

Otolith development in larval and juvenile *Schizothorax davidi*: ontogeny and growth increment characteristics*

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Received May 17, 2016; accepted in principle Jun. 22, 2016; accepted for publication Jul. 11, 2016

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Abstract Laboratory-reared *Schizothorax davidi* larvae and juveniles were examined to assess the formation and characteristics of David's schizothoracin otoliths. Otolith development was observed and their formation period was verified by monitoring larvae and juveniles of known age. The results revealed that lapilli and sagittae developed before hatching, and the first otolith increment was identified at 2 days post hatching in both. The shape of lapilli was relatively stable during development compared with that of sagittae; however, growth of four sagittae and lapilli areas was consistent, but the posterior area grew faster than the anterior area and the ventral surface grew faster than the dorsal surface. Similarly, the sum length of the radius of the anterior and posterior areas on sagittae and lapilli were linearly and binomially related to total fish length, respectively. Moreover, daily deposition rates were validated by monitoring known-age larvae and juveniles. The increase in lapilli width was $1.88 \pm 0.080 \text{ } \mu\text{m}$ at the ninth increment, which reached a maximum and the decreased gradually toward the otolith edge, whereas that of sagittae increased more slowly. These results illustrate the developmental biology of *S. davidi*, which will aid in population conservation and fish stock management.

Keyword: otolith; development; otolith microstructure; growth; *Schizothorax davidi*

1 INTRODUCTION

Since Pannella's (1971) discovery of daily increments in fish otoliths, which occur in numerous larvae and juveniles fish species (Humphrey et al., 2003; Bustos et al., 2015), otolith microstructure has become a powerful tool to study early life stages of fish (Campana, 2005; Joh et al., 2005; Pavlov et al., 2015). Based on the relationship between otolith size and somatic size, daily otolith growth increments have been widely used to estimate daily age and hatch dates, as well as conduct growth trajectory analyses (Tuset, 2003; Burke et al., 2008; Guo et al., 2010; Zeng and Zhang, 2012). Furthermore, daily growth of otoliths is also important for assessing survival and recruitment success in fish (Feet et al., 2002; Begg et al., 2005; Parkinson et al., 2012). However, otolith microstructural characteristics differ among species because of species-specific otolith formation patterns (Campana, 2001; Yamada et al., 2009; Huang et al., 2014). Therefore, it is necessary to select suitable

otoliths to determine the age of wild-caught specimens and validate the periodicity of increment formation through rearing experiments prior to an otolith microstructural analysis.

David's schizothoracin, *Schizothorax davidi*, belongs to the subfamily Schizothoracinae in the family Cyprinidae. The schizothoracine fishes are considered the largest and most diverse ichthyofauna group in the Qinghai-Tibetan Plateau (Chen, 1998). Schizothoracine fish populations have declined dramatically in recent years as a result of overfishing, dam construction, water pollution, and other human interferences. David's schizothoracin is only distributed in mountain rivers of Southwest China (Ding, 1994). Because it is ecologically vulnerable and populations have declined sharply, this species

* Supported by the Double Branch Plan of Sichuan Agricultural University (Nos. 03571421, 03571779)

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has been listed as a key wild animal species for protection in Sichuan Province, China. At present, successful captive breeding and release of hatchery-reared fish into natural waters is an important means to protect and restore wild populations.

Suitable otoliths, the otolith increment formation pattern, and otolith deposition periodicity must be determined to best evaluate recovery of the *S. davidi* resource using an otolith microstructure analysis. In the present study, we describe the morphological features of otoliths in laboratory-reared specimens, establish the most suitable otoliths for aging, and validate otolith microstructural characteristics and growth patterns. These results should benefit resource protection and performance evaluations.

