Deliverable Report

Deliverable No:	D14.1	**Delivery Month:**	30
Deliverable Title	Improved larval rearing protocol for meagre that includes weaning at an earlier age leading to reduced cost in live feed production and better quality juveniles		
WP No:	14	**WP Lead beneficiary:**	P3. IRTA
WP Title:	Larval husbandry - meagre		
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Objective: To reduce costs by early weaning in meagre (*Argyrosomus regius*) larvae and improve growth, survival and larval quality.

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DESCRIPTION

In order to improve the larval rearing protocol for meagre (*Argyrosomus regius*), it is desirable to wean the fish onto a dry feed as soon as possible to improve juvenile quality and reduce costs. The present study tested the effect of advancing the weaning age by feeding available weaning diets such as Gemma Micro (Skretting, Norway) or similar at different larval ages. The most effective weaning age was evaluated in terms of larval growth and size dispersion distribution, quality (typology and incidence of skeletal deformations) and maturation of the digestive track, evaluated as the activity of pancreatic and intestinal enzymes. In addition, survival and larval biochemical composition were analyzed at the end of the experiment and compared to control larvae. The deliverable is an improved protocol that includes the most effective weaning age that results in better quality juveniles.

INTRODUCTION

Larval rearing of meagre is usually carried out following a protocol based on European sea bass (*Dicentrarchus labrax*) and gilthead sea bream (*Sparus aurata*) larval rearing. However, different studies have revealed that these protocols need to be adapted to the biological demands of this species, as meagre larvae are quite sensitive to stress produced by high light intensity (more than 500 lux), long photoperiods or high densities of live prey (Roo et al., 2010; Vallés & Estévez, 2013). Although the precise nutritional requirements are not completely known, larvae show very good growth and survival rates using commercially available enrichment products for live prey (Vallés & Estévez, 2015). Meagre producers do not consider larval rearing to be a major bottleneck for meagre culture (Lazo et al, 2010). On the other hand, cannibalism and variable size distribution in juveniles are considered a main concern, as they reduce production yield and increase the cost of production. Moreover, these problems may be derived from or modulated by the feeding of live food such as *Artemia* nauplii. Therefore, advancing the early weaning of larvae from its dependence on *Artemia* onto a dry feed is a priority and the major focus of the larval work on meagre. Weaning is defined as the switch from live food to inert diet, at a very critical moment during development, which requires gradual and specific dietary protocols for the success of the process (Parma and Bonaldo, 2013). In this sense, a better knowledge of larval digestive physiology under a new feeding protocol will contribute to the optimization of the weaning process and may help to understand functional limitations in the processing capacity of the digestive system to deliver nutrients to the rapidly growing larval tissues under an earlier weaning protocol.

Skeletal deformities in cultured fish are a major factor that reduces production, suppresses growth and increases economic loss, as well as leading to high mortality rates. Most skeletal abnormalities appear during the larval and juvenile stages where several factors can interfere with the normal development of larvae. Several studies have outlined the relationships that exist between skeletal deformities and nutrition, environment, and genetics. In this respect nutritional imbalances are known to play a key role in morphogenesis and skeletogenesis at early stages (Boglione et al., 2013). Thus, the efficiency of early weaning practices and its effects on skeletal development in meagre larvae needs to be considered.

MATERIALS AND METHODS

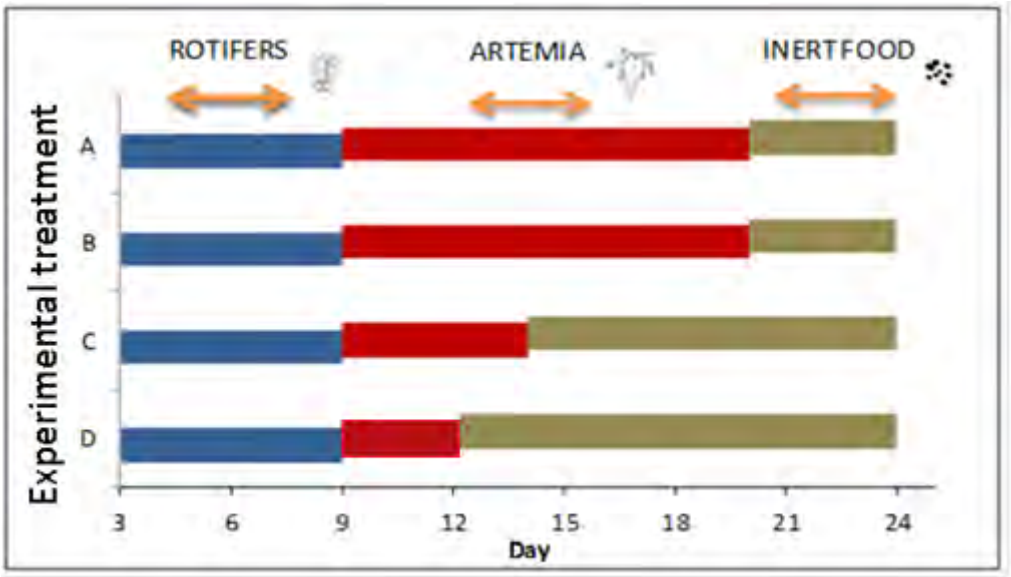
Larval rearing

Fertilized eggs of meagre were obtained in 2014 and 2015 from a wild broodstock maintained in 4000 L circular tanks connected to recirculation units (IRTAMar®) at the P3. IRTA Centre of San Carles de la Rápita (Spain) under controlled conditions and after hormonal induction (Duncan et al., 2012). Floating eggs were stocked in 35 L cylindrical PVC containers provided with airlift systems and high aeration supply. On day 2 post hatching (dph), larvae were stocked into 100 L tanks at a density of 100 larvae L^{-1} and cultured from 2 to 37 dph on different dietary treatments. The 100 L tanks were connected to IRTAMAR® (IRTA, Spain) units with 50% daily water renewal. Temperature (18.2 ± 0.5 ºC), salinity (35.4 ± 0.3 g L^{-1}), dissolved

oxygen (7.9 ± 0.3 mg L^{-1}) and pH (7.9 ± 0.2) were checked daily, whereas nitrites (<0.25 mg L^{-1}) and ammonia (<0.07 mg L^{-1}) were checked once per week (Hach Colorimeter DR/890, USA). Light intensity was maintained at 500 lux at the water surface, and the light regime was 12 h light: 12 h dark. Larvae were fed enriched rotifers (*Brachionus plicatilis*) from 2 dph until 14 dph and *Artemia* metanauplii from 9 dph. Both live preys were enriched using Red Pepper (Bernaqua, Belgium) for 12 h at 28 ºC in the case of rotifers and 6 h at 25 ºC in the case of *Artemia*. Larvae were fed two doses of live prey (morning and evening) every day, whereas dry feed (Gemma Micro, Skretting) was administered by hand every morning at 9h and using automatic feeders every hour, from 9:00 to 20:00h. The amount of feed was adjusted to reach the level of apparent feeding satiation. Every day the bottom of the tanks was siphoned to remove dead fish, uneaten food and faeces.

Two experiments were carried out with meagre larvae in 2014 (Trial 1 with 4 different protocols for early weaning, see scheme below) and 2015 (Trial 2 with 2 protocols for early weaning) as described below and shown schematically.

- Group **A:** Weaning on dry feed started from 20 dph and completed on 30 dph, (control group)
- Group **B:** Weaning started from 20 dph and completed on 30 dph (same as the control but using half the amount of *Artemia* metanauplii)
- Group **C:** Weaning started from 15 and completed on 25 dph
- Group **D:** Weaning started from 12 dph and completed on 23 dph, with three replicates each.



In the experiment carried out in 2014, 10 larvae were randomly collected every week in order to measure growth (standard length and dry weight). Fish were anaesthetized with tricaine methanosulphonate (MS-222, Sigma-Aldrich, Spain). For dry weight, the larvae were placed on glass cover slips, dried in an oven at 60ºC overnight, and weighted in a micro balance (Mettler, MX5, Spain). To examine the development of the digestive system 10 larvae from each of the three replicated tanks were randomly sampled on 10 dph (A,B,C,D groups), 15 dph (D group), 18 dph (C group), 23 dph (A,B,C,D groups) and 35 dph (A,B,C,D groups). Larvae were randomly collected when the weaning started and feed was changed from live to inert diets at 12 dph (D group), 15 dph (C group), 20 dph (A, B groups) and 35 dph, in order to analyse the development of the digestive enzymes, and antioxidant enzyme activities.

Survival was evaluated by counting the animals surviving at the end of the experiment and calculated according to Buckley et al. (1984), which consider the number of sampled individuals during the experiment.

In 2015, the trial was carried out using only two treatments with 5 replicates each.

- Group **A:** Weaning on dry feed started from 20 dph and completed on 30 dph, (control group)
- Group **B:** Started from 10 dph and completed on 23 dph

Considering the results obtained from the trial conducted in 2014, several changes in the rearing protocol were introduced in 2015 to avoid problems associated with larval cannibalism. Thus, light intensity was reduced from 500 lux to 150-200 lux from 13 dph and the number of doses of *Artemia* metanauplii (given at 10, 13, 16 and 18h) and artificial diet were increased in order to ensure providing enough food to the larvae.

Fatty acid analysis

For determination of the fatty acid profile of live prey and larvae, total lipids were extracted from live prey and larvae using chloroform: methanol (2:1, v:v) according to the method of Folch et al. (1957) and quantified gravimetrically after evaporation of the solvent under a nitrogen flow followed by vacuum desiccation overnight. Total lipids were stored in chloroform: methanol (2:1, 20 mg ml^{-1}) containing 0.01% butylated hydroxytoluene (BHT) at −20 °C prior to analysis. Acid catalyzed transmethylation was carried out using the method of Christie (1982). Methyl esters were extracted twice using isohexane diethyl ether (1:1, v:v), purified on TLC plates (Silica gel 60, VWR, Lutterworth, UK) and analysed by gas–liquid chromatography on a Thermo ElectronTraceGC (Winsford, UK) instrument fitted with a BPX70 capillary column (30 m×0.25mm i.d.; SGE, UK), using a two-stage thermal gradient from 50 °C (injection temperature) to 150 °C after ramping at 40 °C min^{-1} and holding at 250 °C after ramping at 2 °C min^{-1}, helium (1.2 ml min^{-1} constant flow rate) as the carrier gas and on column injection and flame ionization detection at 250 °C. Peaks were identified by comparison with known standards (Supelco Inc., Spain) and a well characterized fish oil, and quantified by means of the response factor to the internal standard, 21:0 fatty acid, added prior to transmethylation, using a Chrom-card for Windows (TraceGC, Thermo Finnigan, Italy). Results of fatty acid content are expressed as a percentage of total fatty acids.

