animals

MDPI

Article

Intestinal tissue, digestive enzyme and antioxidant enzyme activities in the early development stage of Endangered *Brachymystax tsinlingensis*

Rongqun Song [1,4,†], Zhenlu Wang [1,4,†], Shaoqing Lin[2], Xingchen Guo [1], Yizhou Wang [1], Lin Zhang [1], Huan Ye[3], Jian Shao[1,4*]

[1] Laboratory of Fishery Resources and Environmental Protection, College of Animal Science, Guizhou University, The Key Laboratory of Animal Genetics, Breeding and Reproduction in the Plateau Mountainous Region, Ministry of Education, Guiyang 550025, China
[2] Tibet Animal Husbandry Service Center, Lhasa 850000, China.
[3] Key Laboratory of Freshwater Biodiversity Conservation, Ministry of Agriculture and Rural Affairs, Yangtze River Fisheries Research Institute, Chinese Academy of Fishery Sciences, Wuhan 430223, China.
[4] Special Fisheries Research Institute, Guizhou University, Guiyang 550025, China
* Correspondence: shaojian5098@163.com
† These authors contributed equally to this work.

Simple Summary: The wild population of *Brachymystax tsinlingensis* is a Grade II protected animal in China. Those were slow growth, harsh habitats, serious individual miniaturization, and a low survival rate of fry during its early developmental stage. In order to improve the survival rate of fry, the present study was conducted to investigate the changes in intestinal tissue structure, digestive enzymes, malondialdehyde content, and antioxidant enzyme activities, when their patterns during the early developmental stage of the *B. tsinlingensis*. By the way to increase the survival rate of fry, protect the species resources, and enrich the species' quantity.

Abstract: In order to explore the development characteristics of the digestive system, paving the way for artificial breeding and population conservation of endangered fish, this study was carried out on intestinal tissue, digestive enzymes and antioxidant enzyme activities in the early development stage of *Brachymystax tsinlingensis*. So, there were ten random samples about endogenous nutrition (7, 10, 11 Days After Hatching), mixed nutrition (13, 19 DAH), and exogenous nutrition (31, 33, 39, 45, 73 DAH), which were collected by histological and biochemical analysis methods. The results show that the intestine of *B. tsinlingensis* already has four layers initially at 7 DAH, and the intestinal gland tissue is evident at 73 DAH. The activity of lipase (LPS) and trypsin (TPS), including TP (3.20±0.26 mg/mL), increased the highest value all at 39 DAH (2.52±0.69 U/g and 2717.45±295.26 U/mg). Catalase (CAT) and glutathione peroxidase (GSH-PX) activity showed the lowest value all at 39 DAH (0.57±0.11 U/mg and 3.35±0.94 U/mg). The activity of amylase (AMS) and malonaldehyde (MDA) increased, and the highest values were 45 DAH (1.32±0.41 U/mg) and 73 DAH (1.29±0.43 nmoL/mg), respectively. Superoxide dismutase (SOD) and GSH-PX activity showed a peak value all at 7 DAH (126.58±20.13 U/mg and 6.47±1.86 U/mg). Overall, the changes in intestinal tissue, digestive enzymes and antioxidant enzyme activities 39 DAH of *B. tsinlingensis* are inseparable from different vegetative stage during developmental period, and these results of this study can provide reference for the proliferation and cultivation of *B. tsinlingensis* resources.

Keywords: *Brachymystax tsinlingensis*; Early development; Intestinal tissue structure; Digestive enzymes; Antioxidant enzymes

Citation:

Academic Editor:

Received:
Revised:
Accepted:
Published:

Copyright: © 2024 by the authors. Submitted for possible open access publication under the terms and conditions of the Creative Commons Attribution (CC BY) license (https://creativecommons.org/licenses/by/4.0/).

1. Introduction

The growth and development of early embryos or juveniles affects the subsequent growth of fish, and the development of digestive system tissues is also different due to the three endogenous, mixed and exogenous vegetative periods. When the transition of fry from endogenous yolk sac nutrition to mixed nutrition, and then to exogenous nutrition is often a period of high mortality rate of fry. The primary challenge in artificial fry breeding is lowering the mortality rate. To address this, we must study the digestive system development of fry and ensure they can effectively digest and absorb nutrients from their food [1]. Besides, it is important to consider the early stages of life in the study of ontogeny to understand nutrient storage and circulation, as most changes occur during this time [2]. And the information about the activity of intestinal enzymes during ontogeny is essential to understand the digestive efficiency of species at specific developmental stages [3]. The expression of digestive enzyme activity can be used as an indicator of the degree of food acceptance of the fry. To some extent, as an indicator of the digestive capacity in relation to the type of feed diet, understanding the digestive enzyme activity and nutrient requirements of the young fry of aquaculture fingerlings, which will help to study the optimal feeding regimen under hatchery conditions, greatly increasing yields [4].

B. tsinlingensis belongs to the order Salmoniformes, Salmonidae, and *Brachymystax*, commonly known as five-color fish and plum fish, which is endemic to China [5-9]. *B. tsinlingensis* is a carnivorous fish, feeding on shrimp, aquatic insects and aquatic invertebrates, also eating small fish and terrestrial insects blown down by the wind, feeding mostly in the morning when the light is weak and in the evening when there are more flying insects [5-6,10-13]. Unfortunately, the wild resources of *B. tsinlingensis* have declined dramatically in recent years, and it has been listed as "vulnerable" in the China Red Data Book of Endangered Animals and classified as a second-class state-protected wild animal in China. [10, 14-18]. In recently years, a large number of scholars have done research on the *B. tsinlingensis*, including the artificial breeding and seed cultivation technology [19-20], genetic diversity of the population [21-26], sperm ultramicrostructure [27], phototaxis behavior [28], digestive tract tissue [13,29], intestinal flora [30-31], and toxicity tests [18]. However, there are unknown information on the activity of intestinal tissue, digestive enzymes and antioxidant enzymes in the early development stage of *B. tsinlingensis*.