2 MATERIAL AND METHOD

In the present study, fish age was defined as the number of days post hatching (dph). All larval and juvenile David's schizothoracin were artificially propagated by the Baojian Breeding Co. Ltd. (Ya'an, Sichuan, China). Fertilized eggs were incubated in a round steel tub (diameter=80.0 cm) at room temperature in the Department of Aquaculture, College of Animal Science and Technology, Sichuan Agricultural University, China. The newly hatched larvae were reared in a biochemical incubator under controlled conditions. Water temperature was maintained at $23.0 \pm 1.0^\circ\text{C}$ during the day and was allowed to drop to ambient temperature at night. The photoperiod matched natural conditions. Water temperature was measured at 8:00 and 20:00 during the experiment, and half of the water was exchanged at that time. The fish were fed small cladocerans before 7 dph, and then with finely chopped *Limnodrilus* sp.

Larvae and juveniles of different ages were sampled during the rearing period. Body length (BL, notochord length for larvae and standard length for juveniles) of all sampled fish was measured with vernier calipers (± 0.02 mm), and the specimens were preserved in 75% ethanol until dissection. Lapilli, sagittae, and asterisci were extracted with a dissecting needle under an anatomical lens (MZ16a; Leica, Heidelberg, Germany). The otoliths were removed, cleaned, dried, and mounted on a microscope slide with resinene.

The digital images were used to measure otolith radius (OR) (from the core along the anterior, posterior, dorsal, and ventral directions) and incremental width (from the centronucleus along the longest axis of lapilli and sagittae). Lapilli and sagittae

increments were examined directly under a light microscope in larvae <42 dph. Older larvae were ground and polished according to a previous report (Song et al., 2008). OR was measured, and the increments were counted using an image analysis system (E800; Nikon, Tokyo, Japan). Incremental widths were measured with the image analysis system described by Zhu et al. (2002). The number of increments in lapilli and sagittae was counted at least three times. If the counts did not vary by >10% from the mean, the mean of the total counts was taken as the final number.

Data are presented as mean \pm standard deviation. A *P*-value <0.05 was considered significant for all statistical analyses. The paired *t*-test and Student's *t*-test were carried out using SPSS 17.0 software (SPSS Inc., Chicago, IL, USA).

3 RESULT

3.1 Otolith development

Lapilli and sagittae appeared before larvae hatched, and the asterisci first developed at approximately 19 dph. Otolith morphology changed as the larvae and juveniles grew, and lapilli and sagittae were almost round at hatching. The lapilli became ovoid at 19 dph, changed to a fan shape at 37 dph, and then changed into the shape of a mussel at 52 dph (Fig.1). Unlike the lapilli, changes in the shape of sagittae were significant during development; they were spindle shaped at 25 dph, irregularly shaped with a rounded anterior and pointed posterior at 37 dph, and finally changed to a long ellipse with a long pointed posterior at 87 dph (Fig.1). Furthermore, the asterisci became quadrilateral at 25 dph, were irregular with some cristae at 37 dph, but remained stellate throughout development, except for some minor differences in the number of cristae (Fig.1).

3.2 Otolith increment deposition rate

No increments were detected on lapilli or sagittae of newly hatched larvae, but a diffuse and obscure increment first appeared at 2 dph (Fig.2a). Thereafter, one increment was deposited per day. Five increments were found on 6-dph-old larvae otoliths (Fig.2b), and otoliths of 20-dph-old larvae had 19 increments (Fig.2c).

Lapilli (I_L) or sagittae (I_S) incremental counts were equal to the number of dph (D), as expressed by the following linear regressions (Fig.3): $I_L = 0.9917D - 0.9458$ ($R^2 = 0.9998$, $n = 158$) and

