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Otolith characteristics and age determination of an endemic *Ptychobarbus dipogon* (Regan, 1905) (Cyprinidae: Schizothoracinae) in the Yarlung Tsangpo River, Tibet

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Abstract We describe the microincrements, checks and annuli in the lapilli of the schizothoracine *Ptychobarbus dipogon*, an endemic species of the Tibetan plateau. We collected samples in the Yarlung Tsangpo River and its tributaries on a monthly basis (from April 2004 to August 2006). We describe the shape features of the three pairs of otoliths and document the full trajectory of lapillus development. We found that five to seven checks were clearly visible in the opaque zone of the first annulus. The pattern of 21–23 daily growth increments within each check might be explained as a lunar-induced deposition. We counted between 137 and 154 increments within the first annulus. Annuli appeared as a sequence of gradually declining increment widths, whereas false rings were characterized by abrupt checks. Our oldest estimates were 23^{+}years for males and 44^{+} for females. The time of annulus completion was clearly between March and April each year using monthly marginal increments analysis. We consider the factors responsible for daily increment formation as an endogenous circadian rhythm. Environmental information, such as strong sunlight and cold water temperatures in the Tibetan Plateau, could reinforce the endogenous daily cycle. Our results provided important data addressing the ecology and population dynamics of *P. dipogon*.

Keywords *Ptychobarbus dipogon* · Otolith · Age determination · Yarlung Tsangpo River

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Introduction

Accurate age determination is imperative for understanding population dynamics and thereby for an optimal resource management in ecological studies of various fish species (Hilborn and Walters 1992). Determination of periodic growth increments in many calcified structures is the most common means of determining age (Casselman 1996; Campana 2001). Otolith growth patterns not only provide a chronological record of early life history, stock structure, growth parameters and migration, but can also record ambient environmental information, such as water temperature, sunlight intensity, and food supply. The annual pattern is typically an alternating sequence of opaque and translucent bands represented differential otolith growth as a result of seasonal changes. Campana and Neilson (1985) suggested that daily microincrements in otoliths of teleost fishes is a widespread phenomenon. Daily increment counts between the nucleus and first annulus had been successfully used to validate annular periodicity (Victor and Brothers 1982).

The subfamily Schizothoracinae has a wide distribution in the Tibetan Plateau and adjacent areas.

Degenerated body scales are one of the prominent characteristics of the subfamily (Cao et al. 1981). Age determination in these species has relied mainly on the irregular anal scales (Tsao and Wu 1962; Zhao et al. 1975). However, it has been difficult to obtain precise and reliable age estimates owing to the slow growth and narrow increments in the anal scales. Zhao et al. (1975) pointed out that it was very difficult to determine ages from the anal scales for *Gymnocypris przewalskii* over 10 years of age. Chen et al. (2002a, b) reported that sectioned lapilli provide more reliable age estimation based on assessing the consistency of age interpretations for *G. selincuoensis*. Thus, the otolith growth mark patterns appeared to be more legible and regular than those of scales for schizothoracine species. In addition, Song et al. (2006) also described otolith microstructure of larval *G. potanini* Herzenstein from the Minjiang River in China.

Ptychobarbus dipogon is an endemic species in the Tibetan Plateau, only distributed in the middle and upper reaches of the Yarlung Tsangpo River and its tributaries at an elevation of about 4000 m (Wu and Wu 1992; Chen and Cao 2000). *P. dipogon* generally inhabits the deeper waters of tributaries and river meanders, and have powerful muscular cylindrical bodies. Although this species was locally abundant, the available information refers mostly to taxonomical species description. Therefore, there is an urgent need for studies of the life-history characteristics and adaptability of *P. dipogon*. It is important to understand its age structure for the reasonable conservation and management of *P. dipogon*.

The objective of our study was firstly to describe the morphological characters of the three types of otoliths of *P. dipogon*; secondly, to validate the daily increments and the presence of checks on lapilli; thirdly, to estimate age and investigate the nature of annuli; and finally, to confirm the time of annuli formation using marginal increment analysis. We also considered the mechanisms underlying the formation of checks and annuli in relation to the plateau environment.

