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Title page

Development of digestive enzymatic activity and gene expression during the early ontogeny of Chinese perch (*Siniperca chuatsi*)

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Abstract

Chinese perch (*Siniperca chuatsi*) is one of the economically important freshwater species fish for aquaculture in China. This study aimed to determine the ontogenetic development of the digestive enzyme activity (trypsin, pepsin, amylase, lipase, chymotrypsin and alkaline phosphatase) and related gene expressions of *S. chuatsi* larvae from hatching to 30 days post-hatching (dph). The larvae were fed with live fry fish twice a day. Results indicated that it was low detection of enzyme activity and gene expression of trypsin, chymotrypsin, lipase, amylase and alkaline phosphatase before mouth opened, the last two enzymes showed an activity close to zero. Different from other carnivorous fish, specific activity and gene expression of trypsin, chymotrypsin and lipase in *S. chuatsi* larvae were not increased after starting the first feeding. Interestingly, the amylase and alkaline phosphatase specific activity progressively increased over development, indicating that the larvae have certain ability to digest carbohydrates. Pepsin activity and gene expression started to sharply increase after 15 dph, other digestive enzyme activity showed downward trends. The

development pattern of digestive enzymes may affect the ability of *S. chuatsi* to digest the zooplankton, which leads to the formation of unique feeding habit of the *S. chuatsi* larvae. This study also will provide the necessary theoretical basis for the artificial opening diet of the *S. chuatsi* larvae.

Key words: *Siniperca chuatsi*; Early ontogenetic development; Gene expression; Digestive enzyme; Feeding habit

1. introduction

The early stages of larval development are an important period as factors such as starvation and subsequent death usually accompany it. Nutrition of larva is an important factor for consideration as it influences growth and survival during the early larval developmental stages^[1]. Larval nutritive need a highly efficient enzymatic digestive machinery which enable to digest the food for supporting high growth. Therefore, a good understanding of age-dependent changes in digestive physiological digestion and digestive tract development during larval stages could help in the improvement of fish larviculture and seed production quality^[2-3].

Chinese perch (or Mandarin fish) *Siniperca chuatsi* is one of the most famous high valued fish species^[4]. To meet the market demands, culture of this species has largely increased with high intensity, but the insufficient or delayed supply of prey fish will cause a large number of larval death, which has become a major bottleneck of *S. chuatsi* larvae rearing^[5-6]. Chinese perch larvae show exclusive piscivory since first feeding stage, they prey on other fish species larvae, but don't eat any zooplankton^[7]. However, live fry as food presents problems that include large investments in maintaining live fry culture, variable supply and disease transmission. As the same time, it has been considered that the zooplankton not necessarily possess the adequate nutrients for *S. chuatsi*, which can cause this species show strong piscivory in the early larval stage^[8]. In this way, if we want to improve the survival during the larval stage, it is necessary to conduct specific studies, based on in digestive physiology of species, that allow for evaluation of the digestive enzymatic capacity of the larvae.

Though morphology of digestive system in Chinese perch larvae has been reported^[9-10], these studies did not indicate the real digestive capacities and functional activities during larval stage developments. It is necessary to evaluate the comprehensively digestive ability of *S. chuatsi* larva from the aspects of gene expression and enzyme activity detection. Recently, the use of molecular biological approaches have complemented traditional methods in expressing the profiles of digestive enzyme precursors in fish^[1,8,11,12] and thus providing insight into both

temporal and spatial expression patterns of genes involved in the development and functionality of digestive systems during early ontogeny. As such, this study looks to investigate the early ontogeny of *S. chuatsi* through an integrative investigation to understand the general patterns of activity through the effect of feeding status, morphological changes, and gene expression on final enzymatic capacity.

2. Materials and methods

2.1 Ethics statement

All experimental procedures followed the ethical guidelines of Ethical Approval: (No. HBAC20091138; Date; 15 November 2009, Wuhan, China) and were approved by the Institutional Animal Care and Institute of Huazhong Agricultural University.

2.2 Larval rearing

Fertilized eggs of *S. chuatsi* were obtained from the Chinese Perch Research Centre of Huazhong Agricultural University. The eggs were incubated at a temperature of 25.0 ± 1.5 °C and an oxygen concentration of 7.8 ± 0.6 mg / L in a 50-L tank. The newly hatched larvae were transferred into three separate replicate 6-L cuboid tanks. During the rearing period, the different water quality parameters like temperature were 24.0 ± 1.0 °C; dissolved oxygen was 7-8 mg / ml, and pH was 7.8-8.3. Throughout the rearing, water quality parameters like dissolved oxygen, water flow, water depth etc. were maintained in normal ranges. Larval exogenous feeding started at 3 dph, the larvae of *S. chuatsi* were fed live fry of *Megalobrama amblycephala* twice a day from 3 dph to 30 dph.

2.3 Fish sampling

Sampling of larvae was conducted at 1, 3, 5, 8, 10, 15, 25, 30 dph. Samples were caught at two hours after feeding and the larvae were individually collected for different analysis in triplicates according to Khoa *et al.*^[1] All *S. chuatsi* samples were euthanised with 1 mg/ml MS-222 (Argent Chemical Laboratories, Redmond, WA, USA) before sampling. To analyse larval growth, 10 individuals per tank were selected and their total lengths and total weights were measured and morphology was observed by a stereomicroscope (Olympus, Tokyo, Japan) equipped with a digital

camera on 1, 3, 5, 8, 10, 15, 25, 30 dph. For quantitative real time PCR, 15 larvae were collected (5 larvae × triplicate) at each sampling day, after being sampled, the specimens were immediately frozen in liquid nitrogen and stored in a cryogenic freezer at -80°C. For the enzyme assay, 45 larvae were collected (15 larvae × triplicate) at each sampling day, rinsed in distilled water, then placed in microtubes and stored at -80 °C.

2.4 Quantitative real time PCR (qRT-PCR) analysis

Quantitative real time PCR analysis followed a modified method of Khoa *et al.*^[1] and He *et al.*^[13] For total RNA isolation, the larvae for each sampling day was homogenized in 1 ml of TRIzol™ reagent (TaKaRa, Dalian, China) in triplicate. The RNA concentration and purity were detected by nucleic acid quantifier (Thermo Scientific, Waltham city, Massachusetts, USA) ensured the OD 260 / 280 of all samples ranged between 1.8 and 2.0. cDNA synthesis was reverse-transcribed using M-MLV reverse transcriptase (TaKaRa, Dalian, China) according to the manufacturer's instructions in a final volume of 20 µl. The expression level of mRNA for trypsin (*try*), α-amylase (*amy*), pepsin (*pep*), pancreatic lipase (*pl*), chymotrypsin (*ctr*), bile salt-activated lipase (*bsal*) and and alkaline phosphatase (*alp*) was determined by quantitative real time PCR (qRT-PCR). Absolute RT-qPCR was performed for each sample with three technical replicates using AceQ® qPCR SYBR® Green Master Mix (Vazyme, Nanjing, China) on a CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA). Each reaction contained 1 µl of cDNA, 0.4 µl of each primer, 8.2 µl of free water and 10 µl of qPCR Mix in a final volume of 20 µl. The three step qPCR program included an enzyme activation step at 95 °C (5 min) and 40 cycles of 95 °C (10 s), annealing temperature of each primer set (30 s) and 72 °C (30 s). The *rpl13a* expression levels among treatments were stable during the larval stages and its average expression was applied as a normalization factor. The primer sequences were listed in Table 1.

