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Age validation, and comparison of otolith, vertebra and opercular bone for estimating age of *Schizothorax o'connori* in the Yarlung Tsangpo River, Tibet

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Abstract A collection of 514 *Schizothorax o'connori* was made between August 2008 and August 2009 from Yarlung Tsangpo River to assess the suitability of three bony structures for age estimation. The annulus characteristics of otolith, vertebra and opercular bone were described. Location of the first annulus was validated by daily growth increment (DGI) analysis in the otoliths. Annual periodicity was verified by marginal increment ratio (MIR) analysis in otoliths and edge analysis in vertebrae and opercular bones. Annuli formed, once a year, between March and May for all three bony structures. Otoliths, vertebrae and opercular bones were examined to determine which structure produced the most precise and accurate age estimates in *S. o'connori*. Vertebrae and otoliths matched closely for the first 21 years of life, while opercular bones appeared to underestimate age. For older fish, the counts diverged and otoliths consistently providing higher age estimates. Sectioned otoliths proved to be the most precise and accurate structure for age estimation. The oldest observed schizothoracine fish was 50, more than twice the longevity previously accepted in *S. o'connori*.

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Keywords Age validation · Otolith · Vertebra · Opercular bone · *Schizothorax o'connori*

Introduction

Accurate fish ages are important for growth analysis, population dynamics estimates and resource management (Campana and Thorrold 2001). The most reliable ageing method may vary among the species, thus, the evaluation of the precision and accuracy of bony structures should be studied (Polat et al. 2001). Comparison of age estimates from various bony structures has been reported in a number of fishes to identify the most suitable structure for a fish population (Khan and Khan 2009).

The subfamily Schizothoracinae distributes widely in the Tibetan Plateau and adjacent areas (Cao et al. 1981). Previously, age estimation in this subfamily has relied chiefly on the anal scales (Singh and Sharma 1995). Nevertheless, some researchers considered that the anal scales might underestimate the age for these species (Chen et al. 2009). Chen et al. (2002a,b) showed that otoliths provide the most reliable age estimation, while the annuli on vertebrae and opercular bones were not very clear in *Gymnocypris selincuoensis*. He (2005) also revealed that vertebrae and opercular bones could underestimate the age of *S. o'connori* beyond 6 years.

As an endemic species in the Tibetan Plateau, *S. o'connori* (Cyprinidae: Schizothoracinae) only resides in the middle and upper reaches of the Yarlung Tsangpo River and its tributaries (Chen and Cao 2000). *S. o'connori* generally inhabits limpid waters with gravel bottom, and has powerful muscular cylindrical body. Commercially, *S. o'connori* is one of the most important species in Tibet. Although this species was locally abundant, catches have recently shown a slightly depletion mainly because of indiscriminate fishing. Attempts to develop an effective population management strategy have been obstructed by a lack of basic biological information. The available information refers mostly to molecular phylogeny (He et al. 2004) and phylogeography (He and Chen 2009). There have been few studies of age and growth of *S. o'connori* (He 2005; Yao et al. 2009). Results from these two investigations may only be applicable to younger fish because these studies did not include older individuals. Difficulties in collecting a sufficient number of older individuals (older than 24 years or standard length >500 mm) made it impossible to establish appropriate aging techniques for older age classes. Furthermore, neither of these studies used a validated methodology. The current study aims to firstly to describe the annulus characteristics of otolith, vertebra and opercular bone; secondly, to validate the annuli and verify annual periodicity in these structures; and finally, to make a comparison of age estimates among the three calcified structures.

Materials and methods

Collection of samples

A total of 514 *Schizothorax o'connori* individuals were collected from the Yarlung Tsangpo River (97.7%) and its tributaries (Xiang Qu and Nyang Qu) monthly from August 2008 to August 2009 (Fig. 1). More than 30 fish were collected for each month. Various sampling techniques were used to ensure that most age classes were sampled; specimens were sampled using floating gillnets; set gillnets (mesh size 7.5 mm); and trap nets (mesh size 1.5 mm). The standard length (SL) and wet body weight (W) were measured to the nearest 1 mm and 0.1 g using tapeline and electronic balance on fresh specimens, respectively.

The lapillus otoliths were extracted from the head (vestibular apparatus) of the fish. After the otoliths were rinsed with water they were air-dried, and then stored in labeled tubes. The 4–9th vertebrae and opercular bones were removed from the fish, stored in polythene zip packets and then frozen before processing in the laboratory (Xiong et al 2006). Both right and left lapillus otoliths and opercular bones were removed from each fish, but generally the right lapillus otoliths were used for analysis. Similarly, the 6–7th vertebrae were used for interpretation. Otoliths, vertebrae and opercular bones were collected from the same fish (465 specimens), to compare the age estimates from each structure.