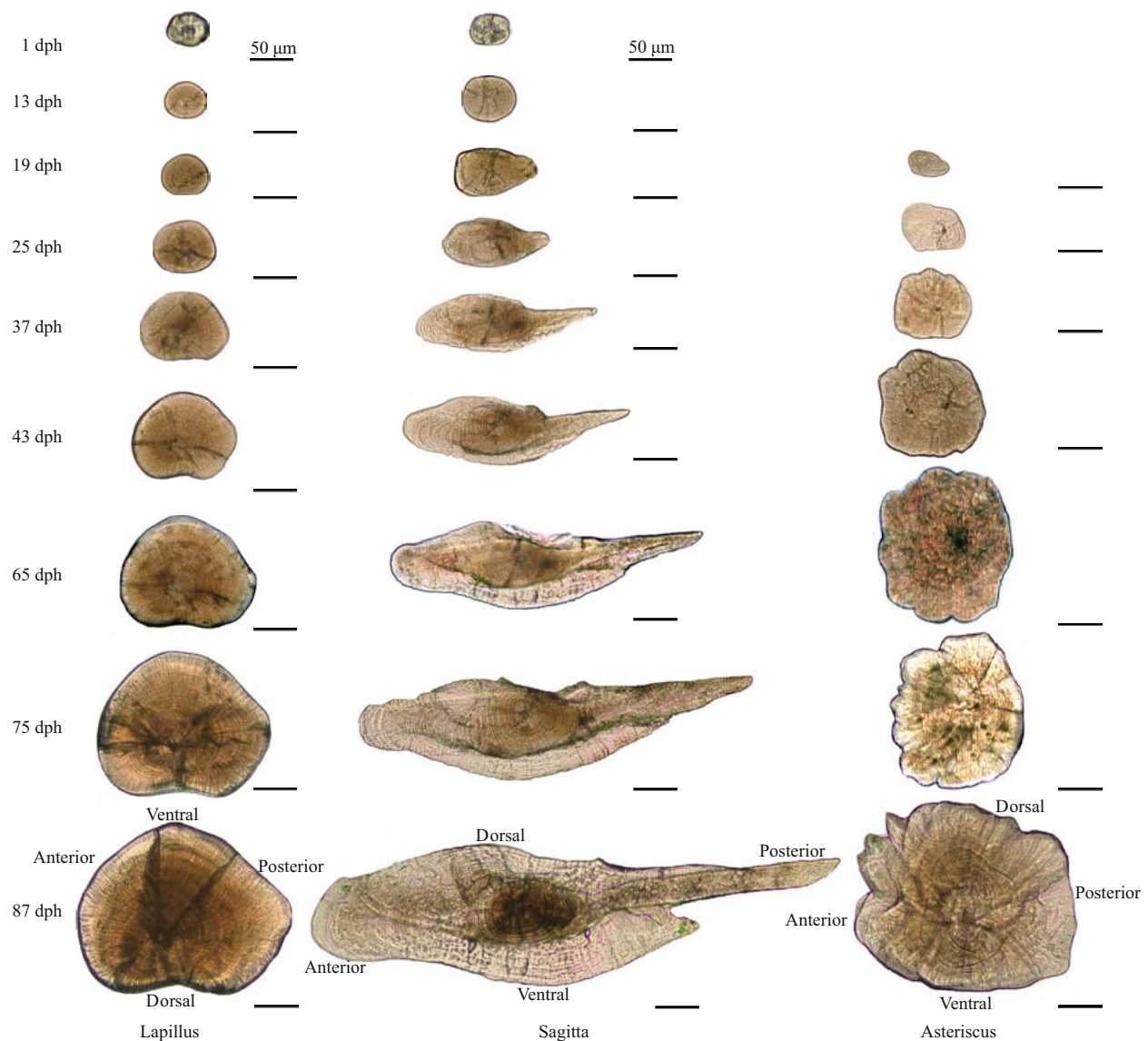


Fig.1 Otolith development in *S. davidi* larvae and juveniles

Scale bar=100 μm , except where otherwise stated.

$I_5=0.992\ 8D-0.9584$ ($R^2=0.999\ 6$, $n=124$), respectively. No differences were detected between the slopes of the equations and 1.0 ($P>0.05$), suggesting that the increments were deposited daily after hatching.

