Black Bullhead Otolith Annual and Daily Increment Validation

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Abstract: Otolith age validation studies are essential to identify the accuracy of using otoliths to age fish; however, black bullhead (*Ameiurus melas*) otolith validation studies have not been conducted for either adult or age-0 individuals. Therefore, the objective of this study was to validate annulus and daily ring formation in lapilli otoliths of black bullheads. We assessed timing of annulus formation using marginal increment analysis on 409 black bullheads caught monthly from July 2015–June 2016 in Lake Carl Etling, Oklahoma. We evaluated daily growth increment deposition by batch-marking 253 age-0 black bullhead by immersion in a solution of 700 mg L^{-1} oxytetracycline (OTC) for 6 hrs to provide a date stamp; thereafter, 10 fish were pulled from the tank every 10 days and had otoliths removed for analysis. We observed that black bullhead produced a single annulus in their lapillus otolith in June. Daily ring deposition was observed in age-0 black bullhead; between reader precision was high for estimates pre-OTC mark, and known ages were accurately estimated up to 37 days post-OTC mark representing fish up to 70 days old. Results of this study demonstrated that lapilli otoliths are reliable aging structures for black bullhead in Oklahoma to determine population characteristics such as recruitment, growth, spawning time, and mortality.

Key words: lapilli, annulus formation, marginal increment analysis

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Accurately aging fish is imperative for fisheries managers when estimating growth, assessing mortality rates, and assigning year classes to understand population dynamics for a particular species (Campana 2001, Buckmeier et al. 2017). Otoliths are a widely used aging structure for many freshwater fish species and can provide more accurate and precise age estimates compared to other calcified structures (e.g., fin rays, opercula, scales, spines, and vertebrate; Buckmeier et al. 2017). It is important for managers to produce quality age data because inaccurate age estimates can result in improper management of fish species (Campana 2001). Unfortunately, sources of error, such as variable reader estimations, inconsistent deposition of growth increments in otoliths, and misinterpretation of annuli, can lead to biased age estimations (Campana 2001, Buckmeier et al 2002).

Otolith validation studies are essential because they can help identify these biases and increase precision and accuracy of age estimates. Therefore, fisheries managers have implemented techniques to validate annulus formation in otoliths to ensure that accurate age estimates are possible. Marginal increment analysis has been commonly used to validate annulus formation of otoliths for many fish species (e.g., Blackwell and Kaufman 2012, Porta and Snow 2017, Phelps et al. 2019). Marginal increment analysis requires repeated samples of fish to be collected during a full year to determine the timing of the annulus formation by measuring the distance between the margin of the otolith and the distal edge of the last opaque annulus (Campana 2001).

Fisheries managers use daily growth increments to estimate many important early life-history characteristics associated with age and growth of age-0 fishes such as hatch-date distribution, growth, and mortality rates (Miller and Stork 1984, Durham and Wilde 2008, Snow and Long 2017). Validation of daily increment formation in fish otoliths is a fundamental process that leads to the understanding of physical and biological factors affecting early life ecology in fishes (Campana and Neilson 1985). Validation studies

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for this purpose are usually conducted by examining otoliths from larvae or juvenile fish of known age (Sakaris and Irwin 2008, Long and Snow 2016, Snow and Long 2017) or by examining otoliths that have been marked on a known date (Yoklavich and Boehlert 1987, Durham and Wilde 2008, Snow and Long 2016). Marking otoliths is usually done by immersing fish in a chemical compound or dye that will incorporate into the otolith and will be visible after preparation (Secor et al. 1992). Validation of daily growth increments have been conducted on various catfish species such as African catfish (*Clarias gariepinus*; Nyamweya et al. 2010), armoured catfish (*Megalechis thoracata*; Mol and Ponton 2003), blue catfish (*Ictalurus furcatus*; Sakaris et al. 2011), channel catfish (*Ictalurus punctatus*; Sakaris and Irwin 2008), eel-tailed catfish (*Tandanus tandanus;* Burndred et al. 2017) and flathead catfish (*Pylodictis olivaris*; Sakaris et al. 2011).