In this study, the intestinal tissues and the activities of digestive enzymes, malondialdehyde content and antioxidant enzymes in the early developmental stages of *B. tsinlingensis* were unfolded at the level of histological and biochemical analysis. To assess digestive function, we measure the total protein concentration of the participating gut, the activity of several key digestive enzymes (Lipase, Trypsin and Amylase), as well as the gut malondialdehyde content, and antioxidant indicator about the activity of several key antioxidant enzymes (Catalase, Glutathione peroxidase and Superoxide dismutase). These results could provide scientific guidance for the artificial seed cultivation technology of *B. tsinlingensis* and help us enrich the population of *B. tsinlingensis*.

2. Materials and Methods

2.1 Laboratory animals

Experiments were conducted from April to July 2023 at the *B. tsinlingensis* Artificial Breeding Experimental Base in the Qinling Mountain region (Shaanxi Province, China). The experimental samples were all from the same batch of parent *B. tsinlingensis* fertilized eggs, which were cultivated in 3.0 m × 0.4 m × 0.25 m seedling tanks, during which the water temperature was 13.66 ± 1.89 °C, pH was 7.08 ± 0.16, and dissolved oxygen was 7.56 ± 0.83 mg/L.

2.2 Specimen collection

According to the changes in intestinal physiology and biology in the early development stage of *B. tsinlingensis*, ten sampling points were collected in three stages: endogenous nutrition (7, 10,11 Days After Hatching), mixed nutrition (13, 19 DAH) and exogenous nutrition (31, 33, 39, 45, 73 DAH), among which 13-31 DAH were fed with harvest worms, 19 DAH yolk sacs disappeared, 33-39 DAH were fed with water earthworms, and 45-73 DAH were fed with feed. Each sample was collected two hours after feeding, and three samples were randomly collected and fixed in 4% paraformaldehyde, and the physiological development of intestinal tissues was observed. There are six tails were collected and stored in liquid nitrogen, and the activities of intestinal digestive enzymes, malondialdehyde content and antioxidant enzymes were subsequently detected.

2.3 H.E stained sections

The fixed tissues were soaked in 4% paraformaldehyde, paraffin-embedded and sectioned, deparaffinized by xylene, stained with hematoxylin-eosin, air-dried and then covered, and observed with an optical microscope (Nikon 80i) [32]. Intestinal villi height, villi width, and muscle thickness were measured using ImageJ software, and 10 points were randomly sampled for each sample and replicated three times.

2.4 Elisa test

Using enzyme activity assay kits, to check the total protein (TP) concentration, lipase (LPS), amylase (AMS), trypsin (TPS) activity, malondialdehyde (MDA) content, catalase (CAT), glutathione peroxidase (GSH-PX), and superoxide dismutase (SOD) activities were measured in all samples. The experimental procedures were carried out according to the manufacturer's instructions, and which were repeated six times to ensure consistency [33-34].

2.4 Data Processing and Analysis

The experimental results were expressed as mean ± standard deviation (Mean ± SD), one-way ANOVA was performed by SPSS 17.0 software, and Duncan's multiple comparison was used to analyze the significant difference between groups. Statistically significant differences were found when $p < 0.05$, and very significant differences when $p < 0.01$.

3. Results

3.1. Microscopic observation of intestinal tissue structure in the early development stage of Brachymystax tsinlingensis

Microscopic observation showed that the intestinal tissue structure of *B. tsinlingensis* had four layers of mucosal layer, submucosa, muscle layer and serous membrane at 7 DAH, which were mucosal folds. The number of goblet cells gradually increased with growth and development, and 73 DAH had intestinal gland structure (Fig. 1).

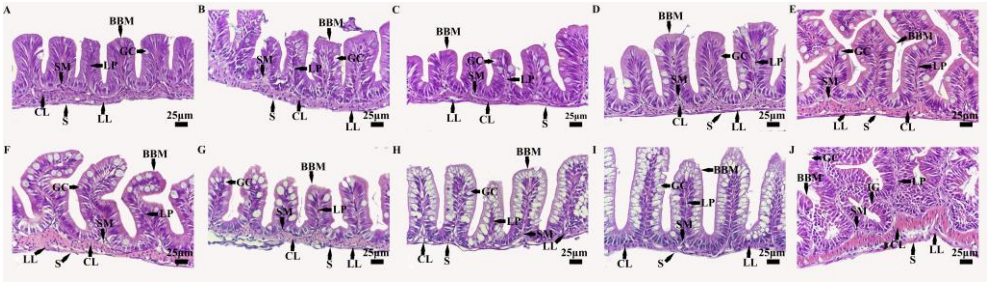


Figure. 1. Intestinal tissue structure of *Brachymystax tsinlingensis*. A: 7 Days After Hatching (DAH), B: 10 DAH, C: 11 DAH, D: 13 DAH, E: 19 DAH, F: 31 DAH, G: 33 DAH, H: 39 DAH, I: 45 DAH, J: 73 DAH. S: serous, LL: longitudinal layer of smooth muscle, CL: circular layer of smooth muscle, SM: submucosa, LP: lamina propria, GC: goblet cells, BBM: brush border microvillus, IG: intestinal glands

The microstructure of intestinal tissue was measured and analyzed (Table 1). The height of intestinal villi was maximum value at 45 DAH (187.31 ± 8.89 μm), and it was significantly greater than that at 7, 10, 11, 13, 39, and 73 DAH ($p < 0.01$). Simultaneously, the maximum value of intestinal villi width was at 45 DAH (69.33 ± 2.42 μm) significantly higher than that of 7, 10, 13, 33, and 39 DAH ($p < 0.01$). The thickness of the intestinal myometrium was maximum value at 73 DAH (30.54 ± 3.63 μm), and it was significantly greater than that at 7, 11, 13, 19, 39and 45 DAH ($p < 0.01$).

