Weaning European glass eels (*Anguilla anguilla*) with plant protein-based diets and its effects on intestinal maturation

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Abstract

Weaning glass eels with compound diets (36% proteins, 16% lipids) differing in their fishmeal (FM) level (50, 75 and 100% FM replaced by a blend of plant proteins, PP) was compared to a group fed cod roe. Weaning lasted for 20 days and then, eels were fed compound diets for 70 days, whereas the other group was only fed cod roe (90 days). Diets were tested with four replicates and evaluated in terms of growth, survival, glass eels metamorphosis into elvers, oxidative stress status and activity of digestive enzymes. Although glass eels are fed with fish roe and progressively weaned onto compound diets, results revealed that this strategy should not be prolonged for a long time, since

feeding glass eels with cod roe for 90 days negatively affected their growth (2 times lower than fish fed compound diets), delayed their metamorphosis, as well as the maturation of their digestive function as the ratio of alkaline phosphatase and leucine-alanine peptidase indicated. Weaning glass eels onto compound diets differing in their FM levels did not affect their growth, metamorphic stage nor the activity of pancreatic enzymes (total alkaline proteases, trypsin, bile salt-activated lipase and α-amylase), although 75% FM replacement by PP sources delayed the level of intestinal maturation in eels. In comparison to glass eels fed the 100% FM diet, survival was negatively affected in groups fed diets with 50 and 75% FM replacement by PP ingredients, which indicated that further improvement is needed in diet formulation for this stage of development.

Keywords: weaning, digestive enzymes, oxidative stress, fishmeal replacement, glass eel, elver

1. Introduction

European eel (*Anguilla anguilla*) farming has long been a worldwide industry based on raising young specimens from the glass eel stage until their commercial size. Until the industry will be capable of reproducing eels in captivity in a commercially viable way, this activity strictly depends on the availability of wild glass eels (FAO, 2004-2018). This practice has been a success in eel farming as glass eels accept readily the early food offered, and they are moderately easy to wean to compound diets with associated high survival rates and easy to transport and keep in captivity. However, the sustainability of this industry (production of 6,098 tonnes in 2016; FEAP, 2016) is directly linked to the health of the European eel stocks, which have declined dramatically in the last century (Jacoby et al., 2015).

Traditionally, some eel culturists recommended feeding *Tubifex* sp., invertebrates or raw meat to the glass eels before giving them artificial diets, whereas this practice has been substituted by the use of frozen fish roe as a first feed to get the eels to start feeding quickly in farm conditions (Heisnbroek, 1991), after which they are successfully weaned onto compound diets containing high fishmeal (FM) levels (46-58% FM; Heinsbroek, 1991; Rodriguez et al., 2005; Hirt-Chabbert et al., 2012) and other high quality marine derived ingredients (FAO, 2004-2018). However, considering that feeding represents up to 50 - 70% of total production costs in intensive fish farms (Rana et al., 2009), and marine raw ingredients, including FM, are among the most expensive ingredients used in aquafeed formulation (Tacon and Metian, 2008), there is a need to find alternative raw materials for aquafeeds. The increasing demand, price, restricted availability and fluctuations of FM supply have directed the most recent research into looking for alternative protein and oil sources (Naylor et al., 2009; Han, 2018). Many studies have shown considerable success in partial or total replacement of dietary FM with plant protein (PP) sources for various marine fish species during the on-growing phase (Hernández et al., 2007; Salze et al., 2010; Moxley et al., 2014; Yaghoubi et al., 2016; Lazzarotto et al., 2018; Kotzamanis et al., 2018 among

62 others). However, there exist few studies addressing this issue at younger stages of development
63 and how these feeding strategies affect their performance, digestive capacities and nutritional
64 condition (El-Saidy and Gaber, 2003; Enyidi and Mgbenka, 2015; Gisbert et al., 2016; Swanepoel
65 and Goosen, 2018).

66 The objective of this study was to evaluate the potential use of PP sources in weaning
67 diets for European glass eels and their impact on growth performance, survival, digestive
68 physiology and oxidative stress condition in this farmed species.

69

70 ## 2. Materials and methods

71 *2.1 Animals and experimental design*

72 Experimental procedures were conducted in compliance with the Guidelines of the European
73 Union Council (86/609/EU) for the use of laboratory animal. As European eel is considered as a
74 critically endangered species (Jacoby et al., 2015), all surviving specimens and those not used for
75 analytical purposes (n = 1,820) were used for restocking purposes in the Ebro River.

76 Wild glass eels (n = 5,000; 180 ± 51 mg in wet body weight, BW) were captured during their
77 onshore migration as described in Gisbert and López (2008) and obtained from Pescados y Mariscos
78 Roset S.L. (Deltebre, Spain). Glass eels were acclimated to IRTA-SCR facilities for two weeks (water
79 temperature were progressively increased from 13.0 to 20.0 in a RAS unit – IRTAmar®) and then they
80 were distributed into 16 tanks (100 L) at an initial density of 200 glass eels (initial body weight (BW_i)
81 = 190 ± 60 mg) per tank connected to a recirculation system IRTAmar®. During their acclimation,
82 glass eels were fed *Artemia* nauplii (EG grade, INVE) and cod roe (mature ovaries of *Gadus morhua*)
83 *ad libitum* on alternating days. In addition, they were treated once with Mebendazole (1 mg L^{-1} for
84 24 h) (Sigma-Aldrich, Alcobendas, Spain) and formalin (100 mg L^{-1} for 5 h) as described in Mellergaard
85 (1990) and Andree et al. (2013). Treatments were conducted during the first week of acclimation in

a three-day interval to avoid potential stress derived from anthelmintic and antibacterial treatments. Mortality at the end of the acclimation period was *ca.* 8.4% (420 individuals). Water quality conditions during the experimental period were as follows: temperature 20.0 ± 0.1 ºC (mean ± standard deviation, SD), dissolved oxygen 6.7 ± 0.3 mg L^{-1} (~96% saturation), salinity 1.3 ± 0.3 ‰; NH_4^+ 0.15 ± 0.1 mg L^{-1}, NO_2^- 0.18 ± 0.1 mg L^{-1}, and the photoperiod was 10L:14D (light:darkness).

In this study, three isoproteic (36%) and isolipidic (16%) diets differing in their FM and PP levels (Diet 1: 100% FM; Diet 2: 50% FM and 50% PP; Diet 3: 25% FM and 75% PP) were evaluated as potential weaning diets for glass eels (Table 1). Experimental diets were compared to a control group that was only fed with natural food (frozen cod roe; proximal composition: 19.4%, crude proteins, 9.2% crude lipids, 1.9% ashes, 69.5% water content). Feed on a dry weight basis was distributed at 5% of glass eel stocked biomass (apparent satiation). Weaning lasted for 20 days; cod roe was progressively replaced by the compound diet (100/0, 75/0, 50/50, 25/75 %) every four days; thus, glass eels were completely weaned into experimental diets at day 25. Each treatment had four replicates, and the trial lasted for 90 days. Extruded diets (pellet size: 0.8 mm) were formulated and manufactured by Sparos Lda. (Portugal). The FM dietary component was partially substituted at 50 and 75% by a blend of PP sources (corn gluten, wheat gluten soybean meal and soy protein concentrate; Table 1), and supplemented with L-lysine and DL-methionine in order to balance their respective amino acid profiles (NRC, 2011).

