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Chemical archives in fishes beyond otoliths: A review on the use of other body parts as chronological recorders of microchemical constituents for expanding interpretations of environmental, ecological, and life-history changes

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Abstract

Microchemical analyses of fish otoliths have revolutionized fisheries science. Molecules deposited within otoliths may originate from ambient water and diet, with molecular concentrations being subject to subsequent physiological alteration after exposure. Analyses of otolith microstructure and incorporation of inorganic elements have led to major advances in stock assessment and fisheries ecology. However, the use of otoliths for microchemical analyses has drawbacks. Specifically, otolith removal from live specimens requires specimen sacrifice, which may be forbidden in the case of protected species. In addition, otoliths rarely contain sufficient concentrations of organic matter to allow reconstruction of food-web relationships via multiple stable isotopes, and otolith microstructure can be difficult to interpret in some species. Here, we review alternatives to otoliths that can provide microchemical analytes for life-history studies in fishes. Our focus is to describe advantages and disadvantages to the use of each alternative structure, with particular attention paid to trace-element analysis for inorganic matrices and stable-isotope analysis for organic ones. In general, the chronological analysis of elemental and isotopic values within each structure depends on the inert nature (or lack of molecular turnover) of the tissue. Structures with high turnover rates or those that are metabolically active will not effectively record elemental or isotopic compositions over time. Here, we provide an assessment of the use of bony endoskeleton, fin spines, fin rays, scales, and eye lenses as alternatives or complements to fish otolith analysis.

Over the past 40 yr, the analysis of calcified structures in fishes has developed into a valuable tool, arguably advancing fisheries science further than any other commonly used technique. Specifically, aging studies on calcified structures have become the accepted norm for stock assessments and continue to lead the way in developing an understanding of population age structures (Francis and Campana 2004; Campana 2005; Kerr and Campana 2014). More recently, microchemical analyses of calcified structures have allowed researchers to investigate pressing issues in fisheries science such as habitat use (Secor et al. 1995; Gillanders and Kingsford 2000), migration patterns (Secor et al. 1995; Elsdon and Gillanders 2003; Elsdon et al. 2008), stock structure (Campana et al. 2000; Patterson et al. 2004), and even dietary patterns (Campana 1999; Elsdon et al. 2010; Tzadik et al. 2015). Otoliths are considered the standard for aging in fishes, comprising the majority of studies on chronological microchemistry (Campana 2005). However, over the past decade, the analyses of numerous alternative structures have proved useful, either singly or in conjunction with otoliths, to document both aging and life-history patterns. As a research tool, otolith microchemistry has previously been reviewed (see Campana 1999), while alternative structures such as endoskeleton, fin spines and rays, and eye lenses have not. One notable exception by Seeley et al. (2015), reviews elemental analyses of fish scales, with a focus on trace elements. The following is a review of alternative structures (both calcified and non-calcified) that can be used as chronological recorders for microchemical analyses in fish. First we provide a brief history of the analysis of alternative structures. Then we review the physiological processes related to each structure, how each has been used in Trace Element Analysis (TEA) and/or Stable Isotope Analysis (SIA), as well as advantages and considerations specific to each type of structure.

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Aging fishes via calcified structures was initially suggested by Aristotle in 340 B.C.E., but the commonly accepted "birth" of aging fishes via calcified structures was attributed to Hans Hederstrom who correctly interpreted annuli in the vertebrae of Pike in 1759. Indeed, the age-growth curve that Hederstrom produced was consistent with modern estimates. Evidence of annulus formation was eventually tested in 1859 by Robert Pell, who analyzed the vertebrae and scales of fishes in a controlled experiment over 2 yr (as reviewed by Jackson 2007). Forty years later, otoliths were first used as an accurate and reliable method to age fishes due to difficulties in aging scales (Reibisch 1899).

Beyond aging, the chemical analysis of calcified structures in fishes has typically concentrated on trace elements in inorganic matrices (TEA) and stable-isotope ratios in organic matrices (SIA) (Elsdon et al. 2010). Information-bearing molecular concentrations are incorporated either during osteogenesis, when deposition occurs at the cellular level for bones, or during protein synthesis for organic structures (Perga and Gerdeaux 2005; Mahamid et al. 2010). The chemical properties documented in the calcified structures can originate from either ambient water-chemistry, variability in chemical-elemental partitioning between water and calcified structure, or from diet (Campana 1999). Both TEA and SIA can inform researchers about geographic origin and fish movement (seasonal migration, ontogenetic habitat shifts, and aperiodic immigration). SIA of organic material can also lead to inferences about diet due to the enrichment of carbon and nitrogen with increasing trophic level or changes in basal resources.

TEA, which is typically conducted on calcified structures, is primarily used to track movements and habitat use in fish (Campana 2005; Mcmahon et al. 2012). The concentrations of certain trace elements in these structures are thought to be representative of ambient water chemistry at the time of deposition (Bath et al. 2000; Walther and Thorrold 2006), although chemical partitioning can also cause variability (i.e., as a function of temperature or carbonate lattice accompidation–substitution space). To date, the majority of studies have concentrated on trace elements that substitute for Ca^{2+} in hydroxyapatite ($Ca_{10}(PO_4)_6(OH)_2$) in bone or calcium carbonate ($CaCO_3$) in otoliths (Campana 1999). Hydroxyapatite, which is alternatively known as bioapatite, crystalline calcium hydroxyapatite, hydroxyl apatite, carbonated apatite, and dahlite, is a hydroxylated polymer of calcium phosphate (Glimcher 1998). Substitutions of trace-metal ions for hydroxyapatite Ca^{2+} are common, but researcher bias toward the study of particular suites of ions is more likely due to limitations in analytical capabilities. Analyses of trace elements are predominantly conducted using mass spectrometers which detect charged particles to determine chemical composition (Pare and Yaylayan 1997). This may be one reason that researchers have mainly focused on analytes that are naturally positively charged (Mokgalaka and Gardea-Torresdey 2006). Recently, advances in the fields of X-ray fluorescence, atomic absorption, and emission optical-spectrometry have expanded and may soon include anions that are difficult to detect using mass spectrometers (Potts et al. 2004). In the body parts of fishes, anion substitution of the hydroxyl group (OH^-) is relatively common and, to a lesser degree carbonate ions ($CO_3^=$) and thus should be given future consideration, if possible (Bogdanova et al. 1998; Trischitta et al. 1998).

To date, most SIA of organic tissues in the marine environment has concentrated on stable-isotope ratios of carbon, nitrogen, and sulfur. Lighter isotopes are typically used preferentially in biological processes so that the heavier isotope is accumulated in tissues (Post 2002). As a result, the measurement of the ratio of the heavy to the light isotope can lead to inferences about the extent of the biological process in question (McMahon et al. 2013). For example, nitrogen isotopic ratios are used as proxies for trophic level (Vanderklift and Ponsard 2003; Galvan et al. 2010), and both carbon and sulfur isotopic ratios are useful for identifying basal-resource dependence (Dawson et al. 2002; Phillips and Gregg 2003). The analysis of these stable-isotope ratios can also be used to study the location of individuals as background levels change across geological "isoscapes" (Bowen 2010; Radabaugh et al. 2013). Spatial signals may also indicate environmental conditions such as hypoxia (Limburg et al. 2015). While nitrogen is unique to organic molecules, carbon and sulfur are integral components of both organic and inorganic matrices (Campana 1999). Due to the inability to differentiate the source, carbon and sulfur SIA present logistical challenges when working with calcified structures that contain robust organic and inorganic components. The challenge to isolate organic from inorganic components may explain why very few studies on these elements have been conducted. In addition, the signal from the organic material is difficult to differentiate between ambient water and dietary sources. Compound-specific amino-acid analyses have the ability to differentiate source material, but remain expensive, time-consuming, and largely untested.

The use of microchemical analyses to determine details of an individual's life history require the assumption that the constituents being analyzed were deposited at the age associated with the annulus where the analytes were found. This assumption is derived from otolith studies that record denser growth bands (opaque annuli) during colder seasons and less dense growth bands (translucent annuli) during warm seasons (Pannella 1971). Otoliths are often described as "inner-ear bones" (Kalish 1989), but are primarily composed of calcium carbonate minerals rather than the hydroxyapatite (mineral) and collagen (structural protein) that dominate bone mass. The calcium-carbonate depositions in otoliths are inert and comprise almost exclusively inorganic material (Campana 1999). There is an organic component in otoliths in the form of a proteinaceous matrix that constitutes only about 3% of otolith mass (Campana 1997, 1999). To date, analyses on the organic component in otoliths have only derived information on the bulk level, lacking temporal resolution (e.g., Gronkjaer et al. 2013). The dominant inorganic

component of the otolith (calcium carbonate) has thus been studied extensively, and many researchers have focused on the chronology of trace elements within the otolith's crystalline calcium carbonate to infer aspects of the fish's life (Campana 2005; Elsdon et al. 2008). However, no studies to date have elucidated microchemical chronologies within the proteinaceous (organic) matrix. Instead, whole otoliths have been studied to infer chemical characteristics of the protein (Elsdon et al. 2010). This methodology eliminates the possibility for temporal inferences and often relies on harsh demineralization techniques that may, in fact, alter the composition of the protein (Rude et al. 2014). Alternative structures that have an increased proportion of organic material would increase our temporal resolution over what otoliths can presently describe.

Given the extensive research on otolith microchemistry and the accepted temporal correlation with material deposition, it would be ideal to compare the trace-element properties of potential alternative structures with those of otoliths (Clarke et al. 2007). Non-vascularized alternative structures are more likely to be temporally conservative than vascularized ones, due to the lack of turnover of the material. Even among non-vascular alternatives, elemental values may not correlate with those of otoliths due to physiological pathway differences (Gillanders 2001). In cases of poor or unknown elemental agreement with other structures, it is still possible that comparisons of a single alternative structure can be made among individuals without correlating its patterns with those of other structures (Arai et al. 2002; Allen et al. 2009; Wallace et al. 2014).