3.3 Increment width

Increment widths were 1.45–1.88 μm in lapilli (Fig.4a) and 2.10–2.90 μm in sagittae (Fig.4b) from samples preserved at 28 dph. Lapilli increment widths were constant down to the third increment at $1.45\pm0.069\ 0\ \mu\text{m}$, and increased thereafter to a maximum of $1.88\pm0.080\ 0\ \mu\text{m}$ for the ninth increment. Thereafter, increment widths dropped gradually almost to the otolith edge. However, sagittae

increment widths increased gradually during growth.

3.4 Otolith growth

A quadratic functional relationship existed between growth of the posterior and anterior areas of lapilli (Fig.5a) and sagittae (Fig.5c), and growth rate of the posterior area was faster than that of the anterior area. Similarly, growth rate of the ventral area was faster than that of the dorsal area in lapilli (Fig.5b) and sagittae (Fig.5d). Furthermore, a clear inflexion point (approximately 12 dph) was detected in the relationship between OR and daily-age of sagittae (Fig.5d); however, the growth trend (quadratic function) did not change before or after that time point.

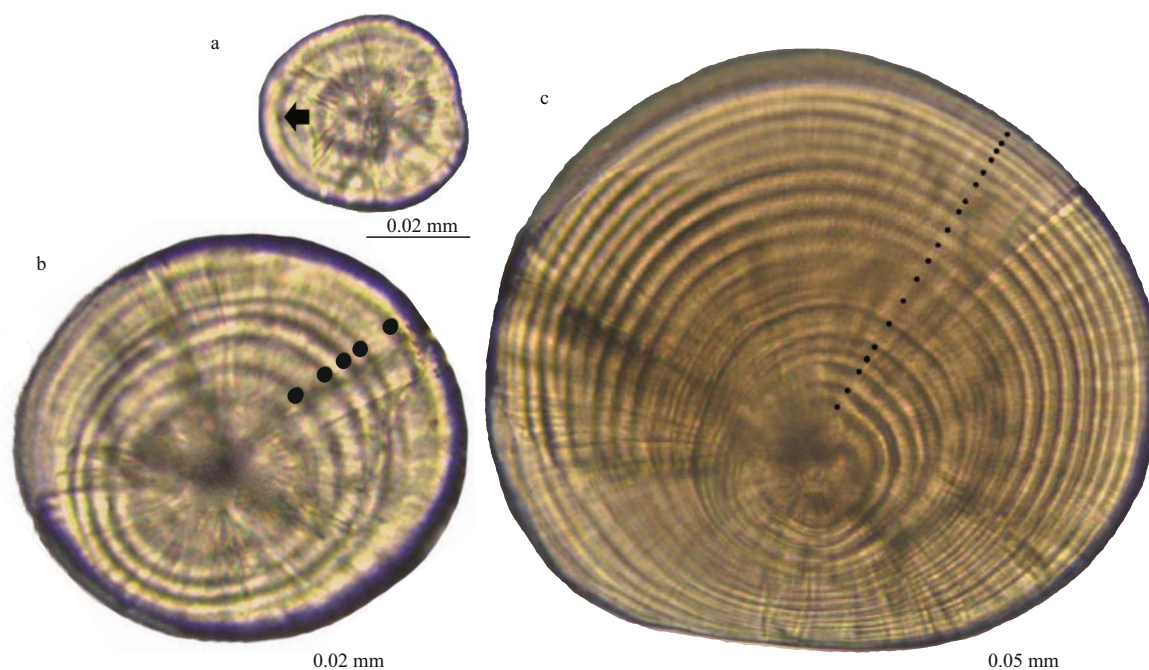


Fig.2 *S. davidi* larvae and juvenile otoliths

a. two-day post hatching (dph) larva lapillus showing the first increment (black arrow); b. six-dph larva sagitta showing five daily increments (black dots); c. twenty-dph larva lapillus showing 19 daily increments (black dots).

