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1 **The ontogeny of greater amberjack digestive system under different rearing conditions: a histological and enzymatic approach**

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24

Abstract

An overall synchronization of morphological and physiological ontogenetic events of the digestive system occurred in greater amberjack (*Seriola dumerili*) larvae reared under intensive (INT) or semi-intensive (MES) conditions for 30 days. The first differentiations of the digestive channel took place at 3-4 days post hatch (dph) (3.6-3.7 mm TL). Differentiation of the endocrine and exocrine pancreas begun at 4-5 dph (4.0-4.1 mm TL), coinciding with a decrease of carbohydrase activity from egg to the onset of exogenous feeding and the maintenance of bile salt-activated lipase and total alkaline proteases. Between 6 and 10 dph (4.1-4.5 mm TL), pepsin remained undetected and pancreatic enzymes raised their activities compared to the lecitotrophic stage (0-5 dph). The first gastric glands and pepsin activity were evident at 17-20 dph (5.5-6.2 mm TL) in both fish groups. The appearance of pyloric caeca had a 5-day delay in the INT-compared to the MES-larvae (28 *vs* 23 dph). Althought both rearing protocols did not globally differ in terms of somatic growth and maturation of the digestive function, oxidative stress appeared to be less severe in larvae reared in MES, which may be attributed to a lower stress and more stable culture conditions with respect to INT.

Key words**:** greater amberjack larvae, ontogeny, digestive system, digestive enzymes, oxidative stress.

44 ## Introduction

45 The greater amberjack *Seriola dumerili* is a marine warm-water large teleost fish with
46 rapid growth and excellent flesh quality, which is receiving increasing research attention
47 in the last years due to its great potential for aquaculture diversification (Rodríguez-
48 Barreto *et al.* 2012, 2017; Mylonas *et al.* 2016; Zupa *et al.* 2017a,b; Jerez *et al.* 2018;
49 Monge-Ortiz *et al.* 2018; Fakriadis *et al.* 2019; Sarih *et al.* 2019). Although knowledge
50 about the optimal feeding regime for larval rearing is essential for a successful
51 production of fry, available information on greater amberjack is still incomplete
52 (Papandroulakis *et al.* 2005; Hamasaki *et al.* 2009; Mylonas *et al.* 2016). In fact, larval
53 rearing is considered one of the major bottlenecks for the flourishing culture of this
54 species, due to the low survival rates obtained during this period, which seriously
55 compromise the availability of juveniles for the on-growing stage. Therefore, there is a
56 great need to evolve culture techniques for mass seed production of the greater
57 amberjack. Although there is scattered information about greater amberjack larviculture
58 and larval development, studies evaluating different rearing technologies through
59 physiological parameters, including the morphophysiological development of the
60 digestive function and oxidative stress condition, are missing. Moreover, a holistic
61 understanding of the feeding ecology and digestive functions of greater amberjack
62 larvae is crucial for the design of specific dietary regimes, as well as for the adaptation
63 of rearing protocols to meet larval requirements. This knowledge will allow the best
64 presentation of prey and microdiets as well as synchronizing the larval stage of
65 development with rearing processes in order to increase the productivity of this species
66 during the first life stages.

67 The digestive system enables fish to capture, ingest, digest and finally absorb
68 nutrients from the food, which are transported across the intestinal epithelium to the

69 circulatory system and then to the whole organism (Rønnestad *et al.* 2013). During the
70 first stages of development until its transformation into a juvenile, numerous changes
71 take place in the digestive system of fish larvae in terms of its morphology and
72 functionality (Przybył *et al.* 2006; Papadakis *et al.* 2009, 2013; Gisbert *et al.* 2018).
73 Over this period, activity of the digestive enzymes is affected by a number of different
74 factors, and their levels are closely related to the state of maturation of the secreting
75 digestive tissues, which show important variations between species, water temperature
76 and rearing conditions (Lazo *et al.* 2011; Koven *et al.* 2019). Therefore, the knowledge
77 of the digestive competence of a fish is essential in order to understand the digestive
78 physiology of larvae and to adjust the feeding protocols to dietary qualitative and
79 quantitative characteristics (Campoverde *et al.* 2017; Gisbert *et al.* 2018) contributing to
80 the optimization of diets (Zambonino-Infante *et al.* 2008; Campoverde *et al.* 2017) and
81 to the proper understanding of functions and limitations in the processing capacity of
82 the digestive system. In this sense, the combined analysis of the ontogeny of the main
83 digestive tract structures and related digestive enzymes during larval development is of
84 special relevance for proper characterization of the plasticity of digestive processes to
85 deliver nutrients to the rapidly growing larval tissues (Rønnestad *et al.* 2013) under
86 changeable feeding and environmental conditions.
87 Early life stages of marine fish are particularly sensitive to environmental stressors
88 and rearing conditions, due to the lack or low functional capacity of some organ systems
89 and to the high rates of metabolism needed to fuel growth and development (Pimentel *et*
90 *al.* 2015). Thus, in order to better elucidate the possible impact of rearing protocols on
91 larval condition and physiological function, oxidative stress biomarkers are broadly
92 considered as reliable indicators (Izquierdo *et al.* 2013; Saleh *et al.* 2014; Garrido *et al.*
93 2017; Suzuki *et al.* 2018). Oxidative stress is a progressive imbalance between reactive

94 oxygen species (ROS) production and the antioxidant defense system (*i.e.* prooxidant/antioxidant imbalance). The production of ROS is controlled by an efficient endogenous antioxidant capacity, characterized by a set of antioxidant enzymes, which can together detoxify ROS (Abele & Puntarulo 2004) and reduce their negative effects at cellular level that finally impact on the health and fitness of the organism (Malanga *et al.* 2004). The antioxidant defense system, which is comprised of endogenous enzymes such as superoxide dismutase (SOD), glutathione peroxidase (GPx), glutathione reductase (GR) and glutathione S-transferase (GST), is designed to maintain the lowest potential level of ROS in cells and is recognized as an essential component of the organism's response to maintain homeostasis (Castex *et al.* 2010; Adeyemi 2014). Moreover, non-enzymatic defenses such as reduced glutathione (GSH) levels are also employed to provide such protection (Harman 1972).

The present study aims to describe the morphological and physiological ontogeny of the digestive system and the response of the antioxidant defences of greater amberjack larvae cultured under two different rearing conditions, intensive and semi-intensive. The intensive rearing conditions and the feeding protocol used is an adaptation from the established methods in Meditteranean hatcheries whereas the semi-intensive methods that allow the use of wild plankton seems to be more appropriate for the culture of this species (Papandrolakis *et al.* 2005). Although there exist several studies describing larval development in other carangid species (Carton 2005; Stuart *et al.* 2011; Martínez-Montaño *et al.* 2016; Plaza *et al.* 2017 among others), there is missing information about greater amberjack. In addition, species-specific conclusions can not be drawn from the above-mentioned studies in order to be applied on greater amberjack larviculture, since each species presents its own developmental patterns and requirements that need to be individually defined. Our results may facilitate the

119 implementation of an adequate feeding strategy adapted to the digestive capacity and
120 nutritional needs of greater amberjack during early development, while also addressing
121 options to reduce cannibalism and size dispersion which are of primary importance to
122 boost larval survival and growth. This information will be of great value for
123 synchronizing the larval stage of development with rearing practices; thus, improving
124 actual larval rearing protocols for greater amberjack.
125

126 **Materials and methods**

127 *Larval rearing*

128 Larval rearing trials were performed in the facilities of the Institute of Marine Biology,
129 Biotechnology and Aquaculture at the Hellenic Center for Marine Research (HCMR,
130 Crete, Greece). Greater amberjack larvae were cultured under two different rearing
131 conditions, the semi-intensive (MES) and the intensive (INT) systems. Eggs used for
132 this study were obtained from induced spawning of wild breeders kept in Argosaronikos
133 SA cage farm (ARGO). After collection, eggs were shipped by air to the hatchery
134 facilities of the HCMR in polystyrene boxes (~12 hours trip).
135

136 *Semi-intensive system (MES)*

137 A total of one hundred and ten thousand eggs were stocked in a 40 m^3 indoor tank (2.75
138 eggs l^{-1}) filled with filtered (5 μm) natural seawater (salinity 37 psu) treated with UV,
139 which was also the water used for subsequent renewal. During the larval rearing,
140 seawater temperature was maintained at 24.0 ± 0.7°C and the pH fluctuated from 8.0 to
141 8.2. Dissolved oxygen varied from 5.8 to 6.8 mg l^{-1}, whereas the rate of water renewal
142 was increased progressively from the initial 15% to 35% of total water volume per day
143 at 17 days post hatching (dph), to 100% at 22 dph and then to 200% at 30 dph. Aeration

144 was provided at 5 points along the perimeter and in the center of the tank. A surface
145 skimmer was operational during the appropriate period (5 to 13 dph) to keep the surface
146 free from lipids, a requisite for good swim bladder inflation. The photoperiod was
147 adjusted to constant light from mouth opening to 20 dph and then reduced to 18L:06D
148 for the remaining experimental period. Light intensity varied according to the weather
149 conditions between 500 lux on cloudy days to 1,000 lux on sunny days. During the
150 night when prolonged photophase was applied, light intensity was about 250 lux. The
151 rearing technology employed here is a semi-intensive technique for production of
152 greater amberjack larvae (Papandroulakis *et al.* 2005).
153
154 *Intensive rearing in closed water recirculation system (INT)*
155 Thirty-six thousand eggs were placed in 0.5 m^3 cylindro-conical tanks (72 eggs l^{-1})
156 connected to a closed water recirculating system coupled to a biological filter. Tanks
157 were filled with borehole 35 psu-water kept at 24.0 ± 0.5°C, pH ranged from 8.0 to 8.2
158 and the dissolved oxygen was maintained between 6.8 and 7.2 mg l^{-1}. Water circulation
159 was achieved through a biological filter during embryogenesis, egg hatching and the
160 autotrophic larval stage with aeration provided at 150-250 ml min^{-1}. After first feeding,
161 water circulation was obtained for each tank by means of an airlift pump in order to
162 maintain stable the rearing environment. The water in the biological filter was used for
163 renewal in the larval rearing tanks at a rate of 3% daily until 15 dph, then increased
164 gradually to 50% until 25 dph. A skimmer was installed at the appropriate period (5-15
165 dph) to keep the surface free from lipids. The photophase was 24L:00D from mouth
166 opening until 20 dph, and 18L:06D for the remaining experimental period. Light
167 intensity varied between 200-800 lux during the day, and was ~200 lux at night.
168

