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# Fatty acid composition and age estimation of wild *Octopus vulgaris* paralarvae

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ABSTRACT

The fatty acid (FA) profile of wild *Octopus vulgaris* paralarvae of estimated age was individually analyzed for the very first time in order to establish a reference for comparison in rearing and nutritional studies. Age of each paralarvae was estimated by analysing daily increments on lateral hood surface of beaks. Wild paralarvae age ranged between 6 and 8 days and their FA composition resembled that from hatchlings produced under culture conditions. However, when compared with the FA composition of up to 20 days old cultured paralarvae described in the bibliography, some striking differences were found. Results showed higher levels of docosahexaenoic acid (22:6n − 3, DHA), lower contents of 18:1n − 9, 18:1n − 7 and 18:2n − 6 and negligible levels of 18:3n − 3 in wild paralarvae, when collated to reared one. These results seem to indicate that preys/diets supplied to cultured paralarvae fail to resemble paralarval natural composition and as a result do not fulfil their FA requirement. The individual applied technique developed in this study will allow to refine the study of wild paralarvae along its development, as well as to compare wild and cultured paralarvae of similar age.
*Statement of relevance:* Artemia does not fulfil paralarval fatty acid requirements.



## 1. Introduction

The species *Octopus vulgaris* is an excellent candidate for aquaculture diversification due to its biological and economic features (Iglesias and Fuentes, 2014; Reis et al., 2015). In spite of this fact, rearing *O. vulgaris* has been particularly difficult due to the total mortalities found during the paralarval stage, which has hampered to close common octopus life cycle under captivity and therefore its commercial production. Based on feeding trials with enriched live food and natural zooplankton, several authors have suggested that this mortality could be caused, in some extent, by nutritional deficiencies of paralarvae (Iglesias and Fuentes, 2014; Navarro et al., 2014; Viciano et al., 2011). Therefore, a better knowledge about nutrition and physiology in wild specimens could help to ascertain the reasons of the high mortalities shown under culture conditions. However, until now, only a few studies dealt on this specific issue due to difficulties in collecting wild paralarvae. Roura et al. (2012) first identified the natural preys of *O. vulgaris* paralarvae collected in the Ría de Vigo (NW Spain), applying molecular markers and finding preference for decapod crustacean zoeae. However, *Artemia* (a non decapod crustacean) is the most commonly used prey for rearing octopus paralarvae, and its nutritional composition could lead to differences between wild and reared individuals. In addition, Estefanell et al. (2013) analyzed the fatty acid (FA) profile of newly hatched paralarvae obtained from eggs collected in the wild, finding differences in the FA profile between cultured and wild hatchlings. Nevertheless, to the best of our knowledge, no studies regarding the FA composition have been done from wild individual paralarvae.

A second challenge is the complexity to determine the age of wild specimens, what has hindered the performance of studies focusing on paralarval development. Hernández-López et al. (2001) studied daily formation of growth increments on the lateral walls of the beaks of *O. vulgaris* paralarvae up to 26 days old. Most recently, Perales-Raya et al. (2014) have validated daily deposition in the beak increments of the same species broadening the range of paralarvae and transition-to-

*Abbreviations:* CV, coefficient of variation; DIC, differential interference contrast; DHA, docosahexaenoic acid; DML, dorsal mantle length; EPA, eicosapentaenoic acid; FA, fatty acid; FAME, fatty acid methyl ester; GC, gas chromatography; LC-PUFA, long-chain polyunsaturated fatty acids; LHS, lateral hood surface; MUFA, monounsaturated fatty acids; PCA, principal components analysis; PC1, principal component 1; PC2, principal component 2.

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settlement individuals up to 98 days old, using the lateral hood surface (LHS) of the beak. These findings allow to refine the study of wild paralarvae along their development as well as to compare wild and cultured individuals of similar age.

Under culture conditions, lipid composition and specifically, the FA profile of reared paralarvae is significantly different from hatchlings, one of the most relevant changes being the progressive decrease of docosahexaenoic acid (22:6n − 3, DHA; Arai et al., 2008; Estévez et al., 2009; Fuentes et al., 2011; Iglesias et al., 2014; Navarro and Villanueva, 2000, 2003; Reis et al., 2015, Seixas et al., 2010a, 2010b). These studies point out the lipid composition of *Artemia* as the major cause of the differences above described since its FA profile seems to be sub-optimal, and may not satisfy paralarval requirements. Moreover, recent studies have shown that *O. vulgaris* has little or no capacity to synthesize long-chain polyunsaturated fatty acids (LC-PUFA), such as DHA, arachidonic acid (20:4n − 6, ARA) or eicosapentaenoic acid (20:5n − 3, EPA) and, as a result, these FA are essential and have to be supplied by the diet (Monroig et al., 2013; Reis et al., 2014). However, there is a lack of studies to determine whether these changes could be related to paralarval development rather than to prey or diet composition. Comparison of wild and reared paralarvae of similar age would allow us to elucidate if the changes in FA profile are related with a non-optimal prey composition or are the result of normal development.

The FA profile of paralarvae has always been analyzed in pooled samples (Fuentes et al., 2011; Iglesias et al., 2014; Kurihara et al., 2006; Navarro and Villanueva, 2000, 2003; Okumura et al., 2005; Reis et al., 2015; Seixas et al., 2010a, 2010b; Viciano et al., 2011), hindering the detection of potential differences among individuals. To obtain FA profiles of *O. vulgaris* paralarvae individually, in the present study we have adapted a direct transmethylation method modified from O'Fallon et al. (2007).