Table 1 Primer sequences for Real time PCR

Gene	Primer forward	Primer reverse	T _m (°C)
<i>try</i>	TCTGCTAAGGTCATCCGTCA	TCGCTCAGGATAGGGGCAT	59
<i>amy</i>	AAAATGGAACGGCGAGAA	GGAATCCCAGAAGGTAACGA	59
<i>pep</i>	AACCCACAGCAGTCCACCAC	CGAAGACAGGCACGACATTG	58
<i>pl</i>	TGTATGTAGCATTTCGTTGGAGA	TGTCACATCAACCAAGTCTACCT	58
<i>ctr</i>	GCCCCAAGTTAGCGGATAC	GAGCAGCAGTGACAACCCA	59
<i>bsal</i>	GGCTCGATGGGTGCTAACT	CAGGGTCTCCTCCAAATGA	58
<i>alp</i>	TACTCCGACAATGAGATGCC	TTTCTCCCGTCTTTTCGTG	55
<i>rpl13a</i>	CACCCTATGACAAGAGGAAGC	TGTGCCAGACGCCCAAG	59

Note: trypsin (*try*), α -amylase (*amy*), pepsin (*pep*), pancreatic lipase (*pl*), chymotrypsin (*ctr*), bile salt-activated lipase (*pl*), and alkaline phosphatase (*alp*).

2.5 Enzyme assay

For enzyme extraction, the samples were removed from the freezer and placed on ice to thaw. After thawing was complete, the samples were homogenized in 10 volumes (w / v) of ice-cold physiological saline solution, then centrifuged at $8000 \times g$ at 4°C for 10 min. The resultant supernatants were collected and stored at -80°C for enzymatic determinations. The soluble protein (mg / ml) contents were determined with the Bradford method using bovine serum albumin as the standard (0.524 g / L)^[14]. Enzymatic activities were expressed as the total activity defined as milli-units per larval fish or units per larval fish (mU / larvae or U/ larvae) based on the whole-fish homogenate. The specific activity was expressed as milli-units per milligram of protein or units per milligram of protein (mU / mg protein or U / mg protein).

2.5.1 Trypsin (EC 3.4.21.4)

The N α -*p*-Tosyl-L-arginine methyl ester hydrochloride (TAME, Sigma-Aldrich, T-4626) was used as the substrate to performed the assay for trypsin activity according to the method described by Lemieux and Blier^[15]. Trypsin catalyzed the hydrolysis of the ester bond of TAME, and released free carboxyl groups neutralize with the sodium hydroxide in the reaction system, resulting in a decrease in the pH

value of the solution. Using phenol red as an indicator, the change in the absorption value of the solution at 555nm is measured. One unit of trypsin activity was defined as the each milligram of protein was catalyzed within one minute, the absorbance at 555nm decreases by 0.5 (37°C).

2.5.2 Amylase (E.C. 3.2.1.1)

Amylase activity was evaluated according to the method described by Li *et al.*^[16]. Amylase activity was determined by the starch hydrolysis method, maltose was used as the standard, and amylase activity was expressed as mmol maltose released from starch ml / min. One unit of amylase activity was defined as the hydrolysis of 10 mg of starch in 30 min under the conditions of the assay (37°C).

2.5.3 Lipase (E.C. 3.1.1)

Lipase activity was assayed by following a modified protocol from Liu *et al.*^[17]. Lipase was measured with a lipase assay kit (NO: A054-2, Nanjing Jiancheng Bioengineering Institute). In the assay, 1, 2-laurelglycerol-3-glutaraldehyde-6'-methyl resorufin could be catalysed by lipase, and chromophore variation was detected at $\lambda = 580$ nm. One unit of lipase activity was defined as the quantity of enzyme that liberated 1 μ mol of butyric acid per min under the condition of the assay (37°C).

2.5.4 Pepsin (E.C. 3.4.23.1)

Pepsin activity was determined by the method of Zhou *et al.*^[18] with slight modification. Pepsin catalyze the hydrolysis of hemoglobin, and the hydrolysate will appear blue after reacting with Folin reagent. One unit of pepsin activity was defined as each milligram of pepsin catalyzes the hydrolysis of hemoglobin to 1nmol tyrosine per minute (37°C).

2.5.5 Chymotrypsin (E.C. 3.4.21.1)

Chymotrypsin activity was assayed with BTEE (N-Benzoyl-L-tyrosine ethyl ester) according to the method described by Hekmatpour *et al.*^[19], chymotrypsin activity was determined at 27 °C and measured at 256 nm using BTEE as substrate in 2 mL of Tris / CaCl₂ buffer, pH 7.8. One unit of enzyme was defined as the amount of enzyme needed to hydrolyze 1 mg of substrate (BTEE) per min per mg protein.

2.5.6 Alkaline phosphatase (E.C. 3.1.3.1)

Alkaline phosphatase activity was assayed by following a modified protocol from Liu *et al.*^[17]. Alkaline phosphatase activity was measured with a lipase assay kit (NO: A059-2, Nanjing Jiancheng Bioengineering Institute). Sodium phenyl phosphate was decomposed to produce free phenol and phosphoric acid by alkaline phosphatase. The phenol reacts with 4-aminoantipyrine in an alkaline solution to form a red hydrazine derivative, and chromophore variation was measured at $\lambda = 520$ nm.

2.6 Data analysis

All data are reported as the mean \pm S.E.M (standard error of the mean). All analyses were conducted using IBM SPSS Statistics 19 (IBM, Armonk, NY, USA). The normal distribution of variables and homoscedasticity was analysed by Kolmogorov-Smirnov and Levene tests, respectively. The level of gene expression and activity of digestive enzyme between different ages were analyzed by one-way ANOVA applied Tukey's HSD (Honestly Significant Difference) test at $p = 0.05$ significant level. All statistical analyses were performed using GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego CA, USA).

3. Results

3.1 Larval growth and morphology

3.1.1 Total length and wet weight

The larval growth in total length (TL) and wet weight (WW) is shown in Fig. 1. The TL and WW of newly hatched larvae (1 dph) were 4.893 ± 0.017 mm and 1.493 ± 0.033 mg, respectively, and those of larvae at 30 dph were 22.020 ± 0.214 mm and 150.400 ± 0.782 mg (Fig. 1). Larval growth in terms of TL and WW increased exponentially by following $y=5.225e^{0.0509x}$ ($R^2 = 0.9793$) and $y=1.9061e^{0.1532x}$ ($R^2 = 0.9619$), respectively.

3.1.2 Morphological development of *S. chuatsi* larvae

Morphological development in *S. chuatsi* during different developmental stages is shown in Figure 2. At 1 dph, the mouth was unopened; yolk sac was full. The oral cavity opened at 2 dph and larvae started exogenous feeding at 3 dph followed by the

yolk sac completely absorbed at 4-5 dph. During the experimental periods, the ontogenetic development of larvae devised into four major stages. The first stage was endogenous nutritional stage, from hatching to mouth opening (1-3 dph). The second stage was from first feeding to yolk sac depletion considered as start of exotrophic stage (3-5 dph). The third stage was after first feeding to the appearance of the gastric glands (5-15 dph), the digestive system increased in volume and the blind sac was expanded when larvae feeding. The fourth stage was the gastric glands appeared in the stomach to the digestion system have well-development and the form of fish was similar to the adult fish. At this stage, the appearance of the fish and the pattern of the body were similar to the adult fish.

