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A meta-analysis approach to the effects of live prey on the growth of *Octopus vulgaris* paralarvae under culture conditions.

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A short running title: Meta-analysis on *Octopus* paralarval growth

1

2 **Abstract**

3 The common octopus (*Octopus vulgaris*, Cuvier 1797) is a promising species for
4 aquaculture diversification, but the massive mortality during the first life stage is the
5 main bottleneck for its commercial production. The aim of the present study was to
6 compare the effects of different live preys (*Artemia* and crustacean zoeae) and/or
7 *Artemia* enrichment protocols in the paralarval growth by using a meta-analysis
8 approach. A total of 26 independent assays were used, including data from the
9 bibliography and from experiments carried out by our group. Three comparisons were
10 established: (i) crustacean zoeae vs *Artemia*, (ii) different crustacean zoeae species and
11 (iii) *Artemia* enriched with marine lecithin (rich in polar lipids-PL and docosahexaenoic
12 acid-DHA) vs previously used *Artemia* enrichments. The meta-analysis approach
13 allowed a quantitatively review of independent studies with reliable conclusions,
14 avoiding the subjectivity inherent to classical reviews. The outputs provided statistical
15 confirmation of the better suitability of crustacean zoeae with respect to *Artemia*.
16 However, not all crustacean species showed the same results, given that the high
17 variability on *Grapsus* zoeae hampered finding significant differences with respect to
18 the control treatment (*Artemia*). Nutrient composition and biometry of the different
19 types of prey are discussed as possible causes of the differences arising from the meta-
20 analysis. Finally, the present results suggest that marine lecithin has a beneficial effect
21 on paralarval growth with respect to previously used enrichments, which could be
22 related to the increase of DHA and PL in *Artemia*, given the essential role of these lipid
23 components in octopus paralarval physiology.

24 **Key words:** Meta-analysis, *Octopus vulgaris*, Paralarvae, Growth, Prey

25

26 Introduction

27 The common octopus (*Octopus vulgaris*, Cuvier 1797) is a species with increasing
28 interest for marine aquaculture diversification, given its high growth rate and easy
29 adaptation to captivity, among other positive features (Iglesias *et al.* 2007; 2014a).
30 However, the massive paralarvae mortalities verified under culture conditions ($\approx 100\%$
31 in most studies) have hampered its commercial production, therefore making this the
32 main bottleneck for industrial farming. According to several authors (Iglesias *et al.*
33 2007, 2014a; Iglesias & Fuentes 2013), the high mortalities could be due to: (i)
34 inadequate and/or unbalanced diets that do not fulfil paralarvae nutritional requirements;
35 (ii) lack of standardized rearing techniques, and (iii) little knowledge about octopus
36 paralarvae physiology and behaviour. Unlike benthic adults, newly hatched paralarvae
37 have a pelagic behaviour that lasts for about two months. Thereafter, octopus
38 progressively acquires benthic habits (Villanueva & Norman 2008).

39 Paralarvae fed crustacean zoeae such as *Maja* or *Pagurus* in co-feeding with *Artemia*
40 have shown the highest growth rates, ranging between 7-8 % dry weight \cdot day $^{-1}$, and
41 attain a development that facilitates their shift from a pelagic to a benthonic life stage
42 (Villanueva 1994; Iglesias *et al.* 2004; Carrasco *et al.* 2006). In addition, Roura *et al.*
43 (2012) has recently shown that, in the wild, paralarvae prey on an wide list of different
44 preys, where crustacean zoeae are preferably selected. However, it is not economically
45 viable to produce crustacean zoeae for feeding octopus paralarvae due to the high
46 commercial value of these crustacean species and the lack technology to produce those
47 (Andrés *et al.* 2007; 2010). As a result, current research has been focused on the use of
48 *Artemia*, which is the standard live prey used in marine larviculture (Sorgeloos *et al.*
49 2001). However, *Artemia* displays a nutritional profile less suitable for octopus
50 paralarvae than zoeae of crustaceans, even after enrichment (Navarro & Villanueva

51 2000; Bell *et al.* 2003; Hormiga *et al.* 2010). Most studies of *O. vulgaris* culture using
52 *Artemia* have promoted paralarvae growth rates between 2-4% dry weight \cdot day⁻¹
53 (Navarro & Villanueva 2000; Villanueva *et al.* 2004; Estévez *et al.* 2009; Seixas *et al.*
54 2010a,b; Reis *et al.* 2014a), while few authors have reported growth rates over 6%
55 (Villanueva *et al.* 2002; Okumura *et al.* 2005; Kurihara *et al.* 2006; Arai *et al.* 2008;
56 Fuentes *et al.* 2011; Viciano *et al.* 2011).

57 *Artemia* nutritional lipid profile presents low levels of polar lipids (PL) and highly
58 unsaturated fatty acids (HUFA), especially docosahexaenoic acid (22:6n-3, DHA)
59 (Navarro *et al.* 1993), and these are of particular relevance for octopus paralarvae
60 development, as initially suggested by Navarro and Villanueva (2000). Recent studies
61 carried out in the research project OCTOPHYS (see acknowledgements section for
62 details) have shown that octopus has little or no ability to synthesize HUFA such as
63 DHA, eicosapentanoic acid (20:5n-3, EPA) and arachidonic acid (20:4n-6, ARA)
64 (Monroig *et al.* 2013; Reis *et al.* 2014b), supporting the essential nature of these fatty
65 acids (FA). In addition, several studies conducted by Guinot *et al.* (2013a,b) have
66 shown an increase of PL and HUFA content in *Artemia*, using marine phospholipids
67 (Marine lecithin LC60, LC) as enrichment. .

68 On the other hand, the high variability in paralarval growth found among studies, using
69 similar diets, is still a main concern that needs to be solved to provide reproducibility
70 under culture conditions. The differences observed among studies could be partially
71 explained by several factors such as: shifts in nutritional live prey composition (e.g.
72 enrichment process, prey origin), rearing conditions (e.g. tank volume, light intensity,
73 density of paralarvae and/or preys) or even spawn quality (e.g. female size, origin, eggs
74 incubation temperature) (Iglesias *et al.* 2007, 2014b; Villanueva & Norman 2008).