2.2 Growth performance and glass eel staging

At the end of the feeding trial, all fish in each tank were anesthetized (100 mg MS-222 L^{-1}, Sigma-Aldrich, Spain) were individually counted in order to assess the impact of diet on their survival and their final body weight (BW_f) measured to the nearest 0.01 g. These values were used for calculating the specific growth rate of eels in BW (SGR_{BW}, % day^{-1}) = [(ln BW_f – ln BW_i) × 100]/time (days), where BW_f and BW_i are the final and initial BW values, respectively.

111 Skin pigmentation in eels fed different diets was used a proxy of their progress of the
112 metamorphosis from the glass eel to the elver stage. In particular, pigmentation stages were
113 determined under a binocular microscope (n = 40-60 per tank) and classified according to the extent
114 of skin pigmentation over the head, tail and body regions, through stages VI_A (VI_{A0}, VI_{A1}, VI_{A2}, VI_{A3} and
115 VI_{A4}) to VI_B as described by Elie et al. (1982). In this study, authors used the term glass eel for all VI_A
116 stages, whereas specimens at the stage VI_B were considered as elvers.

117

118 *2.3 Analysis of digestive enzymes*

119 At the end of the trial, a subsample (n = 10 fish per tank) was used for measuring the intestinal
120 maturity level (alkaline phosphatase and leucine-alanine peptidase ratio) and activity of the main
121 pancreatic digestive enzymes. In particular, elvers were sacrificed with an overdose of MS222 and
122 eels' dissection (separation of the abdominal region containing the hepatopancreas and intestine)
123 was conducted on a prechilled glass plate maintained at 0°C and the abdominal region homogenized
124 in cold 50 mM mannitol, 2 mM Tris–HCl buffer (pH =7.0). Then, 1 ml of the supernatant was pipetted
125 and stored at −20°C for assaying pancreatic (total alkaline proteases, trypsin, chymotrypsin, α-
126 amylase, bile salt activated lipase), gastric (pepsin) and intestinal cytosolic (leucine–alanine
127 peptidase) enzymes. The rest of the homogenate was used for the purification of intestinal brush
128 border membranes (Gisbert et al., 2018), which served to quantify the activity of alkaline
129 phosphatase, aminopeptidase-N and maltase.

130 Pancreatic, gastric and intestinal digestive enzymes were analyzed as previously described
131 by Gisbert et al. (2009) and following the instructions provided by Solovyev and Gisbert (2016)
132 regarding the optimal time of samples' storage before their analyses. All analyses were conducted at
133 25 ºC using standard protocols for the following digestive enzymes: total alkaline proteases (García-
134 Careño and Haard, 1993), trypsin (Holm et al., 1988), α-amylase (Métais and Bieth, 1968), bile salt-
135 activated lipase (Iijima et al., 1998), pepsin (Worthington, 1991), alkaline phosphatase (AP, Gisbert

et al., 2018), aminopeptidase-N (Maroux et al., 1973), maltase (Dahkqvist, 1970) and leucine–alanine peptidase (LAP, Nicholson and Kim, 1975). Soluble protein of extracts was quantified by means of the Bradford's method (Bradford, 1976). All the assays were made in triplicate from each pool of elvers (biological replicate) and absorbance read using a spectrophotometer (Tecan™ Infinite M200, Switzerland). The ratio AP/LAP activities was used as an index for measuring the impact of experimental diets on intestinal maturation (Cahu and Zambonino-Infante, 2001).

2.4 Analysis of oxidative stress enzymes

Levels of lipid peroxidation and activity of oxidative stress enzymes in elvers were assayed in the whole animal (n = 5 per tank) in order to evaluate their health condition. Quantification of lipid peroxidation was conducted by means of the acid reactive substances (TBARs) method described in Solé et al. (2004). The activity of oxidative stress enzymes was measured using the following protocols: catalase (CAT, Aebi, 1974), glutathione reductase (GR, Carlberg and Mannervik, 1975), superoxid dismutase (SOD, McCord and Fridovich, 1969) and glutathione peroxidase (GPX, Günzler and Flohé, 1985). Soluble protein of crude enzyme extracts was quantified by Bradford's method. Enzymatic activities were expressed as specific enzyme activity, in nmol mg^{-1} protein, with the exception of SOD that was expressed as percentage of inhibitory activity. All assays were carried out in triplicate at 25 °C, and the absorbance was read using a Tecan™ Infinite M200 spectrophotometer.

2.5 Proximate composition analysis

Elvers (n = 10 per tank) and diets (n = 2) were homogenized and aliquots dried at 120 °C for 24 h in order to estimate gravimetrically their water content; total fat and protein levels that were determined according to Folch et al. (1957) and Lowry et al. (1951), respectively; and ash determined

159 by heating the sample at 500 to 600 °C for 24 h in a muffle furnace (AOAC, 1990). All analyses were
160 conducted in triplicate (methodological replicates).

161

162 *2.5 Statistics*

163 Data are presented as the mean ± standard error of the mean. Values for different parameters were
164 compared between them by means of one-way ANOVA at a reliability level of 5%. Data expressed as
165 percentage were transformed (arc sine square root transformation). Data were checked for
166 normality (Kolmogorov–Smirnov test) and homogeneity of variances (Bartlett's test) prior to their
167 comparison. When statistical differences were found among data with the ANOVA, the Duncan's
168 Multiple Range test was applied in order to detect which groups differed among each other.

169

170 ## 3. Results

171 *3.1 Survival and growth performance*

172 Elver survival was significantly affected by weaning and type of tested compound diet (Table 2; $P <$
173 0.05). The highest survival rates were observed in elvers fed cod roe (67.5 ± 3.2%), but survival of
174 glass eels weaned onto Diet 1 (100% FM) was 45.8 ± 5.3%. Glass eels weaned onto Diets 2 (50% FM
175 and 50% PP) and 3 (25% FM and 75% PP) showed the lowest survival rate values (31.1 ± 7.4% and
176 27.8 ± 9.8%, respectively). Glass eels weaned onto compound diets achieved larger BW values than
177 those just fed on cod roe (Table 2; $P < 0.05$), whereas no differences in BW and SGR values were
178 found between glass eels weaned onto Diets 1, 2 and 3. Final size distribution in BW also differed
179 among dietary groups (Fig. 1). In particular, glass eels fed cod roe showed higher positive skewness
180 (1.71) and kurtosis (2.25) values than those weaned onto compound diets (skewness = 0.75 – 0.93;
181 kurtosis = -0.11 – -0.37). The above-mentioned results were due to a higher frequency (36.9 ± 2.8%)
182 of smaller animals (201-400 mg) in glass eels fed cod roe than in the other groups (average values

183 ranging from 17.3 to 20.5%) that were weaned onto compound diets. In addition, the feeding
184 strategy also affected glass eel metamorphosis (Table 2; $P < 0.05$). Eels fed cod roe were mainly at
185 the metamorphosis stage of VIA3. Meanwhile, eels weaned onto compound diets showed a higher
186 frequency of specimens at more advanced metamorphosis stages of VIA4 and VIB than eels fed cod
187 roe.

188 *3.2 Proximate composition, lipid peroxidation values and activity of oxidative stress enzymes*

189 There were not statistically significant differences in proximate composition of elvers from different
190 experimental groups (Table 3; $P > 0.05$). No differences in lipid peroxidation (TBARs) values, neither
191 in the activity of oxidative stress enzymes (CAT, GST, GPX, GR and SOD) were found among groups
192 (Table 3; $P > 0.05$).

