The effect of weaning diet type on grey mullet (*Mugil cephalus*) juvenile performance during the trophic shift from carnivory to omnivory

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Abstract

In captive grey mullet (*Mugil cephalus*) juveniles, the weaning stage overlaps the period where there are changes in the ontogeny of digestive enzymes as the fry transit from carnivory to omnivory. The aim of this study was to evaluate growth, survival, weight distribution and the activity of pancreatic and brush border digestive enzymes when fry are fed a carnivorous, herbivorous or omnivorous weaning diet.

Fifteen 17-L aquaria in a flow through system with 40 ‰, UV treated, temperature (24.5 ± 0.5 ºC) controlled seawater were stocked with eighty-five 23 dph grey mullet larvae per aquarium. This allowed the testing of three weaning dietary treatments, differing in their protein and carbohydrate content, in 5 replicate aquaria per treatment from 24-53 dph. Diet 1 was the dried macroalgal species *Ulva lactuca* and was designated as a low protein:high carbohydrate herbivorous diet. Diet 2 was a commercial microencapsulated starter feed designated as a high protein:low carbohydrate carnivorous diet. Diet 3 was a 1:1 w/w mixture of diets 1 and diet 2 representing an omnivorous feeding regime.

The average final weight of the omnivorous feeding fish was significantly ($P < 0.05$) higher (203.9 ± 10.0 mg dry weight, dw) than their carnivorous (163.3 ± 7.1 mg dw) and herbivorous feeding (111.8 ± 14.0 mg dw) cohorts.The population of fish fed the herbivorous diet demonstrated a significantly ($P = 0.02$) higher percentage of smaller fish (<100 mg) than the omnivorous and carnivorous feeding fish. In contrast, there was a markedly ($P = 0.008$ and $P = 0.001$) higher percentage of larger (200-400 mg) fish from the carnivorous and omnivorous treatments, respectively, than fish fed the herbivorous diet. Pancreatic α-amylase, alkaline protease and tripsin activity significantly rose when dietary carbohydrate increased, whereas chymotrypsin and lipase activities were independent of the type of diet ($P > 0.05$). The activity levels of brush border alkaline phosphatase and intracellular leucine alanine peptidase were similar in grey mullet fry fed the carnivorous and omnivorous diets, but were higher than those in fish fed the herbivorous diet ($P < 0.05$). The intestinal maturation index exhibited the highest and lowest values in mullet fry fed the carnivorous and herbivorous diets, respectively, whereas those from the omnivorous group showed intermediate values ($P = 0.03$). This study broadly suggests that aquaculture feeds for juvenile grey mullet should be designed for omnivorous feeding habits.

1. Introduction

Grey mullet (Teleostei, Mugilidae) larvae, similarly to all marine cultured teleost larvae, are strict carnivores feeding mainly on zooplankton such as rotifers and *Artemia* nauplii and metanauplii in commercial hatcheries. However, when mullet larvae metamorphose into juveniles, coinciding with their onshore migration, they begin to change their mode of feeding from a carnivorous to an herbivorous/omnivorous diet as they begin to search out lesser saline environments with higher primary productivity of micro- and macroalgae (Oren, 1981). This contrasts to most marine aquaculture fish species cultured worldwide, such as the gilthead sea bream (*Sparus aurata*), the European sea bass (*Dicentrarchus labrax*) and meagre (*Argyrosomus regius*), which remain carnivorous throughout their life and consume a high protein, low carbohydrate diet.

Koven et al. (2019) demonstrated that in captivity the juvenile mullet's digestive tract reached full maturation at *ca.* 61 days post hatching (dph) when fish were 142.4 ± 10.7 mg (wet weight; ww) and reared at *ca.* 25 °C. At this stage, there is increasing production of pancreatic α-amylase, where at 79 dph (809.8 ± 10.7 mg ww) has reached 5.3 times the level found in 40 dph fish (36.3 ± 2.9 mg ww). At the same time, alkaline protease activity is maintained as the fry adapt to a higher carbohydrate and lower protein diet. It is widely accepted that α-amylase activity is higher in herbivorous and omnivorous fish compared to carnivores (Hidalgo et al. 1999; Solovyev et al., 2015, 2016) and its change in activity has been suggested to occur when there is trophic shift from carnivory to herbivory/omnivory (Koven et al., 2019). Moreover, this age and size parallels the developmental stage that juveniles are migrating to lower salinity estuaries and river mouths (Gisbert et al., 1995; Cardona et al., 1996). This change in digestive capacity would allow grey mullet fry to further exploit estuarine and coastal areas rich in microalgae (Zemke-White and Clements, 1999) and macroalgae (Horn, 1989), as well as benthic organisms living in these waters (Oren, 1981). The subsequent increase in α-amylase activity enables grey mullet fry to properly digest the starch contained in the above-mentioned trophic resources (Gisbert et al., 2016).

On the other hand, the consumption of more plant and less animal protein might also lead to a taurine deficiency as macroalgae generally are taurine deficient, except for some red algae, compared to animal sources (McCluster et al. 2014). Taurine (2-aminoethane sulfonic acid) is a β-amino acid that plays vital roles in bile salt conjugation (Kim et al., 2007), osmoregulation, membrane stabilization (Huxtable, 1992), modulation of neurotransmitters (El Idrissi and Trenkner, 2004), heart and muscular systems (Salze and Davis, 2015) as well as retinal development and function (Militante and Lombardini, 2002), which all contribute to growth.

Interestingly, the fish in this study were grown from larvae to juveniles in the 40 ‰ sea water of the Red Sea, where they are commonly found and suggests that the tropic shift from carnivory to herbivory/omnivory is

genetically determined and not triggered by salinity change when fish are migrating to lower saline estuaries. Nevertheless, although mullet can grow and are found in marine environments worldwide, their growth rate is enhanced in lower salinity environments (De Silva and Perera, 1976).

Currently, captive grey mullet juveniles reared at *ca.* 25 ºC under the present Israel Oceanographic and Limnological Research (IOLR) protocol are weaned from live food onto a dry manufactured diet from 24 to 37 dph, and then exclusively fed this diet from 38 dph onwards, which is earlier than the putative gut maturation age found at *ca* 61 dph (Koven et al. 2019). As this weaning stage appears to overlap with the beginning of the transition period where the mullet fry changes their mode of feeding, the question then arises if an effective weaning diet should be herbivorous, carnivorous or omnivorous in nature. Consequently, the aim of this study was to evaluate the performance of juvenile grey mullet, in terms of growth, survival, weight distribution and the activity of digestive enzymes when fry were fed a carnivorous, herbivorous or omnivorous diet.

