectangular white-walled, fibre glass tanks (2.5m^3, volume) with seawater (37 ppt salinity) supplied by flow though with an

132 average temperature of 22.5 ± 0.5 ºC. Octopuses were reared under natural photoperiod
133 (27º59´28´´N; 15º 22´05´´O); with 10Light:14Dark hours. Tanks were covered by
134 shadowing nets (75% light reduction) in order to promote spawning, and a PVC
135 cylindrical dwelling (length= 60cm, diameter=160 mm) per female was set into each
136 tank as shelter. Octopus broodstock were fed *ad libitum* with a mixed diet consisting of
137 60% crab *Portunus pelagicus* and 40% of bogue (*Boops boops*) (Estefanell, Socorro,
138 Izquierdo & Roo 2014), provided on alternate days. Under these conditions, all females
139 spawned naturally after one month in captivity. Following spawning, each dwelling
140 with the female and its egg mass were removed from the mating tank and transferred to
141 an individual 0.5 m^3 cylinder-conical fibre glass tank in order to estimate the number of
142 paralarvae produced per female. Incubation period lasted from 25 to 31 days. Once
143 hatched, all paralarvae were collected daily and counted volumetrically. Hatching
144 periods lasted from 7 to 10 days, so in order to reduce the effect of the spawning
145 variability, the experimental paralarvae were collected at one time from the same
146 female, during the middle of the hatching period when the number of hatched
147 paralarvae was at its highest. One thousand five hundred newly hatched octopuses (15
148 paralarvae l^{-1}) were stocked in 12 black-walled, cylinder-conical fibre glass tanks of
149 0.12 m^3 volume, filled with natural seawater (37 ppt) filtered through a 50 µm
150 mechanical filter in a flow through system. Octopus paralarvae were maintained under
151 natural photoperiod (10:14 L:D) with water temperature and oxygen levels of 23.3 ± 0.2
152 ºC and 6.4 ± 0.5 ppm, respectively.

153 Three different dietary treatments were tested in quadruplicates tanks, based on the use
154 of 56 hours post hatched *Artemia* metanauplii (EG type, INVE Aquaculture,
155 Dendermonde, Belgium) previously enriched for 48 hours (enrichment started 8 hours
156 after hatching) with A_1 Selco (INVE Aquaculture, Dendermonde, Belgium) and
157 supplied once a day at 9:00 am at 2 indv.ml^{-1}.day^{-1}. The control treatment (T_A) utilised
158 enriched *Artemia* (2 indv.ml^{-1}.day^{-1}) as the sole feed. The second treatment (T_{AG})
159 consisted of feeding enriched *Artemia* (2 indv.ml^{-1}.day^{-1}) supplemented with first zoea
160 stage of *Grapsus adscensionis* (100 zoea.l^{-1}.day^{-1}). Finally, the third treatment (T_{AP}) fed
161 enriched *Artemia* (2 indv.ml^{-1}.day^{-1}) supplemented with first zoea stage of *Plagusia*
162 *depressa* (110 zoea.l^{-1}.day^{-1}). To obtain crab zoeas, gravid female crabs from each
163 species were collected daily along different sampling points from the coast line of Gran
164 Canaria 15 days before the trial started until the end of the experimental period. Gravid
165 specimens were caught using a traditional fishing methodology consisting of a fishing
166 rod with a ball of fishing line wrapped around the tip with a piece of bait (sardines)
167 inserted. Crabs attracted to the bait were rolled in the fishing line, allowing their
168 capture. Only ovigerous females were selected and stocked in separated 1.5 m^3 fibre
169 glass tanks in an open water circuit. Newly hatched zoea were collected daily by
170 overflow in a 500 μm net collector set in the outlet pipe of each tank, volumetrically
171 counted and distributed to the paralarvae tanks. To determine zoea total length (TL) 100
172 individuals from each crab species were measured using a profile projector (Nikon V-
173 12A, NIKON, Tokyo, Japan).
174 *Growth and survival*

Paralarvae growth was assessed by measuring the mantle length (ML) and width (MW) of 15 paralarvae at 7, 16, 22 and 28 days after hatching (dah), using a profile projector. In addition, growth in dry weight (DW) was determined in paralarvae previously measured. Specific dry weight growth rate (% SGR_{Dw}. day^{-1}) was calculated using the following equation: $SGR_{Dw}=([Ln(Dw_t)-Ln(Dw_0)] /t) x 100;$ where t is the time period in days and D_{W0} and D_{Wt} are the paralarval dry weights at the beginning and end of the time period respectively.

Survival was determined at 22 dah by individually counting all remaining paralarvae in culture tanks. From 22 dah until day 28 onwards, mortalities were recorded daily and survival adjusted accordingly.

Biochemical analysis

For biochemical analysis, samples of paralarvae were collected in the morning at hatching, 22 and 28 dah from each tank prior to feeding. In order to determine the nutritional quality of prey, samples of enriched *Artemia* and different crab zoeas were also collected and analyzed (Table 1). All the biochemical analyses were conducted in triplicate. Moisture, protein and lipids were determined according accredited methods (AOAC 2005). Total lipids were extracted as described by Folch, Lees & Stanley 1957. The fatty acid methyl esters were obtained by transesterification with H_2SO_4 (Christie 1982) and purified by adsorption chromatography on NH_2 Sep-Pack cartridges (Waters, S.A., Milford, MA, USA) as described by Fox (1990), and separated and quantified by Gas-Liquid chromatography as described by Izquierdo, Watanabe, Takeuchi, Arakawa & Kitajima (1989).