193

194 *3.3 Activity of digestive enzymes*

195 Results of the specific activity of digestive enzymes in glass eels fed cod roe and weaned onto
196 different experimental diets are shown in Table 4. Concerning pancreatic enzymes, the activity of
197 total alkaline proteases and trypsin differed among groups. In particular, the highest and lowest
198 activities in total alkaline proteases were found in glass eels weaned onto the compound diets and
199 cod roe, respectively, whereas glass eels fed Diet 2 showed intermediate values between the above-
200 mentioned ones ($P < 0.05$). Regarding trypsin, the highest activity values were found in eels fed the
201 compound diets that were significant lower to those found in eels fed cod roe ($P < 0.05$). No
202 differences in the specific activity of α-amylase and bile salt-activated lipase were found between
203 experimental groups ($P > 0.05$). Considering pepsin, the gastric digestive enzyme analysed, no
204 statistically differences were found among different treatments ($P > 0.05$). Regarding the activity of
205 brush border intestinal enzymes, AP activity was higher in glass eels weaned onto Diets 1 (100% FM)
206 and 2 (50% FM replaced by PP sources) in comparison to those fed Diet 3 (75% FM replaced by PP
207 sources) and those fed cod roe ($P < 0.05$). Aminopeptidase-N and maltase activities were higher in

208 all fish weaned onto compound diets in comparison to those eels just fed on cod roe ($P < 0.05$). The
209 activity of leucine-alanine peptidase (LAP), a cytosolic enzyme, followed the inverse pattern in regard
210 to AP, being higher in glass eels weaned onto Diet 3 and those fed cod roe, but lower in glass eels
211 weaned onto Diet 1 and 2 ($P < 0.05$). When considered the index of intestinal maturation calculated
212 as the ratio between a brush border and cytosolic enzyme (AP/LAP), results revealed that glass eels
213 weaned onto Diets 1 and 2 had the highest index values, whereas the lowest ones were found in
214 glass eels weaned onto Diet 3 and those fed cod roe ($P < 0.05$).

215

216 ## 4. Discussion

217 Initial feeding of glass eels in aquaculture facilities after being captured during their onshore
218 migration is the most difficult part of the on-growing process. Generally, there is moderate to high
219 mortality during the first three-month period following their capture and acclimation to farming
220 conditions (Degani et al., 1984; Hirt-Chabbert et al., 2012). During the acclimation process, some
221 glass eels do not learn or adapt to eat the offered food (*i.e.* fish roe, inert diets), and consequently,
222 they progressively lose weight and die, whereas some others that are used to feed tend to grow very
223 slowly and have no economic value. In contrast, the remainder, which are generally the majority
224 of captured glass eels, adapt well to new husbandry conditions and grow very rapidly in body
225 weight (Degani and Levanon, 1983; Degani et al., 1984; Heinsbroek, 1991; Gisbert et al., 2012).
226 Under current experimental conditions, authors found the same adaptive pattern to captive
227 conditions, even though results varied depending on the dietary treatment. In particular, glass
228 eels fed cod roe showed a lower growth performance and higher frequency of specimens at
229 earlier metamorphic stages (VI_{A0-3}) than those weaned onto compound diets. These results may be
230 correlated to the higher survival observed in glass eels fed cod roe, as the inverse trend was found
231 in glass eels weaned onto compound diets. In this sense, weaning may have operated a selection
232 mechanism in rearing tanks, as those specimens not sufficiently adapted to the compound diet
233 would end up dying, meanwhile this would not have occurred in glass eels fed cod roe, as this group

showed a higher frequency of slow growing and less metamorphic advanced specimens. In addition, SGR values of glass eels fed cod roe (SGR = 1.19 % day^{-1}) were similar to those reported in a previous study, when glass eels were fed for 70 days with hake roe (Gisbert et al., 2011). Furthermore, present data illustrated that glass eels could be weaned onto compound diets (36% crude protein, 16% crude fat) with similar somatic growth performances (SGR = 1.82 – 1.75 % day^{-1}) than those fed a compound diet containing 50% crude protein and 22% crude lipids (SGR = 1.86 % day^{-1}) (Hirt-Chabbert et al., 2012). The agreement of present results with previous similar studies regardless of the type of diet used and origin of wild fish confirmed the validity of present data.

In this study, authors showed that it was feasible to wean glass eels onto diets containing different levels of FM with PP sources (Diet 2) when data on somatic growth performance and size distribution in BW were considered. However, weaning success varied depending on the level of dietary FM inclusion. In particular, there was a significant decrease in eel survival with increasing FM substitution levels (50 and 75% FM replacement with PP sources) in comparison to eels from the same cohort fed Diet 1 (100% FM), even though no differences in lipid peroxidation nor activity of oxidative stress enzymes were found among groups. These results may indicate that further improvement is needed in formulating compound diets for glass eels, if PP sources want to be include in feed formulae (Hirt-Chabbert et al., 2012). The results of the present study showed that glass eels weaned onto the three tested compound diets had lower survival rates than those fed cod roe. However, these findings may not be strictly interpreted as negative, as this feeding strategy served to remove slow-growing specimens and those that were not well adapted to culture conditions.

The establishment of an efficient brush border membrane digestion represents a major step in gut maturation (Henning et al., 1994). This process is characterized by an increase in the activity of brush border enzymes such as alkaline phosphatase, aminopeptidase N and/or maltase,

concomitantly with a decrease in the activity of the cytosolic enzymes like leucine-alanine peptidase. Consequently, the ratio AP/LAP is generally considered as a good marker of intestinal maturation in fish larvae and for assessing the acquisition of an adult mode of digestion in juvenile fish (Cahu and Zambonino-Infante, 2001). Regarding anguillid species, there is few and fragmented knowledge on the digestive physiology of this group of species (Kurokawa et al., 1995; Gisbert et al., 2011; Murashita et al., 2013; Hsu et al., 2015). Several studies on Japanese eel (*A. japonica*) at the leptocephalus stage revealed that exocrine pancreatic enzymes needed for proper protein, lipid and carbohydrate digestion are present in leptocephali (Kurokawa et al., 2002; Murashita et al., 2013; Hsu et al., 2015) and their activities substantially increased after the metamorphosis of leptocephali into glass eels (Hsu et al., 2015). Regardless of the eel species considered, there is not available information about gut development and its maturation process. Thus, changes in AP and LAP activities reported in this study suggested the gut maturation in *A. anguilla* during the transition from the glass eel to the elver stage, although transcriptomic (RNAseq) data from *A. japonica* suggested this process may start to occur during the glass eel stage, which attributed with their active costal migration beginnings (Hsu et al., 2015). The former authors postulated that during this period an increase in the number of transcripts linked to amino acid absorption was found in *A. japonica*, an increase that was linked to a higher protein demand to support the fast muscular growth needed for inshore migration (Hsu et al., 2015). In addition to the biological interpretation of changes in gut functionality along eel ontogeny, low activity values of maltase and aminopeptidase N coupled with differences in the AP/LAP ratio indicated that the feeding strategy for glass eel had a direct impact on their gut condition, as feeding glass eels with cod roe for 90 days resulted in a delay in the intestinal maturation process that was associated to lower somatic growth and less advanced metamorphic stage (VI_{A3}). These results may be interpreted as this type of natural food does not cover the nutritional needs of glass eels metamorphosing into elvers for such a long period of time (90 days). Regarding gut maturation of glass eels weaned onto Diet 3 (75% FM replaced by PP sources), the differences in AP/LAP values in those fish in comparison to glass eels weaned onto Diet

285 1 (100% FM) and 2 (50% FM replaced by PP sources) may not be attributed to the above-mentioned
286 factors. In particular, there were no differences in somatic growth, size distribution in BW nor
287 metamorphic stage of eels weaned onto compound diets differing in their level of FM inclusion.
288 Consequently, such differences in AP/LAP were likely a consequence of the high content of
289 phoshoproteins generally found in FM compared to PP sources (Silva et al., 2010). However, the
290 higher activities found in glass eels weaned onto Diets 1 and 2 for the other brush border enzymes
291 (aminopeptidase N and maltase) could not be interpreted as a result of higher content in specific
292 substrates. Thus, this fact strongly suggested an intestinal villi and microvilli better developed in
293 these groups compared to those weaned onto Diet 3, even though no significant effect was noted
294 on growth. Similar results were reported in Atlantic salmon (*Salmo salar*), European sea bass
295 (*Dicentrarchus labrax*) and gilthead sea bream (*Sparus aurata*) fed diets with different FM levels
296 (Bakke-Mckellep et al., 2000; Tibaldi et al., 2006; Silva et al., 2010).