The sections that follow provide reviews of five alternative structures that show promise for microchemical analyses (specifically TEA and SIA), arranged in decreasing order by extent of calcification: bony endoskeleton, fin spines, fin rays, scales, and eye lenses (Fig. 1). The review of these fives structures is not meant to represent a complete list of alternatives, but instead a review of five well-studied and useful structures. Additionally, there is an *Advantages and considerations* section for each structure to identify areas where each structure is useful as well as the limitations of each (also see Table 1). Each section is reviewed for each structure individually, however the use of multiple structures in a single study can often increase inference and reduce the limitations. For reviews of otolith microchemistry, see Campana (1999) and Elsdon et al. (2008). For a review of the microchemistry of the cartilaginous endoskeleton, see Hussey et al. (2012). The literature reviewed here is inclusive of publications from January 1899 to June 2015, from the "web of science" online database (Table 2).

Endoskeleton (up to 40% calcified)

Structure and physiology

Fish endoskeleton (Fig. 2) is composed of organic and inorganic materials. Approximately 90% of the organic matrix of bone is made of collagenous fibrils, which provide the frame of the bone and associated anchoring structures such as ligaments (Nusgens et al. 1972; Veis 1984). The mineral aspect of bone is hydroxyapatite, which is deposited onto collagen fibrils (Vaughan 1975), providing strength to the bone structure and also serving as an ion reservoir (Glimcher 1998). There are three types of cells involved in bone formation and erosion: (1) osteoblasts, which synthesize collagen on the bone surface and aid in mineralization of the organic matrix, (2) osteocytes, star-shaped cells involved in regulation of bone resorption that are formed when osteoblasts are surrounded by collagenous fibrils, and (3) osteoclasts, which destroy bone, thus releasing minerals (reviewed in Panfili et al. 2002). Less derived teleost orders have cellular bone containing osteoblasts, osteocytes, and osteoclasts. In contrast, more derived orders are characterized by acellular bone, formed by osteoblasts which withdraw after bone synthesis, leaving a mineral matrix lacking osteoblasts and osteocytes (Fleming 1967; Parenti 1986).

The collagenous fibrils of the organic matrix are embedded within other proteins such as glycoproteins and osteocalcin (Veis 1984; Nishimoto et al. 2003). These fibrils can form three spatial arrangements that are dependent on the age and metabolism of the fish: (1) woven-fiber bone is typical of fast growth rates and is described as loosely woven and randomly distributed collagen fibrils, (2) parallel fiber or pseudo-lamellar bone is typical of intermediate growth rates and consists of tightly packed collagen fibers with parallel orientation, and (3) lamellar bone is associated with slow growth rates and is composed of successive thin lamellae of tightly packed collagen fibers that change orientation by 90° with each successive lamella (Francillon-Vieillot et al. 1990; Meunier and Huysseune 1992). The orientation of collagen fibrils, which can be viewed using polarized light, forms growth marks in bone that can be used for age estimation (Panfili et al. 2002). Hydroxyapatite forms at the surface of the organic matrix within (Weiner et al. 1999) or on the outside of collagen fibrils (Fritsch and Hellmich 2007). The crystalline structure of the calcium-phosphate mineral exists as small, thin plates (Glimcher 1998). In addition to calcium and phosphate, the mineral material in skeletal tissues contains carbonate, hydroxide, sulfate, fluoride, and small amounts of the macroelements sodium, potassium, magnesium, and chloride (Francillon-Vieillot et al. 1990; Lall 2002; Wopenka and Pasteris 2005; Pasteris et al. 2008).

Bone tissue serves as an ion reservoir and is essential for the regulation of ionic homeostasis in many fish species (Mugiya and Watabe 1977; Takagi and Yamada 1991; Yamada et al. 2002). An increase in osteoclast density has been observed in phosphorus-deficient fishes, suggesting that phosphorus, and not calcium as in terrestrial animals (reviewed by Lall 2002), prompts the release of minerals to maintain homeostasis (Roy et al. 2002; Roy and Lall 2003). Yet, osteoclastic bone resorption resulting in the release of

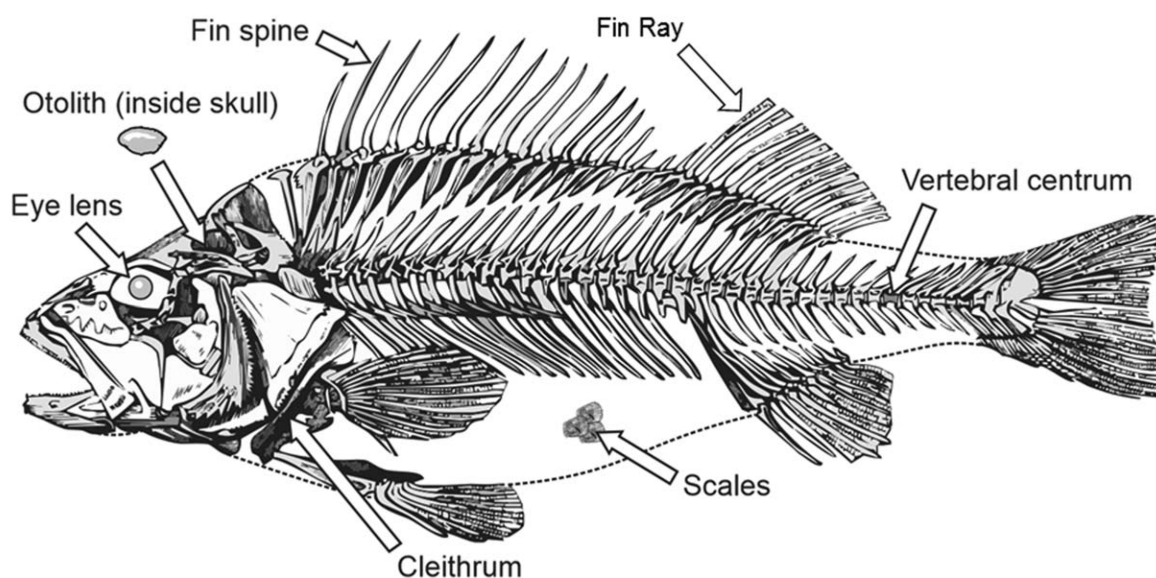

Fig. 1. Basic anatomy of derived fishes. All alternative structures are labeled based on their anatomical locations.

Table 1. A general-reference guide to the alternative structures reviewed in the preceding sections. The descriptions provided here are meant as general guidelines. The "relative ability to address questions" will vary by study species and location.

		Relative ability to address questions		
Structure	**Lethality**	**Aging**	**Migration**	**Diet**
Endoskeleton	Lethal	Yes, with species-specific understanding of bone remodeling	Not recommended due to the mobilization of some trace elements and bone remodeling	May provide a shorter isotopic record than previously assumed, further testing of bone collagen isotopic turnover rates is needed
Fin spines	Non-lethal	Yes, possible loss of early annuli due to vascularization and resorption	Yes, via TEA	Possible but has not been demonstrated
Fin rays	Non-lethal	Yes, possible loss of early annuli due to vascularization and resorption	Yes, via both TEA and SIA	Yes, via SIA
Scales	Non-lethal	Yes, but avoid regenerated or scale margins that display irregular growth or physical erosion	Yes, via both TEA and SIA; However, the metabolic reworking of scales can affect relevant microchemistry and obscure migration patterns	Yes, via SIA. Should be restricted to the outer margins of the scales. Bulk analysis or SIA of targeted annuli can bias results
Eye lenses	Lethal	May require dimensional linkage to other structures	Yes, via SIA	Yes, via SIA

bone minerals occurs both under normal conditions (Sire et al. 1990; Witten 1997) and as a response to ion deficiency (Weiss and Watabe 1979). Following bone resorption, secondary bone can be deposited in eroded areas where antecedent bone has been resorbed due to osteoclast activity; this is known as "bone remodeling" (Francillon-Vieillot et al. 1990). To accurately interpret skeletal records of age and movement patterns, it is necessary to distinguish secondary bone from primary bone, which is new bone deposited by osteoblasts where antecedent bone does not exist (Francillon-Vieillot et al. 1990). Remodeling can be identified by the presence of a cementing line, representing a break in bone growth, in some fish species (Amprino and Engstrom 1952). Osteoclasts in cellular bone typically create resorption

Table 2. The literature reviewed in the preceding sections categorized by topic. The column headers are general categories and the classification of each reference is subject to author discretion. The "total" column represents the number of references that were cited, rather than a total of all the numbers in a particular row as many studies were included in multiple categories.

	Numbers of studies											
Structure	**Freshwater**	**Marine**	**Diadromous**	**Juvenile**	**Adults**	**Juvenile and adults**	**Herbivores**	**Predators**	**Tropical**	**Temperate**	**Polar**	**Total**
Endoskeleton	11	19	9	16	14	9	4	35	2	35	2	39
Fin spines	1	4	2	0	4	3	1	6	0	7	0	7
Fin rays	7	2	5	5	9	0	2	12	0	13	1	14
Scales	26	4	27	6	26	17	9	49	2	41	3	58
Eye lenses	0	7	0	0	7	0	0	7	5	2	0	7

lacunae, but this does not always occur in acellular bone when osteoclasts are mononucleated (Witten 1997).

As vascular canals and cavities form in bony tissue, they are initially filled with fibroblasts, soft connective tissues, and blood vessels (Francillon-Vieillot et al. 1990). These are known as primary vascular canals because they are a part of the intrinsic vascular network of the bone and because they form during initial osteogenesis (De Ricqlès et al. 1991). Secondary vascular canals are formed when osteoclasts resorb bone tissue and the space created is subsequently filled with blood vessels (Francillon-Vieillot et al. 1990). Vascularization of bony tissue is positively related to the metabolic activity of the fish and can be more or less developed, depending on the species (De Ricqlès et al. 1991). Highly vascularized bone is often resorbed, limiting its use for aging and microchemical studies.

