



Persistence of Digestive Enzyme Activities of Sea Cucumber (*Apostichopus japonicus*): Evidence From Diet Switch After Evisceration

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A two-stage diet-switch experiment was conducted to examine the hypothesis that the changes in digestive enzyme activities of sea cucumber (*Apostichopus japonicus*) induced by historic diets might persist in the regenerated intestines. In stage I, *A. japonicus* were treated with two different diets for 56 days, including diet A with 11% crude protein, 1% crude lipid, and 40% carbohydrate, and diet B with 18% crude protein, 2% crude lipid, and 35% carbohydrate. In stage II, each treatment was subjected to evisceration with 0.35M KCl or not (eviscerated and non-eviscerated groups), half of which were then switched to different diets from diet A to B or vice versa for 112 days. The persistence of digestive enzyme activities was evaluated by measuring the changes in digestive enzyme activities before and after evisceration. In stage I, diets B and A increased trypsin and amylase activities, respectively. In stage II, the higher trypsin activities were observed in eviscerated and non-eviscerated *A. japonicus* that had consumed diet B in stage I. The higher amylase activities were observed only in eviscerated *A. japonicus* that had consumed diet A in stage I. It indicated that the historic diets showed long-term effects on the digestion of *A. japonicus*, which led to the persistence of changes in both trypsin and amylase activities in intestines, especially in the regenerated intestines. In addition, the specific growth rates (SGRs) and metabolic rates (MRs) of *A. japonicus* were affected by the long-term effects of historic diets. Meanwhile, the relationships between enzymic activities, SGRs, and MRs were observed in *A. japonicus*, indicating that the historic diets could produce long-term effects on the growth and metabolism of *A. japonicus* through their long-term effects of historic diets on digestive enzyme activities. In conclusion, the present study showed that the changes in digestive enzyme activities induced by different diets in stage I could persist in the intestines and regenerated intestines, leading to long-term effects of historic diets on the growth and metabolism of *A. japonicus*.

Keywords: trypsin activity, amylase activity, specific growth rate, metabolic rate, carbon stable isotope

INTRODUCTION

The stimulus of historic nutrition can induce temporary or long-term changes in digestive ability and subsequent growth potential and metabolic status in later life of organisms (Patel and Srinivasan, 2002; Vera et al., 2017). This phenomenon originating from mammals is termed nutritional programming (Symonds et al., 2009). In recent decades, nutritional programming has been widely studied and reported in numerous aquaculture species (Fang et al., 2014; Gong et al., 2015; Moghadam et al., 2015; Balasubramanian et al., 2016). For instance, early feeding diets enriched in plant-based lipids negatively influenced the growth and survival of gilthead seabream (*Sparus aurata*) in later life (Turkmen et al., 2015). An acute glucose stimulus during the first feeding period induced the long-term repression of weight gains and disturbed the gluconeogenesis regulation of Siberian sturgeon (*Acipenser baerii*) (Gong et al., 2015). Fang et al. (2014) reported that the activities of amylase were significantly enhanced in adult zebrafish (*Danio rerio*) fed a high-carbohydrate diet than those fed a low-carbohydrate diet in the yolk-sac larval stage, indicating the long-term effects of early high-carbohydrate diets on carbohydrate digestion of zebrafish. Similar observations were also detected in Atlantic salmon (*Salmo salar*) (Vera et al., 2017) and rainbow trout (*Oncorhynchus mykiss*) (Balasubramanian et al., 2016).

So far, studies about the long-term effects induced by historic diets remain limited in aquatic invertebrates, especially for those with the regeneration ability. Sea cucumber (*Apostichopus japonicus*) is considered an economically important species (Yang et al., 2015). It has been widely cultured along the coastal regions of several Asian countries for centuries, including China, Japan, Korea, and Russia, because of its high nutritional and medicinal value (Xia et al., 2015a). In 2011, the annual production of *A. japonicus* in the world was more than 138,000 tons, more than 90% of which originated from aquaculture in China. Until 2021, *A. japonicus* in China achieved nearly 200,000 tons of annual production (Ministry of Agriculture and Rural Affairs of People's Republic of China, 2011, 2021). With the expansion of *A. japonicus* cultivation, more artificial diets were applied in commercial aquaculture to improve their production (Ying et al., 2009; Liao et al., 2015b). Because the detritus of macroalgae and sea mud in the sediment are the main food sources for *A. japonicus*, sea mud and powdered macroalgae are usually used as the main component of artificial diets (Ying et al., 2010; Sudong et al., 2012). Besides, Xia et al. (2015b) found that *A. japonicus* fed a diet with fish meal showed the best growth performance, indicating the important effect of animal-source protein on *A. japonicus*. The optimum protein level of diet fed to *A. japonicus* was 18–24%; hence, fish meal is used as the dietary component to improve the protein level of artificial diets (Zhu et al., 2005; Liao et al., 2015a). Numerous studies reported that the digestive physiology of *A. japonicus* would be affected by different diets, suggesting the flexibility of digestive physiology (Liao et al., 2015b; Wen et al., 2016b). In addition, other fascinating biological behaviors were observed in *A. japonicus*, such as autolysis, aestivation, evisceration, and regeneration (Wang et al., 2015; Ru et al., 2019). Evisceration

involves various complex physiological processes and results in the expulsion of the digestive tracts and other viscera (García-Arrarás et al., 1998; Ding et al., 2019). Following evisceration, the new intestine is regenerated from the residual coelomic epithelial cells through the processes of dedifferentiation, migration, redifferentiation, and division (Leibson, 1992; García-Arrarás and Greenberg, 2001; Zang et al., 2012). The persistence of the digestive physiology for the regenerated intestines of *A. japonicus* after evisceration, that is, whether the digestive ability of the regenerated intestine is affected by the historic digestive status before evisceration, remains unclear.

Digestion is one of the crucial metabolic processes in animals because it determines the availability of nutrient requirements (Gisbert et al., 2009). The whole digestive process primarily relies on the types and activities of digestive enzymes (Sveinsdóttir et al., 2006; Nazemroaya et al., 2015). Trypsin is an important serine alkaline protease responsible for protein digestion (Mir et al., 2018). Previous studies examined the trypsin activities to assess the digestion ability of aquatic organisms under different nutritional conditions, such as European sea bass (*Dicentrarchus labrax*) (Parma et al., 2019), olive flounder (*Paralichthys olivaceus*) (Bae et al., 2020), rainbow trout (Kasiga et al., 2020), and sea cucumber (Bai et al., 2015; Wen et al., 2016b). In addition, amylase and lipase are crucial digestive enzymes that can reveal the absorptive capability for dietary carbohydrates and lipids, respectively (Hidalgo et al., 1999; Liao et al., 2015b; Xia et al., 2015a). Previous studies demonstrated that the digestive enzymes, including trypsin, amylase, and lipase, could respond to different diets, which is termed as enzymatic plasticity (Sabat et al., 1999; Liu and Wang, 2007). For example, Bolasina et al. (2006) reported that the changes in diets led to subsequent variations in the trypsin and lipase activities during the ontogenetic development of Japanese flounder (*P. olivaceus*). European sea bass consuming a microparticulate diet showed higher trypsin and amylase activities compared with those consuming natural prey (Infante Zambonino and Cahu, 1994). The adjustment of enzymatic plasticity is crucial for organisms to maintain an appropriate level of digestive ability in response to different environmental conditions and physiological statuses (Fu et al., 2006; Garland, 2011; Kasiga et al., 2020).