297 Regarding pancreatic proteolytic enzymes, total alkaline proteases and trypsin were affected
298 by the diet, whereas pepsin (acid protease produced in the stomach) was not affected. In particular,
299 glass eels fed cod roe showed lower activities of total alkaline proteases and trypsin, the main
300 proteolytic enzymes produced by the exocrine pancreas, in comparison to those fed compound
301 diets, whereas weaning glass eels onto compound diets with different FM levels did not affect the
302 activity of digestive pancreatic enzymes contrary to other studies in carnivorous species (Santigosa
303 et al., 2008). Trypsin cleaves protein at the carboxyl side of basic amino acids, lysine and arginine,
304 which show higher digestibility than other amino acids (NRC, 2011). Both lysine and arginine seem
305 to elevate plasma insulin levels. In salmonids, arginine is the most potent stimulator of insulin
306 secretion (Plisetskaya et al., 1991), whereas lysine has been reported to be more efficient than
307 arginine in stimulating the endocrine pancreas in *A. anguilla* (Ince, 1980). In this sense,
308 Rungruangsak-Torrissen et al. (2006) postulated that an increment in trypsin secretion accompanied
309 by increased plasma insulin levels resulted in growth enhancement in Atlantic salmon (*Salmo salar*).
310 In addition, as Péres et al. (1998) reported, differences in pancreatic proteolytic enzymes may be also

attributed to the higher crude protein content of experimental compound diets in comparison to cod roe (36.0 *vs*. 19.4%). Although there exists limited information about the digestive physiology of eels at early stages of development, the above-mentioned hypotheses may explain how eels fed compound diets exhibiting a higher somatic growth performance as a consequence of higher trypsin activities than those fed cod roe. Concerning pepsin, different feed quality did not affect the activity of this acid protease, since this enzyme is poorly regulated by dietary proteins (Zambonino-Infante and Cahu, 2007); results that were in agreement to those found in other species (Rungruangsak and Utne, 1981; Rungruangsak-Torrissen et al., 2006).

Regarding the activity of the other pancreatic enzymes assayed in the current study, the activity of α-amylase and bile salt-activated lipase was not affected by the diet. In particular, the absence of differences in α-amylase activity might be due to the similar content of carbohydrates between both types of diets (Cahu and Zambonino-Infante, 2001; Yu et al., 2012), although the determination of starch, the substrate for α-amylase, was not conducted in compound diets and cod roe offered to glass eels. Considering lipase activity, regardless of differences in crude lipid content between compound diets and cod roe, the activity of bile salt-activated lipase was similar between both groups. Some studies have found a stimulating effect of dietary lipid content on lipolytic enzymes in fish (Borlogan, 1990; Zambonino-Infante and Cahu, 1999). In European seabass larvae, there exist a direct response of lipase to triglycerides, but evidence was shown by Zambonino-Infante and Cahu (1999) that the maximal capacity of lipase synthesis was reached when diets contained 15% triglycerides. However, other authors have not found the above-mentioned effect (Hoehne-Reitan et al., 2001; Morais et al., 2004). As the former authors suggested, differences in lipid levels between compound diets and cod roe (16.0 vs. 9.2%) might not be as high as for differentially stimulating lipase activity. According to HoehneReitan et al. (2001), the activity of bile salt-dependent lipase in turbot (*Scophthalmus maximus*) larvae appeared to be a function of the ingestion rate rather than dietary lipid levels. Unfortunately, this hypothesis could not be tested under current experimental conditions, since feed intake in glass eels fed different diets was not measured in this

study. Thus, there is a need for further studying and understanding of the underlying mechanisms controlling lipid metabolism and dietary regulation of lipolytic enzymes at glass eel and elver stages in anguillid species, which is reinforced by the recent findings of Gaillard et al. (2016) who found that there existed differences in the regulation of lipid metabolism and lipolytic capacities between glass eels from different geographical locations in American eel (*A. rostrata*).

5. Conclusions

Although traditionally glass eels are fed with cod roe and progressively weaned onto compound diets, present results revealed that this strategy should not be prolonged for a long time, since feeding glass eels with cod roe for 90 days negatively affected their somatic growth, delayed their metamorphosis into elvers, as well as the maturation of their digestive function. Weaning glass eels onto compound diets differing in their FM levels did not affect their growth performance nor metamorphic stage, although 75% FM replacement by PP sources (corn gluten, wheat gluten, soy bean meal and soy protein concentrate) delayed the level of intestinal maturation in eels as indicated by the AP/LAP ratio. When compared to glass eels fed the 100% FM diet, survival was negatively affected in groups fed diets with 50 and 75% FM replacement by PP ingredients, which suggested that further improvement is needed in diet formulation for this stage of development.

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References

Aebi, H., 1974. Catalase. In: Bergmeyer, H.V. (Ed.), Methods in Enzymatic Analysis Vol. 2. Academic Press Inc., New York, NY, pp. 674–684.

Andree, K. B., Rodgers, C. J., Furones, D., Gisbert, E., (2013). Co-Infection with *Pseudomonas anguilliseptica* and *Delftia acidovorans* in the European eel, *Anguilla anguilla* (L.): a case history of an illegally trafficked protected species. J. Fish Dis. 36(7), 647-656. |https://doi.org/10.1111/jfd.12066

Association of Official Analytical Chemists (AOAC), 1990. In: Heldrich, K. (Ed.), Official Methods of Analysis of the Association of Official Analytical Chemists. Arlington, VA, p. 684.

Bakke-Mckellep, A.M., Nordrum, S., Krogdahl, A., Buddington, R.K., 2000. Absorption of glucose, amino acids, and dipeptides by the intestines of Atlantic salmon (*Salmo salar* L.). Fish Physiol. Biochem. 22, 33–44. https://doi.org/10.1023/A:1007872929847

Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein–dye binding. Anal. Biochem. 72, 248–254. https://doi.org/10.1016/0003-2697(76)90527-3

Borlongan, I.G., 1990. Studies on the digestive lipases of milkfish, *Chanos chanos*. Aquaculture 89, 315–325. https://doi.org/10.1016/0044-8486(90)90135-A

Cahu, C., Zambonino-Infante, J.Z., 2001. Substitution of live food by formulated diets in marine fish larvae. Aquaculture 200, 161-180. https://doi.org/10.1016/S0044-8486(01)00699-8

Carlberg, I., Mannervik, B., 1975. Purification and characterization of the flavoenzyme glutathione reductase from rat liver. J. Biol. Chem. 250, 5475–5480.

