

Changes of visceral properties and digestive enzymes in the herbivorous marine teleost *Siganus canaliculatus* fed on different diets

XIE Dizhi^{1†}, XU Shude^{2, 3†}, WU Qingyang², CHEN Fang², WANG Shuqi², YOU Cuihong², LI Yuanyou^{1*}

¹ College of Marine Sciences, South China Agricultural University, Guangzhou 510642, China

² Guangdong Provincial Key Laboratory of Marine Biotechnology, Shantou University, Shantou 515063, China

³ Guangdong VTR Bio-tech Co., Ltd, Zhuhai 519060, China

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Abstract

The rabbitfish *Siganus canaliculatus* is one of the few cultured herbivorous marine teleosts. To better understand the digestive physiology of this fish and provide data for designing formulated feed using macroalgae as an ingredient, the changes of visceral properties and digestive enzyme activities were investigated after the juveniles were fed on different types of food including raw fish (RF), formulated diet (FD) or macroalgae *Enteromorpha prolifera* (EP) and *Gracilaria lemaneiformis* (GL) for eight weeks. The results showed that the hepatosomatic and viscerosomatic indices in the RF and FD groups, as well as the relative intestine length (RIL) in the EP and GL groups, were significantly higher than those in other groups. Additionally, differences in the histological structure of the liver and anterior intestine were also observed among different dietary groups. The hepatic nuclei were displaced to the periphery by lipid inclusions in fish fed RF. The highest levels of mucosal folds were found in the anterior intestines of fish fed macroalgae. Digestive enzyme activity profiles showed obvious fluctuations in the first three weeks, and then leveled off in the following weeks. The levels of protease, lipase and α -amylase in the alimentary tract showed changes related to the levels of dietary protein, lipid and carbohydrate, respectively. Although macroalgae significantly inhibited the activity of protease in the stomach, it increased RIL and the number of mucosal folds in the anterior intestine so as to compensate for the influences on protease activities in the stomach. This study suggests that the digestive tract of rabbitfish can well adapt to different diets, and needs about three weeks to physiologically acclimatize to the nutritional status, thus implying that rabbitfish are somewhat omnivorous.

Key words: digestive enzymes, macroalgae, *Siganus canaliculatus*, visceral property

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1 Introduction

Fish is an important and high-quality protein source, accounting for almost 17% of the global population's intake of animal protein (FAO, 2014). Marine fish is also the main source of long-chain polyunsaturated fatty acids (LC-PUFA) including arachidonic (ARA, 20:4n-6), eicosapentaenoic (EPA, 20:5n-3) and docosahexaenoic (DHA, 22:6n-3) acids (Tur et al., 2012), which have various biological functions in a range of human pathologies, such as cardiovascular and inflammatory diseases, and a vital role in neural development (Calder, 2013; Campoy et al., 2012; Delgado-Lista et al., 2012). Wild fish resources and capture fishery production are limited. Therefore, aquaculture is becoming increasingly important in meeting the demands for fishery products for human beings. Fishmeal is the major dietary protein source for farmed marine fish species owing to its excellent quality. However, its limited availability and high price

severely restrict the development of mariculture, which prompts the urgent search for sustainable alternatives to current aquaculture feeds (Hardy, 2010; Tacon et al., 2011).

To date, most of the studies on fishmeal replacement with alternative proteins in fish diets were mainly focused on terrestrial plant and animal protein sources (Brinker and Reiter, 2011; Sarkar et al., 2012; Parés-Sierra et al., 2012; Hu et al., 2013). Recently, the beneficial effects of marine macroalgae, such as *Gracilaria lemaneiformis* (Xu et al., 2011; Xuan et al., 2013), *G. vermiculophylla* (Valente et al., 2015a; Araújo et al., 2015), *Porphyra dioica* (Silva et al., 2014), and *Ulva lactuca* (Silva et al., 2014; Valente et al., 2015b; Zhu et al., 2015) have been reported in several fish species. The protein levels of macroalgae are not as high as those of terrestrial plant and animal sources, but they contain valuable bioactive substances, such as polyunsaturated fatty acids, minerals and vitamins (Wahbeh, 1997; Oliveira et al., 2009). Many stud-

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*Corresponding author, E-mail: yyli16@scau.edu.cn

†These authors contributed equally to this work.

ies reported promising results using reasonable levels of macroalgae as a feed ingredient and partial replacement for fishmeal in aquafeeds (Xu et al., 2011; Xuan et al., 2013; Zhu et al., 2015; Valente et al., 2015; Araújo et al., 2015; Vizcaíno et al., 2015).

Rabbitfish inhabit widely in the coral reefs of the Indo-Pacific region (Woodland, 1983), among them, *Siganus canaliculatus* and *S. fuscescens* are the two main commercially important species farmed along the coast of Southeast China in recent years (Du et al., 2008; Xu et al., 2011). *Siganus canaliculatus* feed on a wide range of macroalgae, with a preference for *Enteromorpha prolifera* and *G. lemaneiformis* (You et al., 2014). Furthermore, *E. prolifera*, and some by-products of *G. lemaneiformis* have not been well utilized. Incorporating these macroalgae into feed can increase their economic value but also benefit the protection of the oceanic environment. Previously, our preliminary study showed that the growth performance and feed utilization efficiency in rabbitfish fed fresh macroalgae (*E. prolifera* and *G. lemaneiformis*) or formulated feed with 33% dried *G. lemaneiformis* were not as good as for fish fed formulated feed without macroalgae, however fish fed macroalgae or a diet with 33% dried *G. lemaneiformis* had improved non-specific immunity and nutritional quality as evidenced by the increased level of essential amino acid and n-3 polyunsaturated fatty acids (PUFAs) in the dorsal muscle (Xu et al., 2011, 2014). These results suggest that formulated feeds with a proper level of macroalgae may be formulated so as to make good use of the previously wasted macroalgae but also result in benefits to the fish's health and quality.

