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Validation of Scales and Otoliths for Estimating Age of Rainbow Trout from Southern Appalachian Streams

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Abstract.—Annuli counts from scales and otoliths are commonly used to estimate the age of rainbow trout *Oncorhynchus mykiss* in southern Appalachian streams. However, validation of annuli counts from these two structures for rainbow trout has not been reported. Oxytetracycline (OTC) injections (50 mg OTC/kg fish) were used to mark otoliths of visible-implant-tagged rainbow trout in a mark–recapture study. The validation of scales for age estimation was performed by comparing the number of annuli on scales of individual rainbow trout at capture with those collected 12–15 months later upon recapture. Scale accuracy declined from 72% for age-2 fish to 0% for age-4 fish. In contrast, and regardless of age, all fish formed an additional annulus outside the OTC mark on otoliths. We conclude that annuli counts from otoliths will provide accurate age estimates for rainbow trout in the southern Appalachians. The use of otoliths and consequential sacrifice of fish may not be an option in some cases. However, annuli counts obtained from the scales of rainbow trout in southern Appalachian streams may not provide accurate age estimates for fish older than age 2.

Valid age data are necessary for understanding fish life history and assessing population dynamics. A frequently used technique for estimating the age of fish in temperate climates involves examination of calcified structures (i.e., scales and otoliths) for the presence of annuli. Researchers have stressed the importance of critical analysis of all methods for estimating fish age and urged that the techniques be validated for all ages within each species studied, regardless of the structure used (Van Oosten 1923; Beamish and McFarlane 1983).

Counting scale annuli has been the primary means by which age has been estimated for freshwater fish in North America since 1930 (Carlander 1987). Unfortunately, few investigators have validated this method for age determination (Beamish and McFarlane 1983). By the 1950s researchers began to realize that the ages of older fish were frequently underestimated (Carlander 1987). It has been suggested that underestimated ages from scales may be due to indistinguishable annuli during periods of slow growth (Beamish and McFarlane 1987) or resorption of scales during periods of severe stress (Simkiss 1974). Nonetheless, some researchers continue to rely on annuli counts from scales because of the relative ease of collection and preparation and because scale collections do not require fish to be sacrificed (Carlander 1987; Devries and Frie 1996).

As a result of convincing evidence that counting annuli in otoliths provides accurate and precise age data (Neilson and Geen 1981; Marshall and Parker 1982; Campana 1983a, 1983b, Geen et al. 1985; Lowerre-Barbieri et al. 1994), the use of otoliths for age estimation has become increasingly common. Several reports on the preparation, viewing, and interpretation of otoliths have been published (i.e., Brothers 1987; Secor et al. 1992). However, variability in otolith shape and size among species, populations, and individuals has resulted in few standards for viewing and interpreting annuli on otoliths. Furthermore, previous studies have indicated that ages estimated from sectioned otoliths are sometimes different than those estimated from

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whole otoliths (Beamish 1979; Beamish and Chilton 1982; Barber and McFarlane 1987). Otolith morphology differs among species. Therefore, the use of otoliths to age fish requires intraspecific validation of this structure for estimating age over a wide range of fish sizes and at several localities, depending on the degree of habitat variation within a species' range.

Mark–recapture techniques are often used with fish of known age to validate ages estimated from various structures (Beamish and McFarlane 1983). Many of these studies utilize flourochrome labels such as oxytetracycline (OTC), which make a visible mark on otoliths that can be used to verify subsequent annuli (Beamish and McFarlane 1983; Hall 1991). When OTC marking is combined with another tagging technique that provides individual identification, such as visible implant (VI) tags (Haw et al. 1990), use of scales and otoliths for age estimation can be validated when the age at capture is unknown. This can be accomplished, for example, by comparing scales collected from VI-tagged fish at capture and recapture to determine if the number of additional annuli equals the number of years fish were at liberty. For otoliths, the number of annuli that have formed outside of the OTC mark can be compared with the number of years elapsed since the OTC application.

Studies that incorporate OTC marking should document the effect the procedure has on fish health (Gatz et al. 1986; Dwyer and White 1995) because OTC may affect the normal physiological processes of fish (Bumguardner and King 1996). Although fish condition does not always correlate positively with growth (Gutreuter and Childress 1990), it can be used to evaluate the physiological effects of various techniques (Thompson et al. 1997).

Our objectives were to (1) validate the use of annuli counts from scales and otoliths to determine if these two structures provide accurate age estimates for all age groups of rainbow trout *Oncorhynchus mykiss* in two southern Appalachian streams, (2) determine an effective technique for preparing rainbow trout otoliths for age estimation, and (3) evaluate the effects of OTC injections on the relative condition of rainbow trout.