Bullhead catfishes (*Ameiurus* spp.) are native to North America but have been widely introduced outside their native range (Pedicillo et al. 2009, Novomeská and Kováč 2009), where their fast growth and tolerance of poor environmental conditions allows them to readily establish populations (Rutkayová et al. 2013, Copp et al. 2016). Black bullhead (*A. melas*) are one of the most widely distributed bullhead catfishes (Etnier and Starnes 1993), but despite their extensive native and introduced ranges, age and growth information on black bullhead is lacking compared to other ictalurid species (Rypel 2011). Additional information on black bullhead life history (e.g., age and growth) is needed to increase our understanding of this understudied species. Validation of black bullhead otoliths would be useful for researchers to accurately describe life-history characteristics of black bullheads so managers can implement best management practices for this species in their native and introduced range. Thus, the objectives of this study were to 1) validate annulus formation on the lapilli otoliths of black bullhead using marginal increment analysis and 2) confirm otolith daily ring deposition of age-0 black bullhead.

Methods

Marginal Increment Analysis

Black bullhead were collected monthly from Lake Carl Etling, Oklahoma, from July 2015 through June 2016 using boat-mounted electrofishing (pulsed DC, high voltage, Smith Root 7.5 GPP, Smith-Root Inc., Vancouver, Washington) and fyke nets (0.91 x 3.05 m; with 12.7-mm mesh, 0.91- x 1.83-m rectangular cab, 152.4-mm throat, and a 20.12-m lead). We sampled the entire perimeter of the reservoir using boat electrofishing, whereas we set fyke nets haphazardly to avoid the herbaceous and woody vegetation surrounding the lake. We sought to collect a minimum 25 black bullhead each month, although this goal was not met in July 2015 and January 2016. Following capture, each fish was measured for TL (mm) and weight (g), and lapilli otoliths were removed per the procedures of Long and Stewart (2010).

Each pair of lapilli otoliths was cleaned and placed into an individually numbered envelope and left to dry for more than 24 h prior to processing (Secor et al. 1992). Otoliths were processed following methodology similar to Buckmeier et al. (2002). They were then placed into one of 21 individual cells of a silicon mold (Electron Microscopy Sciences, Fort Washington, Pennsylvania) and immersed completely in epoxy for mounting (West System 205-B hardener and 105-B Epoxy resin, Gougeon Brothers Inc., Bay City, Michigan; Sakaris et al. 2017). Once the epoxy cured, the anterior edge of the otolith was cut in the transverse plane using a low-speed IsoMet saw with a 127-mm diameter x 0.4-mm thickness blade (Buehler Model 11-1280-160; Lake Bluff, Illinois) and polished using wet 2000-grit sandpaper until annuli became clear and distinguishable. The prepared otoliths were placed cut-side up in a dish containing black modeling clay, submerged in water, and viewed with a variable-power Olympus SZX16 stereomicroscope capable of 130× magnification (Olympus Corporation, Lake Success, New York) using a 1-mm diameter, single strand fiber-optic filament attached to an external light source to illuminate the annuli. The annuli appeared as dark rings on a lighted background and were counted to assign an age estimate to each fish. Each otolith was evaluated randomly by two independent readers (Hoff et al.1997). When there was a disagreement on an estimated age, a concert reading was conducted by both readers and a final consensus age estimate was determined. Further, percent agreement was calculated to measure between reader precision for age estimations.

After consensus age estimates were determined, marginal increment analysis was conducted by measuring the distance of the hyaline zone on the distal edge of the otolith to the last opaque band (Buckmeier et al. 2017). Increment measurements from otoliths were measured (to the nearest mm) using CellSen Entry (Olympus Corporation, Lake Success, New York) computer software and an Olympus DP74 digital camera attached to the stereomicroscope described above. Marginal increment measurements were made to validate that a single annulus was formed and to determine the timing of annulus formation for ages 1–2, 3–6, and all ages combined (Clayton and Maceina 1999, Blackwell and Kaufman 2012, Porta and Snow 2017, Snow et al. 2018).