Carbon stable isotope analysis is routinely employed to quantify food assimilation, providing details of time-integrated food (Kürten et al., 2013; Vander Zanden et al., 2015; Wen et al., 2016a). The turnover of stable isotope in organisms is generally driven by both growth and metabolism (Macavoy et al., 2006; Tarboush et al., 2006). With known growth performance in terms of specific growth rate (SGR), the metabolic rate (MR) can be estimated based on the simulation of isotopic turnover curves, and the metabolic status of the organism is thus evaluated with stable isotope analysis (Buchheister and Latour, 2010; Antonio and Richoux, 2016). The objectives of the present study were to investigate the long-term effects of historic diets on the digestive enzyme activities of regenerated intestines of *A. japonicus* after evisceration and assess the persistence of digestive physiology and subsequent effects on the growth and metabolism of *A. japonicus* by means of diet switch and stable isotope analysis.

TABLE 1 | Ingredients and nutritional composition of experimental diets fed to *A. japonicus*.

	Diet types	
	Diet A	Diet B
Diet ingredients (dry matter %)		
Fish meal ^a	0	10
Sea mud ^b	20	20
<i>Sargassum thunbergii</i> ^c	80	70
Nutritional composition (dry matter %)		
Crude proteins	10.88 ± 0.31 ^a	17.67 ± 0.51 ^b
Crude lipids	1.07 ± 0.07 ^a	1.65 ± 0.12 ^b
Carbohydrate	40.41 ± 1.25 ^b	34.55 ± 0.72 ^a
Ash	47.64 ± 1.14 ^b	46.13 ± 0.77 ^a

Data are presented as mean ± SD (n = 5). Different letters in the same row indicate significant differences between diet A and diet B (P < 0.05).

^a Fish meal: crude protein 78.75%, crude lipid 8.25%, and carbohydrate 8.05%.

^b Sea mud was burned in the Muffle furnace at 500 °C for 6 h to remove organic, after which only ash was in the sea mud.

^c *Sargassum thunbergii*: crude protein 13.55 %, crude lipid 1.24 %, and carbohydrate 49.74%.

MATERIALS AND METHODS

Diet Preparation

Numerous studies reported that diets consisting of sea mud, fish meal, and macroalgae, including *S. thunbergii*, *Ulva lactuca*, and *Gracilaria lemaneiformis*, could meet the nutritional requirements and lead to the rapid growth of *A. japonicus* (Ying et al., 2009; Sun et al., 2012; Wen et al., 2016a). Meanwhile, two different types of diets were formulated based on previous studies for the following feed experiment, to avoid the effects of dietary additives on the digestive enzyme activities (Shi et al., 2015; Wen et al., 2016a), and identified as diet A and diet B. Diet A comprised sea mud and *S. thunbergii*. Sea mud was collected from the intertidal zone (Qingdao, Shandong province, China), and *S. thunbergii* was obtained from Xiaoheishan Island (Yantai, Shandong province, China). Besides these contents, commercial fish meal (Qingdao Seven Good Biological Technology Co., Ltd.) was added to diets denoted as diet B. The ingredients of these diets were ground, sieved through a 0.25-mm mesh, mixed adequately, slightly watered, stirred, and extruded into pellets (diameter = 1.8 mm) using a feed processing machine (Tairun Equipment, Jining, China). The pelleted diets were dried at 60°C for 48 h and stored at -20°C for the feeding experiment. The concentrations of nitrogen and carbon elements in diets were measured using an elemental analyzer (Vario EL III; Elementar Co., Germany), and the content of crude protein was estimated as nitrogen concentration multiplied by 6.25. The crude lipid content was analyzed using the Soxhlet extraction method with petroleum ether (Luque De Castro and Priego-Capote, 2010). For ash content measurement, dried diets were combusted at 500°C for 6 h in a muffle furnace (Nabertherm, Germany). Carbohydrate = 100 - crude protein - crude lipid - ash (Seo et al., 2011; Xia et al., 2015a; Hassaan et al., 2018). The crude protein, lipid, and carbohydrate contents of diet A versus diet B were 10.88

vs. 17.67%, 1.07 vs. 1.65%, and 40.41 vs. 34.55%, respectively. The detailed ingredients and nutritional composition of these diets are shown in Table 1.