Although, rabbitfish are herbivorous fish, and mainly graze

on macroalgae, they can also feed on formulated feed or trash fish when macroalgae are absent (Li et al., 2008). In order to identify the influence of macroalgae on digestive traits in rabbitfish, the activity of the main digestive enzymes, including protease, amylase and lipase, throughout the digestive tract of rabbitfish, as well as their responses to different diets including raw fish, formulated diet and fresh macroalgae (*E. prolifera* and *G. lemaneiformis*) were investigated in this study. The results will not only enrich our knowledge about the digestive physiological traits of herbivorous marine teleosts fed on macroalgae, but also provide references for the development of high-efficiency and low-cost formulated feed using macroalgae as an ingredient for rabbitfish.

2 Materials and methods

2.1 Experimental diets

Four different diets were used in this study, namely raw fish (RF), formulated diet (FD), macroalgae *E. prolifera* (EP) and *G. lemaneiformis* (GL). FD contained 32% crude protein (from fishmeal) and 8% crude lipid (from fish oil), the formulation of FD was as used before (Xu et al., 2011). The frozen Carangidae raw fish was obtained from local fisherman. EP and GL were the common high-yield macroalgae species preferred by *S. canaliculatus* in the South China Sea (You et al., 2014). The approximate compositions of the four diets are shown in Table 1. Chemical composition analysis followed the methods as our laboratory used before (Li et al., 2008).

Table 1. Nutritional composition of four diets used for feeding the rabbitfish

| Nutritional levels (dry material)/% | Diets | | | |
|-------------------------------------|-------|-------|-------|-------|
| | FD | RF | EP | GL |
| Dry matter | 91.16 | 26.41 | 8.41 | 12.72 |
| Crude protein | 32.03 | 72.24 | 17.96 | 22.12 |
| Ether extract | 8.08 | 8.17 | 0.59 | 0.33 |
| Crude fiber | 5.40 | 0.89 | 5.17 | 5.79 |
| Crude ash | 10.33 | 18.36 | 43.59 | 30.35 |
| Carbohydrate | 45.16 | 0.34 | 32.69 | 41.41 |

Note: FD, RF, EP and GL represent formulated diets, raw fish, seaweed *E. prolifera*, and *G. lemaneiformis*, respectively.

2.2 Experimental design and sampling

Juvenile rabbitfish were captured from the coast near Nan' Ao Marine Biology Station (NAMBS) of Shantou University, Guangdong. They were first reared in an indoor seawater pool for two weeks with an equal mixture of the four experimental diets. Fish with similar size (mean (7.5 ± 0.5) g) were selected and randomly divided into 12 cylindrical tanks (90 cm diameter, 100 cm depth, with 20 fish in each tank) and fed with each experimental diet for eight weeks, respectively. During the trial, oxygen-saturation was maintained by aeration and half of the aquarium water was changed twice a day (morning and evening). Temperature was maintained at $(22 \pm 3)^\circ\text{C}$ and the photoperiod was set at a 12 h light:12 h dark cycle. Fish were fed with the experimental diets three times a day (at 8:00, 12:00 and 16:00) to satiation.

Fish were anaesthetized with 0.01% 2-phenoxyethanol (Sigma-Aldrich Inc., USA) before being sampled. Fish ($n=6$ per treatment) were sampled at the beginning of the trial, then sampled once a week until the experiment ended. The whole viscera and liver were weighed and the total length of intestine was measured. The digestive tracts were excised and dissected to stomach, pyloric caeca, intestine (equally divided as anterior,

middle, and posterior intestine) and liver (hepatopancreas) on an ice tray, gut contents were quickly removed, and then all samples were frozen at -80°C for subsequent assays of enzymatic activity.

2.3 Calculation of visceral properties

The hepatosomatic index (HSI), viscerosomatic index (VSI) and relative intestine length (RIL) were calculated using

$$\text{HSI} = 100 \times [\text{liver weight} / \text{body weight}],$$

$$\text{VSI} = 100 \times [\text{viscera weight} / \text{body weight}],$$

$$\text{RIL} = 100 \times [(\text{intestine length, cm}) / (\text{body length, cm})].$$

2.4 Enzymatic assay

The activities of protease, amylase and lipase in the liver, stomach, pyloric caeca and intestine were determined with spectrophotometry, using protease, amylase and lipase detection kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). The units of the digestive enzymes activities were defined based on Pan and Wang (1997) and the method specified for the detection kit. Enzyme activities were expressed as specific activity (U/mg).

2.5 Histological staining and evaluation

The liver and anterior intestine samples were formalin-fixed, dehydrated and embedded in paraffin. Cross-sections of 5 μm thickness were cut (Leica RM 2035, Leica Microsystems Ltd., UK) and mounted on glass slides. Sections were dewaxed and stained with a combination of haematoxylin–eosin mix and Alcian blue (8 GX, pH 2.5) (Steedman, 1950; Lev and Spicer, 1964), and then observed under light microscopy.