Histological analysis

198 The histological study was performed using 15 paralarvae per tank. Specimens were
199 collected early in the morning, prior to feeding, on days 0, 7, 16, 22 and 28 dah and
200 fixed in 4% buffered formalin, dehydrated through graded alcohols and embedded in
201 paraffin wax. Six paraffin blocks containing five paralarvae per tank were sectioned at 5
202 µm. Serial slides were stained with haematoxylin and eosin (H&E) and Periodic Acid-
203 Schiffs haematoxylin (PAS-H) for histological evaluation (Martoja & Martoja-Pearson
204 1970).
205 *Statistical analysis.*
206 Results are presented as mean ± standard deviation. Data were compared statistically by
207 analysis of variance (ANOVA), and checked for normality and homogeneity of
208 variance, using the Kolmogorov–Smirnoff and the Levene tests, respectively (Sokal &
209 Rohlf 1996). Differences between means were compared using Duncan's test. A
210 significance level of 5% was set for all analysis. All data were analysed using the
211 program SPSS Statistical Software System (ver. 12; SPSS Chicago, USA, 2005).
212 **Results**
213 *Survival and growth*
214 Dietary treatments including crab zoeas showed a significant effect ($P<0.05$) on
215 paralarval survival during the experimental period (Fig.1). Paralarvae fed enriched
216 *Artemia* as single fed (T_A) showed the poorest survival (4.5 ± 1.5 %) at 20 dah, whereas
217 no differences were obtained in *Octopus* paralarvae fed T_{AP} (17.8 ± 11.3 %) and T_{AG}
218 (29.0 ± 9.1 %). By the end of the experimental period (28 dah), no paralarvae survived
219 from T_A, whereas survival rates of 7.3 ± 3.4 % and 20.5 ± 8.9% were recorded for T_{AP}
220 and T_{AG} respectively.

Morphometric characteristics of both species of crab zoea showed that the average total length varied from 1.5 to 2.3mm while maximum thickness ranged from 0.6 to 1.2 mm. Mantle length (ML) of *O. vulgaris* paralarvae at hatching ranged from 1.17 to 1.62 mm (mean 1.41 ± 0.10 mm) and mantle width (MW) from 1.06 to 1.54 mm (mean 1.26 ± 0.16mm). Mean ML of *O. vulgaris* fed different dietary treatments at days 22 and 28 are shown in Table 1. At day 22 ML of T_{AP}-fed paralarvae was significantly higher than those fed $T_{AG,}$ and were also significantly different to T_A. In contrast, at 28dah no significant differences in either ML or MW were observed between T_{AG} and T_{AP}.

Figure 1

Table 1

The effect of crab zoea addition on paralarval growth in DW is presented in Figure 2. Paralarvae fed T_{AG} achieved a significantly higher DW than those fed T_A ($P<0.05$) and slightly, but not significantly higher than those fed T_{AP} during the whole rearing period. In addition, significant differences were observed in terms of SGR_{DW} ($P<0.05$). Thus, from hatching to 22dah the poorest SGR_{DW} values were obtained in paralarvae fed T_A (5.1 ± 0.1%) while similar values were registered in T_{AG} (6.5 ± 0.3%) and T_{AP} (6.0 ± 0.7%) –fed paralarvae. At the end of the study no significant differences were observed between T_{AG} and T_{AP} treatments ($P>0.05$). (Fig. 3).

Figures 2,3.

Biochemical analysis

Protein and lipid content of enriched *Artemia* metanauplii was significantly higher than both tested species of decapods zoea (Table 2). Regarding fatty acid compositions of the different live preys, some significant differences ($P<0.05$) could be addressed. For

instance, enriched *Artemia* metanauplii were richer in monounsaturated, total n-3, n-9 and n-3 PUFA fatty acids including EPA and DHA than both crab species tested. On the contrary, crab zoeas were particularly rich in ARA. Hence EPA/ARA, DHA/ARA and n-3/n-6 ratios in both crab zoeas were significantly lower than in enriched *Artemia* (Table 2).

Table 2

A reduction in moisture was observed in Octopus paralarvae fed enriched *Artemia* in combination with *G. adscensionis* zoea (T_{AG}), although no differences were observed compared to T_{AP}-fed paralarvae (Table 3). No changes were observed in terms of total lipid content between paralarave fed the three different dietary treatments, although an increase in total lipids was observed between hatchling and after 22 days of eating the experimental feeds (Table 3).