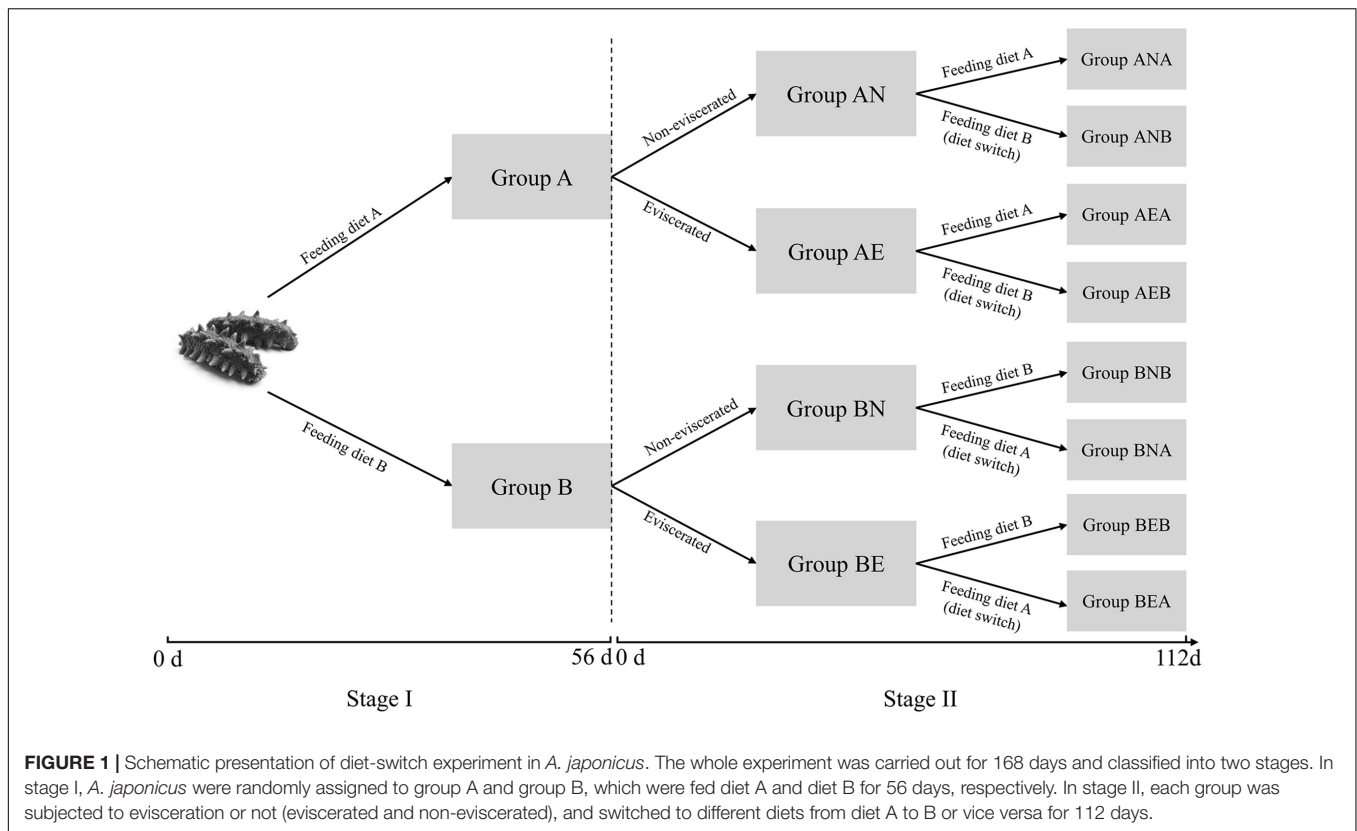
Diet-Switch Experiment

A total of 300 *A. japonicus* juveniles were obtained from Tianheng aquaculture company (Qingdao, Shandong province, China) with an average body weight of 8.80 ± 0.21 g. These individuals were acclimated to the experimental conditions for 2 weeks. Then, the feeding experiment was conducted for two different stages (stage I and stage II). In stage I, all *A. japonicus* were transferred to 2 tanks [2 m × 1.5 m × 0.8 m (L × W × H)], resulting in 150 individuals in each tank. They were classified into two groups, which were fed diet A (group A) and diet B (group B) for 56 days, respectively. Three individuals in each group were randomly sampled and quickly dissected for intestines and body walls after freeze anesthesia at six time points, including days 0, 7, 14, 28, 42, and 56, during the period of the feeding experiment in stage I. The intestines and body walls were immediately stored at -80°C until assayed for digestive enzyme activities and the carbon stable isotope. All *A. japonicus* were starved for 24 h prior to sampling.

Further, 120 individuals in each tank were transferred to 12 glass aquariums [70 cm × 40 cm × 40 cm (L × W × H)] for a further 112-day feeding experiment (stage II). These aquariums from each tank were classified into two groups (six aquariums in each group), which were then subjected to evisceration by the intra-coelomic injection of 0.35M KCl (eviscerated group) or not (non-eviscerated group). The eviscerated *A. japonicus* from groups A and B were named as groups AE and BE while the non-eviscerated individuals from groups A and B were named as groups AN and BN, respectively. Each non-eviscerated and eviscerated group was further divided into two groups (three aquariums in each group), of which one was fed the same diets as in stage I and the other was treated with diets switching from diet A to B or vice versa. Group AN, BN, AE, and BE treated with diet switch or not were renamed as ANA, ANB, BNA, BNB, AEA, AEB, BEA, and BEB, respectively (Figure 1).

Finally, three individuals in each non-eviscerated group, including groups ANA, ANB, BNA, and BNB, were sampled on days 7, 14, 28, 49, 70, 91, and 112 in stage II. Because of 21-day intestine regeneration, the sample collections in eviscerated groups (AEA, AEB, BEA, and BEB) were conducted only on days 21, 28, 35, 49, 70, 91, and 112. The sampling tissues and method in stage II were the same as those in stage I. In addition, the initial and final weights of *A. japonicus* were measured in both stages I and II for the following calculation of the body weight gain (BWG), SGR, and growth rate constant (*k*).

During the whole experiment, *A. japonicus* were fed once a day at 15:00 with a daily ration of 5% wet body weight, based on which the individuals were under the apparent satiation. The residual feed and feces were cleaned by siphoning every day. The temperature was constantly kept at 16.5 ± 0.5°C, the salinity varied between 29 and 31 ppt, the levels of dissolved oxygen were greater than 6.0 mg/L, and a normal photoperiod 14:10 h (L/D) was applied. The water environmental conditions were kept relatively constant for each aquarium during the experiment.



Determination of Enzymatic Activity

The intestinal tissues were homogenized for 2 min in a pre-cooled homogenization buffer using a tissue grinder (JXFSTPRP-CL-24; ShangHaiJingXin Co., China). The supernatants were obtained after centrifugation at 20,000g for 25 min at 4°C, pipetted into clean centrifuge tubes, and immediately stored at 4°C until analysis (less than 12 h).

The protein concentration, trypsin activities, amylase activities, and lipase activities in the intestines of *A. japonicus* were determined using the standard kits (A080-2, C016, A045-4, and A054-2-1) from the Nanjing Jiancheng Bioengineering Institute (Nanjing, China) (Xionggao et al., 2014; Liao et al., 2015b; Ye et al., 2019). The experimental procedures were conducted following the manufacturer's protocols. Both the trypsin and amylase activities were tested using a spectrophotometer (UV5100, METASH, Shanghai, China). The protein concentrations and lipase activities were determined using a microplate reader (Synergy Mx, Bio Tek, VT, United States). The specific trypsin and amylase activities were expressed as trypsin and amylase unit per mg protein, respectively. The specific lipase activity was expressed as lipase unit per g protein.

Growth Performance

The BWG (%) of *A. japonicus* was calculated as (Liao et al., 2015b):

$$\text{BWG (\%)} = \frac{\text{FBW} - \text{IBW}}{\text{IBW}} \times 100$$

where IBW and FBW are the initial body weight and final body weight, respectively.

The SGR of *A. japonicus* was calculated as (Giri et al., 2013):

$$\text{SGR (\% initial body weight/day)} = (\ln W_t - \ln W_0) / t \times 100$$

where W_t and W_0 are the final and initial weights of individuals, respectively, and t is the duration of the experiment.

Carbon Stable Isotope Analysis

The diets and body walls of *A. japonicus* were freeze-dried for 48 h to constant weight and homogenized. A stable isotope mass spectrometer (EA-IRMS, Thermo Finnigan MAT Delta-plus) was used to determine the ratio of carbon isotope ($\delta^{13}\text{C}$) with the accuracy of $\pm 0.1\text{‰}$.

The ratio of carbon stable isotope is generally defined as follows (Peterson and Fry, 1987):

$$\delta^{13}\text{C} = [(R_{\text{sample}}/R_{\text{standard}}) - 1] \times 1000\text{‰}$$

where R is the ratio of ^{13}C and ^{12}C .

The stable isotope turnover model is defined as follows (Hesslein et al., 1993):

$$C_t = C_f + (C_0 - C_f)e^{-(k+m)t}$$

where C_t represents the carbon stable isotope ratio of biological tissue on day t . C_f represents the ratio of stable isotope when diets

and tissue turnover reach a balance after dietary transformation. C_0 represents the carbon stable isotope ratio of the initial biological tissue. k is the growth rate constant, and m is the metabolism rate constant. $k + m$ is the isotopic turnover rate.

The growth rate constant (k) was calculated as follows (Buchheister and Latour, 2010):

$$k \text{ (d}^{-1}\text{)} = (\ln W_f - \ln W_i) / t$$

where W_f and W_i represent the final and initial weights of *A. japonicus*, respectively, and t is the duration of the experiment in days.

The isotopic turnover rate ($k + m$) was obtained by fitting the exponential model to match the observed isotopic data, and the metabolism rate constant (m) was thus obtained by means of $k + m$ minus k (Winter et al., 2019).