Trace-element analysis

Trace-element analysis of fish endoskeleton has been successfully used to classify fish originating from distinct geographical regions (Moreau et al. 1983; Keenleyside 1992; Kennedy et al. 2000). For instance, Kennedy et al. (2000) used Sr isotopic values of salmon vertebrae to distinguish populations from different tributaries of a river. Mulligan et al. (1983) used P, Ca, K, Cu, and Sr to classify salmon stocks with an accuracy of 80–95%. Trace-element concentrations in skeletal tissues have also been used to identify wild vs. cultured fish (Guillou and Delanoue 1987; Yamada and Mulligan 1987; Roy and Lall 2006). Measurement of trace element concentrations and $\delta^{18}O$ in fish bones have been used to track diadromous migrations (Zazzo et al. 2006; Torz and Nedzarek 2013).

The pathway for Sr incorporation into fish bones can involve both diet (Guillou and Delanoue 1987; Gausen and Berg 1988; Kennedy et al. 2000) and ambient water (Yamada and Mulligan 1987; Mugiya and Tanaka 1995). Additionally, temperature and salinity may have positive relationships with skeletal Sr (Balter and Lécuyer 2010; Torz and Nedzarek 2013). Emara et al. (1993) observed a positive relationship between Mn, Fe, and Cu concentrations and trophic level, suggesting that these metals may be actively regulated. Although Mn in bony tissue can increase in fishes exposed to high concentrations of this element (Lockhart and Lutz 1977; Fraser and Harvey 1982; Moreau et al. 1983), Bendell-Young and Harvey (1986) found that this relationship was not clearly defined because Mn is also homeostatically regulated. Direct relationships have been observed between water and skeletal concentrations of Zn, Pb, Ba, As, and Cd (Fraser and Harvey 1982; Moreau et al. 1983; Bendell-Young and Harvey 1986; Bengtsson et al. 1988; Stripp et al. 1990; Miller et al. 1992; Kock et al. 1996); however, Zn concentrations in vertebrae have also been observed to be influenced by genetic factors (Yamada et al. 1987).

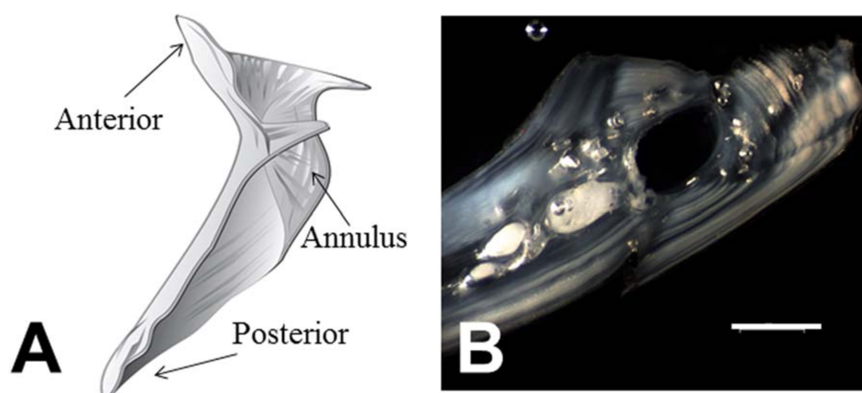

Fig. 2. Anatomical diagram (panel **A**) and cross-sectional photograph (panel **B**) of a cleithrum (a bone in the endoskeleton). Scale bar in panel **B** represents 500 μm. Visible rings in the cross-sectional photograph represent annuli. Sizes and annuli clarity vary by species and study location.

Stable isotope analysis

Fish collagen, which contains most of the C and N isotopes in fish bones, comprises approximately 42% C, 15% N, 7% H, 35% O, and 0.32% S (Szpak 2011). Approximately one-third of all amino acids in collagen are glycine, while proline and alanine comprise a large proportion of the remaining amino acids (Szpak 2011). However, fishes living in colder waters possess a marginally different amino-acid composition than those in warmer waters, and this results in slightly altered bone isotope values (Regenstein et al. 2007).

One general pattern that has emerged from field studies of multiple tissues is that bone collagen is enriched in δ^{13}C and depleted in δ^{15}N relative to muscle tissue (Sholtodouglas et al. 1991; Hobson and Clark 1992; Miller et al. 2010). Studies of bone collagen conducted largely on mammals have identified a 2.0–4.0‰ enrichment in δ^{15}N values per unit increase in trophic level (Deniro and Epstein 1981; Schwarcz et al. 1985). To our knowledge, only one laboratory study of δ^{15}N fractionation of fish collagen exists. Ankjaero et al. (2012) observed bone-collagen δ^{15}N enrichment of 1.0–3.0‰ per trophic step, depending on diet and growth rate (i.e., not metabolic turnover). Other studies identified collagen δ^{13}C trophic enrichment of 3.9–5.3‰ (Deniro and Epstein 1978, 1981; Hobson and Clark 1992). Considered together, these studies indicate collagen can deviate from the 3 : 1 relationship that is often used to represent δ^{13}C : δ^{15}N trophic enrichment in muscle tissue (McCutchan et al. 2003). Additional laboratory studies of organism- and tissue-specific trophic enrichment are necessary to accurately interpret stable isotope data from wild-caught animals (Gannes et al. 1997).

Despite a relatively low C : N value of 2.8 identified by Szpak (2011), the bones of some fish species may serve as lipid reservoirs (Phleger et al. 1976), in which case prior removal (extraction) of lipids is necessary to accurately determine δ^{13}C. Lack of standardization in protocols for lipid extraction from collagen may mean that comparing collagen δ^{13}C among studies that use different extraction methods should be interpreted with caution (Szpak 2011).

Stable isotope analysis has proved useful when using fish bones for a variety of questions beyond life history traits, which may not derive solely from the proteinaceous matrix. For example, Oliveira et al. (2011) used stable isotope abundance in skeletal tissue to identify different gadoid fish species. Additionally, stable isotopes of δ^{18}O and δ^{13}C in fish bones from zooarchaeological assemblages have been used to calculate paleo-temperatures (Longinelli and Nuti 1973) and interpret human diets (Dufour et al. 1999; Miller et al. 2010), respectively.

Advantages and considerations

In fish bodies, skeletal tissue generally has the highest mineral content among tissues (Rosenthal 1963; Emara et al. 1993; Lall 2002; Roy and Lall 2006). Additionally, acellular bone may have a higher mineral content than cellular bone (Roy and Lall 2006); in this regard, fish endoskeleton may be well-suited for microchemistry analysis. Bone is the main tissue involved in the homeostatic regulation of P in fishes, while Ca regulation occurs at the gills and oral epithelial tissues (reviewed by Lall 2002). During periods of high stress such as oogenesis, which involves the production of the Ca-binding protein vitellogenin, Ca mobilization has been observed in scales, but it has not been detected in the endoskeleton (Mugiya and Watabe 1977; Carragher and Sumpter 1991). In the crystalline hydroxyapatite matrix, Ca can be readily replaced with Na and Mg (Vaughan 1975; Driessens and Verbeeck 1986). A reduction in dietary Mg has been shown to increase Na and Ca concentrations in bone (Cowey et al. 1977; Bijvelds et al. 1997), and may reflect the Mg dependence of cellular ion transport mechanisms.

There are a few considerations that should be accommodated when using endoskeleton components for either TEA or SIA. First, we do not yet fully understand collagen turnover rates in fishes. Investigations of turnover rates in other taxa (primarily mammals) have suggested that it is relatively

slow in comparison to other tissues and thus may only be useful for investigating long-term dietary changes (Hobson and Clark 1992; Liden and Angerbjorn 1999). Collagen turnover rates are positively correlated with metabolism (Thorsen et al. 1997; Liden and Angerbjorn 1999) and may vary among bone types (Sealy et al. 1995). Data from field studies imply that bone collagen in fishes has a slow turnover rate (Schoeninger and Deniro 1984; Sholtodouglas et al. 1991; Gaston and Suthers 2004). Additionally, the rate of turnover of S, Ca, and Sr has been observed to be the slowest for bone tissue in comparison to all other tissues measured (Rosenthal 1963). Yet, the only study in which bone collagen isotopic turnover rates were determined experimentally found that the half-lives of δ^{15}N were ranked heart < bone collagen < blood < muscle, suggesting that bone collagen has a faster isotopic turnover than previously thought (Ankjaero et al. 2012). Therefore, the assumption that fish bone is only useful as a long-term isotopic record needs further testing, as the only dedicated study of turnover rates has suggested collagen may provide a shorter isotopic record than previously assumed. These rates were recorded for juveniles with high growth rates where turnover among tissues ranged from 31 d to 78 d, and may therefore not be representative of tissue types among different fish species and ages.

Additionally, standardized methods for demineralization and lipid extraction of collagen (Szpak 2011; Ankjaero et al. 2012) are necessary to make accurate comparisons across studies. Bone remodeling can complicate interpretation of skeletal tissue records and may render some microchemistry applications unreliable (e.g., tracking movement). Nevertheless, a thorough understanding of species-specific skeletal biology may make interpretation of these records possible. For instance, Zazzo et al. (2006) recognized that salmon vertebrae are minimally ossified during the juvenile stage and are potentially resorbed during spawning migration. This knowledge of salmon biology enabled researchers to reconstruct freshwater-to-marine-to-freshwater migrations from fossilized vertebrae. For a list of the unique structures and the species discussed in the TEA and SIA sections, refer to Table 3.

Fin spines (30–35% calcified)

Structure and physiology

The term “fin spines” is applied to the anterior-most structural components of fins that are unsegmented, more rigid, and more calcified than soft, segmented “fin rays” (sensu Lauder and Liem 1983). Kerr and Campana (2014) thoroughly reviewed the structure, composition, metabolic stability, and pathway of incorporation of new material in fin spines. Fin spines (Fig. 3) are composed of dermal bone and can incorporate 1–3 fin-ray substructures into the spine structure (Findeis 1997). Similar to other bones of the endoskeleton, fin spines are mainly composed of calcium phosphate (Gillanders 2001), with the mineral fraction being composed of hydroxyapatite (Ugarte et al. 2011). The incorporation of trace elements and stable isotopes into fish spines appears to be similar to fish vertebrae (Gillanders 2001). However, differences in elemental composition between otoliths and fin spines (Gillanders 2001; Davies et al. 2011) highlight our lack of understanding of metabolic pathways, routes of ion uptake, and differential abilities of structures to incorporate elements and should be taken into consideration when using these structures (Campana 1999; Gillanders 2001).