3.2 Gene expression and activity of digestive enzymes

3.2.1 Trypsin

Gene expression of *try* was observed as early hatching, the expression levels of *try* increased after first feeding (3 dph) and fluctuated considerably until 15 dph. Thereafter, *try* mRNA levels sharply decreased and remained relatively stable throughout the experimental period (Fig. 3 A).

The specific and total activities of trypsin are shown in Figure 3 B & C. The specific activity of trypsin (Fig. 3 B) was detected in the newly hatched larvae (3.757 ± 0.310 U / mg protein), then slightly decreased at 3 dph (2.195 ± 0.104 U / mg protein). The specific activity gradually increased after the larvae start exogenous feeding and peaked at 10 dph (6.735 ± 0.466 U / mg protein). Afterward, the specific activity was sharply decreased at 15 dph (1.887 ± 0.227 U / mg protein). Then, slight fluctuations were measured and showed a decreasing trend to the end of the study (0.799 ± 0.029 U / mg protein).

During initial days, the total activity of trypsin (Fig. 3 C) was detectable at very low levels from 1 dph onwards 5 dph (0.998 ± 0.013 U / larva). Then, the total activity was dramatically increased and peaked at 15 dph (5.000 ± 0.258 U / larva). Thereafter, the total activity was abruptly decreased until 30 dph (2.224 ± 0.111 U / larva).

3.2.2 Pepsin

pep expression was detected at 5 dph and its expression gradually increased over the experimental period (Fig. 3 D). From 15 dph, the expression of *pep* was much higher than those at hatching levels.

The specific and total activities of pepsin are shown in Figure 3 E & F. The specific activity of pepsin was already detected in *S. chuatsi* at hatching, the activity remained fairly constant without a significant variation until 10 dph (37.168 ± 2.301 U / mg protein). Pepsin specific activity was sharply increased at 15 dph (83.496 ± 2.128 U / mg protein) and remaining increasing to the end of the study (438.148 ± 35.404 U / mg protein).

The total activity of pepsin was remained at very low levels from hatching to 10 dph and then rapidly increased at 15 dph (419.737 ± 62.254 U / larva). Thereafter, the total activity remained increasing to the end of the study and peaked at 30 dph (1548.684 ± 130.665 U / larva).

3.2.3 α -Amylase

Low expression levels of *amy* in newly hatched larvae but the gene expression was increased after first feeding at 3 dph. *amy* expression remained increased and peaked at 8 dph. Thereafter, the *amy* expression levels started to decrease at 10 dph, and remained a decreasing trend until 30 dph (Fig. 3 G).

The specific and total activities of α -amylase are shown in Figure 3 H & I. The specific α -amylase activity was undetectable at hatching and first detected after first feeding at 3 dph (0.030 ± 0.005 U / mg protein). The specific activity sharply increasing at 8 dph (0.053 ± 0.008 U / mg protein), and then dramatically decreased at 10 dph (0.013 ± 0.003 U / mg protein) after the first peak. Thereafter, the specific activity gradually increasing again at 15 dph (0.043 ± 0.009 U / mg protein) and then gradual decline until 30 dph (0.009 ± 0.001 U / mg protein).

The total α -amylase activity showed a low value in newly hatched larvae and remained slight fluctuations until 10 dph (1.667 ± 0.333 mU / larva), and then the

total activity sharply increased and peaked at 15 dph (54.250 ± 4.171 mU / larva). Afterward, total amylase activity remaining decreasing until the end of the experiment (25.800 ± 1.319 mU / larva).

3.2.4 Lipase

pl expression levels fluctuated over the larval development (Fig. 4 A). At hatching, the expression of *pl* was observed and then significantly decreased at 5 dph and the peak point of the *pl* expression appeared at 8 dph, and then showed a decreasing trend until 30 dph.

bsal expression levels fluctuated considerably over the larval development (Fig. 4 B). *bsal* expression showed a low value in newly hatched larvae, a slightly increase in expression was observed after first feeding. Thereafter, *bsal* expression levels sharply decreased and remained fairly constant without a significant variation until 15 dph. Expression levels sharply increased and peaked at 15 dph, and then showed a decreasing trend until 30 dph.

The specific and total activities of lipase are shown in Figure 4 C & D. The specific lipase activity was detected in *S. chuatsi* on the first day after hatching (47.304 ± 0.834 mU / mg protein). Then, the specific activity was slightly decreased at 3 dph (27.364 ± 3.301 mU / mg protein) and remained constant until 10 dph (34.449 ± 1.961 mU / mg protein). Thereafter, the specific activity was sharply increased and peaked at 15 dph (96.866 ± 3.627 mU / mg protein), but the specific activity was dramatically dropped at 25 dph (38.948 ± 7.506 mU / mg protein) and remained a decreasing trend until 30 dph (22.208 ± 1.814 mU / mg protein).

The total lipase activity was closest to zero from hatching to 5 dph (3.213 ± 0.252 mU / larva). At 8 dph, a slightly increase on total activity was observed (9.422 ± 0.545 mU / larva). Then, the total activity was sharply increased at 15 dph (55.745 ± 2.087 mU / larva) and remained increasing until 30 dph (63.093 ± 0.619 mU / larva).

3.2.5 Chymotrypsin

ctr expression levels fluctuated over the larval development (Fig. 5 A). At

hatching, the expression of *ctr* was observed and then significantly decreased at 5 dph. Subsequently, a high fluctuation was observed with a peak point at 15 dph, and thereafter a decreasing trend was observed.

The specific and total activities of chymotrypsin are shown in Figure 5 B & C. The specific chymotrypsin activity (Fig. 5 B) remained at low levels from hatching until 10dph and then rapidly increased at 15dph (0.078 ± 0.006 U / mg protein). Thereafter, the specific activity abruptly decreased until 30 dph (0.024 ± 0.002 U / mg protein).

The total chymotrypsin activity (Fig. 5 C) was detected at low levels from hatching to 10 dph (8.186 ± 0.262 U / larva) and increased sharply to 45.146 ± 3.305 U / larva at 15 dph. Then, the total activity progressively rose from 25 dph to its highest activity attained at 30 dph (68.980 ± 4.583 U / larva).

3.2.6 Alkaline phosphatase

alp expression was observed as early hatching, but the expression levels of *alp* dramatically decreased after first feeding (3 dph), and remained low and continued to drop at 5 dph, before sharply increased and peaked at 15 dph. Then, the expression levels of *alp* was decreased at 25 dph and remained a decreasing trend until 30 dph (Fig. 5 D)

The specific and total activities of alkaline phosphatase are shown in Figure 5 E & F. The specific alkaline phosphatase activity was detected at low level (3.224 ± 2.013 U / mg protein) at hatching and increased sharply to 40.848 ± 11.029 U / mg protein at 3 dph at the beginning of exogenous feeding, and remained slight fluctuations until 10 dph. Then, the specific activity was dramatically decreased at 15 dph (5.653 ± 0.663 U / mg protein) and remained low levels until 30 dph.

The total alkaline phosphatase activity closest to zero at hatching, and then gradually increased from 3 dph (2.716 ± 0.180 U / larva) and peaked at 15 dph (16.575 ± 2.433 U / larva). Thereafter, the total activity dramatically decreased at 25dph (5.627 ± 1.352 U / larva) and reached to the highest level again at 30 dph (14.294 ± 2.447 U / larva).