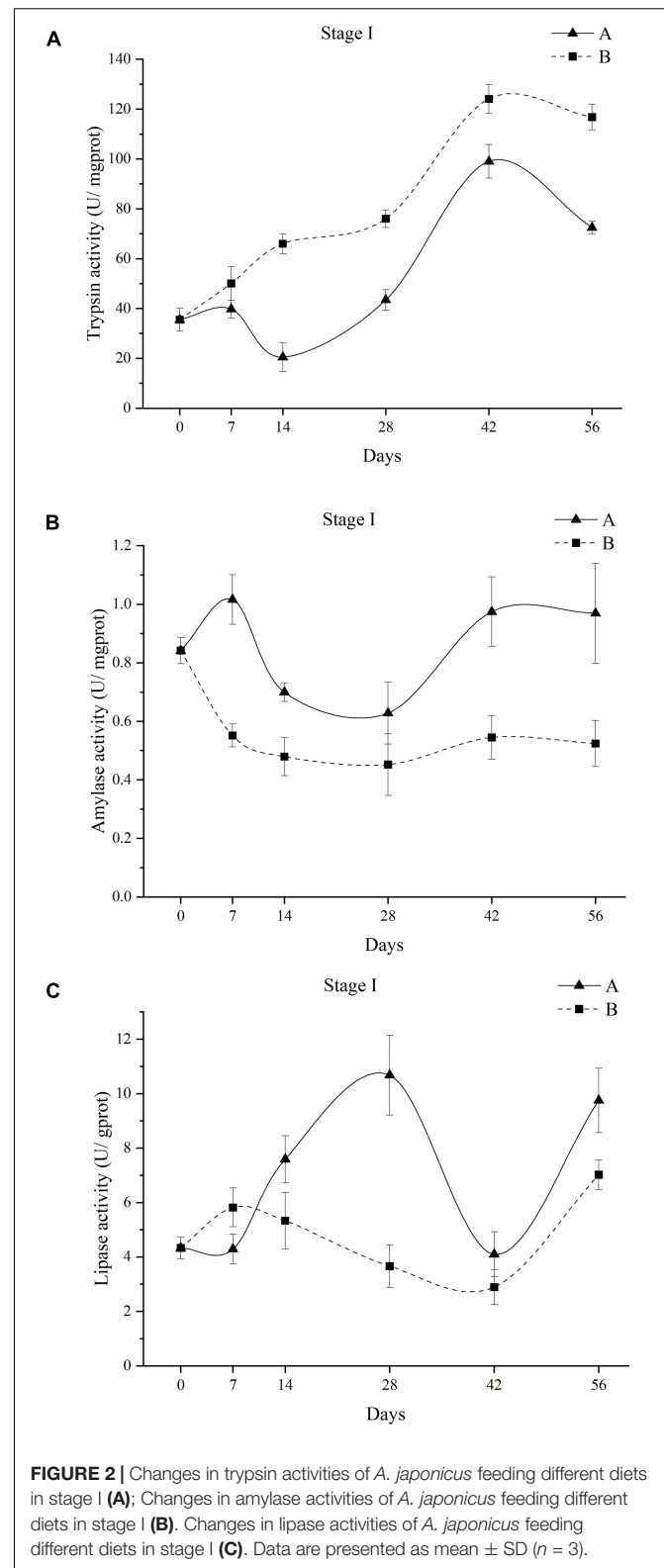
Statistical Analysis

Significant differences in nutritional composition between diet A and diet B were determined using the Student's t -test. For stage I, the differences in the digestive enzyme activities between groups fed different diet types and between sampling times were compared with a two-way analysis of variance (ANOVA) and the differences in the FW, BWG, SGRs, and MRs at the end of stage I between different diet types were compared with the Student's t -test. For stage II, a four-way ANOVA was applied to explore the effects of diet in stage I, diet in stage II, evisceration, and sampling time on the digestive enzyme activities, while a three-way ANOVA was used to compare the differences in FW, BWG, SGRs, and MRs at the end of stage II between diet in stage I, diet in stage II and evisceration. For ANOVA, if the interaction of main factors was detected, a one-way ANOVA or the Student's t -test was conducted to examine the effects of one main factor at the specific level of the other main factor(s). The factors detected to be significant by ANOVAs were further analyzed using a Bonferroni multiple comparison procedure, which adjusted the observed significance level by multiplying it by the number of comparisons being made (Zar, 1999; Norusis, 2008). Pearson correlation analysis was used to examine the relationships of SGRs and MRs to the digestive enzyme activities. A P value of 0.05 was used as the significance level. Before statistical analyses, raw data were diagnosed for the normality of distribution and homogeneity of variance with the Kolmogorov–Smirnov test and Levene's test, respectively. The statistical analysis was conducted using IBM SPSS Statistics (Version 25) and Origin (Version 9.1) software.

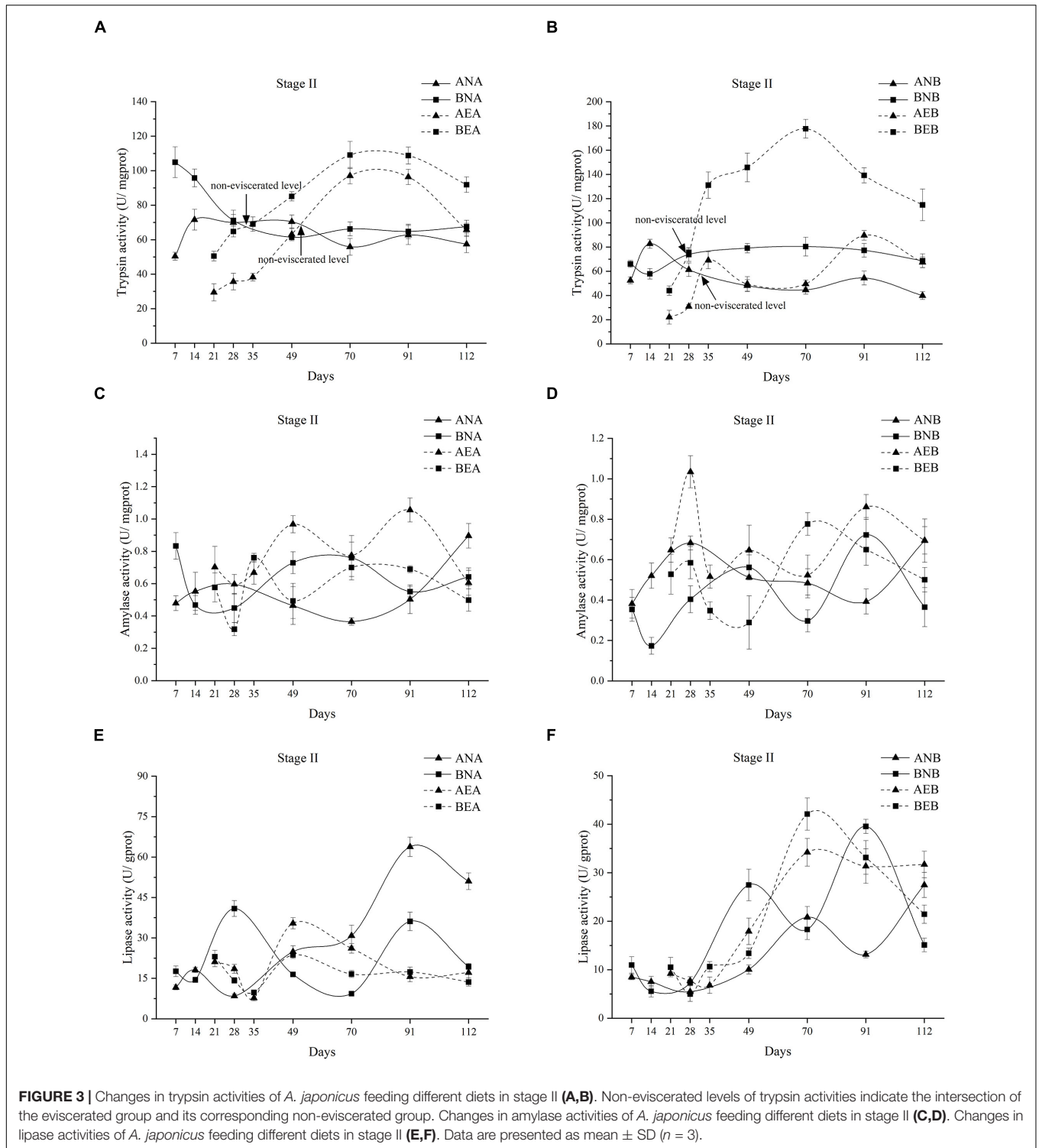
RESULTS

Enzymatic Activities

The digestive enzyme activities of *A. japonicus* fed different diets in stage I are presented in **Figure 2**. Similar trends of trypsin activities of *A. japonicus* were observed in groups A and B (**Figure 2A**). Specifically, the trypsin activities of *A. japonicus* in groups A and B first increased, reaching the