Vascularization and resorption of the fin spine is well documented; it starts at the center of the fin spine and proceeds outward as the fish grows. This process can obscure or destroy annuli associated with early life history (Hill et al. 1989; Drew et al. 2006; Santamaria et al. 2015). Vascularization and resorption is especially prevalent in epipelagic species such as the billfishes and tunas, possibly due to their elevated metabolic activity (Antoine et al. 1983). In addition, elemental mobilization and resorption can occur during periods of nutritional stress (Gillanders 2001).

Trace-element analysis

TEA of fin spines has been effectively used to investigate stock structure (Gillanders 2001), movement (Veinott et al. 1999; Arai et al. 2002; Balazik et al. 2012), environmental histories (Smith 2010), and exposure to pollutants and heavy metals (Ugarte et al. 2011, 2012). Gillanders (2001) described how fin spines have the potential for stock structure analysis when an alternative to otoliths must be used. Because its microchemical structure is correlated with that of the otolith in both juvenile and adult fish, the fin spine has been validated as an acceptable alternative structure for TEA.

Several studies have used TEA to investigate ontogenetic patterns in [Sr] or Sr : Ca ratios of sturgeon. Using separate techniques (i.e., X-ray microprobe analysis, energy dispersive X-ray fluorescence, and inductively coupled mass spectrometry via laser ablation, or LA-ICP-MS) researchers have linked [Sr] with marine derived water sources in fin spines in sturgeon (Veinott et al. 1999; Arai et al. 2002; Balazik et al. 2012). In addition, Smith (2010) used LA-ICP-MS and TEA to investigate the environmental histories of three species of catfish, and combined TEA with SIA to accurately classify individual catfish to their capture locations.

Finally, Ugarte et al. (2011) developed a hydroxyapatite pellet to use as a matrix-matched standard for LA-ICP-MS analysis of trace metals in the fin spines of different tuna species. They presented TEA findings along the pectoral-fin spine of Bluefin Tuna (*Thunnus thunnus*) and Albacore (*Thunnus alalunga*) that indicated temporal variation in analyte concentrations. This was followed by an investigation (Ugarte et al. 2012) of pollutant trace metals in muscle and the first dorsal spine of Bluefin Tuna and Albacore. A positive linear relationship was found between the fin spine and

Table 3. The literature reviewed related to fishes in the Endoskeleton TEA and SIA sections, with the relevant structures and the species that were studied. Other references in these sections were not focused on fishes, and were therefore not included in the table.

Authors	Year	Relevant structure(s)	Species
Ankjaero et al.	2012	Bone collagen	Atlantic Cod (*Gadus morhua*)
Balter and Lécuyer	2010	Backbone, pre-maxillary, mandible, and teeth enamel	Gilthead Seabream (*Sparus aurata*)
Bendell-young and Harvey	1986	Caudal vertebrae	White Sucker (*Catostomus commersoni*)
Bengtsson et al.	1988	Vertebrae	Fourhorn Sculpin (*Myoxocephalus quadricornis*)
Bijvelds et al.	1997	Vertebrae	Mozambique Tilapia (*Oreochromis mossambicus*)
Carragher	1991	Vertebrae and ribs	Rainbow Trout (*Oncorhynchus-mykiss*)
Cowey	1977	Vertebrae	Rainbow Trout (*Oncorhynchus-mykiss*)
Dufour	1999	Bone collagen from skull	Whitefish (*Coregonus lavaretus*), Pike (*Esox lucius*), Zander (*Sander lucioperca*), Burbot (*Lota lota*), Perch (*Perca sp.*), Artic Char (*Salvelinus alpinus*), Omul (*Coregonus migratorius*)
Emara et al.	1993	Unspecified bones	Mediterranean and Red Sea fishes
Fraser and Harvey	1982	Ribs, trunk centra, hypurals, opercula	White Sucker (*Catostomus commersoni*)
Gaston and Suthers	2004	Vertebrae	Mado (*Atypichthys strigatus*)
Gausen and Berg	1988	Vertebrae	Atlantic Salmon (*Salmo salar*)
Glimcher	1998	Vertebrae	Atlantic Salmon (*Salmo salar*)
Guillou and de-la-Nouee	1987	Opercular bone	Brook Trout (*Salvelinus fontinalis*)
Keenleyside	1992	Vertebrae	Northern Redbelly Dace (*Chrosomus eos*)
Kennedy et al.	2000	Vertebrae	Atlantic Salmon (*Salmo salar*)
Kock et al.	1996	Operculum	Artic Char (*Salvelinus alpinus*)
Lockhart and Lutz	1977	Vertebrae	White Sucker (*Catostomus commersoni*)
Longinelli and Nuti	1973	Vertebrae and teeth	Blue Antimora (*Antimora rostrata*), Pacific Grenadier, Blue Shark, etc.
Miller et al.	2010	Operculum	Pupfish (*Orestias ispi)*
Miller et al.	1992	Operculum	White Sucker (*Catostomus commersoni*)
Moreau et al.	1983	Operculum	Brook Trout (*Salvelinus fontinalis*)
Mugiya and Tanaka	1995	Rib	Goldfish (*Carassius auratus*)
Mulligan et al.	1983	Operculum and vertebrae	Sockeye Salmon (*Oncorhynchus nerka*)
Oliveira et al.	2011	Unspecified bones	Atlantic Cod (*Gadus morhua*) and Saithe (*Pollachius virens*)
Rosenthal	1963	Vertebrae	Guppy (*Poecilia reticulate*), Zebra fish (*Danio rerio*), Cloud mountain fish (Tanichthys)
Roy and Lall	2006	Vertebrae	Haddock (*Melanogrammus aeglefinus*)
Roy and Lall	2003	Vertebrae and operculum	Haddock (*Melanogrammus aeglefinus*)
Roy et al.	2002	Vertebrae	Haddock (*Melanogrammus aeglefinus*)
Schoeninger and Deniro	1984	Vertebrae	Many fish species - diadramous, reef, and fresh
Sholtodouglas	1991	Bone collagen	South African Anchovy *(Engraulis capensis)* and Redeye (*Etrumeus whiteheadi)*
Stripp et al.	1990	Unspecified bones	White Sucker (*Catostomus commersoni*) and Yellow Perch (*Perca flavescens*)
Szpak	2011	Bone collagen	Many fish species - diadramous, reef, and fresh
Torz and Nedzarek	2013	Operculum	European Perch (*Perca fluviatilis*)
Weiss and Watabe	1977	All bones and otoliths	Sunfish (*Lepomis macrochirus*) and Golden Shiner (*Notemigonus crysoleucas*)
Yamada and Mulligan	1987	Vertebrae	Sockeye Salmon (*Oncorhynchus nerka*)
Yamada et al.	1987	Vertebrae	Sockeye Salmon (*Oncorhynchus nerka*)
Yamada et al.	2002	Vertebrae	Japanese Eel (*Anguilla japonica*)
Zazzo	2006	Vertebrae, teeth	Sockeye Salmon (*Oncorhynchus nerka*)

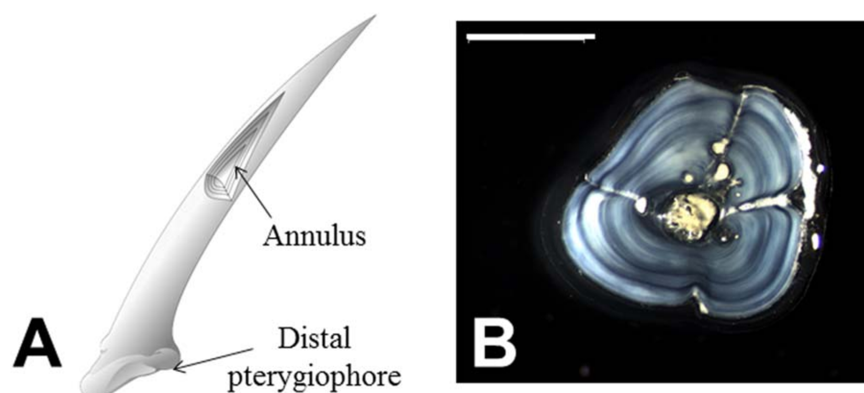

Fig. 3. Anatomical diagram (panel **A**) and cross-sectional photograph (panel **B**) of a fin spine. Scale bar in panel **B** represents 1000 μm. Visible rings in the cross-sectional photograph represent annuli. Sizes and annuli clarity vary by species and study location.

muscle tissue of these two species for Zn, Se, Rb, Cd, As, and Hg. Muscle tissue had higher concentrations of these trace metals, which was attributed to bioaccumulation in muscle.

Stable isotope analysis

SIA of fin spines, while feasible, is rare, and is limited to analyses within the mineral matrix. As mentioned above, Smith (2010) investigated the environmental histories of Channel Catfish (*Ictalurus punctatus*), Blue Catfish (*Ictalurus furcatus*), and Flathead Catfish (*Pylodictis olivarus*), and were able to use δ^{13}C and δ^{18}O to classify individuals to their capture locations. They indicated fin spine chemistry has the potential to be used in areas where geographic differences in water chemistry exist to determine relative recruitment contributions of different geographic areas, to investigate stock mixing, and to characterize movement and dispersal patterns. To our knowledge, the organic matrix in fin spines has not been tested via SIA.