Table 1. Changes in intestinal tissue in the early developmental stage of *Brachymystax tsinlingensis*

Days After Hatching (DAH)	Items		
	Villus height (μm)	Lint width (μm)	Muscular thickness (μm)
7	97.58±13.26^{AB}	53.30±1.71^{AB}	18.82±2.03^{AB}
10	113.58±19.55^{ABC}	58.08±4.98^{ABC}	29.75±0.38^{CD}
11	87.66±7.09^{A}	61.39±8.05^{ABCD}	15.33±2.91^{A}
13	132.88±15.05^{ABC}	52.44±1.32^{A}	20.06±1.21^{AB}
19	154.94±14.70^{CD}	68.59±6.50^{CD}	22.95±1.48^{ABC}
31	153.48±41.42^{CD}	60.10±3.75^{ABCD}	19.97±3.09^{AB}
33	143.58±10.86^{BCD}	57.32±2.37^{AB}	24.54±2.96^{BCD}
39	120.00±6.73^{ABC}	55.72±3.08^{AB}	19.97±1.94^{AB}
45	187.31±8.89^{D}	69.33±2.42^{D}	19.76±5.84^{AB}
73	133.45±16.17^{ABC}	63.97±3.38^{BCD}	30.54±3.63^{D}

^{ABCD} Different superscript letters in the same column indicate that the height, width and thickness of intestinal villi at different stages are extremely different ($p < 0.01$).

3.2. Changes in intestinal digestive enzymes in the early development stage of Brachymystax tsinlingensis

The results demonstrated that the changes of intestinal digestive enzymes in the early development stage of *B. tsinlingensis* showed an upward trend. The total protease concentration showed an overall upward trend, in which the yolk sac disappeared to the stage of feeding earthworm (19-31 DAH) and the stage of switching to feed (39-45 DAH). More importantly, there were significant differences between 7, 10, 11 DAH and other groups ($p < 0.01$). LPS activity increased after the mixed trophic period, which decreased after reaching the peak of 39 DAH. Moreover, there was a significant difference between 39 DAH and 73 DAH ($p < 0.05$). TPS activity showed an upward trend during the exogenous vegetative period, and decreased after reaching the peak of 39 DAH. There was a significant difference between 39 DAH and 73 DAH ($p < 0.01$), while there was no significant difference in the other groups ($p > 0.05$). AMS activity showed an upward trend during the exogenous vegetative period, and decreased after reaching the peak of 45 DAH. There was no greater significant difference between 7, 10, 11, 13, 19, 31 and 73 DAH ($p > 0.01$), and there were extremely significant differences between 33, 39, 45 DAH and other groups ($p < 0.01$), among which there were extremely significant differences between 33 and 45

DAH ($p < 0.01$). The proportion of AMS boost enormously from 33 DAH to 45 DAH in the early development stage of *B. tsinlingensis*, but the relative percentage of TRS was relatively stable in the gross of intestinal digestive enzymes during the experiment (Fig 2, Fig 3).

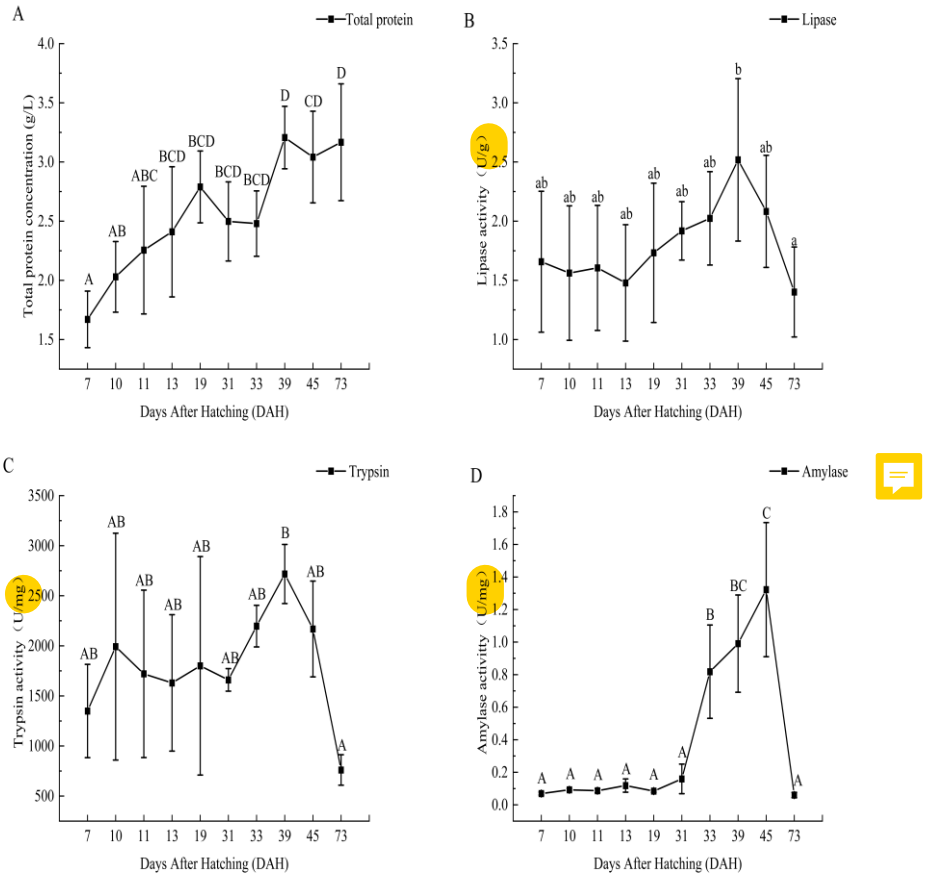


Figure. 2. Trends in intestinal digestive enzymes of Total protein concentration (A), Lipase activity (B), Trypsin activity (C), Amylase activity (D). Uppercase letters indicate extremely significant differences in digestive enzyme activity ($p < 0.01$), and lowercase letters indicate extremely significant differences in digestive enzyme activity ($p < 0.05$).