peak on day 42 and then decreased on day 56. *A. japonicus* fed diet B showed significantly higher trypsin activities than those fed diet A in stage I ($P < 0.05$). The amylase activities



of *A. japonicus* in group A showed an abrupt increase in the first 7 days and then gradually decreased until day 28, followed by an increasing trend to the end of stage I (Figure 2B). The amylase activities of *A. japonicus* in group B decreased first and then showed a slight fluctuation until the end of stage I. Meanwhile, the amylase activities of *A. japonicus* in

group A were higher than those of individuals in group B ($P < 0.05$). As shown in Figure 2C, the lipase activities of *A. japonicus* in group A quite fluctuated throughout stage I, with the highest value on day 28 and the lowest on day 42. For *A. japonicus* in group B, an increase in the lipase activity was observed before 7 days. After gradually decreasing between

TABLE 2 | The growth performance of *A. japonicus* in stage I.

Groups	Final body weight	Body weight gain
A	14.96 ± 0.44 ^a	70.00 ± 1.08 ^a
B	17.21 ± 0.72 ^b	95.52 ± 3.45 ^b

Data are presented as mean ± SD ($n = 3$). The letters *a* and *b* indicate significant differences between different groups at the significance level of 0.05 ($P < 0.05$).

day 7 and day 42, a further increase in lipase activities was observed until the end of stage I. *A. japonicus* fed diet A showed higher lipase activities than those fed diet B from day 14 to the end of stage I.

As shown in **Figure 3A**, the individuals in the non-eviscerated and eviscerated groups presented similar trends. *A. japonicus* in group BNA showed significantly higher trypsin activities than those in group ANA, except on days 28, 49, and 91 ($P < 0.05$, **Figure 3A**). The trypsin activities of *A. japonicus* in group BEA were significantly higher than those of individuals in group AEA ($P < 0.05$, **Figure 3A**), although this difference was non-significant on day 70. The trypsin activities of *A. japonicus* in group BEA increased rapidly and returned to non-eviscerated levels in less time than the activities of those in group AEA. As shown in **Figure 3B**, little changes were found in trypsin activities of *A. japonicus* in groups ANB and BNB. In contrast, the trypsin activities in eviscerated groups, particularly group BEB, greatly changed. *A. japonicus* in groups BNB and BEB exhibited significantly higher trypsin activities than those in groups ANB and AEB ($P < 0.05$). For eviscerated *A. japonicus*, the trypsin activities in group BEB increased rapidly and took 28 days to reach non-eviscerated levels, while the trypsin activities in group AEB returned to non-eviscerated levels after 35 days.

Dramatic fluctuations were observed in the amylase activities of *A. japonicus* in stage II (**Figures 3C,D**). Significant differences were found in amylase activities between *A. japonicus* in group ANA and BNA on days 7, 49, 70, and 112, but the amylase activities of *A. japonicus* in group ANA were higher than those of individuals in group BNA only on day 112 ($P < 0.05$, **Figure 3C**). For eviscerated *A. japonicus*, significantly higher amylase activities were observed in group AEA than in group BEA on days 28, 49, and 91 ($P < 0.05$, **Figure 3C**). As shown in **Figure 3D**, the amylase activities of *A. japonicus* in group ANB were higher than those of individuals in group BNB on days 14, 28, 70, and 112 ($P < 0.05$). *A. japonicus* in group AEB exhibited higher amylase activities than those in group BEB except on days 21 and 70 ($P < 0.05$).

The lipase activities of *A. japonicus* fed diet A in stage II fluctuated; only the lipase activities of *A. japonicus* in group ANA gradually increased during stage II (**Figure 3E**). *A. japonicus* in group ANA showed significantly higher lipase activities than those in group BNA from day 49 to the end of stage II ($P < 0.05$, **Figure 3E**). For eviscerated *A. japonicus*, similar values of lipase activities were observed in groups AEA and BEA during stage II except for days 28, 49, and 70, with higher lipase activities in group AEA than in group BEA ($P < 0.05$, **Figure 3E**). As shown in **Figure 3F**, the lipase

activities of all *A. japonicus* fed diet B in stage II increased during the whole stage II. Notably, the lipase activities of *A. japonicus* in group ANB were significantly higher than those of individuals in group BNB only on day 112 ($P < 0.05$). The higher lipase activities were found in *A. japonicus* in group AEB than those in group BEB only on days 28 and 112 ($P < 0.05$).

Growth Performance

The FBW and BWG of *A. japonicus* in stage I are shown in **Table 2**. *A. japonicus* in group B showed significantly higher FBW and BWG than those in group A ($P < 0.05$). As shown in **Table 3**, diet in stage I, diet in stage II, and evisceration showed an influence on the FBW and BWG of *A. japonicus*, but their interaction did not affect the FBW and BWG. *A. japonicus* fed diet B, regardless of the stage, exhibited significantly higher FBW and BWG compared with those fed diet A in stage I or II ($P < 0.05$). The FBW of non-eviscerated *A. japonicus* was significantly higher rather than that of eviscerated *A. japonicus*, while the BWG of non-eviscerated *A. japonicus* was significantly lower than that of eviscerated *A. japonicus* ($P < 0.05$). The growth rates of *A. japonicus* in stage I are presented in **Table 4**. *A. japonicus* in group B showed a significantly higher growth rate than those in group A ($P < 0.05$). The growth performance of *A. japonicus* in stage II is shown in **Table 4**. The growth rates of *A. japonicus* in groups BNA, BNB, BEA, and BEB were higher than those of individuals in groups ANA, ANB, AEA, and AEA, respectively, despite non-significant differences in *A. japonicus* fed the same diet in stage II ($P > 0.05$).

Time-Dependent Isotopic Turnover and Metabolism

The carbon isotope ratios of diet A and B were measured as -17.97 ± 0.06 and -18.09 ± 0.05 , respectively. The trends of carbon isotope ratios in the body walls of *A. japonicus* were determined and are visualized in **Figure 4**. In stage I, group A not only exhibited higher carbon isotope ratios but also responded more quickly than group B (**Figure 4A**). In stage II, different trends of carbon isotope ratios were observed in different groups, which were caused by the historic and new diets (**Figures 4B–E**). However, the carbon isotope ratios of *A. japonicus* fed diet A in stage I were always higher than those of individuals fed diet B in stage I. The changes in ^{13}C were modeled as functions of time (time models). The related parameters for different groups are presented in **Table 4**. In stage I, the higher MR was observed in *A. japonicus* in group A than in group B ($P < 0.05$). In stage II, *A. japonicus* in groups ANB, AEA, and AEB exhibited significantly higher MR than in groups BNB, BEA, and BEB, respectively ($P < 0.05$).