169 *Feeding protocols*

170 The duration and type of diet for each rearing protocol during the trial is presented in
171 Figure 1. Microalgae (*Chlorella sp*) and rotifers (*Brachionus sp*) enriched with DHA
172 Protein Selco (INVE S.A., Belgium) were daily added in the rearing tanks from 3-4 dph
173 to 23 dph. Rotifers' concentration was kept at 2-3 individuals ml^{-1} in the MES and at 4-5
174 individuals ml^{-1} in the INT. Instar I *Artemia* AF nauplii (12 to 14 dph) and *Artemia* EG
175 instar II nauplii (14 to 30 dph) enriched with A1 DHA Selco (INVE S.A.) were offered
176 to the larvae at a starting concentration of 0.05 to 0.35 nauplii ml^{-1}. Enrichment in all
177 cases was performed according to manufacturer´s instructions. In both rearing systems,
178 microdiets were added progressively according to fish size from 16 dph (MES) and 21
179 dph (INT) (NRD 2/4, grain size 200–300 μm; NRD 3/5 grain size 300–500 μm, INVE
180 S.A., Derdenmonde, Belgium). In the semi-intensive system the feeding was
181 supplemented with potential wild prey developed in the tank (mainly harpacticoida
182 copepods) and also, after 20 dph, with eggs (live or frozen) of gilthead sea bream
183 (*Sparus aurata*) and newly hatched larvae. The egg/larvae addition was based on
184 observations during rearing of other pelagic species.

185

186 *Sampling procedures*

187 As no tank replication was available for the MES treatment, larvae within the 40 m^3
188 tank were collected from three different and distant tank areas in order to allow for
189 larval spatial and temporal heterogenity to attain meaningful significance levels
190 (Gamble 1990). Once captured, fish larvae were sacrificed with an overdose of
191 anesthetic phenoxy ethanol (Sigma-Aldrich, Darmstadt, Germany). For the histological
192 study, random samples of greater amberjack eggs and larvae (n = 10) were collected on
193 the following days: 1 day before hatching, 0 (hatching), 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 12,

194 13, 15, 17, 20, 23, 25, 28 and 30 dph. The total length (TL) of the larvae was
195 determined under graduated stereoscope. Fish samples were preserved in a buffered
196 fixative containing 4% formaldehyde and 1% gluteraldehyde for at least 24 hours
197 (McDowell & Trump 1976). For comparisons of main digestive pancreatic and gastric
198 enzyme activities, eggs and larvae were collected and pooled according to their age-size
199 (3 replicates per experimental condition) at different ontogenic periods: 0-5, 6-10, 11-15
200 and 21-30 dph (1500-2000, 380-450, 50-80 and 5-9 larvae, respectively), rinsed in
201 distilled water to remove external salt and immediately frozen at −80 °C until analysis.
202 Finally, for the study of the oxidative stress status, 125 ± 25 mg wet weight of larvae per
203 stage were collected at 7 dph, 18 dph (flexion), 23 dph and 30 dph. The samples at 23
204 dph included big (B) and small (S) size larvae (TL 8.52 ± 0.65 mm and 5.95 ± 0.49 mm,
205 respectively).
206 All animal experiments were approved by the Ethics Committee of the Institute of
207 Marine Research and the relevant veterinary authorities (Ref Number 255332) and were
208 conducted in certified laboratories (EL91-BIOexp-04) in accordance with legal
209 regulations (EU Directive 2010/63).
210
211 *Histological analyses*
212 Before embedding in methacrylate resin (Technovit 7100®, Heraeus Kulzer, Germany),
213 larvae were dehydrated in gradually increasing ethanol solutions (70-96%). Serial
214 sections of 3 μm were obtained with a microtome (Leica, RM 2245, Germany). Sections
215 were stained with Methylene Blue (Sigma-Aldrich)/Azure II (Sigma-Aldrich)/Basic
216 Fuchsin (Polysciences Inc., Warrington, PA, USA) according to Bennett *et al*. (1976). In
217 order to describe the ontogeny of the digestive system and stomach content analysis, all

218 the sections were examined using a compound microscope (Nikon Eclipse 50i, Melville,
219 NY, USA).
220

221 *Determination of digestive enzyme activities*

222 Determinations of pancreatic (α-amylase, bile salt-activated lipase, total alkaline
223 proteases) and gastric (pepsin) enzyme activities were based on methods previously
224 performed and described by Gisbert *et al.* (2009) and processed as recommended by
225 Solovyev & Gisbert (2016) in order to prevent sample deterioration. Briefly, samples
226 were completely homogenized (Ultra-Turrax T8, IKA©-Werke, Germany) in 5 volumes
227 (v/w) of ice-cold Milli-Q water, centrifuged at 3,300 x *g* for 3 min at 4ºC, and 1 ml-
228 aliquots of supernatant kept at -80ºC until their analysis for enzyme quantification.

229 Larval digestive capacities during early life stages before stomach development and
230 acidic digestion was evaluated by measuring the activity of total alkaline proteases
231 according to the method of García-Careño and Haard (1993). Alkaline proteases activity
232 was determined at room temperature using azocasein (0.5%) as substrate in Tris-HCl 50
233 nmol l^{-1} (pH 9) for 10 min. Reaction was stopped with 20% TCA (trichloroacetic acid)
234 and samples were centrifuged at 10,000 x *g* for 5 min and absorbance of the supernatant
235 was measured at λ = 366 nm. One unit (U) of alkaline proteases activity was defined as
236 1 µmol of azo dye released per minute and per ml of homogenate.

237 Alpha-amylase (E.C. 3.2.1.1) was quantified according to Métais & Bieth (1968)
238 using 0.3% soluble starch dissolved in Na_2HPO_4 buffer (pH 7.4) as substrate. The
239 reaction was stopped with 1 N HCl and, after the addition of 2 ml of N/3000 iodine
240 solution (Merck, Darmstadt, Germany) the absorbance was read at λ = 580 nm. Alpha-
241 amylase activity (U) was defined as the amount of starch (mg) hydrolysed during 30
242 min per ml of homogenate at 37 ºC.

243 Bile salt-activated lipase (BAL, E.C. 3.1.1) activity was assayed for 30 min at 30ºC
244 using *p*-nitrophenyl myristate as substrate dissolved in 0.25 mM Tris-HCl, pH 9.0, 0.25
245 mM 2-methoxyethanol and 5 mM sodium cholate buffer. The reaction was stopped with
246 a mixture of acetone: n-heptane (5:2), the extract centrifuged at 6,000 x *g* and the
247 increase in absorbance of the supernatant determined at λ = 405 nm. BAL activity (U)
248 corresponded to the µmol of substrate hydrolyzed per min per ml of enzyme extract
249 (Iijima *et al.* 1998).

250 Finally, pepsin (E.C. 3.4.23.1) was quantified by mixing the enzymatic extract with
251 the substrate (2% hemoglobin solution in 1 N HCl at pH 2.0) and incubated for 10 min
252 at 37 °C. The reaction was stopped with 5% TCA and the extract centrifuged at 4,000 x
253 *g* for 6 min at 4 °C. The absorbance of the supernatant was read at λ = 280 nm. One
254 unit of activity (U) was defined as 1 µmol of tyrosine liberated per min and ml of
255 homogenate (Worthington Biochemical Corporation, 1972).

256 Enzymatic activities are expressed as the total activity defined as units per larva
257 (U/larva) and specific activity as units per mg protein (U/mg protein). Soluble protein of
258 crude enzyme extracts was quantified by means of the Bradford's method (Bradford
259 1976) using bovine serum albumin as standard. All the assays were made in triplicate
260 from each pool of larvae and absorbance read using a spectrophotometer (Beckman
261 Coulter DU800, Fullerton, CA).

262

263 *Determination of oxidative stress status*

264 To assess the oxidative stress of the developing greater amberjack larvae, the
265 concentration of total GSH and the enzymes' activities of total SOD, GPx, GR and GST
266 were determined. Supernatants were prepared and analyzed for enzyme activities in a
267 manner similar to that described in Hamre *et al.* (2014) with minor modifications. In

268 brief, 25 mg (for GSH) and 100 mg (for SOD, GPx, GR and GST) of frozen samples
269 from each biological sample were placed in Eppendorf tubes (1.5 ml). A 12 x volume of
270 ice-cold homogenization buffer was added in each sample, homogenized with a pellet
271 pestle (cordless motor, Sigma-Aldrich) and centrifuged at 3,000 x *g* for 10 min or at
272 10,000 x *g* for 20 min, for GSH or for all the other enzymes, respectively.
273 Homogenization buffer for GSH was a 5% (w/v) metaphosphoric acid and 0.6%
274 sulfosalicylic acid (w/v) mixture, while for all the other enzymes a 0.1 M phosphate
275 buffer pH 7.4 containing 0.15 M KCl, 1 mM dithiothreitol (DTT), 1 mM EDTA and 0.1
276 M phenyl-methylsulfonylfluoride (PMSF) was used. Supernatants were collected and
277 stored at -80 ºC until analysis. The GSH was analyzed according to Rahman *et al.*
278 (2006) and the GPx, GR and GST according to McFarland *et al.* (1999). The SOD
279 activity was analyzed with a commercial kit (706002, Cayman Chemical Co., MI)
280 according to the manufacturer´s instructions. Total protein concentrations were
281 measured with a Coomassie brilliant blue reagent (Sigma-Aldrich) according to
282 Bradford (1976).
283
284 *Statistical analysis*
285 Data are presented as mean ± SEM unless otherwise stated. For the description of
286 growth performance as total length (TL) as a function of time, an exponential equation
287 was used. Prior to the statistical analysis of the activities of digestive and antioxidant
288 enzymes the data were checked for normality with the one-sample Kolmogorov–
289 Smirnov test and for homogeneity of variances with the Levene's test. A one way
290 analysis of variance (ANOVA) followed by a Tukey post hoc test or a Games-Howell
291 test were performed to determine significant differences among developmental stages
292 (unless otherwise stated). A Student´s *t*-test was applied for comparisons between both

293 rearing systems (MES *vs* INT). The level of significance was established at $P < 0.05$.
294 All statistical comparisons were conducted using SPSS for Windows 21.0 (IBM-SPSS
295 Inc., Chicago, Il, USA).

