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

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Abstract

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

Key words: growth, meta-analysis, *Octopus vulgaris*, paralarvae, prey.

Introduction

The common octopus (*Octopus vulgaris*, Cuvier 1797) is a species with increasing interest for marine aquaculture diversification, given its high growth rate and easy adaptation to captivity, among other positive features (Iglesias *et al.* 2007, 2014a). However, the massive paralarvae mortalities verified under culture conditions (≈100% in most studies) have hampered its commercial production, therefore making this the main bottleneck for industrial farming. According to several authors (Iglesias *et al.* 2007, 2014a; Iglesias & Fuentes 2013), the high mortalities could be due

to: (i) inadequate and/or unbalanced diets that do not fulfil paralarvae nutritional requirements, (ii) lack of standardized rearing techniques, and (iii) little knowledge about octopus paralarvae physiology and behaviour. Unlike benthic adults, newly hatched paralarvae have a pelagic behaviour that lasts for about 2 months. Thereafter, octopus progressively acquires benthic habits (Villanueva & Norman 2008).

Paralarvae fed crustacean zoeae such as *Maja* or *Pagurus* in co-feeding with *Artemia* have shown the highest growth rates, ranging between 7–8% dry weight·day^{-1}, and attain a development that facilitates their shift from a pelagic to a benthonic life stage (Villanueva 1994; Iglesias *et al.* 2004; Carrasco *et al.* 2006). In addition, Roura *et al.* (2012) has recently shown that, in the wild, paralarvae prey on an wide list of different preys, where crustacean zoeae are preferably selected. However, it is not economically viable to produce crustacean zoeae for feeding octopus paralarvae due to the high commercial value of these crustacean species and the lack of technology to produce those (Andrés *et al.* 2007, 2010). As a result, current research has been focused on the use of *Artemia*, which is the standard live prey used in marine larviculture (Sorgeloos *et al.* 2001). However, *Artemia* displays a nutritional profile less suitable for octopus paralarvae than zoeae of crustaceans, even after enrichment (Navarro & Villanueva 2000; Bell *et al.* 2003; Hormiga *et al.* 2010). Most studies of *O. vulgaris* culture using *Artemia* have promoted paralarvae growth rates between 2–4% dry weight·day^{-1} (Navarro & Villanueva 2000; Villanueva *et al.* 2004; Estévez *et al.* 2009; Seixas *et al.* 2010a,b; Reis *et al.* 2015), while few authors have reported growth rates over 6% (Villanueva *et al.* 2002; Okumura *et al.* 2005; Kurihara *et al.* 2006; Arai *et al.* 2008; Fuentes *et al.* 2011; Viciano *et al.* 2011).

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

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

An approach to overcome these problems is to standardise paralarval production and culture protocols among different centres. To reach this goal, different preys, enrichments and rearing conditions were tested under project OCTOPHYS, including the use of *Artemia* enriched with LC as food for *O. vulgaris* paralarvae. Even though, this strategy still produced a large volume of information together with that already available in literature. In this sense, a meta-analysis approach allows the comparison of results from independent studies to get reliable conclusions and avoid subjectivity and variability (Walker *et al.* 2008).

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

Materials and methods

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

Reference papers

A total of 98 and 49 scientific contributions were found in April 2014 in the Web of Science and Scopus, respectively, using the key-word: *Octopus vulgaris* paralarvae. Other bibliography sources such as JACUMAR (Spanish National Advisory Board for Marine Aquaculture) reports, conference communications and PhD theses dealing with paralarval culture, were also considered. However, it should be emphasized that only 5 papers of Web of Science and Scopus, 1 PhD Thesis and 1 conference communication, presented the data as required by the meta-analysis

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Table 1 Studies included in meta-analysis