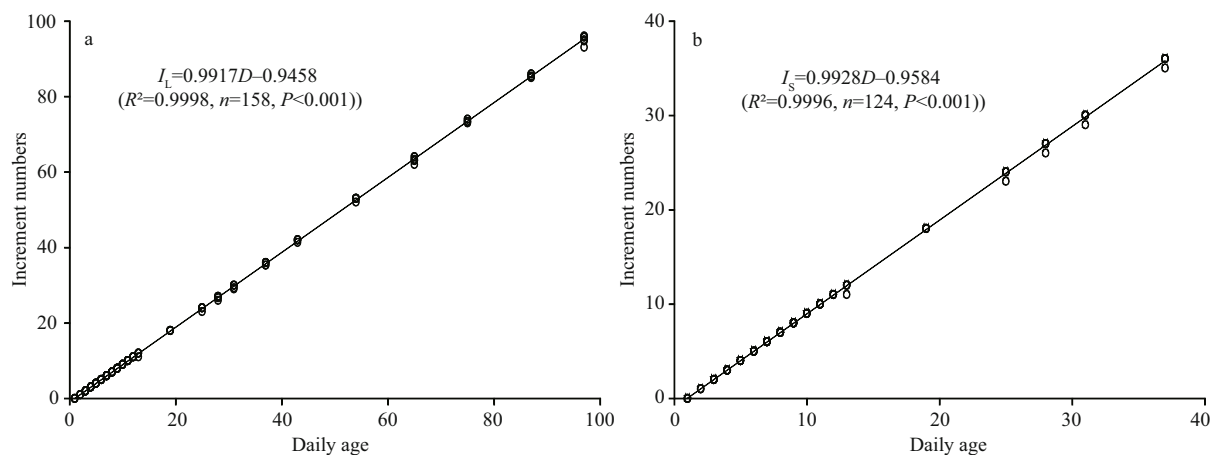


Fig.3 Relationship between incremental numbers in lapilli (a) and sagittae (b) and age of *S. davidi*

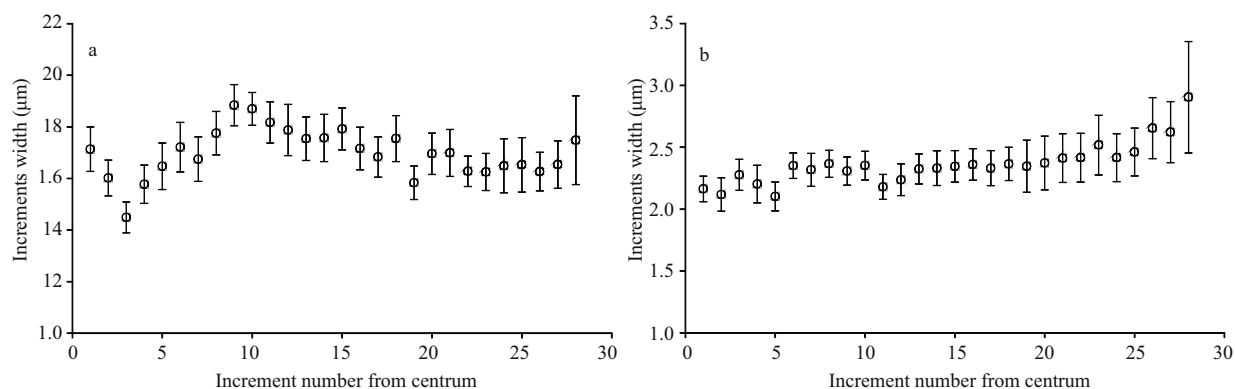


Fig.4 Relationships between mean increment width and increment number from the centrum along the longest axis in *S. davidi* lapilli (a) and sagittae (b)

Larvae at 28 dph (lapillus, $n=43$; sagitta, $n=28$) were used for analysis.

