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RESEARCH PAPER

Comparison of Five Calcified Structures for Estimating the Age of Bream *Abramis brama* (L.) from the Irtysh River in China

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Abstract

We compared five calcified structures (scale, otolith, vertebra, cleithrum, and opercular bone) for estimating the age of bream *Abramis brama* (L.) from the Irtysh River in China. Otoliths consistently had the clearest marks, highest confidence scores, highest between-reader agreement (91.63%), and lowest CVs. Cleithra provided the second-highest between-reader agreement (88.65%) and highest agreement (66.08%) with otoliths. Scales and vertebrae yielded equivalent age estimates that were lower than those from otoliths and cleithra, but the estimates of *A. brama* older than 11 years were consistently underestimated using scales and vertebrae. Opercular bone was inferior to other structures for aging *A. brama*. Opercular bones resulted in the lowest between-reader agreement (84.68%), lowest agreement with otoliths (62.33%), and highest CVs. According to these findings, we recommend the use of ground otoliths for estimating the age of *A. brama* when an accurate age structure assessment is required, and recommend scales if a nonlethal procedure is needed for age estimation in order to analyze simple population metrics of *A. brama*.

Keywords: Age estimation, *Abramis brama*, calcified structures, Irtysh River.

Introduction

Accurate estimation of fish age is essential for growth analysis, population dynamics estimates, and resource management (Khan & Khan, 2009). The use of calcified structures to determine the age of fish is a well-accepted method (Campana, 2001). Comparisons of age estimates based on various ageing structures have been reported for a number of fishes with a view to identify the most suitable structures for determining the ages of individuals in fish populations (Maceina & Sammons, 2006; Phelps, Edwards, & Willis, 2007; Reid, 2007). The most reliable ageing method may differ between species. Therefore, the reliability and accuracy of age estimates based on calcified structures should be evaluated separately for each species (Polat, Bostanci, & Yilmaz, 2001).

Bream, *Abramis brama* (L.), is native to Europe and is one of the most abundantly represented species in freshwater fish communities. The age and growth of *A. brama* have been extensively studied (Goldspink, 1981; Kompowski, 1982; Valoukas & Economidis, 1996; Kakareko, 2001; Neja & Kompowski, 2001; Treer *et al*., 2003; Yilmaz, Erbasaran, Yazicioglu, & Polat, 2015; Zhang *et al*., 2016). Different structures were used for estimating the age of *A. brama*. Unfortunately, no study compared the precision of the age estimation between structures and preparation. Scales are the most common material used for age-growth studies of *A. brama*, although Goldspink (1981) used the opercular bone to study the growth of *A. brama* in three eutrophic lakes in England. Scales are widely used because they are relatively easy to collect and prepare, and their removal are non-lethal. However, annuli may be difficult to discern on scales from older fish (Beamish, 1973; Barbour & Einarsson, 1987; Braaten, Doeringsfeld, & Guy, 1999). Accumulating evidence indicating that scales provide unreliable estimates of age has forced fishery scientists to use other calcified structures, especially otoliths (Hammers & Miranda, 1991), to estimate fish age. Otolith is often the preferred structure for age determination because previous research has shown that otoliths provide reliable and accurate age estimates (Phelps *et al*., 2007; Gunn *et al*., 2008; Ma, Xie, Huo, Yang, & Huang, 2011; Baudouin *et al*., 2016). Other bony structures, such as vertebrae, cleithra and opercular bones, have also successfully been used to determine fish age (Khan & Khan, 2009; Li *et al*., 2009).

The goals of the current study were to describe the annulus characteristics of scales, otoliths, vertebrae, cleithra, and opercular bones from *A. brama*, and to evaluate and compare the five calcified structures for estimating the age of *A. brama* in the Irtysh River in China.