75 An approach to overcome these problems is to standardise paralarval production and
76 culture protocols among different centres. To reach this goal, different preys,
77 enrichments and rearing conditions were tested under project OCTOPHYS, including
78 the use of *Artemia* enriched with LC as food for *O. vulgaris* paralarvae. Even though,
79 this strategy still produced a large volume of information together with that already
80 available in literature. In this sense, a meta-analysis approach allows the comparison of
81 results from independent studies to get reliable conclusions and avoid subjectivity and
82 variability (Walker *et al.* 2008). The methodology used in this study can only be applied
83 in experiments that have experimental and control treatments with their own mean,
84 standard deviation and number of replicates. To compare different studies, the meta-
85 analysis has different phases: (1) search and selection of studies, (2) estimation of
86 treatment effect (effect size), calculated as experimental treatment minus control
87 treatment or *vice versa*, for each study, as well as the effect size across all studies
88 (overall), (3) assessment of data precision measured as confidence interval, which
89 indicates the accuracy of the effect size estimation, and (4) search for data heterogeneity
90 and explore data robustness, quantifying the scattering of the effect sizes across studies
91 (Borenstein *et al.* 2010; Higgins & Green 2011).

92 In the present review, data from published literature regarding *O. vulgaris* paralarvae
93 rearing, as well as data from the OCTOPHYS project and other experiments were
94 considered using a meta-analysis approach aiming to compare: (i) the effects of
95 crustacean zoeae vs *Artemia*, (ii) the effects of different crustacean zoeae species and
96 (iii) the effect of *Artemia* enriched with Marine Lecithin LC60 (LC) vs. other *Artemia*
97 enrichments; on paralarvae growth.

98

99 **Materials and Methods**

100 An integrative meta-analysis was performed with data obtained from published
101 literature and from different trials carried out, under project OCTOPHYS, in three
102 research centres: Institute for Research & Technology Food & Agriculture, IR
103 (Tarragona, Spain); Spanish Institute of Oceanography: Oceanographic Center of the
104 Canary Islands, TF (Tenerife, Spain) and Oceanographic Center of Vigo, VG (Vigo,
105 Spain). Details about the studies included in the meta-analysis are summarized in Tables
106 1, 2, 3 and 4 and in the sections below.

107 ***Reference papers***

108 A total of 98 and 49 scientific contributions were found in April 2014 in the Web of
109 Science and Scopus, respectively, using the key-word: *Octopus vulgaris* paralarvae.
110 Other bibliography sources such as JACUMAR (Spanish National Advisory Board for
111 Marine Aquaculture) reports, conference communications and PhD theses dealing with
112 paralarval culture, were also considered. However, it should be emphasized that only 5
113 papers of Web of Science and Scopus, 1 PhD Thesis and 1 conference communication,
114 presented the data as required by the meta-analysis (experimental and control
115 treatments, mean, standard deviation and number of replicates). These references yield a
116 total of 11 bibliographic inputs used (see Table 1)

117 ***Rearing conditions***

118 Specific experiments were performed and data of paralarval rearing conditions is
119 summarized according to: a) Rearing conditions (Table 2), b) The on-growing *Artemia*
120 (Table 3) and c) Prey enrichment and feeding (Table 4). Broodstock conditions were as
121 described by Reis *et al.* (2014a) for IR and TF and Iglesias *et al.* (2014a) for VG.

122 Newly hatched paralarvae were cultured in fiberglass cylinder-conical tanks (conditions
123 are summarized in Table 2). In IR, tanks were connected to a recirculation unit

124 IRTAMar™. Physicochemical parameters such as oxygen, salinity and temperature
125 were measured daily and nitrite and ammonium once a week. Dissolved oxygen levels
126 were kept close to saturation and nitrite and ammonia were $<0.3 \text{ mg L}^{-1}$ and 0 mg L^{-1} ,
127 respectively in all experiments. Salinity and temperature data are shown in Table 2.

128 Diverse types of commercial *Artemia* were used in trials to compare different *Artemia*
129 enrichment techniques (see experiments 1 to 11 in Table 3) and as the control diet in the
130 experiments with zoeae (see experiments 12 to 15 in Table 3). In all experiments,
131 *Artemia* nauplii were obtained from cysts that hatched in fiberglass cylinder-conical
132 tanks for 24h at 28°C, with 37 PSU, vigorous aeration and 2000lx. Table 4 shows the
133 on-growing *Artemia* parameters used in several experiments. After the on-growing
134 period, *Artemia* enrichments were carried out as described in Table 3 for different
135 experiments. *Artemia* was given to paralarvae once a day in all experiments, except for
136 experiments 1, 2, 3, 4 and 8 where this prey was supplied three times per day. In these
137 experiments, previous to its use as food, *Artemia* were kept at 4°C, without any light,
138 and under gentle aeration to avoid metabolization of the enrichment.

139 Crustacean zoeae of different species were used as experimental diet in experiments 12
140 to 15 (Tables 2 and 3). *Maja brachydactyla* zoeae (experiments 13 and 14) were
141 obtained as described by Iglesias *et al.* (2014a). The production methodology and
142 handling of *Grapsus adscensionis* zoea and *Palaemon sp.* zoea (experiments 12 and 15)
143 were as described in Reis *et al.* (2014a).

144 The *Artemia* cysts were obtained from INVE Aquaculture (Dendermonde, Belgium),
145 fresh *Nannochloropsis sp.* was supplied by Necton, Companhia Portuguesa de Culturas
146 Marinhas, S.A. (Olhão, Portugal), freeze dried *Isochrysis galbana.*, *Nannochloropsis sp.*
147 and *Tetraselmis chuii* by Fitoplancton marino S.L (Cádiz, Spain), *Haematococcus*
148 *pluvialis* was provided by Sainhall Nutrihealth Pte Ltd (Singapour), Marine lecithin

149 LC60 (LC) was supplied by PhosphoTech Laboratories (St. Herblain, France) and
150 Gemma Diamond 0.8 was supplied by Skretting Spain S.A. (Burgos, Spain).