Relationships Between Enzymic Activities, SGRs, and MRs

Linear regression was applied to examine the relationship between enzymic activities, SGRs, and MRs (**Figure 5**). A positive relationship was identified between the trypsin activities and

SGRs ($P < 0.05$), but a non-significant relationship was found between trypsin activities and MRs (Figures 5A,B). The amylase activities of *A. japonicus* negatively correlated with the SGRs but positively correlated with the MRs of *A. japonicus* ($P < 0.05$, Figures 5C,D). No significant relationships between lipase activities, SGRs, and MRs were observed in *A. japonicus* (Figures 5E,F).

DISCUSSION

The present study explored the changes in the digestive enzyme activities, SGRs, and MRs of *A. japonicus* resulting from diet switch before and after evisceration. It revealed that the short-term plasticity of trypsin and amylase in response to historic diets would persist in non-regenerated and regenerated intestines, suggesting the long-term effect of historic diets on the digestion of *A. japonicus*. The correlation between the digestive enzyme activities, SGRs, and MRs suggested the effects of the changes in enzymatic activities induced by different diets on the SGR and MR of *A. japonicus*. This study provided a comprehensive view of the persistence of the plasticity of digestive enzymes in regenerated intestines and revealed the long-term effects of historic diets on digestion, growth, and metabolism of *A. japonicus*.

In stage I, enzymatic activities induced by distinct diets were different. High trypsin and amylase activities were observed in *A. japonicus* fed diet B and *A. japonicus* fed diet A, respectively. It was concluded that the activities of trypsin and amylase were positively affected by the dietary protein and carbohydrate contents, respectively, which were largely consistent with previous studies about white shrimp (*Litopenaeus vannamei*) (Muhlia-Almazan et al., 2003), sea bass (Peres et al., 1996), and large yellow croaker (*Pseudosciaena Crocea*) (Yu et al., 2012). And Xia et al. (2015a) also reported that the amylase activity of *A. japonicus* increased with the elevating dietary carbohydrate levels from 25.61 to 45.31%. However, the lipase activities of *A. japonicus* in group B fed a lipid-rich diet (diet B) were lower than those of group A fed a low-lipid diet (diet A), which was contrary to the previous study that the increasing lipase activities of *A. japonicus* were accompanied by the enhanced dietary lipid levels (Liao et al., 2015a). The positive relationship between lipase activities and dietary lipid content in that study was determined based on the changes in lipase activities of *A. japonicus* fed the isonitrogenous diet. Thus, in the present study, the changes in lipase activities of *A. japonicus* fed different diets might be not only affected by dietary lipids but also other dietary nutrients, which was furtherly supported by previous findings that the dietary proteins, carbohydrates, and lipids had interactive effects on the lipase activities (Xia et al., 2015a; Huang et al., 2019). According to the changes in digestive enzyme activities induced by different diets, great plasticity of the digestive enzymes under exposure to different diets was found in *A. japonicus* in stage I.

In stage II, although *A. japonicus* in groups BNA and ANA, groups BNB and ANB, groups BEA and AEA, as well as groups BEB and AEB were fed the same diet, higher trypsin activities

were observed in groups BNA, BNB, BEA, and BEB than in groups ANA, ANB, AEA, and AEB, respectively. It suggested that the plasticity of trypsin induced by diet in stage I could persist in the non-regenerated and regenerated intestines. The persistence of plasticity was also detected in the amylase activities of eviscerated *A. japonicus*. In detail, *A. japonicus* in group AEA showed higher amylase activities than those in group BEA despite all individuals fed diet A in stage II. Also, *A. japonicus* in group AEB showed higher amylase activities than those in group BEB despite individuals fed diet B in stage II. The persistence of digestion plasticity has already been reported at molecular levels by Geurden et al. (2007), who found that the early hyperglucidic diet could rapidly increase the expression of α -amylase of larvae rainbow trout, and for juveniles subjected to the challenge test with a 25% dextrin diet, the higher expression of α -amylase was observed in rainbow trout that experienced the hyperglucidic stimulus compared with those fed a hypoglucidic diet at first feeding, suggesting that short-term digestive plasticity of fish resulting from dietary carbohydrates in the early feeding stage would persist in the later life. Therefore, in the present study, it was hypothesized that the genes related to trypsin and amylase activities could be affected by diet in stage I and subsequently regulated the trypsin activities and amylase activities in stage II. However, further studies need to be conducted to prove this hypothesis. Additionally, the lipase activities of *A. japonicus* exhibited no persistence of plasticity, which might be explained by the poor demand of dietary lipids and low lipase activities of *A. japonicus* (Liao et al., 2015a; Ye et al., 2019).

Changes in the digestive enzyme activities of *A. japonicus* before and after evisceration indicated the long-term effects of historic diets on digestion; the long-term effects of diets have been reported by numerous studies. For example, rainbow trout fry fed a plant-based diet for a short time enhanced the acceptance and use of a plant-based diet during their later life (Geurden et al., 2013; Balasubramanian et al., 2016). Besides, the early hyperglucidic diet stimuli showed long-term effects on carbohydrate digestion of rainbow trout in later life, which resulted in the persistence of amylase plasticity of the organism (Geurden et al., 2007). Similarly, in the present study, the long-term effects of diet in stage I on trypsin and amylase activities led to the persistence of these two enzymatic activities of *A. japonicus*. In addition, the trypsin activities of eviscerated *A. japonicus* fed diet B in stage I returned to the non-eviscerated levels in less time than those fed diet A in stage I, regardless of which diet was fed to *A. japonicus* in stage II. This finding suggested that the high-protein diet (diet B) in stage I could accelerate the recovery of trypsin activities of eviscerated *A. japonicus*, which further confirmed the positive long-term effects of historic diets on the digestion of *A. japonicus*. Moreover, the trypsin activities of non-eviscerated *A. japonicus* in stage II were affected by the historic diets in stage I, while for eviscerated *A. japonicus*, both trypsin and amylase activities were affected by diet in stage I, suggesting that the digestion of regenerated intestines was more susceptible to the long-term effect of historic diets compared with that of non-regenerated intestines.

For the growth performance of *A. japonicus* in stage I, *A. japonicus* in group B possessed higher FBW, BWG, and

TABLE 3 | The growth performance of *A. japonicus* in stage II.

Source	df	P	Multiple comparison					
			Diet in stage I		Diet in stage II		Non-eviscerated (N) or eviscerated (E)	
			A	B	A	B	N	E
Final body weight								
Diet in stage I	1	< 0.001	35.25 ± 6.09 ^a	47.22 ± 7.97 ^b	37.62 ± 7.56 ^a	44.85 ± 9.64 ^b	45.93 ± 8.46 ^b	36.54 ± 7.68 ^a
Diet in stage II	1	< 0.001						
Evisceration	1	< 0.001						
Diet in stage I*II	1	NS						
Diet in stage I* Evisceration	1	NS						
Diet in stage II* Evisceration	1	NS						
Diet in stage I*II*Evisceration	1	NS						
Body weight gain								
Diet in stage I	1	0.038	214.13 ± 56.52 ^a	240.07 ± 63.86 ^b	205.88 ± 50.27 ^a	248.33 ± 62.56 ^b	185.06 ± 41.98 ^a	269.14 ± 44.04 ^b
Diet in stage II	1	0.002						
Evisceration	1	< 0.001						
Diet in stage I*II	1	NS						
Diet in stage I* Evisceration	1	NS						
Diet in stage II* Evisceration	1	NS						
Diet in stage I*II*Evisceration	1	NS						

See the text for a detailed description of the model. *df* represents the degree of freedom. The letters *a* and *b* indicate significant differences between different groups at the significance level of 0.05 ($P < 0.05$).