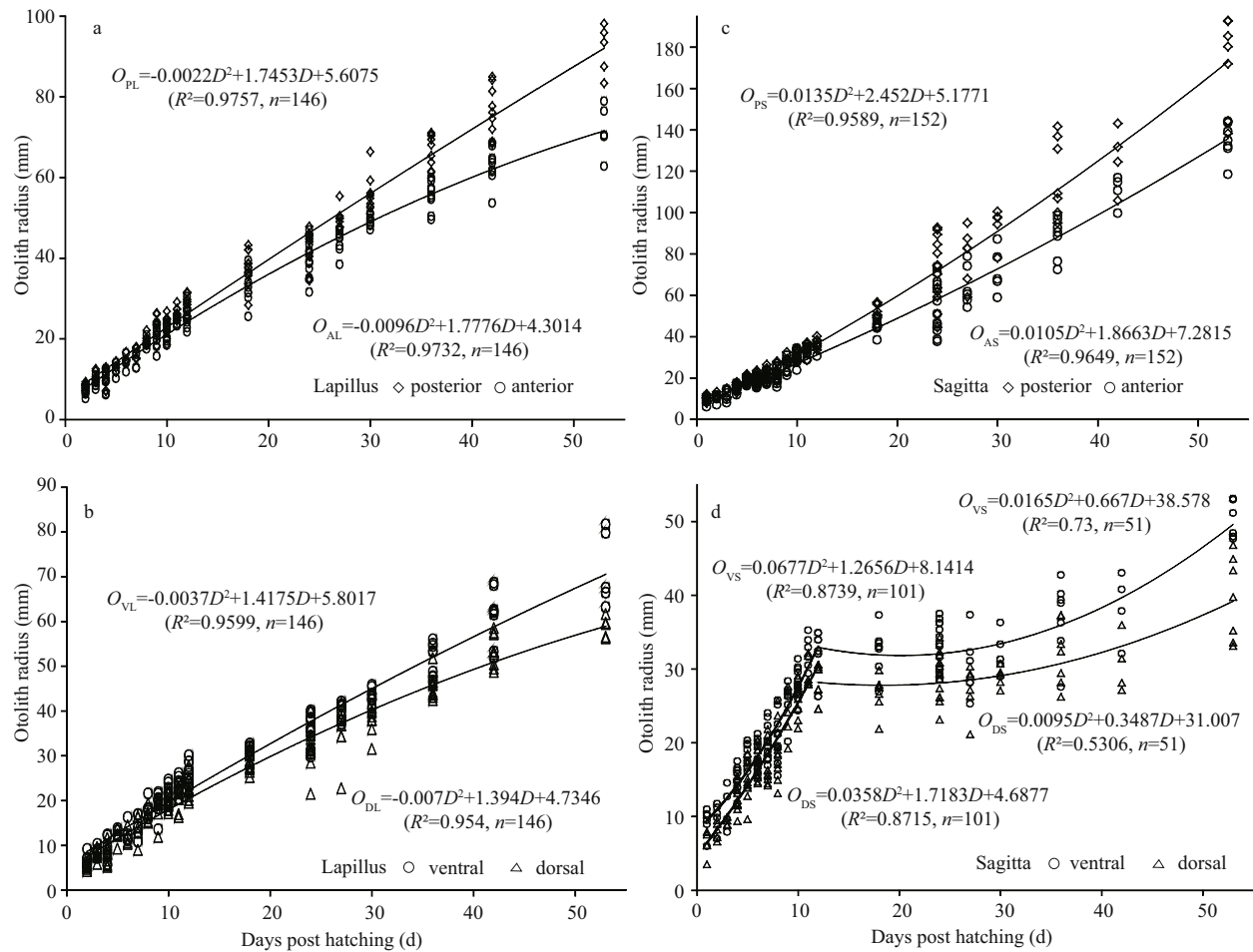


Fig.5 Relationship between otolith radius in different directions on lapilli (a, b) and sagittae (c, d) and *S. davidi* age