Preparation of three bony structures

The lapillus otolith was mounted, proximal face down, on a glass slide using nail polish. The distal face of the otolith was then gound using wet sandpaper (600–2000 grit) and polished with alumina paste (3 μm) until the core was visible under a compound microscope. The section was re-affixed with the polished surface down, ground and polished until the core was again exposed (He et al. 2008).

Vertebrae were placed in boiling water for 10–15 min, cleared of attached tissues, immersed in 1% H_2O_2 for 24 h, and then examined under a dissecting microscope with reflected light after air-drying. Since the vertebra was a bi-concave centrum (ellipse), it was cut in half along the dorsal-ventral axis, and half was put at an optimal angle to make all of the bands obvious (Gunn et al. 2008). The vertebrae were placed on a concave slide of xylol for observation. Opercular bones were put in boiling water for 1 min, cleared of extraneous tissues, immersed in 1% H_2O_2 for 24 h, and then examined under the dissecting microscope with transmitted light after air-drying.

Age validation and annual periodicity

The presumed daily growth increments (DGIs) of the otoliths of seven young fish between 33 and 40 mm SL (caught on 4 January 2009) were counted and the radius of the otoliths was measured (Sequeira et al. 2009). Although DGI periodicity was not validated, the increments were presumed to be daily by analogy with other Schizothoracinae species (Jia and Chen 2009). In addition, the marginal increment ratio (MIR)

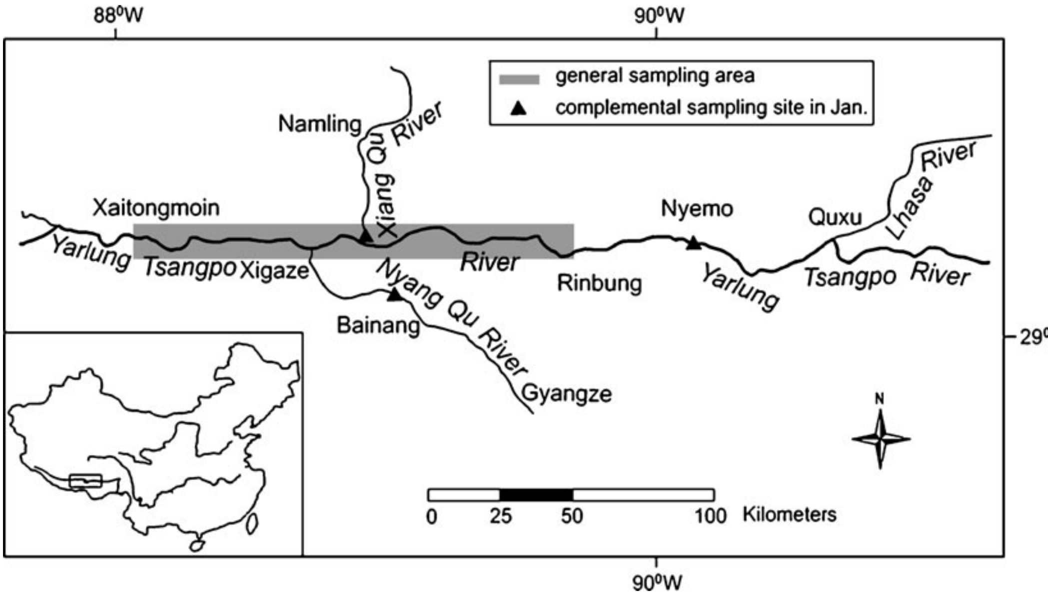

Fig. 1 Sampling locations of *Schizothorax o'connori* in the Yarlung Tsangpo River

analysis was used to verify the period of annulus formation in the otoliths. The monthly changes of MIR (with 1–8 annuli) were estimated with the following formula: MIR = $(R - R_n) / (R_n - R_{n-1})$, where R is the otolith radius, R_n is the radius of the last complete zone, R_{n-1} is the radius of the penultimate complete zone (Haas and Recksiek 1995). Measurements were made along the axis (Fig. 2) using an image analysis system (Ratoc System Engineering, Tokyo) with a direct data feed between the compound microscope and the computer.

Each vertebra and opercular bone (with 1–8 annuli) was assigned to one of the two edge types, translucent or opaque. Monthly changes of type were analyzed to determine the annual periodicity of the translucent/opaque zones. Photos were taken using Leica Application Suite (version 15) with a CCD (charge coupled device) connected to the dissecting microscope and the computer.

Calculations and statistical analyses

Each fish was assigned to an age class assuming 1 January as the designated birthday (Massutí et al. 2000). Annuli were counted without prior knowledge of the size, sex, or collection date for the individual. Each otolith, vertebra and opercular bone was interpreted by two independent readers, and scored subjectively for readability on a five-point scale: 1, excellent; 2, good; 3, acceptable; 4, poor; 5, unreadable (Paul and Horn 2009). The index of average percentage error (IAPE) was calculated to assess the precision of the age determinations between two readers. The equation (Beamish and Fournier 1981) was expressed as follows:

$$\mathrm{IAPE_j} = \frac{1}{N}\sum_{j=1}^{N}\left(\frac{1}{R}\sum_{i=1}^{R}\frac{|X_{\mathrm{ij}} - X_{\mathrm{j}}|}{X_{\mathrm{j}}}\right) \times 100\%$$

where N is the number of fish aged, R is the number of times each fish is aged, X_{ij} is the ith age determination of the jth fish, X_j is the mean age calculated for the jth fish.