2.6 Statistical analysis

Each tank was considered as an experimental unit, and each fish was considered as one repetition ($n=3$). Data is expressed as means \pm SEM. Differences between the different dietary groups were tested using one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison. The significance level was set at $P<0.05$. Statistical analyses and figures were done using the software package Origin[®] Version 7.0 (USA).

3 Results

3.1 Change of visceral properties with feeding time in different dietary groups

In this study, there were no mortalities in any dietary group during the eight-week feeding trial. During the trial, HSI in FD and RF groups gradually increased in the first three weeks and then maintained a relatively stable level, while no significant changes were observed in EP and GL groups (Fig. 1). VSI in fish fed RF showed a significant increase in the first week and then maintained a relatively high level, while that in FD or two macroalgae (EP and GL) groups maintained a relatively low level, although VSI in the EP group decreased in the last two weeks (Fig. 2). RIL in EP and GL groups was higher than that in the FD or RF groups and showed no significant fluctuation, while that in the FD and RF groups gradually decreased during the entire feeding trial (Fig. 3).

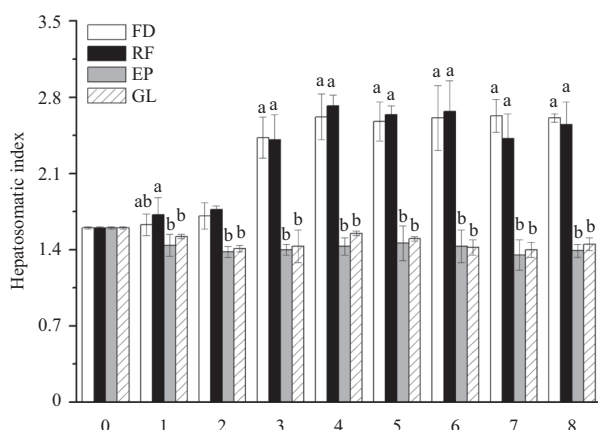


Fig. 1. Response of the hepatosomatic index (HSI) to different diets in rabbitfish during an eight-week feeding period. Data are mean \pm SEM ($n=3$). Bars with different superscripts are significantly different ($P<0.05$, one-way ANOVA test). FD, RF, EP and GL mean rabbitfish fed a formulated diet, iced raw fish, fresh seaweed *E. prolifera* and *G. lemaneiformis*, respectively.

3.2 Changes of digestive enzyme activity with feeding time in different dietary groups

The activities of protease, amylase and lipase in liver and digestive tracts of fish from different groups displayed changes with

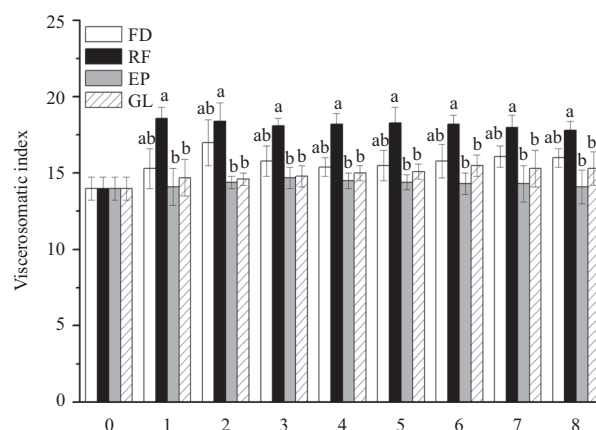


Fig. 2. Changes of the viscerosomatic index (VSI) in response to different diets in rabbitfish during an eight-week feeding period. Data are mean \pm SEM ($n=3$). Bars with different superscripts are significantly different ($P<0.05$, one-way ANOVA test). FD, RF, EP and GL mean rabbitfish fed a formulated diet, iced raw fish, fresh seaweed *E. prolifera* and *G. lemaneiformis*, respectively.

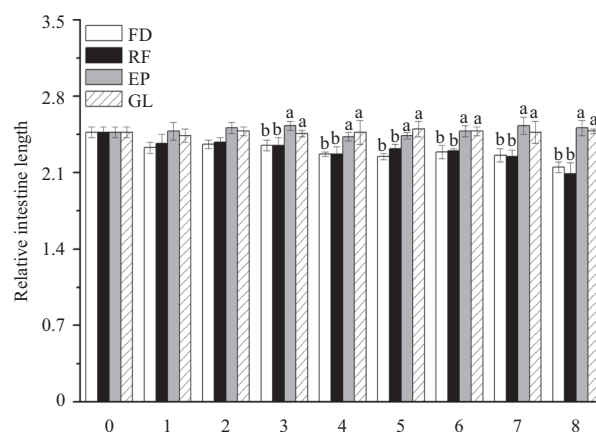


Fig. 3. Changes of relative intestine length (RIL) in response to different diets in rabbitfish during an eight-week feeding period. Data are mean \pm SEM ($n=3$). Bars with different superscripts are significantly different between each diet treatment ($P<0.05$, one-way ANOVA test). FD, RF, EP and GL mean rabbitfish fed a formulated diet, iced raw fish, fresh seaweed *E. prolifera* and *G. lemaneiformis*, respectively.

