Deliverable Report

Deliverable No:	D19.3	**Delivery Month:**	48
Deliverable Title	Determine weaning time and type of feed according to the shift from carnivorous to omnivorous feeding		
WP No:	19	**WP Lead beneficiary:**	P4. IOLR
WP Title:	Larval Husbandry – grey mullet		
Task No:	19.4	**Task Lead beneficiary:**	P4. IOLR
Task Title:	Effect of algal type and concentration on larval performance		
Other beneficiaries:	P3. IRTA		
Status:	Completed	**Expected month:**	36
……..			

Lead Scientist preparing the Deliverable: B. Koven (IOLR)

Other Scientists participating: E. Gisbert (IRTA)

Objective: Determine weaning time and type of feed according to the shift from carnivorous to omnivorous feeding.

Table of Contents

1.0 Introduction

The early developing grey mullet larvae, as in all marine early developing fish, are strict carnivores feeding on zooplankton such as rotifers and *Artemia* in commercial hatcheries. However, after the mullet larvae have metamorphosed into juveniles they begin to change their mode of feeding from a carnivorous to an herbivorous/omnivorous diet as they are programmed, in nature, to search out less saline estuaries with higher primary productivity of micro and macroalgae (Oren et al., 1981). In captivity, research conducted within ***D19.1 Determine most effective type and concentration of algae used in grey mullet larval rearing*** demonstrated that the digestive tract in this species reaches matures fully around 61 days post hatching (dph)

and considerable pancreatic amylase production exists at 79 dph (an increase of 5.3 fold from 40 dph), while maintaining alkaline protease activity as the grey mullet adapt to a high carbohydrate, low protein diet. This contrasts to other marine species such as the gilthead sea bream (*Sparus aurata*) and the European sea bass (*Dicentrarchus labrax*), which remain carnivorous throughout their life and consume a high protein, low carbohydrate diets. From 24-38 dph, grey mullet early juveniles can be weaned off live *Artemia* and onto a dry, a more energy dense starter diet. As the weaning period appears to overlap the transition period where the grey mullet juveniles change their mode of feeding (Gisbert et al., 2016), the question remains if weaning diets should be carnivorous, herbivorous or omnivorous in nature in order to maximize growth and survival.

Consequently, the aim of the present study was to evaluate the efficacy, in terms of growth, survival, weight distribution and digestive tract (DT) enzyme activity, of weaning on to and feeding a carnivorous, herbivorous or omnivorous diet to grey mullet juvenile fish. These fish have been weaned according to a protocol developed at the P4. IOLR in a previous study (Deliverable D19.1) and have begun their trophic shift to a more herbivorous/omnivorous mode of feeding.

2.0 Materials and methods

2.1 Experimental design

Fifteen 17-l aquaria in a flow through system with 40‰, UV treated, temperature -controlled (24.5ºC) seawater were stocked with 85 larvae at 23 dph per aquarium (**Fig. 1**). This allowed the testing of three weaning dietary treatments, differing in their protein and carbohydrate content, in replicates of 5 aquaria treatment^{-1}. Diet 1 was comprised of only dried *Ulva lactuca* produced at the IOLR in Eilat, Israel (34% plant protein, 56% carbohydrate). Diet 2 was the commercial starter diet "caviar" (Bernaqua, Belgium; 55% animal protein, 8% carbohydrate) where the protein fraction is comprised of krill, fish and squid and represented a strictly carnivorous ration. Diet 3, was a 1:1 w/w mixture of the plant diet 1 and the animal diet 2 and represented an omnivorous feed. The time table for supplementing algae (*Nannochloropsis oculata*) to the aquaria and the frequency and type of food (rotifers, *Artemia*, dietary treatments) offered to grey mullet larvae and juveniles is described in **Table 1**. All fish were weaned from the zooplankton diet of rotifer (*Brachionus rotundiformis*) and *Artemia* spp. to the experimental diets over a period from 24-38 dph (**Table 1**). The fish from 38 to 53 dph were hand fed to satiation 1-5 times daily only their respective experimental treatments. At the end of the experimental period, all fish were counted and individually weighed and samples taken for DT enzyme analysis that was carried out at P3. IRTA, Spain. Samples for DT analyses were freeze-dried and shipped to IRTA.