The fatty acid composition of *O. vulgaris* paralarvae at hatching showed a predominance of total saturated, total n-3 and n-3 PUFA including high levels of EPA and DHA acids. Besides, n-6 series were represented by the elevated content of ARA (Table 3). After 22 days of culture, octopus paralarvae clearly reflected the fatty acid profile of their food. Thus, the inclusion of both species of crab zoeas tended to increase the levels of saturated FA, particularly in T_{AP}-fed larvae mainly due to an elevation in palmitic and stearic acid (16:0 and 18:0 respectively) in the paralarvae, although differences were not found between T_A and T_{AG} fed larvae. Besides, the presence of enriched *Artemia* in the diet, lead to an increase in monounsaturated FA from n-9 series, particularly by oleic acid (18:1n-9) in comparison to just hatched paralarvae. Regarding to n-3 PUFA levels, these were maintained in a range between 45-54 $g.kg^{-1}$ DW from

hatching to 22 days of culture, although the proportion of the different fatty acids from this family showed extreme variations. Thus, DHA tended to decrease from 26.1 $g.kg^{-1}$ DW at hatching to a range 15.8 to 22.7 $g.kg^{-1}$ DW when *Artemia* was supplied as single feed or this was supplemented with *P. depressa* zoea, respectively. On the other hand, EPA content tended to increase in a similar proportion for all the groups. Similarly, n-6 series showed a tendency to increase along paralarval rearing, but preferentially in 18:2n-6 when enriched *Artemia* was utilized as single diet while in ARA (20:4n-6) when crab zoeas were supplemented. The effect of the feeding regimes was also reflected in variations of EPA/ARA, DHA/EPA, DHA/ARA and Oleic/DHA ratios after 22dah but also in comparison to initial values recorded at hatching (Table 3).

Histological study

Four different types of vacuoles were identified in the digestive gland (DG; Fig. 4) of the paralarvae. The first type observed as big brown vacuoles under H&E staining and located in the cellular apical region were identified as excretion vacuoles (E_V) (Fig. 4A). A second type of vacuoles, located in the same area but next to the acinar lumen, were integrated by many small eosinophilic vacuoles and identified as enzymatic secretion vacuoles (S_V) (Fig. 4B). The third and fourth types were identified as absorption vacuoles (A_V). But, these showed different locations and characteristics. Those presented in the DG and enterocytes which were not stained by the H&E staining were suggested as lipid absorption vacuoles (A_{VL}) (Fig.4A). While, those groups of eosinophilic absorption vacuoles located in the DG, with slight PAS staining affinity were identified as protein and carbohydrate absorption vacuoles or glycoprotein absorption vacuoles (A_{VG}) (Fig. 4B). Histological evaluation revealed that at 16 dah

100% of the paralarvae showed A_{VG} in their DG, being similar in number for T_{AP} and T_{AG}-fed paralarvae but higher than those found in T_A paralarvae. At 22 dah A_{VG} were identified again in 100% of the evaluated paralarvae from T_{AP} while only 58% and 50% of the paralarvae-fed T_A and $T_{AG,}$ respectively, showed A_{VG}. Similarly, at 28dah, 100% of the paralarvae fed T_{AP} presented A_{VG} in their DG, whereas only 48% of the paralarvae from T_{AG} showed this type of vacuoles (Fig. 4C, 4D). On the contrary, no noticeable differences were observed for the other vacuole types identified among experimental groups.

Figure 4

Table 3

Discussion

The results from the present study showed that dietary treatments where enriched *Artemia* was complemented with crab zoeas at low density (100 indv.l.day^{-1}) provided nutritional sustained benefits during the first month of life observed as enhanced paralarvae survival and growth. These results are in agreement with those previously reported, although higher prey densities than those utilized in the present study were employed (Villanueva 1994; 100-300 zoea.l^{-1}.day^{-1}; Carrasco *et al.* 2006; 700-1000 zoea.l^{-1}.day^{-1}; Iglesias *et al.* 2014; 500 zoea.l^{-1}.day^{-1}). Authors attributed the benefits of crab's zoeas complementation to prey size and biochemical composition. In this sense, the use of crab zoea with 50-100% of the ML of the paralarvae was reported to stimulate first feeding and growth (Villanueva 1994). In the present study *G. adscensionis* and *P. depressa* zoeas TL were 106% and 163% of the paralarvae ML, respectively. However, these values are higher than those suggested by Villanueva

(1994). These differences could be related to the different anatomy of the zoeas utilised in both studies. Indeed, when zoea maximum thickness is related to the paralarvae ML these values were reduced to 43 and 85% of the paralarvae ML, which agreed with the values suggested by those authors. In addition, the zoea species utilized in the present study belong to the brachyura family, which are the most frequent preys reported in wild caught *O. vulgaris* paralarvae (Roura *et al.* 2012) whereas anomura zoea were used by Villanueva (1994).

On the other hand, the use of enriched *Artemia* as single feed for *Octopus* paralarvae has shown highly different degrees of success on larval survival and growth. This variability is a multifactorial response to different causes including *Artemia* density, size and biochemical composition among others (Iglesias, Fuentes, Sánchez, Otero, Moxica & Lago 2006; Okumura, Kurihara, Iwamoto & Takeuchi 2005; Seixas *et al.* 2010; Sánchez, Fuentes, Otero, Lago, Linares, Pazos & Iglesias 2013; Fuentes *et al.* 2011; Iglesias *et al*. 2014). In this study, the poor survival observed in paralarvae fed T_A after 22 days of culture was a consequence of a sustained and progressive mortality along the third week of feeding, which in turn lead to a low number of paralarvae by day 22 in this treatment. These results were confirmed by the histological evaluation of the DG. In this sense, four different types of vacuoles were observed in the DG by day 16 after hatching in paralarvae from all the tested treatments. These results suggest the existence of a functional DG at this sampling point. However, the evaluation performed at 22 dah revealed a lower frequency of absorption vacuoles in the DG, particularly those related to glycoprotein absorption (A_{VG}), in paralarvae fed *Artemia* alone in comparison to those co-fed with crab zoea at the same age. Although limited