Dahkqvist, A., 1970. Assay of intestinal disaccharidase. Enzym. Biol. Clin. 11, 52–66. https://doi.org/10.1016/0003-2697(68)90263-7

384 Degani, G., Levanon, D., 1983. The influence of low density on food adaptation, cannibalism and
385 growth of eels (*Anguilla anguilla* (L.)). Isr. J. Aquac. Bamidgeh 35, 53-60.

386 Degani, G., Levanon, D., Trieger, G., 1984. Preliminary study on the influence of different feeds on
387 mortality and growth of eels (*Anguilla anguilla* L.) in the initial period. Isr. J. Aquac. Bamidgeh
388 36, 47-52.

389 Elie, P., Lecomte-Finiger, R., Cantrelle, I., Charlon, N., 1982. Définition des limites des différents
390 stades pigmentaires durant la phase civelle d'*Anguilla anguilla* L. (poisson téléostéen
391 anguilliforme). Vie Milieu 32, 149–157

392 El-Saidy, D.M.S.D., Gaber, M.M.A., 2003. Replacement of fish meal with a mixture of different plant
393 protein sources in juvenile Nile tilapia, *Oreochromis niloticus* (L.) diets. Aquac. Res. 34, 1119-
394 1127. https://doi.org/10.1046/j.1365-2109.2003.00914.x

395 Enyidi, U.D., Mgbenka, B.O., 2015. Replacement of fish meal with bambara nut waste meal in the
396 diets of larval African catfish *Clarias gariepinus* Burachell (1822). Bri. J. App. Sci. Tech. 5, 526-
397 537. https://doi.org/10.9734/BJAST/2015/12886

398 FAO 2004-2018. Cultured Aquatic Species Information Programme. *Anguilla anguilla*. Cultured
399 Aquatic Species Information Programme. Text by The Danish Aquaculture Development
400 Group (DANAQ). In: FAO Fisheries and Aquaculture Department [online]. Rome. Updated 1
401 January 2004. [Cited 19 July 2018].

402 Folch, J., Lees, N., Sloane-Stanley, G.H., 1957. A simple method for the isolation and purification of
403 total lipids from animal tissues. J. Biol. Biochem. 226, 497–509.

404 Gaillard, M., Pavey, S. A., Côté, C. L., Tremblay, R., Bernatchez, L., Audet, C., 2016. Regional variation
405 of gene regulation associated with storage lipid metabolism in American glass eels (*Anguilla*
406 *rostrata*). Comp. Biochem. Physiol. 196A, 30-37.
407 http://dx.doi.org/10.1016/j.cbpa.2016.02.013

García-Careño, F.L., Haard, N.F., 1993. Characterization of proteinase classes in langostilla (*Pleuroncodes planipes*) and crayfish (*Pacifastacus astacus*) extracts. J. Food Biochem. 17, 97–113. https://doi.org/10.1111/j.1745-4514.1993.tb00864.x

Gisbert, E., López, M.A., 2008. Impact of glass eel fishery on by-catch fish species: a quantitative assessment. Hydrobiologia 602, 87-98. https://doi.org/10.1007/s10750-008-9284-5.

Gisbert, E., Fernández, I., Alvarez-González, C.A., 2011. Prolonged feed deprivation does not permanently compromise digestive function in migrating European glass eels *Anguilla anguilla*. J. Fish Biol. 78, 580-592. https://doi.org/10.1111/j.1095-8649.2010.02879.x

Gisbert, E., Giménez, G., Fernandez, I., Kotzamanis, Y., Estévez, A., 2009. Development of digestive enzymes in common dentex, *Dentex dentex*, during early ontogeny. Aquaculture 287, 381–387. https://doi.org/10.1016/j.aquaculture.2008.10.039

Gisbert, E., Mozanzadeh, M.T., Kotzamanis, Y., Estévez, A., 2016. Weaning wild flathead grey mullet (*Mugil cephalus*) fry with diets with different levels of fish meal substitution. Aquaculture 462, 92-100. http://dx.doi.org/10.1016/j.aquaculture.2016.04.035

Gisbert, E., Nolasco, H., Solovyev, M., 2018. Towards the standardization of brush border purification and intestinal alkaline phosphatase quantification in fish with notes on other digestive enzymes. Aquaculture 487, 102-108. https://doi.org/10.1016/j.aquaculture.2018.01.004

Günzler, A., Flohé, L., 1985. Glutathione peroxidase. In: Greenwald, R.A. (Ed.), CRC Handbook of Methods for Oxygen Radical Research 1. CRC Press Inc., Boca Raton, Florida, USA, pp. 285–290.

Han, D., Shan, X., Zhang, W., Chen, Y., Wang, Q., Li, Z., Shang, G, Xu, P., Li, J., Shouqi, S., Kangsen, M., Tang, Q, De Silva, S.S., 2018. A revisit to fishmeal usage and associated consequences in Chinese aquaculture. Rev. Aquacult. 10, 493-507. https://doi.org/10.1111/raq.12183

Heinsbroek, L.T.N., 1991. A review of eel culture in Japan and Europe. Aquac. Res. 22, 57-72.

Hernández, M.D., Martínez, F.J., Jover, M., García, B.G., 2007. Effects of partial replacement of fish meal by soybean meal in sharpsnout seabream (*Diplodus puntazzo*) diet. Aquaculture 263, 159-167. https://doi.org/10.1111/j.1365-2109.1991.tb00495.x

Hirt-Chabbert, J. A., Skalli, A., Young, O. A., Gisbert, E., 2012. Effects of feeding stimulants on the feed consumption, growth and survival at glass eel and elver stages in the European eel (*Anguilla anguilla*). Aquac. Nutr. 18, 152-166. https://doi.org/10.1111/j.1365-2095.2011.00883.x

Hoehne-Reitan, K., Kjørsvik, E., Reitan, K.I., 2001. Bile salt-dependent lipase in larval turbot, as influenced by density and lipid content of fed prey. J. Fish Biol. 58, 746–754. https://doi.org/10.1111/j.1095-8649.2001.tb00527.x

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. J. Nutr. 118, 515–520. https://doi.org/10.1093/jn/118.4.521

Hsu, H-Y., Chen, S-H., Cha, Y-R., Tsukamoto, K., Lin, C-Y., Han, Y-S., 2015. *De novo* assembly of the whole transcriptome of the wild embryo, preleptocephalus, leptocephalus, and glass eel of *Anguilla japonica* and deciphering the digestive and absorptive capacities during early development. PLoS ONE 10, e0139105.

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 Physiol. Biochem. 18, 59–69. https://doi.org/10.1023/A:1007725513389

Ince, B.W. 1980. Amino acid stimulation of insulin secretion from the in situ perfused eel pancreas; modifications by somatostatin, adrenaline and theophylline. Gen. Comp. Endocrinol. 40, 275–282. https://doi.org/10.1016/0016-6480(80)90276-2

454 Jacoby, D.M., Gollock, M. 2014. *Anguilla anguilla*. The IUCN Red List of Threatened Species 2014:
455 e.T60344A45833138. http://dx.doi.org/10.2305/IUCN.UK.2014-
456 1.RLTS.T60344A45833138.en. Downloaded on 19 July 2018.