296

297 **Results**

298 *Growth performance*

299 No differences in growth of greater amberjack larvae were observed between both
300 culture systems during the experimental period ($P > 0.05$). Growth performance
301 adjusted exponentially to the equation: $y = 3.223\ e^{0.033x}$, $R^2 = 0.9436$, for the MES and y
302 $= 3.3065\ e^{0.0294x}$, $R^2 = 0.9703$, for the INT rearing systems (Figure 1).

303

304 *Ontogeny of the digestive system*

305 Overall, there was a synchronization of the ontogenetic events occurring in greater
306 amberjack larvae from both rearing protocols during the first life stages (Figure 2).

307 *Period 1: 0-5 dph (MES, 3.5 – 4.0 mm TL; INT, 3.5 – 4.0 mm TL)*

308 From hatching (3.5 ± 0.04 mm TL) until 2 dph (3.7 ± 0.05 mm TL), the digestive tract
309 appeared as a closed straight tube located dorsally to the yolk sac (Figure 3a) and it
310 consisted of a single-layer epithelium of cuboidal and columnar cells. The liver
311 developed rapidly. The early hepatic cells appeared at 2-3 dph (3.8 ± 0.1 mm TL) and
312 were initially located behind the yolk sac under the anterior intestine and later
313 surrounding the anterior part of the intestine (Figure 3b). The pancreas appeared as an
314 undifferentiated tissue at 2 dph, but differentiation in endocrine and exocrine regions
315 begun between 4-5 dph (4.0 ± 0.1 mm TL) (Figure 3c).

316 Regardless of the rearing protocol, the first differentiation events of the digestive
317 system took place at around 3-4 dph. During this period, the mouth and the anus opened

318 and the separation of the digestive tract into distinc regions occurred (Figure 3d). The
319 ileorectal valve that separates the midgut from the hindgut also appeared at this stage
320 (Figure 3e).

321 Regarding digestive enzymes, α-amylase, BAL and total alkaline protease activities
322 were detected at this stage, even prior to hatching and to the onset of exogenous feeding
323 (3 dph; 3.8 ± 0.1 mm TL). The specific activity of α-amylase (U/mg protein)
324 significantly decreased from the egg to 5 dph larvae (Figure 4; ANOVA, $P < 0.05$),
325 while that of BAL and total alkaline proteases remained stable (ANOVA, $P > 0.05$).
326 Pepsin activity was not detected during this developmental stage.

327 *Period 2: 6-10 dph (MES, 4.0 – 4.2 mm TL; INT, 3.9 – 4.3 mm TL)*

328 Within this period, both feeding regimes were based mainly on rotifers. At 6 dph (3.9-
329 4.0 mm TL), folding of the oesophageal mucosa occurred (Figure 3f), whereas the
330 pyloric and cardiac sphincters at the intestine indicated the area where the stomach will
331 start developing (Figure 3g). Supranuclear vacuoles were present in the larval hindgut at
332 8 dph in the MES (4.1 ± 0.1 mm TL) and at 10 dph in the INT (4.3 ± 0.1 mm TL)
333 (Figure 3h), being visible up to 30 dph in both rearing systems.

334 In addition, pepsin remained undetected, whereas BAL activity was maintained
335 unvariable and that of amylase and alkaline proteases increased with respect to the
336 previous period when data are expressed as U/mg protein (ANOVA, $P < 0.05$) but
337 remained constant when reported as U/larva (Figure 4; ANOVA, $P > 0.05$).

338 *Period 3: 11-15 dph (MES, 4.5 – 5.0 mm TL; INT, 4.5 – 5.3 mm TL)*

339 The first taste buds were formed along the buccopharyngeal epithelium at 12 dph (4.5
340 mm TL) (Figure 5a), whereas goblet cells appeared at the oesophagus at 15 dph (INT =
341 5.3 ± 0.2 mm; MES = 5.0 ± 0.2 mm), increasing their number over time (Figure 5b). At

342 this age, the first pharyngeal teeth also appeared at the posterior part of the
343 buccopharynx area (Figure 5c).

344 During this period, the activity of pancreatic enzymes (α-amylase, BAL and total
345 alkaline proteases) remained stable compared to the previous stage (ANOVA, $P > 0.05$),
346 whereas pepsin was firstly detected in both rearing systems. When comparing the
347 activity of larval digestive enzymes between treatments at 12 dph, α-amylase was higher
348 in intensive- than in semi-intensive-reared larvae (Figure 6; t-test, $P < 0.05$), BAL and
349 total alkaline proteases presented the opposite trend ($P < 0.05$), and pepsin did not show
350 any significant variation (Figure 6; t-test, $P < 0.05$).

351 *Period 4: 20-30 dph (MES, 6.5 – 8.7 mm TL; INT, 6.0 – 8.2 mm TL)*

352 The first gastric glands at the pyloric portion of the stomach were evident at 20 dph (6.0
353 ± 0.2 mm TL) in the INT group and at 17 dph (5.7 ± 0.4 mm TL) in the MES group
354 (Figure 5d).

355 Supranuclear bodies in the midgut appeared between 20-23 and 20-25 dph in INT
356 and MES-larvae, respectively (Figure 2). The first goblet cells were evident in the
357 midgut at 23 dph (7.2 ± 0.7 mm TL) in the MES group and at 25 dph (7.8 ± 1.0 mm TL)
358 in the INT one (Figure 5e). Moreover, the appearance of pyloric caeca had a 5-day delay
359 in larvae reared under intensive conditions compared to those of the semi-intensive
360 group (28 *vs* 23 dph) where mean fish length were 8.5 ± 1.2 and 7.2 ± 0.7 mm TL,
361 respectively (Figure 5f).

362 As it is shown in Figure 4, pancreatic enzyme activities were similar to those of the
363 previous stage (ANOVA, $P > 0.05$) when given as U/mg protein, but dramatically
364 increased when reported per individual larvae. Regardless of the units considered,
365 pepsin significantly increased in comparison to previous stages (ANOVA, $P < 0.05$). At
366 30 dph, the activity of pepsin was *ca*. 2.5 times higher in internsive-reared larvae than in

367 MES-larvae (P < 0.05) whereas alkaline proteases was higher in the INT group only
368 when expressed as U/mg protein (Figure 6).
369
370 *Oxidative stress*
371 Regarding the antioxidant system and the levels of the oxidative stress biomarkers,
372 GPx, SOD, and GST activities tended to significantly decreased in every developmental
373 stage compàred to 7 dph (Figure 7; ANOVA, P < 0.05). In addition, significantly lower
374 activities of GPx, SOD and GST were also evident in MES-larvae compared to INT-
375 larvae in most developmental stages (Figure 7; t-test, P < 0.05). On the other hand, GSH
376 content was significantly higher at 18 dph than at 7 dph for both rearing systems (Figure
377 7; t-test, P < 0.05), following a trend to a progressive reduction of the enzyme content
378 with larval age. A gradual decrease of GR activities was also recorded with age which
379 was however not significant between culture conditions, with the exception of those
380 measured at 7 dph which were lower for the MES group (Figure 7; t-test, P < 0.05).
381
382 **Discussion**
383 *Ontogeny of greater amberjack digestive system*
384 The ontogeny of the digestive system of greater amberjack can be considered as a rapid
385 process, similarly to other carangid species like *S. lalandi* (Chen *et al.* 2006) and *S.*
386 *rivoliana* (Teles *et al.*, 2017). The development of the digestive capacity is controlled by
387 endogenous factors and generally, it is genetically programmed, which results in similar
388 developmental patterns between teleost fish larvae (Rønnestad *et al.* 2013). However,
389 the time of appearance of the digestive structures and their functionality can be
390 influenced by a number of factors of which temperature and feeding regime are among
391 the most critical issues (Kamler 2002). As developmental rates are correlated with the

392 larval TL, rearing protocols have also to be synchronized with larval size. Generally, in
393 marine fish species, the feeding protocol has to include firstly rotifers, whereas *Artemia*
394 nauplii have to be offered at larger sizes. However, these general principles have to be
395 tailored for each fish species in order to adapt rearing conditions to larval ontogeny. In
396 this sense, the histological description of the main digestive organs, the ontogenetic
397 profile of digestive enzymes, and their dietary adaptation may be used as reliable
398 indicators of larval development, food acceptance, digestive capacity and of their
399 further larval performance (Ueberschär 1993; Rønnestad *et al.* 2013). It is of special
400 relevance to merge data in the same study from two types of approaches, *i.e.*
401 morphological (histological analysis) and functional (assessment of the activity of
402 digestive enzymes) development information on the digestive system; as in some cases
403 the presence of a morphologically distinct organ (*i.e.* stomach) does not match with its
404 functionality (Solovyev *et al.* 2016).