Advantages and considerations

While some other structures are more widely used for TEA and SIA, use of fin spines is advantageous as a non-lethal, minimally invasive sampling method. This makes using fin spines attractive when the fish cannot be sacrificed for their otoliths such as with endangered species (such as rare or threatened) or those of management concern (such as brood stock, protected, or commercially valuable whole fish). In addition, the use of fin spines is useful in species when otoliths are irregularly shaped and annuli are unclear for aging, such as in sturgeons (Arai et al. 2002).

Another factor that must be considered when using fin spines for TEA and SIA is the possibility of contamination, elemental exchange and/or solubilization during storage, removal, and handling that can confound the results of microchemical studies or introduce excess noise to the data. However, Davies (2011) showed that washing fin spines in ultrapure 5% Nitric Acid can effectively remove all external contaminants.

Vascularization and resorption of fin spines may limit their ability to be used in chronological microchemical analyses. As a fish grows, vascularization and resorption begins at the innermost layer of the spine and then proceeds outward over time. The destruction of the interior of the fin spine can obscure and destroy increments of the spine corresponding to the early life history of the individual. Last, Smith (2010) stipulated that there must be a temporal persistence of geographically based differences in water chemistry values in order to effectively characterize a fish's environmental history over time, for example strontium values in the tributaries of the Mississippi river (e.g., Phelps et al. 2012).

Fin rays (23–29% calcified)

Structure and physiology

As in the case of endoskeletal bones, soft fin rays (Fig. 4) have both mineral and organic components. Fin rays are thought to derive from scales or scale-like structures (Lee et al. 2013). The organic matrix of soft fin rays is similar to that of scales and is more robust (by mass) relative to endoskeletal bone or hard fin rays (spines; Mahamid et al. 2010). The organic component in fin rays is higher than that of endoskeletal bone, constituting about 40% by weight, and the organic component consists primarily of type-I collagen (Mahamid et al. 2008, 2010). Despite differences in composition to total mass, the organic and inorganic materials in fin rays are the same as corresponding materials in endoskeletal bones (Mahamid et al. 2010).

Fin rays have a unique growth pattern relative to endoskeletal bones. Osteogenesis occurs in the same manner as in bones, except new layers in fin rays encapsulate old ones, so that a cross section of the ray reveals annuli with the oldest layers at the core (i.e., hatching/birth) and the newest layers at the outer edge (Beamish and Chilton 1977; Mills and Beamish 1980; Rien and Beamesderfer 1994; Rossiter et al. 1995). Fin rays grow in an additive, incremental manner as the existing segments cannot elongate. Instead, new layers

are added over the top of older layers. Upon experimental removal or loss due to other injuries, fin rays exhibit regenerative properties, allowing fish to re-grow damaged fins (Nabrit 1929; Goss and Stagg 1957). Indeed, new osteoblasts are generated after fin-ray excision to re-form the structure and ultimately restore the function of the ray (Singh et al. 2012). This capability is particularly useful to researchers who study life histories of endangered fishes or other fishes of management concern. Fin-ray removal is a nonlethal technique that does not affect growth or survival (Zymonas and Mcmahon 2006).

Fin rays are anatomically similar to fin spines. Vascularization and resorption processes occur in fin rays as in fin spines, although with different frequencies. Vascularization occurs at the center of fin rays, thus resorption processes are most prevalent at the core of fin rays (Beamish and Chilton 1977; Beamish 1981). However, resorption does not seem to affect fish with slower metabolisms, such as fishes with smaller home ranges. Indeed, aging studies have documented strong correspondences between the fin rays and otoliths of reef fishes (which tend to have relatively high site fidelity), including serranids (e.g., Murie et al. 2009), haemulids (e.g., Murie and Parkyn 2005), and lepisosteids (e.g., Glass et al. 2011). In contrast, fishes with higher metabolic demands and larger individual ranges (e.g., billfishes and tunas) are much more difficult to age due to the effects of resorption (e.g., Beamish 1981). In comparison with other calcified structures in the body, fin rays appear to have little to no tissue turnover. This may be due, in part, to their pattern of additive growth that encapsulates old layers instead of the continued growth of a single layer.

Both trace elements and stable isotopes are incorporated into the molecular matrix of fin rays during osteogenesis (Lida et al. 2014). Trace-element molecules are absorbed into the blood from either the ambient environment, the diet, or both, and then attach to larger calcium-phosphate molecules (Mahamid et al. 2008). Amorphous nanospheres of calcium phosphate are dispersed throughout the blood stream until they transform into platelets of crystalline apatite within the proteinaceous matrix (Mahamid et al. 2010). Because the fin ray has substantial organic and inorganic components, both TEA and SIA can be performed to infer life-history characteristics of individual fishes. Trace elements in fin rays typically occur in concentrations that are comparable to other structures (Clarke et al. 2007). In contrast, stable-isotope values tend to be enriched in fin rays compared to muscle tissue, likely as a result of inclusion of the inorganic matrix (Graham et al. 2013).

Trace-element analysis

TEA has traditionally focused on migratory behavior and habitat use in individual fishes (Phelps et al. 2012). Notably, the analysis of divalent ions over time in fin rays has been of interest to distinguish between saltwater and freshwater environments (Arai et al. 2002; Allen et al. 2009; Jaric et al. 2012). In this way, researchers have been able to detect diadromous migrations using the presence of elements such as Ba and Sr, which are used as proxies for fresh water (Tzeng et al. 1997; Guay and Falkner 1998). These indicators have been used in sturgeon to infer anadromous migrations 30 yr after they occurred (Allen et al. 2009). Sturgeon have also been traced back to their rivers of origin by studies seeking to identify juvenile habitats for conservation purposes (Jaric et al. 2012). Similar "river-of-origin" studies via TEA of fin rays have been applied to the nursery habitats of esocids (Rude et al. 2014), centrarchids (Smith and Whitledge 2010), and catastomids (Wolff et al. 2013). To test the efficacy of this technique, controlled experiments have documented the conservation of trace elements in fin rays via "elemental tagging" (e.g., Smith and Whitledge 2011a; Woodcock et al. 2013). Applications also include the identification of wild vs. nursery-hatched individuals (Wolff et al. 2013), stock contributions to fisheries (Rude et al. 2014), and the identification of effective nursery habitat (sensu Beck et al. 2001; Dahlgren et al. 2006) for endangered species and those of management concern (Smith and Whitledge 2011b; Phelps et al. 2012).

Stable isotope analysis

Fin clips can be used for bulk SIA to infer movement and dietary patterns in the same manner as bulk SIA of muscle tissue (Sanderson et al. 2009). SIA of fin clips may be based on the membrane that connects fin rays, on the dorsal tip of one or more fin rays, or on both types of tissues (Johnsen and Ugedal 1988). Relatively little effort has been applied to temporal SIA of fin rays; we are aware of only one study that analyzed a chronology of stable isotope values over time in fin rays to discern lifetime temporal patterns of individual fish (Tzadik et al. 2015). This study suggests stable-isotope chronologies in the proteinaceous matrix is conserved over time and can be used to characterize geographic and trophic histories.

Advantages and considerations

The use of fin rays for both TEA and SIA is a non-lethal alternative to otolith analysis that does not negatively affect growth or survival (Zymonas and McMahon 2006) while providing a conserved chemical matrix that is recorded over the entire lifespan of an individual. The method is well suited for the study of endangered fishes or those of management concern. The use of fin rays in aging studies is well established, while the fields of TEA and SIA on fin rays have had important implications to fisheries science by contributing to the understanding of individual origins, migrations, movements, diet, and stock structure, as discussed above. However, in fishes with high metabolisms, resorption may limit the use of fin rays for chemical archiving purposes. In active fishes such as Bluefin Tuna, fin rays on fish as young as 3 yr old become resorbed (W. Golet pers. comm.). Edge-crowding of annuli is also a concern in older fishes that

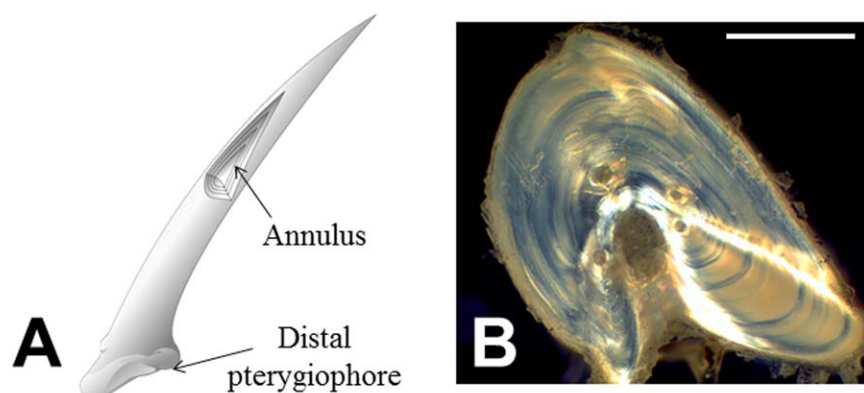

Fig. 4. Anatomical diagram (panel **A**) and cross-sectional photograph (panel **B**) of a fin ray. Scale bar in panel **B** represents 1000 μm. Visible rings in the cross-sectional photograph represent annuli. Sizes and annuli clarity vary by species and study location.

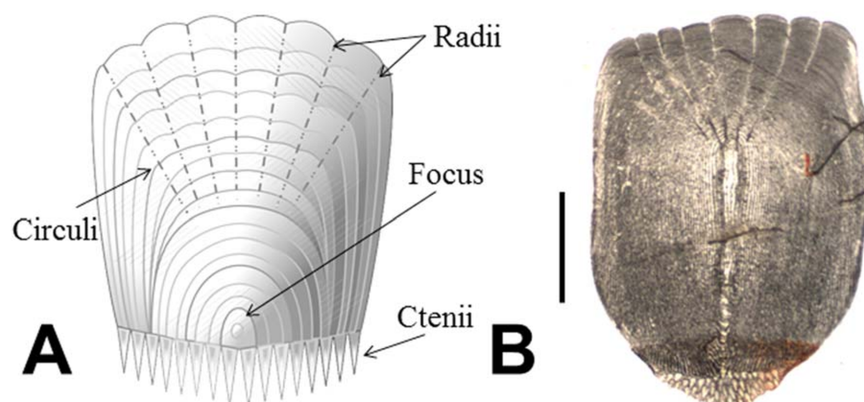

Fig. 5. Anatomical diagram (panel **A**) and photograph (panel **B**) of a ctenoid scale. Scale bar in panel **B** represents 2000 μm. Sizes and circuli clarity vary by species and study location.

have previously been aged via fin-ray analysis. As in other banded structures, fin rays become more difficult to age in older individuals as the annuli become narrower toward the edges of cross sections. Samples from small individuals or species can be difficult to prepare.