4. Discussion

Growth rate is an important parameter for the success of larval rearing^[20]. In the present study, an exponential growth pattern was observed in *Siniperca chuatsi* larvae. The growth rate of the larvae was satisfactory, reaching 17.127 mm and 148.907 mg in TL and WW, respectively. The total length in this experiment corresponds with the results in Doi, Doi *et al.* and Zhang *et al.*^[7,9,21], but behind some previous studies^[22]. This lag might be the size of the tank limit the growth of larvae at the end of study, 3-6 L tanks were used in our study rather than (10 m × 10 m × 0.8 m) tanks in Song *et al.*^[22]. In the present study, the similar patterns of TL and WW were exhibited in *Megalobrama terminalis*^[17], *Pseudosciaena crocea*^[23], *Centropomus nigrescens*^[24] and *Odontesthes bonariensis*^[25].

The present study is the first report of the gene expression of digestive enzyme in *S. chuatsi* larvae, the latest *S. chuatsi* genomics data published by our research team were used to ensure the reliability of genes^[13]. Almost digestive enzyme genes expression (excepted *amy* and *pep*) were detected at hatching, it's concurrent with the digestive enzyme activities. The expression of *try* in *S. chuatsi* larvae were detected at hatching and stained slight fluctuations until 15 dph, these results agree with other reports in larvae of several fish species^[1,11,26]. Then, the expression of *try* gradually decreased until 30 dph, similar findings about *try* expression have been reported in other species such as *Lates calcarifer*^[3], *Clarias magur*^[8], *Lutjanus guttatus*^[27] and *Sphoeroides annulatus*^[28]. The specific activity of trypsin in *S. chuatsi* larvae were detected at hatching and increasing concomitantly with larval development until 10 dph, and then slightly decreased until the end of study. This observation is similar to that reported for *Lates calcarifer*^[3], *Odontesthes bonariensis*^[25], *Miichthys miiuy*^[29], *Alosa sapidissima*^[30], *Anguilla japonica*^[31] and *Trachinotus ovatus*^[32]. One reason for the presence of the *try* expression and trypsin activity was detected at hatching before mouth opening in *S. chuatsi* might be that it is related to a genetically programmed process^[1]. Meanwhile, compared with amylase and chymotrypsin, content of trypsin is the highest in the first-feeding stage and might be the most important enzyme for

digestion in *S. chuatsi* larvae.

The total activity of trypsin in *S. chuatsi* larvae were detected at hatching at very low levels and showed an increasing pattern from 3 to 15 dph and thereafter a decreasing trend was observed until 30 dph. Similar total activity profiles have been reported for *Odontesthes bonariensis*^[25], *Sparidentex hasta*^[33], *Cichlasoma urophthalmus*^[34], *Epinephelus malabaricus*^[35]. In stomach-containing teleosts, the progressive shift in relative activity from alkaline to acid proteases during larval development^[36], So, it is not surprising that trypsin activity decreased at 30 dph after completion of metamorphosis and expression of pepsin.

In *S. chuatsi* larvae, the *pep* gene was almost undetected at hatching, gene expression increased with larval development starting at 5 dph after the larvae opened the mouth and fed live food. From then on, the expression of *pep* gene stained sharply increasing until 30 dph. This finding is in agreement with the early detection of *pep* gene expression in other fish species: *Pagrus major*^[1,37], *Clarias magur*^[8], *Acipenser persicus*^[26]. In the present study, the pattern of specific pepsin activity was similar with the total activity, the pepsin activity was detected at hatching and then showed slight fluctuations until 15 dph. Since most fish larvae lack a morphologically distinct and functional stomach during the early developmental stages, the larvae lack pepsin digestion^[3]. A very low level of pepsin activity was observed during the early developmental stage might be due to the presence of other acidic proteases such as cathepsin (lysosomal enzyme) present in the larva body as whole larvae were used for enzyme assays^[38]. Thereafter, the pepsin activity sharply increased and remained a increasing trend until 30 dph. This result might indicate a maturational digestive ability from the morphological or physiological point of view due to the formation of a functional stomach^[9]. At the same time, other digestive enzymes activity decreases since it is in the course of being partly replaced by pepsin activity in the developing stomach^[39]. The same pattern was observed in *Pagrus major*^[1,37], *Megalobrama terminalis*^[17], *Alosa sapidissima*^[30], *Trachinotus ovatus*^[32], *Sparidentex hasta*^[33], *Seriola rivoliana*^[40], *Acipenser persicus*^[41]. Most studies have suggested that protein digestion mainly depends on acidic digestion after the digestive function matures^[17].

In this study, gene expression of amylase were detected at hatching at very low transcription level and kept rising until 8 dph. thereafter, the *amy* expression showed a decreasing tendency until the end of the experiment. Similar pattern has been documented in *Pagrus major*^[1,37], *Lates calcarifer*^[3], *Lutjanus guttatus*^[42]. The variation trend of specific amylase activity was consistent with gene expression, other fish species with carnivorous feeding habits have also been highlighted to showcase this enzymatic behavior^[1,3,29,33,37,40]. Different from *S. chuatsi*, the specific amylase activity of the carnivorous fish mentioned above reached the highest value at the 3 dph (the first-feeding time). The specific amylase activity in *S. chuatsi* larvae peaked at 8 dph, it means the specific amylase activity was lacking when *S. chuatsi* larvae first foraging (3-5 dph). This indicates that carbohydrates play an important role during early larvae development, but the function of α -amylase at early stages is not completely understood^[43]. It is generally accepted that the ontogeny of the digestive system is a genetically programmed process^[36], because the *S. chuatsi* larvae fed the live pry fish at first-feeding which contain less carbohydrates, but the expression of *amy* in *S. chuatsi* larvae continued to rise until 10 dph before the the increase of tissue proteins.

The total amylase activity was very low levels from hatching to 10 dph, then sharply peaked at 15 dph. Thereafter, slightly decreased at 25 dph and remained level until the end of study. This indicates that the *S. chuatsi* have certain ability to digest carbohydrates after growing up. These results agree with other reports in larvae of several fish species: *Lates calcarifer*^[3], *Alosa sapidissima*^[30], *Anguilla japonica*^[31], *Trachinotus ovatus*^[32].

It's worth noting that the total activity of trypsin, pepsin and amylase were researched in *S. chuatsi* by Doi^[9]. In Doi's study, the three digestive enzymes all detected at 3 dph and remained nearly constant activities from 3 dph larvae to 9 dph, all of three enzyme activities increased at 13 dph larvae. This pattern was similar with our study, the total activities were very low levels after first-feeding to 10 dph, and sharply increased at 15 dph. However, the difference are: (1) In the present study, the pepsin total activity was higher than Doi's study after first-feeding to 10 dph, the

pepsin total activity was about 10 U / larva in our results, much higher than the trypsin and amylase total activity. (2) In the present study, the trypsin total activity started increase at 8 dph with the development. But in Doi's study, the trypsin total activity remained nearly constant activities from 3 dph larvae to 9 dph. (3) In the present study, the the amylase total activity was nearly with trypsin from 1-10 dph, just the amylase not increased until 15 dph. The reasons might be the differences of detection methods and sensitivity lead to more fluctuations in our results, which can better reflect the changes of enzyme activity at early stage.