151 Paralarvae dry weight was determined individually, after oven drying for 20 h at 110°C,
152 as described by Iglesias *et al.* (2014a).

153 All the experiments were performed according to the Spanish Law 6/2013 based on the
154 Directive 2010/63/EU regarding the protection and humane use of animals for scientific
155 purposes.

156 ***Statistical Analysis***

157 The effect of different treatments on dry weight of octopus paralarvae was tested and
158 compared through meta-analysis (Borenstein *et al.* 2010). The methodology used in this
159 study can only be applied in experiments that have experimental and control treatments
160 with their own mean, standard deviation and number of replicates (Table 1). The
161 estimation of treatment effect (effect size) was calculated as the differences on dry
162 weight of paralarvae in the experimental treatment minus control treatment or *vice versa*
163 for each study (See Table 1), as well as the effect size across all studies (overall). The
164 effect size was calculated by standardized mean difference (Hedges's *g*, Hedges 1981).
165 Due to the different origins of prey and paralarvae, and rearing methodologies used in
166 the research centres, it was assumed that each study had its own error. Therefore, the
167 Random effects model (Cochran's *Q*) was used, employing the Comprehensive Meta-
168 analysis software (Biostat, Englewood, USA).

169 In the meta-analysis plots, the effect size on the left from vertical axis indicated that a
170 given experimental treatment improved the dry weight of paralarvae respect to control,
171 when the confidence interval of 95% (CI) rank did not intercept the vertical axis. To
172 confirm the correct choice of the Random effects model, the variability among studies

173 was run as comparable heterogeneity analysis (Q). P value <0.05 was considered
174 significant.

175

176 **Results and Discussion**

177 *Crustacean zoeae vs Artemia*

178 Crustacean zoeae have been tested in different studies as a suitable prey for octopus
179 paralarval culture, generally achieving better results than *Artemia* (Iglesias & Fuentes
180 2013; Iglesias *et al.* 2014b). However, this fact has not been quantified comparing the
181 data sets from different studies through a meta-analysis.

182 A total of 26 inputs, 7 using crustacean zoeae (see Table 1, inputs 12 to 18) and 19
183 using *Artemia* (see Table 1, inputs 1 to 11 and 19 to 26) were analysed. After the
184 bibliographic research, only the references which fulfil to meta-analysis requirements
185 were included in the statistical analysis. Some studies could not be included due to the
186 lack of a control treatment or standard deviation (e.g. Itami *et al.* 1963; Villanueva
187 1995; Navarro & Villanueva 2000; Moxica *et al.* 2002; Iglesias *et al.* 2004, Socorro *et*
188 *al.*, 2004; Carrasco *et al.* 2006; Moxica *et al.* 2006; Iglesias *et al.* 2014a).

189 Results obtained in the meta-analysis are shown in Figure 1. The overall model
190 (Overall) showed a significant increase on paralarval dry weight of ($p=0.001$) derived
191 from the individuals fed with zoeae, which displayed a positive effect ($p=0.001$).
192 Contrarily, *Artemia* was represented on the right side of the vertical axis indicating that
193 this prey did not improve the dry weight of *O. vulgaris* paralarvae ($p=0.654$). Zoeae and
194 *Artemia* showed heterogeneity ($Q=29.05$, $p<0.05$).

195 The meta-analysis results confirm statistically the suitability of crustacean zoeae
196 compared to *Artemia* in paralarval culture. This conclusion is in agreement with

197 previous studies using crustacean zoeae (Itami *et al.* 1963; Villanueva 1995, Moxica *et*
198 *al.* 2002; Iglesias *et al.* 2004; Morote *et al.* 2005, Socorro *et al.* 2005; Carrasco *et al.*
199 2006; Iglesias *et al.* 2007, 2014a) or *Artemia* under different enrichments (Navarro &
200 Villanueva 2000; Moxica *et al.* 2006; De Wolf *et al.* 2011). Similarly, Iglesias and
201 Fuentes (2013) pointed out that the growth obtained adding zoea can be six-fold higher
202 than that achieved with *Artemia*. Furthermore, paralarvae fed with zoeae in some cases
203 reached the benthic stage (Itami *et al.* 1963; Villanueva 1995; Iglesias *et al.* 2004;
204 Carrasco *et al.* 2006). In contrast, settlement of paralarvae fed with *Artemia* has rarely
205 been achieved, requiring a longer rearing period than paralarvae fed with zoeae
206 (Moxica *et al.* 2006; De Wolf *et al.* 2011). Several studies using *Artemia* (Moxica *et al.*
207 2006; Fuentes *et al.* 2011; Viciano *et al.* 2011) displayed a higher dry weight gain at 30
208 days, reaching 1.6-1.8 mg (SGR of 5-6%·DW day⁻¹) but this is still below that achieved
209 with crustacean zoeae (2.5-3.5 mg, SGR of 7-8%·DW day⁻¹; Villanueva 1995; Iglesias
210 *et al.* 2004; Carrasco *et al.* 2006; Iglesias *et al.* 2014a).

211 The better results obtained using zoeae may be due to prey size or prey nutritional
212 composition. Usually, the different zoeae species used in the octopus' culture display
213 greater length (1.3-3.4 mm) than *Artemia metanauplii* (0.8-2 mm) (Villanueva &
214 Norman 2008), which could increase the biomass ingested by paralarvae during each act
215 of feeding thereby reducing energy expenditure of hunting multiple preys to obtain the
216 necessary daily requirements, leading to higher growth. Previous studies have shown
217 the paralarval preference for large prey (Iglesias *et al.* 2006), being able to capture preys
218 between 45 to 118% of paralarvae total length (Villanueva & Norman, 2008).