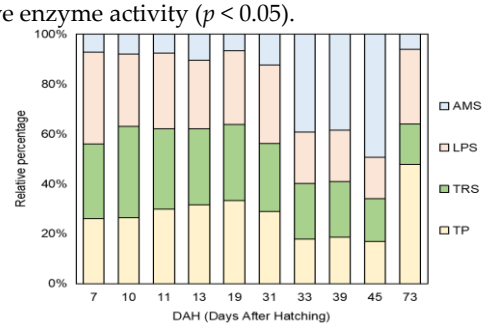


Figure. 3. The relative percentage of total protein concentration (TP), lipase activity (LPS), trypsin activity (TPS), Amylase activity (AMS) in the early development stage of *Brachymystax tsinlingensis*

3.3. Changes of intestinal malondialdehyde content and antioxidant enzymes in the early development stage of Brachymystax tsinlingensis

The results showed that the changes of intestinal antioxidant enzymes in the early development stage of *B. tsinlingensis* were as follows: the content of malondialdehyde showed an overall upward trend. And the content of malondialdehyde increased from the disappearance of the yolk sac in the exogenous vegetative period to the stage of feeding earthworm (19-39 DAH) and the feeding stage (45-73 DAH), which was a significant

difference in 73 DAH ($p < 0.01$). CAT activity decreased after the disappearance of yolk sac (19 DAH), but that increased after reaching the lowest peak at 39 DAH, and there was no significant difference in other groups ($p > 0.05$). The activity of GSH-PX showed a decreasing trend on the whole. Specifically, there was an upward trend from the disappearance of the yolk sac to the stage of feeding earthworms (19-33 DAH), and the stage of switching to feed (39-73 DAH) after the disappearance of the yolk sac in the exogenous trophic period. GSH-PX activity at 7 DAH was significantly higher than 39 DAH ($p < 0.01$). The activity of SOD showed a decreasing trend as a whole. The enzyme activity was slow from the disappearance of the yolk sac to the earthworm feeding stage (19-33 DAH) in the exogenous trophic period. However, the enzyme activity showed an upward trend in the feeding stage (45-73 DAH). There was no significant difference between the groups in the mixed trophic period (13-19 DAH) and the exogenous trophic period (31-73 DAH) ($p > 0.05$). The mixed trophic period and exogenous trophic period (13-73 DAH) were significantly higher than those in the endogenous trophic period (7-11 DAH) ($p < 0.05$). The relative percentage of four intestinal antioxidant enzymes was all relatively stable in the gross of intestinal digestive enzymes even at different vegetative period (Fig4, Fig 5).

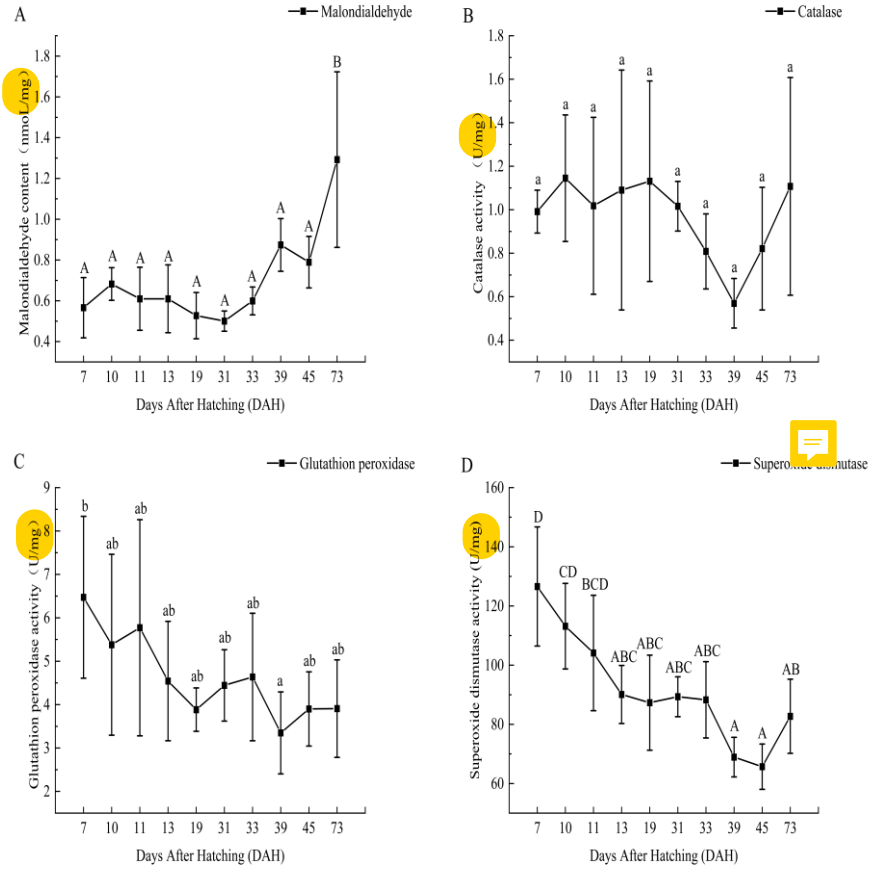


Figure. 4. Trends in intestinal antioxidant enzymes of Malondialdehyde content (A), Catalase activity (B), Glutathione peroxidase activity (C), Superoxide dismutase activity (D). Uppercase letters indicate extremely significant differences in digestive enzyme activity ($p < 0.01$), and lowercase letters indicate extremely significant differences in digestive enzyme activity ($p < 0.05$).