2. Materials and methods

2.1 Experimental design

Fifteen 17-L aquaria in a flow through system with 40 ‰, UV treated, temperature (24.5 ± 0.5 ºC) controlled ambient seawater (7 aquarium exchanges per day) were stocked with eighty-five 23 dph grey mullet larvae per aquarium. This allowed the testing of three weaning dietary treatments, differing in their protein and carbohydrate content, in 5 replicate aquaria per treatment. Diet 1 was comprised of only the dried and ground macroalgal species *Ulva lactuca*, which is produced at the IOLR in Eilat, Israel (29.5% ± 0.0 crude protein, 11.7% ± 0.2 carbohydrate) and was designated as a low protein:high carbohydrate diet (LP-HC). Diet 2 was a commercial microencapsulated starter diet Caviar™ (Bernaqua, Belgium; 58.2% ± 0.2 crude protein, 2.3% ± 0.3 carbohydrate), where the protein fraction is comprised of marine animal sources such as krill, fish and squid, that are considerably high in taurine (Spitz et al., 2003). This dietary treatment was designated as a high protein:low carbohydrate diet (HP-LC). Diet 3 (HP-LC:LP-HC) was a 1:1 w/w mixture of diet 1 (LP-HC) and diet 2 (HP-LC) resulting in 43.8% ± 0.1 crude protein, 7.0% ± 0.1 carbohydrate and represented an omnivorous feeding regime. The aquaria were monitored daily for oxygen saturation (95% or 6.2 mg L^{-1}) and frequently for ammonia levels, which were below detectable levels.

2.2 Diet analyses

The weaning diets were analyzed for protein, lipid, carbohydrate and ash levels (**Table 1**). The average protein: energy ratios (P:E) from 3 replicates of the different diets were calculated assuming that energy values of carbohydrate and protein was 4 kcal g^{-1} and lipid was 9 kcal g^{-1} and are included in **Table 1**. Crude protein was measured using the Kjeldahl technique (Kirk, 1950), while crude lipid was determined

after total lipid was chloroform-methanol extracted (Folch et al., 1957) from the diet and then dried under vacuum before being gravimetrically weighed. Ash was calculated from the weight loss after incineration of the samples for 24 h at 550 C in a muffle furnace while carbohydrate was analysed according to Masuko et al. (2005). In **Table 2**, the amino acid concentrations (% of total amino acids) of the diets 1, 2 and 3 are shown. *Ulva lactuca* analysis (Diet 1) was carried out at a certified pharmaceutical laboratory, Aminolab (Ness-Ziona, Israel) whereas the amino acid composition of weaning diet 2 (Caviar™) was provided by Bernaqua, Belgium. As diet 3 comprised a 1:1 (w/w) mixture of diets 1 and 2, the amino acid composition of this diet (g amino acid 100 g^{-1} protein) was presented as the calculated averages of the consituent amino acids of diets 1 and 2.

The rearing protocol and schedule for supplementing algae (*Nannochloropsis oculata*) to the aquaria and the frequency and type of food (rotifers, *Artemia* and dietary treatments) offered to grey mullet larvae and juveniles is described in **Table 3**. All fish were weaned from the zooplankton diet based on rotifers (*Brachionus rotundiformis*) and *Artemia* spp. to the experimental diets from 24-38 dph (**Table 1**). Then, fish from 39 to 53 dph were hand fed to satiation 1-5 times daily only their respective experimental dietary treatments. At the end of the experimental period, all fish were counted and individually weighed and samples for digestive enzyme analyses were freeze-dried and shipped to IRTA (Spain).

2.3 Taurine and amino acid analyses

Freeze dried diet samples of 2–5 mg for Varian 325–410 HPLC (Agilent Technologies, California, USA) taurine analysis were prepared by adding 3 ml of 6 M HCL and 0.5% phenol. The samples were flushed with nitrogen and placed in a heating block for 24 h at 108–110 °C. After cooling samples to room temperature and filtering (0.45 μm; cellulose nitrate), 0.5 ml carbonate buffer (pH 9), 0.5 ml DMSO (dimethyl sulfoxide) and 0.1 ml DNFB (1-fluoro-2,4 dinitrobenzene) were added and the samples mixed well followed by heating for 15 min at 40 °C then cooled for 10 min. To the samples were added 6.5 ml of 0.01 M of buffered phosphate, vortexed for 30 s and then left to stand for 5 min. The samples were then transferred to HPLC vials and injected (10 μl) into an Acclaim ™120 C18 (5 μm, 4.6 × 150 mm) HPLC column (Thermo Scientific, USA). Column flow rate was 1.5 ml min−1 where specific ratios of buffer phosphate 0.01 M (pH 6) and acetonitrile (90;10, 10:90, 10:90, 90:10, 90:10) were introduced into the column at different times (0, 10, 11, 11.01, 18 min), respectively.

2. 4 Digestive enzyme activities

For quantifying the activity of the pancreatic (trypsin, chymotrypsin, total alkaline proteases, α-amylase and bile salt-activated lipase) and intestinal enzymes (alkaline phosphatase, maltase and leucine-alanine peptidase), 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. The rest of the homogenate was used for brush border purification according to Gisbert et al. (2018).

Quantification of digestive enzyme activities for pancreatic and intestinal enzymes were conducted as previously described in Gisbert et al. (2009). In brief, trypsin 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 min^{-1} mL^{-1} of enzyme extract at λ = 407 nm (Holm et al., 1988). Chymotrypsin activity was quantified at 25 ºC using BTEE (benzoyl tyrosine ethyl ester) as substrate and its activity (U) corresponded to the μmol BTEE hydrolyzed min^{-1} mL^{-1} of enzyme extract at λ = 256 nm (Worthington, 1991). Total alkaline protease activity was measured according to García-Careño and Haard (1993). This method uses azocasein (0.5%) as substrate in Tris-HCl 50 nmol l-1 (pH 9) at room temperature for 10 min. Reaction was stopped with 20% TCA (trichloroacetic acid) and Samples were centrifuged at 10,000 x g for 5 min and absorbance of the supernatant was measured at λ = 366 nm.