457 Jacoby, D. M., Casselman, J. M., Crook, V., DeLucia, M. B., Ahn, H., Kaifu, K., Kurwie, T., Sasal, P.,
458 Silfvergrip, A.M.C., Smith, K.G., Uchida, K., Walker, A.M., Gollock, M.J., 2015. Synergistic
459 patterns of threat and the challenges facing global anguillid eel conservation. Glob. Ecol.
460 Conserv. 4, 321-333. https://doi.org/10.1016/j.gecco.2015.07.009

461 Kotzamanis, Y., Kouroupakis, E., Ilia, V., Haalabous, J., Papaioannou, N., Papanna, K., Richards, R.,
462 Gisbert, E., 2018. Effects of high-level fishmeal replacement by plant proteins supplemented
463 with different levels of lysine on growth performance and incidence of systemic noninfectious
464 granulomatosis in meagre (*Argyrosomus regius*). Aquacult Nutr. In press.
465 https://doi.org/10.1111/anu.12814

466 Kurokawa, T., Kagawa, H., Ohta, H., Tanaka, H., Okuzawa, K., Hirose, K., 1995. Development of
467 digestive organs and feeding ability in larvae of Japanese eel (*Anguilla japonica*). Can. J. of
468 Fish. Aquat. Sci. 52, 1030-1036. https://doi.org/1030-1036. 10.1139/f95-101

469 Kurokawa, T., Suzuki, T., Ohta, H., Kagawa, H., Tanaka, H., & Unuma, T., 2002. Expression of
470 pancreatic enzyme genes during the early larval stage of Japanese eel *Anguilla japonica*.
471 Fish. Sci. 68, 736-744. https://doi.org/10.1046/j.1444-2906.2002.00487.x

472 Lazzarotto, V., Médale, F., Larroquet, L., Corraze, G., 2018. Long-term dietary replacement of
473 fishmeal and fish oil in diets for rainbow trout (*Oncorhynchus mykiss*): Effects on growth,
474 whole body fatty acids and intestinal and hepatic gene expression. PLOS ONE 13, e0190730.

475 Lowry, O.H., Rosebrough, N.J., Farr, A.L., Randall, R.J., 1951. Protein measurement with the Folin
476 phenol reagent. J. Biol. Chem. 193, 265–275.

477 Maroux, S., Louvard, D., Baratti, J., 1973. The aminopeptidase from hog-intestinal brush border.
478 Biochim. Biophys. Acta 21, 282–295. https://doi.org/10.1016/0005-2744(73)90083-1

479 McCord, J.M., Fridovich, I., 1969. Superoxide dismutase: an enzymatic function for erythrocuprein
480 (hemocuprein). J. Biol. Chem. 244, 6049–6055.

481 Mellergaard, S., 1990. Mebendazole treatment against *Pseudodactylogyrus* infections in eel (*Anguilla*
482 *anguilla*). Aquaculture 91, 15-21. https://doi.org/10.1016/0044-8486(90)90174-L

483 Métais, P., Bieth, J., 1968. Détermination de l'α-amylase. Ann. Biol. Clin. 26, 133–142.

484 Morais, S., Cahu, C., Zambonino Infante, J.L., Robin, J., Ronnestad, I., Dinis, M.T., Conceiçao, L.E.C.,
485 2004. Dietary TAG source and level affect performance and lipase expression in larval Sea bass
486 (*Dicentrarchus labrax*). Lipids 39, 449-458. https://doi.org/10.1007/s11745-004-1250-2

487 Moxley, J.D., Rossi, W., Buentello, A., Pohlenz, C., Gatlin, D. M., Tomasso, J.R., 2014. Replacement of
488 fish meal with plant feedstuffs in the diet of red drum, *Sciaenops ocellatus*: effects on
489 production characteristics and tolerance to aquaculture-related stressors. J. World Aquac.
490 Soc. 45, 192-198. https://doi.org/10.1111/jwas.12106

491 Murashita, K., Furuita, H., Matsunari, H., Yamamoto, T., Awaji, M., Nomura, K, Nagao, J., Tanaka,
492 H., 2013. Partial characterization and ontogenetic development of pancreatic digestive
493 enzymes in Japanese eel Anguilla japonica larvae. Fish Physiol. Biochem. 39, 895-905.
494 https://doi.org/10.1007/s10695-012-9749-3

495 Naylor, R.L., Hardy, R.W., Bureau, D.P., Chiu, A., Elliott, M., Farrell, A.P., Forster, I., Gatlin, D.M.,
496 Goldburg, R.J., Hua, K., Nichols, P.D., 2009. Feeding aquaculture in an era of finite
497 resources. PNAS 106, 15103–15110. https://doi.org/10.1073/pnas.0905235106

498 NRC, 2011. Nutrient Requirements of Fish and Shrimp. The National Academic Press, Washington,
499 D.C.

500 Péres, A., Zambonino Infante, J.L., Cahu, C., 1998. Dietary regulation of activities and mRNA levels
501 of trypsin and amylase in sea bass (*Dicentrarchus labrax*) larvae. Fish Physiol. Biochem. 19,
502 145-152. https://doi.org/10.1023/A:1007775501340

503 Plisetskaya, E.M., Buchelli-Narvaez, L.I., Hardy, R.W., Dickhoff, W.W., 1991. Effects of injected and
504 dietary arginine on plasma insulin levels and growth of pacific salmon and rainbow trout.
505 Comp. Biochem. Physiol. 98A, 165–170. https://doi.org/10.1016/0300-9629(91)90595-4

506 Rana, K.J., Siriwardena, S., Hasan, M.R., 2009. Impact of rising feed ingredient prices on aquafeeds
507 and aquaculture production. FAO Fisheries and Aquaculture Technical Paper 541, 63 pp.

508 Rodriguez, A., Gisbert, E., Rodriguez, G. Castelló-ORvay, F. 2005. Histopathological observations
509 in European glass eels (*Anguilla anguilla*) reared under different diets and salinities.
510 Aquaculture 244, 203– 214. doi:10.1016/j.aquaculture.2004.09.039

511 Rungruangsak, K. and Utne, F. 1981. Effect of different acidified wet feeds on protease activities
512 in the digestive tract and on growth rate of rainbow trout (*Salmo gairdneri* Richardson).
513 Aquaculture 22, 67–79. https://doi.org/10.1016/0044-8486(81)90134-4

514 Rungruangsak-Torrissen, K., Moss, R., Andresen, L.H., Berg, A., Waagbø, R., 2006. Different
515 expressions of trypsin and chymotrypsin in relation to growth in Atlantic salmon (*Salmo*
516 *salar* L.). Fish Physiol. Biochem 32, 7-23. https://doi.org/10.1007/s10695-005-0630-5

517 Salze, G., McLean, E., Battle, P.R., Schwarz, M.H., Craig, S.R., 2010. Use of soy protein concentrate
518 and novel ingredients in the total elimination of fish meal and fish oil in diets for juvenile
519 cobia, *Rachycentron canadum*. Aquaculture 298, 294-299.
520 https://doi.org/10.1016/j.aquaculture.2009.11.003

521 Santigosa, E., Sánchez, J., Médale, F., Kaushik, S., Pérez-Sánchez, J., Gallardo, M.A., 2008.
522 Modifications of digestive enzymes in trout (*Oncorhynchus mykiss*) and sea bream (*Sparus*

523 *aurata*) in response to dietary fish meal replacement by plant protein sources. Aquaculture
524 282, 68-74. https://doi.org/10.1016/j.aquaculture.2008.06.007

525 Silva, F.C.P., Jacques R. Nicoli, J.R., Zambonino-Infante, J.L., Le Gall, M.M., Kaushik, S., Gatesoupe,
526 F.J., 2010. Influence of partial substitution of dietary fishmeal on the activity of digestive
527 enzymes in the intestinal brush border membrane of gilthead sea bream, *Sparus aurata*
528 and goldfish, *Carassius auratus*. Aquaculture 306, 233-237.
529 https://doi.org/10.1016/j.aquaculture.2010.05.018

