Chapter | eleven

Chemical Composition of Fish Hard Parts as a Natural Marker of Fish Stocks

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CHAPTER OUTLINE

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11.1 PRINCIPLES OF CHEMISTRY APPLICATIONS TO FISH HARD PARTS

11.1.1 Introduction

The chemical composition of calcified structures in fish has been identified as a useful natural marker to identify fish that inhabit different environments. In some cases, this information can be used to infer stock structure. Considerable work has established the utility of otolith chemistry as a natural tag of fish stocks, and there is growing interest in expanding the application of this method to fish spines, rays, scales, and vertebrae. This application depends on geographic variation in water chemistry or other factors that influence the chemistry of the calcified structure, such as temperature, so that fish that inhabit different environments exhibit differences in the chemical composition of calcified structures (Thresher, 1999; Secor et al., 2001; Campana, 2005; Elsdon et al., 2008). It is important to note that differences in the chemical signature of structures do not imply genetic differences. Therefore, the use of this technique in stock discrimination should be considered carefully because a number of assumptions must be met (Thorrold et al., 1998a; Campana et al., 2000; Elsdon et al., 2008).

The application of structure chemistry for the purpose of stock identification requires that the structure is metabolically inert, such that newly deposited material is neither resorbed nor reworked after deposition (Campana and Neilson, 1985). This has been clearly demonstrated for otoliths, and evidence suggests that at least some components of scales, vertebrae, and fin spines and rays are metabolically stable; however, some questions remain (Campana and Thorrold, 2001). Another requirement is that the uptake of elements into the growing structures reflects the physical and chemical environment experienced by the fish at the time of deposition, although we expect physiological regulation will affect the absolute chemical concentrations incorporated into the fish structure (Kalish, 1989; Farrell and Campana, 1996; Sturrock et al., 2012). This type of environmental response has been established for certain trace elements and isotopes preserved within otoliths (e.g., Fowler et al., 1995; Gallahar and Kingsford, 1996; Kennedy et al., 1997; Thorrold et al., 1997a; Secor et al., 2001; Kerr et al., 2007, 2009). This implies that

the otolith concentration of selected elements and isotopes (the "chemical fingerprint") can be used as a biological tag to discriminate among groups of fish that have spent at least part of their lives in different environments (Figure 11.1). However, less empirical support exists regarding how well the chemical composition of vertebrae, scales, and fin spines and rays reflect the growth environment of the fish.

For those elements and isotopes that reflect exposure to the environment, signatures are expected to differ among groups of fish that have experienced

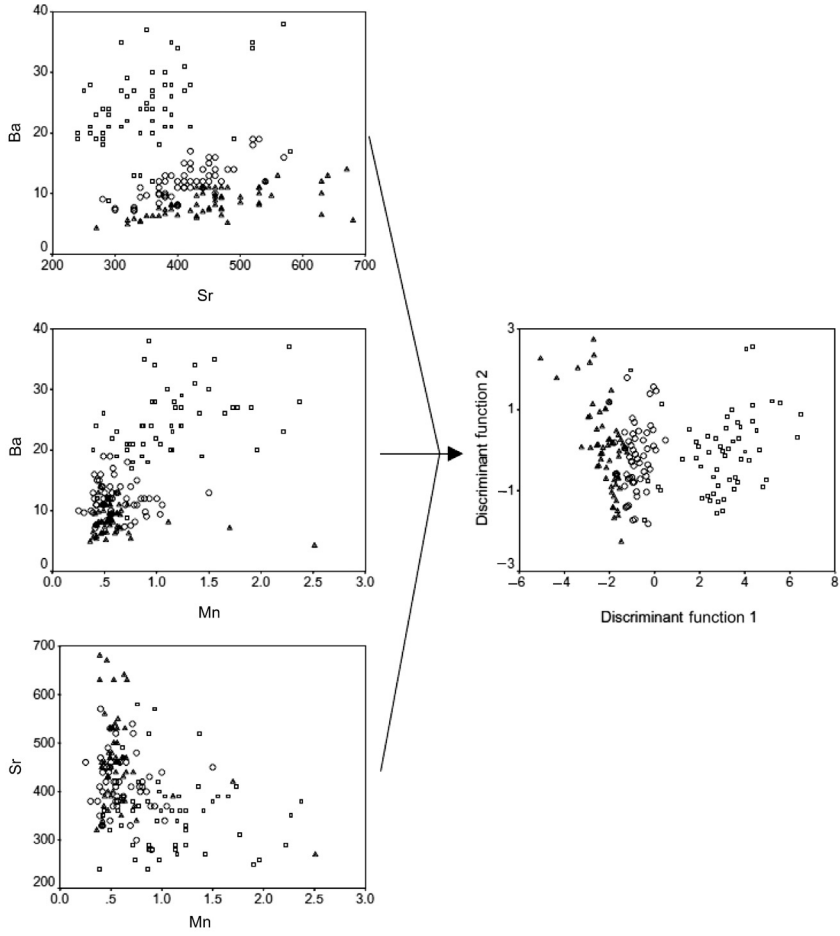

FIGURE 11.1 Example of the preparation of a multivariate otolith elemental fingerprint for shad (*Alosa sapidissima*). The concentrations of three elements (Ba, Mn, and Sr) were measured in otoliths from about 60 shad collected in each of three river systems. When one element is plotted against another (left panels), there are varying degrees of differences among the three rivers (each river is represented by a different symbol). The differences in elemental composition among rivers become more evident when the individual elements are pooled into a multivariate fingerprint (right panel). Full details of the shad study are available in Thorrold et al. (1998a).

different histories (e.g., groups of fish with shared migration history; Secor et al., 2001; Kerr et al., 2009), whether or not the groups come from different populations. Differences in composition cannot be used to infer the length of time that the groups of fish remained separate, because even occasional residency in a different environment can potentially introduce a detectable difference in the chemical composition (Campana, 2005). By corollary, the absence of differences would not necessarily imply that the groups of fish are of common origin (Campana, 2005). Thus, otolith elemental fingerprints can be powerful discriminators of groups when differences exist, but they are of negligible value when differences cannot be detected. Where differences are detected, additional information (e.g., microsatellite DNA; Feyrer et al., 2007) would be required to determine if the groups actually corresponded to stocks or populations. Nevertheless, the presence of different chemical fingerprints among groups of fish of similar age implies different environmental histories. To the extent that populations or stocks of fish inhabit different environments, this signature can then serve as an indicator of stock identity (Campana, 2005).