Daily Ring Deposition

Mature black bullhead were collected in April 2016 from Lake Carl Etling, Oklahoma, using boat electrofishing. Fish were then transported to the Oklahoma Fishery Research Laboratory and reared in two outdoor holding tanks (3398 L) equipped with PVC spawning cavities (30.5 cm diameter x 51 cm long). Spawning cavities were checked once weekly for eggs masses from May through July. Despite weekly monitoring, no egg masses were found in spawning cavities during this time period. However, on 22 August 2016, age-0 black bullheads were observed in one of the tanks swimming near the surface. We collected 253 of these age-0 individuals and batched marked them that same day by immersing fish in a solution of 700 mg L^{-1} oxytetracycline (OTC) and 434 mg L^{-1} sodium phosphate (dibasic) buffer for 6 hrs (Stewart and Long 2011). Fish being marked were kept indoors in the dark to prevent the breakdown of OTC (Brown et al. 2002), and the water in their holding tanks was recirculated instead of aerated to limit foam created by agitation (Snow and Long 2017). This marking technique produced a glowing yellowish orange mark on the otoliths when they were later viewed under a microscope with ultraviolet light (Kuklinski 2013, Snow and Long 2016).

The OTC-marked fish were moved and split evenly into seven 378-L Rubbermaid stock tanks filled with well water and equipped with aeration (Newell Brands Inc., Atlanta, Georgia). Covers were placed over the tanks to block direct sunlight for 48 h after OTC exposure to limit photosensitivity (Stewart and Long 2011). Fish were fed a combination of starter pellets and freeze-dried krill, bloodworms, and brine shrimp to satiation twice daily. The tanks were refilled with fresh well water and cleaned biweekly. Dissolved oxygen averaged 8.4 ppm throughout the experiment and temperatures ranged from 22.3 to 28.4 °C during the 85-day holding period.

Ten OTC-marked individuals were randomly selected from the holding tanks approximately every 10 days and placed in a 1:1 ice to water slurry to be euthanized (Blessing et al. 2010). Once euthanized, each fish was measured for TL (mm) and weighed (g). Lapilli otoliths were removed with fine tipped forceps by positioning the specimen dorsal side down under a dissecting scope (Long and Snow 2016). The removed otoliths were then cleaned and allowed to dry for more than 24 h before processing.

Otoliths were embedded in a two-part epoxy and transverse sections were prepared using a low-speed IsoMet saw as described above. To ensure precise sectioning of each otolith, a bracket that attached to the saw holding a 0.7–3.0x dissecting microscope was positioned with a camera over the blade, which was viewed on a monitor for sectioning purposes (Long and Snow 2016). Otoliths were mounted to glass microscope slides and fixed with thermoplastic cement. Otoliths were polished wet using 2000-grit sandpaper and routinely viewed under a dissection microscope capable of 20x–50x magnification until daily growth increments were visible on the margin of the otoliths. The otoliths then were inverted and polished until the core was visible (<0.5 mm thick depending on otolith size). The otolith was inverted again to expose the otolith core and to make the rings clearly visible for estimating daily growth increments. Otoliths were polished multiple times to reveal growth increments near the nucleus if necessary (Roberts et al. 2004).

Otoliths were examined independently by two readers (Hoff et al. 1997) using a high-resolution monitor connected to an optic-mount digital camera attached to an Olympus BH-2 compound microscope under 100–400x objectives (Olympus Corporation, Lake Success, New York). Otoliths were selected in random order with the readers having no reference to fish size, date, or known age. Growth increments were first estimated from the OTC mark to the outer edge, and then a second count from the outer edge to the OTC mark to verify the first count. Thirdly, readers estimated growth increments from the nucleus margin to the OTC mark and then performed a fourth count from the OTC mark to the nucleus margin to verify the third count.

Linear regression analysis was used to relate increases in length and weight to known days post-OTC mark for age-0 black bullhead. Fish weight was $\log_{10}$ transformed to correct for non-linearity and converted to an integer value. Daily increments counted pre-OTC mark were compared between readers using a paired *t*-test. Additionally, daily increment counts post-OTC mark were compared to the known number of daily increments using a paired *t*-test. To visualize reader bias, the deviation of the mean estimated age from known daily increment post-OTC mark was calculated for each reader (Miller and Stork 1984, Sakaris and Irwin 2008). All analyses were performed using XLSTST 2020 (Addinsoft Inc., New York City, NY) and significance was evaluated at $P \leq 0.05$.