405 The pattern of development of digestive enzyme activities in *S. dumerili* is similar to
406 that reported to other temperate and warm water marine species (Rønnestad *et al.* 2013),
407 and especially to that of *S. lalandi* (Chen *et al.* 2006). In brief, the activity of the main
408 carbohydrases, proteolytic and lipolytic enzymes were detected before and just after
409 hatching. Regardless of detecting BAL activity during the embryonic and at hatching
410 stages in greater amberjack, it does not mean that embryos and/or newly hatched larvae
411 digest lipids contained in their yolk sac reserves by means of this lipolytic enzyme
412 produced by the exocrine pancreas. In fact, these results indicate that the
413 spectrophotometric method for assessing this enzyme, whose activity is enhanced by
414 means of bile salts (sodium cholate), is not specific (Nolasco-Soria *et al.* 2018) and it
415 may also detect lipases hydrolyzing tryglicerides and wax esters contained in the yolk
416 (Heming & Buddington 1988). Yolk protein serves two primary functions: it provides

417 amino acids for tissue growth and supplies energy via catabolic processes (Heming &
418 Buddington 1988). However, the detection of alkaline protease activities before
419 hatching may be mainly attributed to the presence of chorionase, an alkaline proteolytic
420 enzyme involved in hatching (Hagenmaier 1974; Yamagami 1988) rather than to yolk
421 protein digestion. These results would be in agreement to those reported by Segner *et al.*
422 (1989), who stated that trypsin, the main alkaline pancreatic protease, activity was not
423 found in the yolk syncytium of fish larvae. In this sense, yolk amino acids and proteins
424 may be obtained by non-selective bulk endocytosis (Heming & Buddington 1988) and
425 receptor-mediated pinocytosis (Rønnestad & Fihn 1993), respectively. Amylolytic
426 activity have been reported by several authors in the yolk of fish (Gawlicka *et al.* 2000;
427 Naz 2009, present study) and poultry embryos (Ikeno & Ikeno 1991), although this
428 activity may not be correlated to pancreatic α-amylase, since the exocrine pancreas was
429 not fully differentiated during the embryo stage. Amylolytic activity during embryonic
430 development and during the lecitotrophic stage may be attributed to carbohydrate
431 (glycoproteins) utilization contained in yolk reserves as source of energy and structural
432 components (Cetta & Capuzzo 1982; Whyte *et al.* 1993; Kamler 2002).

433 After hatching, the activity of the above-mentioned enzymes tended to increase in
434 parallel to the development of the exocrine pancreas as it has been reported in other fish
435 species (see review by Rønnestad *et al.* 2013). Similar to most fish species described so
436 far, the increment in activity of pancreatic enzymes in *S. dumerili* would be correlated to
437 the morphogenesis of the exocrine pancreas (4-5 dph; 4.0 ± 0.1 mm TL) and the
438 accumulation of zymogen granules in pancreocytes, and to the complete resorption of
439 the yolk sac and transition to exogenous feeding, as our histological data indicated.
440 These results are in agreement to those reported in another carangid species like the
441 golden pompano *Trachinotus ovatus* (Ma *et al.* 2014) and *S. rivoliana* (Teles *et al.*,

442 2017). The decrease in activity of α-amylase from hatching to the onset of exogenous
443 feeding in comparison to values found in embryos may be genetically programmed
444 rather than dietarily induced as it has been reported in most of carnivorous fish larvae,
445 even though the magnitude of the above-mentioned ontogenic decrease in activity is
446 species-specific (Govoni *et al.* 1986; Zambonino-Infante & Cahu 2001; Rønnestad *et al.*
447 2013). In addition, the higher activity of α-amylase found in *S. dumerilii* larvae aged 12
448 dph reared under INT conditions may be attributed to a delay in the maturation of the
449 digestive function (Zouiten *et al.* 2011), as it has been previously reported when data of
450 α-amylase activity has been used as marker of the functionality of the digestive system
451 and accessory glands (Zambonino-Infante & Cahu 2001, 2007). However, the above-
452 mentioned delay in digestive function maturation in *S. dumerilii* larvae was
453 compensated within a few days for intensive-reared specimens as data from larvae
454 sampled at 30 dph from both rearing systems indicated. After the onset of exogenous
455 feeding, total activity of the three assayed pancreatic enzymes, when expressed as
456 U/larvae, sharply increased until the end of the study at 30 dph (8.2-8.7 mm TL).
457 Similarly to other species, total alkaline proteases and BAL were the main pancreatic
458 enzymes during the first days after first feeding in *S. dumerili*, indicating that peptides
459 and proteins, as well as lipids (triglycerides) are the principal sources of energy for
460 sustaining larval growth and development. In particular, alkaline proteolytic enzymes,
461 especially trypsin and chymotrypsin the main pancreatic alkaline proteases, are
462 generally regarded as being particularly significant in the early life stages of fish larvae
463 because of the absence of a functional stomach with its acid protease, pepsin (Rønnestad
464 *et al.* 2013). Under present rearing standard conditions and in agrement with other
465 studies assessing the changes in alkaline protease specific activities along larval
466 development in carnivorous fast-growing species (Chen *et al.* 2006; Ma *et al.* 2014;

467 Solovyev *et al.* 2016; among others), major changes in the activity of total alkaline
468 proteases were found between the onset of exogenous feeding until the early juvenile
469 stage at 30 dph. However, the large deviation values in alkaline protease specific
470 activities found in different sampling points (6-10, 11-15 and 20-30 dph) denoted a
471 heterogeneous larval population, which probably masked somehow the above-
472 mentioned activity peaks associated with pancreas differentiation and changes in food
473 items. Under the current experimental conditions, alkaline proteolytic specific activity
474 was slightly different among the rearing system (MES *vs.* INT) and tended to decrease
475 at late larval stages (21-30 dph), concomitantly to the sharp increase in pepsin activity
476 observed during this period. However, when expressed per larvae, the pancreatic
477 protease activity was still high at day 30 post hatching. These results suggested that
478 even though the stomach was functional at early ages (17 dph, 5.7 ± 0.4 mm TL for
479 MES), and peptic activity appeared and increased due to the presence of abundant
480 gastric glands as histological data revealed, the complete transition to a juvenile-
481 digestion pattern based on acid digestion was not achieved in *S. dumerili* specimens
482 aged 30 dph and measuring 8.2-8.7 mm in TL, as the presence of eosinophilic
483 supranuclear bodies in the hindgut also suggested (Ma *et al.* 2005; Teles *et al.*, 2017). It
484 is really feasible that this process occurred at later stages (Zambonino-Infante & Cahu
485 2001), but current experimental design and sampling schedule did not allow to validate
486 this hypothesis and further longer studies (>30 dph) are needed to elucidate this
487 concern. Regardless of this fact, present results are in agreement to those reported for *T.*
488 *ovatus*, where pepsin activity was detected at higher body sizes of 5 mm in TL and then
489 progressively increased at older ages (Ma *et al.* 2014). When comparing pepsin activity
490 between both rearing protocols, present results show that regardless of the late
491 appearance of gastric glands in larvae from the INT group in comparison to those larvae

492 reared in MES (Figure 2), pepsin activity was higher in *S. dumerili* from the INT group
493 at 30 dph. Such differences in pepsin activity between both systems may be attributed to
494 the feeding protocol and could be considered as dietarily induced rather than differences
495 in the maturation of the stomach, since larvae from the INT were mainly fed on
496 enriched *Artemia* and a compound diet, whereas those from the MES treatment were
497 offered a more diverse diet (*Artemia*, copepods, compound diet, and eggs and fish
498 larvae). Thus, differences in the level of protein, peptide and free amino acid contents
499 between food items may explain such differences in acid protease activity (Zambonino-
500 Infante & Cahu 2007).

501 BAL is an important enzyme for the hydrolysis of a wide range of lipids such as
502 glycerophospholipids, cholesterol esters and lipid-soluble vitamins (Rønnestad *et al.*
503 2013). In *S. dumerili*, BAL total activity sharply increased with the transition to
504 exogenous feeding and remained stable afterwards, whereas there were not clear
505 differences in activity regarding the larval rearing method considered. In *T. ovatus*¸
506 lipase showed two ontogenic activity peaks, one coinciding with first feeding as in the
507 present study in *S. dumerili*, and a second one when golden pompano were fed with
508 *Artemia* nauplii (Ma *et al.* 2014). In contrast, in *S. dumerili* and *S. lalandi* this peak in
509 activity associated to a shift in the type of live prey (rofiter *vs. Artemia* nauplii) (11-15
510 dph) was not detected, which may be associated to the larval sampling procedure used
511 in the present work where individuals were pooled by periods of 5 - 10 days, whereas in
512 other similar studies larvae were sampled more often. Independently of the rearing
513 system considered, histological data revealed that the percentage of area covered by
514 lipid vacuoles in the liver decreased between 11 and 15 dph in comparison to younger
515 ages. These results differed from those observed in *S. rivoliana* (Teles *et al.*, 2017),
516 differences that may be attributed to different larval rearing and *Artemia* enrichment

517 procedures. Under present experimental conditions, the above-mentioned decrease in
518 the level of accumulation of fat stores in the hepatic parenchyma in *S. dumerili* larvae
519 may be correlated to a change in food items (*Artemia* AF *vs.* EG nauplii) coupled with a
520 higher energy demand to support the higher somatic larval growth observed during this
521 period. In addition, the former results were also correlated with a decrease in the activity
522 of GR, GSH, GPX and GST enzymes, which is in agreement with the above-mentioned
523 hypothesis.

524 The comparison between both larval rearing methods, MES *vs.* INT, revealed that
525 there was a large conservation in the ontogenic differentiation of digestive structures
526 during the first developmental stages, independently of the rearing procedure applied as
527 it is shown in Figure 2. The only remarkable variations between both culture procedures
528 were found in the final differentiation of the digestive system and in particular related to
529 the appearance of gastric glands and pyloric caeca. These two digestive structures were
530 detected in *S. dumerili* larvae at smaller sizes in larvae reared in MES (5.7 and 7.2 mm
531 in TL, respectively) in comparison to those from the INT system (6.0 and 8.5 mm in TL,
532 respectively). This pattern was also observed in European sea bass (*Dicentrarchus*
533 *labrax*) (Zouiten *et al.* 2011), as well as in meagre (*Argyrosomus regius*) (Papadakis *et*
534 *al.* 2013; Solovyev *et al.* 2016) when comparing these two rearing systems. Although
535 there are very few studies evaluating the impact of these two different rearing
536 procedures on larval development and quality, the former authors suggested that the
537 proliferation of wild zooplankton on semi intensive tanks besides the presence of
538 enriched live preys (rotifers and *Artemia* nauplii) had a key effect on promoting larval
539 development, although this process was compensated within a few days for the
540 intensive-reared larvae (Zouiten *et al.* 2011). Deviations from these values obtained

541 under standard rearing conditions may be indicative of problems in larval quality,
542 development and/or rearing conditions (Zambonino-Infante & Cahu 2001).