Materials and Methods

Sample Collection

A. brama were collected from the Irtysh River during monthly sampling conducted April-October 2013, using trammel nets (with an inner mesh size of 10 cm and outer mesh size of 23 cm), gillnets (mesh size 2.5 cm) and trap nets (mesh size of 1 cm). For each individual, the standard length (SL), fork length (FL), and total length (TL) were measured to the nearest 1 mm and body weight (BW) to the nearest 0.01 g.

Materials Preparation and Age Estimation

Scales were removed from above the lateral line on the left side of the fish, just posterior to the dorsal fin. Scales were immersed in warm water (30 ℃) for about 5h, and the extraneous matter and mucus were removed by rubbing the scales with fingertips. The cleaned scales were mounted between two glass slides, and were examined under a compound microscope.

Paired lapillus otoliths were extracted from each fish, and only the right one was used in age analysis. Otolith was mounted on a glass slide using nail polish with the proximal face toward the slide. The distal face of each otolith was then ground using wet sandpaper (600-2000 grit) and polished with alumina paste (3μm) until the core was visible under a compound microscope. The section was then re-affixed to a glass slide with the polished surface down, and the proximal face was ground and polished until the core was again exposed (Ma, Xie, Huo, Yang, & Huang 2010).

Vertebrae (4-10th), cleithra, and opercular bones were placed in boiling water for 10-15 min to remove attached tissues, and then were immersed in 1% H_2O_2 for 24h and air-dried. The dry structures were examined under a dissecting microscope. Because the vertebrae had bi-concave centra, they were cut in half along the dorsal-ventral axis. Half of each vertebra was placed at the optimal angle in order to make all of the rings visible (Gunn *et al.*, 2008).

Annuli were counted independently by two readers who did not have access to information about body length, sex, or date of capture. Coinciding estimates were accepted and scored subjectively for readability on a five-point scale: 1, excellent; 2, good; 3, acceptable; 4, poor; 5, unreadable (Paul & Horn, 2009).

Calculations and Statistical Analyses

The reliability of the age determinations made using each structure were compared using age bias plots (Campana, Annand, & McMillan, 1995), calculating the agreement between estimates, and determining the coefficient of variation (CV=100×SD/mean) between the two readers (Chang, 1982). To compare reliability between structures, we constructed age bias plots using otolith ages on the abscissa, as they had the clearest and sharpest annuli of all of the examined structures. Because we used otolith ages as our benchmark for comparison, we removed four fish from analyses that were assigned different otolith ages by the readers. Consensus ages for each structure were regressed on consensus ages for otoliths, and the slope of the regression line was tested to see if it differed significantly from 1. The agreement between the age determinations of each structure and otolith ages from the same fish were calculated. The mean age reading for each structure was subjected to one-way analysis of variance (ANOVA) followed by Tukey's post hoc pair-wise comparisons in order to determine whether the readings from different structures were significantly different from one another (Khan & Khan, 2009).

Data and images were analyzed and processed by means of Microsoft Excel 2007 (Microsoft, Redmond, WA, USA), SPSS 17.0 (IBM, Armnok, NY, USA), Adobe Photoshop 7.0 (Adobe, San Jose, CA, USA), and OriginLab Origin V 8.5.1 (Originlab, Northampton, MA, USA). The data are presented as means ± standard deviation (S.D.) and differences were regarded as significant when $P<0.05$.

Results

Annulus Characteristics

The cycloid scales of the *A. brama* examined had annuli with a cyclic pattern that were separated either by a discordance in the arrangement of the circuli or by a narrow space between adjacent circuli. The annuli were easily distinguishable in young fish aged 2-5 years (Figure 1a). However, in fish >7 years of age, age interpretation was relatively subjective because the annuli were crowded near the scale margin, which sometimes appeared to be eroded (Figure 2a). Furthermore, false marks, indicated by crossing over in only one lateral field, were common on scales, particularly on those of older fish. Additionally, regenerated scales, which were characterized by a large focus with no distinguishable growth marks, were more numerous in old specimens than in young specimens.