Study Sites

Sams Creek (83°62‘N and 35°37‘W) is a second order tributary of the Middle Prong of the Little River in the Great Smoky Mountains National Park (GRSM), in Sevier County, Tennessee. Shining Creek (82°69‘N and 35°22‘W), also second order, is a tributary of the East Prong of the Pigeon River and is in the Shining Rock Wilderness Area in Haywood County, North Carolina. Both study sites began at an elevation of approximately 950 m, and the predominant vegetation was sweet birch *Betula lenta,* yellow birch *Betula allegheniensis,* Eastern hemlock *Tsuga canadensis, Rhododendron* spp., and doghobble *Leucothoe axillaris*. The study area at Sams Creek was approximately 400 m long and contained an allopatric population of rainbow trout. The Shining Creek study area contained syntopic populations of rainbow trout, brown trout *Salmo trutta,* and brook trout *Salvelinus fontinalis,* and extended for approximately 1700 m. These two streams averaged 5–7 m wetted width and conductivity was less than 15 μS/cm. Water temperatures averaged 15°C during July 1997 and 10°C during the October 1997 and 1998 sample periods. Both streams exhibited the characteristic high gradient channel morphology (>5%) and boulder–cobble substrate of many southern Appalachian streams.

Methods

In July 1997, backpack electrofishing units capable of generating 700 V AC were used to capture rainbow trout from Sams Creek ($N = 250$) and Shining Creek ($N = 236$). Fish were anesthetized by immersion in water mixed with a solution of clove oil and ethanol (1 mL clove oil/10 mL ethanol; Anderson et al. 1997; Keene et al. 1998), and total length (mm) and wet weight (g) were measured. Scales were removed from the left side between the dorsal fin and lateral line. All fish were injected intraperitoneally with 50 mg OTC/kg (adapted from McFarlane and Beamish 1987; Hall 1991). Stock OTC (200 mg/mL) was diluted at a 1:10 ratio with an 8% saline solution to reduce viscosity. A 1-mL syringe with a 26-gauge, 9.5 mm-long needle was inserted just inside the abdominal cavity between the pelvic and pectoral fins. The needle was inserted and held at an angle to the body to help prevent penetration and injection of OTC into the internal organs. A VI tag (2.5 × 1.0 × 0.08 mm thick; Northwest Marine Technology, Inc.) was inserted in the adipose tissue behind the eye. The adipose fin was clipped to provide an additional mark for the determination of OTC-injected fish in the event that the VI tag was lost. Upon recovery from the anesthetic all fish were released in the general area of capture.

Rainbow trout marked in July were recaptured ($N = 81$) in October 1997 to ensure that the OTC dosage effectively marked otoliths and to determine if OTC had affected the relative condition of fish. Unmarked fish that were captured in Sams Creek ($N = 49$) and Shining Creek ($N = 34$) during October 1997 were injected with OTC and tagged using the previously described procedure. However, rainbow trout less than 120 total length (TL) were not tagged or marked after the July 1997 sample because of high mortality, probably due to handling stress, and inability to retain VI tags. All other recaptures were collected in October 1998, 12–15 months after receiving an OTC injection.

Recaptures were sacrificed by overdose with clove oil. Scales were collected from between the dorsal fin and the lateral line on the right side to avoid collection of regenerated scales. The sagittae were removed by the ''up through the gills method'' (Secor et al. 1992) and stored dry in light-proof vials to prevent degradation of the OTC mark (Hall 1991).

Age validation.—Annuli were counted on scales taken at initial capture and recapture of rainbow trout that retained a VI tag. Scales were magnified using a microfiche projector (32× magnification), and annuli were counted by an experienced reader without knowledge of the fish's length and weight. A second reader independently reviewed 38% of the scale samples, also without knowledge of length and weight. Unreadable scales were discarded and scales with annuli counts that differed between the two readers were reconciled. The accuracy of annuli counts from scales for age estimation was determined by comparing the number of annuli observed on scales at capture and recapture (October 1998) for each fish and determining the percentage of recaptures with a new annulus.

Whole otoliths were viewed at 40× magnification with a compound microscope. Annuli were difficult to distinguish in the magnified lateral surface of the whole otolith under a variety of lighting and immersion conditions, especially from larger more opaque otoliths, presumably from older fish. We therefore decided to see if grinding to the sagittal midplane (Pannella 1980; Campana and Neilson 1985; Hall 1991) or sectioning along the transverse midplane (Hall 1991; Secor et al. 1992) might improve readability.