The use of chemical signatures in calcified structures as a long-term stock discriminator can present problems if there is not long-term stability of the signature. Use of the fingerprint as a long-term stock discriminator may be justified in instances where environmental differences among stock areas are larger than those within areas or across year-classes, and where the size-related effects on the fingerprint have been statistically removed (Campana, 2005). Analysis of young-of-the-year fish from known stock origin can be used to define a baseline characterization of the chemical fingerprint for a stock, and examination of multiple year-classes can establish the temporal stability of elemental or isotopic signatures in structures (Thorrold et al., 2001; Gillanders, 2002; Rooker et al., 2008, Figure 11.2).

11.1.2 Structure, Composition, Metabolic Stability, and Pathway of Incorporation

Relative to other calcified structures in fish, the chemical properties of otoliths are well studied. Otoliths are also considered to provide superior chronological records to other calcified structures (Campana and Thorrold, 2001). Thus, the otolith is typically the first structure explored for chemical analysis, when available. For those fish that do not possess otoliths (e.g., elasmobranchs), vertebrae and dorsal fin spines have been used for aging purposes and are being explored as structures that may provide insight on stock structure and movement through chemical analysis (Tillett et al., 2011; Werry et al., 2011). Scales, fin rays, and spines may also be useful as alternative structures for chemical analysis in teleost fish whose otoliths are problematic (e.g., vateritic composition) for establishing chronologies (Gillanders, 2001). Furthermore, scales, fin rays, and spines represent a nonlethal alternative to otoliths for reconstructing environmental history of fishes (Coutant and Chen, 1993; Gillanders, 2001; Wells

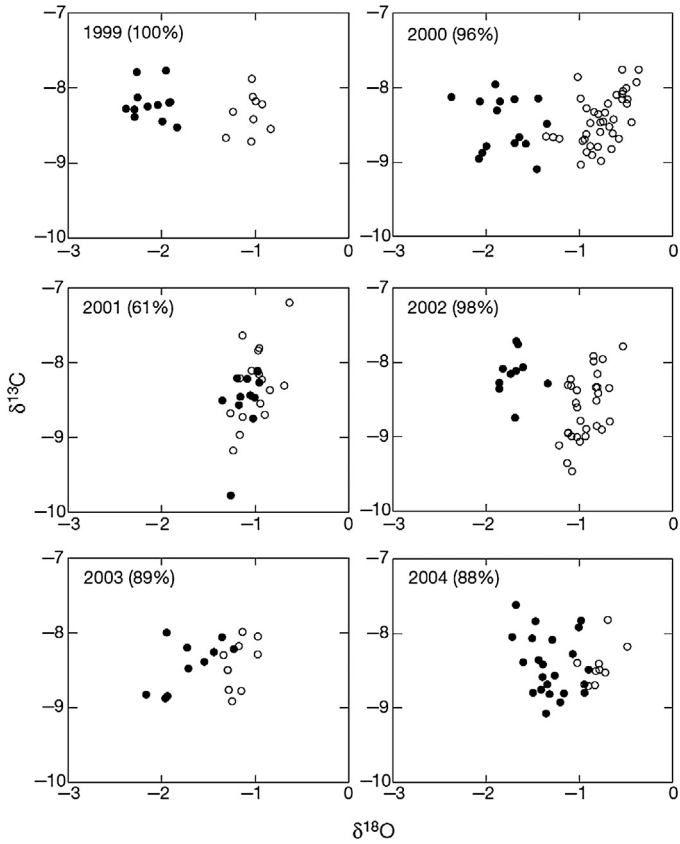

FIGURE 11.2 Example of the use of otolith chemistry of juvenile fish to characterized the isotopic fingerprint for bluefin tuna (*Thunnus thynnus*). Otolith $\delta^{13}C$ and $\delta^{18}O$ values for six year-classes of tuna collected in the eastern Atlantic Ocean/Mediterranean Sea (open circle) and U.S. Atlantic Ocean (closed circle) are shown along with classification success (based on discriminant function analysis). Examination of multiple year-classes can help evaluate the stability of the otolith signature. Full details of the study are available in Rooker et al. (2008).

et al., 2000a,b; Wells et al., 2003; Arai et al., 2002; Jarić, 2011; Phelps et al., 2012), so they are particularly useful for the study of rare or endangered species.

11.1.2.1 OTOLITHS

Teleost (or bony) fish possess three pairs of otoliths (the sagittae, lapilli, and asterisci), with the sagitta being the most frequently used otolith for aging and chemical applications. The otoliths of fish are primarily composed of calcium carbonate (~97%) in a noncollagenous organic matrix (Campana, 1999). This material is precipitated from the endolymphatic fluid and is deposited continuously throughout the lifetime of the animal (Campana, 1999; Campana and Thorrold, 2001). Prior research has established the time-

keeping properties and metabolic and temporal stability of this structure, supporting its use as a record of the chemical environment experienced by an individual fish throughout its lifetime (Campana and Thorrold, 2001).

The predominant source of most elements and isotopes to otoliths is the surrounding water. The diet of the fish can contribute to the chemical composition of the otolith; however, the relative contribution varies for different elements (Kalish, 1991; Farrell and Campana, 1996; Thorrold et al., 1997a,b; Walther and Thorrold, 2006). Elements are incorporated from the water to the fish's blood plasma via the gills or intestines, then into the endolymph, and finally into the crystallizing otolith (Campana, 1999; Sturrock et al., 2012). Discrimination can potentially occur at all of the four interfaces (water−blood, blood−blood binding proteins, blood−endolymph, and endolymph−otolith) and varies for different elements and isotopes (Campana, 1999; Sturrock et al., 2012). Elements may be incorporated directly into the crystal lattice through substitution for calcium, as an inclusion in interstitial spaces, or in the protein matrix of the otolith (Campana, 1999). See Campana (1999) and Sturrock et al. (2012) for further details on the pathways of chemical incorporation into otoliths.

The use of otolith chemistry as a natural marker of stock structure has increased dramatically in the last 20 years and has been applied to various taxa in a variety of environments. This research supports the effectiveness of elemental and isotopic fingerprints as biological tracers of groups of fish in freshwater (Kalish, 1990; Northcote et al., 1992; Bronte et al., 1996; Kennedy et al., 1997, 2000, 2002; Limburg, 1998; Thorrold et al., 1998a; Barnett-Johnson et al., 2008; Walther and Thorrold, 2008; Bradbury et al., 2011) and brackish and saltwater environments (Edmonds et al., 1989, 1991, 1992, 1995; Gunn et al., 1992; Sie and Thresher, 1992; Campana et al., 1994, 1995, 1999; Campana and Gagné, 1995; Thresher et al., 1994; Proctor et al., 1995; Severin et al., 1995; Dove et al., 1996; Gillanders and Kingsford, 1996; Milton et al., 1997; Thorrold et al., 1997b, 1998b, 2001; Begg et al., 1998; Dufour et al., 1998; Newman et al., 2000; Secor and Rooker, 2000; Volk et al., 2000; Gillanders, 2001, 2002; Secor et al., 2001; Miller et al., 2005; Jonsdottir et al., 2006b; Rooker et al., 2008; Longmore et al., 2011; Thorisson et al., 2011).