543 Antioxidant endogenous system plays an important role in providing cell protection
544 from oxidative stress during larval development (Dandapat *et al.* 2003). This period is
545 highly demanding in energy and oxygen uptake, thus the influence of a poor nutritional
546 status or other unfavourable conditions can enhance oxidative damage. There have been
547 a number of studies on the antioxidant defence system in fish, particularly in relation to
548 specific oxidative stress conditions and also in relation to age and development (Peters
549 & Livingstone 1996; Dorval *et al.* 2003; Kalaimani *et al.* 2007; Skjærven *et al.* 2013;
550 Hamre *et al.* 2014; Liravi *et al.* 2014). Generally, the endogenous antioxidant system
551 includes some enzymes which catalyze the reaction of ROS degradation. Therefore,
552 SOD protects against oxidative damage by catalyzing the reaction of dismutation of the
553 superoxide anion to H_2O_2, GRx reduces both hydrogen peroxide and organic
554 hydroperoxides, GR catalyses the reaction to form GSH (glutathione) and maintain a
555 ratio GSH/GSSG (oxidized GSH) under oxidative stress, and GST detoxify some of the
556 secondary ROS produced by reaction with cellular constituents (Rudneva 2013).
557 However, there have not been relevant studies specifically investigating the activity of
558 the antioxidant enzyme systems in developmental larval stages of the greater amberjack.
559 The present study has shown that readily measurable specific activities of most
560 important antioxidant enzymes were present in the first life stages of greater amberjack
561 larvae (from 7 dph to 30 dph) in both rearing technologies. Overall, the results indicated
562 that the activities of GPx, SOD, GR and GST tended to decrease at 18 dph, after the
563 flexion stage, while this reduction was significantly lower in the semi-intensive than in
564 the intensive rearing system. According to literature, a similar progressively reduction in
565 antioxidant enzyme activities with regard, however, to different dph fishes-dependant

566 stages of larval development have been found in Atlantic cod (*Gadus morhua*) for GPx,
567 but not for SOD (Hamre *et al.* 2014), in turbot (*Scophthalmus maximas*) for SOD but
568 not for GR and GPx (Peters & Livingstone 1996), in *Dentex dentex* for SOD (Mourente
569 *et al.* 1999) and in silver carp (*Hypophthalmichthys molitrix*) for GPx and SOD (Liravi
570 *et al.* 2014). Conversely, a progressively induction for GPx have been indicated in the
571 fast-growing Asian seabass (*Lates calcarifer*) (Kalaimani *et al.* 2007) and for SOD in
572 *Salmo iridaeus* (Aceto *et al.* 1994). Depletion in GSH content has been attributed either
573 to higher level of ROS production that convert more reduced GSH to its oxidized form
574 (GSSG) (Ou *et al.* 1996) or to a decreased activity of GR (Costagliola 1991). Thus, the
575 depletion in GSH content observed at 7 dph greater amberjack could be explained by a
576 higher level of ROS production as the activity of GR was significantly higher at this
577 stage compared to all the other stages. The overall patterns of all antioxidant enzymes
578 observed during the developmental stages of *S. dumerili* larvae might suggest specific
579 compensatory mechanisms of antioxidant defense to compensate ROS
580 production/removal (neutralize ROS) and to eliminate the damage of oxidative stress
581 due to a high metabolic rate (Solé *et al.* 2004). Present results also revealed that
582 oxidative stress appeared to be more severe in INT compared to MES rearing systems.
583 Reduced antioxidant enzyme activities in semi-intensive systems could have been
584 related to a lower oxidative stress and more stable rearing conditions than in the
585 intensive system. Thus, we assumed that the decrease/increase of antioxidant enzymes
586 activity indicated during larval stages might be related to the changes in the levels of
587 ROS. It has been reported that oxidative stress in aquatic organisms is more profound
588 during nutritional deficiency (Mourente *et al.* 1999; Hidalgo *et al.* 2002; Tocher *et al.*
589 2003; Morales *et al.* 2004), elevated temperature (Hwang & Lu 2002), hypoxia
590 (Kolkovski *et al.* 2000) and exposure to xenobiotics (Pedrajas *et al.* 1995; Dandapat *et*

591 *al.* 2003; Peňa-Llopis *et al.* 2003; Rudneva & Zalevskaya 2004). Taken into account the
592 two rearing protocols, we hypothesize that the change in the type of food given together
593 with the food availability after the 12 dph stage and after the 20 dph stage (Figure 1)
594 might have influenced the lower antioxidant enzymes activities obtained after 18 dph
595 compared to the 7 dph stage and in the 23 dph stage compared to 7 and 18 dph stages.
596 Besides, the increase of antioxidant and digestive enzymes activities at similar larval
597 stages (*i.e.* 7 dph and 6-10 dph, respectively), as well as the progressive decrease in both
598 antioxidant and digestive enzymes activities after the 7 dph stage revealed that both
599 biomarkers followed the same trend regardless of the physiological process considered.
600 Overall, various aspects of intensive aquaculture stimulate stress responses in fish
601 larvae because artificial systems are frequently exposed to a range of 'unnatural'
602 stressors, which are related to rearing practices (Zouiten *et al.* 2011). Thus, antioxidant
603 enzymes play an important role in inactivation of ROS and thereby control oxidative
604 stress as well as redox signaling. Both processes change across the life span of the
605 organism and thus modulate its sensitivity and resistance against free radical damage.
606

607 **Conclusions**

608 This study is the first one describing the morphoanatomical and functional development
609 of the digestive system in *S. dumerili* and assessing the impact of two different larval
610 rearing systems on the larval digestive function. In addition, the integration of data on
611 oxidative stress was used to evaluate ther impact of both rearing protocols in this novel
612 species. Coupling histological and biochemical data we were able to show that *S.*
613 *dumerili* larvae had a functional digestive system at the onset of exogenous feeding
614 when the digestive process was basically alkaline, regardless of the rearing technology
615 considered. The morphogenesis of the stomach and pyloric caeca were affected by the

616 rearing protocol, appearing earlier in fish cultured in semi-intensive in comparison to
617 those reared in intensive conditions. However, the above-mentioned differences were
618 compensated within a few days for the intensive-reared larvae as data on pepsin activity
619 indicated. Although both tested rearing strategies did not differ in larval performance in
620 terms of somatic growth and maturation of the digestive function, present results
621 showed that oxidative stress appeared to be more severe in larvae reared in intensive
622 than in semi-intensive systems, which may be attributed to more stable rearing
623 conditions in the semi-intensive rearing technology.

624

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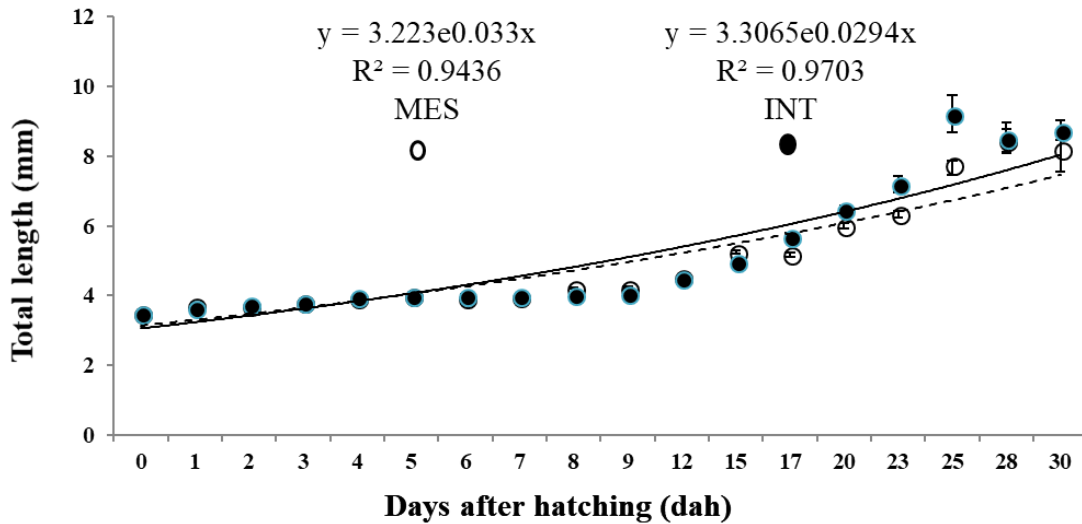
921 **Data Availability Statement**

922 The data that support the findings of this study are available from the corresponding
923 author upon reasonable request.