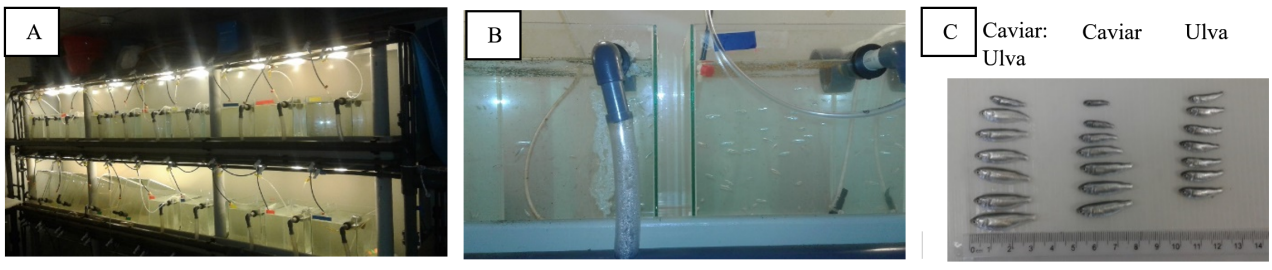

Figure 1. (A) The experimental aquaria system and (B) fish used in the weaning study. (C) Size variability at the end of the study among the experimental treatments *Ulva*, Caviar and Caviar:*Ulva*.

2. 2 Digestive enzyme activities

The digestive enzyme analyses were carried out at P3. IRTA. For quantifying the activity of the pancreatic enzymes (trypsin, chymotrypsin, total alkaline proteases, α-amylase, and bile salt-activated lipase) and intestinal (alkaline phosphatase and leucine-alanine peptidase) enzymes, lyophilized samples were homogenized (Ultra-Turrax T25 basic, IKA©-Werke, Germany) in 5 volumes (v/w) of mannitol (50 mM mannitol, 2 mM Tris-HCl buffer; pH 7.0), centrifuged at 3,300 x g for 3 min at 4 ºC and the supernatant removed for enzyme quantification and kept at -80 ºC until further analysis. After homogenization, 1 ml of the supernatant was pipetted and stored at -20 ºC for cytosolic enzyme (leucine–alanine peptidase) quantification. Then, the rest of the homogenate was used for brush border purification according to Crane et al. (1979).