Determination of antioxidant enzyme activities

Samples of larvae aged 37 dph obtained in Trial 2 (2015) were washed with distilled water and stored at -80ºC until analysis. Approximately 100 mg of tissue per sample (1-4 individuals, depending of age and size) was homogenized and centrifuged; the supernatant was collected for analytical determinations. Homogenized samples, prepared for the determination of the levels of lipid peroxidation, were used to measure antioxidant enzyme activities. Catalase (CAT, E.C. 1.11.1.6) activity was measured in larvae by the decrease in absorbance at 240 nm (e = 40 $M^{\neg}$ $cm^{\neg}$) using 50 mM H_2O_2 as substrate (Aebi, 1974). Glutathione S-transferase (GST, E.C. 2.5.1.18) activity was assayed by following the formation of glutathione chlorodinitrobenzene adduct at 340 nm (e = 9.6 $mM^{\neg}cm^{\neg}$), using 1 mM 1-chloro-2,4-dinitrobenzene and 1 mM glutathione (GSH) as substrates (Habig et al. 1974). Glutathione reductase (GR, E.C. 1.8.1.7.) activity was determined by measuring the oxidation of NADPH at 340 nm (e = 6.22 $mM^{\neg}cm^{\neg}$), using 20 mM glutathione disulphide and 2 mM NADPH as substrates (Carlberg and Mannervik, 1975). Superoxide dismutase (SOD, E.C. 1.15.1.1) activity was using SOD kit (SIGMA) according to manufacturer´s instructions. Lipid peroxidation, based on Malondialdehyde (MDA) levels, was expressed in nmol MDA/g^{-1} wet weight, the enzymatic activities were expressed as specific enzyme activity in nmol of catalysed substrate per milligram of protein (nmol $mg^{\neg}$ protein) for CAT, GST and GR activities. In the case of SOD, activity was expressed as % of inhibition of enzyme activity. All assays were carried out in triplicate at 25 °C, using a spectrophotometer (Tecan TM infinite M200). Soluble protein of crude enzyme extracts was quantified by the Bradford's method (Bradford, 1976).

Determination of digestive enzyme activities

Pooled samples of larvae in both Trials 1 and 2 (5-150 individuals depending on age and size) were collected for enzyme analysis at 12, 15, 20 and 24 dph for the experiment 2014, whereas larvae from experiment 2015 were sampled at 37 dph. Larvae were completely homogenized for analytical purposes, for quantifying the activity of pancreatic and gastric enzymes (tripsine and total alkaline proteases, α-amylase, lipase and pepsin) the samples were homogenized (Ultra-Turrax T25 basic, IKA©-Werke, Germany) in 5 volumes (v/w) of ice-cold Milli-Q water, centrifuged at 3,300g for 3 min at 4 ºC, and the supernatant removed for enzyme quantification and kept at -80 ºC until further analysis. Samples were homogenized in cold 50 mM mannitol, 2 mM Tris-HCl buffer (pH 7.0) in order to evaluate the activity of intestinal (brush border membrane) enzymes that were purified according to Crane et al. (1979). Enzymatic determinations for pancreatic, gastric and intestinal enzymes were conducted as described in Gisbert et al. (2009). In brief, trypsin (E.C. 3.4.21.4) activity was assayed at 25 ºC using BAPNA (N-α-benzoyl-DL-arginine p-nitroanilide) as substrate. One unit of trypsin per ml (U) was defined as 1 µmol BAPNA hydrolyzed per min per ml of enzyme extract at 407 nm (Holm et al., 1988). Bile salt-activated lipase (BALT, E.C. 3.1.1) activity was assayed for 30 min at 30 ºC using p-nitrophenyl myristate as substrate. The reaction was stopped with a mixture of acetone: n-heptane (5:2), the extract centrifuged for 2 min at 6,080 x g and 4 ºC and the increase in absorbance of the supernatant read at 405 nm. Bile salt-activated lipase activity (U/ml) was defined as the nmol of substrate hydrolyzed per min per ml of enzyme extract (Iijima et al., 1998). Regarding intestinal enzymes, alkaline phosphatase (E.C. 3.1.3.1) was quantified at 37ºC using 4-nitrophenyl phosphate (PNPP) as substrate. One unit (U) was defined as 1 µg BTEE released per min per ml of brush border homogenate at 407 nm (Bessey et al., 1946). Finally, pepsin activity (U) was defined as the µmol of tyrosine liberated per min at 37 ºC per ml of tissue homogenate at 280 nm (Worthington, 1991). All enzymatic activities were expressed as specific activity defined as units per milligram of protein (mU/mg protein). Soluble protein of crude enzyme extracts was quantified by means of the Bradford's method (Bradford, 1976) using bovine serum albumin as standard. All the assays were made in triplicate from each pool of larvae and absorbance read using a spectrophotometer (Tecan™ Infinite M200, Switzerland).

Double staining for the analysis of skeletal deformities

To evaluate the impact of different weaning strategies on larval quality assessed by the incidence of skeletal deformities, 20 larvae per tank were randomly sampled at the end of the Trial 2 in 2015. Larvae were preserved in 10% formalin buffered to pH = 7.0 and stored until double staining. Animals were stained with alcian blue and alizarin red to detect cartilaginous and bony tissues (Darias et al., 2010); they were individually examined using stereomicroscope. Quantification of the degree and different typologies of skeletal deformities were performed at 37 dph and expressed in percentage (%). The incidence of skeletal abnormalities was determined in the cranium, vertebral column and caudal fin complex according to the osteological description on this species from Cardeira et al. (2012). Special attention was given to vertebral deformities, which were divided in two categories: severe (fusion, compression, deformed vertebral centrums, torsion and scoliosis) and light (haemal spines and neural spines) deformities and caudal fin complex deformities (modified epural).

Statistical analysis

Data were expressed as mean ± standard deviation (SD) except for skeletal anomalies that were expressed in mean ± standard error of the mean (SEM) and tested by Student's t-test (for 2015 trial with only 2 treatments) or one way ANOVA (for 2014 trial with 4 treatments). When a significant treatment effect was found, Tukey´s test was performed for multiple range comparisons with the level of significant difference set at $P < 0.05$. All the data were tested for normality, homogeneity and independence to satisfy the assumptions of ANOVA.

RESULTS

Trial 1 (2014)

Growth, survival and fatty acid composition

The results in terms of growth performance (standard length and weight) of the larvae are shown in **Figures 1 and 2**, whereas survival values are shown in Figure 3. Group C, weaned at 15 dph and fed half the amount of *Artemia* metanauplii showed similar final results in weight than control group (A) ($P > 0.05$), but exhibited longer length than in all the other groups ($P < 0.05$). Although in this group the incidence of cannibalism, and subsequently the growth in length of some individuals, was higher than the rest of the groups, with the exception of group D that showed the highest mortality rate ($P < 0.05$). On the other hand, group B showed a significantly lower growth in weight and length and the highest survival rate with lower incidence of cannibalism (see photographs in **Fig. 4**) ($P < 0.05$) and a similar distribution of size among the larvae.

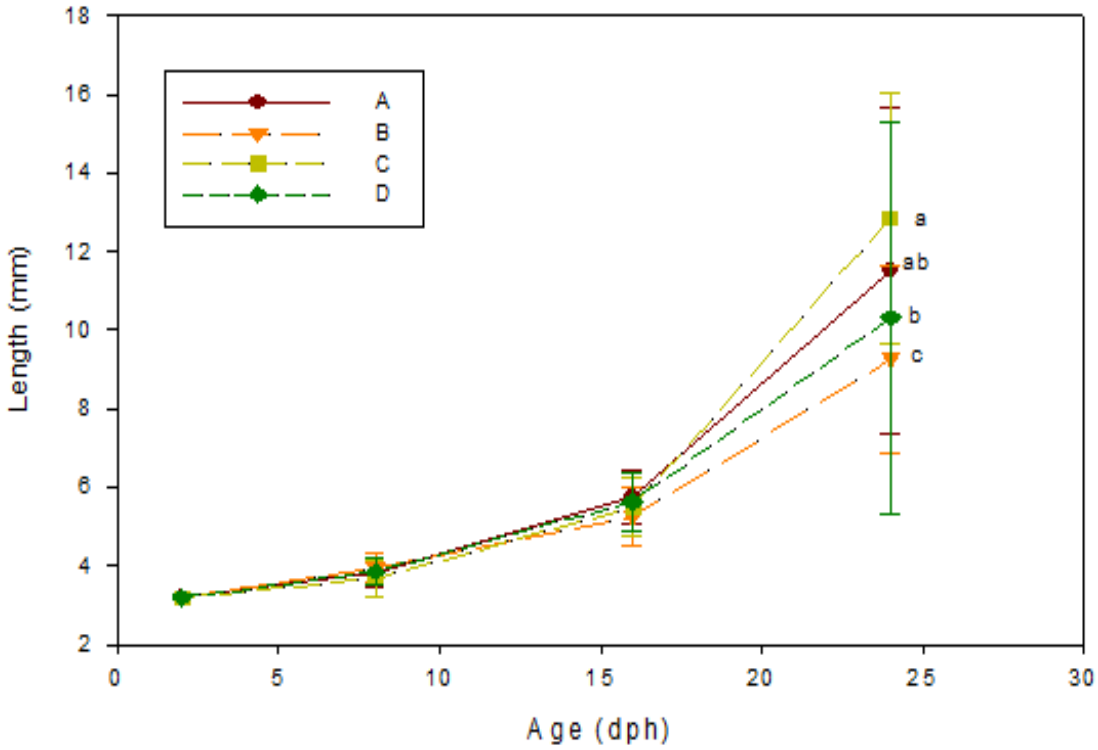


Fig. 1. Growth in standard length (mm, Mean ±SD) of the larvae from the different groups (A,B,C, and D) at different sampling times. Different lowercase letters show significant differences (ANOVA, $P < 0.001$).