For better comparison among different ageing structures, counts of two readers should be consistent. If the two counts were different, then the structures were recounted and the final count then accepted as the agreed age (Liu et al. 2009). Vertebrae and opercular bones were paired with the otoliths (which showed more clear and sharp annuli) in each age class by calculating IAPE (Gunn et al. 2008; Khan and Khan 2009).

Mean age readings obtained from the three bony structures were subjected to one-way analysis of variance (ANOVA) followed by Tukey's post hoc pair-wise comparisons to determine whether readings from different bony structures showed significant differences (Khan and Khan 2009).

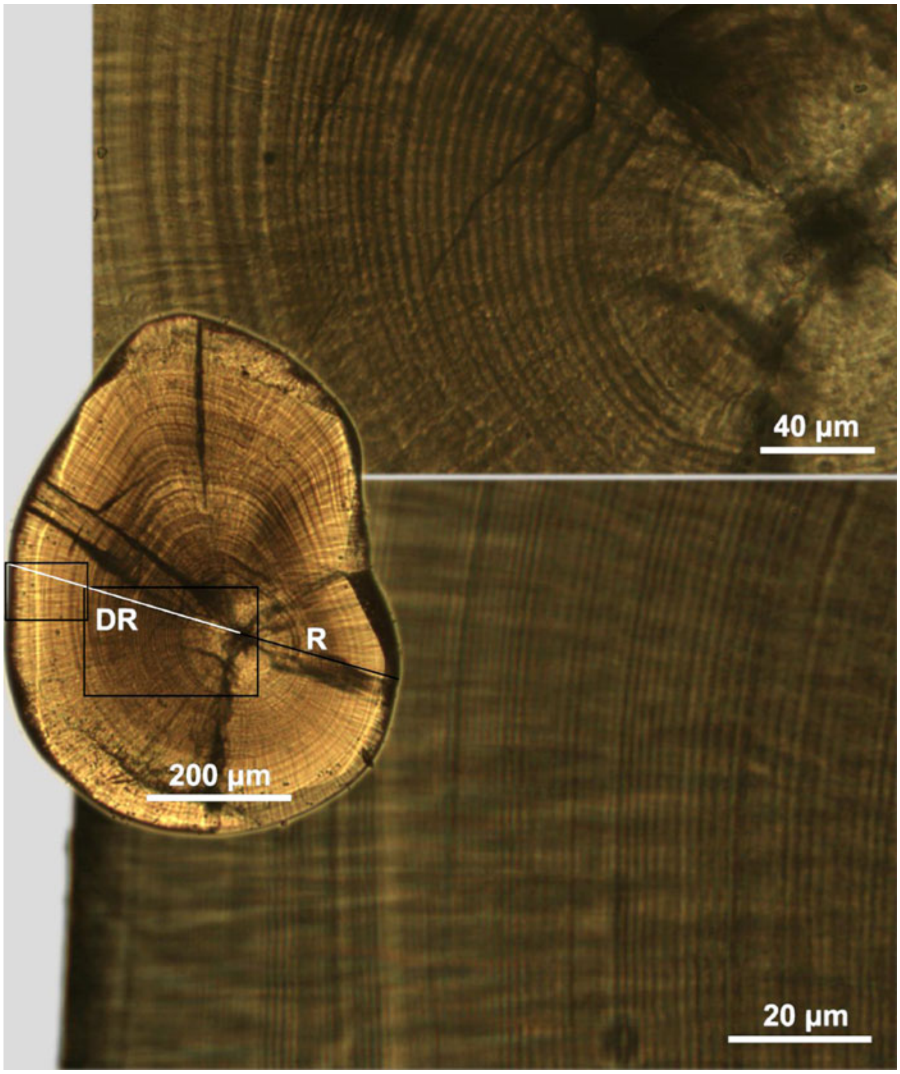

Fig. 2 Daily growth increments (DGIs) in the lapillus of *S. o'connori* with 36 mm SL. The axis *R* (*black line*) was used for ageing and radius measuring, *DR* (*white line*) was used for DGIs counting

The data were presented as mean ± standard deviations (S.D.). Significance was considered when $p<0.05$. The analysis was conducted using SPSS 16.0 and Origin 8.0.

Results

Five hundred fourteen *S. o'connori* specimens ranged from 33 to 553 mm SL (W=0.6–2982.6 g) with the mode size of 400–450 mm SL (Fig. 3).