Enzyme activities for pancreatic, and intestinal enzymes were conducted as described in Gisbert et al. (2009). In brief, trypsin (E.C. 3.4.21.4) activity was assayed at 25 ºC using BAPNA (N-α-benzoyl-DL-arginine p-nitroanilide) as substrate. One unit of trypsin per ml (U) was defined as 1 µmol BAPNA hydrolyzed per min per ml of enzyme extract at 407 nm (Holm et al., 1988). Chymotrypsin (EC. 3.4.21.1) activity was quantified at 25 ºC using BTEE (benzoyl tyrosine ethyl ester) as substrate and its activity (U) corresponded to the µmol BTEE hydrolyzed per min per ml of enzyme extract at 256 nm (Worthington, 1991). Alpha-amylase (E.C. 3.2.1.1) activity was determined according to Métais and Bieth (1968) using 0.3% soluble starch. Amylase activity (U) was defined as the mg of starch hydrolyzed during 3 min per ml of tissue homogenate at 37 ºC at 580 nm. Bile salt-activated lipase (BALT, E.C. 3.1.1) activity was assayed for 30 min at 30 ºC using p-nitrophenyl myristate as substrate. The reaction was stopped with a mixture of acetone: n-heptane (5:2), the extract centrifuged for 2 min at 6,080 x g and 4 ºC and the absorbance of the supernatant read at 405 nm. Bile salt-activated lipase activity (U ml^{-1}) was defined as the µmol of substrate hydrolyzed per min per ml of enzyme extract (Iijima et al., 1998). Regarding intestinal digestive enzymes, alkaline phosphatase (E.C. 3.1.3.1) was quantified at 37 ºC using 4-nitrophenyl phosphate (PNPP) as substrate. One unit (U) was defined as 1 µg BTEE released per min per ml of brush border homogenate at 407 nm (Bessey et al., 1946). All enzymatic activities were expressed as specific activity defined as units per milligram of protein (mU mg $protein^{-1}$). The assay of the cytosolic peptidase, leucine–alanine peptidase (E.C. 3.4.11) was performed on intestinal homogenates using the method described by Nicholson and Kim (1975), using leucine-alanine as substrate in 50 mM Tris-HCl buffer (pH 8.0). One unit of enzyme activity (U) was defined as 1 nmol of the hydrolyzed substrate per min per ml of tissue homogenate at 37 ºC and at 530 nm. Soluble protein of crude enzyme extracts was quantified by means of the Bradford's method (Bradford, 1976) using bovine serum albumin as standard. All the assays were made in triplicate (methodological replicates) from each pool of larvae (biological replicate) and absorbance read using a spectrophotometer (Tecan™ Infinite M200, Switzerland) and data presented in specific activity units (U mg $protein^{-1}$).

2.3 Statistics

Statistical analyses were carried out using GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego California USA, www.graphpad.com). All data are presented as mean ± SEM. Outliers were identified by calculation of the Z value using the Grubbs test (Rousseeuw and Leroy 2003) and removed if calculated Z value was higher than tabulated value. Data were analyzed by one-way ANOVA (percentage data were first arcsine transformed) and Barlett's test for equal variances. If significance ($P<0.05$) was found after ANOVA analysis while Barlett's test was not significant ($P>0.05$), then testing differences between groups was carried out by Newman-Keuls Multiple Comparison test. In cases where ANOVA and Barlett's test were both significant ($P<0.05$), then the non-parametric Kruskal Wallis Test was applied followed by Dunn's multiple Comparison test to determine significant ($P<0.05$) differences among treatments.

2.3 Ethics statement

All animal experimental procedures were conducted in compliance with the Guidelines of the European Union Council (86/609/EU) for the use of laboratory animals.

3.0 Results

In **Figure 2a** the average length of fish fed the carnivorous and omnivorous diets at the end of the study were 2.50 and 2.66 cm, respectively, which were significantly ($P < 0.05$) longer than grey mullet consuming the herbivorous diet (2.22 cm). However, final body weight is generally a more sensitive growth indicator than length. The average final body weight of the fish offered the omnivorous feeding regime (**Fig. 2b**) was significantly ($P < 0.05$) higher (203.94 mg) than those offered the carnivorous (163.32 mg) and herbivorous (111.76) diets. In addition, the fish fed a carnivorous feeding regime were markedly ($P < 0.05$) heavier than the other experimental feeding regimes. Although there was a large size range in each of the treatments (**Fig. 4**), there was no cannibalism and no significant ($P > 0.05$) dietary effect on percent survival. Nevertheless, there was a significant ($P<0.05$) dietary effect on the pattern of weight distribution at the end of the experiment. In **Figure 4**, the population of fish fed the herbivorous diet was skewed to smaller fish so that there were significantly ($P<0.05$) more smaller individuals weighing less than 100 mg, representing an average 47.4% of the population, than the fish fed the carnivorous and omnivorous feeding regimes, representing 23.9 and 14.0%, respectively. In contrast, there were significantly ($P < 0.05$) more 200-300 mg fish fed the carnivorous and omnivorous diets (21.72 and 31.08% of the population, respectively) than those consuming the herbivorous feed (6.02% of the population). In fact, the pattern of increase of larger fish in the population, as a function of diet type, can be described as omnivorous > carnivorous > herbivorous feeding regimes (Fig. 4).