530 Solé, M., Potrykus, J., Fernández-Díaz, C., Blasco, J., 2004. Variations on stress defences and
531 metallothionein levels in the Senegal sole, *Solea senegalensis*, during early larval stages.
532 Fish Physiol. Biochem. 30, 57–66. https://doi.org/10.1007/s10695-004-6786-6

533 Solovyev, M., Gisbert, E., 2016. Influence of time, storage temperature and freeze/thaw cycles on
534 the activity of digestive enzymes from gilthead sea bream (*Sparus aurata*). Fish Physiol.
535 Biochem. 42, 1383-1394. https://doi.org/10.1007/s10695-016-0226-2

536 Swanepoel, J. C., Goosen, N.J., 2018. Evaluation of fish protein hydrolysates in juvenile African
537 catfish (*Clarias gariepinus*) diets. Aquaculture in press,
538 https://doi.org/10.1016/j.aquaculture.2018.06.084

539 Tacon, A.G.J., Metian, M., 2008. Global overview on the use of fishmeal and fish oil in industrially
540 compounded aquafeeds: trends and future prospects. Aquaculture 285, 146–158.
541 https://doi.org/10.1016/j.aquaculture.2008.08.015

542 Tibaldi, E., Hakim, Y., Uni, Z., Tulli, F., de Francesco, M., Luzzana, U., Harpaz, S., 2006. Effects of the
543 partial substitution of dietary fishmeal by differently processed soybean meals on growth
544 performance, nutrient digestibility and activity of intestinal brush border enzymes in the
545 European seabass (*Dicentrarchus labrax*). Aquaculture 261, 182–193.
546 https://doi.org/10.1016/j.aquaculture.2006.06.026

547 Worthington, C.C., 1991. Worthigton Enzyme Manual Related Biochemical. 3th ed. Freehold, New
548 Jersey, USA.

549 Yaghoubi, M., Mozanzadeh, M. T., Marammazi, J. G., Safari, O., Gisbert, E., 2016. Dietary replacement
550 of fish meal by soy products (soybean meal and isolated soy protein) in silvery-black porgy
551 juveniles (*Sparidentex hasta*). Aquaculture, 468, 50–59.
552 https://doi.org/10.1016/j.aquaculture.2016.06.002

553 Yu, H., Ai, Q., Mai, K., Ma, H., Cahu, C., Zambonino-Infante, J.L., 2012. Effects of dietary protein levels
554 on the growth, survival, amylase and trypsin activities in large yellow croaker, *Pseudosciaena*
555 *Crocea* R., larvae. Aquac. Res. 43, 178-186. https://doi.org/10.1111/j.1365-
556 2109.2011.02814.x

557 Zambonino-Infante, J.L., Cahu, C.L., 1999. High dietary lipid levels enhance digestive tract maturation
558 and improve *Dicentrarchus labrax* larval development. J. Nutr. 129, 1195–1200.
559 https://doi.org/10.1093/jn/129.6.1195

560 Zambonino-Infante, J.L., Cahu, C.L., 2007. Dietary modulation of some digestive enzymes and
561 metabolic processes in developing marine fish: applications to diet formulation. Aquaculture
562 268, 98-105. https://doi.org/10.1016/j.aquaculture.2007.04.032

563

564 **Table 1.** Ingredient list and proximate chemical composition of experimental diets tested to evaluate
565 the effects on weaning and performance in glass eels (*Angilla anguilla*) fed experimental diets with
566 different levels of fish meal substitution (FM, no fish meal substitution; PP50, 50% substitution of fish
567 meal with plant protein sources; PP75, 75% substitution of fish meal with plant protein sources).

	Experimental diets		
Ingredient	Diet 1	Diet 2	Diet 3
Fish meal 70 LT	32.0	16.0	8.0
CPSP90	5.0	5.0	5.0
Soy protein concentrate	0.0	5.0	7.0
Wheat gluten	0.0	6.9	10.5
Corn gluten	0.0	5.0	7.0
Soybean meal 48	6.0	6.0	6.0
Rapeseed meal	5.3	5.3	5.3
Sunflower meal	5.3	5.3	5.3
Wheat meal	16.5	12.6	11.0
Pea starch	12.5	12.5	12.5
Fish oil	11.3	12.5	13.1
Vitamin and Mineral premix PV01	1.5	1.5	1.5
Soy lecithin	1.0	1.0	1.0
Binder	1.5	1.5	1.5
Antioxidant	0.2	0.2	0.2
Dicalcium phosphate	1.7	3.0	4.0
L-Lysine	0.0	0.04	0.7
DL-methione	0.2	0.3	0.4
Total	100.0	100.0	100.0
Proximate composition			
Crude protein (%)	36.0 ± 0.2	35.8 ± 0.1	35.9 ± 0.2
Crude fat (%)	15.9 ± 0.1	15.8 ± 0.2	15.9 ± 0.1
Fiber (%)	2.5	2.7	2.8
Starch (%)	14.8	14.2	13.8
Gross energy (J kg^{-1})*	1771.7	1755.8	1757.3

568 * Gross energy content was estimated as: total carbohydrate × 17.2 J kg^{-1}; fat × 39.5 J
569 kg^{-1}; and protein × 23.5 J kg^{-1}.

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572 **Table 2.** Survival and somatic growth performance of European eel (*Anguilla anguilla*) at the elver
573 stage fed cod roe and experimental diets with different levels of fish meal substitution (Diet 1, no
574 fish meal substitution; Diet 2, 50% substitution of fish meal with plant protein sources; Diet 3, 75%
575 substitution of fish meal with plant protein sources). Different letters in the same row denote
576 statistically significant differences among experimental groups ($P < 0.05$). Values are expressed as
577 mean ± standard error.

578

	Diet 1	Diet 2	Diet 3	Cod roe
Survival (%)	45.8 ± 5.3 [b]	31.1 ± 7.4 [c]	27.8 ± 9.8 [c]	67.5 ± 3.2 [a]
Body weight (mg)	892.4 ± 39.9 [a]	949.5 ± 96.9 [a]	832.6 ± 41.6 [a]	479.5 ± 22.0 [b]
SGR (% BW day^{-1})	1.82 ± 0.21 [a]	1.80 ± 0.4 [a]	1.75 ± 0.1 [a]	1.19 ± 0.26 [b]
Metamorphic stages (%)	**Diet 1**	**Diet 2**	**Diet 3**	**Cod roe**
VI_{A0}	0.0 [b]	0.0 [b]	0.0 [b]	2.2 ± 0.5 [a]
VI_{A1}	2.1 ± 0.5	1.6 ± 0.3	1.9 ± 0.4	2.5 ± 1.8
VI_{A2}	7.2 ± 0.9	9.1 ± 2.1	9.3 ± 2.2	11.7 ± 4.1
VI_{A3}	17.8 ± 2.5 [b]	15.4 ± 2.4 [b]	19.1 ± 2.1 [b]	57.8 ± 6.4 [a]
VI_{A4}	22.2 ± 4.0 [a]	22.1 ± 3.1 [a]	20.1 ± 1.8 [a]	6.4 ± 1.1 [b]
VI_{B}	50.7 ± 3.7 [a]	51.8 ± 4.1 [a]	49.3 ± 3.6 [a]	19.4 ± 3.2 [b]

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585 **Table 3.** Proximate composition, lipid peroxidation values and activity of oxidative stress enzymes of
586 European eel (*Anguilla anguilla*) at the elver stage fed cod roe and experimental diets with different
587 levels of fish meal substitution (Diet 1, no fish meal substitution; Diet 2, 50% substitution of fish meal
588 with plant protein sources; Diet 3, 75% substitution of fish meal with plant protein sources). Values
589 are expressed as mean ± standard error.