The ground lapillus otoliths of *A. brama* showed the pattern typical of teleosts, with an alternating sequence of broad translucent and fine opaque zones that progressively narrowed as the number of zones increased. The concentric patterns of zones were

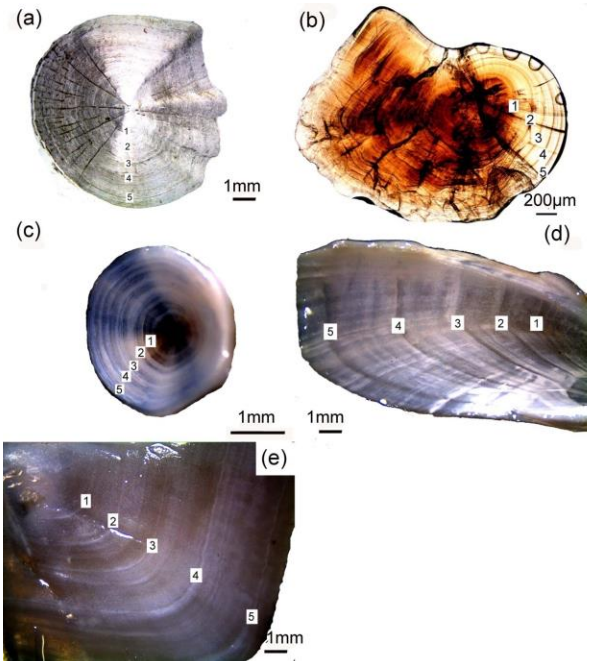

Figure 1. *Abramis brama* (L.) scale (a) under a dissecting microscope using transmitted light; ground lapillus otolith (b) under a compound microscope using transmitted light; vertebra (c), cleithrum (d), and opercular bone (e) under a dissecting microscope using reflected light. All five structures were collected from the same *A. brama* (232mm SL).

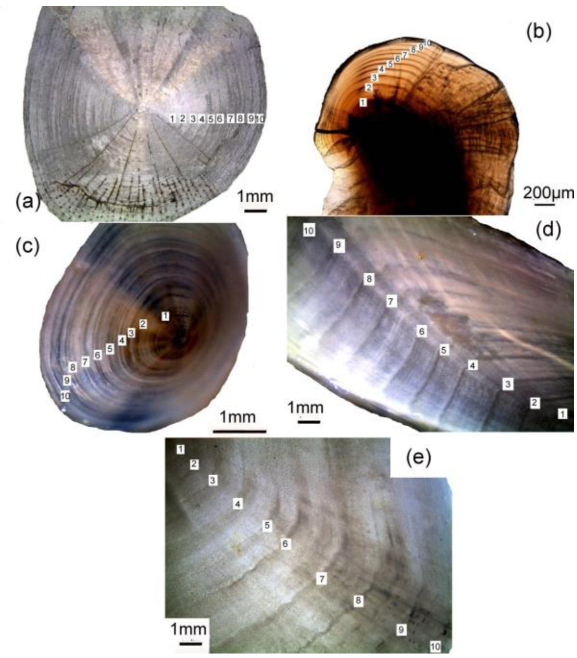

Figure 2. *Abramis brama* (L.) scale (a) and opercular bone (e) under a dissecting microscope using transmitted light; ground lapillus otolith (b) under a compound microscope using transmitted light; vertebra (c) and cleithrum (d) under a dissecting microscope using reflected light. All five structures were collected from the same *A. brama* (263mm SL).

readily distinguishable in the otoliths of young fish aged 1-8 years (Figure 1b), and they were broad and had distinct opaque zones. In otoliths from older fish (>10 years of age), we sometimes saw "pairs" of opaque zones in which two opaque zones were close together (Figure 2b). Pairs of opaque zones were counted as part of the same increment if they coalesced at the margin of the lapillus otolith, and were counted as separate increments if they did not.

In the vertebrae, annuli appeared as a series of concentric zones regularly parallel with the edge of the centrum. In contrast to the other examined structures, the incremental widths increased slightly with age because of the concave centrum (Figure 1c). The first and marginal annuli were too indistinct to identify in the centrums of some older fish (Figure

2c), making age determination of these fish difficult and highly subjective.

