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Role of *try* and *amy* in the feeding, digestion, growth and development of the Japanese medaka (*Oryzias latipes*): insight from a comparative gene knockout study

Xiaodan JIA[1,2*], Shulin TANG[1,2*], Hexiong FENG[1,2], Dimei XU[1,2], Chenyuan ZHU[1,2], Ke LU[1,2] Xufang LIANG[1,2]✉

[1]*College of Fisheries, Chinese Perch Research Center, Huazhong Agricultural University, Wuhan 430070, China*
[2]*Engineering Research Center of Green development for Conventional Aquatic Biological Industry in the Yangtze River Economic Belt, Ministry of Education, Wuhan 430070, China*

Abstract: Digestive enzymes of fish are critical to food digestion at the larval stage, but convincing evidence proving the function and necessity of the associated digestive enzymes remains lacking. In this study, we generated the trypsin gene (*try*) and amylase gene (*amy*) in the Japanese medaka (*Oryzias latipes*) using CRISPR for the first time. *try* deletion significantly decreased the expression of *try* and digestive capacity in *try*$^{-/-}$ medaka larvae; after 8 h digestion, incompletely digested brine shrimp was observed in the digestive tract at 4 and 15 days post-hatching (dph) of *try*$^{-/-}$ medaka larvae. Furthermore, the height of intestinal villi and total body length decreased significantly within 15 dph *try*$^{-/-}$ medaka larvae. However, *amy* deletion did not influence the digestion of medaka larvae at 4 dph. Only a small amount of incompletely digested brine shrimp was observed in 15-dph *amy*$^{-/-}$ medaka larvae. Further analysis of the growth, nitrogen metabolism, and intestinal microbes of *try*$^{-/-}$ adult medaka showed that the body length and weight of adult medaka decreased significantly, while the activity of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) in the blood significantly increased. Pathological observation of liver and intestinal tissues showed that *try* knockout resulted in vacuolar degeneration of liver cells, thinning of the intestinal wall, sparse arrangement of villi, and lower villi height. High-throughput 16s rRNA sequencing revealed that *try* knockout reduced the diversity of intestinal microbes. These findings demonstrated that *try* was indispensable for medaka larvae because it continuously affects their growth, nitrogen metabolism, and intestinal development.

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Key words: *try*; *amy*; Japanese medaka; knockout; feeding; digestion

1 Introduction

Digestion is an important physiological process which directly influences the availability of nutrients required for biological activity in animals; thus the study of digestive physiology has attracted global attention (Gisbert 6et al., 2009; Ma et al., 2014; Nazemroaya et al., 2015; Navarro-Guillén et al., 2015). Digestion processes in fish are poorly understood in comparison to those in mammals, but digestive physiology is critical in fish (Rønnestad et al., 2013; Gisbert et al., 2014). Unlike infant mammals, most fish larvae have a very rudimentary digestive system when they hatch, so their digestive capacity is limited. The types and functions of digestive enzymes largely determine the efficiency of the whole digestive process (Rønnestad et al., 2013; Yúfera et al., 2018; Ueberschär et al., 2018; Aakanchhaa et al., 2020).

✉ Xufang LIANG, xufang_liang@hotmail.com
* The two authors contributed equally to the work
Xufang LIANG, https://orcid.org/0000-0002-6010-3066

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Fish larvae are extremely sensitive to environmental factors when they shift from endogenous to exogenous feeding, and the type and activity of digestive enzymes are crucial in determining digestive capacity, which ultimately affects larval growth performance (Yang et al., 2010; Anderson et al., 2018; Castro-Ruiz et al., 2019). Therefore, fish larvae require suitable, highly efficient digestive enzymes which are able to process the amount of ingested food needed to support rapid growth and development (Yúfera et al., 2018). Knowledge of the digestive enzymes and related genes of fish during the early stages is an invaluable tool for optimizing feeding protocols and increasing seed production, while enhancing the larval survival rate and growth performance (Lazo et al., 2011; Tong et al., 2012; Moguel-Hernández et al., 2016; Khoa et al., 2019; Khoa et al., 2020).

For agastric fish and fish larvae with functional stomachs that are not fully developed, the main enzymes for protein digestion are alkaline proteases in the pancreas and intestine, including trypsin and chymotrypsin (Infante and Cahu 2001; Chong et al 2002; Cara et al 2003; Cahu et al 2004). Trypsin can not only decompose protein components in food, but also rapidly activate chymotrypsinogen secreted by tissues such as pancreas, thus playing an important role in fish protein digestion (Ahsan et al 2001; Darias et al 2007; Halangk et al 2002; Mata-Sotres et al., 2016 a; Hernández-López et al., 2021; Rønnestad et al., 2013; Shen et al., 2018; Srichanun et al., 2013). Amylase is another important digestive enzyme which can catalyze the hydrolysis of starch into smaller carbohydrate molecules, but the function of amylase during early development stages is not completely understood in fish (Srichanun et al., 2013; Mata-Sotres et al., 2016 b; Mir et al., 2019). Up to now, studies on trypsin and amylase have focused on the ontogeny of development and compared gene expression and activity between several enzymes (Lazo et al., 2007; Ma et al., 2012; Rønnestad et al., 2013; Mata-Sotres et al., 2016a; Yúfera et al., 2018; Hernández-López et al., 2021; Khoa et al., 2021a). However, these traditional approaches lacked convincing evidence to illustrate the full function of trypsin and amylase (Srichanun et al., 2013; Khoa et al., 2019).

Recently, molecular biological tools have provided complementary and essential insights into the structure and function of digestive-enzyme genes (Moguel-Hernández et al., 2016; Yúfera et al., 2018; Khoa et al., 2019). The results they provide suggest that synthesis of digestive enzymes is genetically pre-programmed and appears before exogenous feeding in fish larvae (Lazo et al., 2011; Nazemroaya et al., 2015; Pérez-Sirkin et al., 2020). Therefore, in this study, we used the Japanese medaka (*Oryzias latipes*), a useful model organism which has a well-described genome, to explore the function and necessity of trypsin and amylase in fish after the trypsin gene (*try*) and amylase gene (*amy*) were knocked out with clustered regularly interspaced short palindromic repeat (CRISPR)-CRISPR associated 9 (Cas9) gene editing.

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2 Materials and methods

2.1 Larval rearing

Japanese medaka were obtained from the Institute of Hydrobiology of the Chinese Academy of Sciences (Wuhan, China). All medaka were kept in 5-L cuboid tanks at 28.0 ± 1.0 °C and the photoperiod of the fish room was controlled, with 14 h of light and 10 h of dark. Embryos were obtained by natural spawning and incubated with aerated oxygen water. Medaka were fed with brine shrimp (*Artemia nauplii*) from 4 days post -hatching (dph), at least twice a day.

