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Evaluation of the Effectiveness of Marking Juvenile *Takifugu obscurus* Otoliths with Strontium

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Abstract: Strontium marking technology is commonly used for extensive marking in stock enhancement and releasing activities. In order to explore the feasibility of this technology for juvenile *Takifugu obscurus*, five different concentrations of strontium (0, 12, 18, 36, and 72 mg/L) were set up by strontium chloride hexahydrate ($SrCl_2 \cdot 6H_2O$) and *T. obscurus* were immersed for 7 days. Then, *T. obscurus* were reared in non-additive water for 25 days. The results showed that the survival rate of all groups was 100%, except the 72 mg/L group, after 7 days of immersion. Moreover, the growths of all the marked groups were better than that of the control group. There was no significant difference between the control and marked groups, except for the 18 mg/L group, indicating that the appropriate concentration of strontium might have positive effect for *T. obscurus*. The strontium to calcium (*Sr*/*Ca*) ratios of otoliths in the marked groups increased with increasing concentration and time, which were higher than the baseline, respectively. Then, the *Sr*/*Ca* ratios returned to the original level, but the peak value was still retained, suggesting that the success rate of the strontium marking reached 100%. Notably, the residual strontium content of 18 mg/L group was insufficient for causing negative effects for *T. obscurus* after 25 days. Therefore, in consideration of the growth, survival, and effect of strontium marking on otolith, 18 mg/L is an appropriate concentration for strontium marking of juvenile *T. obscurus*. This study confirmed the feasibility of strontium marking for *T. obscurus* and provided a new approach to rationally and scientifically evaluate the stock enhancement and releasing efficiency of *T. obscurus*.

Keywords: *Takifugu obscurus*; otolith; strontium marking; enhancement and release; stock assessment

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1. Introduction

Takifugu obscurus, known as porcupine fish, is a migratory fish belonging to the *Takifugu*. As a traditional aquatic product of Yangtze River in China, *T. obscurus* is widely distributed in the east China Sea, the Yellow Sea, the Korean Peninsula, and Japan [1]. *T. obscurus*, with low fat and high protein, has high nutritional and medical value. Therefore, *T. obscurus* has become more and more popular in China, Korea, and Japan [2]. The production of *T. obscurus* in Jiangsu section of Yangtze River reached 1000 t in the 1950s [3]. However, due to the water pollution and overexploitation for commercial purposes, the wild resources of *T. obscurus* have declined rapidly to cause near extinction [4]. At present, wild *T. obscurus* is hardly easy to catch, compared to the 1950s, which needs attention. To solve the decline of *T. obscurus*, the stock enhancement and releasing activities have been carried out continuously since 2002.

The stock enhancement and releasing activity is considered one of the most effective ways to restore the wild resources of *T. obscurus*. A large number of *T. obscurus* were released into the Yangtze River every year, reaching nearly 10 million tons in just a decade [5].

However, the releasing effect of *T. obscurus* has yet to be evaluated. To estimate the stock enhancement and releasing effectiveness of *T. obscurus*, the mark–recapture monitor is essential, and marking *T. obscurus* is useful before release [6]. The ways of fish marking technique include the coded wire tag (CWT) [7,8], fluorescent marking [9], scutcheon tag [10,11], and microsatellite DNA marker [12]. However, these methods are limited, due to high cost, high mortality, easy shedding of tags, and the inapplicability to small size fish [13,14]. For example, a PIT tag from Biomark, Inc. (Kenilworth, IL, USA) costs US $1.6–2.78 and a T-bar anchor tag from Floy Tag and Manufacturing, Inc. (Seattle, WA, USA) costs US $0.77–1.7, and an operculum hole punch is only applicable to large-size fish [15]. Therefore, it is essential to explore a suitable method for the large-scale marking of *T. obscurus*.