924

925 **Figure 1.**
926



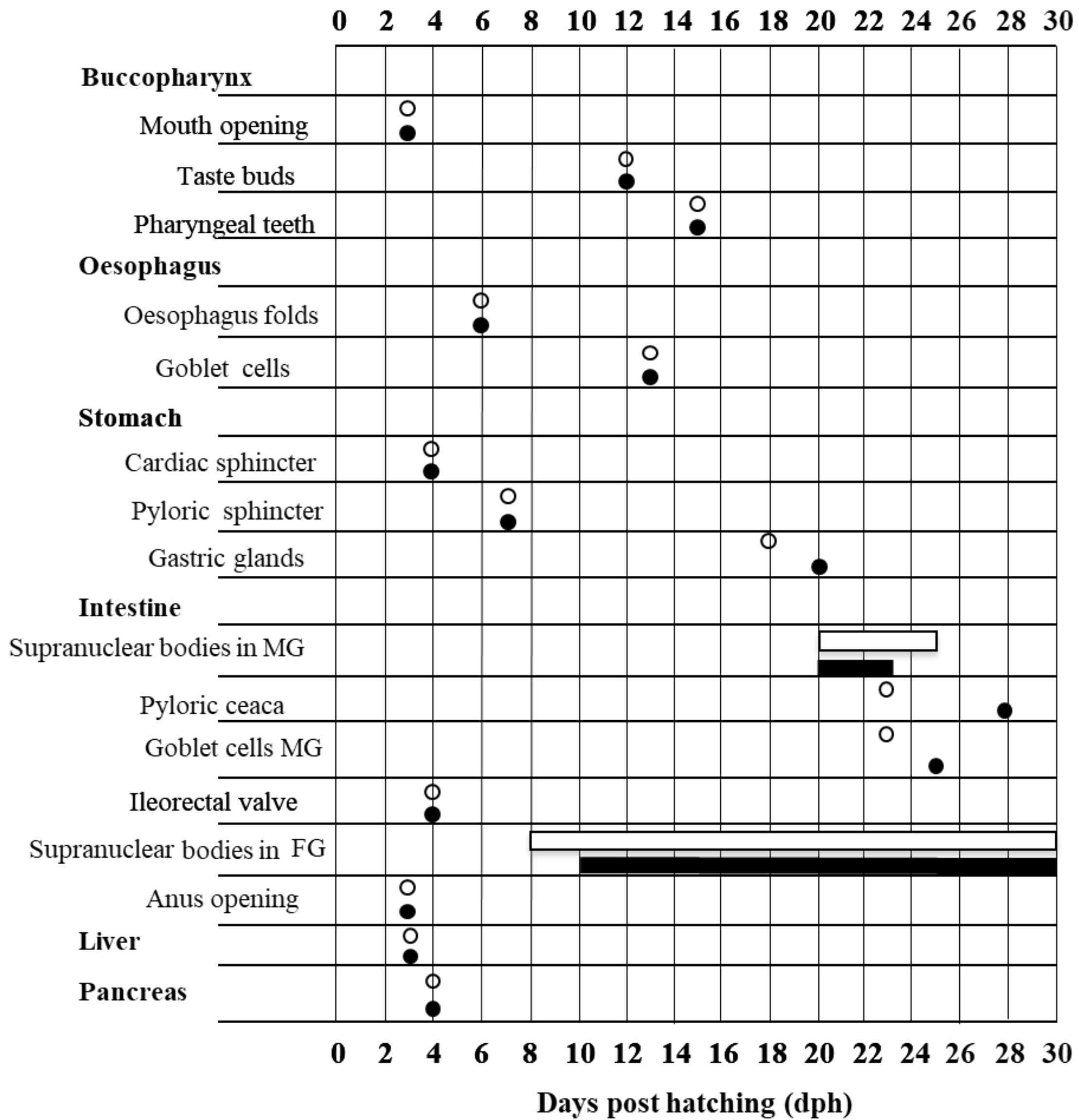
MES

	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30
Phytoplankton																															
Rotifers																															
Artemia nauplii AF																															
Artemia nauplii EG																															
Artificial food																															
Frozen eggs (*Sparus aurata*)																															
Fish larvae (*Sparus aurata*)																															

INT

	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30
Phytoplankton																															
Rotifers																															
Artemia nauplii AF																															
Artemia nauplii EG																															
Artificial food																															

927 **Figure 2.**



	0	1	2	3	4	5	6	7	8	9	12	15	17	20	23	25	28	30
INT	3.52	3.72	3.73	3.81	3.96	4.01	3.94	3.98	4.23	4.23	4.55	5.26	5.19	6.01	6.35	7.77	8.47	8.20
MES	3.52	3.67	3.75	3.83	3.99	4.05	4.03	4.02	4.05	4.09	4.53	5.00	5.69	6.49	7.2	9.23	8.54	8.74

Total length in (mm)

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929 **Figure 3.**
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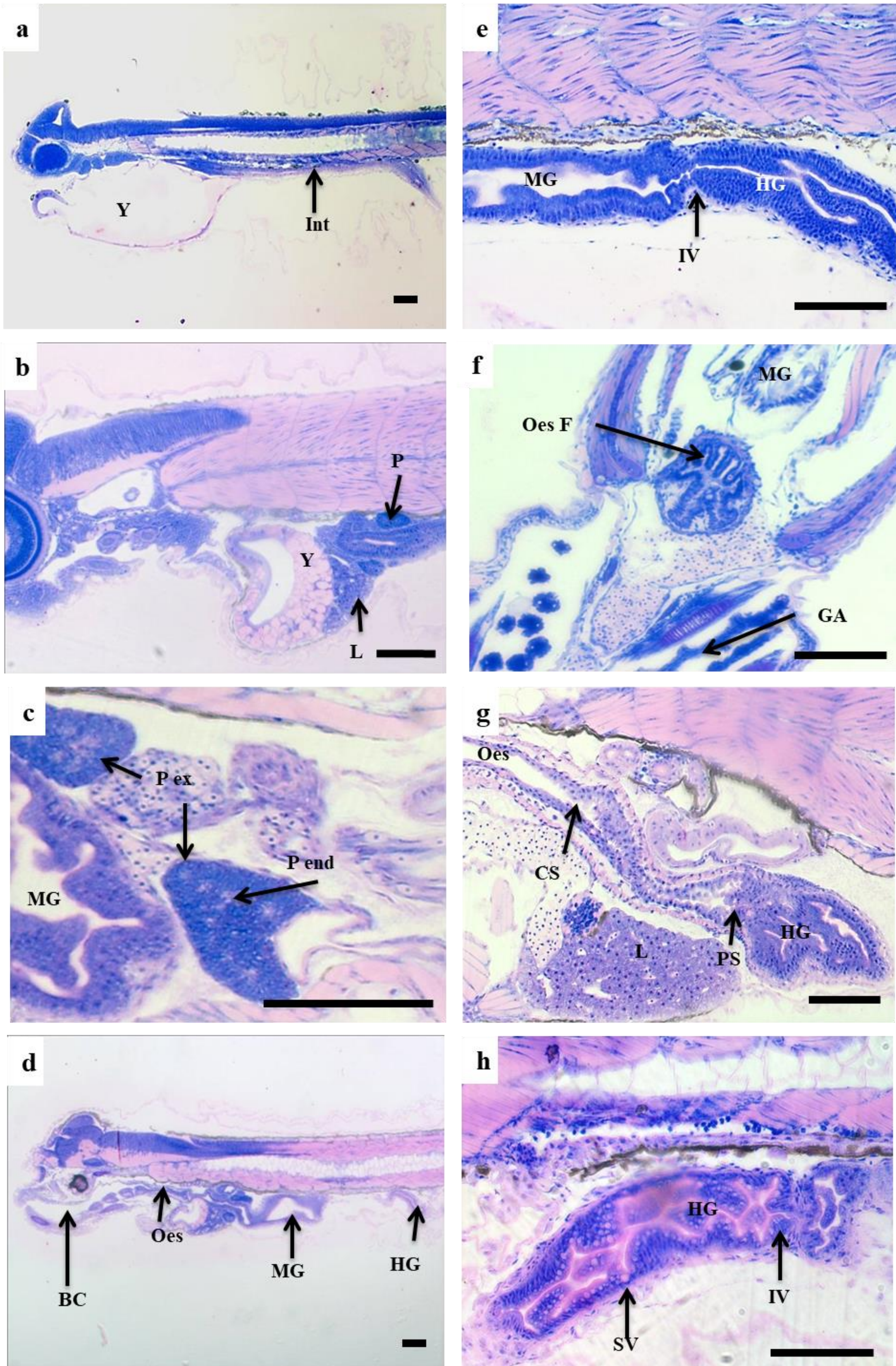