Scales (<20% calcified)

Structure and physiology

For over a century, scales (Fig. 5) have been used as recorders of age, growth, and life history in fishes. Although different morphologies exist, the highly derived elasmoid scale is the most common in modern fish species (Sire and Akimenko 2004). Elasmoid scales consist of two distinct layers: an osseous upper layer and an underlying proteinaceous basal layer (Fouda 1979). The osseous layer is highly calcified and contains collagen fibrils mixed with needle-shaped crystals of hydroxyapatite (Schönbörner et al. 1979; Sauer and Watabe 1984). The basal layer is composed of bundles of partially mineralized collagen arranged into lamellar sheets, forming a plywood like structure known as elasmodin (Sire and Akimenko 2004; Hutchinson and Trueman 2006). After initial deposition, which typically follows larval metamorphosis, scales grow incrementally in proportion to somatic growth (Fisher and Pearcy 1990; Sire and Akimenko 2004). Growth cycles of fish are recorded in scale features called "circuli," which are concentric ridges in the osseous layer comparable to otolith rings (Fisher and Pearcy 1990). Elements taken up through the skin, gills, or diets of fishes can make their way to osteoblasts and fibroblasts at the growing edge of scales, where they are deposited into newly formed hydroxyapatite matrix or collagen fibrils (Bagenal et al. 1973; Wells et al. 2000; Hutchinson and Trueman 2006).

Trace-element analysis

Analysis of scale microchemistry using X-ray spectrometry developed in the 1970s, with demonstrations that scales could be marked by increases in [Sr] in the ambient water (Bagenal et al. 1973) and also by modifying the diet of captive fish (Yamada et al. 1979). Sr has been repeatedly

measured in scales due to its potential to record migrations of diadromous species (notably salmonids) between freshwater and marine environments (e.g., Wells et al. 2003a; Courtemanche et al. 2006; Araya et al. 2014). After Wang et al. (1994) reported the ability of LA-ICP-MS to spatially resolve elemental distributions in teleost scales, the technique was adopted in subsequent microchemical studies (e.g., Farrell et al. 2000; Holá et al. 2009; Campbell et al. 2015). Recent technological advancements include the coupling of laser ablation and atomic fluorescence spectrophotometry to analyze scale mercury concentrations at fine spatial resolution (Beaudin et al. 2010) and to create depth profiles for trace elements within scales using modified LA-ICP-MS (Holá et al. 2011).

The relationship between the microchemical components of scales and other calcified structures, particularly otoliths, is inconsistent across studies. While some findings suggest that scale Sr : Ca correlates with concentrations in ambient water and otoliths (Wells et al. 2003a; Campbell et al. 2015), others have found no such relationship (Clarke et al. 2007). Complicating the matter, Weakfish (*Cynoscion regalis*) scale and otolith Sr : Ca were found to be dissimilar in 1-yr-old fish but comparable at age two, possibly due to elemental remobilization in older individuals (Wells et al. 2003b). Courtemanche et al. (2006) noted that even the subset of scales chosen for analysis affected the detection of a Sr : Ca correlation between otoliths and scales, and suggested scales may become an unreliable indicator of Sr once diadromous fish encounter the marine environment. Several studies by Wells et al. (2000, 2003a,b) have measured depositional patterns of Ba, Mn, and Mg in teleost scales and otoliths. In some cases, scale and otolith Ba : Ca tracked chemical changes in ambient water. However, the effect was not consistent, possibly due to the presence of protein-based Ba or remobilization of scale material in some species. Additionally, despite a significant correlation in Ba : Ca, Ba concentrations in the scales of Spot (*Leiostomus xanthurus*) were six times higher than in otoliths, indicating differences in depositional processes or rates between the two calcified structures. Wells et al. (2000, 2003a,b) also showed inconsistent relationships between Mn : Ca and Mg : Ca in otoliths and scales. Finally, comparing concentrations of trace elements in calcified structures of Arctic Grayling (*Thymallus articus*), Clarke et al. (2007) reported correlations of Sr, Ba, and Mn in otoliths, fin rays, and the environment, but not in scales. Therefore, caution must be exercised in performing microchemical analysis on scales in place of otoliths or other bony structures, as patterns of calcification and deposition may vary on an element- and species-wise basis.

Stable isotope analysis

Stable isotope analysis of scales was uncommon until the 2000s (Estep and Vigg 1985; Wainright et al. 1993 are notable exceptions), but has emerged as a potentially powerful tool to study teleost-fish ecology. The most commonly analyzed stable isotopes (δ^{13}C and δ^{15}N) are primarily deposited into basal-layer collagen, but can also become incorporated into the hydroxyapatite matrix (Hutchinson and Trueman 2006; Ventura and Jeppesen 2010). Depending on the research objective, δ^{13}C and δ^{15}N has been measured in whole scales (Johnson and Schindler 2012), at targeted circuli to isolate life-history events (Dixon et al. 2015), and at the scale's growing edge to describe the most recent activity of an individual fish (Hammond and Savage 2009). Ramsay et al. (2012) found that identification of the site-of-origin of Brown Trout (*Salmo trutta*) improved from 88% to 93% when δ^{13}C and δ^{15}N were included as biogeochemical markers in addition to trace elements. Although measurement of δ^{13}C and δ^{15}N is more commonly used, δ^{34}S in scales has also been used to discriminate the diet and movement of freshwater fishes (Trembaczowski and Niezgoda 2011).

Scale TEA and SIA have been applied to a variety of ecological questions. Scale microchemistry is used to help elucidate patterns of movement and migration in estuarine and freshwater species (e.g., Kennedy et al. 2005; MacKenzie et al. 2012; Araya et al. 2014). Refining decades of morphometric research, Campbell et al. (2015) found that patterns of circuli spacing, commonly used as indicators of habitat transitions, often do not align with diagnostic changes in scale microchemistry. Also, researchers have used scale stable isotopes to describe long-term foraging shifts within species (Pruell et al. 2003; Johnson and Schindler 2012), differences in resource use between sympatric freshwater species (Bašić and Britton 2014), and information about ecosystem-scale changes in food webs (Wainright et al. 1993). Larger-scale studies have drawn connections between information archived in scales to climatic oscillations, changes in the planktonic community, and associated variation in salmonid mortality (Satterfield and Finney 2002; Trueman et al. 2012). Scale microchemistry can also inform aquaculture, conservation, and environmental science research. For example, researchers have used diet-induced Sr tags (i.e., diet enriched with Sr^{89}) and differences in scale Mn concentrations to distinguish farmed and wild salmon (Yamada et al. 1979; Adey et al. 2009). Furthermore, by recording concentrations of dissolved nitrogen, heavy metals, and pollutants, scales can provide historical information on water quality and human health hazards (Rashed 2001; Roussel et al. 2014; Lake et al. 2006).

Advantages and considerations

As a subject for microchemical analysis, scales offer a few distinct advantages over other calcified structures in teleost fish. First, scales can be collected non-lethally, an especially important consideration when sampling endangered or managed species (Cano-Rocabayera et al. 2014). For example, Woodcock and Walther (2014) recently used TEA and SIA on the scales of Atlantic Tarpon (*Megalops atlanticus*) to study

the migrations and diet of this regionally protected species. Scale removal is the least invasive option for collecting calcified tissue from live fish, and this approach often requires less intensive processing before analysis (Adey et al. 2009). Also, since comparable scales are available on each fish, multiple replicates can be collected from the same individual. Repeating measurements on multiple scales from one fish can be used to assess the uniformity of elemental deposition into biological tissue (Courtemanche et al. 2006). Scales may incorporate some trace elements (e.g., Sr, Ba, lanthanides) more readily than other calcified structures (Yamada et al. 1979; Wells et al. 2000) and can serve as useful bioindicators of metals in the environment (Farrell et al. 2000; Rashed 2001). Finally, fish scales are frequently available in historical (preserved) archives and may also be preserved in sediment records, allowing for studies of environmental, ecological, and climatological change over multi-decadal periods (e.g., Wainright et al. 1993; Satterfield and Finney 2002, Johnson and Schindler 2012). Syväranta et al. (2008) verified that after species-specific corrections, stable isotopes in preserved scales are directly comparable to fish-muscle isotopes, and are thus appropriate for ecological interpretation via comparison with muscle isotopes.

Depending on the application, varying levels of mechanical and chemical processing are needed to prepare scales for analysis. To remove mucus and other biological materials, scales are typically soaked in de-ionized water and might require scraping with a scalpel or abrasive pad (e.g., Kennedy et al. 2005; Courtemanche et al. 2006). Also, the mineralized material in fish scales can contain high amounts of calcium carbonate derived from dissolved inorganic carbon (Ventura and Jeppesen 2010), potentially skewing analyses of $\delta^{13}C$ intended to measure organic, trophically derived carbon. To remove the inorganic component, scales are often washed in acid, typically 1.2 M HCl for 2 min (e.g., Syväranta et al. 2008; Torniainen et al. 2014). The effects of the acid-washing technique are highly variable, as measured shifts in post-acidification scale isotope values have been differentially reported as statistically non-significant (e.g., Sinnatamby et al. 2007), significant but small (e.g., Ventura and Jeppesen 2010), and significant and large enough to affect interpretation (e.g., Perga and Gerdeaux 2003). The isotopic effect of acidification is driven by the mineral content of the scale, which is determined by species-dependent depositional processes and environmental variables, particularly CO_2 concentrations (Ventura and Jeppesen 2010). The average acidification effect of six freshwater species was $+0.18‰$ $\delta^{13}C$ and $-0.21‰$ $\delta^{15}N$ (Ventura and Jeppesen 2010), but Perga and Gerdeaux (2003) reported elevations of both isotopic values as high as $+1.3‰$. Because scales do not contain inorganic nitrogen, changes in $\delta^{15}N$ following acidification can likely be attributed to alteration and loss of biogenic proteins within the hydroxyapatite matrix (Perga and Gerdeaux 2003). Due to the inconsistency in scale responses, Sinnatamby et al. (2007) cautioned against general application of acidification and encouraged a case-by-case approach based on species and research goals.