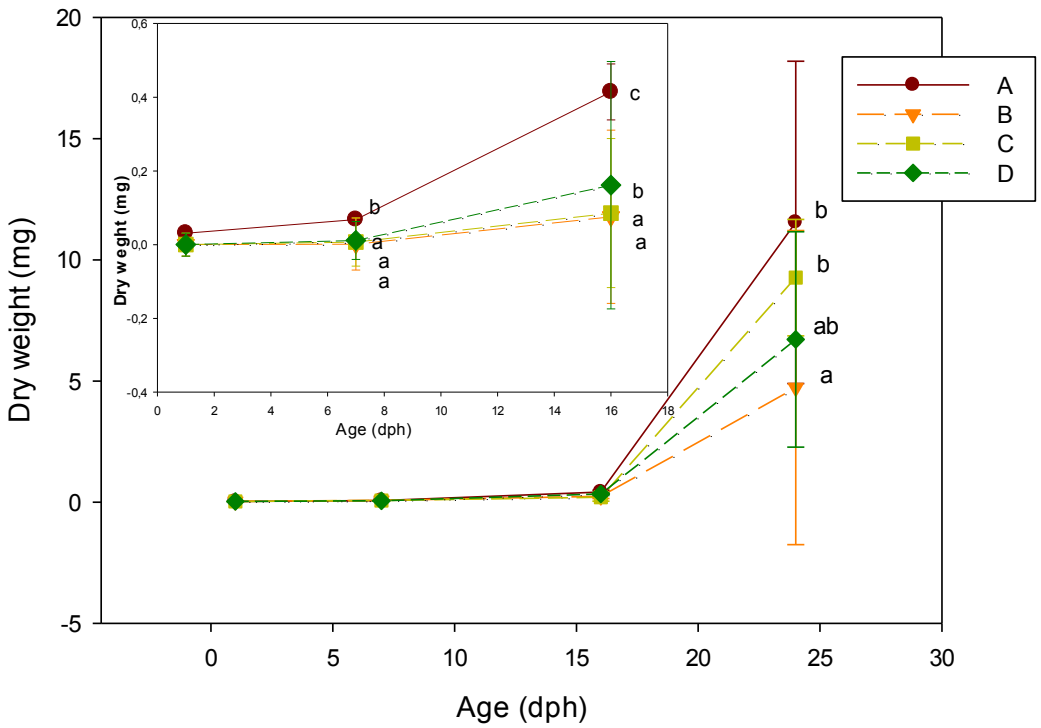
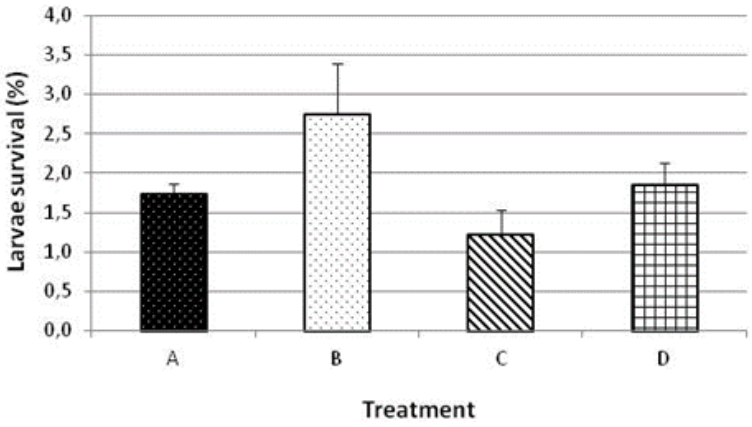


Fig. 2. Growth in dry weight (mg, Mean ±SD) of the larvae from the different groups (A,B,C,D) at different sampling times. Inserted figure shows the larval growth during the first larval period (1-15 dph). Different lowercase letters show significant differences (ANOVA, $P < 0.05$)

Survival rate was very low in all the groups (1.2 to 2.8%, **Fig. 3**), and at the end of the experiment big differences in larval growth were detected in each tank as shown in Figure 4, due to the high incidence of cannibalism. Differences in dry weight among small, medium and large larvae are also shown in **Table 1**, with larvae weighing from 1.28 to 38.28 mg of dry weight depending on the group.



T	%
A	$1,7 \pm 0,1^{b}$
B	$2,8 \pm 0,6^{a}$
C	$1,2 \pm 0,3^{b}$
D	$1,8 \pm 0,3^{b}$

ANOVA P=0,005

Fig. 3. Survival rate (%, mean ± SD) of meagre weaned with different weaning protocols. Significant differences were found (ANOVA, P= 0.005) having the larvae from group B (weaning at 20 dph with half the *Artemia* amounts) a significantly higher survival rate than the rest of the groups

Fig. 4. Photographs showing the differences in the size of the larvae at the end of the experiment (24 dph) in the groups A (left), B (right up) and C (right down), due to the high incidence of cannibalism.

Table 1. Differences in dry weight (mg, mean ±SD) among small, medium and large larvae in the four feeding groups (Groups A, B, C and D).

T	Small	Medium	Large
A	4,52 ± 0,97	16,06 ± 9,58	38,28 ± 8,41
B	1,28 ± 0,16	8,00 ± 7,69	18,67 ± 9,75
C	3,73 ± 1,55	12,27 ± 3,79	23,17 ± 5,11
D	3,01 ± 1,29	8,91 ± 3,89	26,41 ± 2,77

The fatty acid composition of the live prey and larvae at the end of the trial are shown in **Tables 2 and 3**. Differences could be found in the composition of the larvae from trial 1, especially in terms of total monounsaturated fatty acids –reserves of energy- (higher in groups A and B), total n-6 PUFA (higher in groups A, C and D) and DHA content (higher in groups C and D), as a consequence of feeding either the enriched live prey/weaning diet or sibling larvae (cannibalistic behavior). In contrast, in the Trial 2 conducted in 2015, significant differences could be found only in the content of 18:0 (higher in the control group), 16:1, 20:4n-6, total n-6 PUFA and 18:3n-3 that were higher in the early weaned larvae ($P < 0.05$). DHA and EPA content was higher in the larvae of 2015 trial compared to 20.

Table 2. Lipid content (mg/g DW) and fatty acid composition (mg/g lipids) of the live prey enriched with Red Pepper™ and used for larval feeding in the trials (mean ± SD samples 2014 and 2015). Live prey were enriched according to standard procedures following the manufacturer's instructions.

	Rotifer		Artemia	
	Mean	SD	Mean	SD
Total lipids (mg/g DW)	107.9	0.85	160.11	0.79
Total FAs (mg/g lipids)	524.42	22.30	707.66	42.07
Fatty acids (% total)				
14:0	2.49	0.86	1.47	0.07
15:0	0.35	0.07	0.47	0.05
16:0	16.37	3.21	13.79	0.07
18:0	3.11	0.01	5.25	0.05
Total saturated	22.61	1.81	20.99	0.24
16:1	8.43	3.07	1.02	0.02
18:1n-9	13.35	1.35	16.77	0.35
18:1n-7	2.62	0.26	3.23	0.30
20:1	1.73	0.05	0.42	0.04
Total monounsaturated	28.08	2.79	21.44	0.12
18:2n-6	6.02	2.21	6.19	0.05
18:3n-6	0.30	0.21	1.05	0.02
20:4n-6	1.35	0.21	0.82	0.02
22:5n-6	7.83	0.80	2.82	0.06
Total n-6 PUFA	15.83	2.00	10.87	0.16
18:3n-3	1.84	1.02	28.24	0.20
18:4n-3	0.54	0.20	5.17	0.14
20:4n-3	1.61	0.50	1.18	0.08
20:5n-3	3.94	0.95	2.10	0.00
22:6n-3	24.07	0.33	8.59	0.14
Total n-3 PUFA	32.66	1.24	45.26	0.11
Total PUFA	48.48	1.82	56.13	0.27
n-3/n-6	2.22	0.22	4.17	0.05
DHA/EPA	7.75	1.17	4.10	0.06

Table 3. Fatty acid composition (%TFA, mean ± SD) of the larvae at the end of the weaning period in 2014 and 2015, different lowercase letters indicate significant differences.