In **Figure 5**, the activities of pancreatic enzymes showed a dietary-modulated response where the specific activity of amylase increased significantly ($P < 0.05$) when dietary carbohydrate (*Ulva* sp.) was introduced into the omnivorous and herbivorous dietary treatments. Surprisingly, the proteolytic enzymes; alkaline protease and trypsin also increased significantly ($P < 0.05$) as dietary carbohydrates increased. Although lipase showed a non-significant ($P > 0.05$) increase with increased inclusion of dietary carbohydrate, chymotrypsin activity was independent of diet type and composition. On the other hand, the brush border membrane (BBM) enzyme alkaline phosphatase (AP), which is a marker for nutrient absorption and leucine aminopeptidase (AN), an indicator of gut maturation, both increased with more animal protein and less carbohydrate in the diet. In fact, the decreasing ($P < 0.05$) AP/LAP (leucine alanine peptidase-cytosolic enzyme) ratio with increasing Ulva carbohydrates suggests gut maturation decreases with more Ulva and carbohydrates in the diet. The level of maltase activity, which would provide glucose from maltose (originating from starch breakdown) for transport into the enterocyte, was independent of diet type.

Table 1 Timetable for supplementing algae (*Nannochloropsis oculata*) to the aquaria and the frequency and type of food (rotifers, *Artemia*, dry dietary treatments) offered to grey mullet larvae and juveniles.

Dph	Rotifers	*Artemia*	Dietary treatments	Size (µm)	*Nannochloropsis oculata*
23	x2 day	x2 day	0	-	$4x10^6$ cells\ml
24-25	x2 day	x2 day	X1	50-100	$4x10^6$ cells\ml
26-33	0	x2 day	x2 day	100-200	$4x10^6$ cells\ml
34-37	0	x2 day	x3 day	200-300	0
38-53	0	0	x5 day	200-500	0

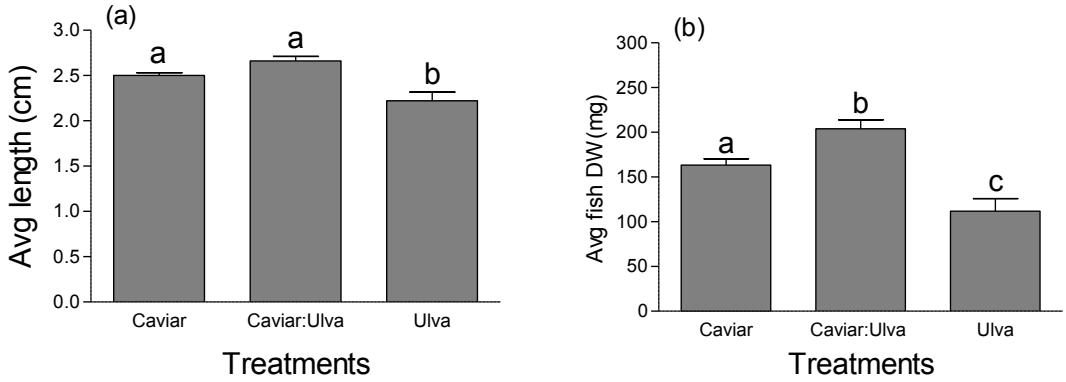

Figure 2. The effect of the commercial starter diet Caviar, macroalgae *Ulva* and the 1:1 mix Caviar: *Ulva* on (a) fish length and (b) dry weight (DW) at the end of the experiment. Length and DW values having different letters were significantly (P<0.05) different.