Annulus characteristics

Microscopically, the three ageing structures of *S. o'connori* showed the typical pattern of teleost fishes, with translucent zones that alternated with opaque zones, attributed to slow and fast growth periods (Figs. 4 and 5). In the otoliths, annuli faded gradually out from the nucleus to the outmost margin (Fig. 4a). The first 4–8 annuli were wide, while the outer annuli decreased gradually in width, generally becoming regular and stable. The increment width abruptly declined, probably at about 20 years old (Fig. 5a). In the vertebrae, annuli appeared as a series of concentric zones regularly parallel with the edge of the centrum. Different from other calcified structures, the increment widths declined slightly with elapsed years because of the concave centrum (Fig. 4b). In some large centrums, the first and the marginal annulus were too indistinct to identify (Fig. 5b). The root of the opercular bone was thick and this thickness fanned out

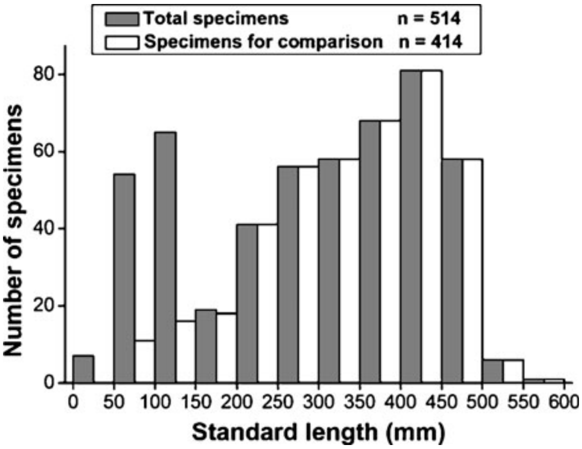

Fig. 3 Distributions of the standard length frequency of *S. o'connori*

into a spongy tissue which often became orange-brown and glutinous. It often made the first and sometimes the second annulus less identifiable (Fig. 4c). The age characteristics then presented a more stable, regular pattern with elapsed years (Fig. 5c).

Age validation and annual periodicity

The lapilli of the seven young *S. o'connori* showed the typical pattern of translucent and opaque zone, which were respectively equivalent to the accretion and the discontinuous zone, composing a daily growth increment. A continuous sequence of concentric rings of decreasing size ranging from 6.57 to 0.72 μm was observed from the otolith core to the margin (Fig. 2). No transition zones (annuli) were observed in any of the specimens aged. The estimated ages were from 130 to 168 days (149±14), thus validating the specimens to be young-of-the-year (YOY) fish. The mean radius of the lapilli was 215.24 (±13.98) μm for the YOY fish, and that of the first annulus was 226.44 (±24.57) μm for the older fish.

For otolith sections with 1–8 annuli, the MIR increased gradually from May to February, and appeared to peak at 0.604 in February. Subsequently, the MIR kept a lower level between March and May, with a minimum of 0.203 in April (Fig. 6a). These results suggested that the opaque band of the otoliths laid down once a year from March to May.

The proportion of translucent edge on the vertebrae increased gradually from March to June (reaching the