	2014												2015						
	Initial		A		B		C		D				Initial		A		B		
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	ANOVA				Mean	SD	Mean	SD	Student's t test
Total lipids (mg/g DW)	152.00	6.30	70.10	9.82	80,90	18.19	76.30	19.92	74.70	17.35			151.50		88.70	4.40	91.70	7.90	NS
Total Fatty acids (mg)	747.48	1.48	518.48	76.70	616.13	82.98	700.56	25.41	588.19	64.61	P=0.056		747.48		576.22	19.81	477.56	28.04	P<0.001
Fatty acids (% total)																			
14:0	1.05	0.00	0.38	0.15	0.26	0.05	0.17	0.04	0.23	0.02	NS		2.5		1.52	0.18	0.82	0.18	NS
16:0	19.72	0.24	15.10	0.74	14.67	1.19	15.31	0.22	15.84	0.32	NS		16.49		22.26	0.41	20.23	0.86	NS
18:0	7.81	0.31	11.63	0.35	11.73	0.69	12.13	0.56	12.53	0.58	NS		3.75		6.83	0.26	9.29	0.43	P<0.001
Total saturated	28.59	0.55	27.26	0.92	26.74	1.94	27.70	0.51	28.66	0.79	NS		23.09		31.22	0.78	30.94	1.22	NS
16:1	5.73	0.48	0.38	0.12	0.30	0.11	0.34	0.04	0.28	0.03	NS		7.61		2.83	0.20	1.92	0.11	P<0.001
18:1n-9	14.12	0.07	17.25	0,16ab	18.24	1,69a	16.44	2,54ab	15.12	0,49b	P=0,016		17.57		13.15	0.48	13.11	0.61	NS
18:1n-7	1.86	0.01	4.29	0.28	2.62	2.28	2.70	2.36	3.71	0.29	NS		3.1		3.38	0.26	3.66	0.18	NS
Total monounsaturated	22.37	0.43	22.55	0,21a	21,71	0,73a	20.17	0,21b	19.81	0,34b	P<0.001		29.36		21.27	0.79	20.17	0.98	NS
18:2n-6	4.60	0.01	5.84	0.22	5.39	0.13	4.56	0.37	4.26	0.48	NS		7.75		11.95	0.44	10.51	0.82	NS
20:4n-6	1.90	0.09	4.68	0,26b	4.53	0,25b	5.99	0,62a	6.39	0,30a	P<0.001		1.2		2.26	0.09	3.62	0.37	P<0.001
22:5n-6	0.37	0.00	3.81	0.15	3.96	0.18	4.31	0.06	4.23	0.12	NS		0.26		1.19	0.07	1.99	0.23	NS
Total n-6 PUFA	9.20	0.33	15.43	0,11b	14.65	0,20a	15.77	0,43b	15.52	0,28b	P=0.006		11.02		15.57	0.41	16.75	0.48	P=0.003
18:3n-3	1.29	0.05	13.70	1,14a	13.12	1,86a	7.77	1,77b	7.71	1,72b	P<0.001		3.11		1.78	0.12	2.39	0.20	P<0.001
20:5n-3	4.46	0.19	5.49	0.44	5.38	0.55	4.25	0.16	4.25	0.09	NS		6.26		6.05	0.24	6.09	0.24	NS
22:5n-3	1.64	0.07	0.67	0.03	0.65	0.09	1.30	0.24	1.32	0.16	NS		1.61		1.41	0.09	1.40	0.05	NS
22:6n-3	27.57	0.39	11.33	0,50a	13.60	0,65a	19.19	2,10b	17.39	1,56b	P<0.001		17.53		21.33	1.65	19.68	0.85	NS
Total n-3 PUFA	35.27	0.30	33.48	1.28	34.97	2.90	34.02	1.01	32.39	0.31			30.15		31.94	1.29	30.76	0.48	NS
Total PUFA	44.47	0.63	48.92	1.23	49.63	2.87	49.79	0.58	47.91	0.59	P=0.355		41.18		47.51	0.99	47.51	0.60	NS
n-3/n-6	3.84	0.10	2.17	0.09	2.39	0,21	2.16	0,12	2.09	0.02	NS		2.74		2.08	0,14	1.84	0.07	NS
DHA/EPA	6.17	0.35	2.08	0,27b	2.53	0,15b	4.53	0,60a	4.10	0,44a	P<0.002		2.80		3.53	0,38	3.24	0,25	NS

Trial 2 (2015)

Growth, survival and fatty acid composition

The lipid content and fatty acid composition of the larvae used in 2015 at the beginning and at the end of the trial have been already discussed and shown in **Table 3**. The results in larval growth in standard length and dry weight and in survival rates are shown in Figs. 5, 6 and 7. In all the cases at 30 dph, the results of the group A, weaned at 20 dph and fed using the standard protocol, were significantly higher than those obtained for group B (larvae weaned at 10 dph) (Student's t test, $P < 0.05$).

Growth in weight and length of the larvae is shown in **Figures 5** and **6**. Growth was significantly higher for the larvae of group A (control) compared to early weaned larvae

Survival rates were around 4-5% at the end of the study; these data were within the range of values observed in commercial fish hatcheries. The use of low light intensity (reduced from 500 to 150-200 lux) and the higher number of feed doses supplied had a clear effect on the reduction of cannibalism that at the same time, allowed a higher survival rate.

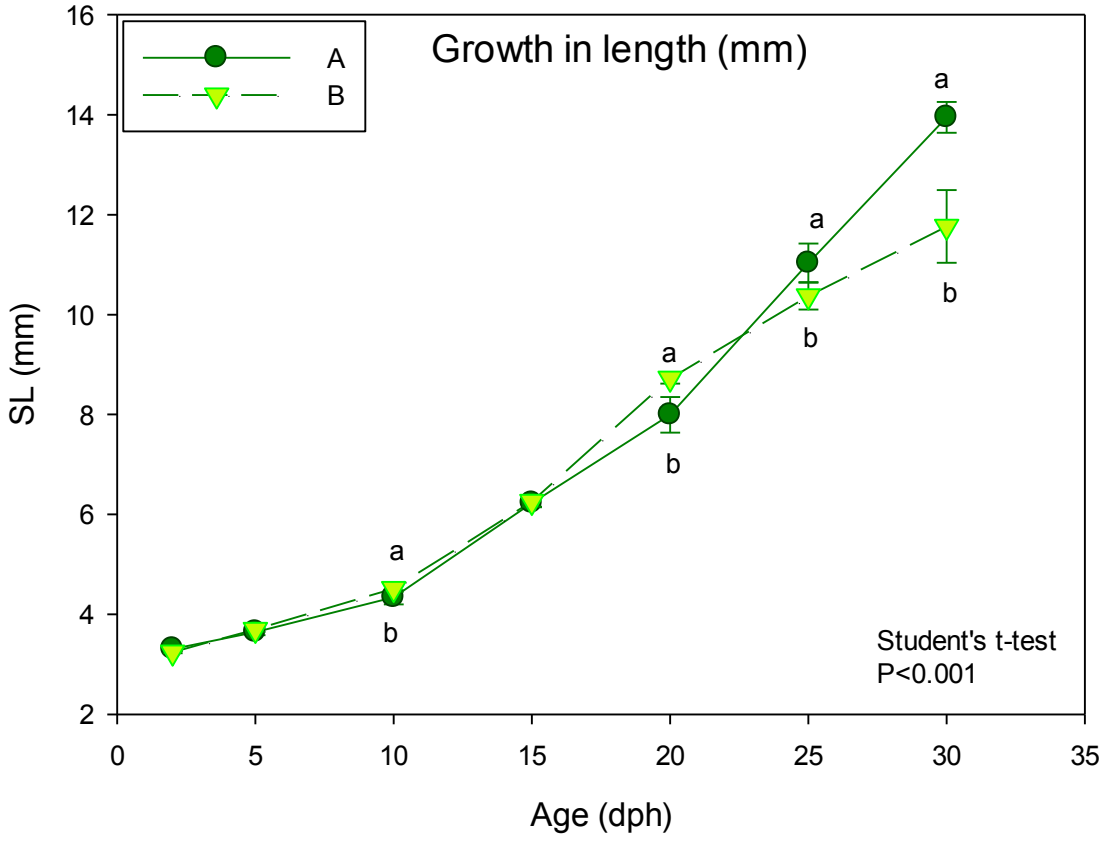


Fig. 5. Growth in length of the larvae weaned at 20 dph (group A) and 12 dph (group B). Different lowercase letters indicate significant differences (Student's t test, $P < 0.001$).

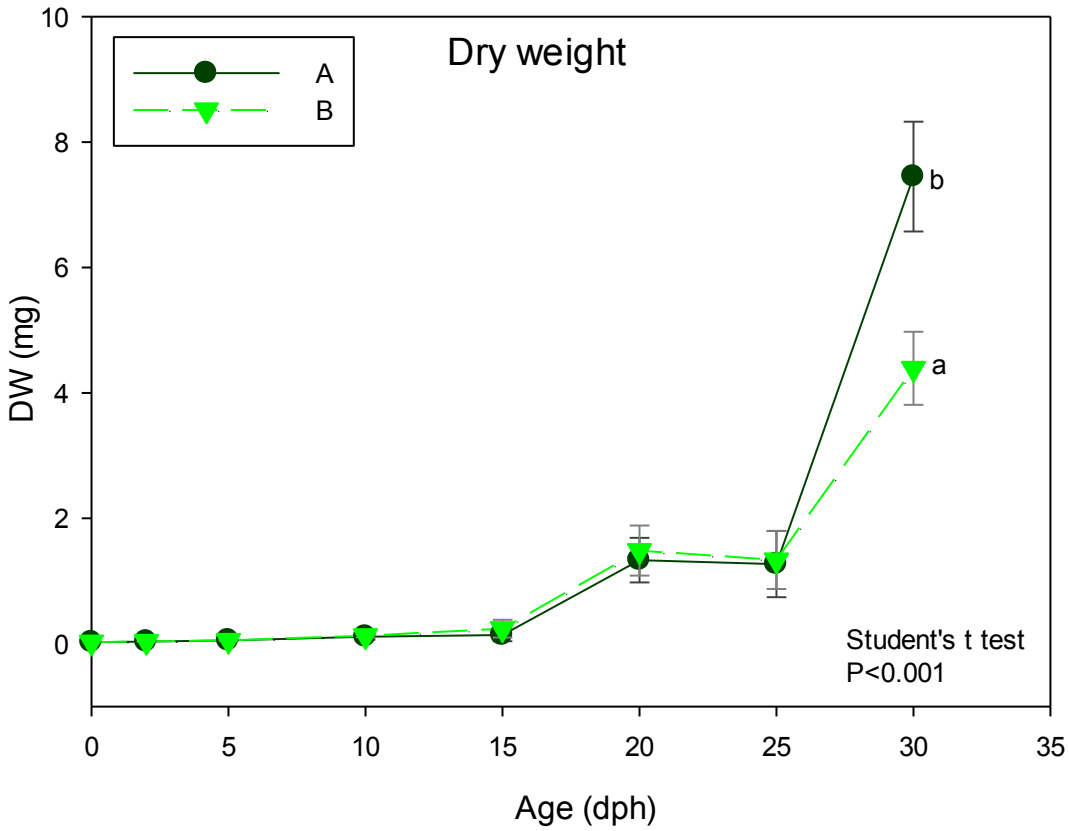
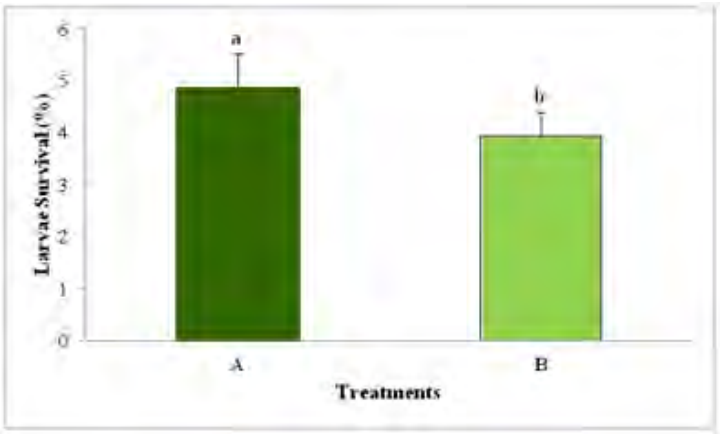


Fig. 6. Growth in dry weight of the larvae weaned at 20 dph (group A) and 10 dph (group B), lowercase letters indicate significant differences (Student's t test, $P < 0.001$).