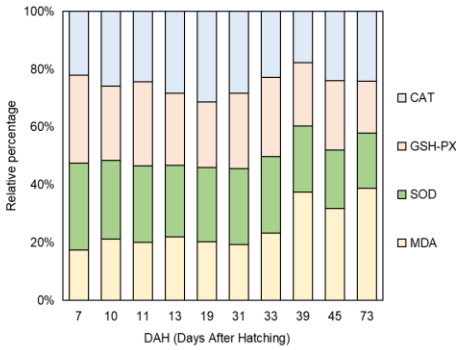


Figure. 5. The relative percentage of Malondialdehyde content (MDA), Catalase activity (CAT), Glutathione peroxidase activity (GSH-PX), Superoxide dismutase activity (SOD) in the early development stage of *Brachymystax tsinlingensis*

4. Discussion

4.1. The development of intestinal tissue structure in the early development stage of Brachymystax tsinlingensis

The intestine is an important place for digestion and absorption. In which that food is constantly digested, absorbed, and transported backwards in the intestine, and finally the residue is excreted as feces [35-36]. Juvenile *B. tsinlingensis* had a four-layer structure of mucosal layer, submucosa, muscle layer and adventitia at 7 DAH, with fold formation to increase the absorption area, as well as the goblet cells in the mucosal layer gradually increased with development and growth [13,29,37-38]. Surprisingly, the intestinal gland tissue was first spotted at 73 DAH, which was inconsistent with the research reports of the digestive system of *B. tsinlingensis* as before [13,29]. Intestinal morphology, such as villi height or epithelial thickness, is an indicator of the intestinal health of animals, including fish [39]. In this study, the height, width and thickness of intestinal villi were very significantly different when the yolk sac began to absorb and disappeared. Some studies have found that the increase in the number of goblet cells will increase the secretion of intestinal mucus in the juveniles of largemouth bass (*Micropterus salmoides*), which under ammonia nitrogen stress, leading to the increase in villi width and muscle thickness may be symptoms of oxidative stress and inflammation in the intestine [40]. A study found that feeding ferulic acid to genetically modified tilapia resulted in a decrease in villi height and width, possibly due to oxidized fish oil in the feed damaging the intestinal structure and reducing nutrient absorption [41]. In this study, the basic intestinal tissue structure of *B. tsinlingensis* was found at the endogenous trophic stage of 7 DAH, and the height, width and thickness of the intestinal villi changed with the growth and development of the fry. It showed that the change in intestinal villi was related to not only species itself but the condition of nutrient absorption mode and bait feeding.

4.2. Changes in intestinal digestive enzyme activity in the early development stage of Brachymystax tsinlingensis

The intestinal tract plays an important role in organ for nutrient absorption and digestive enzymes, importantly that measuring the activity of digestive enzymes in the intestine can reflect the digestion of the body to a certain extent [42]. The development of digestive enzymes reflects the functional development and digestive capacity of the digestive tract of organisms. Therefore, it is often used as a physiological biomarker to assess the nutritional status of fish in the early stages of life development [43]. In this study, the total protein concentration of *B. tsinlingensis* showed an overall upward trend during the early development. Consistent with the study on the change of digestive enzyme patterns in carp (*Cirrhinus mrigala*) and the study on the digestive enzyme development of juvenile perch (*Perca fluviatilis*), it was indicated that juveniles needed a large amount of protein

for growth and development [44-45]. However, with the yolk sac disappearing, the total protein concentration was rapidly decreased last more than 10 days, it may be the adjustment of bait adaptation of juveniles themselves during exogenous nutrition intervention. Meanwhile, the lipase activity began to increase after the mixed trophic period, indicating that the juveniles also absorbed the nutrients of the abundant insects when absorbing the nutrients of the yolk sac, and decreased after reaching the highest value of 39 DAH. Some studies showed an overall upward trend in the ontogeny of digestive enzymes of *Ompok bimaculatus* larvae and the changes in enzyme activity of Nile tilapia (*Oreochromis niloticus*) larvae fed with different CP levels [43,46]. The characteristics in the digestive enzyme pattern of carp (*Cirrhinus mrigala*), it was found that the change trend of LPS activity was consistent, and the LPS activity was affected by dietary changes [45]. In our study, TPS activity showed downward trend at the outset. And the early change of AMS activity was flat, which was consistent with the changes in TPA and AMS activities of greater amberjack developed under circadian rhythm [47]. The activities of TPS and AMS showed an upward trend during the exogenous vegetative period. The enzyme activity increased when the juveniles themselves had exhausted the nutrients in the yolk sac and switched to eating earthworms, it indicated that the digestibility of juveniles to earthworm ingestion increased. However, TPS activity decreased after reaching the peak of 39 DAH, and AMS activity decreased after reaching the peak of 45 DAH. The present study revealed that conversion of endogenous nutrients to exogenous nutrients is critical period in the early development stage of *B. tsinlingensis* as carnivorous fishes.

4.3. Changes in intestinal malondialdehyde content and antioxidant enzyme activity in the early development stage of Brachymystax tsinlingensis

Oxidative stress can cause damage to body cells and tissues [31], and MDA is an indicator of oxidative stress and antioxidants [48-49]. Moreover, GSH-PX, SOD and CAT are essentially the first line of defense against antioxidants, and their efficacy and role are essential for the entire antioxidant defense approach [49-50]. We found that the response of the content of MDA showed an overall upward trend, indicating that the nutrient intake of juveniles had an oxidative effect on the fry itself during growth and development, which continued to increase. CAT activity showed a downward trend after the disappearance of the yolk sac. Moreover, it was speculated that there were fewer free radicals such as hydrogen peroxide in the fry at this time, and increased after the lowest value of 39 DAH. The activities of GSH-PX and SOD showed a decreasing trend as a whole, indicating that the antioxidant response mechanism was not obvious in the whole early developmental stage. GSH-PX showed an upward trend in the stage when the yolk sac disappeared to the stage of feeding earthworms. Furthermore, it is speculated that the fry themselves do not form antioxidant respond ability during development. Effects of hypoxia and reoxygenation on oxidative stress, histological structure, and apoptosis in a new hypoxia-tolerant variety of blunt snout bream (*Megalobrama amblycephala*) [51], and age and sex dependent variation in the activity of antioxidant enzymes in the brown trout (*Salmo trutta*) [52]. Failure to catalyze free radicals such as H_2O_2 in time, resulting in oxidative stress damage. In our study, the feeding of bait also causes oxidative stress to the fish itself, which cannot reduce the damage of oxidative stress and remove the toxic effect of cells in time. Consistent with the results of the changes of oxidative status in yellowfin seabream (*Acanthopagrus latus*) larvae during development [53], juveniles had low antioxidant response and oxidative damage in the early stage, and oxidative damage was greater with growth and development.