One otolith from each of 46 fish was mounted on a glass microscope slide by heating a small drop of thermosetting plastic resin (Neilson and Geen 1981) on a hot plate. The otolith was positioned sulcus side up on the heated liquid resin. The otolith was then hand ground with 600-grit wet sandpaper to the sagittal midplane (Secor et al. 1992). The other otolith was embedded in a thermoplastic epoxide and sectioned with an Isomet saw along the transverse midplane (Secor et al. 1992). The section was then mounted in resin on a glass microscope slide and polished with 600-grit wet sandpaper. The ages estimated from the two techniques for each otolith pair were compared.

Otoliths were viewed under transmitted light to distinguish the opaque and translucent zones. We defined the opaque zone as the portion of the otolith formed during slow growth, referred to as annuli, and the translucent zone as that portion formed during active growth. The number of annuli on each otolith was counted. All otoliths were read independently by two readers without knowledge of fish length and weight. Unreadable otoliths were discarded, and otoliths with annuli counts that differed between readers were reconciled. The number of annuli observed on otoliths and scales from each fish were compared, and agreement was calculated for each age-group.

Otoliths were examined for an OTC mark under a Nikon Optiphot-2 compound microscope equipped with a 100-W ultraviolet light source, a 450–490-nm excitation filter, a 515-nm barrier filter, and a 510-nm dichroic mirror. The percent of fish that had been at liberty for 12–15 months and that had formed an annulus outside of the OTC mark was determined for all age classes. The location of the OTC mark in relation to annuli was noted to determine the approximate time of annulus formation.

Effects of OTC on relative condition.—Rainbow trout collected at Shining Creek and Sams Creek in October 1997 were separated into two groups depending on whether they were recaptures (injected with OTC during July) or first-time captures. Relative condition factors were estimated for both populations from length and weight data from the July and October 1997 samples. The July 1997 sample was used to determine the length-specific weight for rainbow trout in each population because it contained the largest number of fish that had not received an OTC injection. The relative condition factors between captured and recaptured rainbow trout from the October 1997 sample were compared using an unpaired t-test.

Relative condition factors (K_n) were calculated according to the formula:

$$K_n = W/(W' \cdot 100),$$

where W is the observed weight and W' is the expected length-specific weight for a fish in the population, as predicted by a length-weight re-

TABLE 1.—Number of rainbow trout recaptured in Shining Creek and Sams Creek in 1998 that were at liberty for 12–15 months and the number and percent that formed an additinal annulus on scales and otoliths. Ages are the expected age at recapture, based on scale or otolith age at capture plus the length of time fish were at liberty (1 year).

	Shining Creek			Sams Creek		
	Number of	Additional annulus		Number of	Additional annulus	
Age	recaptures	Number	Percent	recaptures	Number	Percent
Scales						
1	0			0		
2	10	7	70	19	15	79
3	7	3	43	15	4	27
4	3	0	0	4	0	0
5	0			0		
Otoliths						
1	1	1	100	0		
2	18	18	100	47	47	100
3	10	10	100	18	18	100
4	3	3	100	2	2	100
5	0			1	1	100

gression estimated for each stream (Le Cren 1951; Murphy et al. 1991):

$$\log W = -5.08 + 3.02 \log \mathrm{TL}$$

for Sams Creek (where TL = total length), and for Shining Creek

$$\log W = -5.24 + 3.10 \log \mathrm{TL}.$$

Results

Age Validation

To validate ages estimated from scales, we recaptured 58 rainbow trout 12–15 months after receiving a VI tag (Table 1). Seventy-six percent of the fish initially estimated to be age 1 had formed a second annulus within the next 12–15 months. Only 32% of those initially estimated to be age 2 had formed a third annulus, and none of those initially estimated to be age 3 had formed a fourth annulus. The percent agreement within scales reviewed by two readers declined from 98% for age 1 to 12% for age 3 (Table 2). No scales were observed that contained more than three annuli.

TABLE 2.—Percent agreement between readers (within scale and otolith), scale and otolith agreement, and ground and sectioned otolith agreement for all age-groups of rainbow trout captured in Shining Creek and Sams Creek, 1997–1998. The sample size (N) is in parentheses.