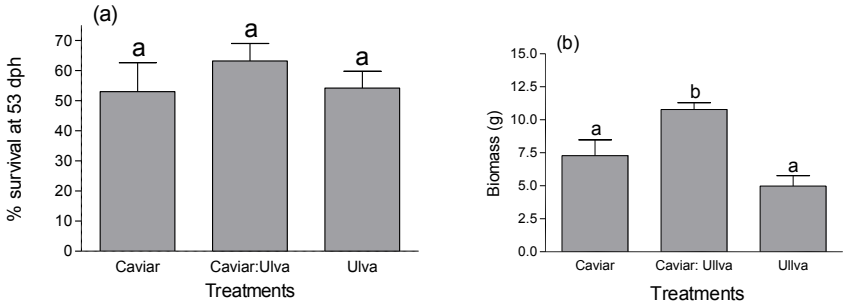

Figure 3. The effect of the commercial starter diet Caviar, macroalgae *Ulva* and the 1:1 mix Caviar: *Ulva* on (a) Percentage (%) of survival and (b) aquarium biomass at the end of the experiment. Percentage and biomass values having different letters were significantly (P < 0.05) different.

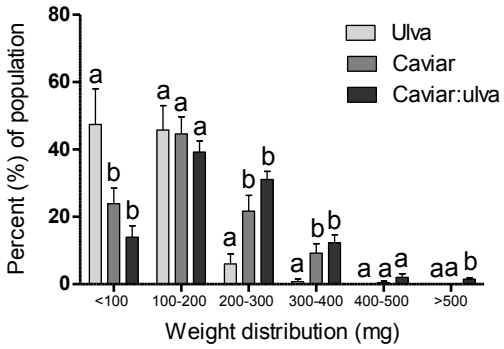

Figure 4. The effect of the commercial starter diet Caviar, macroalgae *Ulva* and the 1:1 mix Caviar: *Ulva* on weight distribution (mg). Percentage values having different letters within a weight class were significantly (P < 0.05) different. All Percent values were arcsine transformed before analysis.

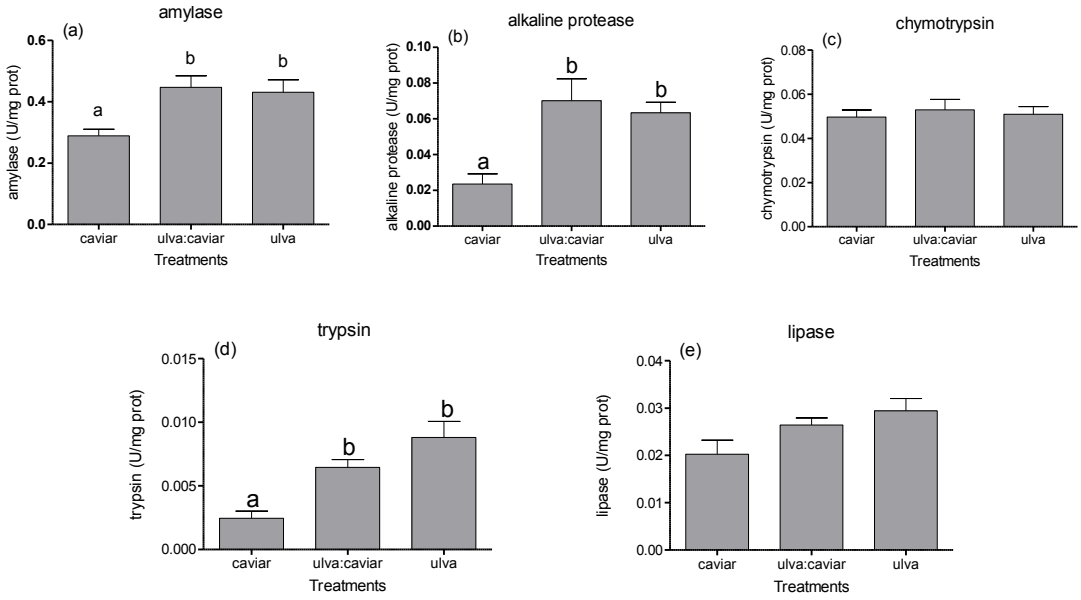

Figure 5. The effect of carnivorous, omnivorous and herbivorous weaning diets on the specific activity of pancreatic digestive enzymes (a) amylase, (b) alkaline protease, (c) chymotrypsin, (d) trypsin and (e) lipase. Enzyme values (U/mg protein) having different letters were significantly ($P < 0.05$) different.