Marks on cleithra were fairly clear in younger fish, but became increasingly difficult to identify in older fish (Figure 1d and Figure 2d). Cleithra of older fish tended to exhibit clear marks at the structure's edge, but the first and second annuli were sometimes blurred or transparent. This did not occur in all older fish, but it was a common source of disagreement between readers when it did occur.

The opercular bones were thick, approximately triangular plates. The rings on opercular bones of younger fish (Figure 1e) were clearer and easier identifiable than older fish (Figure 2e). Roots of opercular bones were thick and fanned out into a spongy tissue that was often orange-brown and glutinous in older fish. This spongy tissue often made the first, and sometimes the second, annulus difficult to identify and may have led to an underestimation of the ages of older fish.

Agreement in Age Determinations for the Same Structure

Of the 458 *A. brama* sampled, 450 specimens were successfully aged using each of the five calcified structures, whereas 8 specimens were discarded due to natural deformations or unidentifiable annulus deposition. The number of specimens in each age class and the mean standard length of the fish in each age class are given in Table 1.

Annuli were clearest and sharpest in otoliths, and thus the fewest errors in age estimation occurred when using these structures. The most readable structures were otoliths, with 88.87% of otolith specimens receiving readability scores of excellent or good. The readability of otoliths was followed by those of cleithra, vertebrae, scales, and opercular bones, of which 79.25%, 79.04%, 64.03%, and 68.89%, respectively, received readability scores of excellent or good (Table 2).The readability of a relatively low percentage of all five structures (all<10%) was scored as acceptable or poor. The otoliths were the structure with the fewest specimens receiving a readability score of acceptable or poor (2.40%). Opercular bones were the structure with the greatest percentage of specimens receiving a readability score of acceptable or poor (8.08%; Table 2).

The between-reader agreement in repeated age determinations varied among calcified structures. Agreement was highest (91.63%) for ground otoliths, followed by cleithra (88.65%), scales (86.87%), vertebrae (86.84%), and opercular bones (84.68%; Table 3). The between-reader agreement for all structures also varied with age. Between-reader agreement was 100% for all structures in age classes 2–5, whereas for older fish (>5 years of age), agreement was much lower. For older fish, ground otolith agreement was the highest (>50% for all ages). In contrast, agreement was less than 50% for the scales, vertebrae, cleithra, and opercular bones of fish >10 years of age, with the exception of the opercular bones of 12-year-old fish (60%; Table 3). Furthermore, age determinations using the five structures exhibited a high level of reliability (CV,<8% in all cases), however, the results indicated that estimates based on scales, vertebrae, cleithra, and opercular bones are slightly less reliable than those based on otoliths, especially for older fish (Table 3).

Comparisons of Calcified Structures from the Same Fish

Agreement between estimates based on otoliths and those based on other structures was 62.33-66.08 % (n = 454; Table 4). The agreement of age determinations based on otoliths and those based on other structures was negatively associated with fish age. Estimates from all structures were in closer agreement with those from otoliths for age classes 2-5 (83.19-85.84%) than for age classes 6-10 (59.55-62.78%), and agreement between estimates decreased greatly for age classes 11-15. The agreement between opercular bone estimates and otolith estimates for age classes 11-15 was especially low at 6.25% (Table 4).