information exists regarding the ontogeny of the enzymatic system in the DG of *Octopus vulgaris*, it is known that paralarvae can adjust their digestive enzymes to different food rations and diets and that this adaptation seems to be correlated with growth (Villanueva, Koueta, Riba & Boucaud-Camou 2002). Thus, the reduction in number of A_{VG} could be an indicator of a lower feeding activity and probably malnutrition when *Artemia* was supplied as the sole prey. As pointed out previously, DG is a key organ in octopus metabolism, with many different functions such as synthesis and secretion of digestive enzymes, nutrients metabolism, synthesis and storage of lipids, glycogen, vitamins and minerals among others. Therefore, the present results indicate that the study of variations in the histological structure of the DG might be a good indicator of the feeding status in octopus paralarvae similarly to what was previously reported for its biochemical composition when adult octopuses were kept under prolonged starvation (García-Garrido, Hachero-Cruzado, Garrido, Rosas & Domingues 2010).

In addition, the positive effects on growth and survival obtained with the supplementation of enriched *Artemia* with decapods zoea could be related to the marked differences in biochemical composition of these live preys. Particularly, lipid content of enriched *Artemia* was significantly higher than that recorded in newly hatched *Octopus*, whereas the lipid contents for both zoeas were closer these levels. Additionally, the differences in fatty acid profiles of the tested preys showed that enriched *Artemia* had a significant lower ARA content than both types of zoea tested. This fatty acid is particularly rich in newly hatched paralarvae (Navarro and Villanueva 2000, 2003). The ARA content in paralarvae fed exclusively enriched *Artemia* was markedly reduced,

359 whereas co-feeding with 100 $indv^{-1}.day^{-1}$ of crab zoeas during critical life stages were
360 good enough to maintain paralarvae ARA contents at similar levels than those observed
361 in newly hatched octopus. The essentiality of ARA for octopus, in contrast to fish,
362 remains an open question. Mainly because ARA levels are not related to dietary inputs
363 in this species (Miliou, Fintikaki, Tzitzinakis, Kountouris & Verriopoulos 2006). This
364 fact led to the presumption that *O. vulgaris* was able of biosynthesizing this fatty acid
365 from their substrates as a Δ-5-like fatty acyl desaturase has been identified in this
366 cephalopod species (Monroig, Navarro, Dick, Alemany & Tocher 2012). However,
367 recent studies using ^{14}C-labelled fatty acids showed no synthesis of ARA from 18:2n-6
368 (Reis *et al.* 2014), results that agree with the present study. These data suggest the
369 importance of supplementation of this fatty acid in paralarval feed at least at similar
370 levels to those found in crab zoeas (>8 $g.kg^{-1}$ DM) to improve growth and survival
371 rates.
372 On the other hand, newly hatched octopus presented high levels of n-3 LC-PUFA (EPA
373 and DHA). The essentiality of these fatty acids has been demonstrated in most of
374 marine fish larvae (Izquierdo 1996; Izquierdo, Socorro, Arantzamendi & Hernández-
375 Cruz 2000) and suggested in *O. vulgaris* paralarvae (Navarro & Villanueva 2000, 2003;
376 Iglesias *et al.* 2007; Monroig *et al.* 2012). The inclusion of enriched *Artemia* as a basal
377 diet for all the experimental groups seemed to be good enough to maintain EPA
378 contents in paralarval tissues at same levels or even higher than those found in the
379 initial paralarvae. In contrast, DHA contents and DHA/EPA ratio tended to decrease
380 from newly hatched octopus to the end of the experimental period. Thus, despite DHA
381 contents in enriched *Artemia* were almost 4-fold times higher than those observed in

crab zoeas, this amount seems to be not sufficient to promote growth and survival at the same levels than when crab zoeas were provided. These results are in agreement with those reported by Seixas *et al.* (2010) and Fuentes *et al.* (2011). These authors suggest that the utilization of diets with a high protein/lipid ratio is more relevant than high dietary DHA levels. However, the relevance of DHA for maintaining structure and functional integrity in cell membranes and its importance for normal neural development and function has been clearly stated in hundred of studies with marine fish larvae (Bell, Batty, Dick, Fretwell, Navarro & Sargent 1995; Izquierdo 1996). Indeed, the relevance of dietary DHA contents is not only related to the total amount of the fatty acid but also to its ratio to other fatty acids and the chemical form in which is provided. Particularly, crab zoea such as spider crab (*Maja brachydactyla;* Andrés, Estévez, Simeó, & Rotllant 2010) are especially rich in DHA presented in the polar lipid fraction (representing even >70%) while DHA content in enriched *Artemia* is usually present in the neutral fraction, reaching values over 80% of the total lipids (author´s data not shown; Guinot *et al*. 2013). These differences are known to modify DHA availability for its biological functions in different marine's species. In this sense, phospholipid digestion appears to be more efficient than that of neutral lipids, regardless of the synthesis of neutral lipase in teleost larvae (Morais, Conceição, Rønnestad, Koven, Cahu, Zambonino Infante, & Dinis), what may account in part for the lower performance observed in T_A-fed paralarvae. In fact, if octopus paralarvae have a limited capability to exchange between neutral and polar lipids, the enrichment of *Artemia* nauplii with LC-PUFA may be worthless and can mask the essentiality/requirements for these fatty acids. In addition, DHA levels are usually present in low levels in the PL of