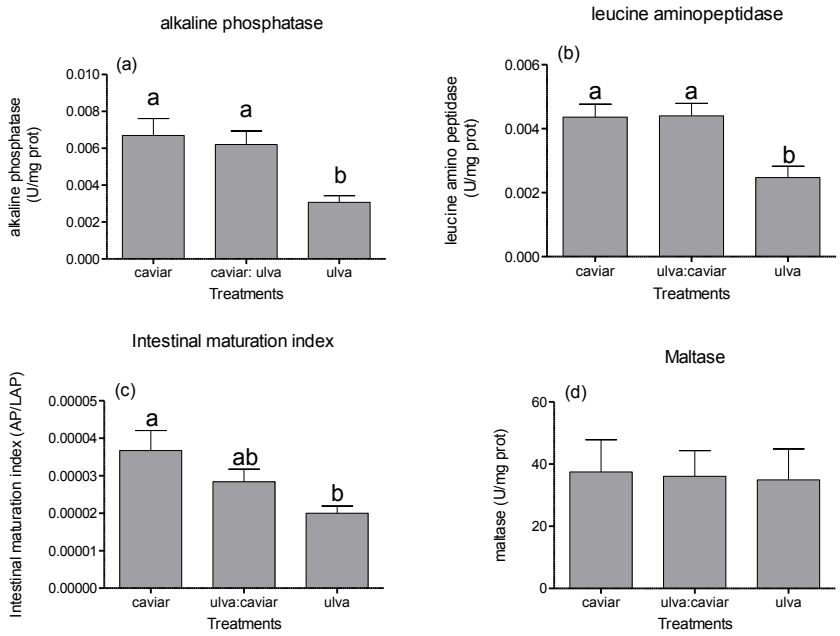

Figure 6. The effect of carnivorous, omnivorous and herbivorous weaning diets on the specific activity of brush border and cytosolic enzymes (a) alkaline phosphatase (AP) and (b) leucine aminopeptidase (LAP), respectively, as well as (c) intestinal maturation index (AP/LAP) and (d) maltase. Enzyme and index values having different letters were significantly ($P < 0.05$) different.

4.0 Discussion

The grey mullet in this study, from 24 to 37 dph, were weaned onto the dry treatment diets and exclusively fed them from 38 to 53 dph. In captivity, the previous work reported in D19.1 demonstrated that the digestive tract reaches full maturation around 61 dph (alkaline phosphatase) and considerable pancreatic amylase production by 79 dph (an increase of 5.3 fold from 40 dph), while maintaining alkaline protease activity as the grey mullet adapt to a high carbohydrate, low protein diet. Nevertheless, it remains unclear if weaning diets should be designed for a carnivorous, herbivorous or omnivorous mode of feeding. The results showed that the fish grew significantly ($P < 0.05$) less, in terms of length and final weight, when fed only an *Ulva* sp. based herbivorous diet (*Ulva* sp.) compared to the carnivorous feed (Caviar diet), while fish fed the 1:1 omnivorous mix of Ulva and Caviar diets exhibited a markedly ($P < 0.05$) superior growth performance than the other dietary treatments. A similar result was shown in aquarium biomass, where the omnivorous diet produced the largest fish. Animal protein has generally a more balanced amino acid profile including free taurine compared to plant based proteins (Pereira and Oliva-Teles, 2003) and the macroalgae *Ulva* (Tabarsa et al., 2012) which can be deficient in methionine and lysine amino acids as well as containing anti-nutritional factors (Azaza et al., 2008).