There are two lipase genes in *S. chuatsi* larvae, *pl* and *bsal* were all detected at hatching, but expression of *pl* showed a downward trend with the *S. chuatsi* larval development until 30 dph. The expression of *bsal* was increased and peaked at the beginning of exogenous feeding, then dramatically decreased and remained low levels until 10 dph. Levels of *bsal* expression dramatically increased and peaked again at 15 dph and then gradual decline until 30 dph. The fluctuating pattern of *bsal* expression was observed in *Lates calcarifer*^[3] and *Clarias magur*^[8]. It was noteworthy that the pattern of *pl* expression was special, this pattern of *pl* expression only reported in *Pagrus major*^[37] which feed the live food.

Interestingly, the lipase activity was similar with many other fish species. The specific lipase activity was detected at hatching but then decreased at 3 dph, it might be related to the end of the process of the digestion and absorption of nutrients derived from the yolk sac. Then, the specific lipase activity remained the low levels until 10 dph. Thereafter, specific lipase activity dramatically increased at 15 dph, but then sharply decreased until end of the study. Similar pattern has been documented in *Lates calcarifer*^[3], *Clarias magur*^[8], *Odontesthes bonariensis*^[25], *Pseudoplatystoma punctifer*^[44], *Mystus nemurus*^[45]. The total lipase activity was also detected at hatching at near zero and remained the low levels until 10 dph. From then on, the total lipase activity sharply increased and remained a increasing trend until 30 dph. The pattern of total lipase activity was observed in *Lates calcarifer*^[3], *Megalobrama terminalis*^[17], *Alosa sapidissima*^[30], *Trachinotus ovatus*^[32], *Sparidentex hasta*^[33], *Pseudoplatystoma punctifer*^[44], *Orthopristis chrysoptera*^[46]. The lipase activity was very low level at

beginning of exogenous nutrition, only the total lipase activity continuously increase with development. This might be related to the special feeding habits of *S. chuatsi*. The live pry fish contained less fat, so the *S. chuatsi* larvae had less demand for lipase.

During the early development of fish larvae, chymotrypsin is another important protease involved in the digestive process^[47]. In this study, the expression of *ctr* was detected at hatching and remained low levels until 10 dph, then increased and peaked at 15 dph. Thereafter, *ctr* expression sharply decreased and back to the low levels until 30 dph. Similar expression profiles have been reported for *Pagrus major*^[1,37], but the *ctr* expression have a slight increase at 3 dph (first-feeding time) in *Pagrus major*. Furthermore, the specific chymotrypsin activity was concurrent with the gene expression in this study. The strong correlation between activity and gene expression of chymotrypsin has been discussed previously in *Pagrus major*^[1,37]. The pattern of specific chymotrypsin activity was observed in *Pagrus major*^[1,37], *Odontesthes bonariensis*^[25], *Mystus nemurus*^[45]. Interestingly, the specific chymotrypsin activity and the gene expression in *S. chuatsi* were not increased at first-feeding, this might directly affect the first feeding of the *S. chuatsi* larvae. Additionally, the total chymotrypsin activity was also detected at hatching at near zero and remained the low levels until 10 dph. From then on, the total chymotrypsin activity sharply increased and remained a increasing trend until 30 dph. These results agree with other reports in larvae of several fish species: *Lates calcarifer*^[3], *Mycteroperca rosacea*^[38], *Pseudoplatystoma punctifer*^[44], *Orthopristis chrysoptera*^[46].

Alkaline phosphatase is produced in the Golgi apparatus of the enterocytes and is mainly distributed in the intestinal epithelial brush border membranes of fish^[20]. Variations in the activity of alkaline phosphatase is considered general indicators of nutrient absorption, as well as enterocyte and intestinal maturation^[48]. Our results showed that the expression of *alp* gene was detected at hatching, but it remained a very low level until 10 dph. Similar pattern has been observed in *Dormitator latifrons*^[11], the expression of *alp* was not high at early stage. This might be related to the development of the gut of fish, as well as other genes involved in the development

of the digestive tract such as leucine aminopeptidase (*lap*). In the present study, *alp* expression sharply increased and peaked at 15 dph and then remained a decreasing trend until 30 dph, this suggested strong digestive capacity at the intestinal membrane level and an almost fully mature intestine. Similar expression profiles have been reported for *Diplodus puntazzo*^[20]. Conversely, the specific activities of alkaline phosphatase was detected at hatching at very low level, but the specific activities sharply increased and peaked after the first feeding (3 dph) and maintained a high levels until 10 dph. The increase in alkaline phosphatase activity might be correlated with the fast development of intestinal mucosa characterised by growth in length and folding (villi) of the intestine, it indicate the intestine is suitable for nutrient absorption at the beginning of exogenous feeding^[50]. A sharp increase in alkaline phosphatase in intestinal brush border membranes characterises normal maturation of enterocytes in developing fish larvae^[51]. In this study, the specific alkaline phosphatase activities showed a low level after 15 dph, the decrease and stabilisation of specific activity values for brush border enzymes in older fish can be mainly explained by the increase of tissue proteins in growing larvae, and it does not correspond to a lowering in the amount of digestive enzymes or dietary shifts^[40,47,49]. Similar pattern has been documented in *Lates calcarifer*^[3], *Megalobrama terminalis*^[17], *Centropomus nigrescens*^[24], *Alosa sapidissima*^[30], *Sparidentex hasta*^[33], *Cichlasoma urophthalmus*^[34], *Seriola rivoliana*^[40], *Acipenser persicus*^[41], *Silurus soldatovi*^[51], *Paralichthys californicus*^[52]. The total alkaline phosphatase activities gradually increased with the development of larvae, this also indicates that the content of alkaline phosphatase enzymes increased and the ability of digestion was enhanced. Similar pattern has been documented in *Lates calcarifer*^[3], *Megalobrama terminalis*^[17], *Odontesthes bonariensis*^[25], *Alosa sapidissima*^[30], *Sparidentex hasta*^[33], *Cichlasoma urophthalmus*^[34], *Mycteroperca rosacea*^[38], *Acipenser persicus*^[41], *Pseudoplatystoma punctifer*^[44], *Paralichthys californicus*^[52].

In conclusion, the ontogenesis of the main digestive enzymes was investigated in *Siniperca chuatsi* during the larval development, using biochemical approaches. In the present study, the expected growth performance and survival rate of shad larvae were

obtained, which showed an exponential growth pattern. According to the results, the specific activities of digestive enzymes in *S. chuatsi* changed constantly from 3 to 30 dph, the content of trypsin, amylase and alkaline phosphatase were higher at first-feeding stage. This indicated that *S. chuatsi* larvae have good protein and carbohydrate utilization, the intestinal absorption function is relatively perfect. However, compared with other similar carnivorous fish, the specific activity of trypsin, chymotrypsin and lipase were not increase significantly at the first-feeding time, and the gene expressions also not changed significantly. This may be the key for *S. chuatsi* to eat live pry fish when it was first-feeding. The digestive enzymes in the live pry fish might compensate for the insufficient amount of enzymes in the *S. chuatsi* larvae. After 15 dph, expression of *pep* gene and pepsin activity were increased and other digestive enzymes started decreased, this indicated the establishment of an efficient acidic digestion. Our findings on the development of the digestive enzymes in *S. chuatsi* provide effective information for the ontogeny of fish larvae, which is useful to improve the seedling cultivation and the technology of healthy breeding.