Materials and methods

Collection of samples

We randomly collected a total of 610 *P. dipogon* individuals from the Yarlung Tsangpo River and its tributaries monthly from April 2004 to August 2006 (Fig. 1). The majority of *P. dipogon* (90.4%) caught were from the Lhasa River. To ensure most age-classes, we captured the samples using floating gill nets and cast nets. The body weight (W), total length (TL) and standard length (SL) were measured to the nearest 0.1 g and 0.1 mm using digital scales and vernier calipers on fresh specimens, respectively. Specimens were classified as male or female or undetermined by macroscopic examination of gonads. We removed the otoliths (sagitta, asteriscus and lapillus) from the head (vestibular apparatus) of fish using the 'guillotine' method (Secor et al. 1992). After rinsing with water, the otoliths were air-dried, and then stored in labelled tubes.

Otolith preparation and microstructural measurement

The size distribution of *P. dipogon* whose lapilli were extracted ranged from 70.6 to 593.0 mm TL. A preliminary analysis of three types of otolith under light microscopy showed that only sectioned lapilli exhibited clear increments. Therefore, the microstructures of sagittae and asterisci were not further examined. The lapillus was mounted on glass slides using thermoplastic resin, grinded transversely using wetted 800–1200 grit abrasive paper and polished with film by hand until close to the core. The section was remounted and grinded with the polished surface down. The thickness of the sectioned otolith was about 0.1–0.2 mm when annuli became clear. Sectioned otoliths were observed under the microscope with transmitted light, and photographed (Carl Zeiss Axioplan 2 Microimaging System). Magnification varied from ×50 to ×400 according to the size of section.

The microstructural variables were measured on all interpretable sectioned otoliths. Increment width measurements were performed along a very clear increment trajectory from the primordium to the lateral and posterior edges. All counts and increment measurements were made along the same reading axis using an image analyzing system (Zhu et al. 2002). The reader had no prior knowledge of length, sex or time of capture before the estimation.

Otolith reading and age validation

Alternating concentric opaque and translucent zones around the lapillus nucleus were considered as annuli and counted. Under light microscopy, microstructure provided a means of distinguishing between the translu-

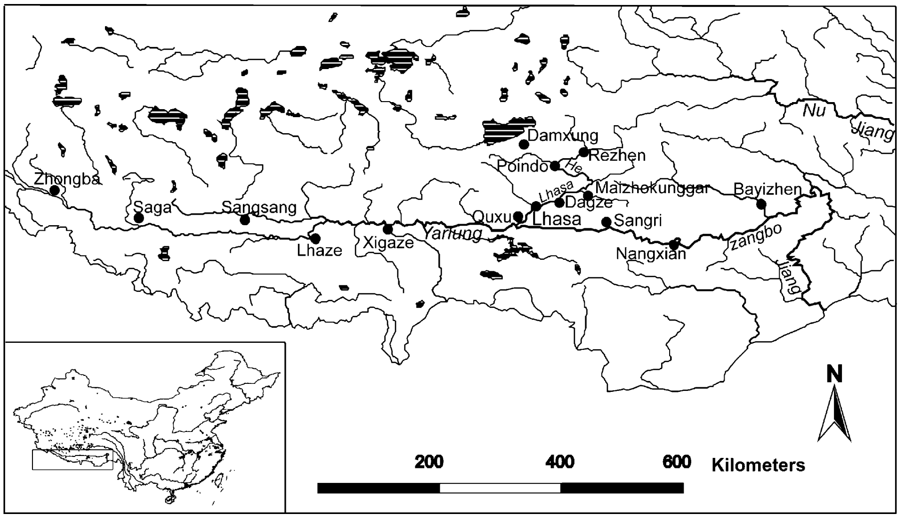

Fig. 1 Sampling locations of *P. dipogon* in the Yarlung Tsangpo River, Tibet

cent material that formed false rings and annuli. Ages were determined twice by the same interpreter after a considerable time (30 days). Age bias plots were used to assess intra-readings bias. Coefficients of variation (CV, %) were calculated as the standard deviation of the corresponding mean estimated ages in each age class between two readings, as it is considered the most valuable coefficient when dealing with comparisons (Campana 2001). The equation (Chang 1982) was expressed as follows:

$$\mathrm{CV}_j = 100\% \times \frac{\sqrt{\sum_{i=1}^{R} \frac{\left(X_{ij}-X_j\right)^2}{R-1}}}{X_j}$$

Where CV_j is the age precision estimate for the jth fish, R is the number of readings, X_{ij} is the ith age determination of the jth fish, and X_j is the mean age calculated for the jth fish. The result is considered good if the CV is lower than 0.05 (Laine et al. 1991). If no precise pairs of readings were found for an otolith, then that otolith was excluded from subsequent analyses.

Marginal increment ratio analysis

The marginal increment (the distal edge of the last translucent zone) ratio analysis (MIR) was used to validate the period of annuli formation. The monthly changes of marginal increment ratio were estimated with the following equation of Hass and Recksiek (1995): $\mathrm{MIR} = (O_c - O_n)/(O_n - O_{n-1})$; where O_c (µm) is the otolith radius at the time of capture, O_n (µm) is the radius to the last formed annulus, respectively. $O_{n\text{-}1}$ (µm) is the radial distance of the penultimate formed annulus, and n denote the number of rings. Typically, the months of mark formation were usually indicated by a bimodal MI frequency distribution because of the occurrence of '0' MIs and maximum marginal increment. The '0' MI was defined as a very narrow opaque margin in the distal edge of the translucent band of otolith section. The margin increment called '0' MI never exceeded 10 µm. In addition, the date of annulus formation of 1^+fishes was validated by back-calculating the new microincrements according to the date of its capture.

All data were analyzed using the Origin 7.0 package. The images were modified with Photoshop 8.0.

Results

Otolith morphometrics and readability

Each otolith of *P. dipogon* has a relatively stable external character. The lapillus was the largest pair of otoliths of *P. dipogon* (Fig. 2a). The increments in the sectioned lapilli were clearly deposited from the primordium to the proximal margin. The asteriscus was a disk with a serrated edge (Fig. 2b). The wavy characters of annuli rings were observed with an ambiguous core, leading to difficulty in discerning age. The sagitta was shaped like an arrowhead (Fig. 2c). Although the anterior part of the sagitta was readable without the grinding and polishing procedure, the sagitta was not appropriate for growth analysis because it was fragile and often broke during the extracting procedures. Consequently, only the lapillus was appropriate for daily increment analysis and age determination on the basis of structural suitability and visibility.

Microstructural features and daily increments

Lapilli showed a regular pattern of increments along the otolith radius when viewed by light microscopy. We observed and measured otolith microstructure of 13 juveniles with TL ranging from 70.6 to 108.5 mm. For the description of internal features, we followed the terminologies of otolith microstructure defined by Campana and Neilson (1985) and Greely et al. (1999). Three distinct regions were apparent within each sectioned lapillus: the larval zone (LZ), postlarval zone (PLZ) and postmetamorphic zone (PMZ). The larval zone included the core out to the metamorphic check which usually located at the last continuous circular increment. The otolith microstructures were shown in some details of primordium (P), nuclear (N) and hatch check (HC). Two primordia formed one nucleus region in most otoliths. The diameter of the nucleus was approximately 72.56±3.40 μm (mean ± SD). The primordium was spherical in shape, and measured 15.72±1.63 μm in diameter (Fig. 3a). The daily growth microincrements (DGI) and monthly-like growth increments (MGI) under light microscopy were clearly showed in Fig. 4. The translucent L-zone and opaque D-zone, which were respectively equivalent to the accretion zone and the discontinuous zone, composed a daily growth increment. The increment width decreased from 3.21 μm in the innermost to 1.84 μm at the margin of the lapilii (Fig. 4c,d). The checks corresponded to a sequence of narrow translucent zones in a annulus. Monthly-like growth increments (MGI) were deposited at about 50 μm spacing with a succession of wide and narrow microincrements. The daily increments varied from approximately 21–23 cases in MGI. The number of daily increments was close to one synodic month, except for the full moon phase (Fig. 4a,b).