219 Another relevant aspect is the composition of prey, specifically the HUFA and DHA
220 contents. Similar to what has been widely demonstrated in fish larvae, the importance of
221 DHA in the physiology of paralarvae may be related with visual and neuronal

222 development as have been suggested by numerous studies (Tocher, 2010, Navarro and
223 Villanueva, 2000; 2003 and Takeuchi, 2014). Newly hatched *O. vulgaris* display a high
224 DHA content ranging between 17-27% of total FA (Navarro & Villanueva 2000;
225 Okumura *et al.* 2005; Kurihara *et al.* 2006; Aria *et al.* 2008; Seixas *et al.* 2010a,b; Reis
226 *et al.* 2014a), similar to the levels observed in recently settled wild juveniles with 15-
227 25% of total FA, (Navarro & Villanueva 2003). In contrast, the DHA content tended to
228 gradually decrease (46-76% from hatching to 30 days old) in paralarvae fed exclusively
229 on *Artemia*, regardless of the enrichment used (Navarro & Villanueva 2000; Estévez *et*
230 *al.* 2009; Seixas *et al.* 2010a,b; Reis *et al.* 2014a). Nevertheless, paralarvae were able to
231 maintain the original levels of DHA throughout development when were fed on a
232 mixture of *Artemia* and sand eel (*Ammodytes personatus*) flakes (Okumura *et al.* 2005).
233 *O. vulgaris* shows little or no ability to synthesise DHA, as reported by Monroig *et al.*
234 (2013) and Reis *et al.* (2014b). Therefore, this FA should be provided in the diet at
235 appropriate levels. While, spider crab zoeae display levels of DHA between 8.7-15.8 %
236 of total FA (Seixas 2009; Andrés *et al.* 2010 and Iglesias *et al.* 2014a), the basal levels
237 of DHA in *Artemia* are negligible (0.1% DHA; Okumura *et al.* 2005; Reis *et al.* 2014a).
238 The use of different enrichment techniques has improved up to 2.3 and 8.0% of DHA
239 (Navarro & Villanueva 2000 and Seixas *et al.* 2010a respectively, among others).
240 Paralarval viability was slightly improved with these *Artemia* enrichments, but it was
241 not enough to maintain DHA levels in paralarvae (Navarro & Villanueva 2000; Estévez
242 *et al.* 2009; Seixas *et al.* 2010a, b; Reis *et al.* 2014a; Takeuchi 2014).

243 These differences between zoea and *Artemia* can be due to other factors related to the
244 bioavailability of DHA. In most species, DHA is mainly esterified in polar lipids (PL),
245 such as phosphatidylethanolamine or phosphatidylcholine (Kanazawa & Shunsuke
246 1994; Salhi *et al.* 1999). However, Bell *et al.* (2003) showed that *Artemia* enriched with

247 DHA accumulated most of this FA in neutral lipid (NL). More recently, Guinot *et al.*
248 (2013b) obtained a similar esterification into NL even when DHA was provided as PL
249 to *Artemia* during enrichment. In fish and cephalopods, diets containing PL have higher
250 apparent lipid digestibility than diets containing high amount of NL, due to the
251 emulsifying properties of PL that improve their digestion and absorption by larvae
252 (Koven *et al.* 1993; Morillo-Velarde *et al.* 2014; Olsen *et al.* 2014). This could be due
253 to the absence of lipid emulsifiers in the digestive tract of cephalopods (Vonk 1962;
254 O'Dor *et al.* 1984). Accordingly, these results suggest that *Artemia* metabolism, which
255 allocates DHA in the NL fraction, could diminish the bioavailability of this FA
256 compared to crab zoeae.

257 Other nutrients such as copper, aminoacids (AA) or vitamins might have an influence
258 on the dry weight of paralarvae. Copper plays an essential role in oxygen transport as a
259 constituent of hemocyanin, the main respiratory pigment in cephalopods. In addition,
260 copper content decreases when paralarvae are fed with *Artemia* nauplii (from 217 $\mu\text{g}\cdot\text{g}^{-1}$
261 DW in hatchlings to 92 $\mu\text{g}\cdot\text{g}^{-1}$ DW in 20 days-old paralarvae (Villanueva & Bustamante
262 2006). This could be related with the low copper content of *Artemia* (7 $\mu\text{g}\cdot\text{g}^{-1}$ DW),
263 which contrast with the values found in *M. brachydactyla* zoea (73 $\mu\text{g}\cdot\text{g}^{-1}$ DW)
264 (Villanueva & Bustamante 2006). On the other hand, the profile of total aminoacids
265 does not seem to be a limiting factor, since the composition of enriched *Artemia*
266 metanauplii, *Pagurus prideaux* zoea and *M. squinado* zoea is similar (Villanueva *et al.*
267 2004). As regards the vitamin content, enriched *Artemia* (DC Super Selco and L-
268 methionine) and *M. brachydactyla* zoea, have similar vitamin E content (428 and 584
269 $\mu\text{g}\cdot\text{g}^{-1}$ DW, respectively) (Villanueva *et al.* 2009). Moreover, the contents of other
270 nutrients not yet evaluated may be important, namely carotenoids, carbohydrates, other
271 vitamins, etc.

272 ***Relation among zoeae from different crustacean species***

273 *O. vulgaris* paralarvae have been fed on several crustacean species such as *M.*
274 *brachydactyla* (Moxica *et al.* 2002; Iglesias *et al.* 2004; 2014a; Carrasco *et al.*, 2006),
275 *Grapsus adscensionis* (Socorro *et al.* 2005; Reis *et al.* 2014a), *Palaemon sp.* (Socorro *et*
276 *al.* 2005; Estevez *et al.*, 2009; Reis *et al.* 2014a), *P. prideaux* (Villanueva 1995),
277 *Linocarcinus depurator* (Villanueva 1995), *Acartia sp.* (Iglesias *et al.* 2007; Estevez *et*
278 *al.*, 2009) and *Palaemon serratus*, *Moina salina* and *Maja squinado* (Morote *et al.*
279 2005). The results obtained among different studies suggest a species-specific effect on
280 paralarval viability, which was tested through the meta-analysis.