In recent years, chemical marking has become an effective method for marking different fish widely and quickly [16,17]. Otolith fluorescent marks [18], stable isotope otolith fingerprint marks [19], strontium marks in otolith [20], and otolith thermal marks [18] are more broadly used. Strontium mark in otolith, as a new method, has been widely applied in various teleost fish, such as *Gadus callarias* [21], *Oncorhynchus keta* [22], *Acipenser fulvescens* [23], and *Anguilla anguilla* [24]. Because otoliths are metabolically inert, it is difficult to change the fingerprints for the element once it is deposited. Strontium, a trace element in the water environment, could form an inert mark on the otoliths, which does not change with time migration [25]. Compared to fresh water, seawater has a higher level of strontium [26], indicating that information about the habitat environment of fish can be acquired [27]. By artificially changing the content of strontium in water, an 'elemental fingerprint' is formed in otoliths, which can be used to distinguish wild populations from released populations, so as to better evaluate the effect of stock enhancement and releasing. At present, the feasibility of strontium marking in some species without negative effects on growth and survival has been proven. However, reports about the application of strontium marking technology in *Takifugu* are scarce. Therefore, strontium chloride hexahydrate was used to mark *T. obscurus* in this study. The aim was to evaluate the effect of strontium marking on the survival, growth, mark quality in otoliths and strontium residue in *T. obscurus*. This study will provide an important reference for the stock enhancement and releasing strategy, implementation, and adaptive management in the future.

2. Materials and Methods

2.1. Experimental Design

The experiment was conducted in an experimental base *with complete facilities* in Shanghai. The juvenile fish of 166 days (average length 10.42 ± 1.29 cm, average weight 40.00 ± 13.14 g) were all healthy and undamaged individuals. In this experiment, 1200 juvenile *T. obscurus* were randomly selected and placed in buckets (300 L of water), with 60 fish in each bucket. According to the content of strontium in water, four marked groups (12 mg/L, 18 mg/L, 36 mg/L, and 72 mg/L) and one control group were set in the experiment, with four parallel samples in each group. Totals of 10.95 g (12 mg/L), 16.43 g (18 mg/L), 32.86 g (36 mg/L), and 65.72 g (72 mg/L) of strontium chloride hexahydrate ($SrCl_2 \cdot 6H_2O$) were added to each bucket, respectively. Strontium chloride hexahydrate, dissolved in fresh water, was obtained from China, Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China).

The experimental water quality parameters were monitored daily to maintain conditions of temperature 27.6–28.6 °C, salinity 2.62–3.93‰, pH 7.18–7.85, and dissolved oxygen above 7 mg/L. The survival of juvenile fish was observed every day, and the dead fish were disposed of in time. Before immersion, all experimental fish were transferred to the bucket used for the experiment without feeding for 24 h to acclimatize. On the fourth day, 50% water was changed. Then, 5.48 g (12 mg/L), 8.22 g (18 mg/L), 16.43 g (36 mg/L), and 32.86 g (72 mg/L) of $SrCl_2 \cdot 6H_2O$ were added to regulate the concentration of Sr^{2+} in the water of each group. After the immersion of 7 days, all groups were cultured for 25 d in water without added strontium.

2.2. Sample Analysis

After 24 h of immersion, samples were taken at Days 1, 3, 5, and 7, with 6 fish randomly sampled each time. After measuring the body length (accurate to 0.01 mm) and weight (accurate to 0.01 g) of samples, the otoliths were taken out and stored at room temperature for testing the effect of marking. Gills, livers, and muscles were frozen and stored to detect residual strontium by inductively coupled plasma mass spectrometry (ICP-MS, the first method of GB5009.268-2016).

According to the size of otolith (sagittaes, asteriscus, and lapillus), the sagittal otolites were selected for analysis. After the embedding, grinding, and polishing of the sagittal otoliths [28], the core of the sagittal otolith can be observed. During immersion, the long axis of otoliths was 1234.19 ± 60.22 μm, and the short axis was 868.66 ± 64.13 μm. After 25 days of culturing, the long axis of otolith was 2278.25 ± 56.86 μm, and the short axis was 1527.86 ± 40.81 μm. Before the detection of otolith elements, USGMACS-3 carbonate was used as a standard sample to check the machine status. The Ar–He gas mixtures were tested using LA-ICPMS without any sample conditions, which was repeated 10 times. The concentration corresponding to 3 times the blank standard deviation was the detection limit of each element, and the element detection limit level of LA-ICPMS was determined accordingly. The element content in the otolith was then determined in the same procedure. The otolith was placed in a closed sample tank and subsequently denudated with a high-energy laser to vaporize them locally by heat. Laser denudation was performed from the core to the edge of the otolith, along the long axis deviation, of which the wavelength was 213 nm, the denudation aperture was 32 μm, the denudation depth was 20 μm, and the laser movement speed was 2 μm/s. The gasified sample was accompanied by a mixture of helium and argon gas with a controlled flow rate into the sample tank until the center tube of the ICP-MS torch. The otolith samples were then further thermally dissociated into molecules, atoms, and ions, which were detected by mass spectrometer. The detection result was substituted into the formula to calculate Sr/Ca the ratio, and the formula was as follows:

$$\left(\frac{Sr}{Ca}\right)_{sam} = \left[\left(\frac{S_{Sr}}{S_{Ca}}\right)_{sam} / \left(\frac{S_{Sr}}{S_{Ca}}\right)_{std}\right] * \left(\frac{Sr}{Ca}\right)_{std} \quad (1)$$

Note: S—signal intensity of isotope mass spectrum of the element to be measured, CPS; $(Sr/Ca)_{sam}$—Sr/Ca ratio of the sample to be tested; $(Sr/Ca)_{std}$—Sr/Ca ratio of standard sample; $(S_{Sr}/S_{Ca})_{sam}$—strontium and calcium isotope CPS ratio of the sample to be tested; $(S_{Sr}/S_{Ca})_{std}$—CPS ratio of strontium and calcium isotopes in standard samples.

2.3. Statistical Analysis

The following steps were taken for otolith element analysis: Firstly, the effective elements were screened according to the minimum detection limit of each element. Secondly, we calculated the ratio of strontium to calcium from the otolith core to the edge. Finally, the curves of Sr/Ca ratio were drawn, and the differences between each concentration group and the control group were compared.

The criterion of successful marking was that the Sr/Ca ratios of the otoliths exceeded the detection limit, which was set to 3.3 standard deviations above the mean of the control group. Marking is successful if the success rate of marking is higher than 99.94% [29].

The survival rate and condition factor of juvenile *T. obscurus* were calculate by Excel, and the formula for condition factor was as follows:

$$K = \frac{W}{L^3} * 100\% \quad (2)$$

L (cm) and W (g) are the body length and body weight, respectively. The condition factor of juvenile *T. obscurus* were analyzed by LSD multiple comparison in SPSS to provide reference for the growth.

3. Results

3.1. Survival and Growth in Different Strontium Concentrations

LSD multiple comparison showed that there were no significant differences in the condition factors of each group at the beginning of immersion ($p > 0.05$); after 7 days of immersion, the condition factor of juvenile *T. obscurus* increased first and then decreased with the increase of concentration (Table 1). There was a significant difference in the condition factor between the control group and 18 mg/L group ($p < 0.05$). The analysis of condition factor showed that strontium had no negative effect on the growth of *T. obscurus*. In addition, abnormalities in swimming and feeding behaviors throughout the experimental period were not observed, indicating no negative effects of the treatment. Throughout the immersing period, death did not occur in all groups, except the 72 mg/L group, which showed that the low concentrations of strontium was safe for *T. obscurus*.

Table 1. The growth and survival of 60 *T. obscurus* during immersion.

Strontium Concentration (mg/L)	Survival Quantity		Survival Rate	Condition Factor
	0 D	7 D		
0	60	60	100	3.1405 ± 0.0839 [a]
12	60	60	100	3.2892 ± 0.3201 [ab]
18	60	60	100	3.5034 ± 0.4666 [b]
36	60	60	100	3.2954 ± 0.2270 [ab]
72	60	59	98.33	3.2543 ± 0.2517 [ab]

Note: The means with different letters in different concentrations in the same index are significantly different ($p < 0.05$), and the same letters mean no significant difference ($p > 0.05$).