Alpha-amylase activity was determined according to Métais and Bieth (1968) using 0.3% soluble starch as substrate. Its activity (U) was defined as the mg of starch hydrolyzed during 3 min mL^{-1} of tissue homogenate at 25 ºC at λ = 580 nm. Bile salt-activated lipase 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 (2 min at 6,080 x g and 4 ºC) and the absorbance of the supernatant read at λ = 405 nm. Bile salt-activated lipase activity (U mL^{-1}) was defined as the μmol of substrate hydrolyzed min^{-1} mL^{-1} of enzyme extract (Iijima et al., 1998).

Regarding intestinal digestive enzymes, alkaline phosphatase was quantified at 25 ºC using 4-nitrophenyl phosphate (PNPP) as substrate. One unit (U) was defined as 1 μmol of pNP released min^{-1} mL^{-1} of brush border homogenate at λ = 407 nm (Bessey et al., 1946). Maltase activity was determined using d (+) - maltose as substrate in 100 mM sodium maleate buffer (pH = 6.0) (Dahkqvist, 1970). One unit of maltase (U) was defined as μmol of glucose liberated per min per ml of homogenate at λ = 420 nm.The assay of the cytosolic peptidase, leucine–alanine peptidase was performed on intestinal homogenates applying the method described by Nicholson and Kim (1975) that utilized L-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 min^{-1} mL^{-1} of tissue homogenate at 25 ºC and at λ = 530 nm. The index of intestinal maturation was calculated as the ratio of the brush border enzyme alkaline phosphatase and the cytosolic enzyme leucine-alanine peptidase, as previously described by Cahu and Zambonino (1995).

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). Data on enzyme activity are 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 ± standard error of the 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 values (percentage data were first arcsine-transformed) analyzed by one-way ANOVA 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.

2.4 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. Results

Table 1 shows that all three diets were significantly ($P<0.05$) different from each other in protein, lipid, carbohydrate and ash content. The P:E ratio of the herbivorous diet 1 (LP-HC) was significantly higher (.629 ± .014) than the carnivorous and omnivorous diets 2 (.554 ± .002) and 3 (.577 ± .004), respectively.

and 3. The dispensable amino acid concentrations in **Table 2** shows that in *U. lactuca*, glutamic acid (17.92 g per 100 g protein) and aspartic acid (11.22 g per 100 g protein) were the most highly represented amino acids and were at greater levels than these amino acids in Caviar ™ (14.18 and 9.38 g 100 g^{-1} protein), respectively. In contrast, the non-dispensable amino acids methionine and lysine were lower in *U. lactuca* (1.89 and 4.6 g 100 g^{-1} protein, respectively) compared to Caviar ™ (3.54 and 9.03 g 100 g^{-1} protein, respectively) (**Table2**). In contrast, the non-dispensable arginine in *U. lactuca* was approximately double the concentration of this amino acid in Caviar ™ (12.12 and 6.04 g 100 g^{-1} protein, respectively) (**Table 2**) **Fig. 1** shows the dietary taurine levels in the LP-HC (0.37%), HP-LC:LP-HC (1.04%) and HP-LC (1.40%) treatments. There was a

significant (ANOVA; P =.002) difference in taurine level between the treatments, according to the level of animal-based protein in the diet, that can be described as HP-LC > HP-LC:LP-HC > LP-HC. At the end of the study, **Fig. 2a** demonstrated differences in total length (TL) between grey mullet fry fed the carnivorous and omnivorous dietary regimes with regard to the herbivorous diet (P = 0.002). In particular, grey mullet fry fed the HP-LC and LP-HC:HP-LC diets were longer (2.50 ± 0.03 and 2.66 ± 0.05 cm, respectively) than those fed the LP-HC diet (2.22 ± 0.10 cm). Final body weights of grey mullet fry fed the different diets are shown in **Fig. 2b**.The average final weight of the omnivorous feeding fish (LP-HC:HP-LC) was significantly (P < 0.05) higher (203.9 ± 10.0 mg dry weight, dw) than their carnivorous (HP-LC) feeding (163.3 ± 7.1 mg dw) and herbivorous (LP-HC) feeding (111.8 ± 14.0 mg dw) cohorts. In addition, the carnivorous feeding fish were markedly (P < 0.05) heavier than the herbivorous ones. Although there was a large size distribution range in each of the treatments, there was no observed cannibalism and no significant dietary effect on the percent of final survival (**Fig. 3a**; P > 0.05), which meant that the significantly (P = 0.002) higher biomass in the omnivorous (LP-HC:HP-LC) feeding group was due to the dietary treatment and was not affected by survival (**Fig. 3b**). Nevertheless, there was a significant dietary effect on the pattern of weight distribution at the end of the experiment (**Fig. 4**; P < 0.05). The population of fish fed the herbivorous (LP-HC) diet demonstrated a significantly (P = 0.02) higher percentage of smaller fish (<100 mg) than the omnivorous (LP-HC:HP-LC) feeding fish, whereas there was no treatment effect on the size group of 100-200 mg . In contrast, there was a significantly (P = 0.008 and P = 0.001) higher percentage of 201-300 and 301-400 mg fish from the carnivorous (HP-LC) and omnivorous (LP-HC:HP-LC) treatments, respectively, than the cohort feeding on the herbivorous (LP-HC) diet. Only in the omnivorous treatment, were the largest individuals (500 mg) found (P = 0.001) (**Fig. 4**).

The activities of pancreatic digestive enzymes showed a dietary-modulated response. α-amylase activity significantly increased when dietary carbohydrate from the green macroalga *U. lactuca* was introduced into the diet (**Fig. 5**; P > 0.05). Surprisingly, the proteolytic enzymes; alkaline protease and trypsin also increased significantly (P < 0.05) as dietary carbohydrate rose, whereas chymotrypsin activity was independent of the type of diet and composition (P > 0.05). Bile salt-activated lipase showed a non-significant (P > 0.05) increase with the increased inclusion of dietary carbohydrates.