2.2 Method of CRISPR/Cas9 gene editing and generation of mutant medaka

The method of CRISPR/Cas9 gene editing was determined based on that described by Cai et al. (2021). We designed sgRNAs for the Japanese medaka *try* gene (ENSORLG00000013582) and *amy* gene (ENSORLG00000020006) using a CRISPR design tool (http://www.e-crisp.org/E-CRISP/). The *try* gene is also called the *prss1* gene. In humans, PRSS1 is the main digestive enzyme secreted by the pancreas (Németh and Sahin-Tóth, 2014; Athwal et al., 2014; Zou et al., 2022). In Japanese medaka, we found that the *prss1* gene was the only orthologous gene with human *PRSS1*. Two targets located in the second exon were designed for the *try*

gene, while one target located in the fourth exon was designed for the *amy* gene. The corresponding sgRNAs and detection primers are displayed in Table S1. We predicted protein domains using Conserved Domain Database in NCBI (https://www.ncbi.nlm.nih.gov/cdd). Amino-acid sequence was aligned using CLC Sequence Viewer 6 (CLC bio, Aarhus, Denmark) (Jesus et al., 2017)

2.3 Feeding experiments on medaka larvae

Twenty WT, *try*$^{-/-}$, and *amy*$^{-/-}$ medaka larvae (4 dph) were kept in separate 300 mL tanks. Larvae were fed with abundant brine shrimp, and 0.5 h after feeding, they were anesthetized with MS-222 (Argent Chemical Laboratories, Redmond, WA, USA) and fixed in 4 % paraformaldehyde (PFA) overnight at 4°C. The specimens were then observed with a stereomicroscope. The same experiment was performed 3 times, and the proportion of feeding and food intake were tracked.

2.4 Growth performance and survival rate

Twenty WT, *try*$^{-/-}$ and *amy*$^{-/-}$ medaka larvae were randomly selected and kept in separate 300 mL tanks. Larvae were fed with abundant brine shrimp twice daily from 4 dph. The total length and survival rate of different genotypes larvae were counted at 15 dph.

2.5 Enzyme assay

The activity of trypsin and amylase in the medaka larvae were determined using kits (Nanjing Jiancheng Bioengineering Institute, trypsin kit, No. A080-2, and amylase kit, No. C016). Activity of ALT and AST in the liver and serum was measured using assay kits (Alanine Aminotransferase Assay Kit, No. C009-2-1 and Aspartate Aminotransferase Assay Kit, No. C010-2-1, Nanjing, China) purchased from Nanjing Jiancheng Bioengineering Institute. The protein content in liver tissue was determined using a Protein Quantitative Assay Kit (No. A045-2, Nanjing, China). All enzyme-activity measurements were completed within 24 h after sacrifice.

2.6 Histological analysis

The medaka larvae, liver, and intestinal tissues preserved in 4% paraformaldehyde were dehydrated, cleared, immersed in wax, and embedded to make paraffin blocks. Next, paraffin sections were prepared using a microtome. The sections were stained with hematoxylin and eosin (H&E) at Wuhan Servicebio Technology Co., Ltd. (Wuhan, China), then examined under a light microscope; and the images were analyzed and annotated with CaseViewer software (Zhang et al 2022).

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2.7 Gene- expression analysis

According to the manufacturer's instructions, we used TRIzol reagent (Takara, Japan) to extract the medaka total RNA of brain and intestinal tissues. Then 1μg RNA was reverse-transcribed into cDNA using the Evo M-MLV RT Mix Kit with gDNA Clean for qPCR Ver.2, AG11728 (Accurate Biotechnology Co., Ltd, Hunan, China) according to the manufacturer's instructions. RT-qPCR was performed with the SYBR Green Premix Pro Taq HS qPCR Kit II AG11702 (Accurate Biotechnology Co., Ltd, Hunan, China). The primers used for qRT-PCR are presented in Table S2. We quantified the mRNA expression levels of target genes relative to the expression of β-actin using the optimized comparative Ct ($2^{-\Delta\Delta Ct}$) value method (Livak and Schmittgen, 2001).

2.8 Gut- microbiota analysis

The QIAamp DNA Mini Kit (Qiagen, Valencia, CA, United States) was used to extract the total DNA of medaka entire gut tissues. Based on the Illumina NovaSeq 6000 sequencing platform (Illumina, San Diego, USA) at Tsingke Biotechnology Co., Ltd., we constructed paired-end libraries for sequencing. Raw reads were deposited into the NCBI Sequence Read Archive (SRA) database. 97% of sequence similarity was identified as the same operational taxonomic units (OTUs). We performed alpha diversity analysis based on Ace, Chao1, PD_whole_tree, and Simpson indexes to reflect the richness and species diversity of gut microbiota. QIIME software was used to generate species abundance tables at different taxonomic levels, and then R language tools were used to draw community structure maps at each taxonomic level of a sample. LDA Effect Size (LefSe) analysis was used to analyze significant difference in gut microbiota.

2.9 Statistical analysis

All data are reported as the mean ± S.E.M (standard error of the mean). All analyses were conducted using

IBM SPSS Statistics 25.0 (IBM, Armonk, NY, USA). We tested statistical differences between two groups using the independent samples *t*-test, and considered a value of $p < 0.05$ to be statistically significant. The data from three medaka larva genotypes were analyzed by one-way ANOVA applied Tukey's HSD (Honestly Significant Difference) test at $p = 0.05$ significance level. All statistical analyses were performed using GraphPad Prism version 8.02 for Windows (GraphPad Software, San Diego CA, USA).

3 Results

3.1 Generation of homozygous try and amy knockout medaka

The genomic target sites of *try* and *amy* were located in exon 2 and exon 4, respectively (Figs. 1a1 and 1b1). After microinjection and generation, we successfully obtained *try* and *amy* mutants. We found that in the *try* mutant line with a fragment of 21 base-pair deletion in the first target site, 3 base-pair insertion, and 1 base-pair deletion in the second target site, the second target site resulted in a pre-stop codon (Fig. 1a1). The *amy* mutant line with a fragment of 10 base-pair deletion in the target site resulted in a pre-stop codon (Fig. 1b1).

Compared with WT medaka, in $try^{-/-}$ medaka insertion and deletion induced truncated protein that had lost the greater part of the trypsin-like serine protease region (Tryp_SPc) (Fig. 1a2). The trypsin of $try^{-/-}$ medaka only comprised 72 amino acids and the amino acids of the catalytic triad (His62, Asp106, and Ser199); the putative start of the mature peptide (amino acid residues 23-26: IVGG) was absent in $try^{-/-}$ medaka (Figs. 1a2 and 1a3). In addition, the deletion in $amy^{-/-}$ medaka induced truncated protein that had lost the part of alpha amylase catalytic domain (AmyAc_bac_euk_AmyA) (Fig. 1b2). The amylase of $amy^{-/-}$ medaka only comprised 194 amino acids. Amino acids of the catalytic triad (Asp212, Glu248 and Asp315) were also absent in $amy^{-/-}$ medaka (Figs. 1b2 and 1b3).

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Fig. 1 Generation of homozygous mutants with the CRISPR/Cas9 system.