11.1.2.2 SCALES

Structurally, teleost scales consist of a well-mineralized outer layer that is composed of a calcium phosphate material similar to hydroxyapatite, as well as a poorly mineralized inner layer that is composed of layers of collagen fibers (Zylberberg and Nicols, 1982; Zylberberg, 2004; Hutchinson and Trueman, 2006). The hard, mineralized outer layer grows in area over time through formation of concentric growth increments, referred to as circuli (Hutchinson and Trueman, 2006). The collagen layer grows in thickness, with new layers added over time (Hutchinson and Trueman, 2006). Thus, older growth can overlie recent growth within the collagen layer of scales.

Some fish are aged by counting aggregates of scale circuli; however, there are issues with scale loss, regeneration, and deposition ceasing at older ages, which can compromise accurate age estimation (Beamish and McFarlane, 1985; Jones, 1992). Thus, scales may not provide a complete temporal record over the lifetime of the fish (Campana and Thorrold, 2001).

Elements are incorporated into fish scales from the surrounding water and diet (Mugiya et al., 1991; Bijvelds et al., 1996; Wells et al., 2000a). The pathway of chemical incorporation into scales depends on the element or isotope. Several studies have documented a high degree of correlation between scale and water chemistry (Bagenal et al., 1973; Coutant and Chen, 1993; Kennedy et al., 2000; Wells et al., 2000a; Ramsay et al., 2011), as well as a high degree of correlation between scale and otolith chemistry for some elements (Wells et al., 2000b; Gillanders, 2001; Ramsay et al., 2011). However, evidence suggests that scales do not exhibit metabolic stability, with resorption of some elements possible during times of stress and high nutritional demand (Bilton and Robins, 1971; Bilton, 1975; Bijvelds et al., 1996). For example, Clarke et al. (2007) found scale strontium concentrations of Arctic grayling were not correlated with water chemistry and suggested remobilization may have occurred. Wells et al. (2003) indicated that the elemental signatures in weakfish scales were not stable over time and not useful as a natural tag of natal origin with weakfish. In a laboratory study, Bijvelds et al. (1996) found magnesium was remobilized from scales to other parts of the body under low magnesium conditions. Despite these limitations, scales have been successfully used to trace fish movement (Courtemanche et al., 2006), understand stock structure (Pender and Griffin, 1996), determine river of origin (van Coillie and Rousseau, 1974; Wells et al., 2003; Muhlfeld et al., 2005), and discriminate farmed versus wild stock fish (Adey et al., 2009). Furthermore, scales offer a distinct advantage over otoliths in that they permit nonlethal sampling of the fish. Scales may be useful tracers of stock structure and movement when chemical signatures remain sufficiently stable throughout a fish's life and the degree of scale alteration is small relative to the differences being compared between groups.

11.1.2.3 ELASMOBRANCH VERTEBRAE

The vertebral centra of elasmobranchs are composed of calcified cartilage, primarily the calcium phosphate mineral hydroxyapatite [$Ca_{10}(PO_4)_6(OH)_2$] deposited within an organic matrix of proteins (proteoglycan and collagen, Urist, 1961; Porter et al., 2006). In most elasmobranchs, this mineralization process occurs incrementally, resulting in vertebral growth bands (Cailliet, 1990). One translucent and one opaque band comprise a band pair that is often assumed to represent 1 year of growth (Cailliet et al., 1983; Cailliet and Goldman, 2004). The degree of metabolic stability of vertebrae remains unclear. Unlike cancellous or "true" bone, which undergoes remodeling over the lifetime of the animal, hydroxyapatite (the primary component of shark cartilage) is thought to grow by accretion with little remodeling;

therefore, it records information throughout the animal's life (Koch et al., 1994). Evidence suggests that chemical signatures are not transported across growth bands; if there is metabolic reworking, it is likely minimal (Campana et al., 2002; Hale et al., 2006; Kerr et al., 2007; Tillett et al., 2011). Further research is needed to conclusively resolve the metabolic stability of vertebrae. If there is resorption or reworking that alters the chemical composition of the vertebrae, this will limit the usefulness of this structure for stock identification.

The pathway of element incorporation into vertebrae is believed to be similar to that of otoliths, in that some elements are absorbed across the skin and gills into the blood and substituted for calcium or trapped within the protein matrix of the vertebrae (Dean and Summers, 2006; Hale et al., 2006; Tillett et al., 2011). There is also a dietary contribution to vertebrae, although the relative contribution of water and diet sources and the degree of discrimination that occurs in elemental incorporation of vertebrae are not well studied (Campana et al., 2002; Kerr et al., 2006).

No studies have explicitly applied chemical methods to vertebrae for the purpose of stock identification of elasmobranchs to date. However, chemical analysis of vertebrae has proven useful in tracking the movement of bull sharks *Carcharhinus leucas* and pig-eye sharks *Carcharhinus amboinensis* (Tillett et al., 2011; Werry et al., 2011). Chemical analysis of the jaw cartilage of the gummy shark *Mustelus antarcticus* suggests that it may be possible to use elemental composition of shark cartilage to distinguish populations (Edmonds et al., 1996). Furthermore, application of radiocarbon age-validation techniques to shark vertebrae suggests that the requirement of metabolic and temporal stability of vertebrae is likely satisfied (Campana et al., 2006; Kerr et al., 2006).

11.1.2.4 FIN RAYS AND SPINES

Teleost fin rays and spines are composed of dermal bone, consisting primarily of calcium phosphate (Findeis, 1997). These structures have been used for aging purposes, and comparative analysis has shown that ray and spine ages are in close agreement with otolith ages for some species (e.g., goliath grouper *Epinephelus itajara*, Brusher and Schull, 2009; bull trout *Salvelinus confluentus*, Zymonas and McMahon, 2009). However, early growth increments in spines of some species can be destroyed as the core-matrix expands, which is particularly a problem in older fish (Prince and Pulos, 1983; Hill et al., 1989). Furthermore, fin rays and spines may be subject to elemental mobilization and resorption during periods of nutritional stress, which may limit their utility as indicators of stock structure (Veinott and Evans, 1999; Gillanders, 2001).