The activity of brush border membrane enzymes such as alkaline phosphatase and maltase, as well as that of the cytosolic enzyme leucine alananine peptidase are shown in **Fig. 6a, b, c**, respectively. In addition, the ratio between alkaline phosphatase and leucine alanine peptidase (AP/LAP), which evaluates the level of gut maturity or intestinal maturation index (IMI), is shown in **Fig. 6d**. The activity levels of alkaline phosphatase and leucine alanine peptidase were similar in grey mullet fry fed the HP-LC and LP-HC:HP-LC diets, but were higher than those recorded in fish fed the LP:HC diet (P < 0.05). However, there were no differences in maltase activity among dietary treatments (P > 0.05). In the gut maturation index, the highest and lowest values were

found in grey mullet fry fed the HP:LC and LP:HC diets, respectively, whereas those from the LP-HC:HP-LC group showed intermediate values ($P = 0.03$).

4. Discussion

Optimizing weaning protocols and diets in cultured fish are key elements for improving larviculture practices, especially for new aquaculture species. The current study suggested that an omnivorous weaning diet for grey mullet juveniles resulted in markedly better growth and a higher percentage of the population skewed to larger fish compared to cohorts feeding on strictly herbivorous or carnivorous feeds. Importantly, the larger individuals from the omnivorous diet were not the result of reduced survival in this treatment, which can lead to improved weight gain in fish due to reduced competition for space and resources (Sahoo et al. 2004), as survival rates were relatively high (53-63.2%) in all dietary treatments. This suggests that differences in growth performances among treatments may be attributed to dietary regimes.

It is important to point out that the fish from this study were sampled at 58 dph, which is slightly prior to the putative gut maturation age (*ca.* 61 dph; 142.4 ± 10.7 mg ww) reported in a previous study conducted under similar rearing conditions by our team, and considerably before the reported peak in α-amylase activity that occurs at ≥ 79 dph (809.8 ± 10.7 mg ww) (Koven et al., 2019). Consequently, it could be argued that the requirement for animal protein is a carry-over from larval carnivory and that juvenile mullet would eventually become more herbivorous, due to the increasing amylase production. This means that juveniles would require higher levels of plant-based grow-out diets containing high levels of starch. On the other hand, we contend that omnivory at this stage more likely describes the permanent trophic status in mullets from juveniles to adults. The ability to effectively digest both protein and carbohydrates provides distinct advantages and reduces trophic competition in estuarine and coastal areas where this species inhabits (Cardona, 2001). Indeed, the advantage of the dietary inclusion of animal protein is that it represents a more balanced essential amino acid profile (Pereira and Oliva-Teles, 2003). The non-dispensable amino acids; methionine and lysine in the carnivorous diet 2 were 3.54 and 9.03 (g 100 g^{-1} protein) , respectively, compared to 1.89 and 4.6 (g 100 g^{-1} protein), respectively, in the herbivorous *U. lactuca* diet 1. Lysine and methionine are often the first limiting amino acids in protein synthesis (Nunes et al., 2014) and are generally higher in animal than plant protein (Refstie and Storebakken, 2001). Moreover, an *in vitro* study showed (Berge et al., 2004) that the uptake of low concentrations of methionine from the digestive tract was inhibited by the other amino acids present in the incubation medium. This would exacerbate further the efficient use of the lower levels of dietary plant-based methionine for protein sythesis. In support of the importance of methionine and lysine in the weaning diet of juvenile grey mullet, Jana et al. (2012) reported successfully replacing fishmeal in a grey mullet diet with processed full-fat soybean, in terms of growth and digestibility, provided that the diet was supplemented with lysine and methionine. Nevertheless, in our study the omnivorous diet 3 gave the best juvenile mullet

308 growth suggesting that its moderate methionine and lysine levels (2.72 and 6.82 g 100 g^{-1} protein,
309 respectively) were sufficient for protein synthesis.
310
311 Another potential advantage of animal protein is that includes the amino sulfonic acid taurine, which is
312 lacking in plant-based proteins such as *U. lactuca* (Tabarsa et al.2012; Pallaoro et al. 2016). Taurine has
313 been shown to promote fish growth in a number of species such as juvenile yellowtail (*Seriola*
314 *quinqueradiata*; Takagi et al., 2008), bluefin (*Thunnus thynnus*; Yokoyama et al., 2001), skipjack
315 (*Katsuwonus pelamis*; Yokoyama et al., 2001), Japanese flounder (*Paralichthys olivaceus*; Kim et al., 2005)
316 and red sea bream (*Pagrus major*; Matsunari et al., 2008). Taurine was reported to be a limiting factor when
317 replacing fish protein with plant-based meals in a range of species such as grouper (*Epinephelus aeneus*;
318 Koven et al., 2016), juvenile cobia (*Rachycentron canadum*; Lunger et al., 2007) and common dentex
319 (*Dentex dentex*; Chatzifotis et al., 2008). At first glance, this would suggest that feeding the HP-LC diet,
320 with the highest taurine level (1.4% dw diet), should result in the fastest growing fish. However, the
321 omnivorous diet (HP-LC: LP-HC) promoted the best growth, with only a moderate taurine level (1.0 % dw
322 diet) suggesting that this nutrient was not a major player in promoting weigh gain in this study.
323
324 In fact, the superior performance of the omnivorous diet may be due to a more favorable protein :carbohydrate
325 and lipid ratio which spares protein the most effectively, leading to enhanced protein synthesis and growth.
326 The constituent amino acids of dietary protein will initially be catabolized for maintenance energy and then
327 directed to growth until the fish's anabolic requirements have been met (Phillips 1972). However, excessive
328 levels of protein in the diet will be catabolized to produce energy (Wilson, 1984), which is undesirable as this
329 is a costly dietary component (Cho and Kaushik 1985). Lipid and carbohydrate are geneerally excellent and
330 relatively cheap energy alternatives that can spare the catabolism of amino acids, which will then be mobilized
331 for protein synthesis, provided that dietary protein is not given in excess (Cho and Kaushik 1985). This is
332 because deaminated amino acids are the preferred energy substrate over lipids and carbohydrates (Stone,
333 2003), which would reduce any protein sparing effect. The relatively low protein level in the herbivorous LP-
334 HC diet may not have provided sufficient amounts of indispensable amino acids for optimal protein synthesis,
335 due to the reduced protein quality and digestibility of plant sources (Neighbors and Horn, 1991; Miles and
336 Chapman, 2015). All these factors would have contributed to a lower performing diet.
337
338 In support of this, the herbivorous diet exhibited a significantly higher P:E ratio than the similar P:E ratios of
339 the carnivorous and omnivorous diets, which is an indicator of reduced protein efficiency. However, despite
340 the similar P:E ratios, body lengths and weight distributions of the carnivorous and omnivorous diets, the
341 omnivorous diet consuming fish grew significantly better thant the other treatments. The advantage of the
342 omnivorous diet may have been due to its higher levels of carbohydrate being a superior protein-sparing
343 substrate than lipid, which may have accumulated in the fish. In addition, the higher α-amylase than bile salt-