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932 **Figure 4.**
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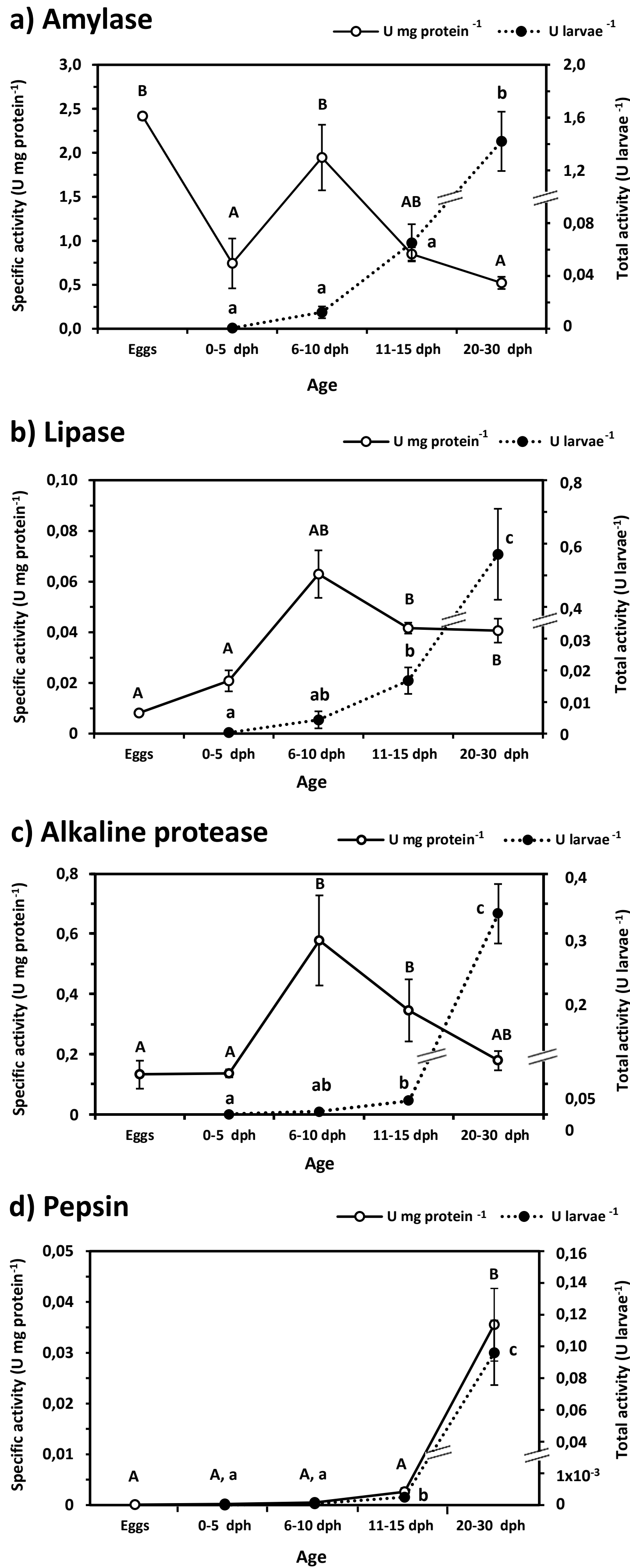
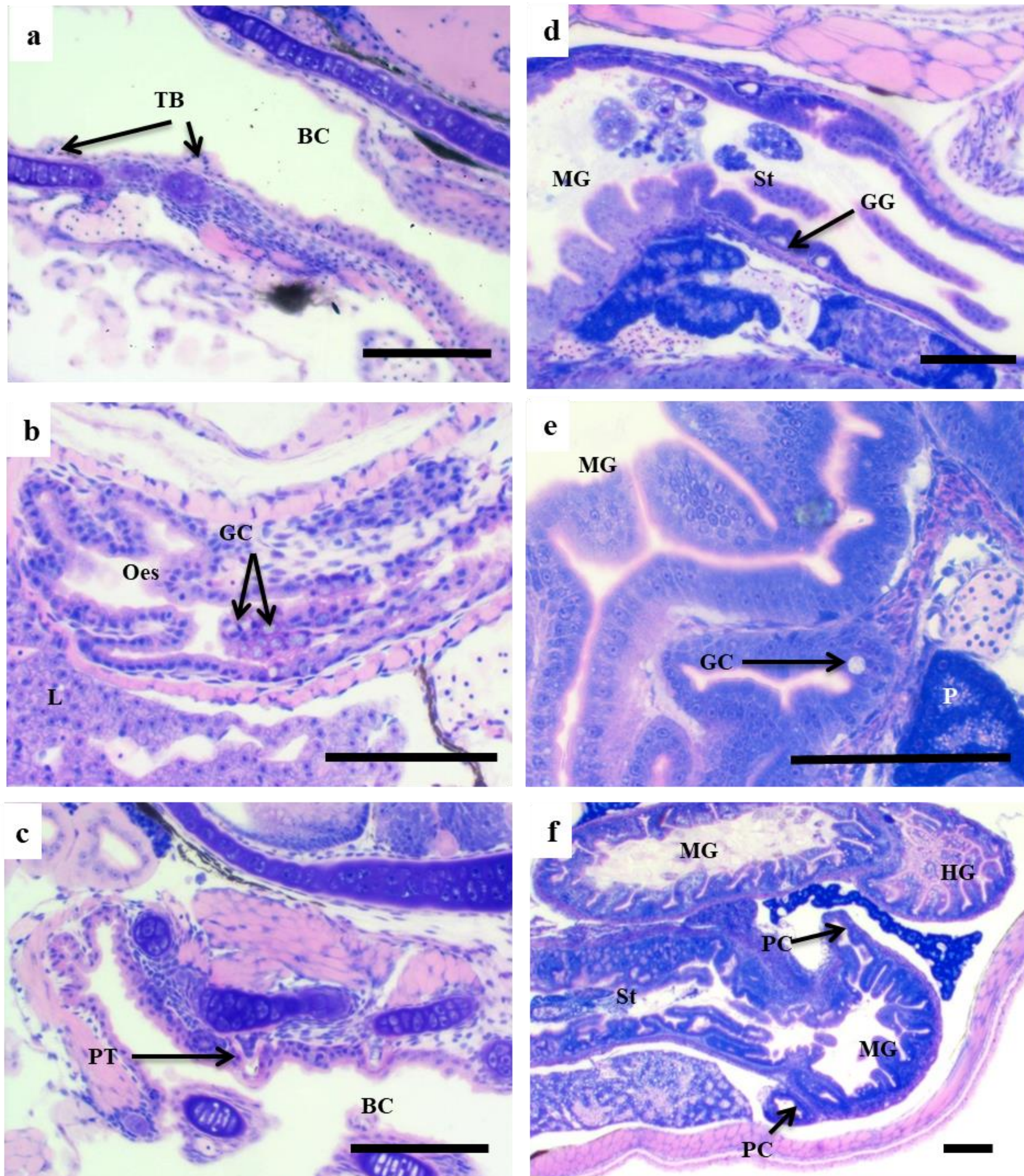


958
959 **Figure 5.**



960
961 **Figure 6**.
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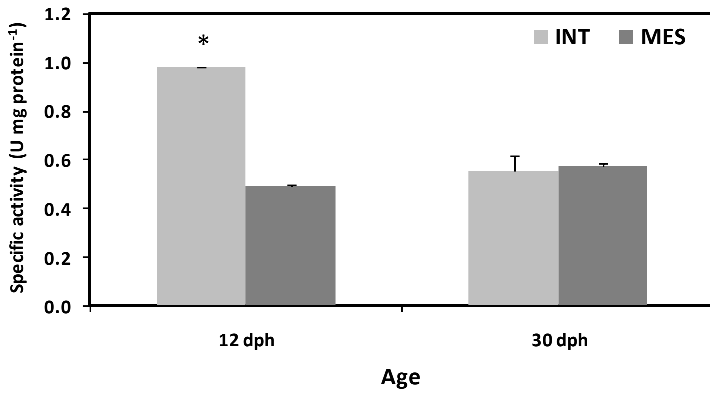
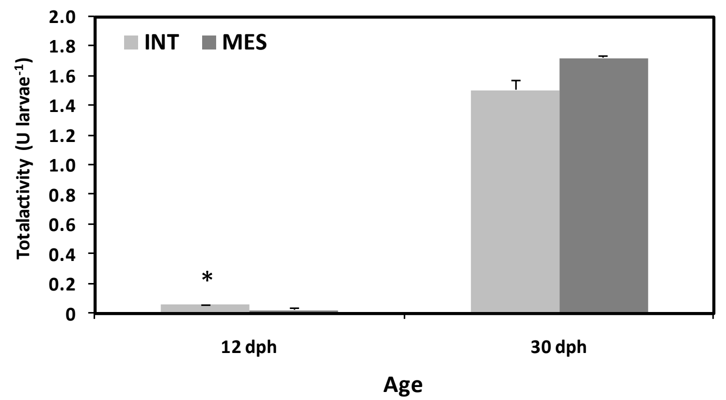
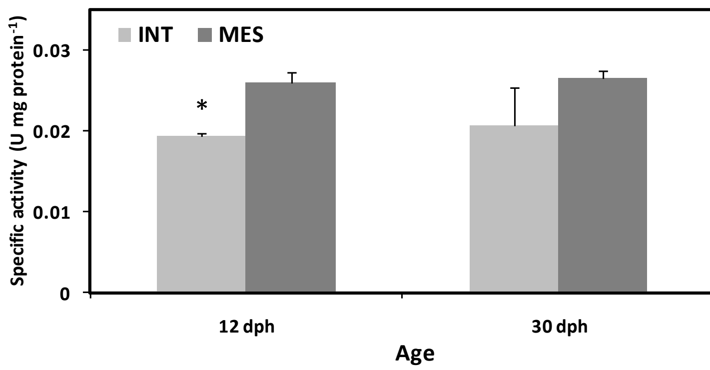
a) Amylase