281 Nevertheless, the lack of fulfilment of experimental requirements for the meta-analysis
282 comparison in many of these studies entail that only 7 inputs from 4 crustacean genera
283 (*Maja*, *Palaemon*, *Grapsus* and the copepod *Acartia*) could be used to compare the
284 effects of different species within the zoea group (see Table 1, inputs 12 to 18). Results
285 are presented in Figure 2. The overall model confirmed the positive effect of feeding
286 octopus paralarvae with crustacean zoea species ($p=0.001$). However, not all crustacean
287 species showed the same results, with *Grapsus* zoeae displaying no significant
288 differences with respect to the control treatment, probably due to the high variability in
289 the confidence interval. It also has to be considered that this analysis did not show
290 heterogeneity ($Q=5.08$, $p=0.166$), due to the size effects showing similar values and
291 their confidence interval (CI) overlapping among studies.

292 These results obtained in the meta-analysis related to *G. adscensionis* zoeae were
293 probably due to its lower nutritional value, given that this species showed a lower DHA
294 content (2.6% of total fatty acids, Reis *et al.* 2014a) when compared with *M.*
295 *brachydactyla* (12.8%-15.1%, Andrés *et al.* 2010; Iglesias *et al.* 2014a), *P. elegans*
296 (13.4%, Reis *et al.* 2014a), *P. prideaux* (18.1%, Navarro & Villanueva 2000) or the

297 mysid *Acanthomysis longicornis* (24.0%, Navarro & Villanueva 2000). It should be
298 noted also that *G. adscensionis* is a species with relatively lower copper content
299 ($7.4 \pm 2.5 \mu\text{g g}^{-1}$ DW, Martin *et al.* 2011) when compared with *M. brachydactyla* (50.0-
300 $72.5 \mu\text{g g}^{-1}$ DW, Andrés *et al.* 2010; Villanueva & Bustamante 2006). In addition, the
301 size of *G. adscensionis* could influence the results obtained, since this species has a
302 smaller carapace length (CL) and lower DW (0.45 mm and 0.02 mg, respectively) than
303 other zoeae species, such as *L. depurator* (CL 0.52 mm), *P. prideaux* (CL 1.18 mm),
304 *Dardanus arrosor* (CL 1.44 mm) (Villanueva 1994) and *M. brachydactyla* (CL 1.01
305 mm and DW 0.109 mg) (Andrés *et al.* 2007).

306 Paralarvae fed on *Maja* and *Palaemon* zoeae as well as *Acartia* showed increased DW
307 with respect to the control group (*Artemia*), confirming the positive effects of these
308 zoeae in paralarval growth. However, the fluctuations in quality regarding biochemical
309 composition (among other features) of newly hatched zoeae or copepods throughout the
310 year, the lack of specific culture technology, and the economic value of these species
311 (many of them used for human consumption) have hampered its commercial production
312 for paralarvae culture (Andrés *et al.* 2007, 2010). In consequence, further studies are
313 necessary with the aim to produce high quality enriched *Artemia* with appropriate
314 nutritional profiles to meet the requirements of *O. vulgaris* paralarvae.

315 ***Effects of marine phospholipids on Artemia enrichment using Marine lecithin LC60***
316 ***vs other enrichments***

317 As previously mentioned, DHA and PL seem to be essential in the physiology of
318 octopus paralarvae. However, *Artemia* shows a profile poor in these lipid components.
319 Guinot *et al.* (2013a,b) have demonstrated that the use of marine phospholipids such as
320 marine lecithin LC60[®] (LC) as enrichment improved the content of DHA and PL in
321 *Artemia*. Therefore, the next step was to compare the effect of this product on paralarval

322 DW gain with other *Artemia* enrichments, tested either individually or in combination.
323 The enrichments considered were different phytoplankton species (*Isochrysis galbana*,
324 *Nannochloropsis* sp., *Haematococcus pluvialis*, *Tetraselmis chuii*, *Rhodomonas lens*),
325 free L-amino acids (lysine, arginine, and methionine), commercial enrichments (Ori-
326 Gold[®], DC Super Selco[®], Easy DHA-Selco[®]), M70 (a lipid enrichment used by Viciano
327 *et al.* 2011) and crushed wild zooplankton (see Table 1 and 4). Other enrichments such
328 as *Phaeodactylum tricornutum*, Krill powder, Red-pepper[®], Algamac[®], Multigain[®], Ori-
329 Prot[®], Ori-Culture[®] and Ori-Green[®] have been cited in the literature, but they were not
330 included in the meta-analysis due to the lack of statistical requirements.

331 Finally, a total of 19 inputs were used, 9 for LC (see Tables 1 and 4, inputs 1 to 9) and
332 10 for other *Artemia* enrichments (see Tables 1 and 4, inputs 10, 11 and 19 to 26).
333 *Artemia* fed with LC improved paralarvae DW ($p=0.014$), whereas other *Artemia*
334 enrichments showed a decreased in DW ($p=0.044$) (Figure 3). Results from the overall
335 model (which include LC as well as other enrichments) did not show any significant
336 effect on paralarval DW ($p=0.259$), since differences between LC and other *Artemia*
337 enrichments displayed high heterogeneity ($Q=8.84$, $p=0.003$). These results suggest that
338 marine phospholipids (LC) seem to have a beneficial effect on paralarvae, with respect
339 to other enrichments, improving their growth.