(a) Establishment of medaka *try* mutant line. (a1) The two genomic target sites of CRISPR located in exon 2, and sequencing results of WT medaka and homozygous mutants at two targets, showing 21 base-pair deletion in the first target site, 3 base-pair insertion, and 1 base-pair deletion in the second target site. (a2) The trypsin-like serine protease region (Tryp_SPc) was disrupted

in the predicted truncated trypsin mutant protein. (a3) Amino-acid sequence alignments of trypsin for WT and *try*$^{-/-}$ medaka. Active-site triad residues His62, Asp106. and Ser199 (marked with red) were absent in *try*$^{-/-}$ medaka, and the putative start of the mature peptide (residues 23-26: IVGG) (marked with green box) was disrupted. (b) Establishment of medaka *amy* mutant line. (b1) The genomic target site of CRISPR located in exon 4, and sequencing results of WT medaka and homozygous mutants at this target, showing 10 base-pair deletion in the target site. (b2) The alpha amylase catalytic domain (AmyAc_bac_euk_AmyA) was disrupted in the predicted truncated mutant amylase protein. (b3) Amino-acid sequence alignments of trypsins for WT and *amy*$^{-/-}$ medaka. Active-site triad residues Asp212, Glu248, and Asp315 (marked with blue) were absent.

3.2 First -feeding and digestion of medaka larvae

At 4 dph, the larvae were fed brine shrimp for the first time. Compared with WT medaka larvae, the *try*$^{-/-}$ medaka larvae performed low proportion of feeding; after 8 h digestion, the incompletely digested brine shrimp was observed in *try*$^{-/-}$ medaka larvae digestive tracts, and expression of *npy* had significantly decreased (Fig. 2a, 2c, 2e). The proportion of feeding, digestive capacity, and expression of *npy* in *amy*$^{-/-}$ medaka larvae showed no significant differences with WT medaka larvae (Fig. 2a-c, e). Moreover, expression of *pomc* was not significantly different between the three genotypes (Fig. 2d).

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Figure 2. First feeding and digestion of the three genotypes of medaka larvae in 4 dph larvae. (a) The proportion of feeding in the three genotypes compared. (b) Quantification of food intake of the three genotypes. (c-d) Expression of *npy* and *pomc* in the three genotypes. (e) Images of 4 dph larvae fed brine shrimp after 0.5 h and then starved for 8 hours. Red arrow indicates incompletely digested brine shrimp. The results are expressed as mean ± SEM (n=6). Different superscript letters indicate significant differences between the genotypes, $P < 0.05$. Scale bar = 1000 μm.

3.3 Histological analysis of the digestive tract in the three genotypes of medaka larvae

Microphotographs of the gut of the 15 dph medaka larvae showed significant alterations of the intestinal villi between the different genotypes (Fig. 3). One can see that compared with WT medaka larvae (Fig. 4a), *try*$^{-/-}$ medaka larvae had a much lower height of the intestinal villi (Fig. 4b, 4d), but no significant change was observed in *amy*$^{-/-}$ medaka larvae (Fig. 4c-d).

Figure 3. Histological analysis of the digestive tract in 15 dph medaka larvae. (a-c) Microphotographs of the digestive tract and intestinal villi in WT, *try*$^{-/-}$, and *amy*$^{-/-}$ medaka larvae. Height of intestinal villi is marked with double yellow arrows. (d) Statistical analysis of the height of intestinal villi in WT, *try*$^{-/-}$, and *amy*$^{-/-}$ medaka larvae. The results are expressed as mean ± SEM (n=6). Different superscript letters indicate significant differences between the genotypes (a < b < c, P < 0.05). Scale bar: 100 μm.

3.4 Digestive capacity, growth performance, and survival rate in 15 dph medaka larvae

There was a large quantity of incompletely digested brine shrimp in 15 dph *try*$^{-/-}$ medaka larval digestive tracts after 8 h digestion. In contrast, only a small amount was observed in 15 dph *amy*$^{-/-}$ medaka larvae (Fig. 4a). Compared with WT medaka larvae, *try*$^{-/-}$ medaka larvae had much shorter total length. The *amy*$^{-/-}$ medaka larvae showed a decreasing trend at 15 dph but no statistical differences (Fig. 4b). In addition, expression of *gh* and *igf1* significantly decreased in *try*$^{-/-}$ medaka larvae, but increased significantly in *amy*$^{-/-}$ medaka larvae (Fig. 4d-e). However, the survival rates were not statistically different between the three genotypes (Fig. 4c).

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Figure 4. Analysis of digestive capacity, total length, survival rate, and expression of growth-performance-related genes in 15 dph medaka larvae. (a) 15-dph larvae fed brine shrimp after 0.5 h and then starved for 8 hours. Red arrows indicate incompletely digested brine shrimp. (b) Total length of WT, *try*$^{-/-}$, and *amy*$^{-/-}$ medaka larvae. (c) Survival rate of WT, *try*$^{-/-}$, and *amy*$^{-/-}$ medaka larvae. (d-e) Expression of *gh* and *igf* in WT, *try*$^{-/-}$, and *amy*$^{-/-}$ medaka larvae. The results are expressed as mean ± SEM (n=6). Different superscript letters indicate significant differences between the genotypes (a < b < c, P < 0.05). Scale bar: 1000 μm.

3.5 Growth performance of *try* $^{-/-}$ adult medaka

Our previous description showed that the digestive capacity of *try*$^{-/-}$ medaka larvae to brine shrimp was significantly reduced, and so were the total length and intestinal-villi height of the larvae. Although 15dph *amy*$^{-/-}$ medaka larvae showed a slight decrease in their ability to digest brine shrimp, there was no significant difference in digestive-tract development or total length between *amy*$^{-/-}$ medaka larvae and WT medaka larvae. Therefore, we next focused on the effects of *try* knockout on the growth, nitrogen metabolism, and gut microbiota of adult medaka.

We found that total length (Fig. 5a) and body weight (Fig. 5b) of *try*$^{-/-}$ adult medaka decreased significantly compared with the WT.

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Figure 5. Total length and body weight of medaka at 4 months of age. (A): total length. (B) body weight. (WT, n=24; *try*$^{-/-}$ medaka, n=29) The results are expressed as mean ± SEM, $^{**}P < 0.01$.

3.6 Genes- expression level of *try* $^{-/-}$ adult medaka

We detected expression levels of the appetite-stimulating genes *npya* and *agrp* in the brain, and of *ctra*, *ctrb*, and *mtor* in the intestine, using RT-qPCR. The results showed that expression of *npya* and *agrp* were much lower in *try*$^{-/-}$ adult medaka compared with the WT (Fig. 6a-b). Similarly, expression levels of *ctra*、*ctrb*, and *mtor* in the intestine of *try*$^{-/-}$ adult medaka were significantly lower than those of the WT (Fig. 6c-e).

Figure 6. Gene-expression level of *try*$^{-/-}$ adult medaka. Quantitative analysis of *npya* (a), *agrp* (b), *ctra* (c), *ctrb* (d), and *mtor* (e) gene mRNA expression levels. mRNA levels of *try*$^{-/-}$ adult medaka are expressed as values relative to WT in each gene analysis, n=6. Results are shown as means ± SEM. **P*< 0.05, ***P* < 0.01, vs. WT.

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3.7 Enzyme activity of *try* $^{-/-}$ adult medaka

The results of AST and ALT enzyme activity in the blood and liver are shown in Fig. 3. In liver tissue, there was no significant difference in AST and ALT activity between WT and *try*$^{-/-}$ adult medaka (Fig. 7a-b). The enzyme activity of AST and ALT activity in the blood of *try*$^{-/-}$ adult medaka was significantly increased compared with the WT (Fig. 7c-d).