Results

Marginal Increment Analysis

We collected 409 black bullhead for age estimation and marginal increment analysis. Black bullhead length ranged from 95 to 318 mm TL (Figure 1); our lapilli otolith age estimates ranged from 1 to 12 years old (Table 1). Agreement between readers was 95.6%, with 98.8% agreement within one year and 100% agreement within two years. The sample was dominated by fish estimated to be 2 and 5 years old, comprising approximately 50% of the total sample and contributing on average 21.3% and 25.1% respectively to each monthly sample (Table 1). Marginal increment analysis clearly indicated that a single opaque band was formed annually in all three age groups. Annulus formation occurred during June for our black bullhead aged 3–6 and all ages combined; however, annulus formation started in May and finished in June for black bullhead aged 1–2 (Figure 2). In lapilli otoliths of black bullhead across all ages, the distance to the edge of the otolith increased monthly after annulus formation (Figure 2).

Daily Increment Analysis

Minimal mortality was observed (5.6%; 14 of 253) during the OTC marking process. One-hundred black bullhead were collected over 10 weeks, ranging in known days post OTC mark from 8 to 85 days (Table 2). The OTC marks were identified in 100% of lapilli otoliths used to validate daily increment deposition (Figure 3). Number of days after being marked with OTC was related to black bullhead growth in TL ($r^2 = 0.81$, $P < 0.01$) and weight ($r^2 = 0.71$, $P < 0.01$); mean daily growth was 0.51 mm day^{-1} and 0.01 g day^{-1} in TL and weight, respectively.

Table 1. Estimated age distribution of black bullhead collected monthly at Lake Carl Etling, Oklahoma, from January 2015–December 2016.

	Age (years)												
Month	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**Monthly total**
January	1	3	5	–	1	9	–	–	–	–	–	–	19
February	1	17	5	–	4	15	–	–	1	–	–	–	43
March	–	5	9	–	1	20	1	–	–	–	–	–	36
April	4	4	7	6	4	3	2	–	1	1	–	1	33
May	5	7	5	3	1	5	–	1	2	–	3	–	32
June	9	7	–	–	9	1	–	–	–	–	–	–	26
July	6	3	–	2	7	–	–	–	–	–	–	–	18
August	5	10	–	–	15	–	–	1	–	–	–	–	31
September	3	3	7	5	3	1	2	2	–	2	–	–	28
October	7	3	4	3	14	3	–	1	1	–	–	2	38
November	14	13	1	–	25	–	–	–	–	–	–	–	53
December	8	17	–	–	27	–	–	–	–	–	–	–	52
Total	63	92	43	19	111	57	5	5	5	3	3	3	409

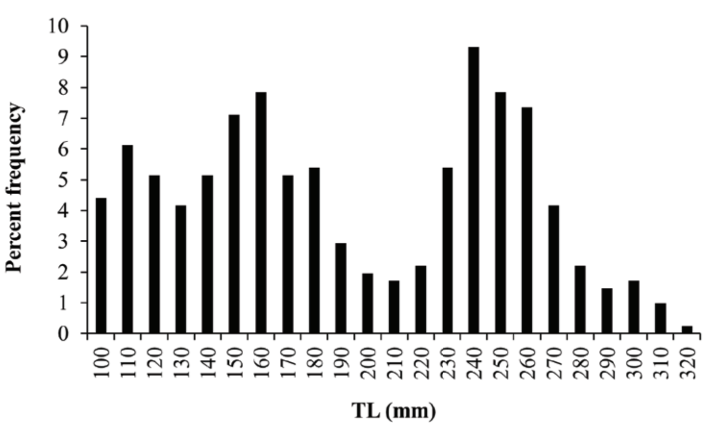

Figure 1. Length-frequency distribution of black bullhead aged and used for marginal increment analysis ($n = 409$).

Table 2. Mean TL and weights (g) of tank-raised age-0 black bullhead batch marked using oxytetracycline (OTC). OTC marks were used for validating daily deposition of growth increments in black bullhead lapilli otoliths.

Sample week (days post OTC mark)	*n*	Mean TL (mm)	SD	Mean weight (g)	SD
1 (8)	10	26.2	1.62	0.274	0.05
2 (16)	10	31.5	2.84	0.508	0.14
3 (23)	10	37.6	2.46	0.981	0.11
4 (27)	10	39.5	3.37	1.018	0.36
5 (37)	10	43.7	5.74	1.215	0.51
6 (47)	10	47.3	4.30	1.381	0.42
7 (57)	10	47.6	5.82	1.351	0.50
8 (67)	10	51.0	4.85	1.693	0.47
9 (78)	10	54.7	4.76	2.246	0.74
10 (85)	10	58.0	5.44	2.821	0.97

Figure 2. Mean marginal increment measurements by month from black bullhead lapilli otoliths. Error bars indicates standard errors.