5. Conclusions

In summary, the intestinal tissue of *B. tsinlingensis* had formed a relatively complete digestive structure at 7 DAH in the early development stage, and there was little change in the subsequent development. It founded the formation of intestinal gland tissue at 73

DAH. During the early development stage of *B. tsinlingensis*, the digestive enzymes and antioxidant enzyme activities trend has unique characteristics that different from other species. Namely, the activities of digestive enzyme and antioxidant enzyme changed with the growth and development and the transfer of bait for a long time. *B. tsinlingensis* could not reduce oxidative stress damage, especially in the early development period. It suggested that the absorption and digestibility of fish fry are improved to better adapt to the slow growth and harsh living environment. The results showed that it is necessary to pay close attention to the change of nutrient absorption mode of fish fry before 19 DAH, and we suggested that timely feed exogenous bait (live food) to solve the problem of high lethality rate of fish fry during the vital feeding period (19-45 DAH) of *B. tsinlingensis*.

Author Contributions: R. S: Conducted experiment and drafted paper. Z. Wang and X. G: Completed the data collection and analysis. Y. W and L. Z: Carried out the experiment. X. P: Assisted in designing study and analyzing data. D. X: Assisted in the experiment. H. Ji: Polished the language. J. S: Designed study and modified paper. All authors have read and agreed to the published version of the manuscript.

Funding: This research was supported by National Natural Science Foundation of China (grant/award no: 32002392), the Fund for Youth Science and Technology Talents Growth of Guizhou Education Department of China (grant/award no: 202437) and the Open Project for Key Lab of Freshwater Biodiversity Conservation, Ministry of Agriculture of China (grant/award no: LFBC1109).

Institutional Review Board Statement: In this study, all experimental procedures were conducted according to Inspection Form for Guizhou University Experimental Animal Ethics (Approval Code: EAE-GZU-2020-T041; Approval Date: April 14, 2020).

Informed Consent Statement: Not applicable.

Data Availability Statement: Data will be made available on request

Acknowledgments: We gratefully acknowledge J. Li, X. Liu and X. Ye for their unselfish help during the experiment. We also thank Dr. J Li, H Jiang and Prof. M. An for assistance in the preparation of this manuscript.

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

References

1. Gawlicka, A.; Parent, B.; Horn, M.; Ross, N.; Opstad, I.; Torrissen, O. Activity of digestive enzymes in yolk-sac larvae of Atlantic halibut (*Hippoglossus hippoglossus*): indication of readiness for first feeding. *Aquaculture* **2000**, 184, 303-314.
2. Downs, K.; Kelly, P.; Ascanio, A.; Vanni, M. Ontogenetic variation in the ecological stoichiometry of fish species during early development. *Ecology* **2023**, 104, e4176.
3. Rathore, R.; Kumar, S.; Chakrabarti, R. Digestive enzyme patterns and evaluation of protease classes in Catla (family: *Cyprinidae*) during early developmental stages. *Comparative biochemistry and physiology. Part B, Biochemistry and Molecular Biology* **2005**, 142, 98-106.
4. Suzer, C.; Kamac, H.; Coban, D.; Yıldırım, S.; Fırat, K.; Saka, S. Functional changes in digestive enzyme activities of meagre (*Argyrosomus regius*; Asso, 1801) during early ontogeny. *Fish Physiology and Biochemistry* **2013**, 39, 967-977.
5. Li, S. On new subspecies of fresh-water trout, *Brachymystax lenok tsinlingensis*, from taipaishan，shensi，China. *Acta Zootaxonomica Sinica* **1966**, 3, 92-94.
6. Li, S. Discussion on the geographic distribution of the fishes of the family Troglophilidae in China. *Journal of Zoology 1984*, 1, 34-37.
7. Meng, Y.; Wang, G.; Xiong, D.; Liu, H.; Liu, X.; Wang, L.; Zhang, J. Geometric morphometric analysis of the morphological variation among three lenoks of genus brachymystax in China. *Pakistan journal of zoology* **2018**, 50, 885-895.
8. Xing, Y.; L., B.; Ye, E.; Fan, E.; Li, S.; Wang, L.; Zhang, C.; Zhao, Y. Revalidation and redescription of *Brachymystax tsinlingensis* Li, 1966 (Salmoniformes: Salmonidae) from China. *Zootaxa* **2015**, 3962, 191-205.
9. Zhao, Y., Zhang, C. Threatened fishes of the world: *Brachymystax lenok tsinlingensis* Li, 1966 (Salmonidae). *Environmental Biology of Fishes* **2009**, 86, 11-12.
10. Yue, P.; Chen, Y. China red data book of endangered animals. *Beijing: Pisces. Science Press* **1998**, 107-109.
11. Gao, X.; Liu, Z.; Li, Q.; Qin, Y.; W. L.; Tang, P. Embryonic and larval development of *Brachymystax lenok tsinlingensis* in Weihe River. *Journal of Gansu Agricultural University* **2014**, 57, 43-50.