No 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	PE
3	A	1.47 ± 0.36	8	A	2.38 ± 0.35	8	30	PE
4	A	0.66 ± 0.07	11	A	0.76 ± 0.22	10	30	PE
5	A	0.41 ± 0.05	15	A	0.45 ± 0.05	15	14	PE
6	A	0.48 ± 0.08	30	A	0.47 ± 0.08	30	14	PE
7	A	0.60 ± 0.11	30	A	0.67 ± 0.14	30	14	PE
8	A	0.43 ± 0.05	15	A	0.46 ± 0.07	16	14	PE
9	A	0.33 ± 0.08	12	A	0.33 ± 0.05	12	14	PE
10	A	0.33 ± 0.08	12	A	0.32 ± 0.36	12	14	PE
11	A	0.48 ± 0.18	6	A	0.45 ± 0.17	6	14	PE
12	A	0.48 ± 0.18	6	GZ	0.58 ± 0.11	6	14	PE
13	A	0.77 ± 0.12	30	MZ	1.11 ± 0.13	30	14	PE
14	A	0.78 ± 0.12	30	MZ	1.31 ± 0.30	30	30	PE
15	A	0.31 ± 0.02	30	PZ	0.34 ± 0.04	30	9	PE
16	A	0.22 ± 0.03	40	PZ	0.27 ± 0.02	40	9	Reis *et al.* 2015;
17	A	0.22 ± 0.03	40	GZ	0.30 ± 0.03	40	9	Reis *et al.* 2015;
18	A	0.90 ± 0.03	6	PZ/Ac	1.10 ± 0.08	6	30	Estévez *et al.* 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 *et al.* 2004;
21	A	0.65 ± 0.02	24	A	0.57 ± 0.02	24	20	Villanueva *et al.* 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 *et al.* 2010b;
24	A	0.80 ± 0.09	30	A	0.74 ± 0.10	30	25	Seixas *et al.* 2010a;
25	A	1.62 ± 0.39	20	A	0.93 ± 0.08	20	30	Fuentes *et al.* 2011;
26	A	1.76 ± 0.28	10	A	1.88 ± 0.22	10	28	Viciano *et al.* 2011

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).

(experimental and control treatments, mean, standard deviation and number of replicates). These references yield a total of 11 bibliographic inputs used (see Table 1).

Rearing conditions

Specific experiments were performed and data of paralarval rearing conditions is summarized according to: a) Rearing conditions (Table 2), b) The on-growing *Artemia* (Table 3) and c) Prey enrichment and feeding (Table 4). Broodstock conditions were as described by Reis *et al.* (2015) for IR and TF and Iglesias *et al.* (2014a) for VG.

Newly hatched paralarvae were cultured in fiberglass cylinder-conical tanks (conditions are summarized in Table 2). In IR, tanks were connected to a recirculation unit IRTAMar™. Physicochemical parameters such as oxygen, salinity and temperature were measured daily and nitrite and ammonium once a week. Dissolved oxygen levels were kept close to saturation and nitrite and ammonia were <0.3 mg L^{-1} and 0 mg L^{-1}, respectively, in all experiments. Salinity and temperature data are shown in Table 2.

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

Crustacean zoeae of different species were used as experimental diet in experiments 12 to 15 (Tables 2 and 3). *Maja brachydactyla* zoeae (experiments 13 and 14) were obtained as described by Iglesias *et al.* (2014a). The production

Table 2 Rearing conditions of performed experiments

No study	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Research Centre	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 ($mL{\cdot}s^{-1}$)†	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 ($ind{\cdot}L^{-1}$)	5	6			10			10	3	3	3	3	10	11	1.5
Green water sp.	I+N	N			–	–	N	–	–	–	Ch	Ch	I + N	I + N	Ch
Green water (10^6 cells/mL)	0.3 + 1	0.25			–	–	1	–	–	–	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

IR, Research & Technology Food & Agriculture Center; TF, Oceanographic Center of the Canary Islands; VG, Oceanographic Center of Vigo; B, black; W-B, white bottom and black walls; C, Gentle and central; L, Gentle and lateral; F1, OSRAM Dulux superstar 21W/840; F2, OSRAM Dulux Superstar 36W/840; I-B, 40 W Incandescent bulb; I, *Isochrysis galbana*; N, *Nannchloropsis* sp.; Ch, *Chlorella* sp.

†Closed seawater system was just used in IR centre.

‡Open 4 h from 5^{th} to 15^{th} and 24^{th} until 30^{th} day.