	Survival rate (%)
Group A	$4{,}86 \pm 0{,}65^{a}$
Group B	$3{,}93 \pm 0{,}47^{b}$

Student's t test P=0,032

Fig. 7. Larval survival rate obtained in the trial carried out in 2015 and Student t-test showing significant differences, indicated by lowercase letters, between the two groups (Groups A and B) (Student's t test, $P = 0.032$).

Digestive enzyme activities

Results of enzymatic activity expressed as specific activity (U mg protein^{-1}) of the larvae from Trial 1, at the beginning of the weaning (12 dph group D, 15 dph group C and 20 dph groups A and B) and at the end of the trial (24 dph) as well as at the end of Trial 2 (37 dph) are shown in **Figures 8 and 9**. The results clearly show that all the larvae show a similar enzymatic activity when they are fed live prey (**Fig. 8**) and differences could be found only in the alkaline phosphatase production, being higher in the younger larvae (groups C and D). At the end of the weaning phase (**Fig. 9**) pancreatic enzymes tended to be higher in the early weaned larvae, especially in the larvae from Trial 2 and in larvae from the group C larvae Trial 1 with a significantly higher lipase activity compared to the control group (A) ($P < 0.05$). Alpha-amylase did not show differences between early weaned and control larvae and trypsin also showed higher activity in early weaned larvae ($P < 0.05$).

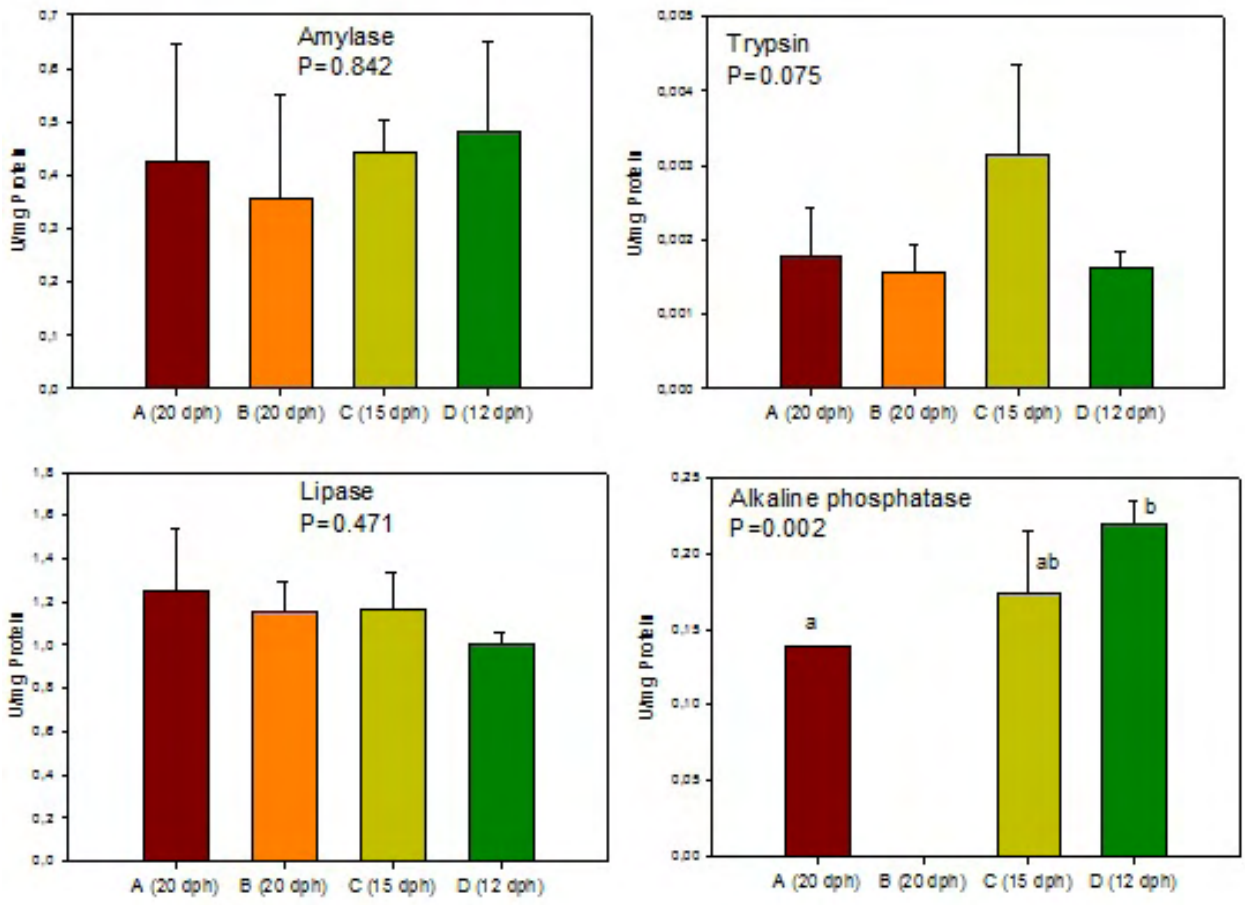


Fig. 8. Results of digestive enzyme activity measured in the larvae from 2014 at the end of the live prey feeding period. Different lowercase letters indicate significant differences (ANOVA, $P < 0.05$)

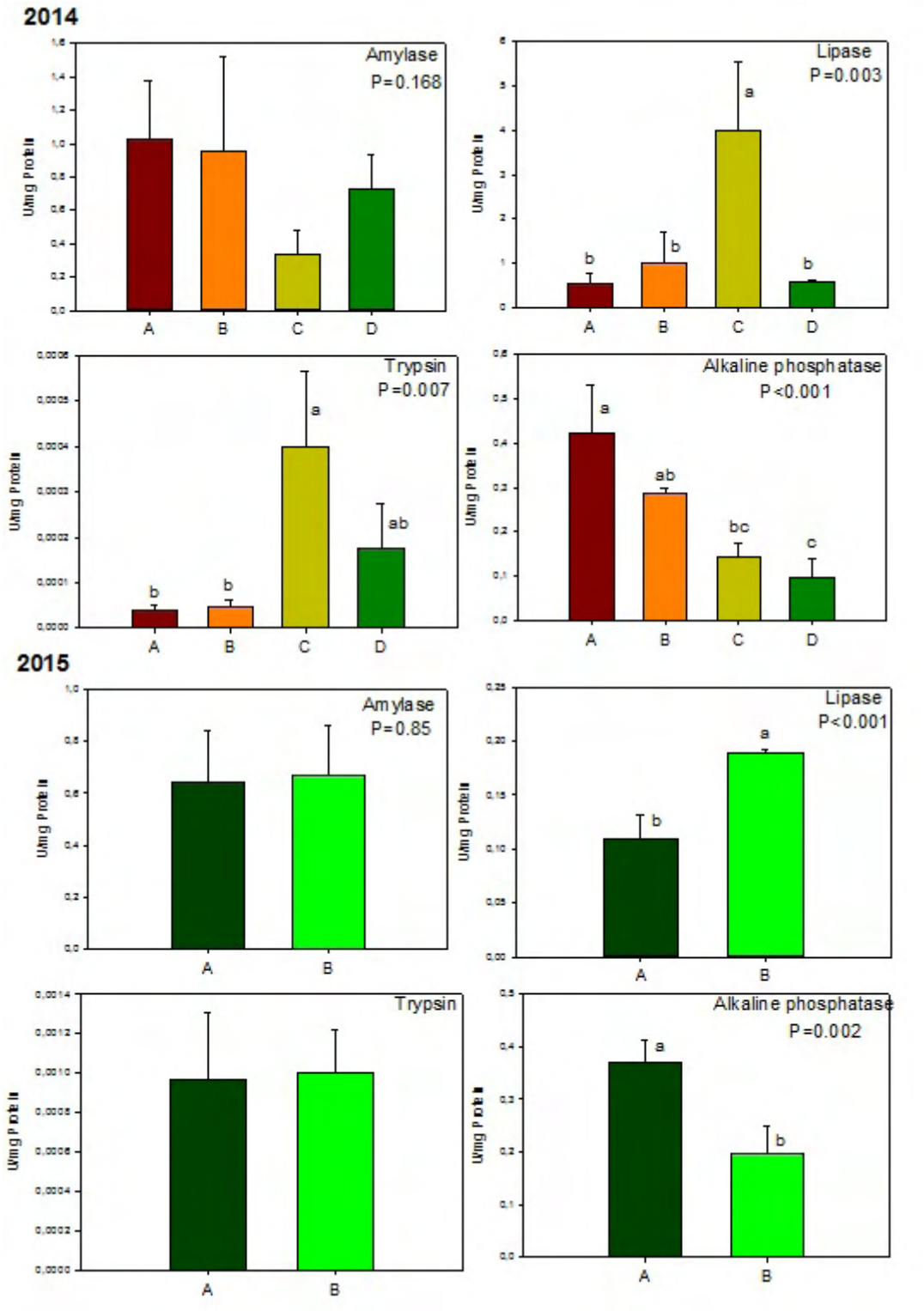


Fig. 9. Results of digestive enzyme activity measured in the larvae from 2014 and 2015 trials at the end of the experiments. Different lowercase letters indicate significant differences (ANOVA, data from 2014 and Student's t test, data from 2015, $P < 0.05$).