Although the postlarval zone of the lapillus was extremely dark, the checks and daily growth increments encircling the nucleus region were prominently visible. The postmetamorphic zone was the region from the terminal increment of the PLZ to the outermost edge of the lapillus. The PMZ was abruptly demarcated by a transition in elemental composition. Under transmitted light, the PMZ represented a regular pattern of alternating continuous and discontinuous zones. The first annulus was observed at intervals of five to seven distinct discontinuous zones (check C), and had an average semidiameter of 205.11±25.78 μm (Fig. 3b). The number counted from regular microincrements ranged from 137 to 154, with a maximum of 220. The pattern of microincrements was the same in the sectioned lapilli from individuals of different sizes. Checks often clearly occurred on the first three annuli. However, the deposition of daily microincrements and the pattern of checks between successive annulus could not be observed in peripheral regions of the otoliths of adults.

Annuli characteristics and age validation

Macroscopically, the sectioned otolith showed a clear pattern of opaque-and translucent-zones under transmitted light (Fig. 5). Annuli appeared as a sequence of concentric zones of gradually declining increment widths. The annuli faded gradually out from the nucleus to the outmost edge (Fig. 5b). This phenomenon commonly occurred in individuals prior to 10 years of age. The characteristics of lapilli presented a more stable, regular pattern with elapsed years. During the early growth phase, the opaque zone contained wider increments relative to the translucent zone, and was composed of five to seven successive growth interrup-

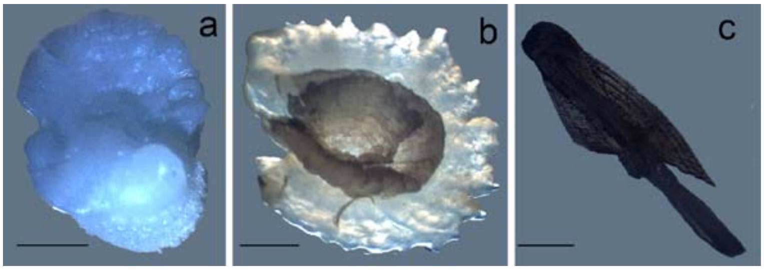

Fig. 2 Morphology of three types of otoliths of *P. dipogon*. **a** lapillus, **b** asteriscus, **c** sagitta (scale bar=1 mn)