12. Meng, Y.; Wang, G.; Xiong, D.; Liu, H.; Zhang, J.; Wang, J.; Wang, L.; Liu, X. The validity of subspecies of *Brachymystax lenok tsinlingensis* Li based on morphological difference analysis. *Acta Hydrobiologica Sinica* **2018**, 42, 550-560.
13. Xiong, D.; Meng, Y.; Yu, H.; Liu, X.; Liu, H. Structure and ultrastructure studies of the digestive tract of the endangered Qinling lenok (*Brachymystax tsinlingensis* Li, 1966). *Agricultural Research,Education and Extension Organization* **2019**, 18, 981-995.
14. Ren, j. Liang, G. Resource survey report of *Brachymystax lenok tsinlingensi* in Qianhe river valleys of Qinling mountains. *Journal of Shaanxi Normal University (Natural Science Edition)* **2004**, S2, 165-168+174.
15. Wu, J.; Yang, H.; Shao, J.; Du, H.; Wang, C.; Wei, Q. Habitat environmental characteristics of *Brachymystax lenok tsinlingensis*. *Acta Hydrobiologica Sinica* **2017**, 41, 214-219.
16. Yang, D.; Li, J.; Wu, J.; Wang, F.; Wen, S.; Xiong, D.; Shao, J. Autumn survey of aquatic biological resources in the Qianhe River Basin. *Chinese Journal of Fisheries* **2021**, 34, 49-58.
17. Zhang, J.; B, K.; Ma, J.; Gou, N.; Wang, K. Fish resource status and assessment of protection measures in the Qinling Ganyu River. *Journal of Hydroecology* **2019**, 40, 30-34.
18. Guo, X.; Chu, Z.; Song, R.; Wang, Z.; Li, R.; Xiong, D.; Zhang, M.; Jiang, H.; Shao, J. Toxicity of common biocides used in aquaculture to embryos and larvae of *Brachymystax tsinlingensis* Li. *Journal of Fish Biology* **2023**, 30, 1–12.
19. Shao, J.; Wei, Q.; Wu, J., Sun Q. A method of artificial propagation of *Brachymystax tsinlingensis*. Hu bei (China): CN104041457A, **2016.**
20. Shao, J.; Chu, Z.; Lu, B.; Wei, Q.; Wang, F. Effects of different baits on the growth and survival of seedling *Brachymytax tsinlingensis*. Scientific Fish Farming, **2018**, 8, 26-28.
21. Liu, H.; Li, Y.; Liu, X.; Zou, G.; Wei, Q. Isolation and characterization of eleven novel microsatellite loci of *Brachymystax lenok tsinlingensis*, a threatened fish endemic to Shaanxi, China. *Conservation Genetics Resources* **2013**, 5, 389-391.
22. Huang, J.; Zhang, J.; Fang, C.; Jin, T.; Wang, K. A review of research progress and individual miniaturization of *Brachymystax tsinlingensis*. *Chinese Journal of Fisheries* **2021**, 34, 16-21.
23. Shao, J.; Xiong, D.; Chu, Z.; Wu, J.; Du, H.; Wang, F.; Wen, S.; Wei, Q. Population differentiation and genetic diversity of endangered *Brachymystax tsinlingensis* Li betweenYangtze River and Yellow River in China based on mtDNA. *Mitochondrial DNA A DNA Mapp Seq Anal* **2019**, 30, 695-701.
24. Shao, J.; Luo, W.; Wei, Q.; Wang, F.; Guo, W.; Ye, H.; Chu, Z.; Wu J.; Zhang S. Assignment of parentage by microsatellite analysis in the endangered *Brachymystax lenok tsinlingensis* (Salmonidae). Aquatic Biology, **2017**, 26, 69-73.
25. Wen, S.; Li, P.; Wang, F.; Li, J.; Liu, H.; Li, N. De novo assembly and microsatellite marker development of the transcriptome of the endangered *Brachymystax lenok tsinlingensis*. *Genes & Genomics* **2020**, 42, 727-734.
26. Zhu, W.; Wang, Z.; Li, H.; Li, P.; Ni, L.; Jiao, L.; Ren, Y.; You, P. A chromosome-level genome of *Brachymystax tsinlingensis* provides resources and insights into salmonids evolution. *G3 (Bethesda, Md.)* **2022**, 12, 162.
27. Guo, W.; Shao, J.; Li, P.; Wu, J.; Wei, Q. Morphology and ultrastructure of *Brachymystax lenok tsinlingensis* spermatozoa by scanning and transmission electron microscopy. *Tissue & Cell* **2016**, 48, 321-327.
28. Zhang, L., Lan, K.; Song, R.; Xiong, D.; Li R.; Li, J.; Jiang, H.; Shao, J. Selective behavior of juvenile *Brachymystax tsinlingensis* depends on substrate color, light intensity, and light color. *Progress in Fishery Sciences* **2023**, 44, 179-187.
29. Qing, S.; Zhang, W.; Qiang, X.; Wang, G. The morphological observation on digestive system of *Brachymystax lenok tsinlingensis* Li. *Chinese Journal of Veterinary Science* **2012**, 32, 889-893.
30. Huang, M.; Deng, B.; Xie, X.; Wu, X.; Li, Y. Analysis of microbial diversity and enzyme activity in the gut of wild *Brachymystax lenok* from Qinling mountains. *Chinese Journal of Zoology* **2018**, 53, 114-125.
31. Ma, F.; Ma, R.; Zou, Y.; Zhao, L. Effect of astaxanthin on the antioxidant capacity and intestinal microbiota of tsinling lenok trout (*Brachymystax lenok tsinlingensis*). *Mar Biotechnol (NY)* **2022**, 24, 1125-1137.
32. Rawski, M.; Mazurkiewicz, J.; Kieronczyk, B.; Jozefiak, D. Black soldier fly full-fat larvae meal is more profitable than fish meal and fish oil in Siberian sturgeon farming: the effects on aquaculture sustainability, economy and fish GIT development. *Animals (Basel)* **2021**, 11, 604.
33. Jia, P.; Guo, H.; Liu, B.; Guo, L.; Zhang, N.; Zhu, K.; Jiang, S.; Zhang, D. Effects of cryopreservation on the physiological characteristics and enzyme activities of yellowfin seabream sperm, *Acanthopagrus latus* (Houttuyn 1782). *Aquaculture* **2022**, 549, 737-739.
34. Pan, J.; Zhu, K.; Liu, J.; Guo, H.; Liu, B.; Zhang, N.; Xian, L.; Zhang, D. Cryopreservation of Goldlined seabream *Rhabdosargus sarba* (Forsskal, 1775) sperm: CASA observation and enzyme activity evaluation. *Aquaculture*, **2023,** 740494.
35. Guan, H.; Xu, W.; Kuang, Y.; Zhao, C.; Yin, J. Histological comparison and observation on the digestive system of the baby fish in two kinds of salmonid fishes. *Journal of Shanghai Ocean University* **2009**, 18, 282-288.
36. Zhang, Y.; Jia, Z.; Liu, Y.; Tong, G.; Yin, J. Studies on morphology and histology of digesting system of *Hucho taimen*. *Freshwater Fisheries* **2011**, 41, 30-35.
37. Li P.; Liu W.; Lu, W.; Tang F.; Wang, J. Morphology and histology observation of the digestive organs and liver of Chum salmon (*Oncorhynchus keta*). *Journal of Southern Agriculture* **2022**, 53, 1457-1465.
38. Ren, Y.; Nie, Z.; Yang, Z.; Zhang, S.; Wei, J. Morphology and histology studies of the digestive system of *Schizothorax biddulphi*. *Progress in Fishery Sciences* **2020**, 41, 49-57.
39. Jenabi, H.; Sarvi, M.; Mohseni, M.; Imani, A. Effect of dietary soybean lecithin on fish performance, hemato-immunological parameters, lipid biochemistry, antioxidant status, digestive enzymes activity and intestinal histomorphometry of pre-spawning Caspian brown trout (*Salmo trutta caspius*). *Fish & Shellfish Immunology* **2019**, 91, 50-57.