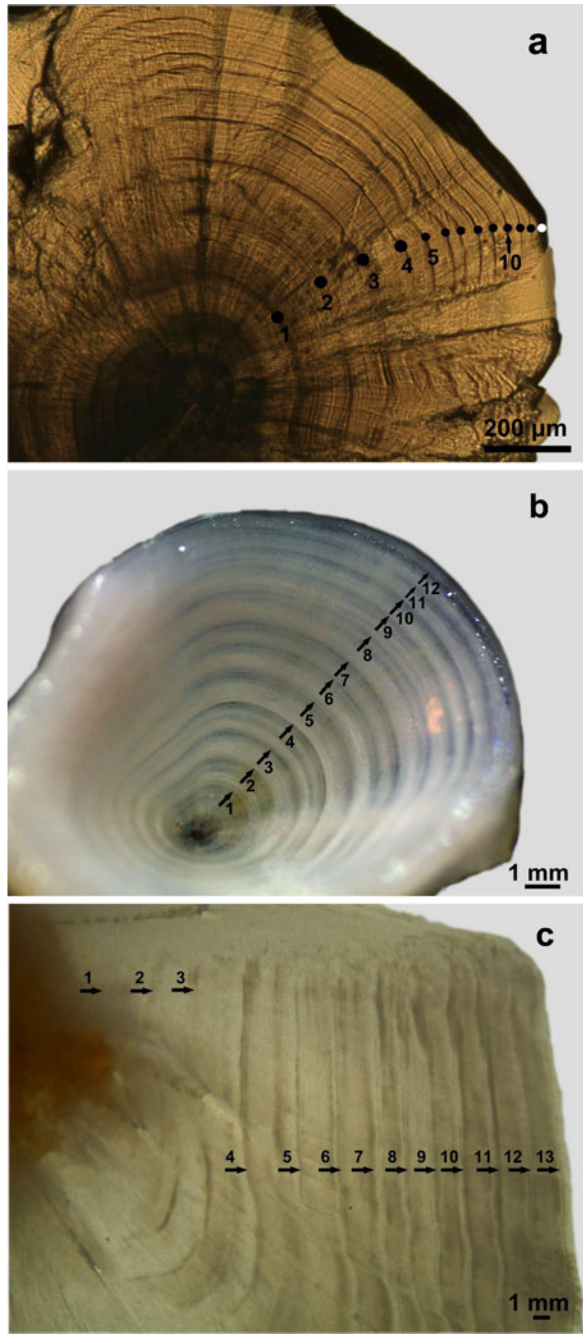

Fig. 4 Sectioned lapillus (**a**) under transmitted light using the compound microscope, vertebra (**b**) under reflected light and operclar bone (**c**) under transmitted light using the dissecting microscope. The three structures collected from the same *S. o'connori* with 402 mm SL. *Dots* (**a**) and *arrows* (**b** and **c**) indicate annuli

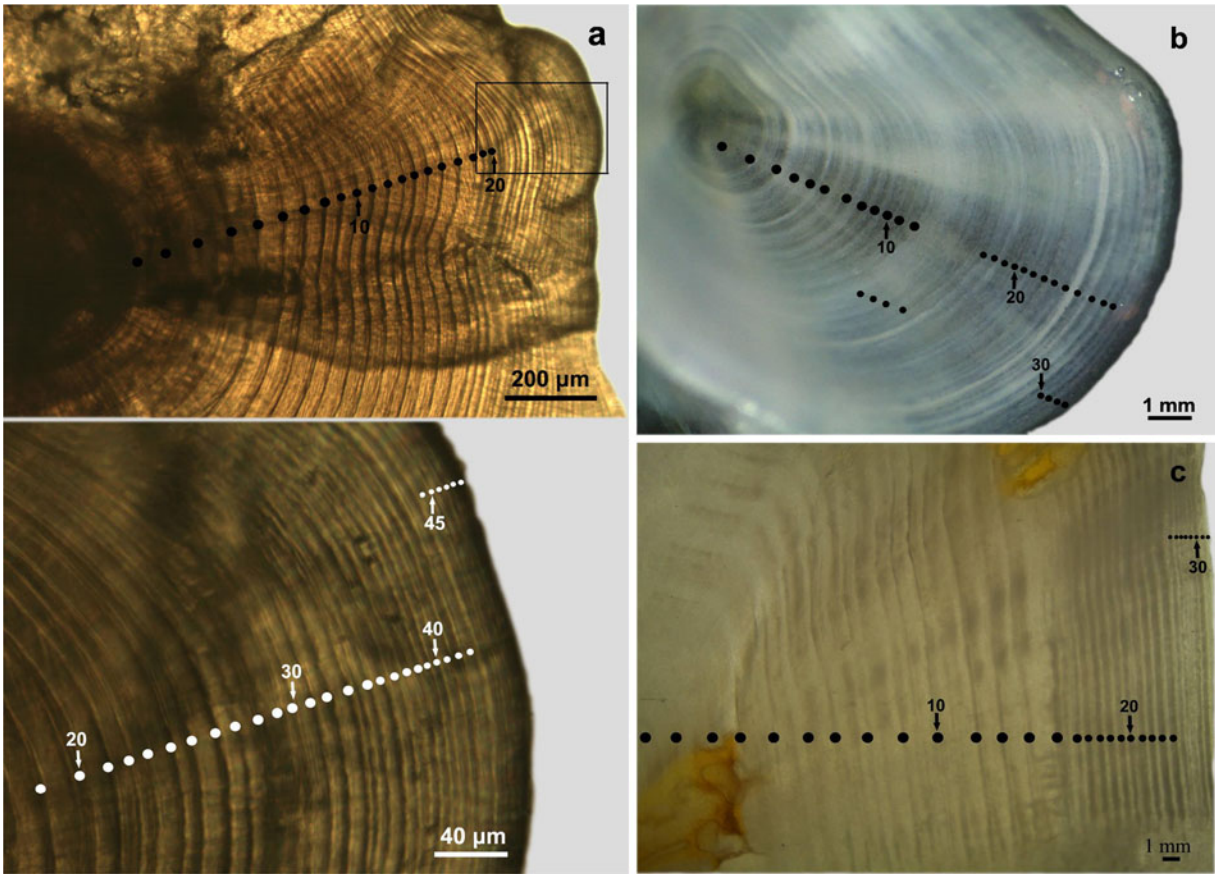

Fig. 5 Sectioned lapillus (**a**) under transmitted light using the compound microscope, vertebra (**b**) under reflected light and operclar bone (**c**) under transmitted light using the dissecting microscope. The three structures collecting from the same *S. o'connori* with 479 mm SL, estimated to be 49, 33 and 32 years old, respectively. *Dots* represent annuli

peak at 100%), and maintained a high level in July and August, then decreased from September to November, and stayed a lower level between December and February (Fig. 6b). In February, the edge was opaque for all fish. A similar finding was observed on the opercular bones (Fig. 6c). The shift in growth pattern of the two bony structures from opaque zone to translucent zone was therefore from March to May.