405 supplemented *Artemia* nauplii (~2.5%), whereas 18:3n-3 and EPA are the major PUFA
406 in the PL (~12% each; Sargent, McEvoy, Estevez, Bell, Bell, Henderson & Tocher
407 1999). Thus, octopus paralarvae must replace the dominant 18:3n-3 in their ingested PL
408 with EPA and DHA present in the neutral lipids fraction. In the present study, sufficient
409 amount of EPA was provided in the enriched *Artemia* nauplii to guarantee this
410 replacement, although this was not true for the DHA what indicated that in the case of
411 using enriched *Artemia* for the early life stages of *O. vulgaris*, DHA levels over 25 $g.kg^{-1}$
412 DM (2.5% TFA in DM) would be necessary to ensure adequate incorporation of this
413 fatty acid into the paralarvae PL in order to fulfil DHA requirements.
414 Furthermore, the high number of sensory-related systems including visual, mechanical
415 and chemical receptors, or chromatic elements under development during planktonic
416 life stages suggests that an adequate provision of DHA should also be critical for a
417 successful metamorphosis to the benthic life stage of the octopus paralarvae. In this
418 study, the supplementation of enriched *Artemia* with different crab zoeas seemed to be
419 good enough to meet EPA and ARA needs, but limited in DHA content, albeit better
420 than feeding exclusively enriched *Artemia*. These results, suggest that for covering
421 DHA demand and given that paralarvae are specialist predators, higher amounts of
422 zoeas or another type of prey different than those utilized in present study might be
423 necessary to reach higher paralarvae survival in later stages. The dietary treatments
424 assessed, particularly those using *Grapsus* zoea, seemed to be a suitable diet to overpass
425 the first month of life of *O.vulgaris* paralarvae with a high survival rate (up 25%),
426 providing good quality paralarvae for further studies in the next life stages.

In summary, results from the present study showed that supplementation with *G. adscensionis* and *P. depressa* zoeas stimulated paralarval feeding, improving absorption mechanisms and feed utilisation, confirmed by the results obtained on growth, survival and DG histology. In addition, zoea supplementation acted as a good source of ARA for *O. vulgaris* paralarvae, while its low DHA contents suggests that and additional supply of DHA might be necessary in order to sustain the high levels of this FA recorded in newly hatched paralarvae. Providing adequate levels of ARA seems to be crucial to enhance paralarval growth and survival, overcoming the importance for DHA at least at this developmental stage. Furthermore, both the content and the lipid class of FA supplemented through the enriched *Artemia* as single diet, suggest that this is not appropriate to meet the demand of ARA and DHA for *O. vulgaris* paralarvae. Further studies are needed to determine the importance of these FA, particularly ARA, on the development of octopus paralarvae and if these FA can avoid mass mortalities in later developmental stages.

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608 **Figure legends**

609 Figure 1. Survival on the 22nd and 28th dah of *O. vulgaris* paralarvae fed enriched *Artemia*
610 (T_A) or on enriched *Artemia* either supplemented with *G. adscensionis* zoeas (T_{AG}) or *P.*
611 *depressa* zoeas (T_{AP}) (mean + S.D.; n=3). The different superscript letter over the bars with
612 same the color shows significant differences ($P \leq 0.05$).

613

614 Figure 2. Dry weight of *O. vulgaris* paralarvae fed on enriched *Artemia* (T_A) or on enriched
615 *Artemia* either supplemented with *G. adscensionis* zoeas (T_{AG}) or *P. depressa* zoeas (T_{AP})
616 (mean + S.D.; n=3) at each sampling point. Data are fitted to a quadratic regression analysis
617 ($f=y_0+ax+bx^2$).

618

619 Figure 3: Specific Growth Rate (SGR_{DW}) of *O. vulgaris* paralarvae fed enriched *Artemia*
620 (T_A) or enriched *Artemia* either supplemented with *G. adscensionis* zoeas (T_{AG}) or *P.*
621 *depressa* zoeas (T_{AP}) (mean + S.D.; n=3). The different superscript letters over each age
622 show significant differences ($P \leq 0.05$).

623

624 Figure 4: Transversal sections of *O. vulgaris* paralarvae showing the morphology of the
625 digestive gland (DG), haematoxilin and eosin (H&E; Fig. 4a) or periodic acid-reactive-
626 Schiffs-haematoxilin (PAS-H; Fig. 4b-d) staining (x400): a) Octopus paralarvae fed T_A at
627 22dah b) T_{AG}-fed paralarvae at 22dah c) T_{AP}-fed paralarvae at 22dah d) T_{AP}-fed paralarvae
628 at 28d. Excretion vacuoles (E_V); Secretion vacuoles (S_V), lipid absorption vacuoles (A_{VL})
629 and glycoprotein absorption vacuoles (A_{VG}).