activated lipase activity found in the digestive tract of the mullet broadly hints that carbohydrates may be preferred over lipids as a protein sparing substrate. Diets containing excess non-protein energy substrates, such as lipid, can reduce fish intake, produce fatty fish and interfere with the utilization of other nutrients (Ali and Al-Asgah, 2001; Hemre et al., 2002). Taking this one step further, it is conceivable that the low carbohydrate and high lipid content of the carnivorous diet would not efficiently spare the catabolism of any of the high protein in this diet, which would lead to decreased growth.

Having said all of the above, there is a cautionary note here that although the dietary treatments are representative of herbivorous, carnivorous and omnivorous diets, micronutrients not taken into account would also vary among the udy and have some impact on the results. Nevertheless, the authors believe that dietary type is the dominant factor influencing fish perfomance in this study.

Different studies on several freshwater omnivorous species like Nile tilapia, *Oreochromis nilotiucs* (Siddiqui et al., 1988) and common carp, *Cyprinus carpio* (Ogino and Saito, 1970; Hasan et al., 1997) indicated that a optimum dietary protein level of about 40% was found for these species which largely approximates the dietary protein level of 43.8% found in the omnivorous diet. The ability to utilize elevated dietary protein levels was alluded to in a recent grey mullet study (Koven et al. 2019). These authors suggested that the capability to breakdown proteins may be enhanced in 79 dph juvenile grey mullet as both enterocyte-based intracellular digestion, indicated by leucine-alanine peptidase (LAP) activity, as well as brush border membrane digestion, where alkaline phosphatase (AP) is an absorption marker, increased from that age onwards. This expanded protein digestion capability may serve to compensate for the lack of acid proteases in grey mullet and resulted in more effective protein digestion. This capability is somewhat at odds with the prevailing wisdom in marine carnivorous fish species, where intracellular protein digestion decreases while brush border membrane enzymes increases as gut maturation proceeds (Cahu and Zambonino Infante, 1995; Zambonino Infante and Cahu 1999).

Fish have shown some plasticity in their digestive enzyme production in response to diet, as the metabolic expense of producing larger than necessary amounts of digestive enzymes would be wasted, if their substrates are at low levels (German et al., 2014). Intuitively, this means that digestive enzyme activities will vary according to dietary composition (German et al., 2014). Thus, herbivorous fish species generally exhibit higher α-amylase activities in order to digest the storage carbohydrates (starch) of macroalgae, which can reach as high as 50% of their dry mass (Horn, 1989). In contrast, carnivorous fishes frequently show greater proteolytic enzyme activities in order to digest high dietary 40-55% protein levels (Hasan, 2001). The activity of α-amylase in an herbivorous species such as *Barbus sharpeyi* was higher than the omnivorous species *Cyprinus carpio* where both were greater than the carnivorous *Aspius vorax* (Al-Tameemi et al., 2010). However, when there is a trophic shift during fish ontogeny from larval carnivore to juvenile herbivore or omnivore, there will

be a subsequent exposure to profound changes in food composition, where enzyme activity will be substrate and/or developmentally modulated. The ontogeny of α-amylase activity in grey mullet juveniles was reported to be largely genetically based (Koven et al. 2019). This assumption was reinforced by similar high α-amylase activities found in grey mullet fry that were weaned onto starch poor diets that were rich in fishmeal or with a high level of fish meal substitution by plant carbohydrate containing meals (Zouiten et al., 2008; Gisbert et al., 2016). In the present study, the activity of α-amylase significantly increased with the inclusion of dietary carbohydrates from macroalgae (*U. lactuca*), but not in a dose dependent manner. This is demonstrated since LP-HC and HP-LC:LP-HC diets, although differing in their carbohydrate content (11.7 and 7.0%, respectively), demonstrated similar α-amylase activities. This reinforces our hypothesis that the production of α-amylase is modulated by available substrate but mainly influenced by larval developmental stage (Koven et al., 2019).

The effects of the weaning dietary treatments on proteolytic enzymes showed an increase in total alkaline proteases and trypsin activities in weaned grey mullet juveniles fed the omnivorous (HP-LC: LP-HC) and herbivorous (LP-HC) diets in comparison to those individuals fed the high protein and low carbohydrate carnivorous (HP-LC) diet. Initially, this seems counter intuitive as proteolytic activities are generally correlated to increasingly higher dietary protein and not carbohydrate levels as was reported in Tambaqui, *Colossoma macropomum* (de Almeida et al., 2006). Trypsin activity was positively correlated to soluble protein content in *Brycon guatemalensis* during the switch from insectivorous to frugivorous feeding habits (Drewe et al., 2004), while Zambonino-Infante et al. (1997) found that the activity of pancreatic alkaline proteases was linked to the level of non-hydrolysed protein in the digesta in European sea bass (*Dicentrarchus labrax*). The correlation between protease activity and the higher carbohydrate in weaning diets in the present study may be attributed to a greater need of proteolytic activity to digest less available proteins from the macroalga *U. lactuca.* In fact, our results, on closer scrutiny may not be at odds afterall with the notion correlating substrate and enzyme activity. In other words, the increased α-amylase activity from the high levels of carbohydrate may have exposed more protein substrate leading to increased proteolytic activity, as a non-negligible fraction of macroalgal protein and carbohydrate compounds are in the form of glycoproteins. On the other hand, Azaza et al. (2008) found that increasing levels of *Ulva* spp. meal were less available to the omnivorous *Oreochromis niloticus*, possibly resulting from the dietary content of indigestible fiber that prestented a physical barrier to enzyme activity (Potty, 1996). Nevertheless, starch can be highly represented component in *Ulva spp*. (Prabhu et al. 2019) and it is conceivable that the activity of α-amylase in the digestive tract of tilapia may not be high enough to expose increased protein substrate. In contrast, Gisbert et al. (2016) showed that the activity of alkaline proteases did not increase in grey mullet larvae weaned on to compound diets having different levels of plant-protein sources (a blend of corn gluten, wheat gluten, soy bean meal and soy protein concentrate). This may have been due to the higher digestibilities of raw materials used in these feed formulations. Nonetheless, the higher protease activity in the herbivorous weaning diet to maximize