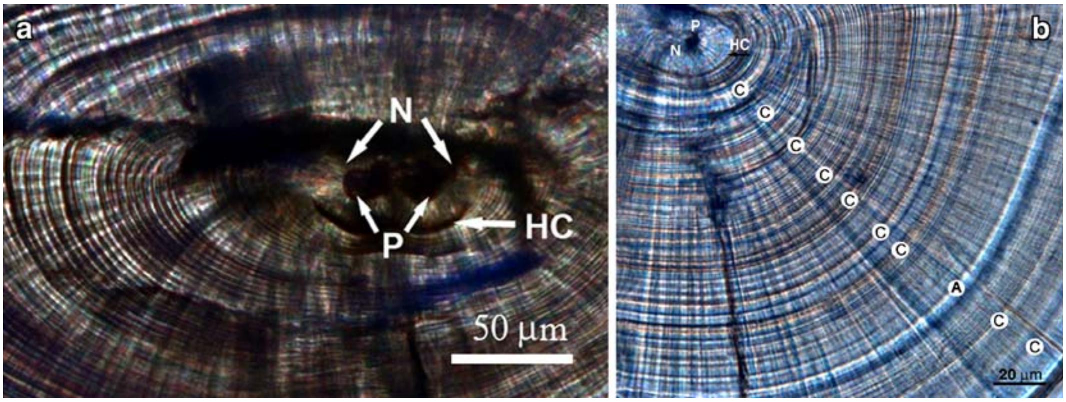

Fig. 3 Microstructures near the nucleus region of the *P. dipogon* on lapillus viewed under light microscopy, showing **a** primordia (P), nucleus (N), hatch check (HC). **b** monthly-like check (C) and annual ring (A) of lapillus from a 70.6 mm TL *P. dipogon*

tions (checks) (Fig. 5a). However, the pattern of depositions was reversed for the oldest age classes. In a word, the translucent zone became much broader than the opaque one (Fig. 5b). The annulus characters in the ventrolateral area of the otolith appeared to be more reliable because of the legibility and regularity of its growth marks (Fig. 5c). Otoliths grew at a measurable rate throughout the life of the fish. However, the increment width abruptly declined, probably at about 19 years old.

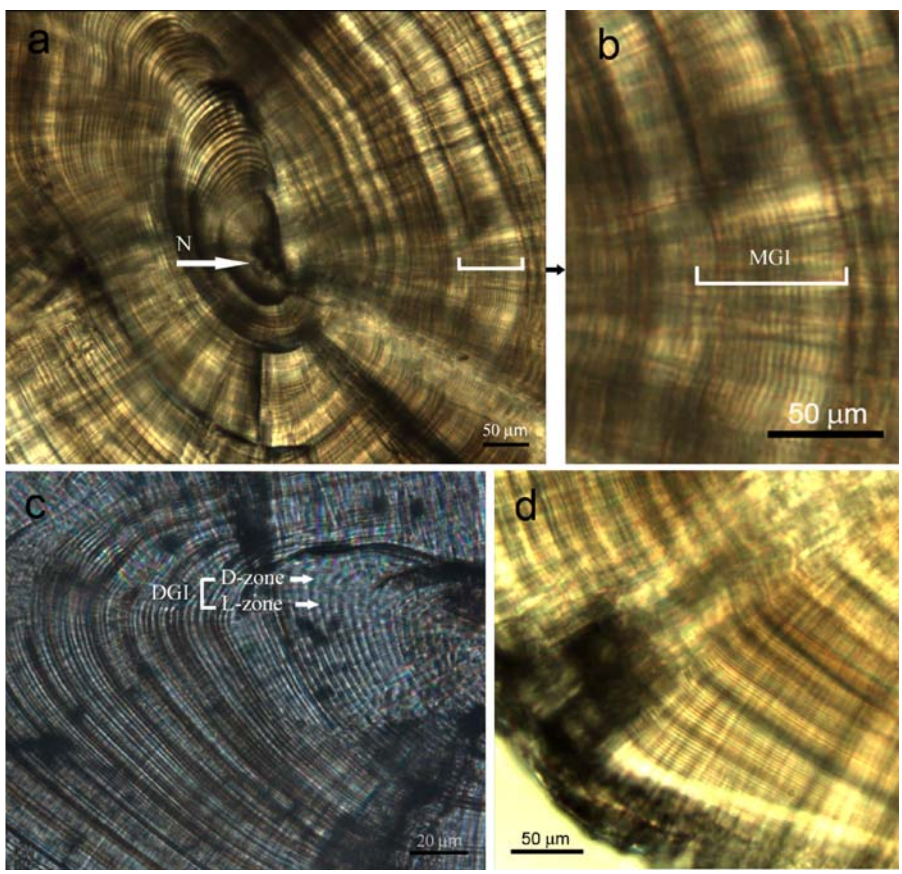

Fig. 4 Daily growth increments and monthly-like growth increment in the lapillus of *P. dipogon* exhibited under transmitted light, showing **a** area surrounding the core. **b** Monthly-like growth increments (MGI). **c** Daily growth increments (DGI) of core region. **d** Daily growth increments (MGI) of peripheral area. *Arrow head* indicates the translucent zones (L-zone) and opaque zone (D-zone)