TABLE 4 | Summary of time-based stable isotope turnover models and related growth and metabolism parameters for body walls of *A. japonicus*.

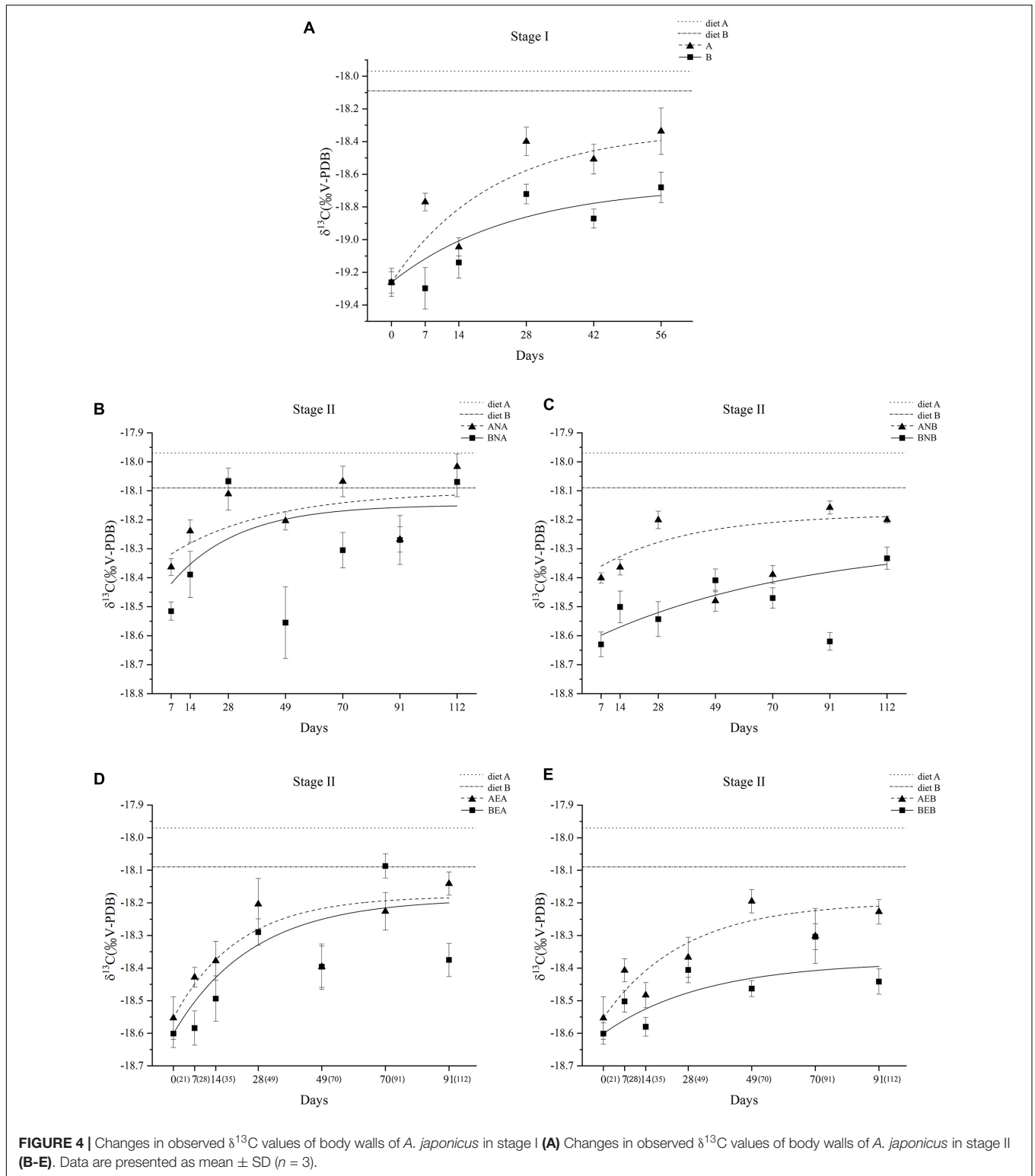
	Groups	Equation	R ²	k + m	k (d ⁻¹)	m (d ⁻¹)
Groups in stage I	A	$C_t = -18.33 - 0.94e^{-0.04704t}$	0.55*	0.04704	0.0095 ± 0.0005 ^a	0.0375 ± 0.0005 ^b
	B	$C_t = -18.67 - 0.60e^{-0.03970t}$	0.80*	0.03970	0.0120 ± 0.0007 ^b	0.0277 ± 0.0007 ^a
Non- eviscerated groups in stage II	ANA	$C_t = -18.10 - 0.26e^{-0.02696t}$	0.56*	0.02696	0.0082 ± 0.0007 ^a	0.0187 ± 0.0007 ^a
	BNA	$C_t = -18.15 - 0.36e^{-0.04052t}$	0.54*	0.04052	0.0088 ± 0.0007 ^a	0.0317 ± 0.0007 ^b
	ANB	$C_t = -18.18 - 0.22e^{-0.02933t}$	0.86*	0.02933	0.0090 ± 0.0008 ^a	0.0203 ± 0.0008 ^b
	BNB	$C_t = -18.27 - 0.36e^{-0.01299t}$	0.78*	0.01299	0.0109 ± 0.0009 ^a	0.0021 ± 0.0009 ^a
	Eviscerated groups in stage II	AEA	$C_t = -18.18 - 0.37e^{-0.04645t}$	0.81*	0.04645	0.0130 ± 0.0009 ^a
	BEA	$C_t = -18.19 - 0.41e^{-0.03838t}$	0.71*	0.03838	0.0140 ± 0.0012 ^a	0.0244 ± 0.0012 ^a
	AEB	$C_t = -18.20 - 0.35e^{-0.03773t}$	0.79*	0.03773	0.0149 ± 0.0010 ^a	0.0228 ± 0.0010 ^b
	BEB	$C_t = -18.38 - 0.22e^{-0.03007t}$	0.61*	0.03007	0.0160 ± 0.0009 ^a	0.0141 ± 0.0009 ^a

t represents the experimental time in days. C_t represents the carbon stable isotope ratio on day *t*. R^2 represents the goodness of fitting for time-based stable isotope turnover model. * means significant correlation at the significance level of 0.05 ($P < 0.05$). *k* + *m* represents the isotopic turnover rate. *k* represents growth rate constant and *m* metabolic rate constant. See the text for a detailed description of the model. The letters *a* and *b* indicate significant differences between different groups at the significance level of 0.05 ($P < 0.05$).