340 In addition, the use of *Artemia* enriched with LC promoted a slight increase of the
341 HUFA content (including DHA) in paralarvae when compared with other *Artemia*
342 enrichments (8.3 vs 6.2 % DHA of the total FA, respectively) (Garrido *et al.* 2013).
343 Moreover, the use of the LC enrichment promoted an increase of the PL fraction in
344 *Artemia* (Guinot *et al.* 2013b). Therefore, the beneficial effects of LC on paralarval dry
345 weight gain could be related to an improvements in lipid composition of *Artemia*.
346 However, further studies are necessary to establish the lipid requirements of paralarvae

347 during their pelagic stage (especially in HUFA and PL) as well as the metabolism and
348 bioavailability of these lipid components in *Artemia* and in other suitable types of prey
349 for *O. vulgaris* paralarvae.

350

351 **Conclusions**

352 In summary, using selected data from independent studies, the meta-analysis showed
353 significant differences in paralarvae fed with crustacean zoeae vs *Artemia*, where the
354 use of zoeae resulted in a better performance of *O. vulgaris* paralarvae displaying a net
355 positive effect on growth (dry weight). Nevertheless, not all the zoeae species displayed
356 a similar growth enhancement, given that the high variability on *Grapsus* zoeae
357 hampered finding significant differences with respect to the control treatment. Finally,
358 results suggest that *Artemia* enrichment with marine lecithin has a beneficial effect on
359 paralarval growth compared to other *Artemia* enrichments, which could be related to the
360 increase of DHA and PL, given the essential role of these lipid components in the
361 paralarval physiology.

362

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545 **Tables**

546

547

Table 1. Studies included in meta-analysis

N° study	CONTROL			EXPERIMENTAL			Age	Ref.
	Prey 1	DW (mg)	N	Prey 2	DW (mg)	N		
1	A	0.82 ± 0.15	30	A	0.80 ± 0.36	15	30	PE
2	A	0.94 ± 0.15	5	A	1.21 ± 0.25	5	30	“
3	A	1.47 ± 0.36	8	A	2.38 ± 0.35	8	30	“
4	A	0.66 ± 0.07	11	A	0.76 ± 0.22	10	30	“
5	A	0.41 ± 0.05	15	A	0.45 ± 0.05	15	14	“
6	A	0.48 ± 0.08	30	A	0.47 ± 0.08	30	14	“
7	A	0.60 ± 0.11	30	A	0.67 ± 0.14	30	14	“
8	A	0.43 ± 0.05	15	A	0.46 ± 0.07	16	14	“
9	A	0.33 ± 0.08	12	A	0.33 ± 0.05	12	14	“
10	A	0.33 ± 0.08	12	A	0.32 ± 0.36	12	14	“
11	A	0.48 ± 0.18	6	A	0.45 ± 0.17	6	14	“
12	A	0.48 ± 0.18	6	GZ	0.58 ± 0.11	6	14	“
13	A	0.77 ± 0.12	30	MZ	1.11 ± 0.13	30	14	“
14	A	0.78 ± 0.12	30	MZ	1.31 ± 0.30	30	30	“
15	A	0.31 ± 0.02	30	PZ	0.34 ± 0.04	30	9	“
16	A	0.22 ± 0.03	40	PZ	0.27 ± 0.02	40	9	Reis <i>et al.</i> 2014a
17	A	0.22 ± 0.03	40	GZ	0.30 ± 0.03	40	9	Reis <i>et al.</i> 2014a
18	A	0.90 ± 0.03	6	PZ/Ac	1.10 ± 0.08	6	30	Estévez <i>et al.</i> 2009
19	A	0.83 ± 0.09	30	A	0.80 ± 0.10	30	25	Seixas, 2009
20	A	0.68 ± 0.02	24	A	0.68 ± 0.03	24	20	Villanueva <i>et al.</i> 2004
21	A	0.65 ± 0.02	24	A	0.57 ± 0.02	24	20	Villanueva <i>et al.</i> 2004
22	A	0.83 ± 0.09	30	A	0.87 ± 0.08	30	25	Seixas, 2009
23	A	0.50 ± 0.07	15	A	0.44 ± 0.06	15	15	Seixas <i>et al.</i> 2010
24	A	0.80 ± 0.09	30	A	0.74 ± 0.10	30	25	Seixas <i>et al.</i> 2010
25	A	1.62 ± 0.39	20	A	0.93 ± 0.08	20	30	Fuentes <i>et al.</i> 2011
26	A	1.76 ± 0.28	10	A	1.88 ± 0.22	10	28	Viciano <i>et al.</i> 2011

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Abbreviations: DW: dry weight. N: number of data. Age: paralarvae days old. Ref.: bibliographic references/ PE: data of performed experiments. A: *Artemia*. GZ: *Grapsus adscensionis* zoea. MZ: *Maja brachydactyla* zoea. PZ: *Palaemon sp.* zoea. Ac: *Acartia sp.* Data are presented as mean±SD (Standard Deviation)

554 **Table 2. Rearing conditions of performed experiments**

N° study	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Research Center	VG	VG	IR	TF	TF	IR	IR	TF	TF	TF	TF	TF	VG	VG	TF
Trial days	30	30				14		14	14	14	14	14	14	30	9
Tank volume (L)	800	500				100		100	100	100	100	100	500	500	100
Tank colour	B	B				B		B	W-B	W-B	W-B	W-B	B	B	W-B
Flow (mL·s⁻¹)[†]	56	17				10		10	4	4	1	1	56	56	1
Renovation (h)	‡	14				14		14	24	24	24	24	‡	‡	24
Aeration	C	C				L		L	L	L	L	L	C	C	L
Skimmer	Yes	Yes				-		-	-	-	-	-	Yes	Yes	-
Exit mesh (µm)	500	500				500		363	363	363	363	363	500	500	363
Light (h)	12	12				12		12	12	12	12	12	24	24	12
Light (lux)	1000	700				200		200	200	200	200	200	1000	1000	200
Light type	F2	F2				F1		F1	F1	F1	I-B	I-B	F2	F2	I-B
Replicates (n° tanks)	2	3				6		5	4	4	6	6	2	2	3
Paralarval density (ind·L⁻¹)	5	6				10		10	3	3	3	3	10	11	1.5
Green water sp.	I+N	N				-		-	-	-	Ch	Ch	I+N	I+N	Ch
Green water (10⁶ cells/mL)	0.3+1	0.25				-		-	-	-	0.2	0.2	0.3+1	0.3+1	0.2
Temperature (°C)	21.5	21.5	21.5	22.7	19.8	21.5	21.5	22.1	24	24	21.6	21.6	21.5	21.5	21
Salinity (PSU)	35.0	35.0	35.5	36.8	36.8	35.0	35.0	36.8	36.8	36.8	36.8	36.8	35.0	35.0	36.8