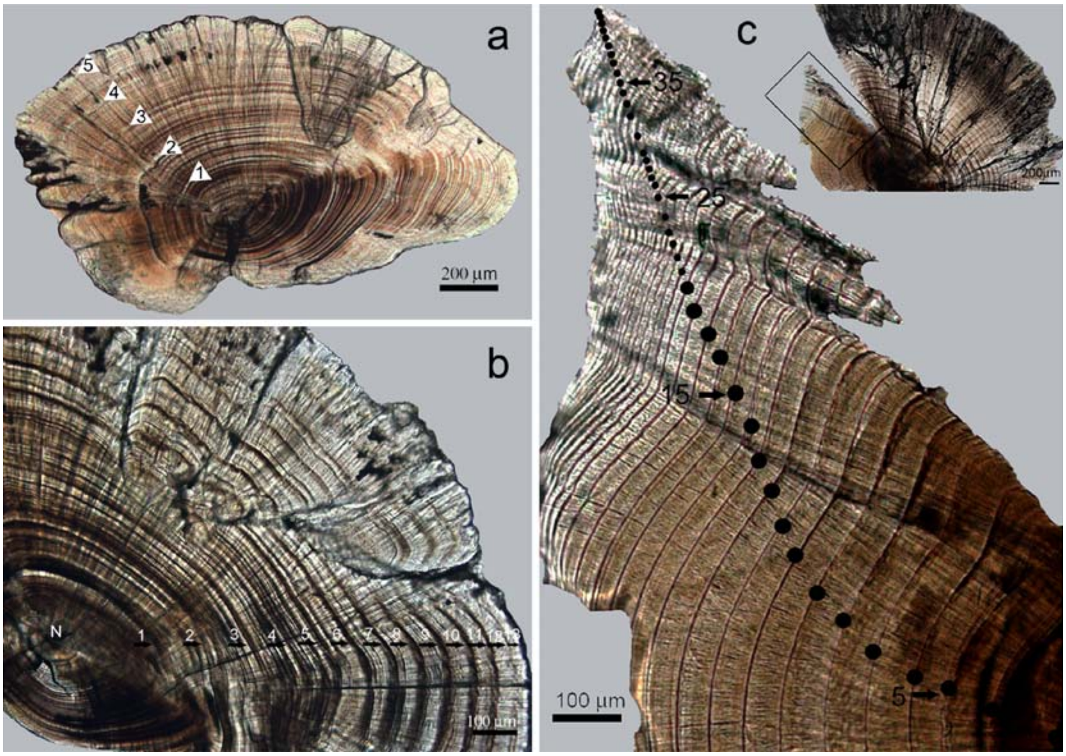

Fig. 5 The typical magnification appearance of a *P. dipogon* transverse cross-section lapillus under transmitted light. Annual growth increments are denoted as annuli with *triangles*, *black dots* and *arrows* from first to fifth in (**a**), 1st–13th in (**b**) and 5th, 15th, 25th and 35th annulus in (**d**), respectively

Ages were determined in 420 lapilli, only 28 (6.67%) were discarded due to natural deformations and unidentifiable annulus deposition. Ages ranged from 1+ to 23+ years for males and 1+ to 44+ for females. The deviations observed between successive readings were ±3 years for 5≤ ages ≤8 years and >19 years. The low CV (3.14%) of age estimates reflected good concordance in the readings.

Marginal increment ratio and annuli formation

For otolith sections with 1+–8 annuli, their marginal increments followed the same pattern. A fish with four annual marks and a 0 MI was observed in April (Fig. 6c), a new mode of very small increments appeared during June, indicating individuals had formed a new mark during March or April (Fig. 6a,b). Monthly MI frequency plots showed marked deviations through March or April (Fig. 7), showing that 0 MI's and maximum margin growth co-occurred. In general, the progression of the mode was consistent over long periods of time (from May to November) when otolith margin growth resumed. The MI remained stable in winter, indicating very slow winter otolith growth. These consistent patterns of MI growth indicated that the time of mark formation was restricted to the same short time period each year, March and April.