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Table 1. Growth in ML at 22 and 28dah of *O. vulgaris* paralarvae fed enriched *Artemia* (T_A) or enriched *Artemia* either supplemented with *G. adscensionis* zoea (T_{AG}) or *P. depressa* zoea (T_{AP}). Data represent means ± standard deviation (SD), (n=3).

Treatment	Age	ML (mm)	n	Age	ML (mm)	n
T_A	22	2.00±0.14[a]	30	---	---	---
T_{AG}	22	2.44±0.18 [b]	45	28	2.32±0.04	30
T_{AP}	22	2.21±0.18 [c]	45	28	2.32±0.09	30

*Means in columns with different superscript letter are significantly different ($P<0.05$)

Table 2.- Proximate composition (% dry matter) and fatty acids (FA) content (% DW) of different live preys. Data represent means ± SD, (n=3). Different superscripts within each row indicate a significant difference between live preys (ANOVA, $P \leq 0.05$; Duncan´s).

	Enriched Artemia	*G. adscensionis Zoea*	*P. depressa Zoea*
Proximate analysis (g kg^{-1} dry matter)			
Lipids	282.0	120.6	121.7
Proteins	493.6	362.5	391.9
Dry matter	71.5	73.8	69.9
Fatty acid content (g kg^{-1} dry matter)			
Saturated	56.8	37.7	49.7
ΣMonounsaturated	107.1	33.3	31.1
Σn-3	85.5	20.9	18.4
Σn-6	26.4	22.6	16.7
Σn-9	71.1	23.0	19.4
Σn-3PUFA	56.7	18.3	15.8
14:0	5.7	2.3	3.2
16:0	33.9	21.5	30.2
16:1 n-7	18.1	3.1	5.1
18:0	12.3	10.3	13.2
18:1 n-9	68.0	21.1	17.3
18:1 n-7	16.1	6.1	5.4
18:2 n-6	17.9	6.9	4.0
18:3 n-3	23.1	1.9	1.6
20:1 n-9	1.4	1.3	1.3
ARA	3.8	12.3	9.1
EPA	36.6	11.1	9.9
DHA	16.7	4.9	4.2
DPA (22:5n-6)	0.9	0.3	0.4
DHA/22:5 n-6	195.3	156.9	104.0
EPA/ARA	96.5	9.1	10.9
DHA/EPA	4.6	4.4	4.2
DHA/ARA	44.1	4.0	4.6
Oleic/DHA	40.7	42.7	40.0
Oleic/n-3PUFA	12.0	11.5	10.6
n-3/n-6	32.6	9.3	11.1

652 Table 3.- Proximate composition (% dry matter) and fatty acids (FA) content (% DW) of *O.*
653 *vulgaris* paralarvae fed enriched *Artemia* (T_A) or enriched *Artemia* either supplemented
654 with *G. adscensionis* zoea (T_{AG}) or *P. depressa* zoea (T_{AP}) at 22dah. Data represent means
655 ± SD, (n=3). Different superscripts within each row indicate a significant difference
656 between octopus fed live preys only (not initial; ANOVA, $P \leq 0.05$; Duncan´s).
657