3.2. Quantitative Analysis of Otolith Marking

The effect of marking was quantified by the ratio of strontium-to-calcium. The *Sr*/*Ca* ratio in otolith in the control group was 0.86 ± 0.09 mmol/mol (Table 2), with no significant fluctuation throughout the experiment. Accordingly, if the ratio of *Sr*/*Ca* in otolith was higher than the baseline of detection (1.16 mmol/mol), the marking would be considered successful. After 1 d of immersion, the *Sr*/*Ca* ratios from the core to the edge of the otoliths (0.5–0.7 mm) in all marked groups, except 12 mg/L increased, and the *Sr*/*Ca* ratio of 12 mg/L group started to increase until 3 d after immersion (Figure 1). Thereafter, the peak value of each marked group was higher than the detection baseline and increased with the prolongation of the immersion time.

Table 2. The change of *Sr*/*Ca* ratio in the otoliths of *T. obscurus* in different concentrations.

Strontium Concentration (mg/L)	*Sr*/*Ca* Ratios in Otoliths (mmol/mol)			
	1 D	3 D	5 D	7 D
0	0.81 ± 0.09	0.84 ± 0.13	0.82 ± 0.08	0.86 ± 0.09
12	0.87 ± 0.06	1.34 ± 0.17	1.76 ± 0.05	1.90 ± 0.09
18	1.12 ± 0.14	1.66 ± 0.07	1.99 ± 0.06	2.29 ± 0.02
36	1.66 ± 0.05	2.13 ± 0.17	2.49 ± 0.03	3.05 ± 0.22
72	2.00 ± 0.10	2.95 ± 0.13	3.03 ± 0.06	3.75 ± 0.33

The *Sr*/*Ca* ratios in the otoliths of *T. obscurus* were analyzed 25 d after immersion, which verified the persistence of the marking with strontium (Figure 2). The ratios of marked groups were 1.86 ± 0.06 mmol/mol (12 mg/L), 2.39 ± 0.06 mmol/mol (18 mg/L), 3.02 ± 0.08 mmol/mol (36 mg/L), and 3.61 ± 0.34 mmol/mol (72 mg/L), which were 1.6, 2.05, 2.6, and 3.1 times the detection baseline, respectively. The *Sr*/*Ca* ratios in the marked groups showed crests with a width of approximately 0.1 mm in the region of 0.5–0.7 mm from the core to the edge of otoliths. When the fish were reared again in water without

added strontium, the *Sr*/*Ca* ratio returned to the previous level. Thus, the success rate of marking by exogenous strontium was 100%.

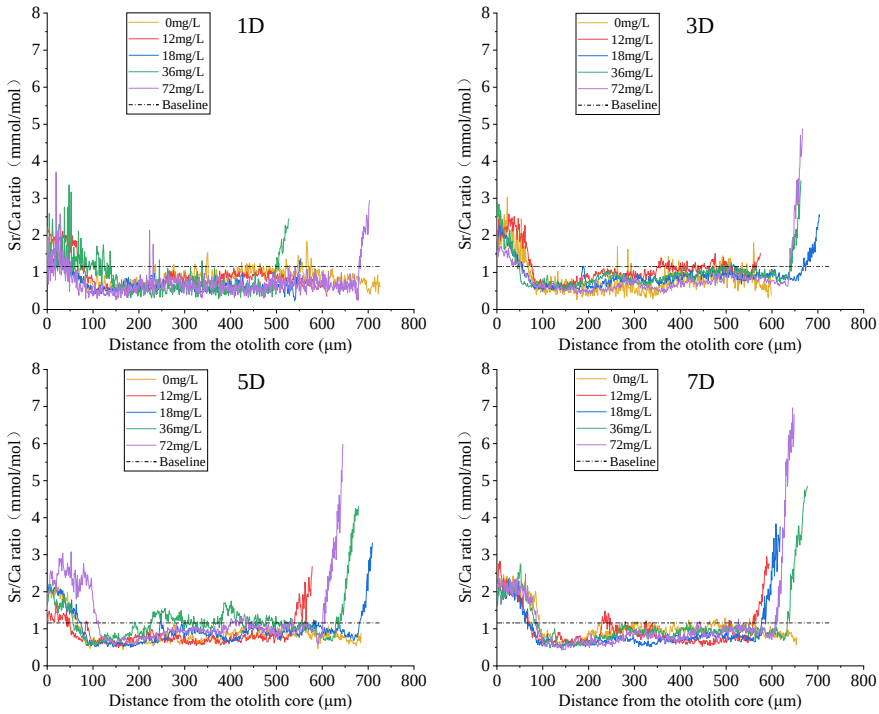

Figure 1. The *Sr*/*Ca* ratios in the otoliths of *T. obscurus* in different concentrations.