590

Proximate composition	Diet 1	Diet 2	Diet 3	Cod roe
Crude protein (%)	56.3 ± 3.96	54.8 ± 1.01	55.7 ± 2.32	59.6 ± 2.11
Crude lipid (%)	23.9 ± 1.06	24.4 ± 0.89	24.7 ± 2.29	22.6 ± 0.92
Carbohydrate (%)	3.1 ± 0.06	2.9 ± 0.14	3.1 ± 0.10	3.2 ± 0.12
Ash (%)	2.2 ± 0.09	2.3 ± 0.08	2.1 ± 0.04	2.2 ± 0.2
Oxidative stress				
TBARS (nmol MDA g^{-1} tissue)	158.4 ± 36.4	126.9 ± 22.6	111.2 ± 6.0	115.4 ± 4.0
CAT (µmol mg $protein^{-1}$)	1.84 ± 0.13	1.83 ± 0.08	1.90 ± 0.12	2.03 ± 0.09
GPX (µmol mg $protein^{-1}$)	1.84 ± 0.32	1.36 ± 0.31	1.10 ± 0.35	1.62 ± 0.44
GST (µmol mg $protein^{-1}$)	8.38 ± 0.62	8.29 ± 0.59	8.05 ± 0.30	8.40 ± 0.45
GR (µmol mg $protein^{-1}$)	0.81 ± 0.05	0.76 ± 0.06	0.76 ± 0.03	0.82 ± 0.02
SOD (% inhibition activity)	40.0 ± 4.65	50.7 ± 3.23	43.7 ± 1.34	49.2 ± 1.71

591

592

593 **Table 4.** Specific activity (mU mg protein^{-1}) of digestive enzymes in European eel (*Anguilla anguilla*)
594 at the elver stage fed cod roe and experimental diets with different levels of fish meal substitution
595 (Diet 1, no fish meal substitution; Diet 2, 50% substitution of fish meal with plant protein sources;
596 Diet 3, 75% substitution of fish meal with plant protein sources).

Pancreatic enzymes	Diet 1	Diet 2	Diet 3	Cod roe
Total alkaline protesases	0.98 ± 0.27 [a]	0.86 ± 0.15 [ab]	1.57 ± 0.29 [a]	0.27 ± 0.10 [b]
Trypsin	0.081 ± 0.011 [a]	0.090 ± 0.022 [a]	0.110 ±0.023 [a]	0.023 ± 0.006 [b]
α-amylase	19.7 ± 4.6	13.1 ± 4.16	17.7 ± 1.42	15.4 ± 1.65
Bile-salt activated lipase	6.04 ± 0.74	5.70 ± 1.55	6.01 ± 0.92	5.57 ± 0.64
Gastric enzyme				
Pepsin	0.004 ± 0.0004	0.003 ± 0.0003	0.004 ± 0.0004	0.003 ± 0.0010
Intestinal enzymes				
Alkaline phosphatase	2.81 ± 0.43 [a]	2.35 ± 0.30 [a]	1.48 ± 0.17 [b]	1.37 ± 0.11 [b]
Aminopeptidase- N	0.056 ± 0.011 [a]	0.049 ± 0.008 [a]	0.049 ± 0.007 [a]	0.028 ± 0.005 [b]
Maltase	524.9 ± 81.9 [a]	593.4 ± 94.6 [a]	560.6 ± 68.5 [a]	226.7 ± 49.1 [b]
Leucine-alanine peptidase	388.1 ± 46.49 [a]	381.1 ± 51.16 [a]	506.98 ± 36.99 [b]	413.5 ± 51.28 [b]
Intestinal maturation index				
AP/LAP (*1000)	7.12 ± 0.88 [a]	6.3 ± 0.89 [a]	2.95 ± 0.28 [b]	3.34 ± 0.26 [b]

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599 Figure caption

600

601 Figure 1. Size dispersion in body weight (mg) of European eel (*Anguilla anguilla*) at the elver stage
602 fed cod roe and experimental diets with different levels of fish meal substitution (Diet 1, no fish meal
603 substitution; Diet 2, 50% substitution of fish meal with plant protein sources; Diet 3, 75% substitution
604 of fish meal with plant protein sources). Distribution skewness (S) and kurtosis (K) values for each of
605 the experimental groups are included. Frequency values are expressed as mean ± standard error.

606

607 Figure 1

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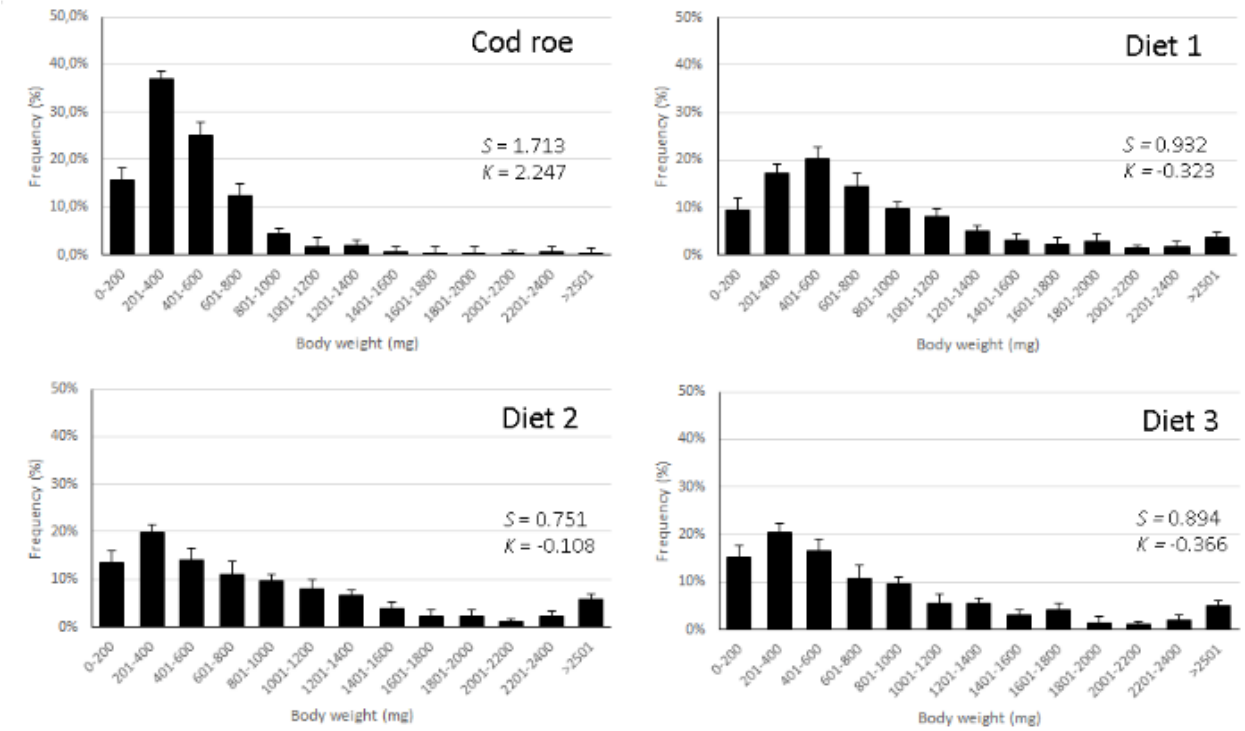

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