40. Wei, M.; Zheng, T.; Lu, S.; Qiang, J.; Tao, Y.; Li, Y.; Xu, P. Ammonia-N stress on tissue structure, enzyme activity and intestinal microbiota of *Macropterus salmoidest*. *Acta Hydrobiologica Sinica* **2024**, 48, 10-22.
41. Yu, L.; Wen, H.; Jiang, M.; Wu, F.; Tian, J.; Lu, X.; Xiao, J.; Liu, W. Effects of ferulic acid on intestinal enzyme activities, morphology, microbiome composition of genetically improved farmed tilapia (*Oreochromis niloticus*) fed oxidized fish oil. *Aquaculture* **2020**, 528, 735543.
42. Xu, A.; Shang, G.; Li, Z.; Gao, Z.; Huang, Y.; Chen, Q. Effects of dietary asafoetida (*Ferula sinkiangensis* K. M. Shen) levels on feeding attraction activity, growth performance, healthiness, and digestive enzyme activity in juvenile Lateolabrax japonicus. *Fish Physiology and Biochemistry* **2020**, 46, 1991-2003.
43. Pradhan, P.; Jena, J.; Mitra, G.; Sood, N.; Gisbert, E. Ontogeny of the digestive enzymes in butter catfish *Ompok bimaculatus* (Bloch) larvae. *Aquaculture*, **2013**, 372-375: 62-69.
44. Cuvier, P.; Kestemont, P. Development of some digestive enzymes in Eurasian perch larvae *Perca fluviatilis*. *Fish Physiology and Biochemistry* **2001**, 24, 279-285.
45. Chakrabarti, R.; Rathore, R. Ontogenic changes in the digestive enzyme patterns and characterization of proteases in Indian major carp *Cirrhinus mrigala*. *Aquaculture Nutrition* **2010**, 16, 569-581.
46. Silva, W.; Costa, L.; Lopezolmeda, J.; Costa, N.; Santos, W.; Ribeiro, P.; Luz, R. Gene expression, enzyme activity and performance of Nile tilapia larvae fed with diets of different CP levels. *Animal: an international journal of animal bioscience* **2019**, 13, 1376-1384.
47. Gamberoni, P.; Yufera, M.; Las, H.; Siguero, I.; Gilannejad, N.; Martinez, R.; Navarro, G. Ontogeny and diurnal patterns of molecular gene expression and activity of digestive enzymes in developing greater amberjack. *Aquaculture* **2021**, 534, 736330.
48. Xi, J.; Shao, J.; Wang, Y.; Wang, X.; Yang, H.; Zhang, X.; Xiong, D. Acute toxicity of triflumizole to freshwater green algae Chlorella vulgaris. *Pesticide Biochemistry and Physiology* **2019**, 158, 135-142.
49. Wang, Z.; Guo, X.; Tu, J.; Shi, X.; Gan, L.; Zhang, M.; Jiang, H.; Zhang, X.; Shao, J. Responses of *Micropterus salmoides* under ammonia stress and the effects of a potential ammonia antidote. *Animals* **2023**, 13, 397.
50. Amenyogbe, E.; Luo, J.; Fu, W.; Abarike, E.; Wang, Z.; Huang, J.; Ayisi, C.; Chen, G. Effects of autochthonous strains mixture on gut microbiota and metabolic profile in cobia (*Rachycentron canadum*). *Scientific Reports* **2022**, 12, 17410.
51. Shuang, L.; Chen, S.; Ren, C.; Su, X.; Xu, X.; Zheng, G.; Zou, S. Effects of hypoxia and reoxygenation on oxidative stress, histological structure, and apoptosis in a new hypoxia-tolerant variety of blunt snout bream (*Megalobrama amblycephala*). *Comparative biochemistry and physiology. Part A, Molecular and integrative physiology* **2023**, 278, 111358.
52. Parolini, M.; Iacobuzio, R.; De Felice, B.; Bassano, B.; Pennati, R.; Saino, N. Age and sex dependent variation in the activity of antioxidant enzymes in the Brown trout (*Salmo trutta*). *Fish physiology and biochemistry* **2019**, 45, 145-154.
53. Morshedi, V.; Noori, F.; Prokic, M.; Petrovic, T.; Jafari, F.; Moradian, S. Changes of oxidative status in yellowfin seabream larvae (*Acanthopagrus latus*) during development. *Comparative biochemistry and physiology. Part A, Molecular & Integrative Physiology* **2023**, 281, 111433.

Disclaimer/Publisher's Note: The statements, opinions and data contained in all publications are solely those of the individual author(s) and contributor(s) and not of MDPI and/or the editor(s). MDPI and/or the editor(s) disclaim responsibility for any injury to people or property resulting from any ideas, methods, instructions or products referred to in the content.