Table 3 Preys enrichment and feeding

No Study	1†	2†	3†	4†	5	6	7	8	9	10	11	12	13	14†	15
Research Centre	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	1/4	8
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	–	–	–	–

IR,TF,VG, I and N, see Footnote Table 2; AF, *Artemia* AF; AG, *Artemia* EG; T, *Tetraselmis chuii*; GZ, *Grapsus adscensionis* zoea; MZ, *Maja brachydactyla* zoea; PZ, *Palaemon elegans* zoea; LC, Lécithine Marine Naturelle LC60 ($g{\cdot}L^{-1}$); Nr, Haematococcus pluvialis ($g{\cdot}L^{-1}$).

Prey age (days). Feeding rate ($invidual{\cdot}mL^{-1}{\cdot}day^{-1}$). Diet concentration (Phyto (I. N and T): $x10^6$ $cells{\cdot}mL^{-1}$/other enrichments (LC and Nr): $g{\cdot}L^{-1}$). Prey density ($individual{\cdot}mL^{-1}$).

†Experiments carried out in two phases (0–15/16–30 days).

‡AG, *Artemia* Sept-Art EG.

§See Table 4 for the details of the on-growing *Artemia* (≥4 days-old).

**Co-feeding: values showed below correspond to Zoea. *Artemia* values as the control treatment.

††Gemma diamond 0.8 from 24 days-old (1 g/day).

§§*Artemia* was starved for 12 h before enrichment.

¶¶12 h with I + 8 h with I +LC.

Table 4 On-growing *Artemia* parameters

No study	1	2	3	4	11	14	15
Research Centre	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

See Footnote Table 3.
Diet concentration, 10^5 cells·mL^{-1}.

methodology and handling of *Grapsus adscensionis* zoea and *Palaemon sp.* zoea (experiments 12 and 15) were as described in Reis *et al.* (2015).

The *Artemia* cysts were obtained from INVE Aquaculture (Dendermonde, Belgium), fresh *Nannochloropsis* sp. was supplied by Necton, Companhia Portuguesa de Culturas Marinhas, S.A. (Olhão, Portugal), freeze dried *Isochrysis galbana*., *Nannochloropsis* sp. and *Tetraselmis chuii* by Fitoplancton marino S.L (Cádiz, Spain), *Haematococcus pluvialis* was provided by Sainhall Nutrihealth Pte Ltd (Singapour), Marine lecithin LC60® (LC) was supplied by PhosphoTech Laboratories (St. Herblain, France) and Gemma Diamond 0.8 was supplied by Skretting Spain S.A. (Burgos. Spain).

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

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

Statistical analysis

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

In the meta-analysis plots, the effect size on the left from vertical axis indicated that a given experimental treatment improved the dry weight of paralarvae respect to control, when the confidence interval of 95% (CI) rank did not intercept the vertical axis. To confirm the correct choice of the Random effects model, the variability among studies was run as comparable heterogeneity analysis (*Q*). *P* value <0.05 was considered significant.

After the bibliographic research, only the references which fulfil meta-analysis requirements were included in the statistical analysis. Some studies could not be included due to the lack of a control treatment or standard deviation (e.g. Itami *et al.* 1963; Villanueva 1995; Navarro & Villanueva 2000; Moxica *et al.* 2002, 2006; Iglesias *et al.* 2004, 2014a; Socorro *et al.* 2004; Carrasco *et al.* 2006). First, to compare the effects of Crustacean zoea vs. *Artemia* a total of 26 inputs, 7 using crustacean zoeae (see Table 1, inputs 12 to 18) and 19 using *Artemia* (see Table 1, inputs 1 to 11 and 19 to 26) were analysed. Then, in zoeae from different crustacean species comparison, seven inputs from genera *Maja, Palaemon, Grapsus* and the copepod *Acartia* were used (see Table 1, inputs 12 to 18). Finally, a total of 19 inputs were used to compare the effect of *Artemia* enriched with LC vs. other *Artemia* enrichments, 9 for LC (see Tables 1 and 4, inputs 1 to 9) and 10 for other *Artemia* enrichments (see Tables 1 and 4, inputs 10, 11 and 19 to 26).