Therefore, the aim of this study was to analyze individually, for the very first time, the FA profile of wild *Octopus vulgaris* paralarvae, and to estimate their age through daily deposition of increments on LHS of the beaks in order to establish a baseline age-FA profile for comparison in nutritional studies of reared paralarvae.

## 2. Materials and methods

### 2.1. Sample collection

Zooplankton samples were collected in the Ría de Vigo (NW Spain) at night between 7th and 8th of October 2013 onboard R/V "Mytilus" (IIM, CSIC). A multinet sampling gear (0.7 m × 0.7 m) was used to carry out a stratified sampling, collecting samples at the surface, 10, 20, 30 and 40 m of depth during 10 min at a speed of 2 kn. Ten wild paralarvae were sorted on board from surface samples collected in two stations around Cies Islands. Paralarvae were slaughtered into dry ice and kept at −80 °C until their analysis.

### 2.2. Length and age of paralarvae

Dorsal mantle length (DML) of each paralarvae was determined with a stereomicroscopes (Leica MS 5, Leica Microsistemas S.L.U., Barcelona, Spain) prior to beak extraction. Beaks were extracted, cleaned, and preserved in distilled water at approximately 4 °C, according to the procedure of Perales-Raya et al. (2010). Due to the difficulty of the procedure, only 6 beaks of a total of 10 paralarvae were correctly extracted undamaged. Age of paralarvae was estimated reading daily growth increments on LHS of upper beaks according to Perales-Raya et al. (2014), using a transmitted light microscope with Nomarski differential interference contrast (DIC) and 400× magnification (Nikon AZ 100, Tokyo). The DIC system generates a three-dimentional image in which increments are revealed on LHS. Beak reading was performed three times for each paralarvae, being their age estimation the mean value of them. The age precision among readings was assessed with the coefficient of variation (CV; standard deviation divided by the mean number of increments in each sample) (Campana, 2001; Chang, 1982).

### 2.3. Fatty acid analysis

A modification of O'Fallon et al. (2007) method basically based on a downscaling for small amounts of sample was used to analyze the fatty acid methyl ester (FAME) profile of each paralarvae individually without addition of internal standard. The method had been previously tested in hatchlings samples already analyzed by traditional methods (Christie, 1982; Folch et al., 1957). Briefly, each specimen was introduced into a crew capped 2 mL vial with 70 μL of 10 N KOH in distilled water plus 660 μL of methanol, that was tightly closed. Samples were incubated at 55 °C during 1.5 h, being shaken during 5 s every 20 min. Then the vial was cooled at room temperature, and 72.5 μL of 24 N $H_2SO_4$ in distilled water were added. Once again the mix was incubated in the conditions above mentioned (55 °C for 1.5 h, shaking every 20 min). After cooling again at room temperature, 187.8 μL hexane were added, and the mix was shaken and centrifuged at 2000 *g* for 2 min. Finally, the upper hexane layer, which contained the FAME, was transferred to GC vials, evaporated under nitrogen current and redissolved in 300 μL of hexane. FA composition was determined using an Agilent 6850 Gas Chromatograph coupled to a 5975 series Mass Selective Detector (MSD, Agilent Technologies, Santa Clara, CA, USA, equipped with a fused silica 30 m × 0.25 mm open tubular column (Tracer, TR-WAX, film thickness: 0.25 μm, Teknokroma, Sant-Cugat del Vallés, Spain). Injection of 1 μL samples was carried out in splitless mode, using helium as carrier gas (1.5 mL/min constant flow), and a thermal gradient from 50 to 220 °C, and reported in % of total fatty acids.

### 2.4. Data analysis

Age and FA composition (16:0, 18:0, 18:1n − 9, 18:1n − 7, 18:2n − 6, 18:3n − 3, 20:4n − 6, 20:5n − 3, 22:5n − 3 and 22:6n − 3) of wild paralarvae, together with hatchlings and reared paralarvae (under or equal to 20 days old) obtained from previous studies (Almansa et al., 2012, Navarro and Villanueva, 2000, 2003; Reis et al., 2015; Seixas et al., 2010a, 2010b; Socorro et al., 2004) were analysed by principal components analysis (PCA). Factor scores from PCA 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 by arcsine when needed (Fowler et al., 1998). After that, one-way ANOVA followed by a Tukey's post hoc test (Zar, 1999) was assessed. 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). The FA with striking differences among wild, hatchlings and culture paralarvae (18:1n − 9, 18:1n − 7, 18:2n − 6, 18:3n − 3 and 22:6n − 3) were also analyzed by one-way ANOVA following the procedure described above. Statistical analyses were carried out using SPSS for Windows 15.0 statistical package (SPSS Inc., Chicago, IL, USA).

## 3. Results and discussion

The FA composition of the age-estimated wild *Octopus vulgaris* paralarvae was achieved individually. It was only possible in 10 specimens, since collecting of wild paralarvae presents serious difficulties in terms of getting a reasonable number of individuals mainly due to their dispersion patterns, linked to marine dynamics, and their possible vertical migration (Otero et al., 2009). Some studies focused on prey identification and microbiome (Roura et al., 2012, 2015) as well as nutritional composition (Lourenço, 2014) have been also carried out recently in wild paralarvae, however, in these studies the age of the paralarvae was unknown, so accurate comparisons with reared paralarvae could not be performed. In addition, the analysis of