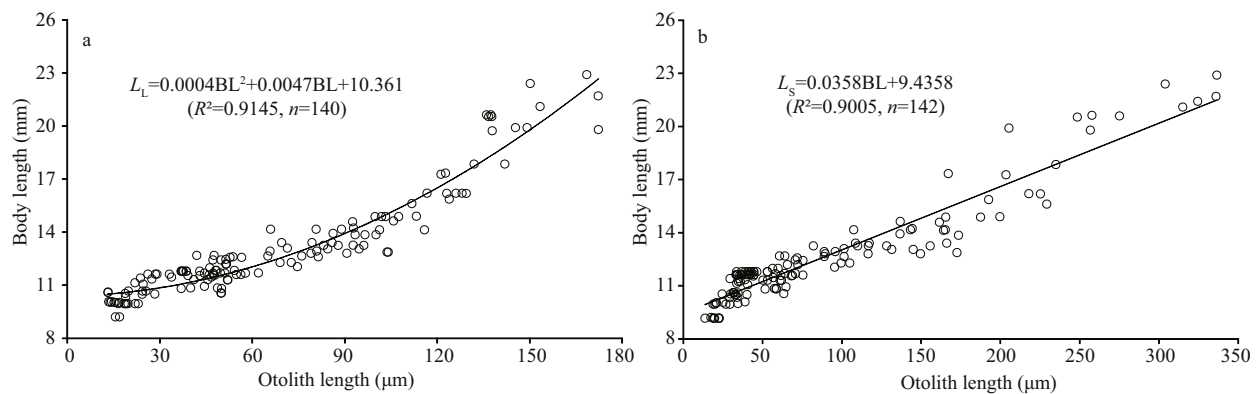


Fig.6 Relationships between otolith (a, lapillus; b, sagitta) radius and body length of *Schizothorax davidi* larvae and juveniles

3.5 Relationship between otolith growth and fish growth

OR and BL of lapilli and sagittae were binomially and linearly related, respectively (Fig.6). The regression equations between otolith length (sum length of radius anterior and posterior areas; L_L , length of lapillus; L_S , length of sagittae) and BL were:

$$L_L = 0.0004BL^2 + 0.0047BL + 10.361 \quad (R^2=0.9145, n=140) \quad (\text{Fig.6a}), \text{ and } L_S = 0.0358BL + 9.4358 \quad (R^2=0.9005, n=142) \quad (\text{Fig.6b}).$$

4 DISCUSSION

The morphology of sagittae changed significantly during development, which agrees with previous

reports on Cyprinidae (Huang and Chiu, 1997; Song et al., 2008; Yan et al., 2014). The development of lapilli was relatively stable, but the changes in morphology varied in four areas. Allometric growth of the OR in lapilli and sagittae was very clear; the growth rate of the posterior area was faster than that of the anterior area, and the growth rate of the ventral surface was faster than that of the dorsal area. The differences in growth rates among the four areas may be the main reason for the morphological differences. Moreover, OR may be useful to back-calculate the growth trajectory during the early life history using the relationship between growth and morphology.

In the present study, lapilli and sagittae appeared before hatching, and the daily increment first appeared at 2 dph. Larvae asterisci emerged at 19 dph, but their increments were unclear and irregular. In contrast, both lapilli and sagittae are useful for assessing age in this species (Morioka and Matsumoto, 2007; Song et al., 2008). However, sagittae are easily damaged during extraction and grinding because of elongation along the anterior-posterior area. The increments along the anterior-posterior direction were obscure, and the peripheral increments of the dorsal-ventral area were pressed together because of the slow growth and were unable to be counted. The usefulness of lapilli rather than sagittae for determining the daily increment has been reported for other Schizothoracinae species in our laboratory (unpublished results), as well as for other cyprinids (Mugiya and Tanaka, 1992; Song et al., 2008; Yan et al., 2014), but the lapilli were more suitable for determining age of *S. davidi*.