Elasmobranch dorsal fin spines are composed of dentine with slightly different structure across taxa (Squaliformes, Heterodontiformes, and Chimaeriformes; Clarke and Irvine, 2006). Dogfish spines are composed of three main components: (1) the interior layer, composed of cartilage and pulp tissue; (2) the stem layer, composed of three dentine layers; and (3) the mantle,

composed of a dentine layer, a pigment layer, and an outer, mineralized enamel layer (Holden and Meadows, 1962; Beamish and McFarlane, 1985). Growth occurs from the base of the spine as well as internally (Holden and Meadows, 1962; Beamish and McFarlane, 1985). Dark ridges on the mantle of the spine have been confirmed to represent annual growth rings (Beamish and McFarlane, 1985; Campana et al., 2006). However, spines often experience wear, particularly with increasing size, which can prevent preservation of a complete temporal record over the lifetime of the fish (Holden and Meadows, 1962).

The sources and pathways of elements to fin rays and spines are believed to be similar to that of fish vertebrae (Gillanders, 2001). The degree of discrimination in the absorption of elements and whether resorption occurs for these structures are not well characterized (Gillanders, 2001). The chemical composition of spines and otoliths of damselfish (*Parma microlepis*) were positively correlated, particularly for strontium and barium, indicating that spines could potentially provide estimates of stock structure similar to that of otoliths (Gillanders, 2001). Fin ray chemistry has been successfully used to examine movement and stock structure of several species of sturgeon (Veinott and Evans, 1999; Arai et al., 2002; Allen et al., 2009; Jarić, 2011; Phelps et al., 2012). Additionally, trace element analyses of fin rays have been used to reconstruct the environmental history of a variety of other species, such as Arctic grayling *Thymallus arcticus* (Clarke et al., 2007), smallmouth bass (Smith and Whitledge, 2010), channel catfish *Ictalurus punctatus*, blue catfish *Ictalurus furcatus*, and flathead catfish *Pylodictis olivarus* (Smith, 2008).

11.1.3 Factors Influencing the Chemical Composition of Structures

The chemical composition of calcified structures in fish is influenced by several factors, including water chemistry, environmental conditions (e.g., temperature), dietary sources, and fish physiology (Kalish, 1991; Kennedy et al., 1997; Thorrold et al., 1997a; Campana, 1999; Secor and Rooker, 2000; Elsdon and Gillanders, 2002; Sturrock et al., 2012). Recent research also suggests that genetic differences among populations can be a determinant of otolith chemistry (Clarke et al., 2011).

Water chemistry is regulated by physical and chemical processes, and the sources of distinct signatures of water masses (e.g., underlying geology, precipitation, runoff, microbial processes, anthropogenic influence, and mixing of water masses with different compositions) differ for each element (Campana, 1999; Kraus and Secor, 2004; Kerr et al., 2007; Elsdon et al., 2008). Variability in water chemistry and otolith chemistry have been identified across a range of temporal and spatial scales (Elsdon et al., 2008). Otoliths have recorded spatial variability in water chemistry on the order of tens to hundreds of kilometers (Secor and Zdanowicz, 1998; Gillanders and Kingsford, 2000; Gillanders, 2002; Dorval et al., 2005; Miller, 2007; Walther and Thorrold, 2008) and temporal variability ranging from seasonal to interannual (Campana et al., 2000; Gillanders, 2002; Mateo et al., 2010). Temporal variability in water chemistry

can result in age-related differences in exposure history and different fingerprints for fish of different size classes from the same population (Edmonds et al., 1989; Hoff and Fuiman, 1993; Campana et al., 1995, 2000; Begg et al., 1998; Begg and Weidman, 2001).

The influence of dietary contributions to the chemical signature of fish hard parts varies by element and structure. The contribution from dietary sources to otoliths is assumed to be minor for many elements (e.g., Na, Mg, Mn, K, Ca, and Cu; Buckel et al., 2004; Marohn et al., 2009), although there are conflicting results regarding the influence of diet on concentrations of Sr and Ba (Limburg, 1995; Gallahar and Kingsford, 1996; Buckel et al., 2004; Walther and Thorrold, 2006). Further research is needed to determine the relative contribution of dietary sources to the chemical composition of fish vertebrae, scales, spines, and rays.

Physiological differences can result in differences in the discrimination and incorporation of elements and isotopes in calcified structures of fish. For example, ontogenetic differences in growth rates have been shown to influence Mg, Sr, and Ba concentrations in otoliths (e.g., Kalish, 1989; Sadovy and Severin, 1992, 1994; Fowler et al., 1995; Secor et al., 1995). However, other studies have shown mixed or no effects of growth rate on elemental concentration (e.g., Martin et al., 2004; Martin and Thorrold, 2005; Miller, 2009). Additionally, a relationship between metabolic rate and carbon isotope ratios in otoliths has been documented, leading to changes across size groups of fish due solely to metabolic rate differences (Radtke et al., 1987; Kalish, 1991; Thorrold et al., 1997a). Reproductive activity has also been identified as an influence on elemental composition of otoliths (e.g., Fuiman and Hoff, 1995). Thus, changes in the physiological state can confound the interpretation of the chemical composition of calcified structures, such as otoliths (Elsdon et al., 2008).

Temperature has been identified as an external factor that has a significant influence on the concentration of many elements and isotopes in fish structures (Campana, 1999). Several elemental and isotopic ratios have been found to vary systematically with temperature, including Sr:Ca, Ba:Ca, ^{18}O:^{16}O, and ^{13}C:^{12}C (Campana, 1999; Thorrold et al., 1997a; Elsdon and Gillanders, 2002). The effect of temperature on chemical incorporation may be indirect through its influence on fish metabolism or direct through kinetic effects, which results in differential uptake of elements or isotopes with temperature (Campana, 1999). Additionally, interactive effects of temperature and salinity on otolith chemistry have been documented, which can affect our interpretation of the environmental histories of fish (Elsdon and Gillanders, 2002).

An understanding of factors contributing to chemical differences and variability in chemical signatures can be helpful; however, for the purpose of stock identification, recognition of between group differences in chemical signatures is sufficient (Campana et al., 2000; Elsdon et al., 2008). Irrespective of the cause of differences in chemical fingerprints among groups, the fingerprints become the natural distinguishing feature of those groups at a given point in

time (Campana, 2005). Accordingly, chemical fingerprints in calcified structures appear to be well suited as biological tracers of groups of fish, requiring relatively few assumptions for confident application to difficult tracking or stock mixing situations.

11.1.4 Assumptions of Approach

Use of the chemical composition of fish hard parts as natural tags to infer stock structure makes several assumptions; these are listed in detail in Elsdon et al. (2008). Two central assumptions of the application of structure chemistry for the purpose of stock identification are discussed here.