	Percent agreement			
Age	Within scale	Within otolith	Scale versus otolith	Ground versus sectioned otolith
1	98 (60)	91 (35)	92 (35)	100 (1)
2	70 (19)	88 (118)	79 (112)	92 (25)
3	12 (17)	82 (40)	6 (33)	100 (13)
4	(0)	87 (8)	0 (6)	33 (6)
5	(0)	100 (3)	0 (1)	100 (1)

We recaptured 100 rainbow trout with otoliths that contained an OTC mark in October 1998. All 100 fish formed an additional annulus outside the OTC mark during the 12–15 months that they were at liberty, regardless of age. No fish older than age 5 were captured, suggesting that this age may be the maximum life expectancy for rainbow trout in Sams Creek and Shining Creek, and possibly other southern Appalachian streams. The mean sagittal section agreement between two experienced readers for all harvested recaptures (N = 204) was 88% and did not decline with age. Of the 204 recaptures, 156 contained at least one otolith with a distinguishable OTC mark. The mean percent agreement between scales and otoliths for all ages combined was 66%. Within each age-class, standard deviations for total length often overlapped previous and successive age classes for fish recaptured in October 1997 and 1998 (Figure 1).

The first annulus on whole otoliths was sometimes hard to distinguish for fish older than age 2. Grinding to the sagittal midplane and sectioning along the transverse midplane were both useful techniques for locating the first and second annuli on older fish. Identical ages were obtained in paired comparisons of ground and sectioned otoliths from 40 of 46 fish (87%). Of the six differences, ground sagittal surfaces had one less annulus than sectioned transverse surfaces in two instances and one more annulus in the four in-

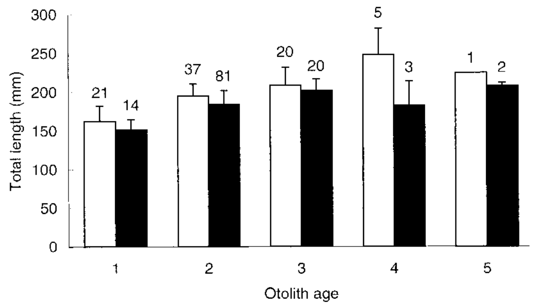

FIGURE 1.—Mean total length of each age-class of rainbow trout recaptured from Shining Creek (white bars) and Sams Creek (black bars) in October 1997 and 1998. Vertical lines represent standard deviation, and the sample size is indicated above each line.

stances. In all six cases, age varied by only 1 year between the two techniques.

Of 77 rainbow trout recaptured with visible OTC marks that were injected during July 1997, all marks were located within a translucent zone. The OTC mark was just before or just within an opaque zone on the otoliths of the 23 recaptured rainbow trout that received an OTC injection in October 1997.

Effects of OTC on Relative Condition

Mean relative condition factors did not differ significantly between recaptured and captured rainbow trout from Shining Creek (t = 1.39; df = 64; P = 0.17) or from Sams Creek (t = 0.94; df = 96; P = 0.35) in October 1997. In Shining Creek, the mean relative condition factor and standard deviation for captured rainbow trout was 1.009 ± 0.043 (N = 34) and for recaptured rainbow trout was 0.994 ± 0.046 (N = 32). In Sams Creek, the mean relative condition factor and standard deviation for captured rainbow trout was 0.991 ± 0.023 (N = 49) and for recaptured rainbow trout was 0.987 ± 0.020 (N = 49).

Discussion

We conclude that annuli counts from otoliths will provide accurate age data for rainbow trout in Shining Creek and Sams Creek, and possibly other southern Appalachian streams. Furthermore, our results indicate that scales were unreliable for estimating the age of rainbow trout older than age 2. Similar observations have been documented by other researchers (Alvord 1953; Carlander 1987) and have been attributed to scale erosion (Alvord 1953) or resorption (Simkiss 1974). We did not investigate any of these phenomenon, and the reason why annuli did not form on rainbow trout scales for all age groups during this study is unclear. In addition to the problems we observed in the accuracy of annuli counts from scales, the precision obtained for both structures indicates less variability will occur between multiple reader estimates of age based on otoliths than if scales are used.

In some cases, the sacrifice of fish may not be feasible. We conclude that scales may be used with accuracy and precision for rainbow trout up through age 2, but it may be difficult to accurately estimate the age of rainbow trout once they have formed two annuli. Although 76% of the age-1 fish formed a second annulus after 12–15 months at liberty, only 32% of age-2 fish formed a third annulus. Therefore, rainbow trout labeled as age 2 may actually be older because of the low frequency of age-3 fish that had scales with more than 2 annuli. This conclusion could be due, in part, to reader error, but no trends in reader error were observed (i.e., the second reader did not consistently overestimate or underestimate the ages assigned by the first reader). As indicated previously, we suggest that rainbow trout age estimates from scales are more variable between multiple readers than ages estimated from otoliths. As a result, annuli counts from otoliths should allow more confidence in the estimated ages obtained, regardless of the age-group investigated.