Figure 7. Nitrogen-metabolizing enzyme activity in the liver and blood of try $^{-/-}$ adult medaka. (a) AST enzyme activity in the liver. (b) ALT enzyme activity in the liver. (c) AST enzyme activity in blood. (d) ALT enzyme activity in blood. Results are shown as means ± SEM. n=6, **P*<0.05, vs. WT, *n.s*, not significant.

3.8 Analysis of intestinal and liver- tissue histology in *try* $^{-/-}$ adult medaka

Compared with the WT, the intestinal wall of *try*$^{-/-}$ adult medaka was thinner, and the villi were sparsely arranged and lower in height. The red arrows in Fig. 8 show where the striatum was damaged and imperfect (Fig. 8a). However, in the *try*$^{-/-}$ group, cells showed vacuolar degeneration (as indicated by the arrow). Cells were also significantly enlarged, the cytoplasm was blank, and the nucleus was squeezed to one side (Fig. 8b).

Figure 8. Analysis of intestinal and liver tissue histology in *try*$^{-/-}$ adult medaka (a) H&E staining of intestine, scale bar: 50 μm. Black double arrow indicates intestinal-wall thickness and yellow arrow indicates striatum. (b) H&E staining of liver. Scale bar: 20 μm.

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3.9 Gut microbiota diversity in *try* $^{-/-}$ adult medaka

High-throughput 16s rRNA sequencing was performed on the intestinal tissues of *try*$^{-/-}$ and WT adult medaka. A Venn diagram of OUTs shows that 316 OUTs were shared by the two groups, 3,575 OUTs specific to the KO group and 2,042 OUTs specific to the WT group (Fig. 9a). The species richness and species diversity of gut microbiota in the medaka were analyzed in terms of alpha diversity. WT and *try*$^{-/-}$ adult medaka, as assessed by the ACE index, Chao1 index, and PD_whole_tree index, had no significant differences (Fig. 9b-d). However, compared with the WT Simpson index, the KO medaka index rose clearly (Fig. 9e), indicating that KO had no significant effect on the species richness of intestinal flora, but led to an increase in species diversity.

Figure 9. Effects of *try* knockout on intestinal microbial diversity of medaka. (a) OUT Venn diagram. (b) ACE index. (c) Chao1 index; (d) PD_whole_tree index. (e) Simpson index. Results are shown as means ± SEM. n=4, $P<0.05$.

3.10 Intestinal microbial composition in *try* $^{-/-}$ adult medaka

Based on relative abundance, *try* knockout significantly affected the dominant flora in medaka intestine. At

the phylum level (Fig. 10a), the dominant flora for *try*$^{-/-}$ adult medaka was Firmicutes, followed by Proteobateria. At the genus level (Fig. 10b), although the dominant flora of WT and *try*$^{-/-}$ adult medaka were Thauera and Calorithrix, in contrast the proportion of dominant intestinal flora of *try*$^{-/-}$ adult medaka in the overall flora was significantly reduced. An LDA value distribution histogram showed that 25 species were significantly different, and *try*$^{-/-}$ medaka had changes in 10 groups, including p_Firmicutes、c_Clostridia、o_Oscillospirales, and seven other groups (Fig. 10c). An evolutionary branching diagram showed that Proteobacteria and Calditrichota played a major role in the WT, while Firmicutes played a major role in *try*$^{-/-}$ adult medaka (Fig. 10d).

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Figure 10. Relative abundance of intestinal microorganisms in medaka at the phylum and genus levels. (a): Relative abundance at phylum level; (b): Relative abundance at genus level; (c): LDA analysis columnar graph; (d): Evolutionary branching diagram. Results are shown as means ± SEM. n=4.

4 Discussion

The early stage is an important period in animal development, and one of the principal factors influencing survival of larvae is the effective digestion of their diet. The efficiency of digestion depends on the type and function of the digestive enzymes (Kolkovski, 2001; Cahu et al., 2004; Lazo et al., 2007; Ma et al., 2014; Ghasemi et al., 2020). Trypsin and amylase are two important digestive enzymes, and reports about these two enzymes are focused on the ontogeny of development and comparison (Rønnestad et al., 2013; Khoa et al., 2019; Hernández-López et al., 2021). Convincing evidence to support the importance of trypsin and amylase in fish is still lacking. This is the first study to generate digestive enzymes in knockout fish. Using the genome-editing system CRISPR/Cas9, we disrupted *try* and *amy* in the medaka.

In this study, *try*$^{-/-}$ medaka larvae showed significantly low digestive capacity at 4 and 15 dph. We observed incompletely digested brine shrimp in *try*$^{-/-}$ medaka larval digestive tracts after 8 h digestion (Fig. 2, 4). This result demonstrates that *try* really plays the key role in food digestion. Other studies also suggest that trypsin is the main digestive enzyme during the early development of the piscine digestive system, so it can be used as an excellent indicator of protein digestion in larvae before the development of the functional stomach and pepsin activity (Nazemroaya et al., 2015; Mir et al., 2018; Hernández-López et al., 2021; Khoa et al., 2021 a). Amylase is another important digestive enzyme in fish, which participates in the first stages of starch digestion (Kortner et al., 2011; Murashita et al., 2014; Mata-Sotres et al., 2016 b; Mir et al., 2019; Pérez-Sirkin et al., 2020). Unexpectedly, compared with the WT medaka larvae, *amy*$^{-/-}$ medaka larvae show normal digestive capacity at 4 dph. Previous studies proved that trypsin has a strong capacity to digest brine shrimp (Arndt et al., 2015), and our study suggests that *amy*$^{-/-}$ medaka may digest brine shrimp by trypsin at 4dph.

The length of intestinal villi significantly decreased in 15 dph *try*$^{-/-}$ medaka larvae (Fig. 3), which might be related to low trypsin activity. A previous study also demonstrated that low digestive capacity could decrease the length of intestinal villi in longsnout seahorse *Hippocampus reidi* larvae (De Souza et al., 2020). Furthermore, low digestive capacity is also reflected in larval growth (Wang and Gu, 2010; Karasov and Douglas, 2013; Zokaeifar et al., 2012; De Souza et al., 2020). Results in this study show that the total body length of 15 dph *try*$^{-/-}$ larvae is significantly shorter, which might be related to the lack of nutrition due to low digestive capacity Fish larvae obviously need sufficient nutrition to support their growth (Hamre et al., 2013; Gisbert et al., 2022). This study indicates that low digestive capacity and incomplete intestinal villi might influence the obtainment of essential nutrients by *try*$^{-/-}$ medaka larvae, limiting larval development and growth. *gh* and *igf1* have been considered as possible markers for evaluation of growth rate in fish (Pérez-Sánchez et al., 2018; Khoa et al., 2021 b). The decrease in expression of *gh* and *igf* in fish might also be due to low digestive capacity (Zhou et al., 2015; Butt and Volkoff, 2019; Khoa et al., 2020). In addition, our study shows that expression of *gh* and *igf* decreases markedly in 15 dph *try*$^{-/-}$ larvae. This indicates that the absence of *try* not only influences larval digestive capacity, but also whole-larva development. However in 15 dph *amy*$^{-/-}$ larvae, the intestinal villi and total length are no different from those of WT larvae, while expression of *gh* and *igf* are significantly increased. It could be that larvae increase expression of *gh* and *igf* to support faster growth.