Several physiological features of scales must be considered before performing TEA or SIA to avoid inappropriate inferences. Importantly, scales can regenerate after loss, and thus contain material deposited over an incomplete subset of a fish's lifespan (Neave 1940; Hammond and Savage 2009). Even non-regenerated scales cannot reliably describe early life history, as most larval teleosts lack scales (Sire and Akimenko 2004). Although regenerated scales can be identified by an absence of circuli and avoided (Fouda 1979), non-regenerated scales may not always be available for analysis (Wells et al. 2000). Regenerated scales may be useful, however, in interpreting microchemistry over a recent, short timespan if the age of regenerated material is known (Hammond and Savage 2009). While calcified material in scales is often assumed to be metabolically inert (Yamada et al. 1979; Johnson and Schindler 2012), it has been repeatedly demonstrated that scales can undergo biological, mechanical, and chemical erosion (e.g., Bilton and Robins 1971; Khanna et al. 2007). For example, post-depositional ionic exchange for fish migrating between chemically distinct environments, marine to fresh water, (Seeley et al. 2015), or during periods of physiological stress or extreme environmental conditions (particularly low Ca), resorption of calcium and magnesium from the scales can occur, altering the composition of previously deposited material (e.g., Mugiya and Watabe 1977; Metz et al. 2014). Such reworking fundamentally violates the assumption of conservative preservation required to recreate chemical chronologies from incrementally calcified structures.

Any description of diet using scale stable isotopes should include a correction factor, drawn empirically or from the literature, to make measurements directly comparable to muscle tissue, the most commonly used material by fish-isotope ecologists. The presence of ^{13}C-depleted lipids in muscle tissue contributes to a relative elevation of $\delta^{13}C$ in scales, which have low fat contents (Estep and Vigg 1985; Pinnegar and Polunin 1999). Various authors have reported species-specific differences between scale and muscle $\delta^{13}C$ ranging from $+0.2$ to $4.0‰$ (Satterfield and Finney 2002; Perga and Gerdeaux 2003; Kelly et al. 2006). Conversely, $\delta^{15}N$ is generally slightly lower in scales than muscle tissue, with a reported maximum difference of $-1.5‰$ (Perga and Gerdeaux 2003; Kelly et al. 2006). Even within a species, variation may exist in the isotopic difference measured between scale and muscle tissue, based on population-level differences and differences in analytical method (Kelly et al. 2006). Once adjusted, however, scale stable isotope abundances can correlate well with muscle, allowing scales to serve as a nonlethal proxy for muscle (Satterfield and Finney 2002; Syväranta et al. 2008).

Hutchinson and Trueman (2006) described a major limitation of SIA on fish scale collagen, demonstrating that

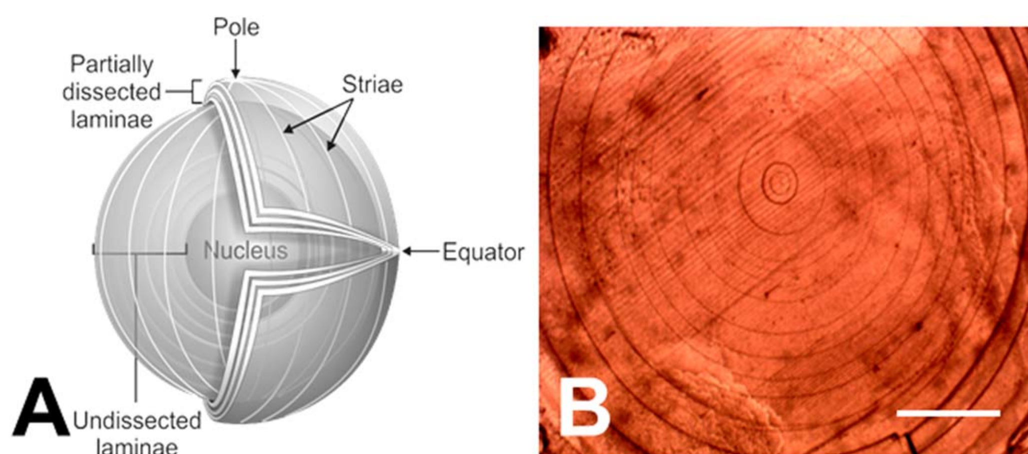

Fig. 6. Anatomical diagram (panel **A**) and cross-sectional photograph (panel **B**) of an eye lens. Scale bar in panel **B** represents 2000 μm. Visible rings in the cross-sectional photograph do not represent annuli. Sizes vary by species and study location.

measurements made in the "juvenile" portion of the scale could be contaminated by subsequently deposited material. Through a process called underplating, newly deposited collagen extends along the margin between the osseous and basal layer away from the scale's growing end. Thus, a cross section taken at a particular circulus will contain collagen created more recently than the targeted life history period. The study found that expected differences in δ^{15}N between juvenile and adult salmon were dampened by up to 75% due to incorporation of post-juvenile collagen. Additionally, interpretations of diet based on whole scale analysis may be skewed by the disproportionate abundance of material created at later life stages. The authors conclude that neither chronological isotopic measurements of scale collagen nor whole-scale analysis are possible without risking bias and subsequent misinterpretation. However, Woodcock and Walther (2014) suggest that the derivation of a correction factor may be possible based on the level of underplating bias, to account for this limitation. Due to the chemical variability and the availability of fish scales, many samples are often used to increase statistical power and inference, albeit at a substantial increase in costs.

Eye lenses (0% calcified)

Structure and physiology

Eye lenses (Fig. 6) are included in this review as a recently introduced alternative structure that can be used in TEA and SIA. This tissue has been studied extensively with respect to formation and function, but the potential for using eye lenses for life-history reconstruction is undergoing initial evaluation.

During the gastrula stage of embryonic development, eye lenses develop as surface ectoderm that differentiates into two cell types, lens epithelial cells and lens fiber cells (Dahm et al. 2007; Wride 2011). Lens epithelial cells form a single layer (lens epithelium) on the anterior (lateral) surface of the lens (but not on the medial surface); the lens epithelium is adjacent to underlying lens fiber cells (Nicol 1989). Lens epithelial cells secrete a protective, transparent layer (the lens capsule) that surrounds the entire lens surface; the lens capsule is a permeable membrane that allows aqueous humor to enter and leave the lens (Berman 1991). The lens epithelium also produces the protein crystallin that is the primary constituent of lens fiber cells (Nicol 1989; Berman 1991; Horwitz 2003). The different types of crystallin that exist within vertebrate eye lenses (e.g., α-, β-, and γ-crystallin) are not directly synthesized; they instead result from post-translational modification, and vary in their degree of water solubility (Berman 1991; Horwitz 2003). As the fish grows, new lens fiber cells are produced at the interface between the lens epithelium and the adjacent (underlying) lens cortex, which consists of existing lens fiber cells. Newly forming lens fiber cells initially possess a full complement of DNA and organelles, and maintain capabilities for DNA repair and protein synthesis (Berman 1991). Lens fiber cells have remarkably uniform dimensions and shapes (they are hexagonal in cross-section) and accumulate in well-ordered, overlapping geometries that produce distinct layers (laminae) that are multicellular in cross-section (i.e., multiple lens fiber cells contribute to total laminar thickness).

Newly forming lens fiber cells become highly elongated before they take their final position within the geometric microstructure of the growing lens lamina. After taking position, lens fiber cells undergo a form of "attenuated" apoptosis, in which DNA, mitochondria, and all other organelles are expelled, leaving behind the cell membrane (Nicol 1989; Dahm etal. 2007; Vihtelic 2008; Wride 2011). This is different from "classical" apoptosis, where everything, including the cell membrane, degrades. At this point, no new protein synthesis is possible and the cells are considered

metabolically inert. Attenuated apoptosis, when coupled with the geometric cellular shape and precisely structured cellular arrangement, creates laminae that minimize the absorbance and scattering of light (Berman 1991). Laminae are added to the outside of the lens much like the layers of an onion (Nicol 1989; Dahm et al. 2007; Wride 2011). The outer, more water-soluble laminae (in the outer lens cortex) are younger than laminae at the center of the lens (the lens nucleus), which become hardened and much less water-soluble with age.

Trace-element analysis

Trace elements in eye lenses are studied more than stable isotopes, but might not be as useful due to metal-bearing humor that continues to perfuse into and out of the lens (particularly in the outer cortex) after de novo protein synthesis has ceased. Metals likely bond to the sulfhydryl groups in crystallin instead of substituting for Ca^{2+} (as in otoliths) because Ca is not usually abundant except when cataracts are present (Berman 1991). Although measurable trace elements in eye lenses include Ba, Co, Cu, Fe, Hg, Mn, Pb, Rb, and Sr, elements that are widely studied in otoliths (for example: Cu, Mg, and Fe) are metabolically regulated within the eye and thus concentrations will be altered in newly forming (pre-apoptotic) lens fiber cells and also within the aqueous humor that perfuses the lens (Kuck 1975; Dove and Kingsford 1998). Dove and Kingsford (1998) attribute physiological regulation to the uniformity of Mn in eye lenses, which contrasts with the spatial variability present in the otoliths.