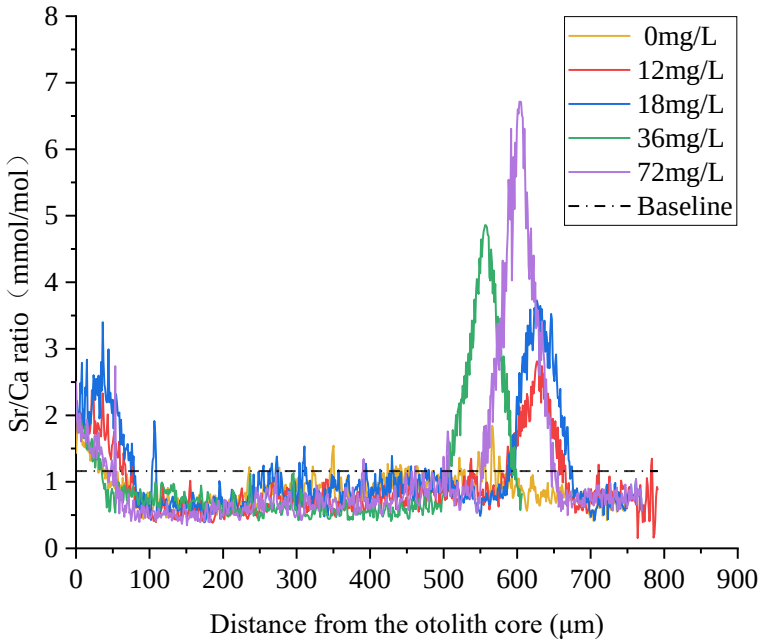

Figure 2. *Sr*/*Ca* ratios in the otoliths of *T. obscurus* in all groups after 25 d of immersion.

3.3. Changes in the Strontium Content of Fish

Marking was successful in all concentration groups. There was no difference in survival in all groups, except the 72 mg/L group, and the growth of the 18 mg/L group was the best, indicating that 18 mg/L may be the most suitable concentration. Therefore, the change of strontium residue in the 18 mg/L group was investigated.

3.3.1. Changes in the Strontium Content in Muscle

With the extension of marking time, the strontium content in the muscle of the 18 mg/L group increased gradually. At the end of the immersion, the independent sample T tests revealed a significant difference in strontium content between the 18 mg/L group and control group in the muscle ($p < 0.05$). After immersing, the decrease trend of strontium content in 18 mg/L group was first rapid and then slow (Figure 3). When reared in water without added strontium for 25 d, the strontium content in the muscle decreased to 0.254 ± 0.0429 mg/L, which was not significantly different from the control group ($p > 0.05$).

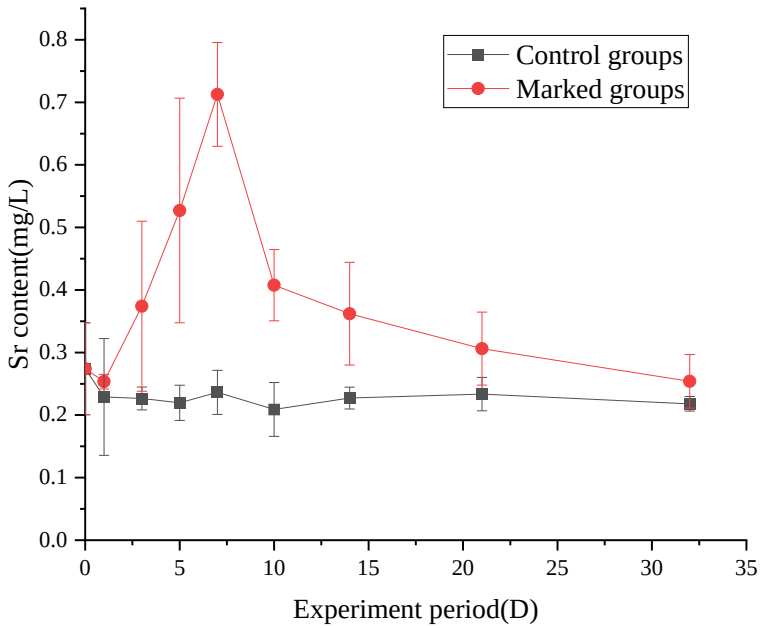

Figure 3. The variation trend of Sr^{2+} in muscle of *T. obscurus*.