feeding period (Figs 4–6). From the influence of feeding time, the changes of digestive enzyme activity were obvious between the first two weeks and the last six weeks, and tend to smooth during the last six weeks. From the influence of diets, the level of protease activities in RF groups were significantly higher than in other dietary groups ($P<0.05$) during the last six weeks, and their lipase activities were significantly higher than the EP and GL groups ($P<0.05$), while their amylase activities were significantly lower than other dietary groups ($P<0.05$). In the EP and GL groups, the amylase activities remained high throughout the digestive tracts (Fig. 4), and the level of stomach protease activity was significantly lower than that in the FD and RF groups (Fig. 5), while the lipase activity was significantly lower than in the other two dietary groups, and undetectable in the stomach, middle intestine and posterior intestine during last three weeks (Fig. 6). As shown in Figs 4–6, these three digestive enzyme activities stay at

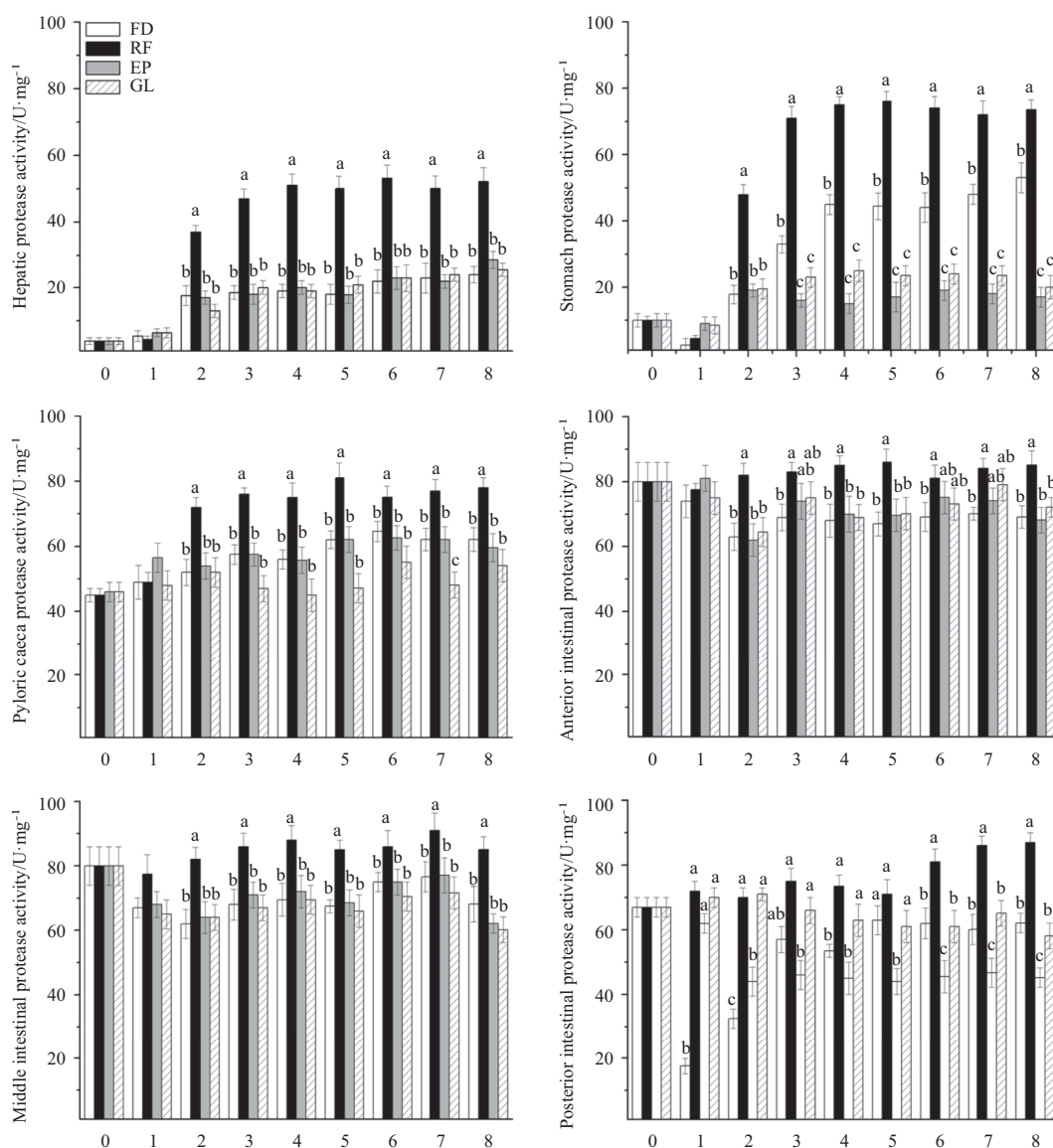


Fig. 4. Changes of the total protease activity in the liver, stomach, pyloric caeca, anterior intestine, middle intestine and posterior intestine, in response to different diets in rabbitfish during an eight-week feeding period. Data are mean±SEM ($n=3$). Bars with different superscripts are significantly different between each diet treatment ($P<0.05$, one-way ANOVA test). FD, RF, EP and GL mean rabbitfish fed on formulated diet, raw fish, fresh seaweed *E. prolifera* and *G. lemaneiformis*, respectively.

a high level in the FD group, suggesting that the FD is a more suitable diet for juvenile rabbitfish.