Comparison of three bony structures

Of the 465 *S. o'connori* sampled, 414 specimens (with 63–553 mm SL) were successfully aged from three calcified structures, while 51 specimens were discarded due to natural deformations and unidentifiable annulus deposition. The specimen number and size of each age class are given in Table 1 and Fig. 3. Annuli were clearer and sharper in otoliths thereby producing fewer errors in age estimation. Relatively more otolith sections were scored as excellent and good in readability, while relatively more from vertebrae and opercular bones were scored as acceptable and poor. Less than 50% of the opercular bones were scored as good in readability (Table 2). IAPE between the two independent readers was highest for otoliths (2.58%), followed by vertebrae (5.13%) and opercular bones (7.92%). The IAPE value of vertebra vs. otolith was 9.3%, while that of opercular bones vs. otolith was 11.4%. There were significant differences among mean values of age estimates from three bony structures (ANOVA, F=5.023, $P<0.05$). Mean values of age estimates from vertebra and opercular bone showed significant underestimation (1.28 and 1.40, $P<0.05$), when compared to that from otolith (Table 3).

The IAPE value of vertebra vs. otolith and opercular bone vs. otolith in each age class indicated vertebrae were more accurate than opercular bones

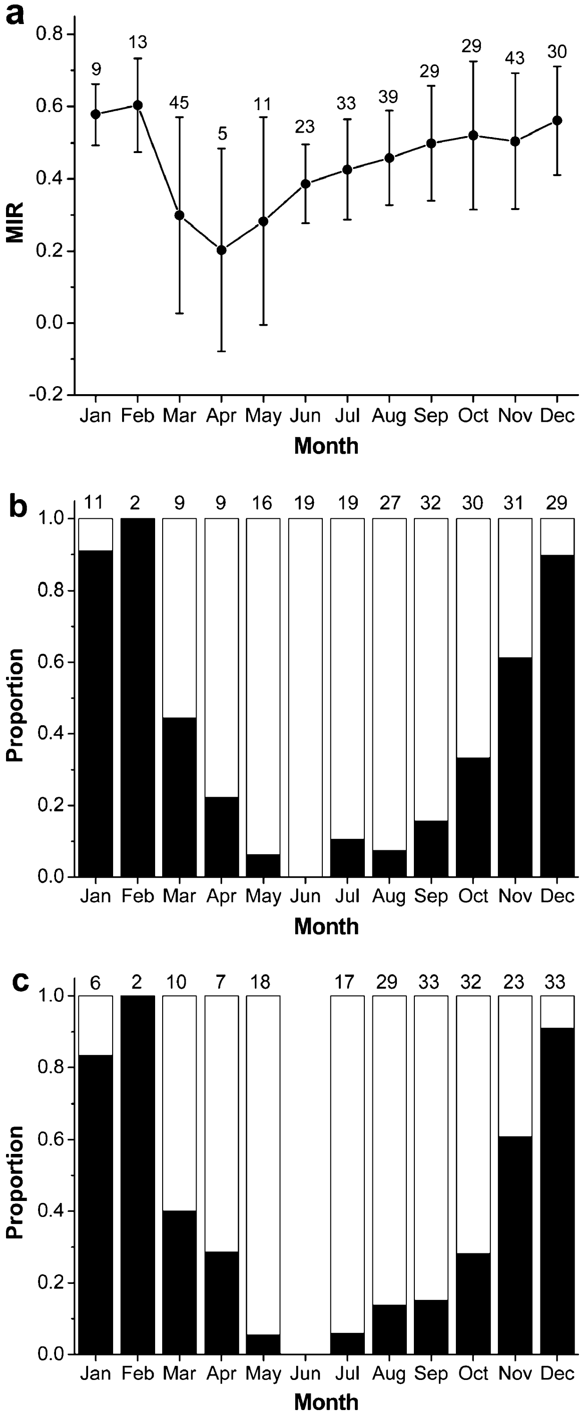

Fig. 6 **a** Mean monthly MIR for *S. o'connori* lapillus otoliths with 1–8 annuli, error bars represent the S.D, (**b**) and (**c**) the proportion of *S.o'connori* vertebrae and opercular bones (1–8 annuli) with opaque and translucent margin zone. *Black bars*, opaque margin zone; *white bars*, translucent margin zone. Numbers above bars indicate the number of samples