555 *Abbreviations:* IR: Research & Technology Food & Agriculture Center. TF: Oceanographic Center of the Canary Islands. VG: Oceanographic
556 Center of Vigo. B: black. W-B: white bottom and black walls. C: Gentle and central. L: Gentle and lateral. F1: OSRAM Dulux superstar
557 21W/840. F2: OSRAM Dulux Superstar 36W/840. I-B: 40 W Incandescent bulb. I: *Isochrysis galbana*. N: *Nannchloropsis* sp. Ch: *Chlorella* sp.
558 *Symbols:* † Closed seawater system was just used in IR centre. ‡ Open 4h from 5th to 15th and 24h until 30th day.
559

560 **Table 3. Preys enrichment and feeding**

N° Study	1 [†]	2 [†]	3 [†]	4 [†]	5	6	7	8	9	10	11	12	13	14 [†]	15
Research Center	VG	VG	IR	TF	TF	IR	IR	TF	TF	TF	TF	TF	VG	VG	TF
Trial days	30	30			15			15	15	15	15	15	15	30	9
CONTROL															
Larval Feeding	Prey	AF	AG [‡]		AG [‡]			AG [‡]	AG	AG	AG	AG	AG	AG	AG
	Prey age[§]	1/4	1/4		1			1	1	1	1	1	1	1	1/4
	Feeding rate	0.3/0.3	0.3/0.15		0.3			0.3	0.08	0.08	0.07	0.07	0.5-1	0.5-1	0.04
Prey Enrichment	Diet	I/N	I/N		I			I	N	N	N	N	I	I/N	T
	Diet concentration	1/10	1/10		1			1	63	63	10	10	0.5	0.5/10	0.4
	Prey density	10/5	10/5		8			50	250	250	7	7	0.5	0.5/0.5	10
	time (h)	20/20	20/20		20			20	8	8	20	20	20	20/20	20
EXPERIMENTAL															
Larval Feeding	Prey	AF	AG [‡]		AG [‡]			AG [‡]	AG	AG	AG	GZ	MZ+AG [¶]	MZ+AG ^{¶,»}	PZ+AG [¶]
	Prey age[§]	1/4	1/4		1			1	1	1	8	1	1	1	1
	Feeding rate	0.3/0.3	0.3/0.15		0.3			0.3	0.08	0.08	0.06	0.07	0.01	0.01/0.001	0.001
Prey Enrichment	Diet	LC/LC	LC/LC		LC			I+LC	LC	Nr	N	-	-	-	-
	Diet concentration	0.6	0.6		0.6			1+0.6	0.6	0.24	10	-	-	-	-
	Prey density	125/50	250/50		250			50	250	250	7	-	-	-	-
	time (h)	3/3 [*]	8/6 [*]		8 [*]			20 [▲]	8 [*]	8	20	-	-	-	-

561 *Abbreviations:* IR,TF,VG, I and N: see Footnote Table 2. AF: *Artemia* AF. AG: *Artemia* EG. AG[‡]: *Artemia* Sept-Art EG. T: *Tetraselmis chuii*. GZ:
562 *Grapsus adscensionis* zoea. MZ: *Maja brachydactyla* zoea. PZ: *Palaemon elegans* zoea. LC: Lécithine Marine Naturelle LC60 (g·L⁻¹). Nr:
563 *Haematococcus pluvialis* (g·L⁻¹).
564 *Units:* Prey age (days). Feeding rate (individual·mL⁻¹·day⁻¹). Diet concentration (Phyto (I, N and T): x10⁶ cells·mL⁻¹/other enrichments (LC and Nr): g·L⁻¹).
565 Prey density (individual·mL⁻¹).

566 *Symbols:* †Experiments carried out in two phases (0-15/16-30days). § See Table 4 for the details of the on-growing *Artemia* (≥ 4 days-old). ¶ Co-feeding:
567 values showed below correspond to Zoea. *Artemia* values as the control treatment. ”Gemma diamond 0.8 from 24 days-old (1g/day). **Artemia* was starved
568 for 12h before enrichment. ^ 12h with I + 8h with I +LC.
569

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570 **Table 4. On-growing *Artemia* parameters**

N° study	1	2	3	4	11	14	15
Research Center	VG	VG	IR	TF	TF	VG	TF
Strains	AF	AG [‡]			AG	AG	AG
Prey age	3	3			7	3-5	7
Prey density	5	5			10	5	10
Diet	I	I			T	I	T
Diet concentration	4	4			4	5	4

571 *Abbreviations*: see Footnote table 3572 *Units*: see Footnote Table 3. Diet concentration: (10^5 cells·mL⁻¹).

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573 **Figure legends**

574

575 **Figure 1.** Meta-analysis results comparing effect of paralarvae fed crustacean zoeae (n=7) vs
576 *Artemia* (n=19). They are presented as effect (symbol) plus 95% confidence interval (horizontal
577 bar). Heterogeneity between studies (Q-test values) has been included.

578

579 **Figure 2.** Meta-analysis results comparing effect of paralarvae fed different zoeae species
580 (n=7). They are presented as effect (symbols) plus 95% confidence interval (horizontal bar).
581 Heterogeneity between studies (Q-test values) has been included.