Results and discussion

Crustacean zoeae vs *Artemia*

Results obtained on the effects of crustacean zoeae vs. *Artemia* using a meta-analysis approach are shown in Fig. 1. The overall model (Overall) showed a significant increase on paralarval dry weight of ($P = 0.001$) derived from the individuals fed with zoeae, which displayed a positive effect ($P = 0.001$). Contrarily, *Artemia* was represented on the right side of the vertical axis indicating that this prey did not improve the dry weight of *O. vulgaris* paralarvae ($P = 0.654$). Zoeae and *Artemia* showed heterogeneity ($Q = 29.05$, $P = 0.000$).

The meta-analysis results confirm statistically the suitability of crustacean zoeae compared with *Artemia* in paralarval culture. This conclusion is in agreement with previous studies using crustacean zoeae (Itami *et al.* 1963; Villanueva 1995; Moxica *et al.* 2002; Iglesias *et al.* 2004; Morote *et al.* 2005; Socorro *et al.* 2004; Carrasco *et al.* 2006; Iglesias *et al.* 2007, 2014a) or *Artemia* under different enrichments (Navarro & Villanueva 2000; Moxica *et al.* 2006; De Wolf *et al.* 2011). Similarly, Iglesias and Fuentes (2013) pointed out that the growth obtained adding zoea

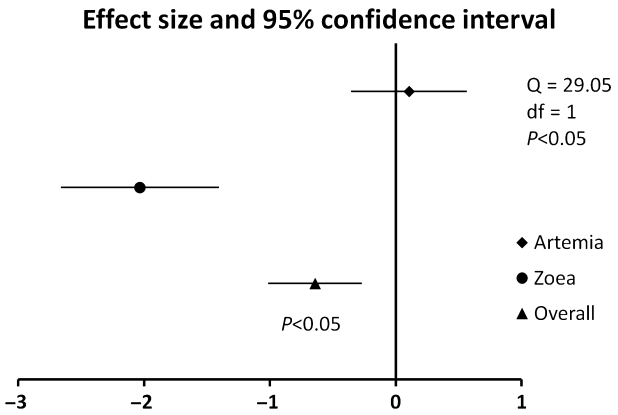

Figure 1 Meta-analysis results comparing effect of paralarvae fed crustacean zoeae (n = 7) vs. *Artemia* (n = 19). They are presented as effect (symbol) plus 95% confidence interval (horizontal bar). Heterogeneity between studies (Q-test values) has been included.

can be six-fold higher than that achieved with *Artemia*. Furthermore, paralarvae fed with zoeae in some cases reached the benthic stage (Itami *et al.* 1963; Villanueva 1995; Iglesias *et al.* 2004; Carrasco *et al.* 2006). In contrast, settlement of paralarvae fed with *Artemia* has rarely been achieved, requiring a longer rearing period than paralarvae fed with zoeae (Moxica *et al.* 2006; De Wolf *et al.* 2011). Several studies using *Artemia* (Moxica *et al.* 2006; Fuentes *et al.* 2011; Viciano *et al.* 2011) displayed a higher dry weight gain at 30 days, reaching 1.6–1.8 mg (SGR of 5–6%·DW day^{-1}) but this is still below that achieved with crustacean zoeae (2.5–3.5 mg, SGR of 7–8%·DW day^{-1}; Villanueva 1995; Iglesias *et al.* 2004; Carrasco *et al.* 2006; Iglesias *et al.* 2014a).

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

Another relevant aspect is the composition of prey, specifically the HUFA and DHA contents. Similar to what has been widely demonstrated in fish larvae, the importance of DHA in the physiology of paralarvae may be related with visual and neuronal development as have been suggested by numerous studies (Navarro & Villanueva 2000, 2003; Tocher 2010 and Takeuchi 2014). Newly hatched *O. vulgaris* display a high DHA content ranging between 17–27% of total FA (Navarro & Villanueva 2000; Okumura *et al.* 2005; Kurihara *et al.* 2006; Arai *et al.* 2008; Seixas *et al.* 2010a,b; Reis *et al.* 2015), similar to the levels observed in recently settled wild juveniles with 15–25% of total FA (Navarro & Villanueva 2003). In contrast, the DHA content tended to gradually decrease (46–76% from hatching to 30 days old) in paralarvae fed exclusively on *Artemia*, regardless of the enrichment used (Navarro & Villanueva 2000; Estévez *et al.* 2009; Seixas *et al.* 2010a,b; Reis *et al.* 2015). Nevertheless, paralarvae were able to maintain the original levels of DHA throughout development when were fed on a mixture of *Artemia* and sand eel (*Ammodytes personatus*) flakes (Okumura *et al.* 2005).