11.1.4.1 Assumption 1: There Are Characteristic and Reproducible Markers for Each Group

The chemical signature of the groups of interest must be significantly different for this approach to be applicable. Initial investigations into water chemistry can be helpful to inform the likelihood that groups will differ in their chemistry (Elsdon et al., 2008). Group-specific variation in elemental composition has been documented in many otolith applications (e.g., Edmonds et al., 1989, 1991, 1992, 1995; Northcote et al., 1992; Campana and Gagné, 1995; Campana et al., 1995, 1999; Bronte et al., 1996; Thorrold et al., 1998a). It is important to recognize that differences in elemental concentration among groups can be confounded by size differences among groups because elemental concentrations have been observed to vary with fish size (Edmonds et al., 1989; Hoff and Fuiman, 1993). However, statistical removal of the effect of otolith weight on elemental concentration can resolve this problem (Campana et al., 2000).

Temporal stability of chemical signatures needs to be considered for assays of whole structures. Long-term stability of an environmentally induced marker is not expected due to continued growth, nor has it been observed in otoliths (Edmonds et al., 1995; Campana et al., 2000; Begg and Weidman, 2001). However, short-term stability over the interval between characterization (e.g., spawning group) and mixing is both expected and observed, particularly with respect to differences among groups, allowing for whole otolith assays over short intervals (Campana et al., 1995, 2000; Kennedy et al., 1997, 2000; Thorrold et al., 1998a). The interval between sampling spawning fish to characterize their elemental fingerprints and sampling the stock mixture should be on the order of a 1- to 2-year period (and shorter for a young or fast-growing species); beyond this time, a noticeable change in the elemental fingerprint may occur due to the addition of new material (Edmonds et al., 1995; Campana et al., 2000). An analysis that targets core material or a specific growth band in a structure alleviates concerns associated with the continued growth of a whole structure. However, if the chemical signature of cores or growth bands from fish of unknown origin are being compared to a reference (e.g., a baseline characterization of the chemical signature of a population using young-of-the-year fish), then matching the temporal period for the reference

and sample is important unless the chemical signature in the environment is stable over time (Elsdon et al., 2008).

11.1.4.2 ASSUMPTION 2: ALL POSSIBLE GROUPS CONTRIBUTING TO THE GROUP MIXTURE HAVE BEEN CHARACTERIZED

This assumption applies as much to genetic studies as it does to chemistry applications, with the implication being that uncharacterized groups of fish present in the mixture could be mistakenly interpreted as one or more of the reference groups (Wood et al., 1987, 1989; Wirgin et al., 1997). Careful selection of reference groups can help to minimize this problem, particularly if they are sampled at a time when the groups are known to be discrete (e.g., on the spawning or nursery grounds). Additionally, one of the available stock mixture analyses provides the ability to recognize the presence of uncharacterized reference groups (Smith and Campana, 2010).

Additional assumptions include the following: (1) there is no reworking of the chemical signature of the fish structure, (2) there is accurate aging of the fish, (3) there is no effect of the chemical tag on survival rates, and (4) similar methods are used to detect chemical tags across groups (Elsdon et al., 2008).

11.1.5 Limitations of Application

In principle, the chemical composition of fish hard parts can be used to identify and track groups of fish with different environmental histories. In practice, there are three limitations to their use. The first limitation is that many of the most easily measured elements are under strict physiological regulation, and thus they may be unsuitable for use as environmental indicators. For otoliths, this list includes the major elements calcium, oxygen, and carbon (which make up the calcium carbonate matrix), as well as the minor (>100 ppm) elements Na, K, S, P, and Cl, with the exception of Sr (Thresher et al., 1994; Proctor et al., 1995; Schwarcz et al., 1998). Nevertheless, some physiologically regulated elements and isotopes can prove useful as biological tracers of a group of fish, as long as the concentrations of those elements vary significantly among groups. The second limitation concerns the analysis of the less abundant trace (<100 ppm) elements, which appear to be more suitable as environmental indicators. Although their lower concentrations make them less likely to be osmoregulated by the fish, it also makes them more difficult to assay with accuracy and without contamination during handling. Finally, few if any trace elements (even when normalized to Ca) are likely to be incorporated into calcified structures in direct proportion to availability in the environment (Hanson and Zdanowicz, 1999). Both temperature and growth rate are known to be at least as influential as ambient concentration in modifying otolith elemental composition. With these caveats in mind, the elements most likely to serve as environmentally influenced stock markers include Sr, Ba, Mn, Fe, and Pb (and perhaps Li, Mg, Cu, Ni, Se, and Hg), in which both ambient element:Ca concentrations and/or temperature produce significant effects on

otolith composition (Fowler et al., 1995; Farrell and Campana, 1996; Geffen et al., 1998; Campana, 1999; Bath et al., 2000; Milton and Chenery, 2001; Lochet et al., 2010). The isotope ratios that are most useful as natural tags are Sr, S, Pb, C, and O (Kennedy et al., 1997, 2000; Spencer et al., 2000; Thorrold et al., 2001; Weber et al., 2002; Kerr et al., 2007).

11.2 METHODOLOGY

11.2.1 Collection and Preparation of Materials

The choice of scale of sampling within the fish structure has important implications. One approach is analysis of the whole structure. Another approach is targeted analysis of a core region representative of the early life history of the individual. For structures that grow continuously throughout the life of the fish, the whole-structure fingerprint integrates across the entire lifetime and thus serves as a marker for groups of fish that have experienced different overall environmental exposures (Campana, 2005). In contrast, analysis of the core of a structure is generally intended as a more direct measure of stock or nursery origin (Campana, 2005). Both of these approaches are discussed further below.

The most robust application of whole-structure fingerprints is one that is targeted at questions of stock mixing or for tracking stock migrations, in which the fingerprints are used as natural tags of predefined groups of fish over short periods of time (Campana et al., 1995, 1999, 2000; Gillanders and Kingsford, 1996; Kennedy et al., 1997, 2000). Application of a chemical fingerprint as a natural tag takes advantage of the fact that the size and composition of a calcified structure cannot change appreciably over a brief time period. Once the chemical fingerprint of all potential source groups has been determined, fish should remain identifiable as to their source group, despite any mixing with other groups, until the elemental composition of later growth has significantly altered overall chemical composition of the structure. The fingerprint would not be expected to remain stable over extended periods of time (e.g., years) because interannual variation in the habits and environments of the fish would eventually produce a detectable change in the overall chemical composition. However, short-term stability is both expected and observed, particularly with respect to differences among groups (Campana et al., 1995; Kennedy et al., 1997; Thorrold et al., 1998b). An appealing feature of this application is that the fingerprint need not be linked to potential sources or locations in the environment.