Discussion

The morphometrics and daily rhythm of otolith microincrement of *P. dipogon* are the first successful validation in schizothoracine fishes. Under the microscope, the microincrements of *P. dipogon* lapillar otoliths were very clear. However, the reproducibility of annulus determination relied on the section orientation during otolith preparation and the exclusion of false annuli. The reading errors mainly involved the first annulus because the center of the section was relatively opaque. In addition, marginal annulus estimates became subjective due to the very narrow and limited visibility in the outermost edge of some older ones.

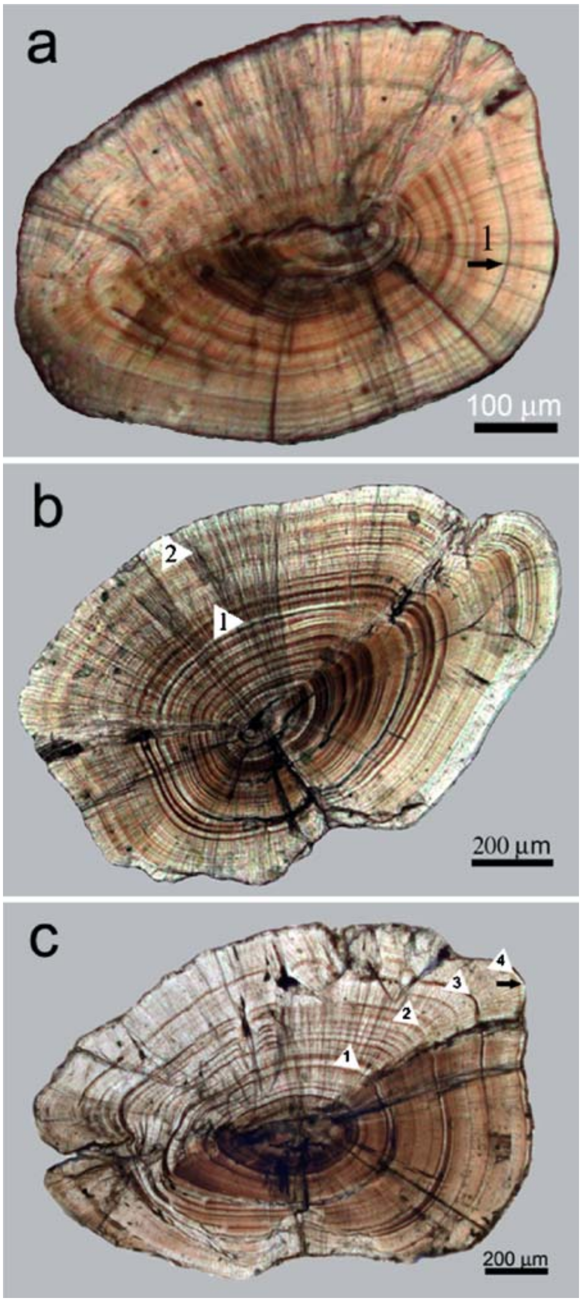

Fig. 6 The edge condition for a lapillus of *P. dipogon* under the light microscope. The *arrowhead* indicates the annual ring formation: **a** Otolith from first year fish with opaque edge (age=1+, 70.6 mm TL and 2.3 g, June 2006); **b** Otolith with a wide opaque edge (age=2+, 162.6 mm TL and 36.1 g, June 2006); **c** Otolith with a translucent edge (age=4, 231.2 mm TL and 98.0 g, April 2004)