protein digestion did not compensate for the overall low level of dietary protein in this treatment, which likely led to poor growth. Chymotrypsin activity, the other serine protease analyzed in our study, was unlike trypsin activity, in that it was independent of weaning dietary treatment. This was unexpected as this protease is activated by trypsin and therefore should show similar enzymatic activity (Rungruangsak-Torrissen et al., 2006). On the other hand, these results agreed with those reported by Rungruangsak-Torrissen et al. (2006) who similarly found that trypsin and chymotripsin activities were not correlated under normal developmental and nutritional conditions.

Although dietary lipid levels significantly differed from each other among the weaning treatments, bile salt-activated lipase activity appeared to be statistically independent from experimental diets. On the other hand, the patterns of lipase and amylase activities (Fig. 5a and e) look strikingly similar. This may suggest, similarly to alkaline protease, that the higher amylase activity in the digestive tract of mullet fed the Ulva diet was revealing more lipid substrate and therefore initiating more lipolytic activity, although not markedly.

The activity of the intestinal enzymes of the brush border membrane (BBM) and cytosolic enzyme activities indicated that fish fed the *U. lactuca* herbivorous (LP-HC) diet exhibited delayed gut maturation and mucosal absorptaat oddsion. This was revealed by the IMI computed from the ratio of BBM and cytosolic intestinal enzymes (AP/LAP and MAL/LAP) described by Zambonino-Infante et al. (1997). A protracted maturation of the gut would be a contributing factor to the observed sub-optimal growth performance in fish feeding on this diet. This would also lead to the prevalence of smaller fish in the population compared to their omnivorous feeding cohorts. It has been previously reported that gut maturation may be accelerated by dietary supplementation of protein hydrolysates, particularly di- and tripeptides (Zambonino-Infante et al., 1997). As the weaning diet Caviar^{TM} included in the HP-LC and HP-LC:LP-HC diets contained 2% dw yeast hydrolysate, the gut maturation may have been hastened in mullet juveniles feeding on these weaning diets. In fact, yeast hydrolysate was found to be superior or equally effective as fish hydrolysate in improving gut nutrient absorption in *Sparus aurata* (Fronte et al., 2019). This was supported by Gisbert et al. (2012) who also worked on the larvae and juveniles of this species and reported that microdiets containing either yeast or pig blood hydrolysate showed a lower incidence of skeletal deformities and enhanced maturation of enterocytes compared with microdiets containing fish protein hydrolysates.

When comparing the activity of both glucosidases, the pancreatic α-amylase and brush border maltase, we found that the activity of maltase was *ca.* 100 times higher than α-amylase in mullet juveniles. Generally, data from different enzymes are not directly comparable due to the use of different substrates and analytical methods. However, in this case, α-amylase and maltase are comparable, since both methods are based on the molecules of glucose released by the action of these two enzymes. Consequently, the results reveal the important role of maltase in the digestion of starch-type carbohydrates, where pancreatic α-amylase would

participate in the first stages of starch digestion, while its hydrolysis products (disaccharides such as maltose) are finally digested by maltase in the brush border of enterocytes. These results are consistent with those reported by Quezada-Calvillo et al. (2007) who found that the α-amylase contributed less than 15% to starch digestion in *in vitro* studies with human enterocytes. Taken together, our findings recommend the quantification of both enzymes when assessing the carbohydrate digestive capacities of fish larvae and juveniles. Interestingly, the activity of BBM maltase was independent of dietary treatment. This was unexpected since it is widely believed that α-amylase activity is a function of dietary carbohydrate content in herbivores and omnivores, where increased levels would provide a higher number of available disaccharide substrates and consequently promote maltase activity. (Gisbert et al., 2016). Interestingly, a study on rabbits found that maltase activity was similarly not affected by the level of dietary starch (Debray et al., 2003), whereas the opposite results were found in mice (Bustamante et al., 1986).

In conclusion, the results from this study on growth performance and digestive physiology broadly suggest that aquaculture feeds for grey mullet developing juveniles should be designed for omnivorous feeding habits where feeds should include moderate levels of proteins, as well as considerable amounts of starch or other low cost amylolytic energetic compounds.

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Table 1. Proximate composition and protein: energy ratio (P:E) of the weaning diets LP-HC, HP-LC and HP-LC:LP-HC. Dietary component values (%), after arcsin transformation, within diets having different letters were significantly ($P < 0.05$) different.

Diet composition	**Diet 1 LP-HC**	**Diet 2 HP-LC**	**Diet 3 HP-LC: LP-HC**
Protein	$29.5^{a} \pm 0.0$	$58.2^{b} \pm 0.2$	$43.8^{c} \pm 0.1$
Lipid	$2.5^{a} \pm 0.0$	$19.8^{b} \pm 0.0$	$11.2^{c} \pm 0.2$
Carbohydrate	$11.7^{a} \pm 0.2$	$2.3^{b} \pm 0.3$	$7.0^{c} \pm 0.1$
Ash	$29.9^{a} \pm 0.3$	$11.1^{b} \pm 0.0$	$20.5^{c} \pm 0.1$
P:E	$.629^{a} \pm .014$	$.554^{b} \pm .002$	$.577^{b} \pm .004$

Table 2. The amino acid composition (g 100 g^{-1} protein) of weaning diets 1, 2 and 3 (LP-HC, HP-LC and HP-LC:LP-HC, respectively).