Analysis of the core of structures is generally intended as a more direct measure of stock origin than analysis of the whole structure. As is the case with whole structure analysis, the presence of fingerprint differences implies differences in the history of environmental exposure that may or may not correspond to genetic differences (Campana, 2005). In this application, however, the environmental exposure is limited to the period of growth represented by the material assayed, whether that is the period around hatch, the first few months of life, or some other period (Campana, 2005). The subsequent life

history is not sampled and therefore is irrelevant. To the extent that spawning or nursery grounds are characterized by different temperature or chemical environments, this approach has proved effective in distinguishing among groups of fish with different origins (Kalish, 1990; Sie and Thresher, 1992; Campana et al., 1994; Thresher et al., 1994; Proctor et al., 1995; Severin et al., 1995; Dove et al., 1996; Gillanders and Kingsford, 1996; Milton et al., 1997; Thorrold et al., 1997b, 2001; Gillanders, 2002; Ashford et al., 2006; Bradbury et al., 2008; Rooker et al., 2008).

To prepare materials for chemical analysis, the structure of interest (otolith, vertebrae, scale, fin spine, or ray) must first be extracted from the fish. Once extracted, structures may be frozen or cleaned of adhering tissue and dried, then stored prior to preparation for analysis. With analytical sensitivity comes the potential for contamination from unwanted sources. Factors such as the mode of fish or structure preservation, composition of the instruments used to remove the structure from the fish, cleaning methods, handling, and even household dust are potential modifiers of the perceived chemical composition (Milton and Chenery, 1998; Thresher, 1999). Contamination or modification is of particular concern for elements present at micro levels (100−5000 ppm, including Na, Sr, K, S, and C1) or trace levels (less than 50−100 ppm, including Zn, Br, Se, Ni, and Pb) and for elements incorporated through occlusion rather than bound within the structure (Campana, 1999). Therefore, chemical analysis of structures stored dry or frozen appears to be safest. Preservation in fluids such as ethanol and formalin appears to have the greatest potential for influencing concentrations of elements that are not tightly bound in the calcium carbonate matrix (e.g., Na, Mg, and K; Milton and Chenery, 1998; Proctor and Thresher, 1998). However, elements that replace calcium in the calcium carbonate matrix (e.g., Sr and Ba) do not appear to be influenced by storage in fluids, such as ethanol (Milton and Chenery, 1998; Proctor and Thresher, 1998; Hedges et al. 2004).

Protocols for handling and preparing otoliths for trace element analysis reflect a high level of concern for contamination. However, such stringent precautions may not be necessary for elements and isotopes that occur at higher concentrations. These methods are drawn from the water analysis literature and always involve isolation from skin, metallic instruments, and solutions that are of other than trace metal grade. In general, decontamination based on brushing and sonification in ultrapure water, followed by storage in acid-washed polyethylene vials, results in minimal contamination (Campana, 1999). Minor elements such as Na, K, Cl, and S appear to be affected by the water sonification stage (Proctor and Thresher, 1998), perhaps because these elements are incorporated by occlusion and are not lattice bound. However, it is equally probable that such poorly bound elements would be severely affected by exposure to any fluid, including the endolymph if it shifts its composition during the death of the fish. As a result, such elements would probably not be well suited for use as stable biological tracers (Campana, 2005). Acid washing of otoliths does not appear to be necessary for elements such as Ba,

Mg, Sr, and Li (Campana et al., 2000; Secor et al., 2001), despite the fact that it is an important step in the decontamination of sediment-laden forams. Complete protocols for handling and preparing otoliths for elemental assay are presented elsewhere (Campana et al., 2000; Campana, 2005).

Of particular relevance to inductively coupled plasma mass spectrometry (ICPMS), but applicable to all analytical techniques, is the likelihood of instrument drift (change in sensitivity) during the analysis of large numbers of samples or between instrument days. Because the estimated elemental concentration can be significantly affected by this drift despite the analysis of analytical standards, it is important that the analysis sequence be blocked and randomized so that the order of analysis for any one sample group is spread over the entire analysis sequence (Campana and Gagné, 1995). Use of isotope dilution-ICPMS minimizes (although it may not eliminate) instrumental drift (Campana et al., 1995).

Differences in the composition of calcified structures among groups of fish may be statistically significant, but they will not necessarily be large. Artifactual but significant differences among groups of otolith elemental assays are not uncommon (Campana et al., 1997). Therefore, calibration of separate assay runs or laboratories against a reference powder specific to the calcified structure is highly recommended to ensure that any observed differences among runs are real rather than artifactual (Campana, 1999; Thresher, 1999; Yoshinaga et al., 2000; Sturgeon et al., 2005).

11.2.2 Chemical Analysis

There are a wide variety of sophisticated instruments available for elemental assays of calcified structures, but the most frequently used include the energy-dispersive (ED-EM) and wavelength-dispersive (WD-EM) electron microprobes (Gunn et al., 1992), proton-induced X-ray emission (PIXE) (Sie and Thresher, 1992; Limburg et al., 2003), and laser ablation inductively coupled plasma mass spectrometry (LA-ICPMS) (Campana et al., 1994; Thorrold and Shuttleworth, 2000). In a detailed experimental comparison among the above instruments, Campana et al. (1997) noted that no one instrument type was sensitive to each element, nor was any one instrument preferred for use in all assays. In general, however, the minor elements such as Na and K could only be measured accurately with an electron microprobe, whereas the trace elements generally used in stock identification studies required PIXE or LA-ICPMS. Strontium was measured accurately and precisely with either WD-EM, PIXE, or LA-ICPMS.

Probe-based assay techniques are also useful for the analysis of certain isotopes in calcified structures, such as the use of multicollector ICPMS for analysis of strontium isotopes (Barnett-Johnson et al., 2005) and the use of an ion microprobe for analysis of sulfur (Weber et al., 2002) and carbon and oxygen isotopes (Weidel et al., 2007). An alternative approach involves microsampling or coring techniques that physically remove a portion of the structure for subsequent solution-based analysis of material using an isotope ratio ("light" elements) or

thermal ionization mass spectrometry ("heavy" elements). Computerized micromilling machines have been proven effective in several studies, whereby seasonal or annual growth zones visible in structure cross-sections are milled to a discrete depth and the powder collected for assay (Wurster et al., 1999; Weidman and Millner, 2000). Controlled acid dissolution of overlying material in otoliths has also been reported, although the acid apparently leached some material from the core (Dove et al., 1996). The disadvantage of microsampling is one of limited sampling resolution. The temporal resolution of the extracted sample is typically seasonal at best, although higher resolution sampling has been achieved (Weidman and Millner, 2000). It appears unlikely that microsampling or coring would introduce contaminants that would confound stable isotope assays as long as the extracted samples were treated carefully. On the other hand, there is potential for contamination from the sampling process on trace element assays, despite the fact that Dove et al. (1996) reported no artifacts due to sectioning with an IsoMet saw.