3.3.2. Changes in the Strontium Content in Gill

During the whole experiment, the strontium content in the gills of the control group was about 4.0337 ± 0.4667 mg/L, while the strontium content in the 18 mg/L group increased gradually (Figure 4). At the end of the immersion, there was a significant difference in the strontium content between the 18 mg/L group and control group in the gills ($p < 0.05$). In the 25 d of treatment without added strontium, the decreasing trend of strontium content in the gills was similar to that in the muscle, but the trend in gills was slower. After 25 d of treatment in water without added strontium, the strontium content decreased to 4.8605 ± 0.7695 mg/L, which had no significant difference with the control group ($p > 0.05$).

3.3.3. Changes in the Strontium Content in Liver

The strontium content in the liver of 18 mg/L group increased gradually during the immersing period, which changed from slow to rapid (Figure 5). A significant difference in strontium content was observed between the 18 mg/L group and control group in the liver at the end of immersion ($p < 0.05$). However, the excess strontium in the liver was eliminated by the metabolism after 25 days of treatment in water without added strontium. The content of strontium in the liver of 18 mg/L group was only 0.1159 ± 0.0316 mg/L, which was lower than the control group (0.1280 ± 0.0764).

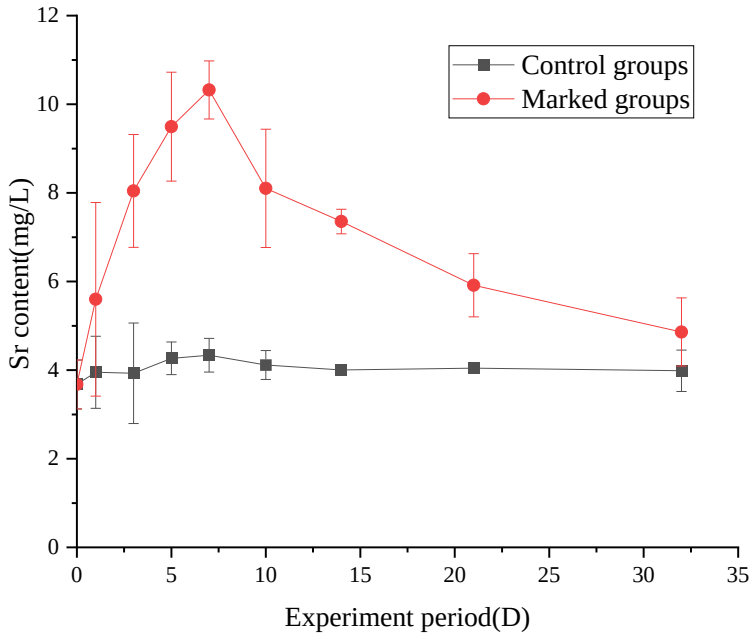

Figure 4. The variation trend of Sr^{2+} in gill of *T. obscurus*.