3.3 Effects of diet on the histological structure of the liver and intestine

After fish were fed with the experimental diets for eight weeks, histological structure in the liver and anterior intestine showed an obvious difference among different dietary groups. In the EP and GL groups, the hepatocytes were closely spaced and ruleless, while the nuclei were unremarkable (Figs 7c and d). The hepatic squamous cells were big and regular, while the cell interstices were large in FD treatments (Fig. 7a). Fish in the RF group had small hepatic squamous cells, the hepatocyte nuclei were displaced to the periphery, and the nuclei were larger than for other dietary treatments (Fig. 7b). Representative micrographs showing the morphological changes encountered across the anterior intestine from fish fed the different experimental diets are shown

in Fig. 8. Fish fed the macroalgae displayed the highest levels of mucosal folds in the anterior intestine, and the fish fed FD diets had moderate levels of mucosal folds. Few mucosal folds and mucosal cells were seen in the fish fed RF diets.

4 Discussion

In recent years, the application of macroalgae as a feed ingredient and fish meal substitute in aquatic feed has been reported in several papers. Among them, the influence of macroalgae on growth performance, immunity and stress resistance of teleosts have been widely observed (Xu et al., 2011; Wassef et al., 2013; Güroy et al., 2013; Valente et al., 2015a, b; Araújo et al., 2015), however, the influence on visceral properties and digestive enzyme activities were comparatively less investigated.

In this study, the visceral properties including HSI, VSI and RIL were observed in rabbitfish fed different diets. The results showed that the visceral properties in both the FD and RF groups

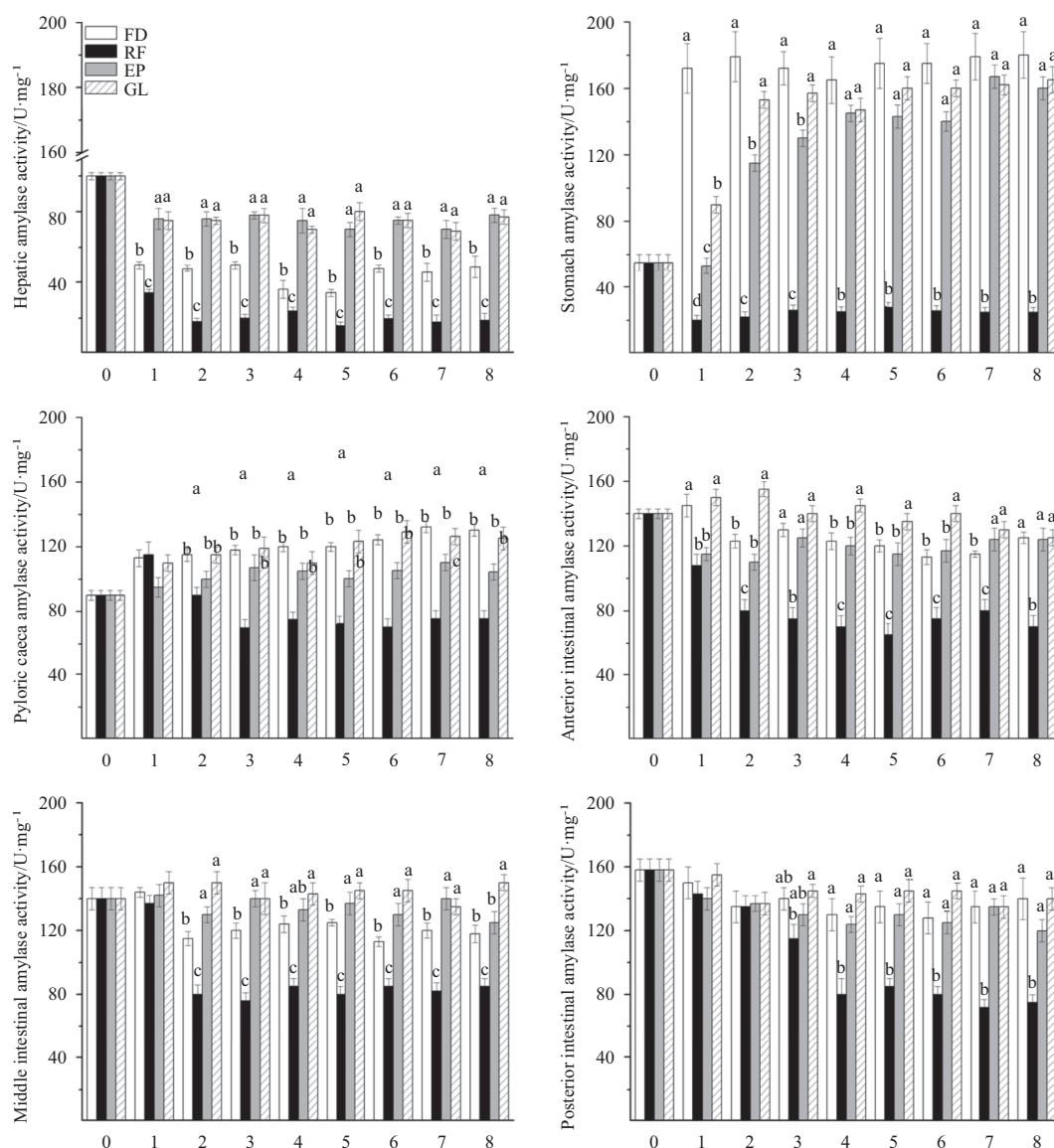


Fig. 5. Changes of the amylase activity in the liver, stomach, pyloric caeca, anterior intestine, middle intestine and posterior intestine, in response to different diets in rabbitfish during an eight-week feeding period. Data are mean \pm SEM ($n=3$). Bars with different superscripts are significantly different between each diet treatment ($P<0.05$, one-way ANOVA test). FD, RF, EP and GL mean rabbitfish fed on formulated diet, raw fish, fresh seaweed *E. prolifra* and *G. lemaneiformis*, respectively.