(Fig. 7). For age classes 1–12, the IAPE of vertebra vs. otolith ranged from 0 to 8.3%; in age classes 13–21, the IAPE ranged from 7.4 to 10.9%; but in age classes 22–50, the IAPE rose linearly from 9.5 to 34.5%. In opercular bones, the IAPE presented a fluctuation of around 11% for age classes 2–22; while in age classes 23–50, the IAPE rose from 6.5 to 37.5%. For age classes 1–21, mean values of age estimates from different structures showed no significantly difference ($P>0.05$) between otolith and vertebra, but showed significant difference (1.00, $P<0.05$) between otolith and opercular bone. Beyond age 21, mean values of age estimates from vertebra and opercular bone both showed significant underestimation (5.35 and 4.44, $P<0.05$), when compared to that from otolith (Table 3). For example, the age obtained from vertebra and opercular bone was 16 and 17 years younger than otolith for age 49 (Fig. 5). In addition, the ages read from different structures presented more variability for individuals beyond 400 mm SL (Table 1).

Discussion

According to the capture date (4 January) and the hatch time (April and May, Fishery Bureau of Tibet Municipality 1995), the DGIs of the YOY fish should be about 240, but there were only 130–168 observed in this study. This phenomenon was also described in other Schizothoracinae fishes. The DGIs within the first annulus were 121–184 in *Oxygymnocypris stewartii* (Jia and Chen 2009) and 137–154 in *Ptychobarbus dipogon* (Li et al. 2009). It seems that optical resolution would be insufficient to keep track of daily increments in winter (Chen et al. 2002a,b). The mean number of microincrements represented the number of days (about seven or eight months in one year) when Schizothoracinae fishes grew rapidly (Jia and Chen 2009). And the narrower DGI width might be due to decreasing growth rates during the cold winter in Tibetan plateau (Li et al. 2009). From November to February, water temperature is below 5°C, and there is ice floating in the Yarlung Tsangpo River in December and January. *S. o'connori* often inside crevices or underneath boulders, and rarely goes out for feeding. It is possible that *S. o'connori* grows slowly for about four months in the cold winter, and their otoliths also undergo a greatly reduced growth.

Table 1 Number of specimens and mean ± S.D. of standard length (SL) at age of *S. o'connori* estimated from otoliths, vertebrae and opercular bones

Age (years)	Otoliths		Vertebrae		Opercular bones	
	n	Mean ± S.D (mm)	*n*	Mean ± S.D. (mm)	*n*	Mean ± S.D. (mm)
1	3	68.8±6.0	4	71.1±6.7	5	74.5±9.7
2	9	91.3±18.0	10	95.9±19.1	11	101.4±20.8
3	14	127.1±26.9	18	139.9±27.8	22	153.2±39.7
4	24	178.8±34.1	32	200.2±34.0	30	213.1±37.6
5	34	235.6±26.3	35	249.3±24.6	36	259.1±35.9
6	32	262.5±35.7	37	280.9±35.1	37	283.5±34.6
7	31	294.9±28.4	31	309.1±32.7	32	313.7±40.4
8	32	318.1±33.0	29	337.1±25.2	37	345.6±41.1
9	21	345.8±42.7	21	363.0±49.0	33	374.0±48.1
10	29	376.7±37.0	42	389.1±41.9	28	408.7±39.6
11	29	381.7±42.0	26	407.9±42.0	22	416.8±50.7
12	26	410.6±37.6	21	418.3±47.9	14	411.1±48.1
13	16	426.6±45.4	8	444.3±38.1	12	413.9±33.7
14	14	425.8±56.5	10	436.9±33.3	5	460.8±18.6
15	5	437.4±21.0	6	451.0±33.8	9	445.7±39.7
16	4	457.5±47.9	6	431.5±25.5	4	425.8±23.5
17	7	447.4±35.5	10	417.6±36.2	8	430.0±33.6
18	11	427.6±30.9	10	438.4±47.1	10	456.3±37.7
19	5	430.2±49.1	6	418.7±25.2	10	419.9±34.7
20	11	432.4±39.7	16	446.8±35.0	9	439.3±39.3
21	12	446.3±26.5	10	457.9±40.3	5	461.0±31.0
22	2	426.0±73.5	4	469.5±31.2	7	447.4±49.0
23	3	454.3±17.9	2	458.5±21.9	4	439.5±29.3
24	8	450.5±43.6	8	460.5±39.3	9	458.4±49.0
25	6	430.8±29.9	3	425.0±48.0	2	454.0±33.9
26	2	422.0±35.4	1	482	1	461
27	5	466.4±60.9			3	474.0±69.5
28	3	470.0±84.5	3	483.3±65.9	2	445.0±28.3
29	1	427				
30	2	489.0±48.1			1	475
31	3	441.7±28.0	1	457	1	455
32	1	454			1	479
33	1	473	3	463.7±13.3	2	469.5±17.7
34	2	478.5±4.9	1	480		
35	1	478				
36					1	457
38	1	457				
39					1	
40	1	422				
42	1	457				
49	1	479				
50	1	480				
Total	414		414		414	