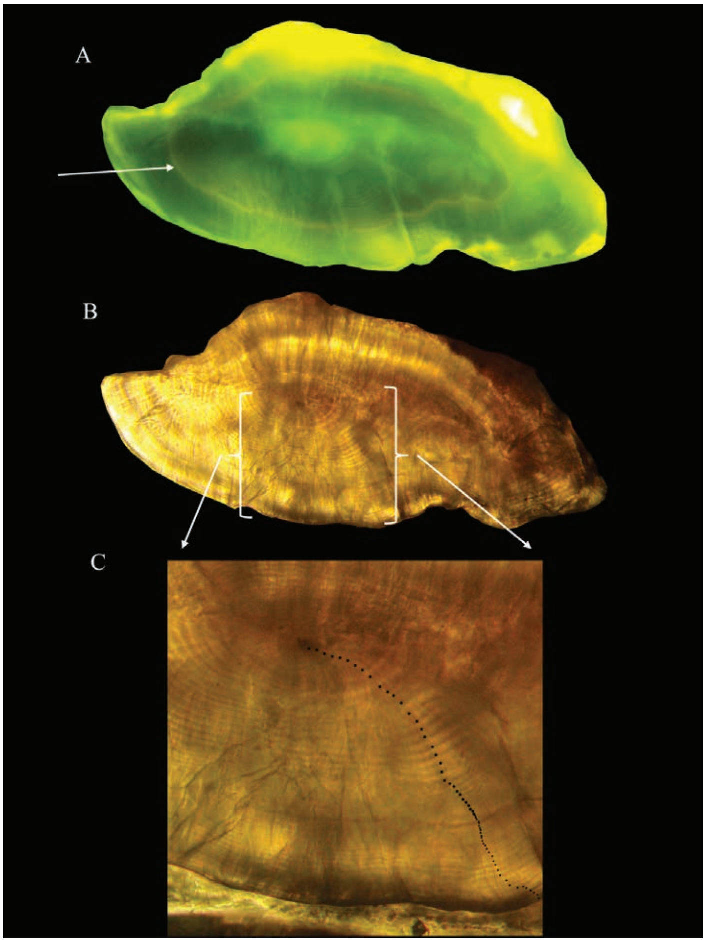

Figure 3. Photograph depicting (A) oxytetracycline (OTC) mark, (B) whole view of the lapilli otolith, and (C) a close up of B with dots indicating daily growth increments from a 42-mm TL black bullhead that was estimated to be 62 days old (35 days pre- plus 27 days post-OTC mark).

Table 3. Outcomes of paired *t*-test for both readers examining differences between known days post oxytetracycline (OTC) mark and estimated daily growth increments in lapilli otoliths of black bullhead.

Sample week (days post OTC mark)	Reader	*n*	Mean daily growth increment estimates post OTC mark (SD)	*t*	df	*P*
1 (8)	1	10	8.1 (0.7)	–0.43	9	0.68
	2	10	7.9 (0.9)	0.36	9	0.72
2 (16)	1	10	15.9 (1.1)	0.29	9	0.78
	2	10	16.1 (0.9)	–0.36	9	0.73
3 (23)	1	10	23.4 (1.1)	–1.18	9	0.27
	2	10	24.8 (1.1)	0.56	9	0.59
4 (27)	1	10	27 (1.5)	0.00	9	1.00
	2	10	28.8 (1.3)	0.69	9	0.51
5 (37)	1	10	37 (1.7)	0.00	9	1.00
	2	10	36.9 (1.6)	0.29	9	0.78
6 (47)	1	10	45.4 (2.9)	2.95	9	0.01
	2	10	44.2 (2.7)	1.79	9	0.02
7 (57)	1	10	50.7 (3.4)	8.44	9	0.00
	2	10	52.2 (3.1)	7.24	9	0.00
8 (67)	1	10	57.5 (4.7)	12.21	9	0.00
	2	10	54.8 (5.0)	7.88	9	0.00
9 (78)	1	10	57.7 (4.7)	13.61	9	0.00
	2	10	56.9 (4.5)	14.80	9	0.00
10 (85)	1	10	62.9 (5.9)	24.90	9	0.00
	2	10	61.8 (4.8)	15.28	9	0.00