demonstrated obvious fluctuations, i.e., VSI and especially HSI, gradually increased, while these indices were smooth in two macroalgae groups (EP and GL). A similar result showed that the increasing supplementation of macroalgae meal in diets effectively reduced the HSI in gilthead bream (*Sparus aurata*), rainbow trout (*Oncorhynchus mykiss*) and black sea bream (*Acanthopagrus schlegelii*) (Xuan et al., 2013; Güroy et al., 2013; Vizcaino et al., 2015). Consistent with this, the changes of liver histological parameters were significantly affected by experimental diets, the hepatocytes of fish fed fresh macroalgae were closely spaced, and the nuclei were inconspicuous, while the liver histological parameters in the FD and RF groups were abnormal. Additionally, the positive role of macroalgae meal supplement in lipid metabolism was found in black sea bream, red sea bream (*Pagrosomus major*) and gilthead bream (Nakagawa, 1997; Nakagawa et al., 1987; Xuan et al., 2013). However, the potential mechanism of the macroalgae on fish lipid metabolism deserves future study.

Additionally, RIL was also affected by dietary categories. Compared to fish fed macroalgae, the RIL in the FD and RF groups gradually decreased, and was significantly lower at the end of the feeding trial. These observations are in agreement with previous studies, which showed that the digestive tracts in vertebrate classes tend to be shortest in carnivores, intermediate in omnivores and longest in herbivores species (Stevens and Hume, 1995; Ricklefs, 1996; Karasov et al., 2011). Sibly (1981) suggested that the consumption of food with a high content of indigestible material results in an increase in gut dimensions. Diets FD and RF contained higher levels of protein and lipid, while diets EP or GL contained more material indigestible to rabbitfish. Furthermore, diets also affected the histological structure of the anterior intestine. Rabbitfish fed macroalgae displayed the highest levels of mucosal folds in the anterior intestine. The functional changes of guts were a consequence of the ingestion of large volumes of low-quality food and maximized digestive efficiency (German,