Table 2 Distribution of readability scores for different calcified structures of *S. o'connori*; the values are percentages of the sample size (n=465)

Structure	Readability scores				
	1	2	3	4	5
Otoliths	5.0	78.8	13.3	1.4	1.5
Vertebrae	2.4	65.0	21.7	9.7	1.2
Opercular bones	1.2	48.3	28.5	19.6	2.4

DGIs between presumed annuli can provide strong corroboration of the frequency of annuli formation. DGIs can be analyzed to estimate the expected radius of the first annulus in the otoliths (Campana 2001). The mean radius of the YOY fish was approached that of the first annulus in the older fish. Thus, the first annulus of lapilli was considered to be validated in *S. o'connori*. And the daily increment would be further validated in our studies to confirm this result.

In this study, the MIR and edge analysis validated that annuli formed once a year up to age 8 in all of the three ageing structures. Campana (2001) mentioned that the MIR and edge analysis can validate the annuli for young fish, but can not be applied to older fish. Hence, the ageing of the older fish (after age 8) in *S. o'connori* should be further validated by tagging or radiometric methods in the future.

Otoliths have been favored over other ageing structures because otoliths are acellular and not subjected to resorption (Sponaugle 2009) and also because otoliths are metabolically inert and thus do not reflect physiological changes that may occur throughout the life of the fish (Phelps et al. 2007). Furthermore, in slow-growing or old fish, otoliths

Table 3 Comparison of mean values of age estimates from different calcified structures in *S. o'connori*. Values having different superscripts in each column are significantly different (P<0.05) from each other

Bony structures	Mean values of age estimates		
	Total	Age 1–21	Age >21
Otoliths	11.66±7.86^{a}	9.57±4.91^{a}	27.30±4.40^{a}
Vertebrae	10.38±6.41^{b}	8.82±4.60^{ab}	21.95±3.44^{b}
Opercular bones	10.26±6.74^{b}	8.57±4.65^{b}	22.86±4.12^{b}
n	414	369	37

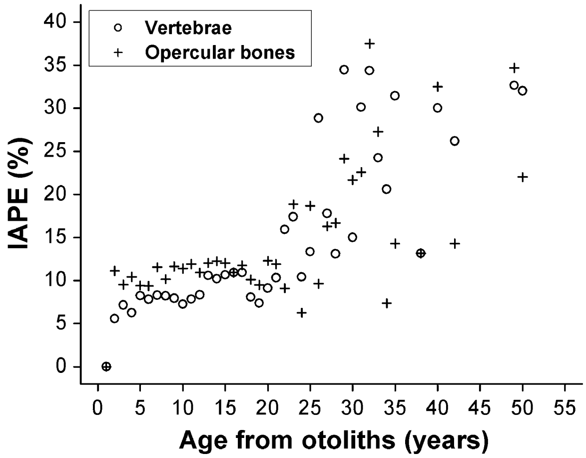

Fig. 7 The index of average percentage error (IAPE) between vertebrae, opercular bones and otoliths age estimates from samples collected from the same specimen, plotted as a function of the otolith-based age

grow more rapidly than the other structures and continue to record cyclic seasonal growth and age (Casselman 1990). A great deal of research has documented that sectioned otoliths provides the most precise and accurate age estimates (Phelps et al. 2007; Gunn et al. 2008).

Vertebrae have been used for age determination and growth analysis in many studies (Alves et al. 2002; Liu et al. 2009). Polat et al. (2001) showed that vertebra was the most reliable structure having minimal ageing error in *Pleuronectes flesus luscus*. In the current study, vertebrae provided age estimates similar to those from otoliths up to age 21, but consistently underestimated age of *S. o'connori* after age 21. The findings were similar to those of Gunn et al. (2008), who reported that age estimates of *Thunnus maccoyii* from vertebrae and otoliths matched closely up to age 11, and then the counts diverged for older individuals. Nargia (2006) and Khan and Khan (2009) suggested that annuli in opercular bone can be used to determine the age for *Catla catla*. However, opercular bone was found less reliable than both otoliths and vertebrae for age estimate in the current study.

Vertebrae and opercular bones could underestimated the age of older *S. o'connori* (especially after age 21), which may be attributed to the following factors: (1) the first annulus on vertebrae and opercular bones could not be validated in this study, which may have resulted in estimate errors; (2) the dense root of opercular bones often obscured the first and sometimes the second annulus; (3) crowding and blurring at the edge of the vertebrae made the annuli difficult to distinguish.

It was concluded that annuli were formed in the otoliths, vertebrae and opercular bones once a year up to age 8, and otoliths would be the most precise and accurate structure for age determination in *S. o'connori*. However, when completing age estimates to analyze simple population metrics of *S. o'connori*, we recommend using vertebrae for fish up to age 21, if the otoliths can not be obtained easily.

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