Otolith microstructural analyses are very valuable for relating biotic and abiotic factors to life-history traits. However, periodicity of increment formation must be validated when studying otolith microstructure in different species (Humphrey et al., 2003). Validation methods include monitoring known-age fish under laboratory conditions (Song et al., 2008; Yamada et al., 2009; Pavlov et al., 2012; Yan et al., 2014), using fluorescent markers (Sugeha et al., 2001; Joh et al., 2005), performing a marginal incremental analysis (Moku et al., 2005), and using statistical inferences (Morley et al., 2005). Of these, the most direct method to validate increment deposition periodicity is to hatch eggs and rear the larvae (Campana, 2001; Sponaugle, 2009), and this method has been used to validate daily increment deposition in many species (Mendiola and Álvarez, 2008). By analyzing larvae of known-age and counting the increments under laboratory conditions, we have demonstrated that the

first regular increment formed the 2 dph, and that the number of increments was equal to the actual age in days–1. These findings suggest that increments were deposited daily, which agrees with previous results obtained with a fluorescent marker in fish reared under different temperatures (He et al., 2008). However, the method of monitoring known-age larvae used in this study was easier and more direct for validating daily increments compared to the aforementioned. Additionally, the fish were less affected by endocrine-driven endogenous rhythms and environmental conditions.

The relationship between fish length and OR helps with the back-calculation of the growth trajectory during early life history, but this can be limited by many factors. Although fish body length and OR are closely related, the relationship varies with growth, and the slope of this relationship is affected by temperature (Aldanondo et al., 2008). Additionally, starvation, infrequent feeding, and other environmental conditions may cause similar problems (Folkvord et al., 2000; Fox et al., 2003). Furthermore, ontogenetic shifts in growth patterns, which are controlled by endogenous factors, can also affect the OR-body size relationship, as demonstrated in perch when they begin exogenous feeding (Kristensen et al., 2008). The relationship between fish length and OR of clupeoids also changes from an allometric relationship during the larval stage to a linear relationship during the juvenile stage (Watanabe and Kuroki, 1997; Takahashi et al., 2001). However, the relationship between OR and body length in *S. davidi* was either linear or binomial, and the effects of environmental conditions and ontogenetic shifts had no obvious impact under laboratory-rearing conditions. Therefore, these species-specific differences should be considered during early life stages when back-calculating growth using the OR-body size relationship.

Notably, the incremental changes in width differed between lapilli and sagittae. The first lapilli increment width was less than that in sagittae, and peaks and troughs were observed in the increment widths of lapilli, whereas those of sagittae tended to increase slowly during growth. This result may be closely related to otolith and fish growth (Ivarjorda et al., 2008; Yamada et al., 2009). In this study, lapilli were more regular in shape than sagittae, and growth was relatively consistent between different areas. In contrast, sagittae changed shape significantly, particularly in the ventral and dorsal areas, and growth

in the posterior area also differed significantly from that in other areas. Furthermore, the maximum lapilli increment width appeared at 9 dph, and then decreased, which has been associated with exogenous food intake during the feed transition. Therefore, the change in lapillus increment microstructure may be a true reflection of fish growth when compared to the steady increase in sagitta growth.

5 CONCLUSION

In summary, the morphological features and developmental characteristics of *S. davidi* otoliths were species-specific; the first regular increment formed at 2 dph, and deposition was clearly periodic. Lapilli and sagittae development and growth were closely related to age and body length of fish. These results will advance the research and protection of wild resources and fisheries management of this species.

6 ACKNOWLEDGMENT

We thank LIU Xiaoshuai, WANG Yufeng, and YANG Ting for their help with sample collection. We are grateful to Professor SONG Zhaobin for evaluating the manuscript.

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