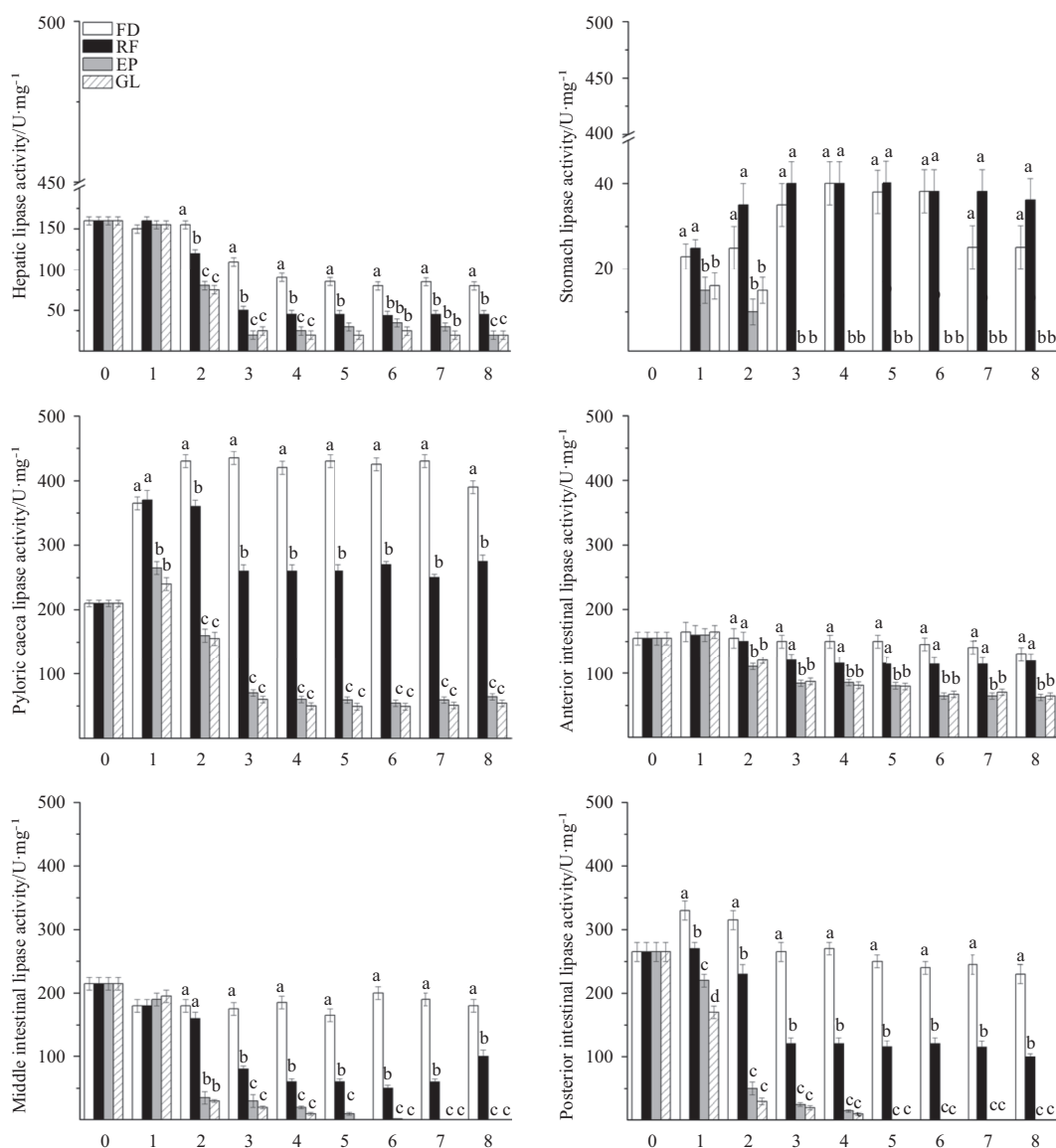


Fig. 6. Changes of lipase activity in the liver, stomach, pyloric caeca, anterior intestine, middle intestine and posterior intestine, in response to different diets in rabbitfish during an eight-week feeding period. Data are mean \pm SEM ($n=3$). Bars with different superscripts are significantly different between each diet treatment ($P<0.05$, one-way ANOVA test). FD, RF, EP and GL mean rabbitfish fed on formulated diet, raw fish, fresh seaweed *E. prolifra* and *G. lemaneiformis*, respectively.

2011).

With regard to the profile of digestive enzyme activities, this study demonstrated the activity distributions of protease, α -amylase and lipase throughout the alimentary tract in rabbitfish. In fish, however, nutrient absorption is known to take place in the pyloric caeca and anterior intestine, and to a lesser extent, in the middle and posterior intestine. In this study, protease, amylase and lipase showed high activity in the pyloric caeca and intestine, with the unusually high activity in the posterior intestine. Our results coincide with the data in dentex (*Dentex dentex*) reported by Pérez-Jiménez et al. (2009), who suggested that the product of a possible drag of the secreted mucous displaced to posterior intestine caused the higher digestive enzyme activity. The knowledge of how different diets affect digestive enzymatic activity is important, because this would revealed the ability of fish to modulate digestive mechanisms (Deguara et al., 2003). In our study, the profile of digestive enzyme activities showed obvi-

ous fluctuations in the first three weeks, and then leveled off during the last several weeks, which indicated that rabbitfish may need about three weeks to adapt to the nutritional environment. The period of acclimatization is necessary to teleosts. For instance, the acclimatization period was one week for dentex (Pérez-Jiménez et al., 2009), and 30 days for tambaqui (*Colosoma macropomum*) (Corrêa et al., 2007).

Digestive enzymatic activities often reflect the feeding habits of teleosts (German et al., 2010). For example, herbivorous fish tend to have higher carbohydrase activities, and some carnivores possess higher protease activities (Gawlicka and Horn, 2006; Horn et al., 2006; German et al., 2010). In the initial sample of this study, higher activities of α -amylase than those of protease were detected throughout the intestinal tract, and were also evident at the end of feeding trial. The α -amylase activities were higher in the pyloric caeca and intestine in the RF groups. The characteristics of digestive enzymatic activities coincide with the herbivor-

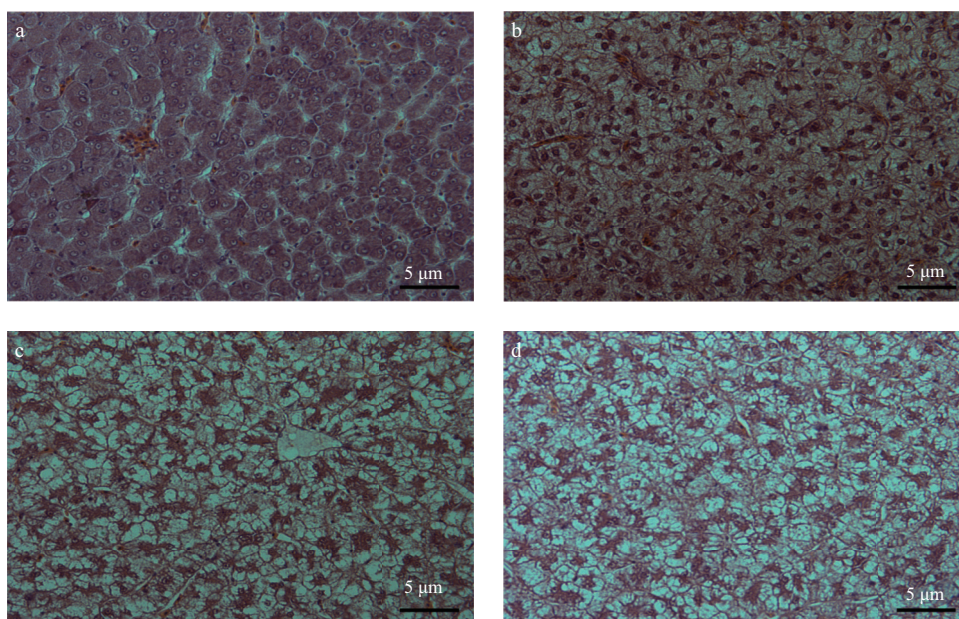


Fig. 7. Images of morphological variations encountered across the liver of rabbitfish fed different experimental diets: a. formulated diets, b. raw fish, c. *E. prolifra*, and d. *G. lemaneiformis*.