For all structure comparisons, regression slopes between age estimates based on otoliths and those based on other structures were all significantly less than 1 (scales 0.86, vertebrae 0.88, cleithra 0.90, opercular bones 0.85; $P<0.05$; Figure 3). Disagreements between ages assigned using otoliths and those assigned using the other four structures were not consistent. For age classes 2-10, ages assigned using the four structures both underestimated and overestimated otolith ages. However, when ages assigned using otoliths and those assigned using the four other structures disagreed for age classes 11-15, estimates from all four structures tended to be lower than estimates from otoliths by 1, 2, or 3 years. The maximum age estimated using otoliths was 15 years; the maximum age estimated using scales, vertebrae and cleithra was 14 years; and the maximum age estimated using opercular bones was 13 years. The mean values of age estimates differed between structures, although the differences were not statistically significant (ANOVA, $F = 1.045$, $P>0.05$). The mean age from ground otoliths was the highest (7.04 ± 2.44), followed by cleithra (6.85 ± 2.27), vertebrae (6.81 ± 2.26), scales (6.80 ± 2.23), and opercular bones (6.77 ± 2.20). Relative to age estimates based on otoliths, estimates based on the four other structures underestimate fish age to different degrees.

Discussion

Ground otoliths yielded the most reliable age estimates for *A. brama*. Otoliths were superior to the other examined structures in all criteria used to

Table 1. Number of specimens and mean standard length (SL) at each age of *Abramis brama* (L.), as estimated from scales, otoliths, vertebrae, cleithra, and opercular bones

Age	Scales		Otoliths		Vertebrae		Cleithra		Opercular bones	
	N	Mean ± S.D. (mm)	N	Mean ± S.D. (mm)	N	Mean ± S.D. (mm)	N	Mean ± S.D. (mm)	N	Mean ± S.D. (mm)
2	11	68.18 ± 5.55	11	68.18 ± 5.55	11	68.18 ± 5.55	11	68.18 ± 5.55	11	68.18 ± 5.55
3	30	98.47 ± 12.64	29	97.69 ± 11.91	32	99.48 ± 15.63	31	99.23 ± 15.84	32	99.48 ± 15.63
4	54	121.44±15.85	58	121.95±16.54	51	121.41±14.55	52	121.13±14.55	50	120.94 ± 14.30
5	21	159.95±33.22	17	156.71±25.59	22	159.64±33.02	24	159.70±32.26	24	159.25 ± 31.72
6	41	197.02±28.52	23	201.52±33.56	46	205.00±33.43	41	204.86±33.55	49	205.82 ± 31.92
7	127	223.17±30.17	106	221.47±28.12	123	219.84±30.01	119	220.34±29.74	124	221.97 ± 29.89
8	96	235.97±28.84	115	233.23±33.14	85	236.29±31.20	96	238.40±33.01	79	235.00 ± 32.17
9	42	249.07±37.24	46	240.74±37.75	46	249.09±36.96	41	242.71±35.87	45	247.58 ± 36.43
10	12	253.54±38.02	17	227.35±29.64	20	250.15±35.20	18	241.72±38.89	25	251.28 ± 39.34
11	13	261.38±29.87	12	246.50±41.88	10	266.90±38.21	12	258.25±36.55	11	273.64 ± 35.77
12	5	304.80±45.96	12	290.67±34.43	6	278.83±45.64	9	291.11±40.15	5	297.40 ± 45.00
13	3	302.33±25.40	3	269.67±44.38	2	306.00±33.87	3	292.67±24.50	2	318.50 ± 36.06
14	2	306.00±53.74	2	310.00±48.08	2	318.50±36.06	1	344.00 ± 0.00		
15			3	278.67±12.90						
Total	457		454		456		458		457	

Table 2. Percentage of *Abramis brama* (L.) samples with readability scores of 1, excellent; 2, good; 3, acceptable; 4, poor; and 5, unreadable (n =458)

Structure	Readability scores				
	1	2	3	4	5
Scales	28.82	46.07	21.40	3.49	0.22
Otoliths	43.89	44.98	8.73	1.53	0.87
Vertebrae	29.48	49.56	16.59	3.93	0.44
Cleithra	33.62	45.63	16.38	4.37	0.00
Opercular bones	25.55	39.08	27.29	7.86	0.22

Table 3. Comparison of percent agreement and coefficients of variation (100 × SD /mean) among age determinations for *Abramis brama* (L.) made using scales, otoliths, vertebrae, cleithra and opercular bones