	Diet 1[1]	**Diet 2[2]**	**Diet 3[3]**
AMINO ACIDS	**Ulva (LP-HC)**	**Caviar™ (HP-LC)**	**HP-LC:LP-HC (1:1)**
Aspartic acid	11.22	9.38	10.30
Serine	4.59	4.58	4.59
Glutamic acid	17.92	14.18	16.05
Proline	3.98	5.49	4.74
Glycine	6.55	6.11	6.33
Alanine	8.16	7.25	7.71
Tyrosine	3.60	3.64	3.62
Threonine*	4.71	4.91	4.81
Valine*	5.36	5.63	5.50
Methionine*	1.89	3.54	2.72
Isoleucine*	3.8	5.03	4.42
Leucine*	6.09	8.69	7.39
Phenylalanine*	4.41	4.2	4.31
Histidine*	1	1.99	1.50
Lysine*	4.6	9.03	6.82
Arginine*	12.12	6.04	9.08

*Non-dispensable amino acids.

[1]Shpigel et al., 2018, [2]Bernaqua, Hagelberg 3, B-2250 Olen, Belgium.[3]Calculated average between diets 1 and 2.

Table 3. Time table 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.

Age (dph)	**Rotifers 10 mL^{-1}**	***Artemia* 1.5 mL^{-1}**	**Dietary treatments**	**Size (µm)**	***Nannochloropsis oculata***
23	x2 day	x2 day	0	-	4 x 10^6 cells ml^{-1}
24-25	x2 day	x2 day	x1 day	50-100	4 x 10^6 cells ml^{-1}
26-33	0	x2 day	x2 day	100-200	4 x 10^6 cells ml^{-1}
34-37	0	x2 day	x3 day	200-300	0
38-53	0	0	x5 day	200-500	0

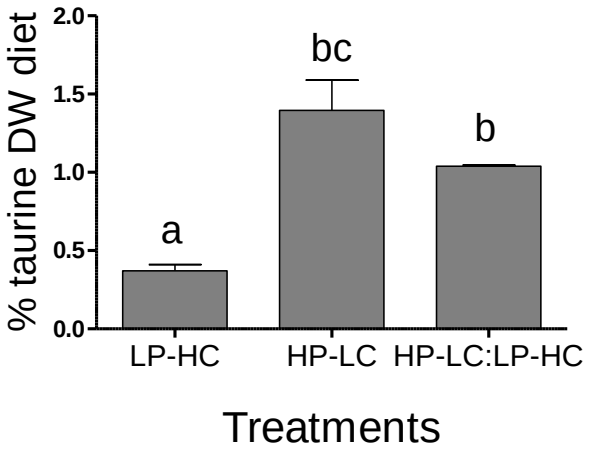

Figure 1 The percent (%) taurine DW diet in the LP-HC, HP-LC and HP-LC:LP-HC diets. Bar values having a different letter were significantly (different (ANOVA, P = 0.004; n=3).

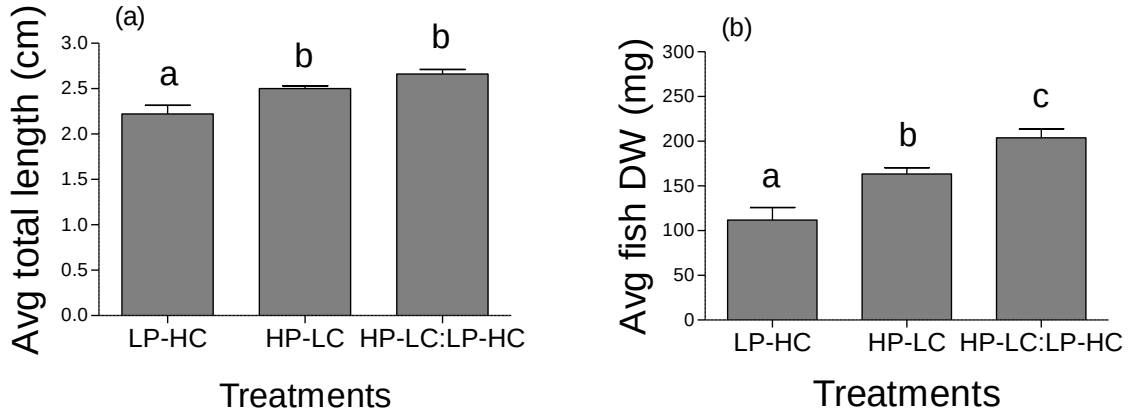

Figure 2. The effect of LP-HC, HP-LC and HP-LC:LP-HC diets on (a) total fish length (TL) and (b) dry weight (DW) at the end of the experiment. Values having different letters were significantly different (ANOVA, $P < 0.05$, n=5).

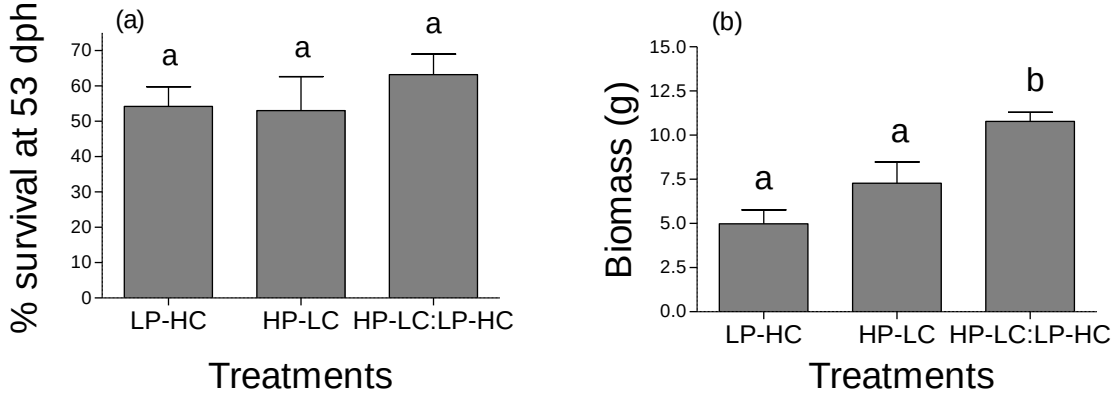

Figure 3. The effect of LP-HC, HP-LC and HP-LC:LP-HC diets on (a) survival and (b) tank biomass at the end of the experiment. Values having different letters were significantly different (ANOVA, $P < 0.05$, n=5).