11.2.3 Data Analysis

Although elemental concentrations are generally reported in terms of moles per mole of Ca or micrograms per gram of otolith (or other structure), many studies have noted size-specific concentrations for some elements that could otherwise be confused for stock-specific differences (Figure 11.3). To ensure that differences in fish length and/or structure weight among samples do not confound any stock-specific differences in elemental composition, it is important to remove the effect of structure weight from the statistical analysis (Campana, 2005). In steelhead trout (*Oncorhynchus mykiss*), for example, Mg varied significantly with otolith weight, whereas Ba did not (Figure 11.3). Subtraction of the common within-group linear slope (derived from the analysis of covariance (ANCOVA)) from the Mg data removed the trend from the element–otolith

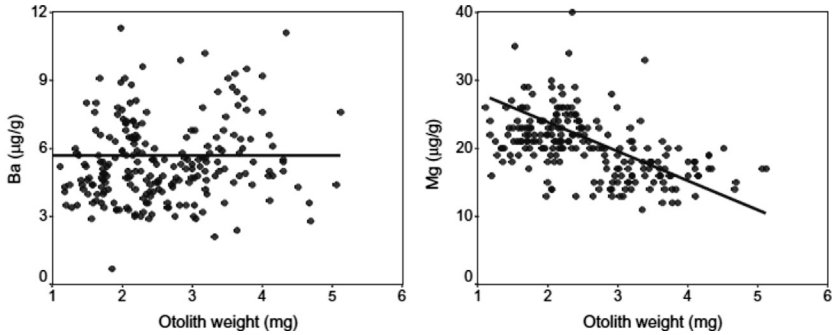

FIGURE 11.3 Examples of the relationship between elemental concentration and otolith weight in steelhead trout. A significant negative relationship was noted for Mg, but no relationship was evident for Ba. To ensure that variations in otolith weight among samples do not confound the interpretation of differences among areas, the effect of otolith weight should be removed statistically from elements where the relationship exists. Campana, unpublished.

weight relationship (Campana, 2005). In instances where the element−otolith weight relationship is markedly nonlinear, alternative detrending procedures are possible (Campana et al., 2000). Detrended elemental concentrations should show no obvious residual relationship with otolith weight; but in any event, relative differences among detrended samples should be similar to those based on original data. Additionally, within-group distributions of elemental concentrations are sometimes skewed and thus must be transformed prior to statistical analysis.

In many studies, calcified structures are characterized by a suite of several elements; therefore, multivariate statistics are used to distinguish among samples. Multivariate analysis of variance (MANOVA) is used to test for significant differences among samples, whereas discriminant analysis can be used to prepare two-factor elemental fingerprints for illustrative purposes. Discriminant analysis is not usually the best method to classify samples of unknown stock affinity because it performs poorly when the stock markers are similar and requires prior knowledge of stock proportions in the mixture (Campana, 2005). In contrast, stock composition analysis using a maximum likelihood-based method provides maximal discriminatory power in mixed stock situations (Wood et al., 1987; Campana et al., 1999; Gillanders, 2002). Increasingly, Bayesian methods are being used in stock classification (Munch and Clarke, 2008; Smith and Campana, 2010; Pflugeisen and Calder, 2011). These models allow for simultaneous analysis of multiple data sources (e.g., otolith and genetic data; Smith and Campana, 2010) and enable one to account for and analyze several sources of uncertainty (Pflugeisen and Calder, 2011).

A simplified protocol for the statistical analysis of chemistry data follows (Campana, 2005):

1. Examine frequency histograms of the concentration of each element or isotope in each group. For elements where the distribution is nonnormal in most or all groups, transform that element appropriately (e.g., ln transform; transformation must be applied to that element in all groups). Remove clearly aberrant outliers (>5 standard deviations away from the mean) from the transformed data if it is not associated with particularly small or large fish.
2. Visually and statistically assess each element and isotope within each group for a relationship with fish size or otolith weight. Where a relationship is evident in most or all groups, the effect of the relationship must be removed statistically by subtracting the common, within-group slope (obtained from the ANCOVA of the element or isotope with group as the factor and otolith weight as the covariate) from the observed value in each group. Nonlinear relationships must be removed differently, as in Campana et al. (2000). It is important to note that simple use of a regression is not appropriate for weight detrending because it does not account for group effects.
3. Test for univariate differences in the concentration of each element or isotope across groups (e.g., through ANOVA). Error bar plots or box and whisker plots help visualize the intergroup differences.

4. Test for overall differences in the chemical fingerprint among groups using MANOVA.
5. Use stepwise discriminant function analysis to identify the elements and isotopes that contribute the most to fingerprint differences among groups. Visually assess the differences among groups by plotting the first two discriminant function axes against each other. Note that classification of unknown fish using discriminant analysis can give highly inaccurate results and is not recommended.
6. Classify an unknown mixture using a maximum likelihood-based (Figure 11.4) or Bayesian mixture analysis (Munch and Clarke, 2008), using the known identity fish as the reference. Reference fish must be completely comparable to the unknown fish, as per the assumptions of the method discussed earlier.

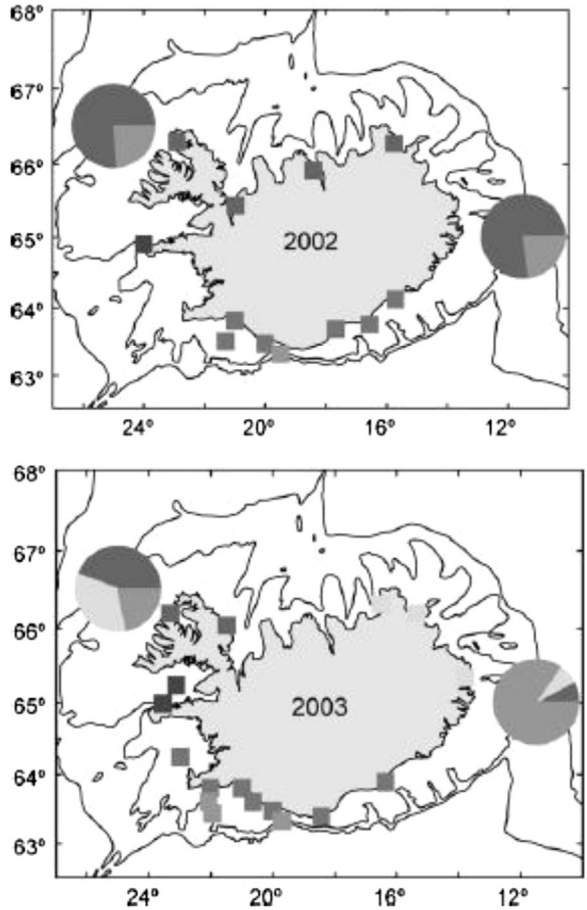

FIGURE 11.4 An example of stock mixture analysis whereby cod of unknown stock origin sampled at feeding grounds northeast and east of Iceland were classified using a maximum likelihood-based approach (proportions represented in pie graphs). Figure in black and white represent unique spawning groups, characterized by baseline sampling. See Jonsdottir et al. (2007).