A wider body weight distribution was demonstrated in fish fed the different treatments (**Fig. 4**). Nevertheless, fish fed the herbivorous diet demonstrated significantly ($P<0.05$) higher numbers of smaller fish (<100 mg), than fish fed the carnivorous and omnivorous diets, thus in general, fish fed the herbivorous diet exhibited a population skewed to slower growing individuals. Conversely, 200-300 mg specimens fed the carnivorous and omnivorous dietary treatments had a significantly ($P < 0.05$) higher percentage of the population in comparison to the herbivorous diet fed fish. Moreover, the weight percentages of the omnivorous fish population appeared to be representative of a normal population distribution. In aquaculture, populations that are skewed to smaller fish would mean a poor food conversion ratio (FCR), a serious limitation as feed represents the largest single expense in commercial operations. However, the present results showed that population weight distribution can be modulated by diet type. Taken together, the results broadly suggest that aquaculture feeds at this developmental stage should be designed for omnivorous feeding fish and include higher levels of starch or other low cost amylolytic energetic compounds.

The previous deliverable (D19.1) demonstrated that juvenile grey mullet were genetically programmed to incrementally increase amylase activity from 25 to at least 79 dph. However, the present study showed that increasing dietary carbohydrate significantly augmented this predisposition further. Amylase activity is much higher in herbivorous and omnivorous fish compared to carnivores (Hidalgo et al. 1999; Solovyev et al. 2015). Interestingly, the proteolytic capacity to breakdown protein also markedly ($P < 0.05$) increased with increasing dietary carbohydrate content. Intuitively, we would expect decreased alkaline proteases and typsin synthesis as dietary protein level declines. However, enhanced protease capability might be necessary to digest less available plant proteins, and the higher alkaline protease levels found in this group may be considered as a digestive compensatory mechanism for digesting macroalgae (*Ulva s*p.). Azaza et al. (2008) found that *Ulva* meal was less available to the omnivorous tilapia (*Oreochromis niloticus*) possibly due to indigestible fiber presenting physical barriers to enzyme activity on their substrates (Potty, 1996). On the other hand, increased amylase activity and starch breakdown would potentially expose more macroalgae protein for protease digestion. Nevertheless, grey mullet juveniles retaining high amylase and considerable protease capability would be well suited to digest the relatively starch rich microalgae (Zemke-White and Clements, 1999) and macroalgae (Horn, 1989), as well as benthic protein rich organisms characterizing the lower salinity estuarine waters (Oren, 1981; Gisbert et al., 2016). Furthermore, the high amylase and maltase activity in the omnivorous diet would provide glucose as an energy substrate, which could be protein sparing

resulting in improved growth, results that are in agreement with those reported in D23.1 and published in Gisbert et al. (2016).

The results of the BBM and cytosolic enzyme activity supported the hypothesis that the *Ulva* high carbohydrate diet delayed gut maturation and mucosal absorption, which would also contribute to the sub-optimal growth performance of fish feeding on this diet and the prevalence in the population of smaller fish compared to their omnivorous feeding cohorts. Poorly formulated diets for larvae and juvenile fish have been shown to delay gut maturation, which would reduce growth performance with potentially lethal results (Cahu and Zambonino Infante, 2001).

Taken together, the present IOLR (P.4) weaning protocol is most effective when juvenile grey mullet are weaned onto an omnivorous diet and not herbivorous or carnivorous diets.

5.0 References

Azaza, M.S., Mensi, F., Ksouri, J., Dhraief, M.N., Brini, B., Abdelmouleh, A., Kraïem, M.M., 2008. Growth of Nile tilapia (*Oreochromis niloticus* L.) fed with diets containing graded levels of green algae ulva meal (*Ulva rigida*) reared in geothermal waters of southern Tunisia. Journal of Applied Ichthyology 24, 202–207.