Eye lenses appear to have trace elemental fingerprints. The elemental fingerprint for eye lenses is distinct from that of otoliths, scales, and spines (Dove and Kingsford 1998; Gillanders 2001). Dove and Kingsford (1998) demonstrated environmental spatial correlation when compared with otoliths, while Kingsford and Gillanders (2000) observed a correlation with water depth. It seems Hg and Pb in eye lenses, both of which are often below detection limits in otoliths, can complement trace element data from otoliths (Dove and Kingsford 1998; Kingsford and Gillanders 2000). This area of study remains relatively unexplored.

Stable isotope analysis

The large amount of protein (primarily crystallin) within eye lenses makes them suitable for analysis of $\delta^{13}C$ and $\delta^{15}N$ (Wallace et al. 2014); crystallin comprises up to 50% of eye lens mass (de Jong 1981). Wallace et al. (2014) evaluated eye lenses as long-term stable isotope recorders in fishes, and examined variations among species and among individuals. The observed magnitude of isotopic variation matched the magnitude of spatial variability in isotopic baselines reported by Radabaugh et al. (2013). In addition, isoscape-based interpretations of isotopic histories from eye lenses were consistent with known life-history trends. Other exploratory work has been conducted on squid lenses; squid have a complex eye much like vertebrates (Parry 2003; Hunsicker et al. 2010). Wallace et al. (2014) also called for further experimentation with respect to aging eye lenses as well as conducting compound specific isotope analyses.

Advantages and considerations

Eye lenses appear to provide long-term records for $\delta^{13}C$ and $\delta^{15}N$. When used in conjunction with other calcified structures, researchers can couple eye-lens TEA with SIA to study lifelong trends in the geographic and trophic histories of individual fishes. Eye lenses differ from bone tissue in having no known tissue turnover or reworking, and differ from otoliths in providing nitrogen-rich material for $\delta^{15}N$ analysis. Temporal resolution of eye-lens isotope data appears to be seasonal or possibly better (Wallace et al. 2014), whereas subdividing otolith annuli after age-0 requires models or untested assumptions that often vary among year classes (e.g., Campana 1996). Present technologies require micromilling to obtain isotope samples from otoliths, whereas eye-lenses can be delaminated for stable-isotope analysis using a pair of forceps and a dissecting microscope (Wallace et al. 2014). Thus, the method can be used in remote areas and at locations that do not have access to micromills. Lens delamination is a meticulous process that requires considerable manual dexterity, but it is nevertheless a rapid method relative to micromilling. In addition, one lens can be coarsely subdivided to identify general lifetime trends ("screening" method, Wallace et al. 2014), and the second lens can later be examined in more detail if needed ("high resolution" method, Wallace et al. 2014), reducing both the time and cost required to meet research objectives.

The present drawbacks to eye-lens use for TEA or SIA are aging the sampled laminae or groups of laminae, and the lethal nature of the sampling process. The radial midpoint of each removed lamina can be readily measured using ocular micrometers or analysis of digital photographs, and these midpoints can be related to corresponding points on an otolith or other source of known-age microstructure (Campana 1999), thus providing age information for eye-lens data.

Future directions

Since the time of Aristotle, anatomical structures have been used to discern life history patterns of fishes. Rapid development of microchemical analyses over the last 40 yr has greatly expanded our abilities (Table 1). Not only has the technology advanced, allowing more precise analysis of chemical properties and isotopic ratios, but we are discovering that various structures found in fishes can be used to inform research about development, ecology, behavior, management, and conservation. In this regard, it is clear that alternative structures that are rich in structural or optical proteins can be at least as useful as mineral-rich structures. Mineral-rich structures, if vascularized, are subject to turnover and reworking that disrupts conservation of elemental

and isotopic records. We have identified seven activities that would advance the use of alternative structures:

1. Combine multiple structures for TEA and SIA and develop a means for evaluating the results. Events, patterns, or relationships identified through TEA and SIA can be further evaluated using other structures, such as teeth, baleen, and scutes, and growth rate could be used as an indicator of significance (e.g., Fraser-Lee method, Campana 1999).
2. Compare different groups of fishes. Comparisons could be made for freshwater vs. marine, deep-sea vs. neritic, herbivores vs. predators, tropical vs. temperate vs. polar, ontogenetic stages, and other groups.
3. Identify turnover rates of structures to allow ready determination of whether a given structure is appropriate for meeting specific research goals.
4. Search for alternative analytes that potentially document movement, diet, physiological events, or environmental conditions. For example, anion (e.g., Cl^- and Fl^-) substitution of the hydroxyl group (OH^-) is relatively common and should be given consideration (Bogdanova et al. 1998; Trischitta et al. 1998).
5. Document the effects of dynamic environment conditions on incorporation of isotopes and trace elements into these structures. External environmental conditions and the internal milieu can affect incorporation through stress (Kalish 1992; Fuller et al. 2005).
6. Develop element maps and isoscapes for comparison with analytes of interest (Bowen 2010). Background values change relatively slowly in the terrestrial environment, but dynamic spatial heterogeneity (lack of spatial stationarity) is an area of concern in marine systems for both TEA and SIA (Deegan and Garritt 1997; Carlier et al. 2009). While background levels may change in some marine systems on the order of days to weeks, seasonal trends in other areas are detectable (Graham et al. 2010). These trends can in turn be used to create element maps and isoscapes in the marine environment for specific time periods (e.g., Radabaugh et al. 2013).
7. Continue to search for new, alternative structures that may harbor conserved analyte records.

Glossary

Acellular – A structure that lacks cells. In bone, this specifically refers to the lack of osteocytes and osteoblasts.

Annuli – Ring-like features deposited yearly on calcified structures of fishes. Typically used for aging.

Aqueous humor – Fluid in the eyes that is positioned between the cornea and the crystalline lens. This fluid maintains the pressure within the eye and provides nutrition for the cornea and the lens, which lack a blood supply.

Basal layer – The base layer of skin of scales which provides the base for all other layers.

Bioapatite – Synonymous with hydroxyapatite, crystalline calcium hydroxyapatite, hydroxyl apatite, carbonated apatite, and dahlite. These terms refer to the inorganic matrix of bone and many other calcified structures. The structure is formed of calcium cations, phosphate and a hydroxide anion in the chemical formula $Ca_5(PO_4)_3(OH)$, which typically exists as $Ca_{10}(PO_4)_6(OH)_2$.

Bone remodeling – Following bone resorption, secondary bone can be deposited in eroded areas where antecedent bone has been resorbed due to osteoclast activity.

Carbonated apatite – See "bioapatite."

Circuli – Similar to annuli, although not necessarily yearly depositions. Typically used in relation to scales.

Collagen – The primary structural protein in the majority of bone and other tissues in fishes. There are 28 known "types" of collagen, but there are five that are much more common than the rest.

Crystallin – A globulin-class protein that is present in the eye lens.

Crystalline calcium hydroxyapatite – See "bioapatite."

Dahlite – See "bioapatite."

Dermal Bone – Also known as membrane bone. A bony structure derived from intramembranous ossification forming components of the vertebrate skeleton.

Edge-crowding – The compression of annuli toward the outer layer of a calcified structure. Typically problematic in particular species over others and frequent in otoliths, endoskeleton, vertebrae, fin spines, fin rays, and scales.

Elasmodin – Previously called "isopedin." A layer within the scale that consists of several stratum of collagen fibrils organized into a plywood-like structure.

Fibroblasts – A cell that synthesizes collagen. Fibroblasts are similar to osteoblasts in bone, but they are specifically allocated to proteinaceous matrices, specifically collagen.

Hydroxyapatite – See "bioapatite."

Isotope fractionation – The enrichment of one isotope to another, usually within a single element. Enrichment can occur via biological, chemical, or physical processes.

Lamellar – A type of bone that is described by slow growth rates and is composed of successive thin lamellae of tightly packed collagen fibers that change orientation by 90° with each successive lamellae. Also called "secondary bone."

Lens cortex – The tissue that surround the lens nucleus.

Lens epithelial cells – Membranous tissue composed of several layers of cells that cover the eye lens.

Lens fiber cells – Transparent, biconvex layer that separates the posterior chamber and the vitreous body of the eye. The layer refracts light so that it can be focused by the retina.

Mineral phase – In bones and other calcified structures, the mineral phase is the inorganic matrix within the structure. This matrix is made of hydroxyapatite.

Osteoblast – One of the three types of cell involved in bone formation and erosion. The osteoblast synthesizes collagen on the bone surface and aids in mineralization of the organic matrix. Osteoblasts are usually absent from bones in fishes due to withdrawal after done synthesis.

Osteoclast – One of the three types of cell involved in bone formation and erosion. Osteoclasts destroy bone, thus releasing minerals.

Osteocyte – One of the three types of cell involved with bone formation and erosion. Osteocytes are star-shaped cells formed when osteoblasts are surrounded by the organic matrix and involved in regulation of bone resorption. Osteocytes are usually absent from bones in fishes.

Osteogenesis – The formation of bone.

Parallel fiber – A type of bone that is typical of intermediate growth rates and consists of tightly packed collagen fibers with parallel orientation.

Primary bone – New bone that is deposited by osteoblasts where antecedent bone does not exist.

Primary vascular canals – Form during initial osteogenesis and are part of the intrinsic bone vascular network. They are initially filled with fibroblasts, soft connective tissues, and blood vessels.

Pseudo-lamellar – Synonymous with "parallel fiber."

Resorption – The process whereby bone tissue is eroded and absorbed into the ambient blood.

Resorption lacunae – Small irregularities in bone or other calcified tissues such as pits or divots that are actively being resorbed by osteoclasts.

Secondary bone – See "lamellar" bone.

Secondary vascular canals – Formed when osteoclasts resorb bone tissue and the space created is subsequently filled by blood vessels.

Vascularization – Formation of blood vessels; the process of becoming vascularized.

Woven fiber – Typically repaired bone that has collagen fibers with irregular orientations. They are weaker than most other forms of bone.

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Conflict of Interest

None declared.

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