Pepsin activity was only analyzed in larvae from Trial 2 (2015) and the results are shown in **Fig. 10**. Early weaned larvae (10 dph) showed a significantly lower activity of this gastric enzyme when compared with larvae weaned at 20 dph.

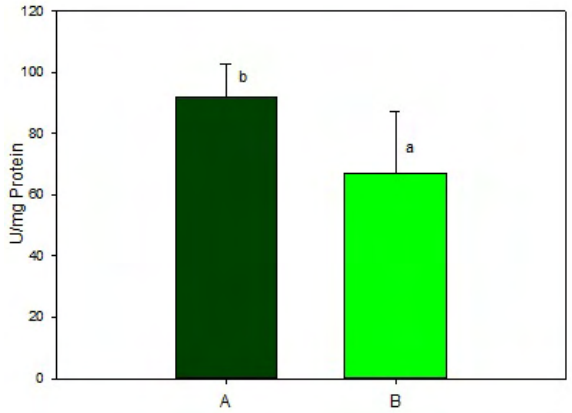


Fig. 10. Results of digestive enzyme activity measured in the larvae from 2015 at the end of the experiments. Lowercase letters indicate significant differences (Student's t test, $P < 0.05$).

Activity of antioxidative stress enzymes and levels of lipid peroxidation

Lipid peroxidation levels in larvae (based on MDA levels) were significantly affected by early weaning ($P < 0.05$, **Table 4**). Thus, MDA values were significantly higher in Group A (15.46 ± 2.85 nmol MDA/g) than in Group B (9.89 ± 1.86 nmol MDA/g) as a consequence of the different feeding regime. However CAT, SOD and GPX specific activities did not show any significant variation among the larvae reared under different weaning strategies. In contrast, GR and GST specific activities were higher in fish from the group B ($P < 0.05$)

Table 4. Quantification of lipid peroxidation LPO (nmol MDA g^{-1} w.w.) and specific activities of catalase CAT (in mol min^{-1} mg^{-1} protein) superoxide dismutase SOD (U mg^{-1} protein), glutathione reductase GR (nmol min^{-1} mg^{-1} protein), glutathione peroxidase GPX (nmol min^{-1} mg^{-1} protein) and glutathione S-transferase GST (nmol min^{-1} mg^{-1} protein) in meagre larvae at different weaning. Values are expressed as mean ± SEM (*n = 5*). Different superscript letters denote significant differences among groups ($P < 0.05$).

	A	B
LPO	15,46 ± 2,85^{a}	9,89 ± 1,87^{b}
CAT	28,16 ± 4,97	27,61 ± 3,72
SOD	26,73 ± 6,95	29,38 ± 8,24
GR	14,13 ± 3,37^{a}	18,49 ± 1,66^{b}
GST	39,30 ± 9,66^{a}	56,47 ± 7,47^{b}
GPX	124,77±33,46	141,66 ± 27,06

Skeletal deformities

Early weaning of larvae did not have any effect on the incidence of total skeletal deformities in meagre early juveniles. The frequency of deformed fish was low and not significantly different between the two groups of fish obtained from Trial 2 (Student's t test, P = 0.621). In particular, the incidence of skeletal deformities ranged from 17.6% to 21.1% (**Fig. 11A**). However, most of the detected skeletal anomalies were considered as not severe and were categorized as light (12.4 ± 7.3% in Group A and 16.8 ± 2.5% in Group B; **Fig. 11B**), since these anomalies did not affect the external morphology of specimens nor their quality. In addition, the incidence of severe skeletal deformities was only 4.6 ± 1.8% in Group A and 4.3 ± 2.2% in Group B (P > 0.05).

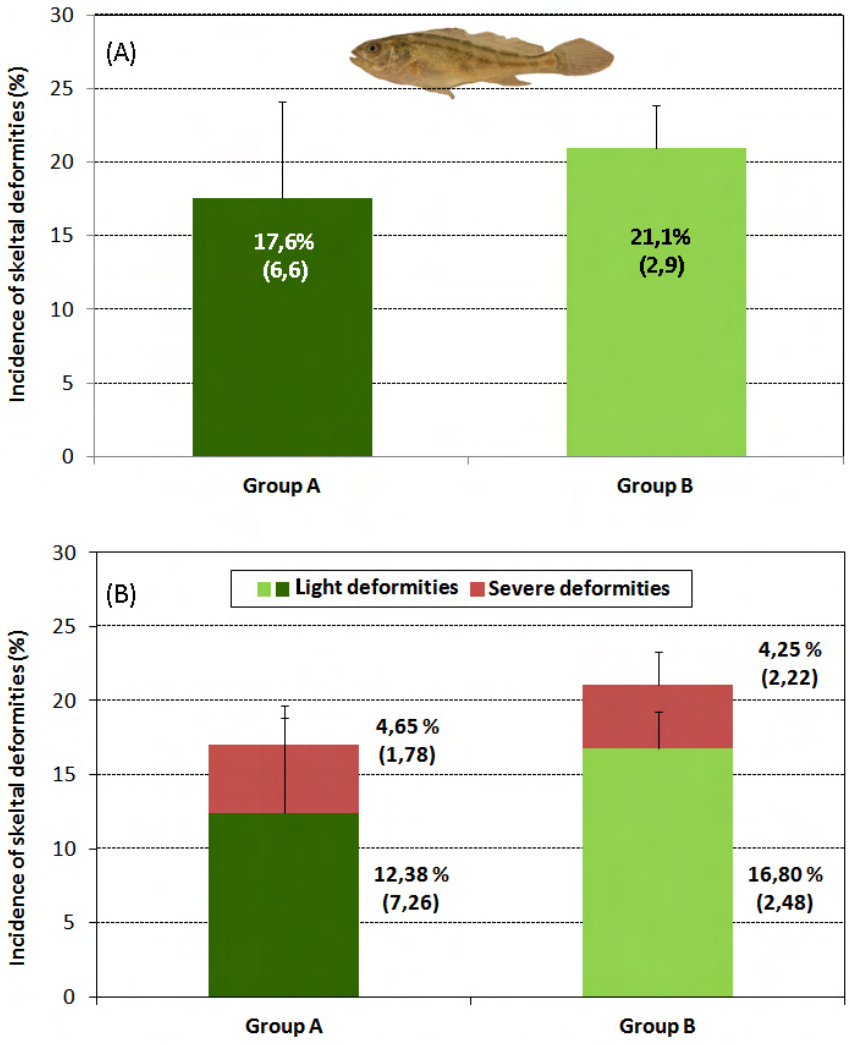


Fig. 11. Incidence of skeletal deformities (A) and incidence of light and severe skeletal deformities (B) in meagre early juveniles (Trial 2015, Group A: control, Group B: early weaned). Data are expressed as mean ± SEM in brackets (n = 5).

Regarding the impact of early weaning on the incidence of skeletal deformities found in different body regions (**Fig. 12**), results indicated that no skeletal deformities were found in the cranial region of meagre early juveniles which may be indicative of the earlier ossification of this part of the skeleton with regard to

the rest of skeletal elements. Regarding the vertebral column, this part of the skeleton is composed of 25 vertebrae, divided in 5 cephalic, 5 pre-haemal, 11 haemal and 3 caudal vertebrae, including the urostyle. In particular, the age of weaning did not affect the number of vertebral bodies ($P > 0.05$), since all examined animals had 25 vertebral bodies.

No significant differences in the frequency of skeletal abnormalities could be found in the vertebral column (pre-haemal and haemal regions) between both groups ($P > 0.05$), whereas any deformity was found in the cephalic vertebrae. The fusion of adjacent vertebral bodies was the most abundant skeletal deformity found in the haemal region of the vertebral column in both experimental groups, whereas kyphosis and lordosis were mainly affected in the pre-haemal region (ca. 0.8% of examined animals).

The skeletal elements forming the caudal fin complex were almost not affected by the weaning time; we were able to only detect some anomalies with regard the number and level of differentiation of the three epurals (9.3 ± 6.3% in Group A, 16.0 ± 3.0% in Group B) that in some specimens were completely or partially missing. Although it may exist a trend with regard to the incidence of deformities affecting the epurals and the age of weaning, there were not significantly differences between Group A and B ($P > 0.05$), which was mainly due to the large variability between tanks (replicates). It should be also mention that this anomaly was not considered as severe as it did not dramatically impacted the external shape of the animal nor its performance.

Some examples of different skeletal anomalies detected in both groups are shown for illustrative purposes (Annex).