Author Contributions

X.-F.L. and S.H. designed the research. S.-L.T. carried out the experiments and analyzed the data. Y.P.Z. provide the fertilized eggs of *S. chuatsi*, D.P. and H.X.F. helped to rear larvae, S.-L.T. wrote the paper. All authors have approved the final version of the manuscript.

Competing interests

The authors declare no competing interests.

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Figure legends

Figure 1. Growth in total length (A) and body weight (B) of *S. chuatsi* larvae from 1 to 30 days after hatching. The data was presented as mean \pm SEM. Different superscript letters indicate significant differences on different days (ANOVA, Tukey's HDS test, $a < b < c < d < e < f < g < h$, $p < 0.05$).

Figure 2. Morphology change of *S. chuatsi* larvae during the ontogenetic development. The scale bar = 1000 μm .

Figure 3. Dynamics of the gene expression and activity of trypsin (*try*), pepsin (*pep*) and α -amylase (*amy*) of whole-body *S. chuatsi* larvae during development. (A) expression of *try* gene; (B) specific activity of trypsin; (C) total activity of trypsin; (D) expression of *pep* gene; (E) specific activity of pepsin; (F) total activity of pepsin; (G) expression of *amy* gene; (H) specific activity of α -amylase; (I) total activity of α -amylase. Data are given as mean ($n = 3$ larval pools) \pm SEM. Different letters denote significant differences between larval stages, $p < 0.05$.

Figure 4. Dynamics of the gene expression and activity of lipase (*pl* and *bsal*) of whole-body *S. chuatsi* larvae during development. (A) expression of *pl* gene; (B) expression of *bsal* gene; (C) specific activity of lipase; (D) total activity of lipase. Data are given as mean ($n = 3$ larval pools) \pm SEM. Different letters denote significant differences between larval stages, $p < 0.05$.

Figure 5. Dynamics of the gene expression and activity of chymotrypsin (*ctr*) and alkaline phosphatase (*alp*) of whole-body *S. chuatsi* larvae during development. (A) expression of *ctr* gene; (B) specific activity of chymotrypsin; (C) total activity of chymotrypsin; (D) expression of *alp* gene; (E) specific activity of alkaline phosphatase; (F) total activity of alkaline phosphatase. Data are given as mean (n = 3 larval pools) \pm SEM. Different letters denote significant differences between larval stages, $p < 0.05$.

Figures

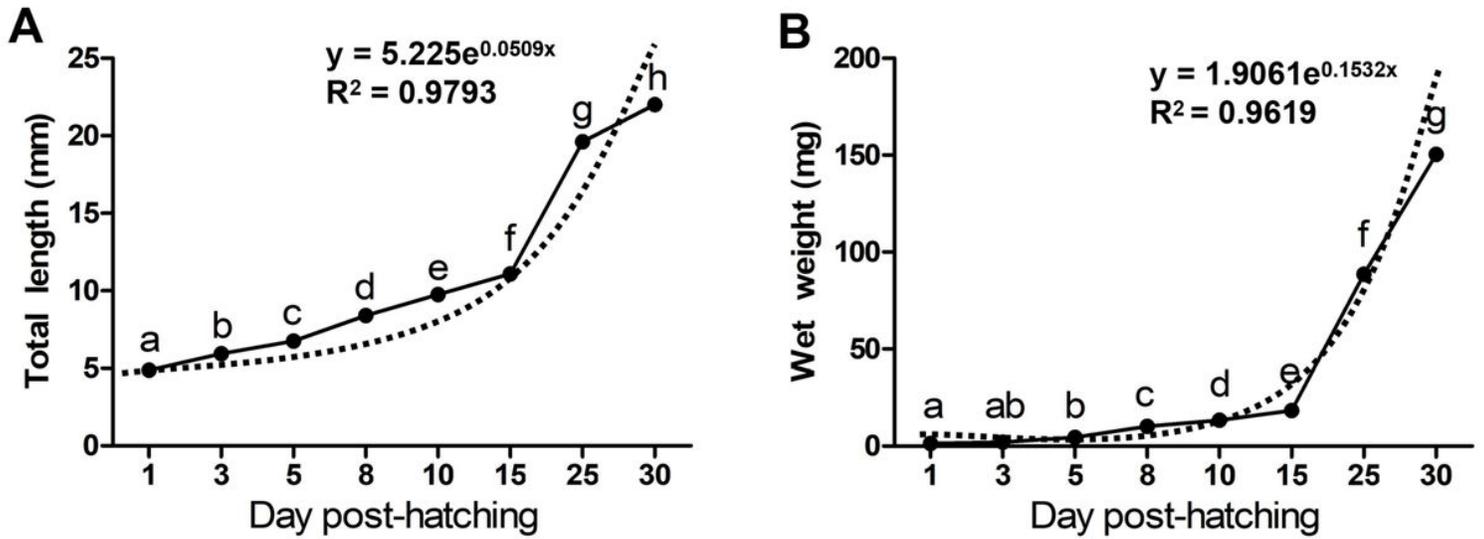


Figure 1

Growth in total length (A) and body weight (B) of *S. chuatsi* larvae from 1 to 30 days after hatching. The data was presented as mean \pm SEM. Different superscript letters indicate significant differences on different days (ANOVA, Tukey's HDS test, $a < b < c < d < e < f < g < h$, $p < 0.05$).