	Hatching	T_A paralarvae	T_{AG} paralarvae	T_{AP} paralarvae
Proximate analysis (g kg^{-1} dry matter)				
Dry matter	119.3 ± 2.5	52.45 ± 5.85^{a}	99.6 ± 19.9^{b}	79.7 ± 23.6^{ab}
Lipids	142.0 ± 12.1	210.1 ± 11.3	205.5 ± 5.9	204.4 ± 7.0
Fatty acid content (g kg^{-1} dry matter)				
ΣSaturated	42.0 ± 7.5	44.8 ± 0.9^{a}	51.3 ± 5.5^{ab}	55.0 ± 2.8^{b}
ΣMonounsaturated	33.4 ± 4.1	66.2 ± 1.1^{a}	64.0 ± 0.6^{a}	55.0 ± 3.6^{b}
Σn-3	47.4 ± 8.3	62.5 ± 12.1	62.4 ± 10.7	65.2 ± 7.3
Σn-6	11.1 ± 3.5	20.0 ± 0.8	20.1 ± 1.3	20.0 ± 0.1
Σn-9	26.7 ± 2.5	46.9 ± 0.4^{a}	44.2 ± 0.5^{a}	37.1 ± 2.1^{b}
Σn-3PUFA	46.0 ± 8.9	45.4 ± 11.1	49.3 ± 8.7	54.4 ± 5.2
14:0	1.7 ± 0.4	2.1 ± 0.0	2.1 ± 0.1	2.2 ± 0.1
16:0	30.3 ± 5.0	24.1 ± 0.5^{a}	28.8 ± 2.7^{b}	31.6 ± 1.2^{b}
16:1 n-7	0.6 ± 0.2	8.5 ± 0.7	8.2 ± 0.3	7.1 ± 0.9
18:0	7.0 ± 1.3	14.4 ± 0.3	17.4 ± 2.4	17.6 ± 1.5
18:1 n-9	18.3 ± 2.0	42.4 ± 0.1^{a}	40.0 ± 0.6^{a}	33.0 ± 2.4^{b}
18:1 n-7	2.9 ± 0.5	10.3 ± 0.3^{ab}	10.8 ± 0.2^{a}	9.8 ± 0.3^{b}
18:2 n-6	0.5 ± 0.1	11.9 ± 2.1^{a}	8.5 ± 0.5^{b}	7.2 ± 0.8^{b}
18:3 n-3	0.3 ± 0.0	15.3 ± 0.8^{a}	11.7 ± 1.7^{ab}	9.3 ± 2.0^{b}
20:1 n-9	5.9 ± 1.4	3.1 ± 0.0	3.3 ± 0.2	3.3 ± 0.1
ARA	7.7 ± 1.9	5.4 ± 0.9^{a}	8.7 ± 0.6^{b}	9.6 ± 0.8^{b}
EPA	15.6 ± 2.3	26.0 ± 6.4	26.4 ± 5.0	27.7 ± 4.2
DHA	26.1 ± 6.2	15.8 ± 4.1	18.9 ± 3.2	22.7 ± 0.9
DPA (22:5n-6)	0.7 ± 0.1	0.6 ± 0.2	0.6 ± 0.1	0.7 ± 0.0
DHA/22:5 n-6	39.57 ± 17.02	25.12 ± 1.11^{a}	29.61 ± 2.41^{b}	32.55 ± 0.84^{b}
EPA/ARA	2.03 ± 0.95	4.77 ± 0.38^{a}	3.00 ± 0.35^{b}	2.94 ± 0.69^{b}
DHA/EPA	1.67 ± 0.15	0.61 ± 0.01^{a}	0.72 ± 0.01^{ab}	0.83 ± 0.09^{b}
DHA/ARA	3.41 ± 1.96	2.89 ± 0.27^{a}	2.15 ± 0.21^{b}	2.39 ± 0.29^{ab}
Oleic/DHA	0.70 ± 0.19	2.81 ± 0.76^{a}	2.16 ± 0.41^{ab}	1.46 ± 0.05^{b}
Oleic/n-3PUFA	0.40 ± 0.10	0.97 ± 0.24	0.83 ± 0.16	0.61 ± 0.01
n-3/n-6	4.28 ± 2.96	3.14 ± 0.74	3.09 ± 0.33	3.27 ± 0.38

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659 Values (mean ± SD) followed by different superscript letters within a row shows significant differences ($P < 0.05$).

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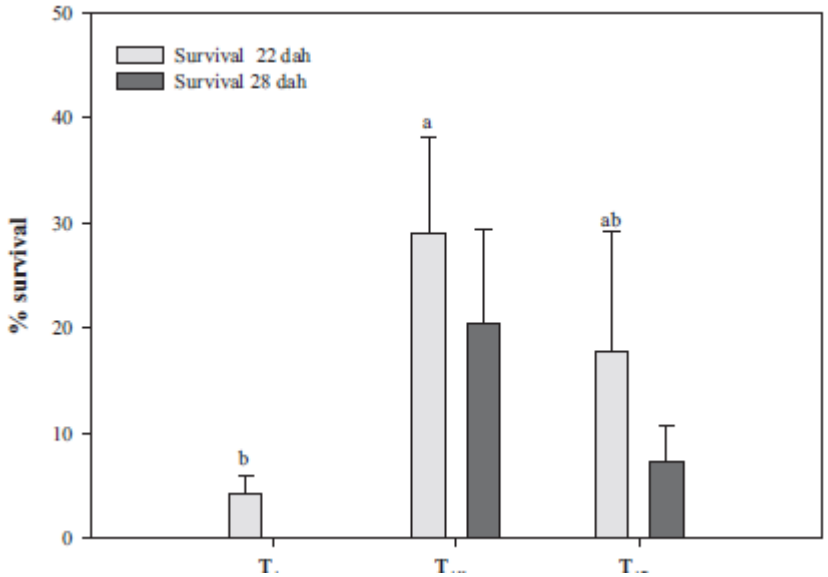

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667 Figure 1
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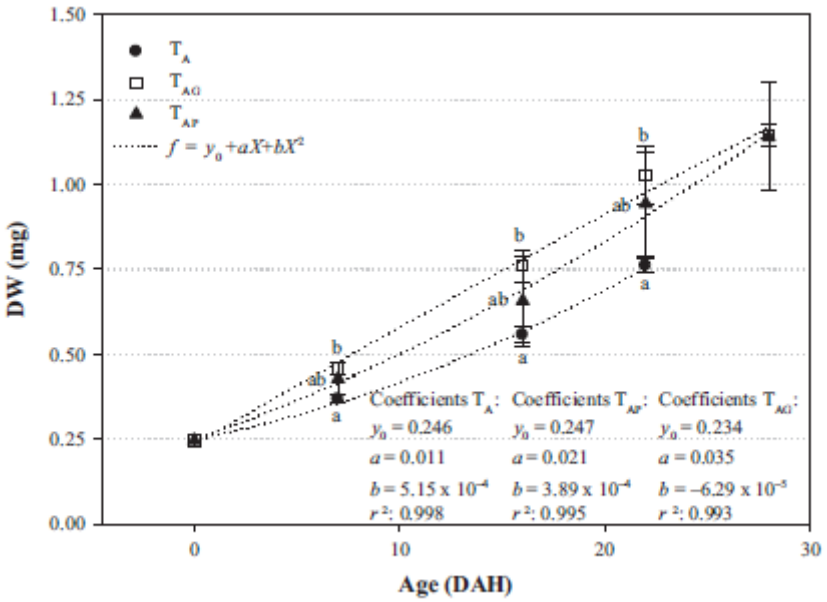