Age	Scales			Otoliths			Vertebrae			Cleithra			Opercular bones	
	N	Percent agreement (%)	CV	*N*	Percent agreement (%)	CV	*N*	Percent agreement (%)	CV	*N*	Percent agreement (%)	CV	*N*	Percent agreement (%)
2	11	100.00	0.00	11	100.00	0.00	11	100.00	0.00	11	100.00	0.00	11	100.00
3	30	100.00	0.00	29	100.00	0.00	32	100.00	0.00	31	100.00	0.00	32	100.00
4	54	100.00	0.00	58	100.00	0.00	51	100.00	0.00	52	100.00	0.00	50	100.00
5	21	100.00	0.00	17	100.00	0.00	22	100.00	0.00	24	100.00	0.00	24	100.00
6	41	95.12	4.43	23	100.00	0.00	46	97.83	1.72	41	97.56	1.81	49	91.84
7	127	93.70	3.10	106	94.34	2.70	123	90.24	3.48	119	94.12	3.16	124	83.87
8	96	84.38	4.13	115	89.57	3.10	85	88.24	7.61	96	87.50	7.27	79	88.61
9	42	64.29	5.17	46	84.78	3.07	46	65.22	6.32	41	73.17	4.60	45	71.11
10	12	50.00	4.80	17	76.47	4.61	20	55.00	6.51	18	61.11	5.35	25	44.00
11	13	38.46	6.70	12	66.67	4.92	10	50.00	4.17	12	50.00	5.65	11	45.45
12	5	40.00	6.47	12	83.33	2.37	6	50.00	4.32	9	44.44	6.46	5	60.00
13	3	33.33	6.69	3	66.67	0.00	2	0.00	4.08	3	33.33	3.87	2	0.00
14	2	50.00	3.64	2	50.00	3.64	2	0.00	4.28	1	0.00	5.24		
15				3	66.67	2.69								
Total		86.87			91.63			86.84			88.65			84.68
N	457			454			456			458			457	

Table 4. Comparison of percent agreement between age determinations for *Abramis brama* made using scales, vertebrae, cleithra, opercular bones, and otoliths

Structures	Percent Agreement (%)			
	Total	Age 2-5	Age 6-10	Age 11-15
Scales	65.20	85.84	61.81	25.00
Vertebra	63.88	84.07	60.84	21.88
Cleithra	66.08	85.19	62.78	37.50
Opercular bones	62.34	83.84	59.55	6.25

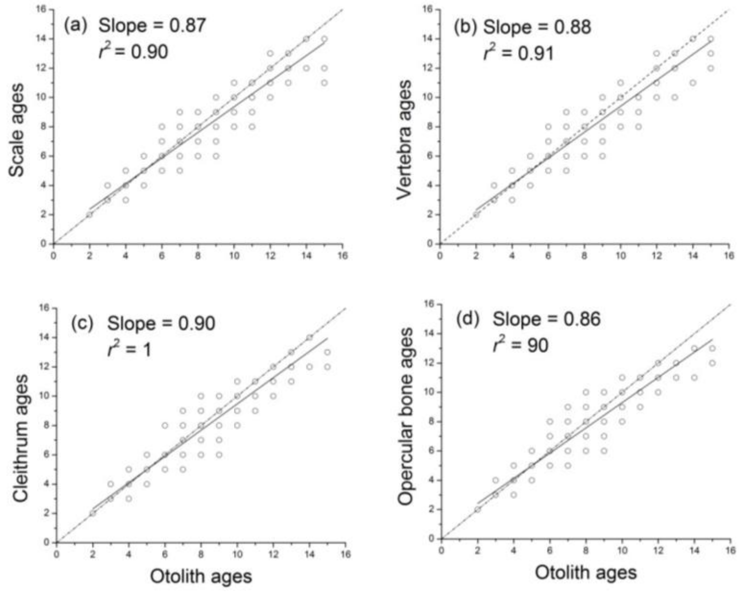

Figure 3. Comparisons between age estimates of *Abramis brama* (L.) based on ground otoliths and those based on scales (a), vertebrae (b), cleithra (c), and opercular (d) bones. The dashed line represents 100% agreement.