582

583 **Figure 3.** Meta-analysis results comparing the effect of paralarvae fed marine phospholipids
584 (Marine lecithin LC60) (n=9) vs other *Artemia* enrichments (n=10). They are presented as effect
585 (symbols) plus 95% confidence interval (horizontal bar). Heterogeneity between studies (Q-test
586 values) has been included.

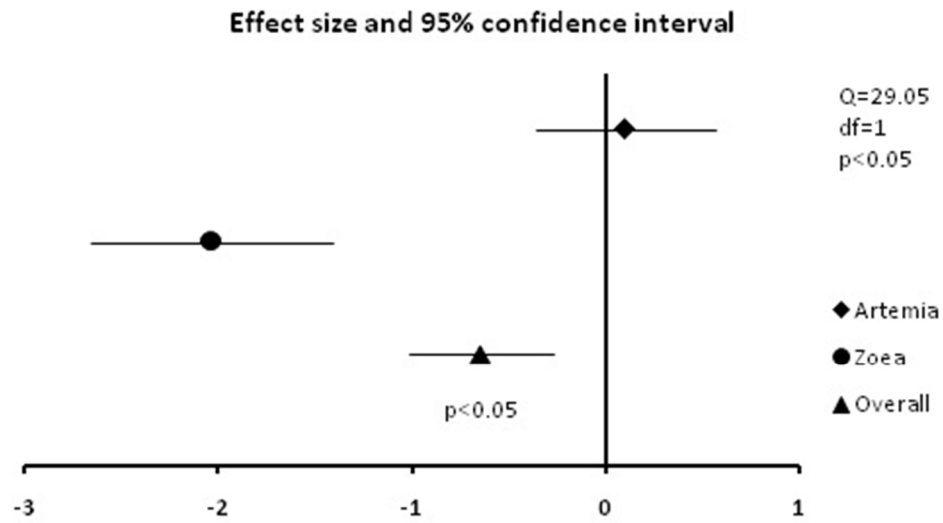


Figure 1. Meta-analysis results comparing effect of paralarvae fed crustacean zoeae (n=7) vs Artemia (n=19). They are presented as effect (symbol) plus 95% confidence interval (horizontal bar). Heterogeneity between studies (Q-test values) has been included.
135x78mm (96 x 96 DPI)

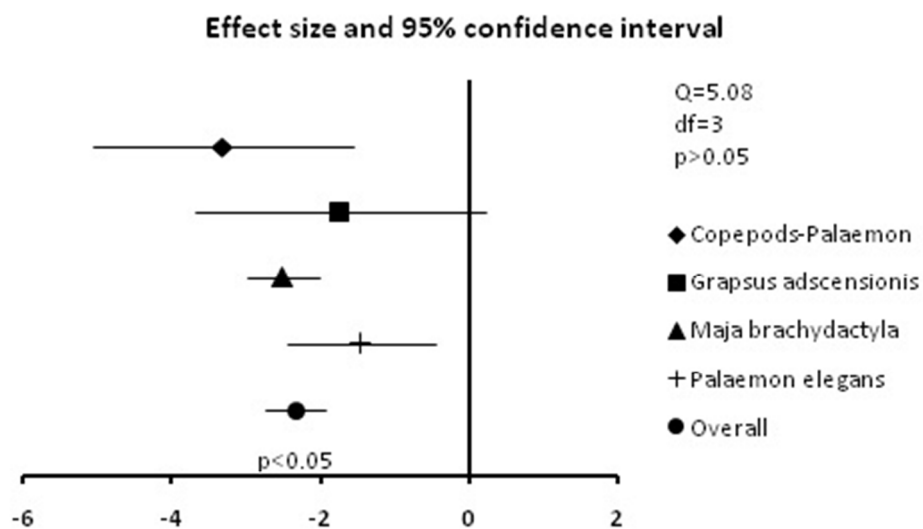


Figure 2. Meta-analysis results comparing effect of paralarvae fed different zoeae species (n=7). They are presented as effect (symbols) plus 95% confidence interval (horizontal bar). Heterogeneity between studies (Q-test values) has been included.

128x76mm (96 x 96 DPI)

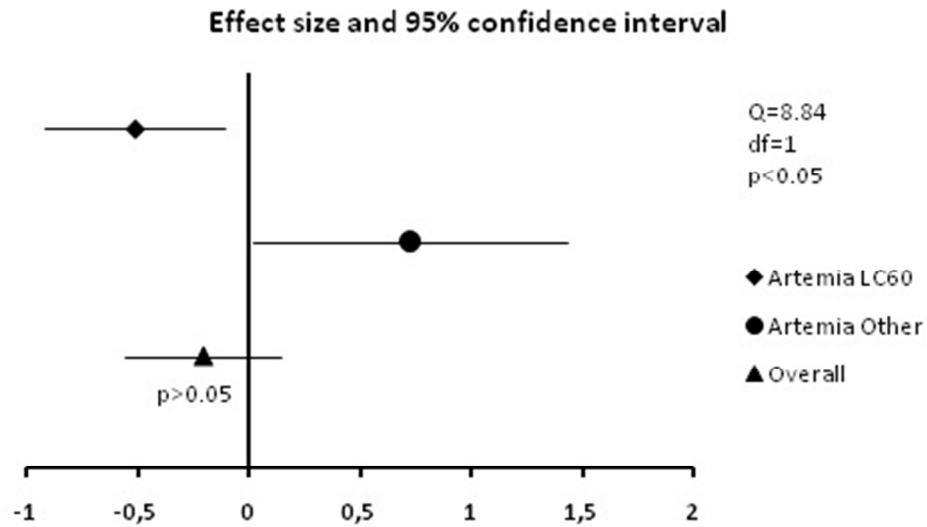


Figure 3. Meta-analysis results comparing the effect of paralarvae fed marine phospholipids (Marine lecithin LC60) (n=9) vs other Artemia enrichments (n=10). They are presented as effect (symbols) plus 95% confidence interval (horizontal bar). Heterogeneity between studies (Q-test values) has been included.
128x77mm (96 x 96 DPI)