Daily ring deposition occurred in lapilli otoliths before and after observed OTC marks (Figure 3). Mean daily ring estimates pre-OTC mark were similar between readers ($t = -0.35$, df = 99, $P = 0.72$; reader 1: $\bar{x} = 34.97$ days [range 32 to 39]; reader 2: $\bar{x} = 35.03$ days [range 32 to 38]). Mean reader daily ring estimates were closely related to known days post-OTC mark up to 37 days post-OTC mark. Ages older than this were progressively underestimated as ages increased (Table 3). Deviation of estimated daily ages from known age was minimal and not significant up to 37 days post-OTC mark with all daily ring estimates within 3 days of known age past the OTC mark (Table 3).

Discussion

Marginal Increment Analysis

Marginal increment analysis for black bullhead lapilli otoliths indicated that a single opaque band formed once annually, thus validating this structure for age estimation. We found that for black bullhead aged 3–6 years these otoliths had the smallest marginal increment measurement from May to June, indicating that their annular growth marks were fully completed in June. However, black bullhead aged 1–2 years had annular growth marks starting in April, suggesting that annulus formation may begin earlier in younger fish.

Identifying timing of annulus formation in otoliths for a particular species can provide useful information to fisheries managers because it shows the time of year when precise readings can be taken for age and growth analysis (Kerns and Lombardi-Carlson 2017). For example, Porta and Snow (2017) found white perch produced a single annulus during the period of April–June and precision of readings was highest in the month of July, suggesting that the month immediately after annulus formation is ideal for discerning age estimates (Snow et al. 2018). Based on our findings, we recommend collecting adult black bullhead lapilli otoliths from Oklahoma waters in July to obtain the most precise age estimates for age and growth analysis.

Daily Increment Analysis

Daily increments in age-0 black bullhead lapilli otoliths were observed from the core to the margin throughout the 10-week duration of our study. Despite not knowing the exact spawn date, precision of pre-OTC daily growth increment estimates was high between our readers, with both estimating ~35.0 days. Accurate aging of daily rings was determined to be 37 days post-OTC mark. After 37 days post-OTC mark, black bullhead daily age began to be underestimated. Therefore, given the pre-OTC increment estimate of 35 days and the post-marking threshold of 37 days, we estimated that readers began to underestimate the ages of fish older than 72 days. This is similar to other daily age studies that examined the accuracy of post-hatch North American ictalurids such as channel catfish and blue catfish (≤60 days post hatch; Sakaris and Irwin 2008, Sakaris et al. 2011) and flathead catfish (≤72 days post hatch; Sakaris et al. 2011). Thus, aging accuracy of black bullhead lapilli otoliths is similar to what has been found for other catfishes and provides additional support of our estimates for black bullhead daily growth increments.

There were difficulties during otolith preparation and estimating daily growth increments which likely contributed to the lack of accuracy past ~72 days post-hatch in age-0 black bullhead. Although the morphology of the lapilli otolith facilitated preparation and viewing under the microscope, we found that readers had difficulty viewing older fish on a single plane. The nucleus margin often required multiple sanding efforts to reveal increments near the nucleus, which resulted in the loss of outer daily rings. We hypothesize that as the age of the fish increased, their growth slowed and became more variable leading to the narrowing of daily growth increments, thus leading to discrepancies in the estimation of daily rings. Similar results have been observed in a variety of other fish species (Ponton et al. 2001, Sakaris and Irwin 2008, Sakaris et al. 2011). Due to the effects of age on accuracy of age-0 catfishes, future research should examine the accuracy and precision associated with sectioning lapilli otoliths in different planes (i.e., sagittal and frontal sections). Without such refinement, studies using daily ages of catfishes should ensure their fish are not older than about 80 days to ensure acceptable accuracy.

This study provides insight into the utility of the lapilli otoliths for obtaining annular and daily age estimates that provide valuable life history information for informing management of this understudied species in its native and introduced range. Results of this study suggest that lapilli otoliths should be used for aging black bullhead to accurately assess population rate functions such as recruitment, growth, and mortality. However, the accuracy of otolith age interpretation is still in need of further validation in adult black bullhead. Also, future research should be conducted on age-0 otoliths to determine when the first daily growth increment is discerned.

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