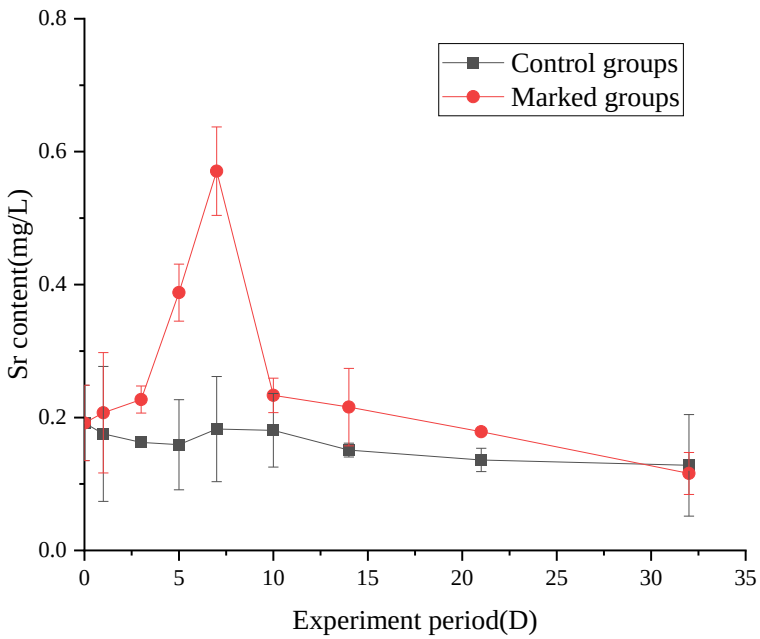

Figure 5. The variation trend of Sr^{2+} in liver of *T. obscurus*.

4. Discussion

4.1. Survival and Growth in Different Strontium Concentrations

Strontium is widely found in nature. As an essential microelement in the body of organisms, strontium has low toxicity risk [30]. The egg hatchability and hatching time were correlated with Sr, but Sr^{2+} did not affect the survival and growth of *Fathehead minnows* [31]. Sr^{2+} would not cause harm to juvenile crucian carp on strontium marking [32]. Not only that, strontium can even enhance bone strength by promoting the formation of bone cells to promote the growth of organisms [33]. A certain concentration of Sr^{2+} could contribute to cell proliferation [34]. However, when the concentration of strontium exceeds the appropriate range, it will not be conducive to the growth and survival of the organisms. The high concentrations of strontium could cause growth retardation in the animals [35].

The Ca^{2+} absorption capacity of fish was inhibited by the high concentrations of strontium, which would affect the growth and survival of juvenile fish [36]. In this experiment, the survival rate and condition factor of the 72 mg/L group decreased, indicating that the 72 mg/L had exceeded the optimum concentration for survival and growth. However, the condition factor of all marked groups was higher than that of the control group, which indicated that the strontium concentration set in this experiment might have a positive effect on the growth of *T. obscurus*. In addition, the strontium concentrations designed for this study were all several times that of the maximum in natural water, because it is usually difficult to distinguish between conditions at releasing and in the wild. Therefore, the concentrations we used, except 72 mg/L, are safe and reliable.

4.2. Quantitative Analysis of Otolith Marking

In this study, with the increase of immersing time and concentration, the *Sr*/*Ca* ratio of each marked group increased significantly. As expected, the peak ratio of *Sr*/*Ca* in each marked group exceeded the detection baseline (1.16 mmol/mol) after 7 days of immersion, indicating a 100% marking success rate. The high values of *Sr*/*Ca* ratio within 0.1mm from the core of otolith were found in most individuals, such as *Larimichthys crocea*, which may be caused by the maternal effect of the parental body [37].

In this study, the ratio of *Sr*/*Ca* in the 12 mg/L group began to increase after immersing for 3 days, while the ratio of other groups immediately increased after immersing for 1 day, which was different from the findings of previous studies. Different degrees of time lag may be caused by the different deposition rates of strontium in the otoliths of different fish species [38]. The strontium deposition on the otoliths of *Acanthopagrus butcheri* had a delay of 20 days [39]. A survey showed that it took 30–60 days for the elements in the environment to fully equilibrate with the otolith composition of juvenile eel [40]. A high strontium ring could be found on the edge of bighead only after it was raised for 28 days after strontium marking treatment [41]. However, time lag was not reflected when marking *Coilia nasus* [42]. Compared to these fish species, it is speculated that the deposition rate of strontium on otoliths is not only related to the fish species, but also related to the salinity and experimental temperature. Salinity was the main factor affecting the deposition of strontium on otoliths [43,44]. Both *T. obscurus* and *C. nasus* belong to anadromous fish, and there is a transition stage from fresh water to seawater in their growth and development. In contrast, carp and bighead are freshwater fish, while juvenile eel, juvenile salmon, and Briny snapper are still in the mariculture stage. In addition, the experimental temperatures of *T. obscurus* and *C. nasus* both reached 27.5 °C, while that of others were less than 24 °C. When the water temperature exceeds 20 °C, the partition coefficient of strontium in otoliths increase with the increase of water temperature, which affects the absorption rate of strontium in otoliths. The difference of temperature, fish species, and salinity may produce synergistic effects for increasing the deposition rate of strontium in otoliths [45]. Other factors, such as food, developmental stage, fish genetics, and growth rate, may also contribute to the degree of time lag [46,47]. The specific mechanism of time lag is not clear, so the rationality of this hypothesis needs to be further verified.