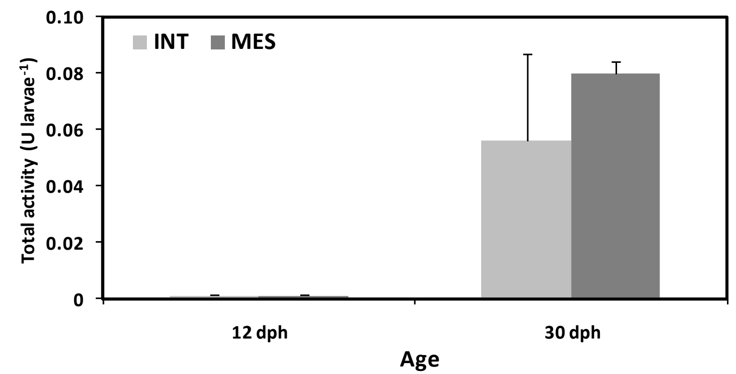
a) Amylase



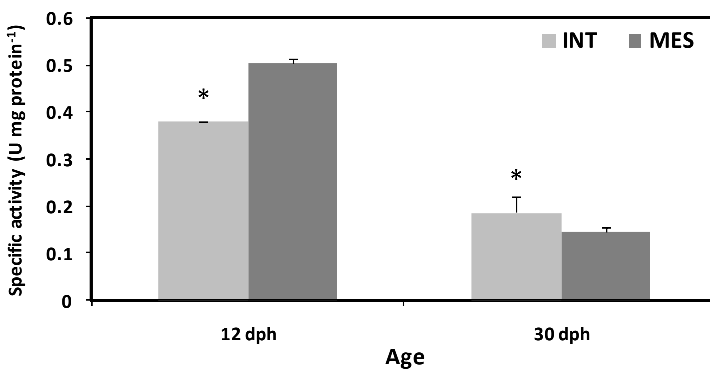
b) Lipase



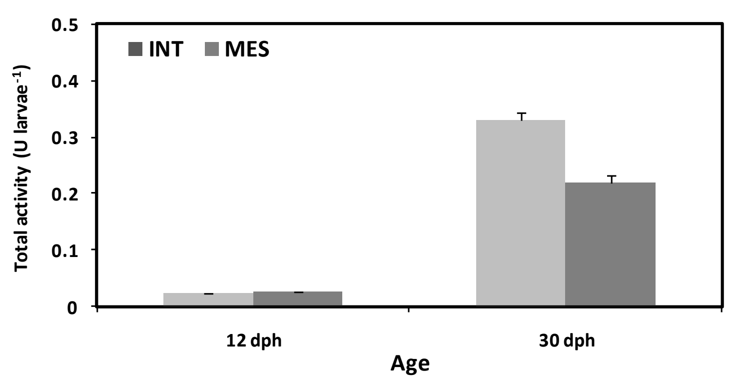
b) Lipase



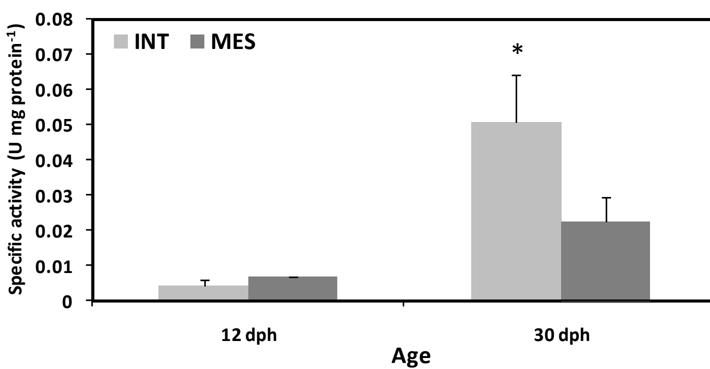
c) Alkaline protease



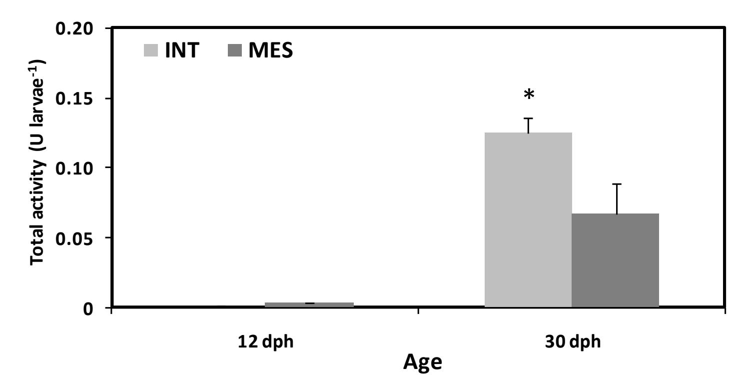
c) Alkaline protease



d) Pepsin



d) Pepsin



963 **Figure 7.**
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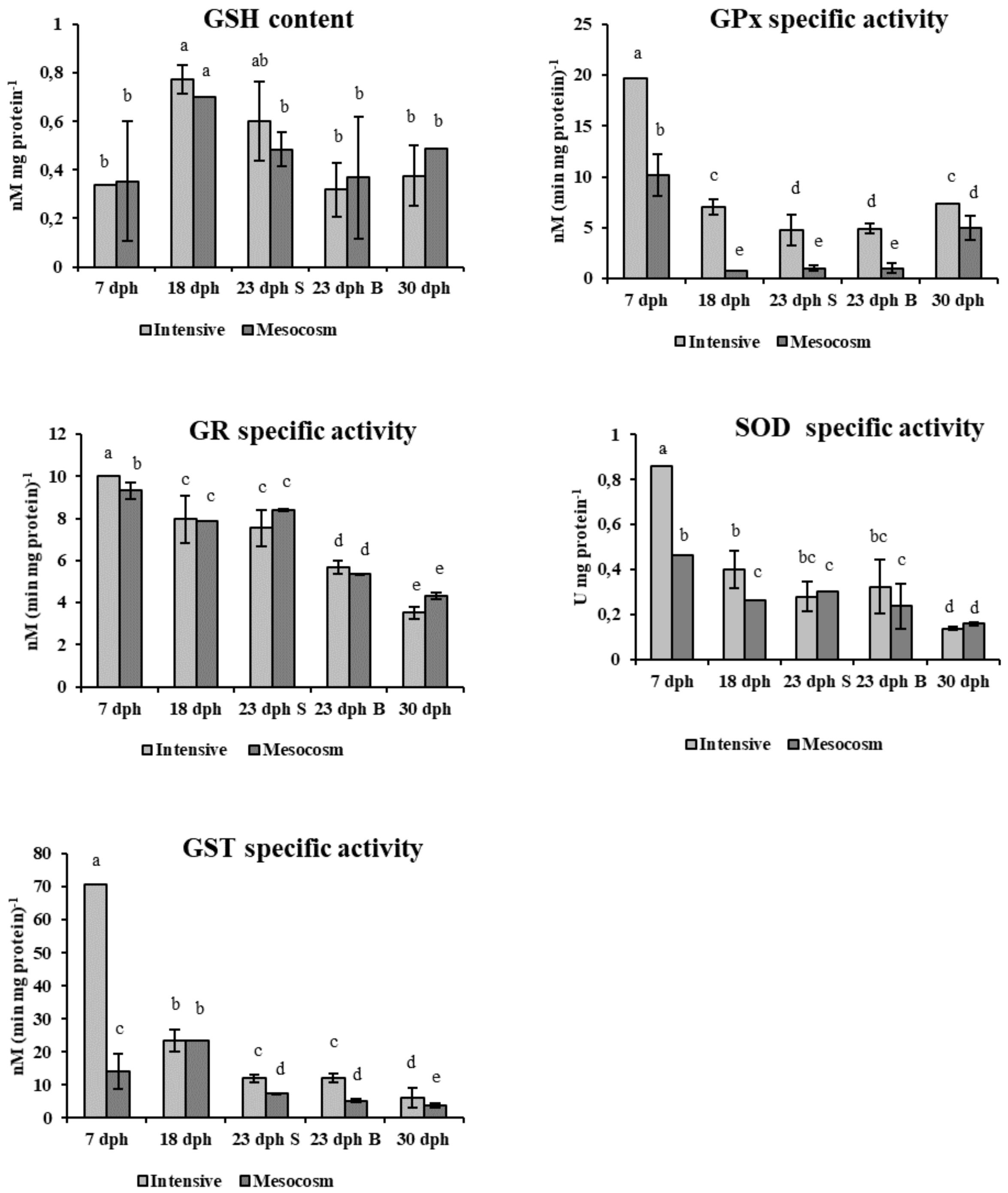

965 **Figure 1.** Growth performance of greater amberjack larvae (mean ± SD of total length)
966 cultured in intensive (INT) and semi-intensive (MES) rearing systems. Below the graph,
967 the rearing protocols used during the rearing procedure are presented, including type
968 and duration of food items provided.
969 **Figure 2.** Schematic representation of the appearance (open solid circles indicate the
970 MES, black solid circles indicate the INT system) of the main developmental structures
971 examined in greater amberjack larval digestive system, as a function of days after
972 hatching (dph, horizontal axis). Horizontal bars (white MES and black INT) indicate the
973 period that supranuclear bodies (vacuoles) were present in the anterior-median intestine
974 (mid gut, MG) and hindgut (HG). Below, mean values of the total length of greater
975 amberjack larvae for each sampling day and rearing system are presented.
976 **Figure 3.** Microphotographs of histological sections from greater amberjack larvae at
977 different developmental stages. (a) At 1dph showing digestive tract as a closed straight
978 tube located dorsal to the yolk sac. (b) At 2 dph when the liver and pancreas appeared.
979 (c) At 4 dph when the pancreas differentiated to endocrine and exocrine parts. (d) At 4
980 dph showing the different characteristic areas of the digestive system. (e) At 4 dph when
981 the ileo-rectal valve appeared. (f) At 6 dph showing the formation of folds at the
982 oesophagus. (g) At 6 dph showing the formation of the stomach area. (h) At 8 dph
983 showing the supranuclear vacuoles at the hindgut (MES). BC = buccopharynx; CS =
984 cardiac sphincter; GA = gill arches; HG = hindgut;Int = intestine; IV = ileo-rectal valve;
985 L = liver; MG = midgut;Oes = oesophagus;Oes F = oesophageal folds; P= pancreas; P
986 end = endocrine pancreas; P ex = exocrine pancreas; PS = pyloric sphincter; SV =
987 supranuclear vacuoles; Y = yolk. Bar represents 100 µm.
988 **Figure 4.** Specific activity (U mg protein^{-1}, white circles) and total activity (U larvae^{-1},
989 black circles) of digestive enzymes during the ontogeny of the digestive tract of greater
990 amberjack larvae cultured under intensive conditions. Different letters indicate
991 significant differences (P<0.05) between larval ages.
992 **Figure 5.** Microphotographs of histological sections of greater amberjack larvae at
993 different developmental stages. (a) At 12 dph when the taste buds appeared. (b) At 15
994 dph when the goblet cells appeared at the oesophagus. (c) At 15 dph showing the
995 pharyngeal teeth at the buccopharynx. (d) At 17-20 dph showing the gastric glands at
996 the stomach (MES *vs* INT). (e) At 23-25 dph showing the goblet cells at the midgut
997 (MES *vs* INT) and (f) at 23-28 dph showing the formation of the pyloric caeca (MES *vs*
998 INT). BC = buccopharynx; GC = goblet cells; GG = gastric glands; HG = hindgut; IV =
999 ileo-rectal valve; L = liver; G = midgut, Oes = oesophagus; PT = pharyngeal teeth; PC =
1000 pyloric caeca; St = stomach; TB = taste buds. Bar represents 100 µm.
1001 **Figure 6.** Comparison of specific activity (U mg protein^{-1}) and total activity (U larvae^{-1})
1002 of digestive enzymes of greater amberjack larvae between intensive and semi-intensive
1003 rearing systems at 12 and 30 dph. Asterisks (*) indicate significant differences (P<0.05)
1004 between INT and MES rearing systems for a particular enzyme and larval age.
1005 **Figure 7.**Changes in antioxidant defense systems (GSH content and GPx, GR, SOD and
1006 GST specific activities) of developmental larval stages of *Serioladumerili*cultured in
1007 intensive and semi-intensive rearing systems (S and B refer to small and large larvae,
1008 respectively).Different letters indicate significant differences (P<0.05) between larval
1009 ages.
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