O. vulgaris shows little or no ability to synthesise DHA, as reported by Monroig *et al.* (2013) and Reis *et al.* (2014). Therefore, this FA should be provided in the diet at appropriate levels. While, spider crab zoeae display levels of DHA between 8.7–15.8% of total FA (Seixas 2009; Andrés *et al.* 2010 and Iglesias *et al.* 2014a), the basal levels of DHA in *Artemia* are negligible (0.1% DHA; Okumura *et al.* 2005; Reis *et al.* 2015). The use of different enrichment techniques has improved up to 2.3 and 8.0% of DHA (Navarro & Villanueva 2000 and Seixas *et al.* 2010a; respectively, among others). Paralarval viability was slightly improved with these *Artemia* enrichments, but it was not enough to maintain DHA levels in paralarvae (Navarro & Villanueva 2000; Estévez *et al.* 2009; Seixas *et al.* 2010a,b; Takeuchi 2014; Reis *et al.* 2015).

These differences between zoea and *Artemia* can be due to other factors related to the bioavailability of DHA. In most species, DHA is mainly esterified in polar lipids (PL), such as phosphatidylethanolamine or phosphatidylcholine (Kanazawa & Shunsuke 1994; Salhi *et al.* 1999). However, Bell *et al.* (2003) showed that *Artemia* enriched with DHA accumulated most of this FA in neutral lipid (NL). More recently, Guinot *et al.* (2013b) obtained a similar esterification into NL even when DHA was provided as PL to *Artemia* during enrichment. In fish and cephalopods, diets containing PL have higher apparent lipid digestibility than diets containing high amount of NL, due to the emulsifying properties of PL that improve their digestion and absorption by larvae (Koven *et al.* 1993; Morillo-Velarde *et al.* 2014; Olsen *et al.* 2014). This could be due to the absence of lipid emulsifiers in the digestive tract of cephalopods (Vonk 1962; O'Dor *et al.* 1984). Accordingly, these results suggest that *Artemia* metabolism, which allocates DHA in the NL fraction, could diminish the bioavailability of this FA compared with crab zoeae.

Other nutrients such as copper, aminoacids (AA) or vitamins might have an influence on the dry weight of paralarvae. Copper plays an essential role in oxygen transport as a constituent of haemocyanin, the main respiratory pigment in cephalopods. In addition, copper content decreases

when paralarvae are fed with *Artemia* nauplii from 217 $\mu g \cdot g^{-1}$ DW in hatchlings to 92 $\mu g \cdot g^{-1}$ DW in 20 days-old paralarvae (Villanueva & Bustamante 2006). This could be related with the low copper content of *Artemia* (7 $\mu g \cdot g^{-1}$ DW), which contrast with the values found in *M. brachydactyla* zoea (73 $\mu g \cdot g^{-1}$ DW) (Villanueva & Bustamante 2006). On the other hand, the profile of total aminoacids does not seem to be a limiting factor, since the composition of enriched *Artemia* metanauplii, *Pagurus prideaux* zoea and *M. squinado* zoea is similar (Villanueva *et al.* 2004). As regards the vitamin content, enriched *Artemia* (DC Super Selco and L-methionine) and *M. brachydactyla* zoea, have similar vitamin E content (428 and 584 $\mu g \cdot g^{-1}$ DW, respectively) (Villanueva *et al.* 2009). Moreover, the contents of other nutrients not yet evaluated may be important, namely carotenoids, carbohydrates, other vitamins, etc.

Relation among zoeae from different crustacean species

O. vulgaris paralarvae have been fed on several crustacean species such as *M. brachydactyla* (Moxica *et al.* 2002; Iglesias *et al.* 2004, 2014a; Carrasco *et al.* 2006), *Grapsus adscensionis* (Socorro *et al.* 2004; Reis *et al.* 2015), *Palaemon sp.* (Socorro *et al.* 2004; Estévez *et al.* 2009; Reis *et al.* 2015), *P. prideaux* (Villanueva 1995), *Linocarcinus depurator* (Villanueva 1995), *Acartia sp.* (Iglesias *et al.* 2007; Estévez *et al.* 2009) and *Palaemon serratus*, *Moina salina* and *Maja squinado* (Morote *et al.* 2005). The results obtained among different studies suggest a species-specific effect on paralarval viability, which was tested through the meta-analysis.