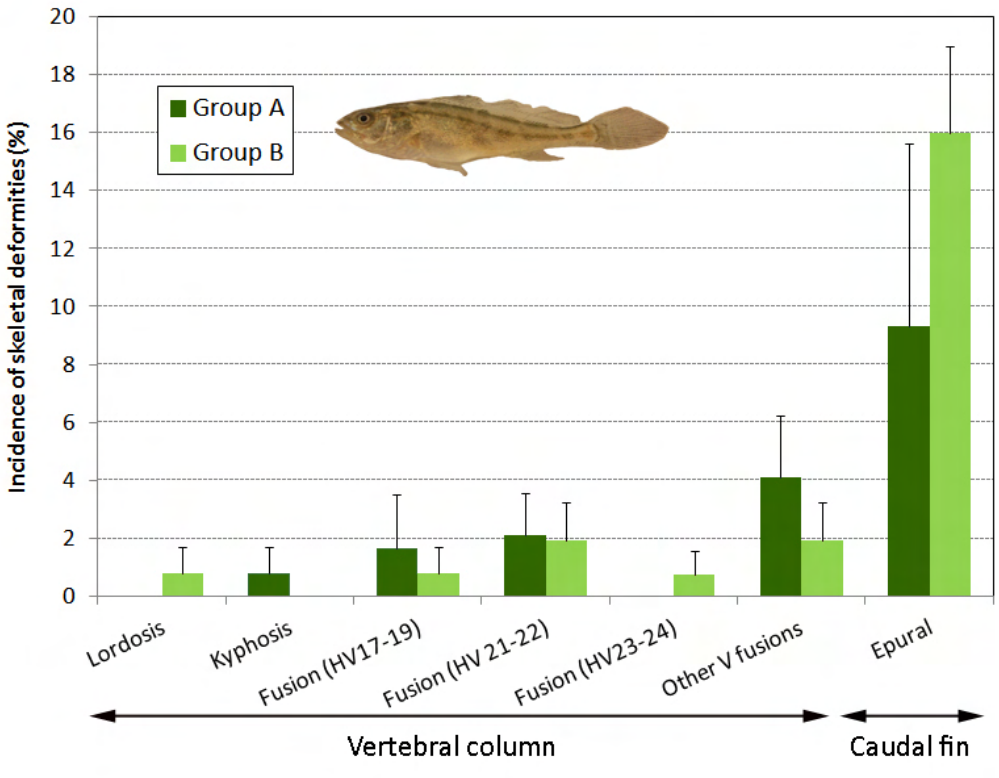


Fig. 12. Different typologies of skeletal deformities (%) found in 37 dph meagre *Argyrosomus regius* early juveniles (Group A, control group, Group B, early weaned larvae), considering the number of abnormal skeletal elements per fish. The number of vertebral body affected counted from the cephalic area is including within brackets. Abbreviation: HV, haemal vertebrae.

DISCUSSION AND CONCLUSIONS

Weaning, the transfer from live food to an artificial diet is successful with most marine fish with a completely developed digestive tract (Person Le Ruyet et al., 1993). In the current study weaning was carried out with a commercial weaning diet (Gemma Micro, Skretting) using a gradual transfer from live prey to this artificial diet over a minimum period of five days, although in some other marine species like sea bass there is an abrupt replacement (Person Le Ruyet, 1990). Duran et al (2009) using a weaning protocol similar to the one used in Trial 2 (2015) obtained similar results in growth and survival. Thus, early weaning can be carried out with meagre larvae if several measures to reduce cannibalism are in place.

Cannibalism is regarded as an alternative feeding strategy, more likely to be adopted by larvae and early juveniles, which are carnivorous, when resources become limiting (Hecht and Pienaar, 1993). It is a major problem in the culture of many marine fish larvae. Size variation is the primary cause of cannibalism in larval fish (Katavic et al., 1989). Other factors influencing cannibalism include food availability and larval density, feeding frequency, light intensity, water turbidity, and shelter (see review by Hecht and Pienaar, 1993). Cannibalism was controlled in Trial 2 by increasing feeding frequencies, removing dominant individuals, grading regularly and keeping the larvae in the dark when food was unavailable or in short supply. The use of low light intensity before feeding in the morning increased survival of the larvae in the trial carried out in 2015 by reducing cannibalism.

The weaning success of any finfish larvae from live feeds onto a formulated diet is partly dependent on the composition of the diet and the ability of the larvae to select and digest an inert diet. In this sense, the proper development of feeding protocols and diet formulations under fish larval culture conditions requires a deep knowledge and understanding of the digestion processes occurring during early ontogeny in order to synchronize different types of feeds (live prey and microdiets) with production of different digestive enzymes. Animals are adaptively plastic in their digestive enzyme production in response to diet, because the metabolic expense of producing large amounts of digestive enzymes would be wasted by animals ingesting low levels of the substrates for those enzymes. Therefore, the assessment of the presence and level of activity of digestive enzymes may be used as a comparative indicator of the rate of development of fish larvae, food acceptance, digestive capacity, as well as for survival and growth rate predictions. Thus, the synthesis and secretion of digestive enzymes are regarded as indicators for the transition from live feeds to microdiets. Pancreatic enzyme synthesis and secretion appear to be particularly sensitive to food deprivation and dietary composition in teleost larvae and, consequently, pancreatic enzyme activity provides a reliable biochemical marker of larval fish development and condition. The pancreatic secretory process matures during the first three or four weeks after hatching in temperate marine fish larvae. This maturational process can be disrupted when larvae are fed diets that do not meet their specific needs: the earlier the feeding with such inadequate diets, the lower the pancreatic secretion level (Gisbert et al., 2013). In the present study, the activity of pancreatic enzymes tended to be higher in the early weaned larvae, with a significantly higher lipase activity compared to the control group. Proteolytic enzymes from the exocrine pancreas are regarded as being particularly significant in the early life stages of precocious and altricial fish because of the absence of a functional stomach with pepsin. Lipase plays an active role in lipid digestion, especially in the breakdown of triacylglycerol to diacylglycerol and then to monoacylglycerol (Zambonino Infante and Cahu, 2001). In many fish species, including meagre, lipase is active during resorption of the oil globule and the complete transition to exogenous feeding being relevant for the digestion of high levels of triacylglycerols present in the enriched life prey such as *Artemia*. On the contrary, the capacity to digest proteins in the stomach (pepsin activity) was significantly lower in the early weaned larvae, which coincided with the significantly lower growth rate achieved by this group. Having in mind that pepsin activity is detected after the formation of a functional stomach that in meagre generally occurs between 15 and 20 dph (Papadakis et al., 2013; Suzer et al., 2013), several authors have suggested this age is the best for weaning meagre larvae. This is supported in the present study that indicated that 10-12 dph (weaning ages used in the experiments) might be premature for larval weaning. The weak ability of early weaned larvae for denaturizing and proteolytic cleavage of proteins from the artificial food could be the reason for the poor larval growth achieved at early weaning.

Some fish seem to activate antioxidant defense mechanisms in response to dietary peroxidized lipids to prevent alterations in their physiological processes. Studies carried out in gilthead sea bream during larval development have shown a diminution of the specific activity of most oxidative stress enzymes with the inclusion of additives in the diet (Mourente et al., 2002). In the present study, the specific activity of most of the oxidative stress enzymes was lower for the standard weaning protocols (treatment A, weaned at day 20) compared to early weaned larvae (Treatment B, day 10). However, the lower activity of these enzymes might be related to larval development and possibly considered as an adaptation to dietary oxidative stress over time. Overall, the activities of GST-GR were significantly different for early weaned larvae and may be related to the lower levels of lipid oxidation in the larvae.

Several studies have shown that nutrients are responsible for the appearance of skeletal deformities when their levels in the diet are insufficient or unbalanced (Cahu et al., 2003; Lall and Lewis, 2007, Afonso et al., 2000). During early larval development as well as during weaning a change to an inappropriate diet or in the hydrodynamic conditions of the rearing tank might cause problems in some still developing skeletal structures. In the present study, malformation rate of the larvae was not affected by the feeding regime, which indicates that the weaning protocol used (co-feeding for several days enriched *Artemia* metanauplii with commercial microdiets) can supply adequate nutrients for the larvae during skeletal development without compromising their quality. Nevertheless, similar deformities were observed in both treatments (larvae weaned at 20 dph and at 10dph) and in this case we cannot discard other factors such as environmental (Sfakianakis et al., 2004), genetic background, or broodstock nutrition (Afonso et al., 2000; Cahu et al., 2003) as the main causative agents.

In conclusion, based on these results meagre larvae can be weaned from live feed to an artificial diet at as early as 10 dph, but other important aspects for production success including larval performance and survival should be considered.

REFERENCES

Aebi, H., 1974. Catalase. In Bergmeyer, H.V. (Ed) Methods in enzymatic analysis, Vol. 2, Academic Press Inc., NY, Usa, pp 674-684

Afonso, J.M., Montero, D., Robaina, L., Astorga, N., Izquierdo, M.S. and Ginés, R. 2000. Association of a lordosis-scoliosis-kyphosis deformity in gilthead seabream (*Sparus aurata*) with family structure. Fish Physiol. Biochem., 22: 159-163

Ballagh, D.A., Fielder, D.S. and Pankhurst, P.M. 2010. Weaning requirements of larval mulloway, *Argyrosomus japonicus*. Aquaculture Research, 41: e493-e504

Bessey, O.A., Lowry, O.H., Brock, M.J., 1946. Rapid coloric method for determination of alkaline phaphatase in five cubic millimeters of serum. The Journal of Biological Chemistry, 164: 321-329

Bradford, M.M., 1976. A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem., 72: 248-254

Buckley, L.J., Turner, S.I., Halavik, T.A., Smigielski, A.S., Drew, S.M., Laurence, G.C., 1984. Effects of temperature and food availability on growth, survival and RNA-DNA ratio of larval sand lance (*Ammodytes americanus*). Marine Ecology Progress Series, 15: 91-97

Cahu, C., Zambonino Infante, J., Takeuchi, T., 2003. Nutritional components affecting skeletal development in fish larvae. Aquaculture, 227: 254-258

Cardeira, J., Vallés, R., Dionísio, G., Estévez, A., Gisbert, E., Pousao-Ferreira, P., Cancela, M.L., Gavaia, P.J. 2012. Osteology of the axial and appendicular skeletons of the meagre *Argyrosomus regius* (Sciaenidae) and early skeletal development at two rearing facilities. J. Appl. Ichthyol. 28: 464-470.