Based on our study on medaka larvae, *try* plays a more important role in food digestion than *amy*, and lack of *try* leads to more food-digestion problems. Therefore, we went on to investigate the effects of *try* knockout on the growth, nitrogen metabolism, and gut microbes of adult medaka. The total length and body weight of 4-month-old *try*$^{-/-}$ medaka are shorter and lower compared with the WT, and expression levels of *npy* and *agrp* genes are significantly reduced in the brain tissue of these larvae, suggesting that a deficiency of *try* may continuously reduce the feeding intake of *try*$^{-/-}$ medaka and thus affect their growth and development.

Chymotrypsin, a digestive enzyme, is present in the pancreatic tissue and intestine of vertebrates. There are only two types of chymotrypsin in fish, namely *ctra* and *ctrb* (Zhou et al 2011). Chymotrypsin activity is influenced by many factors, including trypsin concentration, growth and development stage, feeding, and nutritional status of fish (Zhou et al 2011; Seo et al 2022). In the present study, mRNA expression of *ctra* and *ctrb* genes in the intestine of *try*$^{-/-}$ medaka decreased significantly compared with that in the WT. Studies have shown that chymotrypsin plays a major role in growth restriction or inhibition in fish (Rungruangsak-Torrissen et al 2006). Normally, chymotrypsin activation is regulated by trypsin (Ahsan et al 2001; Darias et al 2007).

The liver is an important location of protein metabolism, and the activity of transaminase in liver can reflect the state of protein metabolism (Li et al 2013). AST and ALT are the two most important transaminases involved in amino-acid metabolism in fish liver. They play an important role in the synthesis of non-essential amino acids and protein catabolism (Zhao et al 2012). In this study, medaka *try*$^{-/-}$ resulted in a significant increase in AST and ALT activity in serum. Under normal conditions, serum transaminase activity is low, but when fish are affected by adverse environmental stress or malnutrition, especially liver damage, it often leads to large amounts of transaminase from liver cells being released into the blood, resulting in a significant increase in AST and ALT activity in serum (Sun et al 2013; Cai et al 2017). H&E staining of *try*$^{-/-}$ medaka liver tissue showed vacuolar degeneration of hepatocytes, significantly enlarged cells, blank cytoplasm, and nuclei squeezed to one side. The structure of healthy fish liver is uniform, and the liver cells are arranged regularly and tightly, unlike the damaged liver (Matsumoto et al 2010; Topić Popović et al 2023). Therefore, this study suggests that *try* deficiency may cause liver-cell damage, thereby affecting the normal physiological function of the liver.

Protein synthesis in intestinal tissue is an important basis for fish growth and development (Jiang et al 2009; Zhao et al 2012). The ability of fish to digest and absorb food is largely determined by the development of the digestive organs. In addition, translation initiation is a rate-limiting step in protein synthesis, and the mTOR signaling pathway can regulate translation initiation and act as a regulatory center for intestinal protein synthesis (Feng et al 2013). RT-qPCR showed that *try*$^{-/-}$ medaka significantly reduced expression of the *mtor* gene in medaka intestine, suggesting that *try*$^{-/-}$ medaka had adverse effects on development of the intestine. Studies have

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shown that reduced protein deposition may affect the growth and development of intestinal tissues to some extent (Zhang et al 2013). The integrity of vertebrate intestinal structure is an important basis for intestinal health and normal development (Zhang et al 2013; Ullah et al 2022). Observation of the intestinal tissue section showed that the intestinal wall was thinner, the intestinal villi were sparsely arranged, they were shorter, the microvilli were less numerous, and the striatum margin was damaged and incomplete.

We further investigated the effect of *try*$^{-/-}$ medaka on the gut microbiota of adult medaka using 16s rRNA high-throughput sequencing. The gut is a complex system, with obligate symbiosis and facultative parasitism between the microbes in the gut and the host, which play an indispensable role in growth and development, nutritional metabolism, immune function, and resistance to the invasion of pathogens (Wang et al 2018). Microbial interactions in the gut are dynamic, not static. Studies have shown that factors such as environment, nutrient levels, and growth stage can affect the intestinal microbial composition of fish (Roeselers et al 2011; Blaut 2015). Here, Alpha Diversity analysis showed that the diversity of gut microbiota is significantly reduced in *try*$^{-/-}$ medaka. This suggests that damage to the intestinal structure will affect the colonization of intestinal flora and then lead to a reduction in diversity. At the phylum level, although Proteobacteria and Firmicutes are the dominant phyla of *try*$^{-/-}$ and WT medaka, they have different composition ratios. Compared with the WT, the proportion of Proteobacteria in *try*$^{-/-}$ medaka is lower, while that of Firmicutes is higher. Studies have shown that the intestinal flora of fish mainly consists of Proteobacteria, Firmicutes, actinobacteria, and Bacteroidetes, and Proteobacteria is the most important flora (Kormas et al 2014). Firmicutes can effectively obtain energy from substrates, resulting in increased fat content. A large number of studies have shown that increased Firmicutes abundance is related to body weight and fat deposition (Fujisawa et al 2019; Zhang et al 2021). Therefore, changes in the diversity of *try*$^{-/-}$ medaka intestinal flora may affect amino-acid-metabolism and lipid-metabolism pathways, and thus affect the growth and development of fish.

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5 Conclusion

In conclusion, in this study we established *try* and *amy* knockout medaka using CRISPR/Cas9 system. Our results demonstrate that *try* is indispensable for medaka larvae and also continuously affects the growth, nitrogen metabolism, and intestinal development of medaka.

Availability of data and materials

All data and materials are available from the corresponding author by request.

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Author contributions

Xiaodan JIA: drafted the manuscript, designed the experiment and performed the experiment. **Shulin TANG:** drafted the manuscript, designed the experiment and participated in some experiments. **Hexiong FENG:** carried out the gene knockout. **Dimei XU:** participated in some experiments. **Chenyuan ZHU:** participated in some experiments. **Ke LU:** Check and revised the manuscript. **Xufang LIANG:** designed the experiment and checked the manuscript. All authors read and approved the final manuscript.

Compliance with ethics guidelines

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

All experimental procedures involving chickens were ap- proved by the Institutional Animal Care and Use Committee of Huazhong Agricultural University (approval number: HZAUFI-2020-0042).

References

Aakanchhaa, A. K., Guptac, R. K., Kumard, A., Ranad, S., Kumarie, K., and Chande, G. B. (2020). Genetic divergence and phylogenetic analysis of fish digestive enzymes in carnivores, herbivores and omnivores. *Anim. Sci.* 15, 21-28.

Ahsan, M., Funabara, D., Watabe, S. (2001). Molecular cloning and characterization of two isoforms of trypsinogen from anchovy pyloric ceca. Marine biotechnology. 3(1), 80-90.