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

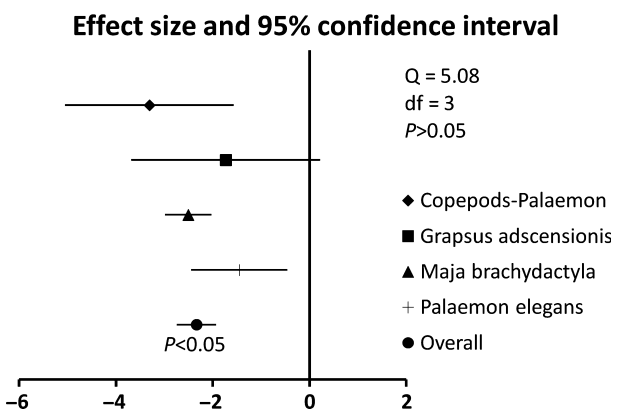

Figure 2 Meta-analysis results comparing effect of paralarvae fed different zoeae species ($n = 7$). They are presented as effect (symbols) plus 95% confidence interval (horizontal bar). Heterogeneity between studies (*Q*-test values) has been included.

These results obtained in the meta-analysis related to *G. adscensionis zoeae* were probably due to its lower nutritional value, given that this species showed a lower DHA content (2.6% of total fatty acids, Reis *et al.* 2015) when compared with *M. brachydactyla* (12.8–15.1%, Andrés *et al.* 2010; Iglesias *et al.* 2014a), *P. elegans* (13.4%, Reis *et al.* 2015), *P. prideaux* (18.1%, Navarro & Villanueva 2000) or the mysid *Acanthomysis longicornis* (24.0%, Navarro & Villanueva 2000). It should be noted also that *G. adscensionis* is a species with relatively lower copper content (7.4 ± 2.5 $\mu g\ g^{-1}$ DW, Martín *et al.* 2011) when compared with *M. brachydactyla* (50.0–72.5 $\mu g\ g^{-1}$ DW, Andrés *et al.* 2010; Villanueva & Bustamante 2006). In addition, the size of *G. adscensionis* could influence the results obtained, since this species has a smaller carapace length (CL) and lower DW (0.45 mm and 0.02 mg, respectively) than other zoeae species, such as *L. depurator* (CL 0.52 mm), *P. prideaux* (CL 1.18 mm), *Dardanus arrosor* (CL 1.44 mm) (Villanueva 1994) and *M. brachydactyla* (CL 1.01 mm and DW 0.109 mg) (Andrés *et al.* 2007).

Paralarvae fed on *Maja* and *Palaemon* zoeae as well as *Acartia* showed increased DW with respect to the control group (*Artemia*), confirming the positive effects of these zoeae in paralarval growth. However, the fluctuations in quality regarding biochemical composition (among other features) of newly hatched zoeae or copepods throughout the year, the lack of specific culture technology, and the economic value of these species (many of them used for human consumption) have hampered its commercial production for paralarvae culture (Andrés *et al.* 2007, 2010). In consequence, crustacean zoeae do not seem to be suitable for the commercial productions of *O. vulgaris* paralarvae. However, as mentioned before, an economically feasible prey as *Artemia* has displayed serious limitations in the paralarval culture when conventional enrichments (such as lipid emulsions or phytoplankton) were used. This issue lead us to contemplate alternative *Artemia* enrichments, taking into account other relevant factors such as copper, vitamins, essential aminoacids, fatty acid ratios,

non-protein nitrogen substances as taurine, as well as alternative ways to supply DHA and polar lipids to paralarvae.