Carlberg, I. and Mannervik, B., 1975. Purification and characterization of the flavoenzyme glutathione reductase from rat liver. Journal of Biological Chemistry, 250: 5475-5780

Christie, W.W., 1982. Lipid Analysis. Pergamon Press, oxford, UK, 207 pp

Darias, M.J., Lan Chow Wing, O., Cahu, C., Zambonino-Infante, J.L., Mazurais, D., 2010. Short communication: Double staining protocol for developing European sea bass (*Dicentrarchus labrax*) larvae. Journal of Applied Ichthyology, 26: 280-28

Duncan, N., Estévez, A., Porta, J., Carazo, I., Norambuena, F., Aguilera, C., Gairin, I., Bucci, F., Vallés, R., Mylonas, C.C., 2012. Reproductive development, GnRHa-induced spawning and egg quality of wild meagre (*Argyrosomus regius*) acclimatized to captivity. Fish Physiology and Biochemistry, 38: 1273-1286

Durán, J., Pastor, E., Grau, A., Massuti-Pascual, E., valencia, J.M., Gil, M.M. 2009. Total replacing or Atemia by an artificial diet in larval rearing feeding protocol of meagre (*Argyrosomus regius*, Asso, 1801). Aquaculture Europe, Trondheim August 14-17, 2009. EAS Spec. Publi, 38

Folch, J., Lees, N., Sloane-Stanley, G.H., 1957. A simple method for the isolation and purification of total lipids from animal tissues. Journal of Biological Chemistry, 226: 497-509

Gisbert, E., Giménez, G., Fernández, I., Kotzamanis, Y., Estévez, A., 2009. Development of digestive enzymes in common dentex *Dentex dentex* during early ontogeny. Aquaculture, 287: 381-387

Gisbert E, Morais S, Moyano FJ (2013) Feeding and Digestion. In: Qin JG (ed) Larval fish aquaculture. Nova Publishers, New York, pp 73–124

Habig, W.H., Pabst, M.J., Jacoby, W.B., 1974. Glutathione S-transferases: the first enzymatic step in mercapturic acid formation. Journal of Biological Chemistry, 249: 7130-7139

Hecht, T. and Pienaar, A.G., 1993. A review of cannibalism and its implications in fish larviculture. Journal of the World Aquaculture Society, 241: 246-261

Holm, H., Hanssen, L.E., Krogdahl, A., Florholmen, J., 1988. High and low inhibitor soybean meals affect human duodenal proteinase activity differently: in vivo comparison with bovine serum albumin. Journal of Nutrition, 118: 515-520

Katavic, I., Jug-Dujakovic, J. and Glamuzina, B., 1989. Cannibalism as a factor affecting the survival of intensively cultured sea bass (*Dicentrarchus labrax*) fingerlings. Aquaculture, 77: 135-143.

Iijima, N., Tanaka, S., Ota, Y. 1998. Purification and characterization of bile-salt activated lipase from the hepatopancreas of red sea bream, *Pagrus major*. Fish Physiology and Biochemistry, 18: 59-69

Lazo, J.P., Holt, J.G., Fauvel, C., Suquet, M., Quemener, L., 2010. Drum-fish or croakers (Family: Sciaenidae). In N.R. Le Francois, M. Jobling. C. Carter and P.U. Blier (Eds) Finfish Aquaculture Diversification, CABI Publishing, UK, pp.397-405

Métais, P. and Bieth, J., 1968. Determination de l'alpha-amylase. Ann. Biol. Clin., 26: 133-142

Mourente, G., Díaz-Salvago, E., Bell, J.G. and Tocher, D.R. 2002. Increased activities of hepatic antioxidant defence enzymes in juvenile gilthead sea bream (*Sparus aurata* L.) fed dietary oxidised oil: attenuation by dietary vitamin E. Aquaculture, 214: 343-361

Papadakis, I.E., Kentouri, M., Divanach, P., Mylonas, C.C. 2013. Ontogeny of the digestive system of meagre *Argyrosomus regius* reared in a mesocosm, and quantitative changes of lipids in the liver from hatching to juvenile. Aquaculture, 388-391: 76-88

Person-Le Ruyet, J., 1990. Early weaning of marine fish larvae. onto microdiets: constraints and perspectives. In: Advances in Tropical Aquaculture (Barret, J. ed.), pp. 625–642. IFREMER, Actes de Colloque 9. Tahiti, French Polynesia.

Person Le Ruyet, J., Alexandre, J.C., Thebaud, L, Mugnier, C. 1993. Marine fish larvae: formulated diets or live prey? Journal of the World Aquaculture Society, 24: 211-224

Person-Le-Ruyet, J., Alexandre, J.C., Thébaud, L. and Mugnier, C.,1993. Marine fish larvae feeding: Formulated diets or live prey?. J. World Aquacult. Soc., 24: 211–224

Roo, J., Hernández-Cruz, C.M., Borrero, C., Schuchardt, D., Fernandez-Palacios, H., 2010. Effect of larval density and feeding sequence on meagre (*Argyrosomus regius*, Asso, 1801) larval rearing. Aquaculture, 302: 82-88

Sfakianakis, D.G., Koumoundouros, G., Divanach, P., Kentouri, M. 2004. Osteological development of the vertebral column and of the fins in Pagellus erythrinus (L. 1758). Temperature effect on the developmental plasticity and morpho-anatomical abnormalities. Aquaculture, 232: 407-424

Suzer, C., Kamaci, H.O., Coban, D., Firat, K., Saka, S., 2013. Functional changes in digestive enzyme activities of meagre (*Argyrosomus regius*, Asso, 2801) during early ontogeny. Fish Physiol. Biochem., 39: 967-977

Vallés, R. and Estevez, A., 2013. Light conditions for larval rearing of meagre (*Argyrosomus regius*). Aquaculture, 376-379: 15-19

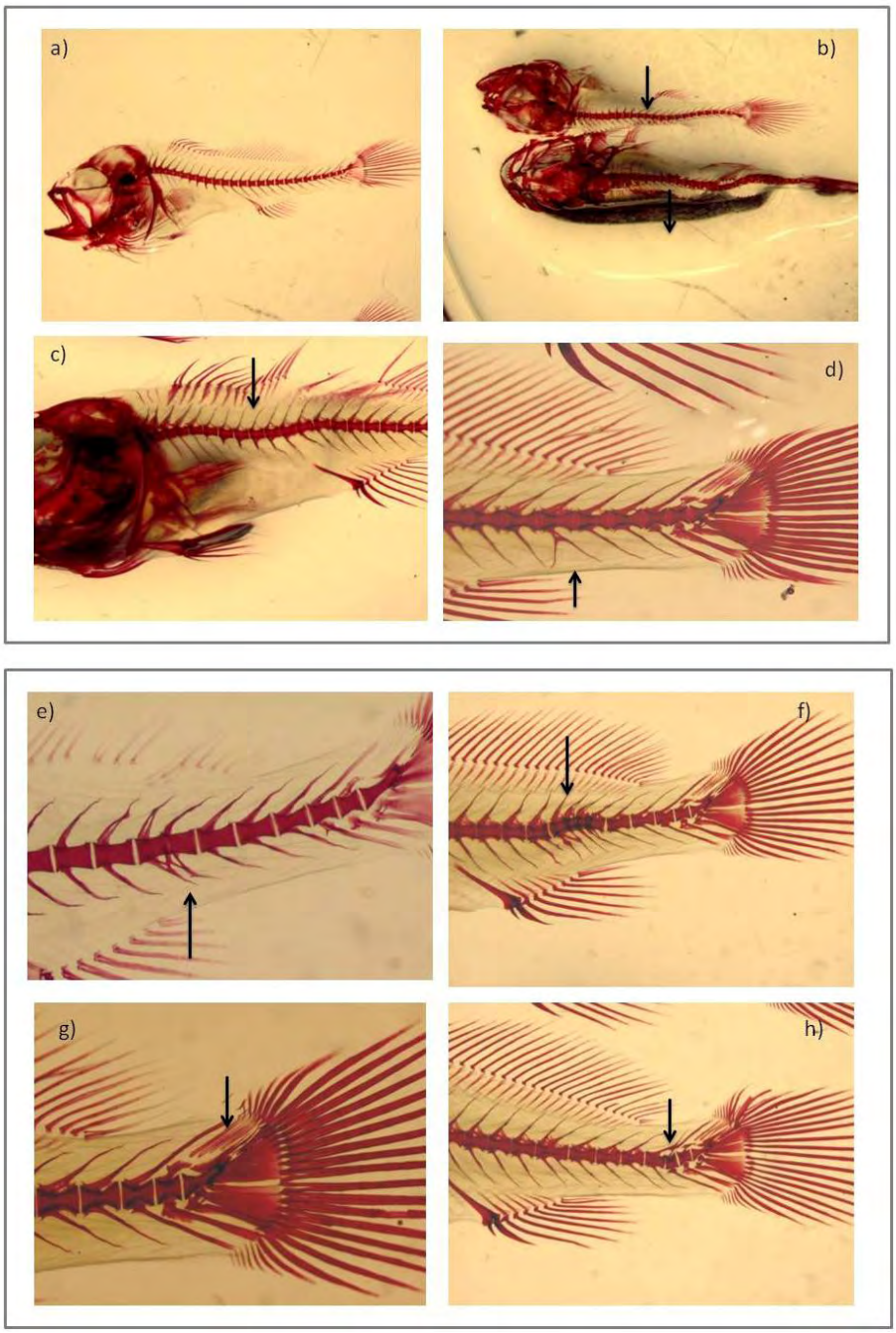
Vallés, R. and Estevez, A., 2015. Effect of different enrichment products rich in docosahexaenoic acid on growth and survival of meagre, *Argyrosomus regius* (Asso, 1801). Journal of the World Aquaculture Society, 42: 191-200

Worthington Biochemical Corporation, 1991. Worthington Enzyme Manual: Enzymes, Enzyme reagents, Related biochemical. Worthington Biochemical Corp., Freehold, N.J.

Zambonino Infante, J. and Cahu, C., 2001. Substitution of live food by formulated diets in marine fish larvae. Aquaculture, 200: 161-180

Deviations: The deliverable was delayed several months to allow finishing the biochemical and histological analyses, due to equipment failure (the centrifuge was broken and we had to buy a new equipment).

ANNEX.- Examples of different typologies of skeletal deformities found in 36 days post hatch *Argyrosomus regius*. a) View of the larvae without abnormalities. (b) (c) Observation of the vertebral region of a larva exhibiting scoliosis. (d) Modification haemal spine. (e) Partial fusion in the haemal region 17-18 haemal vertebra (HV) and modification haemal spines (Hs). (f) Strong fusion in the haemal region 17 to 19 HV and presence of fusion of two haemal spines closely parallels. (g) 2º Epural Modificated. (h) Torsion in caudal vertebra (23 -24 Ca V).



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