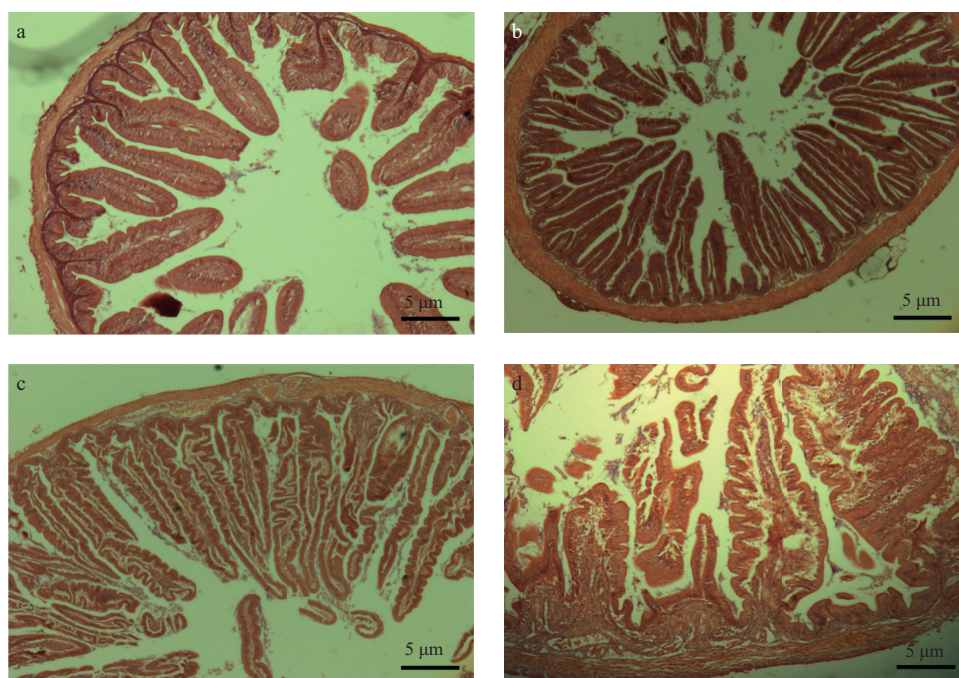


Fig. 8. Images of morphological variations encountered across the anterior intestine of rabbitfish fed different experimental diets: a. formulated diets, b. raw fish, c. *E. prolifra*, and d. *G. lemaneiformis*.

ous wild rabbitfish, which prefers to feed on macroalgae. Indeed, our previous researches on rabbitfish showed the efficient use of plant protein and/or lipid, as partial substitutes for fishmeal and fish oil, without negative effects on growth performance or food utilization in this species (Xu et al., 2011, 2012). Compared with these terrestrial plant feedstuffs, macroalgae, being rich in polyunsaturated fatty acids (Wahbeh, 1997; Oliveira et al., 2009), improved the levels of essential amino acids and PUFA in rabbitfish (Saito et al., 1999; Xu et al., 2014). These results indicated that macroalgae such as *E. prolifra* and *G. lemaneiformis* have

emerged as interesting candidates for rabbitfish feed ingredients.

According to the protease enzymatic profiles observed, the present results suggest that the levels of dietary crude protein markedly affected protease enzymatic activity in stomach, but exerted less influence on protease enzymatic activity in the intestine, which implies the existence of a compensation mechanism in this species. Consistently, the protease activity of postlarvae white shrimp (*Litopenaeus setiferus*) (Guzman et al., 2001), silver barb (*Puntius gonionotus*) fingerlings (Mohanta et al., 2008a) and juvenile gibel carp (*Carassius auratus gibelio*) (Ye et al., 2015)

were also affected by dietary protein level. Interestingly, the activity of protease in the stomach was significantly lower than that in the intestine in macroalgae groups, while this difference was not detected in RF and FD groups. Similarly, lower stomach protease activity was observed in juvenile black sea bream and white spotted snapper fed the highest level of macroalgae diets (Xuan et al., 2013; Zhu et al., 2015). These results supported the view of Horie et al. (1995) that dietary fiber would inhibit the stomach protease activity.

Regarding lipase activity, although little study has reported the influence of diets on lipase activity, most studies indicated that lipolytic activity in teleosts has nothing to do with dietary protein and carbohydrate content, but with dietary lipid levels (Gangadhara et al., 1997; De Almeida et al., 2006; Mohanta et al., 2008b). Our results indicate that macroalgae diets, containing less crude lipid, induced the lowest lipase activity throughout the alimentary tract. Consistently, lipase was not significantly affected by iso-lipid diets with different macroalgae levels (Xuan et al., 2013; Zhu et al., 2015). These results suggested that the addition of macroalgae has no negative impact on lipase activity.

Overall, the present study investigated the characteristics of visceral properties and digestive enzyme activities in rabbitfish fed different diets. The results suggest that this fish, with a bias to omnivore, needs about three weeks to modulate its digestion mechanism in response to the nutritional environment. Furthermore, macroalgae exerted functional changes on visceral properties, such as improving RIL and the levels of mucosal folds in the anterior intestine to compensate for the influence on protease activities in stomach. The results provide a reference for rabbitfish aquaculture, and lay a foundation for further study on macroalgae as a valuable alternative feed ingredient source for fish meal in aquaculture.

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