4.3. Changes in the Strontium Content of Fish

While selecting the appropriate marker concentration, the negative effects should be taken into account, while ensuring the marking effect, such as the impact of strontium on the fish and the side effects of strontium residue. Considering the growth, survival, and enzyme activity in muscle, it was best to select 10 mg/L as the optimum concentration of strontium when *Oncorhynchus keta* was marked with strontium [36]. When the safety of the water quality and marking effect was considered, 4 mg/L was selected as the optimum concentration for *Aristichys nobilis* [41]. Some fish species choose strontium concentration ignoring the effect of strontium on growth and the possible negative effects of strontium residue. In this study, on the basis of ensuring the marking effect, 18 mg/L was selected,

according to the growth and survival situation, to explore the change of strontium residue in *T. obscurus*.

Excessive concentrations of strontium are toxic [34]. There are occasional incidents of *T. obscurus* being caught and eaten by fishermen after releasing. In order to avoid the potential harm caused by excessive strontium, it is necessary to investigate strontium residue in the tissues of *T. obscurus.* All the concentration groups could achieve the expected effect after 7 days of immersion. Notably, the survival rate of the 18 mg/L group reached 100% and the growth was the best. Therefore, the changes of strontium residue in the tissues of 18 mg/L group were studied. The results showed that the level of Sr^{2+} increased significantly after immersion for 7 d, and then decreased gradually. Compared with muscles and gills, the strontium content in the liver of *T. obscurus* took the shortest time to return to the level of the control group, which may be related to the strong detoxification ability of liver [48]. Studies have shown that 8 mg/L, as the limit of strontium for drinking natural mineral water, is appropriate [49]. When *T. obscurus* were reared in water without added strontium for 25 d, strontium residue in the tissues of *T. obscurus* was lower than 8 mg/L, which is not enough to cause food safety issues. Therefore, after strontium marking, *T. obscurus* should be reared in natural water for a period of time to avoid the adverse consequences of high strontium.

5. Conclusions

The study found that the appropriate concentration of strontium had no negative effect on survival of *T. obscurus* and could promote growth. After 7 d immersion in strontium-rich water, strontium can be stably deposited on the otoliths of *T. obscurus.* When reared in water without added strontium, the content of strontium in the body of *T. obscurus* decreased gradually. Strontium could be used to mark *T. obscurus* before releasing. After recapture, the ratio of Sr/Ca in otoliths were detected to distinguish the wild population from the released population, and then the recapture rate can be obtained. The effect of releasing can be evaluated according to the recapture rate to provide an important reference for stock enhancement, releasing strategy, implementation, and adaptive management in the future, which also can promote releasing activities effectively.

Author Contributions: Conceptualization, L.G. and G.F.; data curation, L.G. and Y.L.; formal analysis, L.G. and H.Z.; funding acquisition, G.F.; investigation, L.G., W.H. and C.S.; project administration, G.F.; visualization, L.G.; writing–original draft, L.G. and H.Z.; writing—review and editing, G.F., Z.H., F.Z. and Q.Y. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: The *T. obscurus* in the experiments belonged to cultured fish, which is not a rare protected species and does not involve animal welfare. Nevertheless, the applicable institutional and governmental ethical regulations concerning the use of experimental animals were also followed.

Informed Consent Statement: Not applicable.

Data Availability Statement: The data presented in this study are available in the article. Further information is available upon request from the corresponding author.

Conflicts of Interest: The authors declare no conflict of interest.

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