Anderson, K., Kuo, C. Y., Lu, M. W., Bar, I., and Elizur, A. (2018). A transcriptomic investigation of digestive processes in orange-spotted grouper, *Epinephelus coioides*, before, during, and after metamorphic development. *Gene* 661, 95-108.

Arndt, C., Sommer, U., and Ueberschär, B. (2015). A comparative in-vitro-test on the digestibility of live prey for fish larvae under specific consideration of trypsin. *Aquaculture* 446, 12-16.

Athwal, T., Huang, W., Mukherjee, R., Latawiec, D., Chvanov, M., Clarke, R., et al. (2014). Expression of human cationic trypsinogen (PRSS1) in murine acinar cells promotes pancreatitis and apoptotic cell death. *Cell Death Dis.* 5, e1165-e1165.

Blaut, M. (2015). Gut microbiota and energy balance: role in obesity. *Proceedings of the Nutrition Society*. 74(3), 227-234.

Butt, R. L., and Volkoff, H. (2019). Gut microbiota and energy homeostasis in fish. Front. Endocrinol. 9.

Cahu, C. L., Ronnestad, I., Grangier, V., Zambonino-Infante, J. L. (2004). Expression and activities of pancreatic enzymes in developing sea bass larvae (*Dicentrarchus labrax*) in relation to intact and hydrolysed dietary protein; involvement of cholecystokinin. *Aquaculture* 238, 295-308.

Cai, G., Mo, C., Huang, L., Li, J., and Wang, Y. (2015). Characterization of the two CART genes (CART1 and CART2) in chickens (*Gallus gallus*). *PLoS One* 10, e0127107.

Cai, W.C., Li, X.F., Jiang, G.X., Liu, S.Q., Liu, W.B. (2017). Effects of fish meal replacement by rice protein concentrate on growth，intestinal digestive and absorptive capability and amino acid metabolismof blunt snout bream (*Megalobrama amblycephala*). Journal of Nanjing Agricultural University. 40(3), 529-538.

Cai, W. J., Li, J., Li, L., Chen, X., Wei, J. R., Yin, Z., et al. (2021). Knockout of t1r1 gene in zebrafish (*Danio rerio*) by CRISPR/Cas9 reveals its roles in regulating feeding behavior. *Aquaculture* 545, 737189.

Castro-Ruiz, D., Mozanzadeh, M. T., Fernández-Méndez, C., Andree, K. B., García-Dávila, C., Cahu, C., et al. (2019). Ontogeny of the digestive enzyme activity of the Amazonian pimelodid catfish *Pseudoplatystoma punctifer* (Castelnau, 1855). *Aquaculture* 504, 210-218.

Chong, A., Hashim, R., Lee, L. C., Ali, A.B. (2002). Characterization of protease activity in developing discus *Symphysodon aequifasciata* larva. *Aquaculture Research*. 33(9), 663-672

Cara, J.B., Moyano, F.J., Cárdenas, S., Fernández‐Díaz, C., Yúfera, M. (2003). Assessment of digestive enzyme activities during larval development of white bream. Journal of Fish Biology. 63(1), 48-58.

Darias, M.J., Murray, H.M., Gallant, J.W., Douglas, S.E., Yúfera, M., Martínez-Rodríguez, G. (2007). The spatiotemporal expression pattern of trypsinogen and bile salt-activated lipase during the larval development of red porgy (*Pagrus pagrus, Pisces, Sparidae*). *Marine Biology*. 152(1), 109-118.

De Souza, A. P. L., Ferreira, T. H., Mouriño, J. L. P., Martins, M. L., Magenta Magalhaes, A. R., Tsuzuki, M. Y. (2020). Use of Artemia supplemented with exogenous digestive enzymes as sole live food increased survival and growth during the larviculture of the longsnout seahorse *Hippocampus reidi*. *Aquacult. Nutr.* 26, 964-977.

Feng, L., Peng, Y., Wu, P., Hu, K., Jiang, W.D., Liu, Y., Zhou, X.Q. (2013). Threonine affects intestinal function, protein synthesis and gene expression of TOR in Jian carp (*Cyprinus carpio var. Jian*). *PloS one*. 8(7), e69974.

Fujisawa, K., Takami, T., Nagatomo, T., Fukui, Y., Hoshida, H., Saeki, I., Sakaida, I. (2019). Usefulness of adult medaka fish as a model for the evaluation of alcoholic fatty liver. *Alcohol*. 77, 147-154.

Ghasemi, N., Imani, A., Noori, F., and Shahrooz, R. (2020). Ontogeny of digestive tract of stellate sturgeon (*Acipenser stellatus*) from hatching to juvenile stage: Digestive enzymes activity, stomach and proximal intestine. *Aquaculture* 519, 734751.

Gisbert E., Giménez G., Fernández I., Kotzamanis Y. and Estéveza A. (2009). Development of digestive enzymes in common dentex *Dentex dentex* during early ontogeny. *Aquaculture* 287, 381-387.

Gisbert, E., Luz, R. K., Fernández, I., Pradhan, P. K., Salhi, M., Mozanzadeh, M. T., et al. (2022). Development, nutrition, and rearing practices of relevant catfish species (Siluriformes) at early stages. *Rev. Aquacult.* 14, 73-105.

Gisbert, E., Moreira, C., Castro-Ruiz, D., Ozturk, S., Fernández, C., Gilles, S., et al. (2014). Histological development of the digestive system of the Amazonian pimelodid catfish *Pseudoplatystoma punctifer*. *Animal* 8, 1765-1776.

Halangk, W., Kruger, B., Ruthenburger, M., Sturzebecher, J., Albrecht, E., Lippert, H., Lerch, M.M. (2002). Trypsin activity is not involved in premature, intrapancreatic trypsinogen activation. *American Journal of Physiology-Gastrointestinal and Liver Physiology*. 282(2), G367-G374.

Hamre, K., Yúfera, M., Rønnestad, I., Boglione, C., Conceição, L. E., and Izquierdo, M. (2013). Fish larval nutrition and feed formulation: knowledge gaps and bottlenecks for advances in larval rearing. *Rev. Aquacult.* 5, S26-S58.

He, S., Li, L., Lv, L. Y., Cai, W. J., Dou, Y. Q., Li, J., et al. (2020). Mandarin fish (Sinipercidae) genomes provide insights into innate predatory feeding. *Comms. Biol.* 3, 1-13.

Unedited

Hernández-López, I. A., Ibarra-Castro, L., Álvarez-González, C. A., Martínez-Brown, J. M., Maytorena-Verdugo, C. I., and Peña-Marín, E. S. (2021). Characterization of digestive enzymes during early ontogeny of white Snook (*Centropomus viridis*). *Aquaculture* 535, 736399.

Infante, J.Z., Cahu, C.L. (2001). Ontogeny of the gastrointestinal tract of marine fish larvae. *Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology*, 130(4), 477-487.

Jesus, T. F., Moreno, J. M., Repolho, T., Athanasiadis, A., Rosa, R., Almeida-Val, V. M., et al. (2017). Protein analysis and gene expression indicate differential vulnerability of Iberian fish species under a climate change scenario. *PloS One* 12, e0181325.