evaluate their suitability for estimating the age of *A. brama*. Otoliths were consistently clearer and easier to interpret than scales, vertebrae, cleithra, and opercular bones, despite some difficulties for older fish. Many studies have shown that the use of otoliths provides the most reliable and accurate age estimates (Hammers & Miranda, 1991; Phelps *et al*., 2007; Gunn *et al*., 2008; Ma *et al*., 2011). Otoliths are often used as a benchmark to compare age estimates from other structures (Niewinski & Ferreri, 1999). Reliable age estimation using otoliths is also supported by the fact that otoliths do not undergo resorption and their growth is acellular, rather than a result of calcification (Secor, Trice, & Hornick, 1995). Otoliths are reported to be metabolically inert and do not reflect physiological changes that may occur throughout the life of fish (Phelps *et al*., 2007). However, extraction and processing of otoliths are time consuming and require the fish to be sacrificed. The sacrifice of fish may not be feasible in certain situations.

Cleithra were found to be the second-best structures for estimating the age of *A. brama*, followed by vertebrae and scales, and the least suitable structures for estimating the age of *A. brama* were opercular bones. Although cleithra were the second-best structures for age estimation in the current study, few researchers have reported the use of this structure for ageing fish. In contrast, vertebrae have been used to age fish in a variety of other studies (Alves, Barros, & Pinho, 2002; Liu, Lee, Joung, & Chang, 2009). Polat *et al*., (2001) showed that vertebrae were the most reliable structure for age determination of *Pleuronectes flesus luscus* and resulted in minimal ageing error. However, in the current study, vertebrae provided age estimates similar to those from otoliths only up to age 5 (between-reader agreement, 84.07%), and consistently underestimated the age of *A. brama* after age 11. These results are 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 10, but the counts diverged for older individuals. As reported by Hill, Calliet, and Radtke, (1989) for blue marlin (*Makaira nigricans* Lacepede) and Khemiri, Gaamour, Zylberberg, Meunier, and Romdhane (2005) for *Boops*, we found that the rings on vertebral centra were not very clear and showed numerous minute marks unrelated to cyclic events.

Marks on scales were often difficult to interpret using objective ageing criteria, as false marks and regenerated scales were common and marks at the edge of scales from older fish were crowded. The use of scales for estimating age fish has been criticized because of the frequent underestimation of the ages of older fish (Beamish & McFarlane, 1987). Unreliable and inaccurate age determinations from scales have been attributed to resorption and deposition of false annuli due to stress and food limitation, and to obfuscation of annuli due to the cessation of scale growth as fish grow older (Beamish & McFarlane, 1987; Maceina & Sammons, 2006). Whereas, scales were the only calcified structure can be sampled without sacrificing the fish, compared to the other examined structures. The general consensus is that while scales are inferior to otoliths for age estimation (Sipe & Chittenden, 2001; Maceina *et al*., 2007), their efficient removal, simple preparation process and non-destructive sampling continues to make scales a desirable and suitable structure from which to determine age for some fast growing, short lived species.

Opercular bones were inferior to ground otoliths, cleithra, scales, and vertebrae for estimating the age of *A. brama*. The rings on opercular bones of younger fish were clearer and easier identifiable than older fish. Similar observations have also been reported by other researchers (Shafi & Maitland, 1971; Nargis, 2006). The use of opercular bones for age determination may result in underestimates of the ages of older *A. brama*. This might be attributed to the thick root of opercular bones, which often obscures the first, and sometimes the second, annuli.

According to present study, we recommend the use of ground otoliths for age estimation in *A. brama* when an accurate age structure assessment or mortality rate estimation is required. However, we recommend using scales if a nonlethal procedure is needed to analyze simple population metrics of *A. brama* up to the age of 10 years.

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