SGR than those in group A. Previous studies documented that adequate dietary protein contributed to the growth of *A. japonicus* (Seo et al., 2011; Li et al., 2021). For MR in stage I, *A. japonicus* fed diet B exhibited lower MR than those fed diet A, which might be related to the low carbohydrate content of diet B. Increased MR in response to high-carbohydrate diets was observed in the present study, which was consistent with that in Southern catfish (*Silurus meridionalis*) and black carp (*Mylopharyngodon piceus* Richardson) (Shijian and XieXiao, 2007; Cai et al., 2010). Moreover, the negative relationship between MR and SGR of *A. japonicus* was reported by Xia et al. (2015a), in which *A. japonicus* fed a diet consisting of 75% *S. thunbergii* and 25% soybean meal showed higher MR but lower

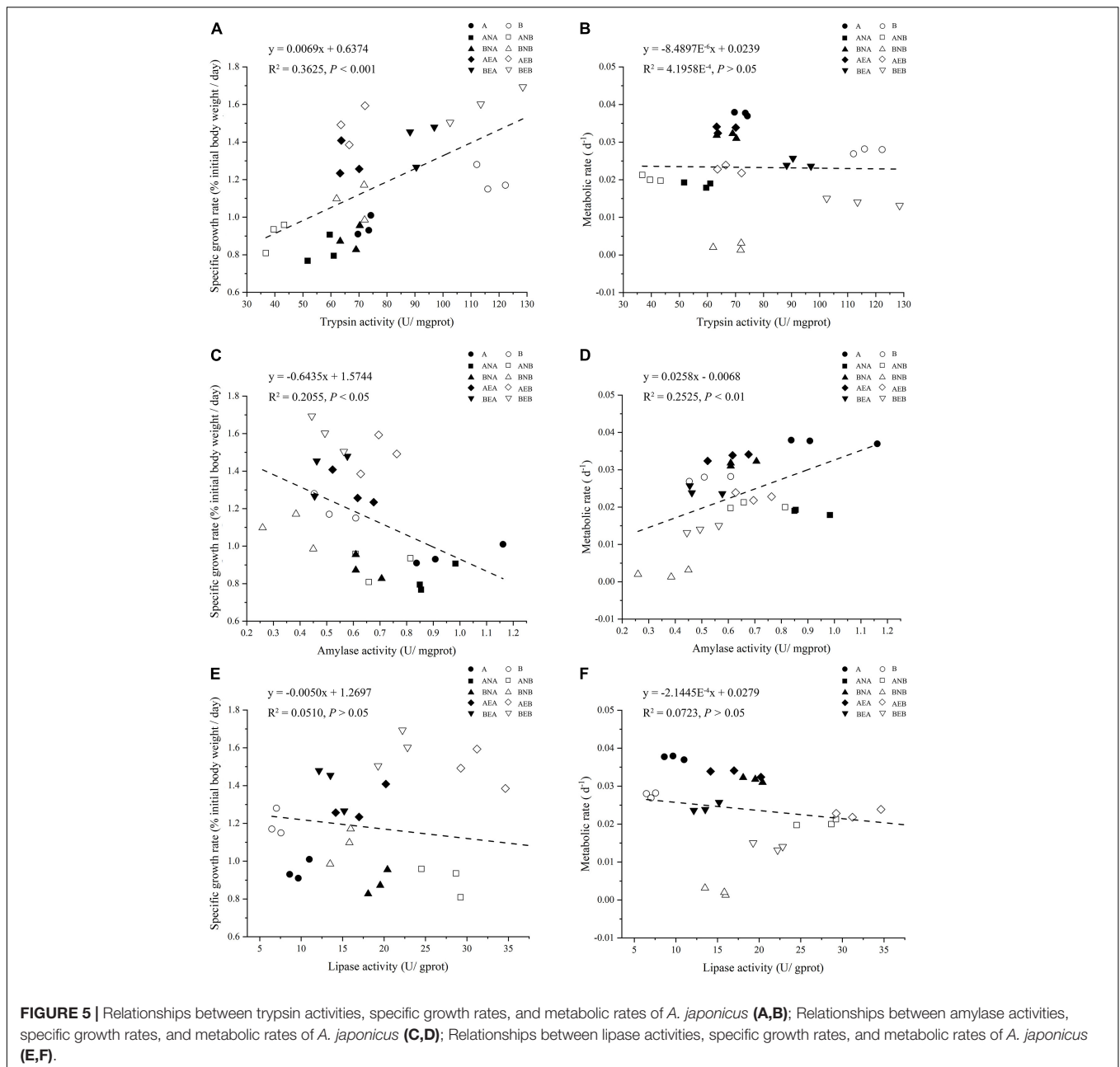
SGR than those of *A. japonicus* fed a diet consisting of 100% *S. thunbergii*. It suggested that the growth rate of *A. japonicus* would be restricted when more energy was provided to meet metabolic requirements.

In stage II, eviscerated *A. japonicus* exhibited higher BWG but lower FBW compared with the non-eviscerated *A. japonicus*. It was consistent with the previous finding that the growth performance would be rapidly improved after the intestinal regeneration of *A. japonicus*, although the body weights of eviscerated *A. japonicus* could not exceed that of non-eviscerated individuals due to huge loss weights caused by evisceration (Zang et al., 2012). The growth performance of *A. japonicus* in stage II was affected by not only evisceration



but also the diet types in different stages. Diet B as diet in stages I or II would enhance FBW, BWG, and SGRs of all *A. japonicus* in stage II. Especially for *A. japonicus* fed diet B in stage II, it was more beneficial for their subsequent

growth performance if diet B was included in stage I. However, for non-eviscerated or eviscerated groups, the differences in SGRs of *A. japonicus* fed the same diet in stage II were non-significant. It suggested that *A. japonicus* showed similar



SGRs when they consumed the same diet, and the SGRs of *A. japonicus* were slightly affected by the long-term effects of the historic diets.

The changes in MRs of *A. japonicus* fed the same diet in stage II indicated that the MRs of *A. japonicus* seemed to be affected by diet in stage I, which was in agreement with previous findings that the nutritional history had major impacts on metabolic processes to improve fish performance (Vera et al., 2017). Besides, Lage et al. (2020) also demonstrated that the early dietary restriction resulted in a long-term modification of the metabolism of white shrimp by regulating the mRNA levels. In addition, the enzymatic activities are important for digesting and assimilating nutrients and are

closely associated with growth performance and metabolic status (Sunde et al., 2001; Rungruangsak-Torrissen et al., 2006). In the present study, the SGRs and MRs of *A. japonicus* were positively affected by trypsin and amylase activities, respectively, which were consistent with the positive relationship between trypsin activities and body weight observed in Atlantic salmon (Rungruangsak-Torrissen et al., 2006). Besides, Rungruangsak-Torrissen et al. (2006) and Murashita et al. (2013) reported that the changes in digestive enzyme activities could be applicable as the indicators for growth and metabolic studies of fish to reflect the growth and metabolism during the development of organisms. Therefore, in the present study, based on the relationship between digestive enzyme activities, growth, and

metabolism, the historic diets could produce long-term effects on the growth and metabolism of *A. japonicus* by affecting the enzyme activities in stage II.

In summary, the persistence of digestive enzyme activities induced by historic diets was found in non-eviscerated and eviscerated *A. japonicus*, indicating the long-term effects of historic diets on the digestion of *A. japonicus*. The digestion of regenerated intestines was more susceptible to the long-term effects of historic diets than those of non-regenerated intestines. The changes in SGRs and MRs, as well as their relationships with digestive enzyme activities, revealed that the historic diets showed long-term effects on the growth and metabolism of *A. japonicus* through long-term effects of historic diets on digestive enzyme activities. Further studies are required to investigate the molecular mechanism of the persistence of digestive enzyme activities and the long-term effects of historic diets on *A. japonicus*.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

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AUTHOR CONTRIBUTIONS

XL was in charge of feeding experiments, data analysis, and manuscript writing. QG and YT were in charge of experimental guidance and manuscript revision. YM, YX, and YC were in charge of feeding experiments. All authors designated in this manuscript actively participated in this research.

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Conflict of Interest: YS and WJ were employed by Homey Group Co., Ltd.

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