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

As previously mentioned, DHA and PL seem to be essential in the physiology of octopus paralarvae. However, *Artemia* shows a profile poor in these lipid components. Guinot *et al.* (2013a,b) have demonstrated that the use of marine phospholipids such as marine lecithin LC60® (LC) as enrichment improved the content of DHA and PL in *Artemia*. Therefore, the next step was to compare the effect of this product on paralarval DW gain with other *Artemia* enrichments, tested either individually or in combination. The enrichments considered were different phytoplankton species (*Isochrysis* galbana, *Nannochloropsis* sp., *Haematococcus pluvialis*, *Tetraselmis chuii*, *Rhodomonas lens*), free L-amino acids (lysine, arginine, and methionine), commercial enrichments (Ori-Gold®, DC Super Selco®, Easy DHA-Selco®), M70 (a lipid enrichment used by Viciano *et al.* 2011) and crushed wild zooplankton (see Table 1 and 4). Other enrichments such as *Phaeodactylum tricornutum*, Krill powder, Red-pepper®, Algamac®, Multigain®, Ori-Prot®, Ori-Culture® and Ori-Green® have been cited in the literature, but they were not included in the meta-analysis due to the lack of statistical requirements.

Results showed that *Artemia* fed with LC improved paralarvae DW ($P = 0.014$), whereas other *Artemia* enrichments showed a decreased in DW ($P = 0.044$) (Fig. 3). Results from the overall model (which include LC as well as other enrichments) did not show any significant effect on paralarval DW ($P = 0.259$), since differences between LC and other *Artemia* enrichments displayed high heterogeneity ($Q = 8.84$, $P = 0.003$). These results suggest that marine phospholipids (LC) seem to have a beneficial effect on paralarvae, with respect to other enrichments, improving their growth.

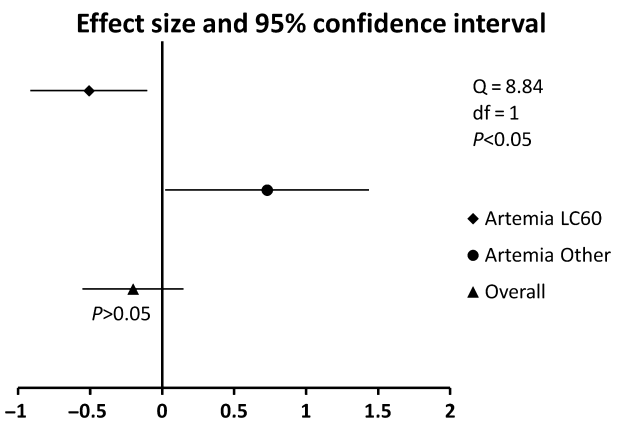

Figure 3 Meta-analysis results comparing the effect of paralarvae fed marine phospholipids (Marine lecithin LC60) ($n = 9$) vs. other *Artemia* enrichments ($n = 10$). They are presented as effect (symbols) plus 95% confidence interval (horizontal bar). Heterogeneity between studies (*Q*-test values)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

In addition, the use of *Artemia* enriched with LC has been reported to promote a significant increase ($P < 0.05$) of the HUFA content (including DHA) in paralarvae when compared with other *Artemia* enrichments (8.3 vs. 6.2% DHA of the total FA, respectively) (Garrido *et al.* 2013). Moreover, the use of the LC enrichment promoted an increase in the PL fraction in *Artemia* (Guinot *et al.* 2013b). Therefore, the beneficial effects of LC on paralarval dry weight gain could be related to improvements in lipid composition of *Artemia*. However, further studies are necessary to establish the lipid requirements of paralarvae during their pelagic stage (especially in HUFA and PL) as well as the metabolism and bioavailability of these lipid components in *Artemia* and in other suitable types of prey for *O. vulgaris* paralarvae.

Conclusions

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

In consequence, we consider that the future research lines in paralarval culture should include fresh approaches focused in new *Artemia* enrichments. In this sense, we consider interesting to test several parameters such as the copper levels, fatty acids ratios (mainly EPA/AA ratio), other vitamins with antioxidant roles, non-protein nitrogen substance like taurine as well as alternative ways to supply DHA and polar lipids. The development of artificial diets seems to be another reasonable approximation, although some key parameters as an adequate acceptability, binder and composition are serious defiances nowadays. In addition, the effects of environmental and zootechnical factors such as temperature (in eggs and paralarval development) or light conditions should be tested. Finally, a deeper knowledge of the nutrients metabolism as well as the selection of biomarkers capable to an early detection and quantification of the nutritional condition and physiological

stress are imperative, in order to advance in the successful culture of *O. vulgaris* paralarvae.

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