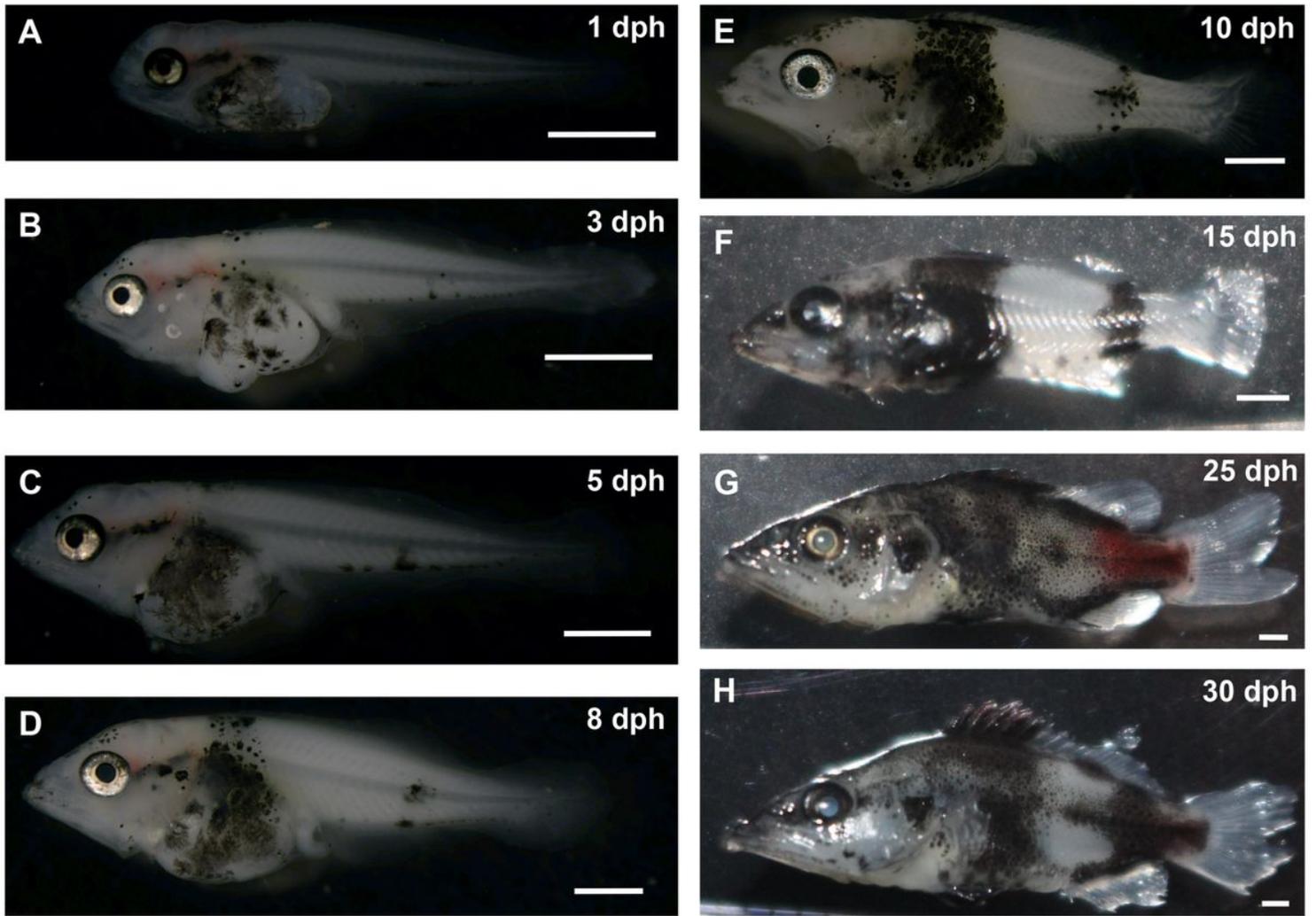


Figure 2

Morphology change of *S. chuatsi* larvae during the ontogenetic development. The scale bar = 1000 μm .

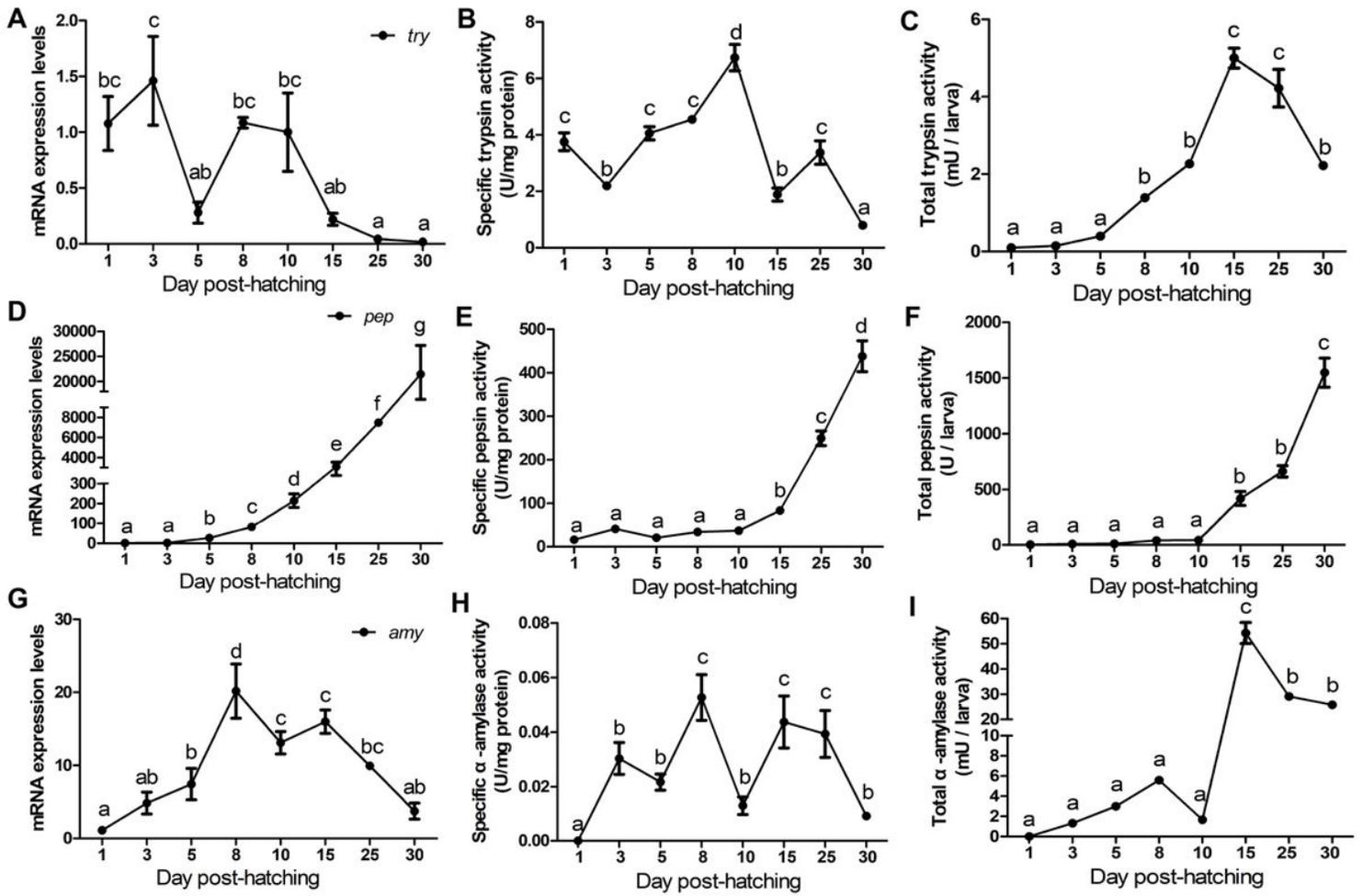


Figure 3

Dynamics of the gene expression and activity of trypsin (try), pepsin (pep) and α -amylase (amy) of whole-body *S. chuatsi* larvae during development. (A) expression of try gene; (B) specific activity of trypsin; (C) total activity of trypsin; (D) expression of pep gene; (E) specific activity of pepsin; (F) total activity of pepsin; (G) expression of amy gene; (H) specific activity of α -amylase; (I) total activity of α -amylase. Data are given as mean ($n = 3$ larval pools) \pm SEM. Different letters denote significant differences between larval stages, $p < 0.05$.