Jiang, J., Zheng, T., Zhou, X.Q., Liu, Y., Feng, L. (2009). Influence of glutamine and vitamin E on growth and antioxidant capacity of fish enterocytes. *Aquaculture Nutrition*. 15(4), 409-414.

Karasov, W. H., and Douglas, A. E. (2013). Comparative digestive physiology. *Compr. Physiol.* 3, 741.

Khoa, T. N. D., Hayasaka, O., Matsui, H., Waqalevu, V., Honda, A., Nakajima, K., et al. (2021 a). Changes in early digestive tract morphology, enzyme expression and activity of Kawakawa tuna (*Euthynnus affinis*). *Aquaculture* 530, 735935.

Khoa, T. N. D., Waqalevu, V., Honda, A., Shiozaki, K., and Kotani, T. (2020). Comparative study on early digestive enzyme activity and expression in red sea bream (*Pagrus major*) fed on live feed and micro-diet. *Aquaculture* 519, 734721.

Khoa, T. N. D., Waqalevu, V., Honda, A., Shiozaki, K., and Kotani, T. (2021 b). An integrative description of the digestive system morphology and function of Japanese flounder (*Paralichthys olivaceus*) during early ontogenetic development. *Aquaculture* 531, 735855.

Khoa, T. N. D., Waqalevu, V., Honda, A., Shiozaki, K., Kotani, T. (2019). Early ontogenetic development, digestive enzymatic activity and gene expression in red sea bream (*Pagrus major*). *Aquaculture* 512, 734283.

Kolkovski, S. (2001). Digestive enzymes in fish larvae and juveniles-implications and applications to formulated diets. *Aquaculture* 200, 181-201.

Kormas, K.A., Meziti, A., Mente, E., Frentzos, A. (2014). Dietary differences are reflected on the gut prokaryotic community structure of wild and commercially reared sea bream (*Sparus aurata*). *Microbiologyopen.* 3(5), 718-728.

Kortner, T. M., Overrein, I., Oie, G., Kjorsvik, E., Bardal, T., Wold, P. A., et al. (2011). Molecular ontogenesis of digestive capability and associated endocrine control in Atlantic cod (*Gadus morhua*) larvae. *Comp. Biochem. Physiol. A* 160, 190-199.

Lazo J. P., Mendoza R., Holt G. J., Aguilerab C. and Arnoldc C. R. (2007). Characterization of digestive enzymes during larval development of red drum (*Sciaenops ocellatus*). *Aquaculture* 265, 194-205.

Lazo, J. P., Darias, M. J., Gisbert, E. (2011). "Ontogeny of the digestive tract," in *Larval Fish Nutrition*, ed G. J. Holt (Wiley-Blackwell, West Sussex), 3-46.

Li, Y., Li, J., Lu, J., Li, Z., Shi, S., Liu, Z. (2017). Effects of live and artificial feeds on the growth, digestion, immunity and intestinal microflora of mandarin fish hybrid (*Siniperca chuatsi*♀× *Siniperca scherzeri*♂). *Aquac. Res.* 48, 4479-4485.

Li, B., Liang, X.F., Liu, L.W., Yuan, X.C., Zhu, W.H., Li, J. (2013). Effects of dietary protein levels on growth, feed utilization and enzymes activity on nitrogen Metabolism of grass carp (*Ctenopharyngodon Idella*). Acta Hydrobiologica Sinica. 38(02),233-240.

Matsumoto, T., Terai, S., Oishi, T., Kuwashiro, S., Fujisawa, K., Yamamoto, N., Sakaida, I. (2010). Medaka as a model for human nonalcoholic steatohepatitis. *Disease models & mechanisms*, 3(7-8), 431-440.

Ma Z., Guo H., Zheng P., Wang L., Jiang S., Qin J. G. et al. (2014). Ontogenetic development of digestive functionality in golden pompano *Trachinotus ovatus* (Linnaeus 1758). *Fish Physiol. Biochem.* 40, 1157-1167.

Ma Z., Qin J. G., Nie Z. (2012). "Morphological Changes of Marine Fish Larvae and Their Nutrition Need," in *Larvae: Morphology, Biology and Life Cycle*, eds K. Pourali and V. N. Raad (Nova Science Publishers, Inc., New York, USA), 1- 20.

Mata-Sotres, J. A., Martos-Sitcha, J. A., Astola, A., Yúfera, M., Martínez-Rodríguez, G. (2016 b). Cloning and molecular ontogeny of digestive enzymes in fed and food-deprived developing gilthead seabream (*Sparus aurata*) larvae. *Comp. Biochem. Physiol. B* 191, 53-65.

Mata-Sotres, J. A., Moyano, F. J., Martínez-Rodríguez, G., and Yúfera, M. (2016 a). Daily rhythms of digestive enzyme activity and gene expression in gilthead seabream (*Sparus aurata*) during ontogeny. *Comp. Biochem. Physiol. A* 197, 43-51.

Mir, I. N., Bhat, I. A., Dar, S. A., Jain, K. K., Varghese, T., Kumari, R., et al. (2019). Expression of alpha-amylase and growth-related genes during early larval developmental stages of *Clarias magur*. *Aquaculture* 507, 69-74.

Mir, I. N., Srivastava, P., Bhat, I., Muralidhar, A., Varghese, T., Gireesh-Babu, P., et al. (2018). Expression and activity of trypsin and pepsin during larval development of Indian walking catfish (*Clarias magur*). *Aquaculture* 491, 266-272.

Moguel-Hernández, I., Peña, R., Andree, K.B., Tovar-Ramirez, D., Bonacic, K., Dumas, S., et al. (2016). Ontogeny changes and weaning effects in gene expression patterns of digestive enzymes and regulatory digestive factors in spotted rose snapper (*Lutjanus guttatus*) larvae. *Fish Physiol. Biochem.* 42, 1319-1334.

Murashita, K., and Kurokawa, T. (2011). Multiple cocaine-and amphetamine-regulated transcript (CART) genes in medaka, *Oryzias latipes*: cloning, tissue distribution and effect of starvation. *Gen. Comp. Endocr.* 170, 494-500.

Murashita, K., Matsunari, H., Kumon, K., Tanaka, Y., Shiozawa, S., Furuita, H., et al. (2014). Characterization and ontogenetic development of digestive enzymes in Pacific bluefin tuna *Thunnus orientalis* larvae. *Fish Physiol. Biochem.* 40, 1741-1755.

unedited

Navarro-Guillén, C., Moyano, F. J., and Yúfera, M. (2015). Diel food intake and digestive enzyme production patterns in *Solea senegalensis* larvae. *Aquaculture* 435, 33-42.

Nazemroaya, S., Yazdanparast, R., Nematollahi, M.A., Farahmand, H., Mirzadeh, Q. (2015). Ontogenetic development of digestive enzymes in Sobaity Sea bream *Sparidentex hasta* larvae under culture condition. *Aquaculture* 448, 545-551.

Németh, B. C., and Sahin-Tóth, M. (2014). Human cationic trypsinogen (PRSS1) variants and chronic pancreatitis. *Am. J. Physiol.-Gastr. L.* 306, 466-473.