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670 Figure 2
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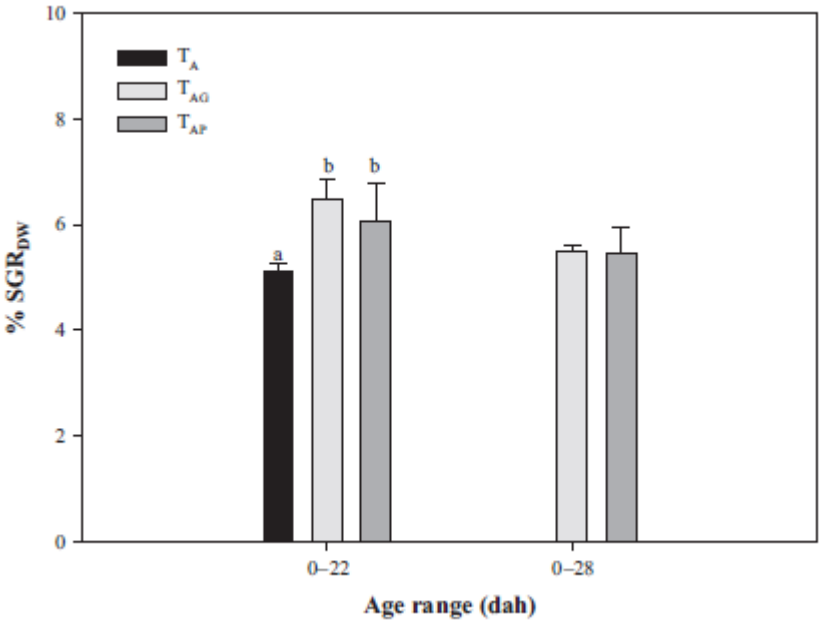

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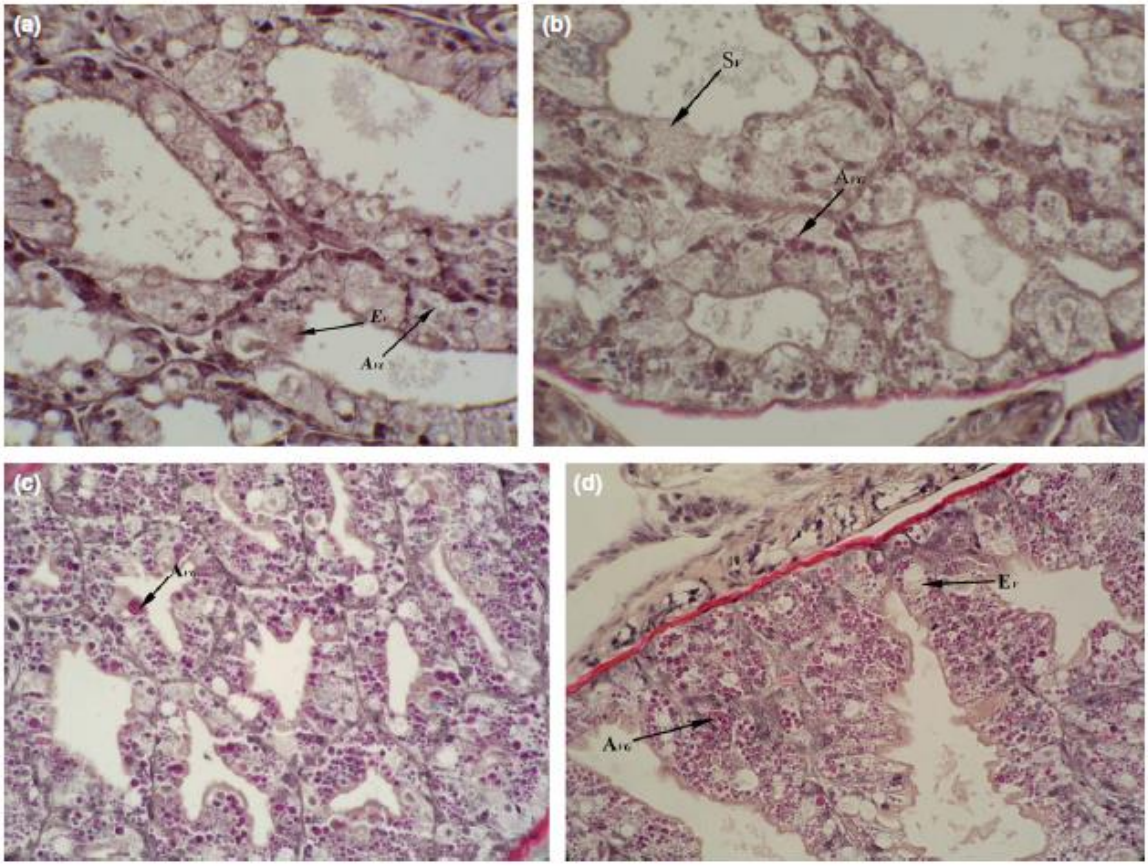

Figure 4