Bessey, O.A., Lowry, O.H., Brock, M.J., 1946. Rapid coloric method for determination of alkaline phosphatase in five cubic millimeters of serum. J. Biol. Chem. 164, 321–329.

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.

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

Crane, R. K., Boge, G., Rigal, A., 1979. Isolation of brush border membranes in vesicular form from the intestinal spiral valve of the small dogfish *Scyliorhinus canicula*. Biochim Biophys Acta 554, 264–267.

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.

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.

Hidalgo M.C., E. Urea, and A. Sanz. 1999. Comparative study of digestive enzymes in fish with different nutritional habits: proteolytic and amylase activities. Aquaculture 170, 267–283.

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.

Horn, M.H., 1989. Biology of marine herbivorous fishes. Oceanogr. Mar. Biol. Annu. Rev. 27, 167–272.

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.

Métais, P., Bieth, J., 1968. Détermination de l'α-amylase. Annal. Biol. Cliniq. 26, 133-142.

Nicholson, J.A., Kim, Y.S., 1975. A one-step l-amino acid oxidase assay for intestinal peptide hydrolase activity. Anal. Biochem. 63, 110–117.

Oren, O.H., 1981. Aquaculture of Grey Mullets. Cambridge University Press, Cambridge

Pereira, T.G., Oliva-Teles, A., 2003. Evaluation of corn gluten meal as a protein source in diets for gilthead sea bream (Sparus aurata L.) juveniles. Aquaculture Research 34, 1111–1117.

Potty, H. V.1996. Physio-chemical aspects, physiological functions, nutritional importance and technological significance of dietary fibres – a critical appraisal. J. Food Sci. Technol. 33, 1–18.

Rousseeuw, P.J., Leroy, A.M. 2003. Robust regression and outlier detection. Wiley Hoboken, p. 195

Solovyev, M.M., Kashinskaya, E.N., Izvekova, G.I., Gisbert, E., Glupov, V.V. 2015. Feeding habits and ontogenic changes in digestive enzyme patterns in five freshwater teleosts. J. Fish Biol. 85, 1395-1412.

Tabarsa, M., Rezaei, M., Ramezanpour, Z., Waaland, J.R., 2012. Chemical compositions of the marine algae *Gracilaria salicornia* (Rhodophyta) and *Ulva lactuca* (Chlorophyta) as a potential food source. Journal of the Science of Food and Agriculture 92, 2500–2506.

Worthington, C.C., 1991. Worthington Enzyme Related Biochemicals Manual, 3rd ed., Freehold, New Jersey, USA.

Zemke-White, W.L., Clements, K.D., 1999. Chlorophyte and rhodophyte starches as factors in diet choice by marine herbivorous fish. J. Exp. Mar. Biol. Ecol. 240, 137–149.

Deviation from DOW

The co-feeding of rotifers and copepods task has been postponed due to technical problems culturing sufficient numbers of copepods. The P4. IOLR rearing protocol has been modified since the writing of the DOW and now begins on 24 dph, which is the earliest that the fish will accept any dry weaning diet, and the juveniles are completely weaned on to dried *Ulva* by 38 dph. The *Artemia* control was not included in this study as fish performance on extended feeding of *Artemia* is very poor compared to the weaning diets and its removal allowed more replicates for the weaning diet treatments. The results of the turbidity study (***D19.1 Determine most effective type and concentration of algae used in grey mullet larval rearing***) clearly showed that the incremental increase in amylase activity, gut maturation and the shift from carnivorous to omnivorous feeding started during the weaning period. Consequently, the question of the deliverable "type of feed according to the shift from carnivorous to omnivorous feeding" is very relevant and whether the feed should be carnivorous, herbivorous or omnivorous based is addressed in the present study.

Co-funded by the Seventh Framework Programme of the European Union