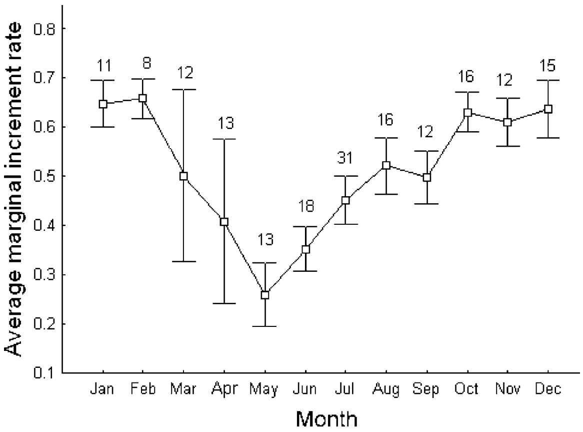

Fig. 7 Monthly changes in mean marginal increments rates of otoliths from pooled sexes and size classes of *P. dipogon*. The *vertical bars* indicated standard errors (n=177)

The microstructure of sectioned otoliths of *P. dipogon* revealed the bipartite nature of otolith increments, each of which consisted of a translucent zone (calcium-rich component) and an opaque zone (organic-rich component) under transmitted light. The sectioned lapilli demonstrated a distinct pattern of five to seven checks in an annulus, and the daily increments of *P. dipogon* contained approximately 21–23 cases within each check. The daily growth increments of *P. dipogon* are regulated by an endogenous circadian rhythm. The otolith checks of *P. dipogon* reflect the influence of lunar cycles on the formation of increments. However, the number of monthly checks never approached 12, nor did the number of daily growth increments within these regions approach 365. Chen et al. (2002b) described this phenomenon in *G. selincuoensis*. The average size per increment would have gradually decreased to about 0.1 µm day^{-1} in winter. It seems that optical resolution would be insufficient to keep track of daily increments.

We presume that the narrower microincrements might be due to decreasing growth rates during the cold winter (Feet et al. 2002; Chen et al. 2002a; Guibbolini et al. 2006). Geffen (1982) stated the growth rate limitation hypothesis that translucent zone formation was triggered at a threshold of metabolic stress, and that the combined energetic requirements of reproduction and migration might maintain translucent zone formation. The climate of the region is strongly influenced by the elevation of the Tibetan Plateau. Water temperatures of the Lhasa River averaged 7.5°C, fluctuating from 0.8 to

13.9°C (Xiong and Tang 1998). It is possible that *P. dipogon* grows slowly for about 5 months in winter and their otoliths also underwent a greatly reduced increment width of the translucent zone when the water temperatures drop below 5°C (Casas 1998). In addition, Hayashi et al. (2001) reported otolith microincrements growth in the myctophid (*Myctophum asperum*) increased during the night and early morning and stopped growth during the day, and also exhibited lunar periodicity in the deposition of growth increments. The mean increment widths around the time of a full moon were significantly narrower than those around a new moon in 29 cases. The moonlight in the Tibetan Plateau is as strong as the daytime sunlight in the full moon period. The appearance of checks could also have resulted from the slower growth caused by staying in deeper and colder water in response to the light of the full moon.

The annual mark consisted of a bipartite set of bands, a wide opaque band formed in summer and a narrow translucent band formed in winter. A full trajectory of lapillus growth was seen with gradually declining increment widths. Age determination of older fish confirmed 23+ years for males and 44+ for females. Thus, we believe that *P. dipogon* is a long-lived and slow growing coldwater fish based on the correlation of the otolith and somatic growth.

Despite the differences in sampling locations and time, all samples presented the same pattern of growth in their otoliths. Therefore, this deposition pattern could be explained that *P. dipogon* did not subject to migration but physiological transformation. In addition, *P. dipogon* in the Yarlung Tsangpo River lived substantially longer and grew more slowly than previously reported in this subfamily (Tsao and Wu 1962; Zhao et al. 1975; Chen et al. 2002b). Therefore, as a highly vulnerable fisheries resource, it is essential to take adaptive management to maintain the sustainability of this species and stability of the fish community.

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