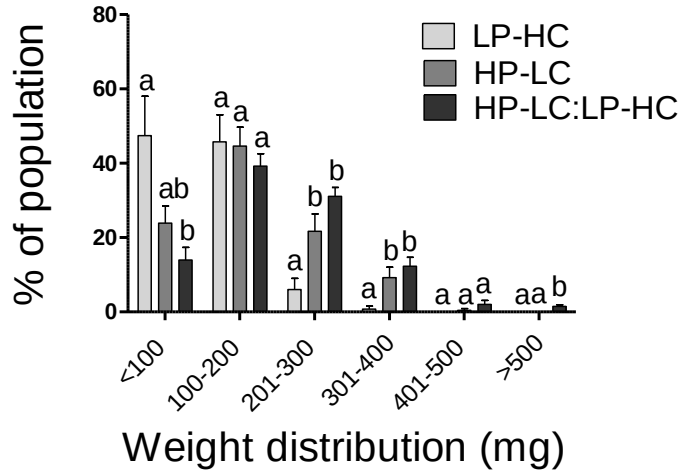

Figure 4. The effect of herbivorous, omnivorous and and carnivorous weaning diets (LP-HC, HP-LC and HP-LC:LP-HC, respectively) on weight distribution (mg). Values having different letters were significantly different (ANOVA, $P < 0.05$, n=5). All Percent values were arcsine transformed before analysis.

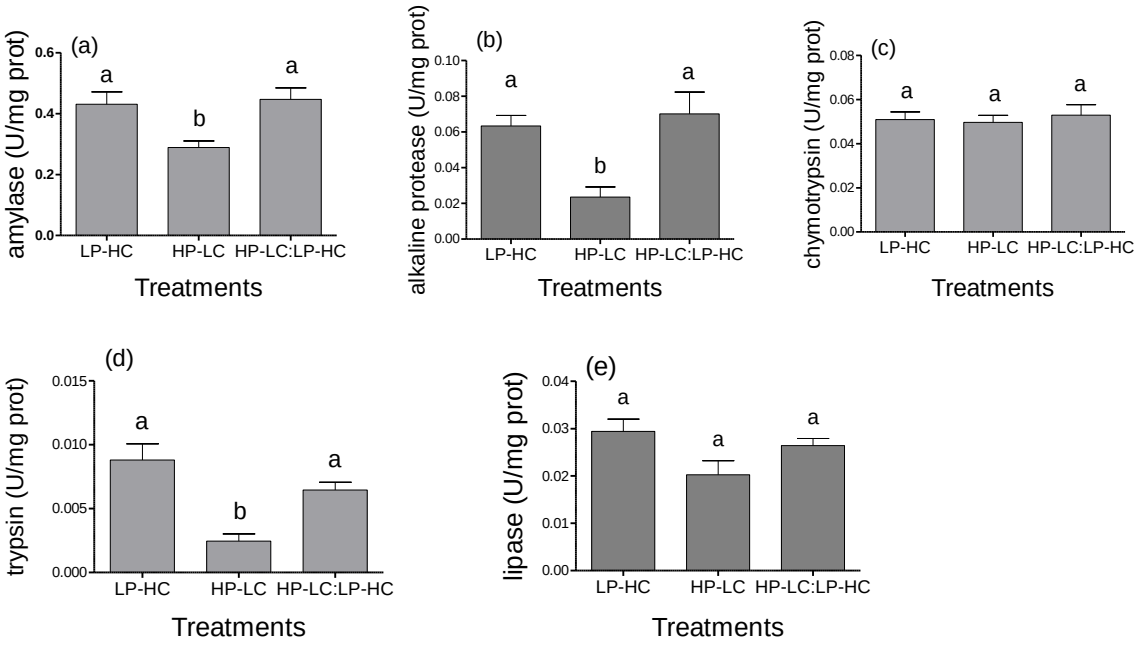

Figure 5. The effect of herbivorous, omnivorous and and carnivorous weaning diets (LP-HC, HP-LC and HP-LC:LP-HC, respectively) on the pancreatic enzymes (a) amylase, (b) alkaline protease, (c) chymotrypsin, (d) trypsin and (e) bile salt-acivated lipase. Enzyme values (U/mg protein) having different letters were significantly different (ANOVA, $P < 0.05$, n=5).

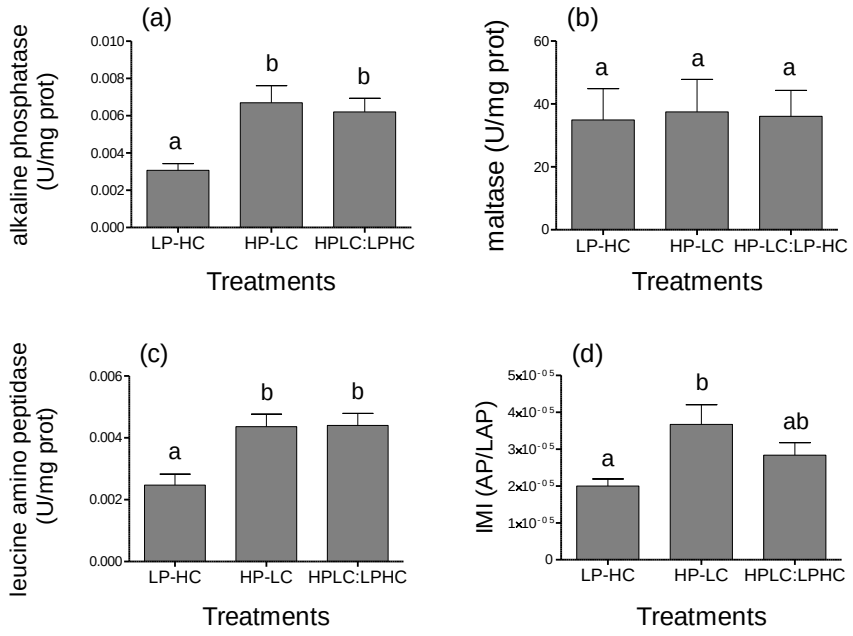

Figure 6. The effect of herbivorous, omnivorous and and carnivorous weaning diets (LP-HC, HP-LC and HP-LC:LP-HC, respectively) on the brush border enzymes (a) alkaline phosphatase (AP) and (b) maltase and the cytosolic enzyme (c) leucine aminopeptidase (LAP) as well as (d) the intestinal maturation index (IMI) determined by AP/LAP ratio. Enzyme and index values having different letters were significantly different (ANOVA, $P < 0.05$, n=5).