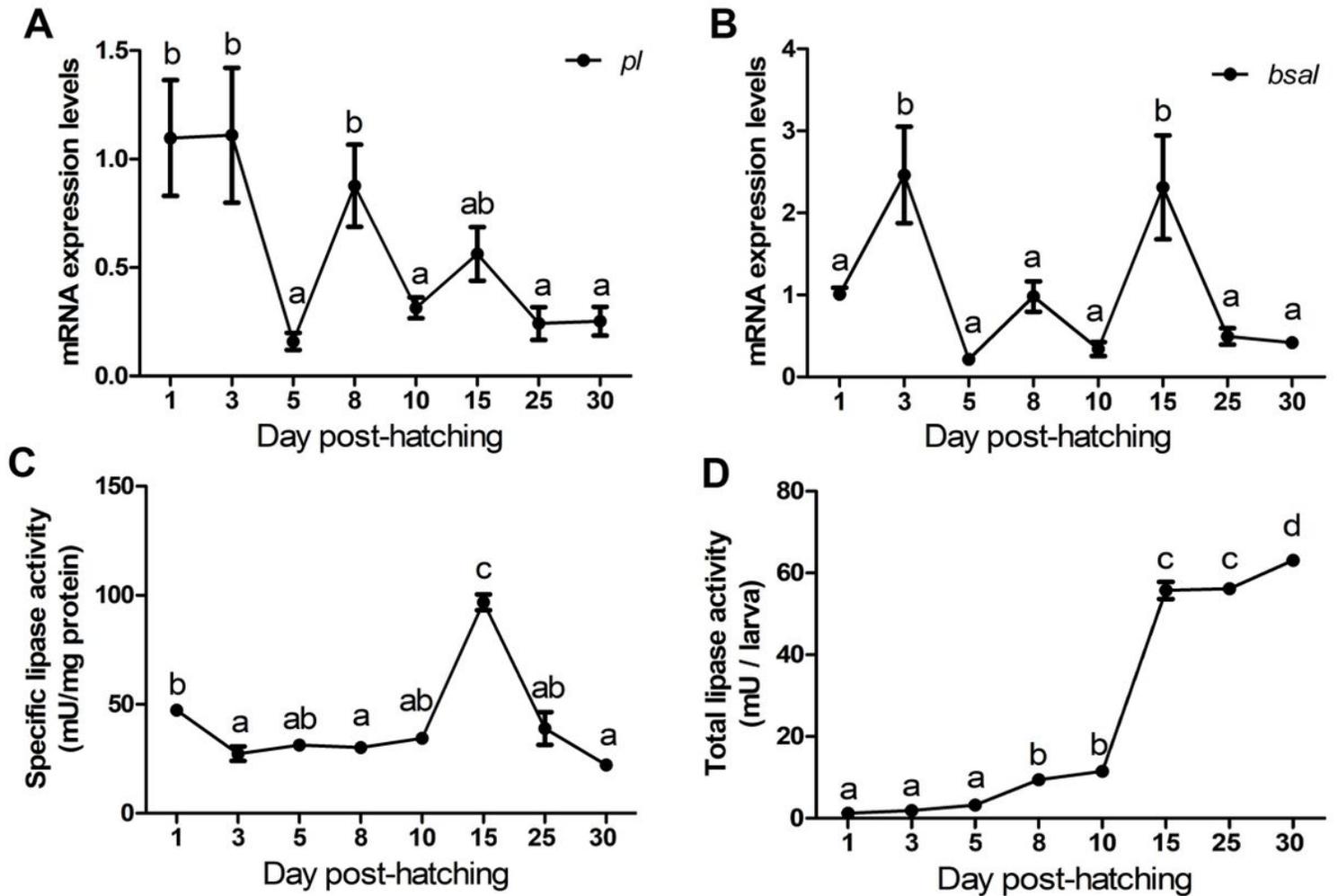


Figure 4

Dynamics of the gene expression and activity of lipase (*pl* and *bsal*) of whole-body *S. chuatsi* larvae during development. (A) expression of *pl* gene; (B) expression of *bsal* gene; (C) specific activity of lipase; (D) total activity of lipase. Data are given as mean ($n = 3$ larval pools) \pm SEM. Different letters denote significant differences between larval stages, $p < 0.05$.

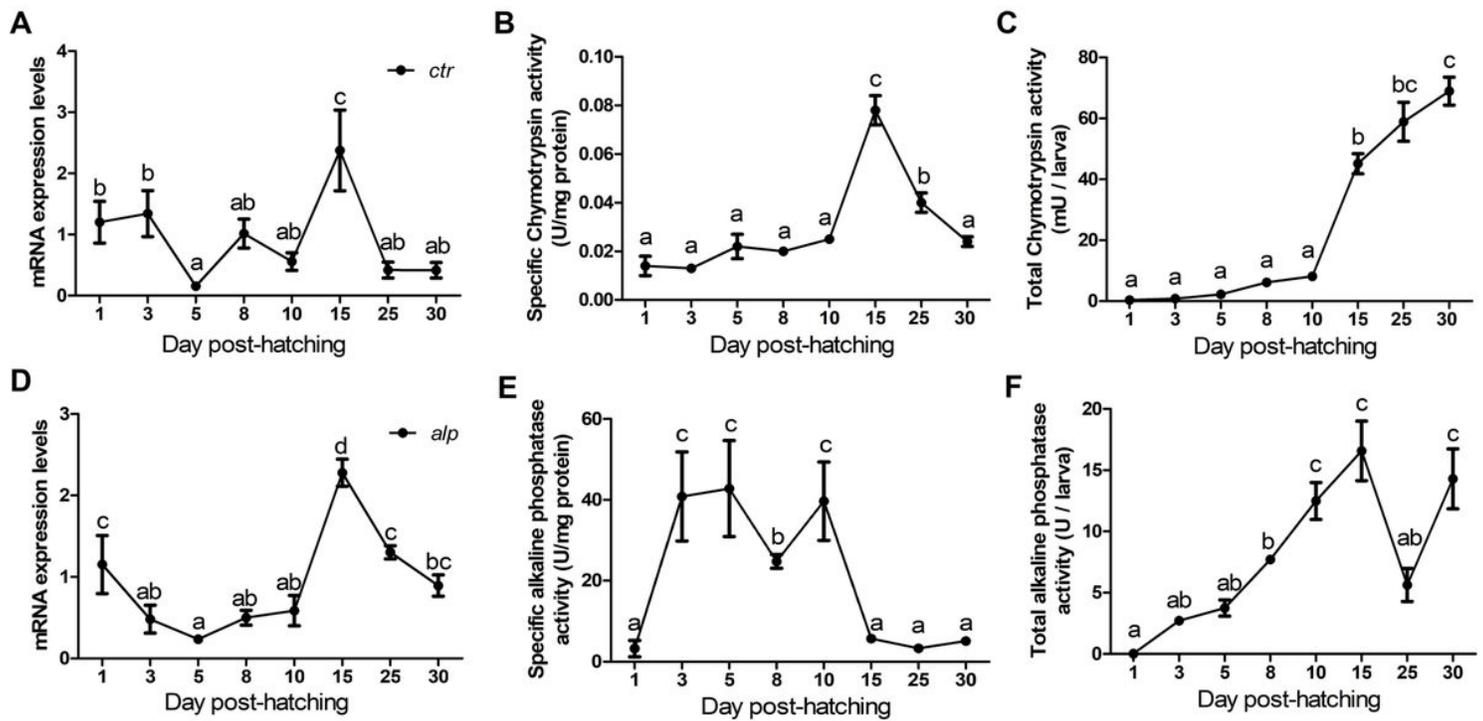


Figure 5

Dynamics of the gene expression and activity of chymotrypsin (*ctr*) and alkaline phosphatase (*alp*) of whole-body *S. chuatsi* larvae during development. (A) expression of *ctr* gene; (B) specific activity of chymotrypsin; (C) total activity of chymotrypsin; (D) expression of *alp* gene; (E) specific activity of alkaline phosphatase; (F) total activity of alkaline phosphatase. Data are given as mean ($n = 3$ larval pools) \pm SEM. Different letters denote significant differences between larval stages, $p < 0.05$.