Pérez-Sánchez, J., Simó-Mirabet, P., Naya-Català, F., Martos-Sitcha, J. A., Perera, E., Bermejo-Nogales, A., et al. (2018). Somatotropic axis regulation unravels the differential effects of nutritional and environmental factors in growth performance of marine farmed fishes. *Front. Endocrinol.* 9, 687.

Pérez-Sirkin, D. I., Solovyev, M., Delgadin, T. H., Herdman, J. E., Miranda, L. A., Somoza, G. M., et al. (2020). Digestive enzyme activities during pejerrey (*Odontesthes bonariensis*) ontogeny. *Aquaculture* 524, 735151.

Roeselers, G., Mittge, E.K., Stephens, W.Z., Parichy, D.M., Cavanaugh, C.M., Guillemin, K., Rawls, J.F. (2011). Evidence for a core gut microbiota in the zebrafish. *The ISME journal*. 5(10), 1595-1608.

Rungruangsak-Torrissen, K., Moss, R., Andresen, L.H., Berg, A., Waagbø, R. (2006). Different expressions of trypsin and chymotrypsin in relation to growth in Atlantic salmon (*Salmo salar L.*). Fish Physiology and Biochemistry. 32(1), 7-23.

Rønnestad, I., Yúfera, M., Ueberschär, B., Ribeiro, L., Sæle, Ø., Boglione, C. (2013). Feeding behaviour and digestive physiology in larval fish: current knowledge, and gaps and bottlenecks in research. *Rev. Aquac.* 5, S59-S98.

Seo, B.S., Park, S.J., Hwang, S.Y., Lee, Y.I., Lee, S.H., Hur, S.W., Choi, Y.H. (2022). Effects of Decreasing Fishmeal as Main Source of Protein on Growth, Digestive Physiology, and Gut Microbiota of Olive Flounder (*Paralichthys olivaceus*). *Animals*. 12(16), 2043.

Shen, F., Li, C., Teng, T., Chen, Y., Guo, J., Zhu, C., et al. (2018). Ontogenetic development of digestive tract and digestive enzymatic activities in *Squaliobarbus curriculus* larvae. *Aquac. Res.* 49, 3158-3166.

Srichanun, M., Tantikitti, C., Utarabhand, P., Kortner, T. M. (2013). Gene expression and activity of digestive enzymes during the larval development of Asian seabass (*Lates calcarifer*). *Comp. Biochem. Physiol. B* 165, 1-9.

Sun, R.J., Zhang, W.B., Xu, W., Mai, K.S. (2013). Effects of dietary protein level and feeding frequency on the growth performance, body composition and protein metabolism of juvenile large yellow croakers. *Acta Hydrobiologica Sinica*. 37(02),281-289

Tang, S., Liang, X. F., He, S., Alam, M. S., Luo, H., and Kuang, Y. (2020). Activin A affects feeding by promoting the inner diameter and muscle development of the pharynx and oesophagus in zebrafish (*Danio rerio*) larvae. *J. Fish Biol.* 97, 1624-1631.

Tong, X. H., Xu, S. H., Liu, Q. H., Li, J., Xiao, Z. Z. and Ma, D. Y. (2012). Digestive enzyme activities of turbot (*Scophthalmus maximus* L.) during early developmental stages under culture condition. *Fish Physiol. Biochem.* 38, 715-724.

Topić Popović, N., Čižmek, L., Babić, S., Strunjak-Perović, I., Čož-Rakovac, R. (2023). Fish liver damage related to the wastewater treatment plant effluents. Environmental Science and Pollution Research, 30(17), 48739-48768.

Ueberschär, B., Navarro-guill, C., Gomes, A., Ivar, R., Rojas-garcia, C., Hanke, I. (2018). "Variability in digestive enzyme capacity in early stages of marine fish larvae: ontogenetic variations, biorhythms, hormonal control and nutrient sensing mechanisms," in *Emerging Issues in Fish Larvae Research*, ed M. Yúfera (Springer International Publishing AG), 87-129.

Ullah, S., Zhang, J., Xu, B., Tegomo, A.F., Sagada, G., Zheng, L., Shao, Q. (2022). Effect of dietary supplementation of lauric acid on growth performance, antioxidative capacity, intestinal development and gut microbiota on black sea bream (*Acanthopagrus schlegelii*). *Plos one*, 17(1), e0262427.

Wang, A. R., Ran, C., Ringø, E., & Zhou, Z. G. (2018). Progress in fish gastrointestinal microbiota research. *Reviews in Aquaculture*, *10*(3), 626-640.

Wang, Y., and Gu, Q. (2010). Effect of probiotic on growth performance and digestive enzyme activity of Arbor Acres broilers. *Res. Vet. Sci*. 89, 163-167.

Yang R., Xie C., Fan Q., Gao C., Fang L. (2010). Ontogeny of the digestive tract in yellow catfish *Pelteobagrus fulvidraco* larvae. *Aquaculture* 302, 112-123.

Yúfera, M., Moyano, F. J., and Martínez-Rodríguez, G. (2018). "The Digestive Function in Developing Fish Larvae and Fry. From Molecular Gene Expression to Enzymatic Activity," in *Emerging Issues in Fish Larvae Research*, ed M. Yúfera (Springer, Cham.), 51-86.

Zhang, J.X., Guo, L.Y., Feng, L., Jiang, W.D., Kuang, S.Y., Liu, Y., Zhou, X.Q. (2013). Soybean β-conglycinin induces inflammation and oxidation and causes dysfunction of intestinal digestion and absorption in fish. *PloS one*. 8(3), e58115.

Zhao, J., Liu, Y., Jiang, J., Wu, P., Chen, G., Jiang, W., Zhou, X. (2012). Effects of dietary isoleucine on growth, the digestion and absorption capacity and gene expression in hepatopancreas and intestine of juvenile Jian carp (*Cyprinus carpio var. Jian*). Aquaculture. 368, 117-128.

Zhou, L., Budge, S.M., Ghaly, A.E., Brooks, M.S., Dave, D. (2011). Extraction, purification and characterization of fish

unedited

chymotrypsin: A review. *American journal of Biochemistry and Biotechnology*. 7(3), 104-123.

Zhou, C., Ge, X., Niu, J., Lin, H., Huang, Z., and Tan, X. (2015). Effect of dietary carbohydrate levels on growth performance, body composition, intestinal and hepatic enzyme activities, and growth hormone gene expression of juvenile golden pompano, *Trachinotus ovatus*. *Aquaculture* 437, 390-397.

Zokaeifar, H., Balcázar, J. L., Saad, C. R., Kamarudin, M. S., Sijam, K., Arshad, A., et al. (2012). Effects of Bacillus subtilis on the growth performance, digestive enzymes, immune gene expression and disease resistance of white shrimp, *Litopenaeus vannamei*. *Fish shellfish Immun*. 33, 683-689.

Zou, W. B., Cooper, D. N., Masson, E., Pu, N., Liao, Z., Férec, C., et al. (2022). Trypsinogen (PRSS1 and PRSS2) gene dosage correlates with pancreatitis risk across genetic and transgenic studies: a systematic review and re-analysis. *Hum. Genet*. 1-12.

Supplementary information

Tables S1 and S2

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