Because rainbow trout less than 120 mm TL were not tagged or marked after the July 1997 sample, we were unable to validate the formation of the first annulus on scales and otoliths by the 1997 cohort in Sams and Shining creeks. However, in a preliminary study we collected age-0 rainbow trout from Little River, a fourth order stream in the Great Smoky Mountains National Park, each month from September 1996 to September 1997. An annulus was observed on scales and otoliths from the 1996 cohort during March and April 1997, approximately 1 year after these fish had hatched, and reader agreement between scales and otoliths was 93.7% (N = 391).

One or both of the first two annuli were often overlooked on whole, unprepared otoliths because of the deposition of calcium on the proximal surface (sulcus). Consequently, the number of annuli on otoliths would have been underestimated in this study without a method of preparation such as grinding or sectioning. Although annuli were clearly visible on both ground and sectioned otoliths, ground sagittal surfaces were much easier and less costly to prepare. Hall (1991) also suggested grinding to the sagittal plane over transverse sections for brook trout *Salvelinus fontinalis* because of the ease of preparation and ability to distinguish annuli. However, otolith preparation techniques should be compared because the size and shape of otoliths are intraspecific. The life expectancies of rainbow trout and other fish species may also vary among geographic areas, and the ability to distinguish individual annuli with these techniques should be investigated if used on older age groups.

Hall (1991) used the anterior tip of ground brook trout otoliths to estimate age. In this study, rainbow trout annuli were counted between the posterior and dorsal portion of ground otoliths. The OTC marks and subsequent annuli were visible on the anterior, dorsal, and posterior portions, but the annuli of older fish were often hard to distinguish on the ventral portion of ground otoliths. Furthermore, previous studies have suggested that otolith growth may be allometric (Beamish and Chilton 1982; Fargo and Chilton 1987; Hall 1991). We observed that OTC marks and annuli were not visible on the distal portion of transverse sections taken from fish older than age 2, but were easily distinguished on the proximal portion of transverse sections. This may indicate that growth on the distal portion of the otolith is reduced in older fish.

Annular formation on otoliths began in early fall and continued until spring, at which time otolith growth increased. Therefore, ages of rainbow trout collected during the spring and early summer in the southern Appalachians may be easier to estimate because the annulus should be complete at this time. Beckman and Wilson (1990) noted that the predominant period of annulus formation in both the northern and southern hemisphere occurred in the spring and summer months. Although the data reported by Beckman and Wilson (1990) contradicts what we observed, they did report that at higher latitudes in the northern hemisphere annulus formation occurred later in the year and over a longer period.

Length-frequency distributions are sometimes used to help distinguish age classes within fish populations (Macdonald 1987), but the accuracy of this technique relies on an unimodel growth rate within each age-class (Devries and Frie 1996). In this study, the standard deviation for total length within each age-class overlapped previous and successive age classes within both streams, and the amount of overlap tended to increase with age. Similar results are evident in length-frequency data from a study by Whitworth and Strange (1983) that investigated the growth and production of rainbow trout in a southern Appalachian stream. There are a variety of statistical and graphical approaches for length-frequency analyses (McNew and Summerfelt 1978; MacDonald 1987; Devries and Frie 1996), and future progress with this technique may provide a low-cost method of distinguishing many age groups. However, variation in growth rates among and within age-classes and between years within a population limits the usefulness of this technique for older fish.

When using OTC for age and growth studies, it is important that enough OTC be injected to mark the structures of interest without impairing fish physiology (McFarlane and Beamish 1987). In this study, oxytetracycline injections did not affect the relative condition of rainbow trout over a 3-month period at a dosage of 50 mg OTC/kg and provided a visible mark on at least one otolith from 77% of the recaptured fish. Injected dosages of OTC at levels exceeding 50 mg OTC/kg may provide more intense and frequent OTC marks on rainbow trout otoliths, but mortality may increase as dosage levels approach and exceed 100 mg OTC/kg (Weber and Ridgway 1967). Future studies would be helpful in determining what effect OTC injections have on fish physiology over a longer period than we investigated, including the late spring and early summer months when we suspect that rainbow trout experience active growth.

We conclude that annuli counts from otoliths ground to the sagittal midplane will provide accurate and precise age data for rainbow trout in southern Appalachian streams. The use of otoliths and consequential sacrifice of fish may not be an option in some cases. However, annuli counts obtained from the scales of rainbow trout in southern Appalachian streams may not provide accurate age estimates for fish older than age 2, and with scales, it may be difficult to distinguish between age-2 and age-3 rainbow trout. Future studies are needed to validate the use of annuli counts from scales and otoliths for rainbow trout in other geographic areas and to determine why annular formation is not always consistent between both structures.

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