11.3 CASE STUDIES

11.3.1 Evidence of Trans-Atlantic Movement and Natal Homing of Bluefin Tuna from Stable Isotopes in Otoliths

The purpose of this study by Rooker et al. (2008) was to use otolith chemistry to determine the extent of population mixing and natal homing of bluefin tuna (*Thynnus thynnus*), a highly migratory species that inhabits the North Atlantic Ocean. Bluefin tuna is currently managed as two stocks—an eastern and western stock, separated by the 45°W meridian. Tagging studies suggest that adults are capable of trans-Atlantic migration; however, questions existed regarding the degree of mixing between and natal homing within eastern and western stocks. Otoliths from yearling bluefin tuna of eastern and western origin were collected in their respective nursery areas and used to establish baseline otolith signatures representative of stock origin. This study used stable isotope ratios in otoliths ($\delta^{18}O$ and $\delta^{13}C$) as natural markers of stock origin. Otoliths from multiple year-classes of yearling fish were examined to understand the stability of the otolith signature. The cores of otoliths from adolescent fish of unknown origin collected in a foraging area off the east coast of the United States and from adults collected in the Mediterranean Sea were analyzed for their stable isotope composition. Due to regional differences in water chemistry, otolith $\delta^{18}O$ was an effective marker of nursery origin for bluefin tuna; however, otolith $\delta^{13}C$ did not differ between regions and was not useful in stock identification.

A maximum likelihood approach to mixed-stock analysis was used to predict the stock origin of adolescent and adult tuna. Mixed-stock analysis estimated that 60% of adolescent fish collected off the east coast of the United States were of eastern stock origin. The analysis also indicated that 94% of fish spawning in the Mediterranean Sea originating from the eastern stock. Thus, otolith chemistry indicated a high degree of natal homing in the eastern stock as well as a high level of trans-Atlantic movement of eastern stock fish at younger ages.

11.3.2 Resolving Natal Tags Using Otolith Geochemistry in an Estuarine Fish, Rainbow Smelt *Osmerus mordax*

Bradbury et al. (2011) explored the utility of elemental and isotopic ratios in otoliths as natural tracers of the natal habitat of rainbow smelt (*O. mordax*) in southeastern Newfoundland. The study focused on analysis of juvenile fish otoliths from known spawning locations to identify the suite of elements most useful in classifying fish to spawning location. Juvenile fish were collected from nine estuaries along the Newfoundland coast and the core material in otoliths was analyzed for a suite of elemental (Mg:Ca, Mn:Ca, Sr:Ca, Ba:Ca) and stable isotope ($\delta^{13}C$, $\delta^{18}O$, $^{87:86}Sr$) ratios.

All element and isotope ratios exhibited significant differences across sites, and the average correct assignment of individuals to known natal site using all ratios in quadratic discriminant function analysis ranged from 63−100% and

averaged 83%. However, by using only isotopic ratios, the average correct assignment of fish to natal origin was highest (87%). The authors suggested that the higher classification rate using isotope ratios was attributable to stable differences in salinity among regions, likely the primary factor driving isotopic differences. The characteristics of the spawning locations influenced classification success of fish based on otolith chemistry; closed estuaries with limited marine exchange had higher classification rates compared to open estuaries, which were subject to more marine influence.

11.3.3 Contribution of Different Spawning Components to the Mixed Stock Fishery for Cod in Icelandic Waters

Jonsdottir et al. (2007) used otolith chemistry and length-at-age information to estimate the contribution of different spawning components of Icelandic cod to a mixed stock fishery. Spawning cod were collected at sites around Iceland in 2002 and 2003 and classified into five groups based on differences in otolith shape and chemistry identified in previous studies (Jonsdottir et al., 2006a,b). Cod were also collected from the two main feeding grounds during this same period. Using maximum likelihood-based integrated stock mixture analysis, cod of unknown origin were assigned to their spawning group of origin, with known stock (spawning) cod used as reference data.

Length-at-age and otolith chemistry information provided the highest classification accuracy of unknown samples, whereas incorporation of otolith shape did not improve classification rates. Mixed-stock analysis indicated that most of the cod collected on the feeding grounds in 2002 and 2003 originated from spawning locations north and northwest of Iceland and from deep offshore spawning areas south of Iceland. This study revealed that inshore cod from the main spawning area south of Iceland did not contribute to the major cod fisheries at two of the main feeding grounds in 2002 and 2003. The spawning locations identified through mixed-stock analysis had previously been considered to be minor contributors to the productivity of the stock.

11.3.4 Identifying River of Origin for Age-0 *Scaphirhynchus* Sturgeons in the Missouri and Mississippi Rivers Using Fin Ray Microchemistry

Phelps et al. (2012) used the chemical signature of *Scaphirhynchus* (pallid and shovelnose) sturgeon pectoral fin rays to determine the natal origin of fish. The specific objective was to determine the relative contribution of sturgeon from the Upper Mississippi and Missouri Rivers to the middle Mississippi River. Determining the relative contribution of these rivers to productivity in the region is important to rebuilding efforts for pallid and shovelnose sturgeon populations.

First, a laboratory experiment was conducted to establish the relationship between water and pectoral fin ray chemistry and to verify that short-term

shifts in water chemistry were recorded in the structure. This study established the utility of Sr:Ca values in pectoral fin rays in tracing the origin of fish given that water sources differ in their chemical composition. Water sampling in the Missouri and Mississippi Rivers provided evidence of differences in water Sr:Ca between rivers. Analysis of Sr:Ca in pectoral fin rays of age-0 fish in 2007 and 2008 revealed that the majority of fish collected in the middle Mississippi River and the lower Missouri River originated within the respective river segments in which they were captured. However, the fin ray chemistry data did reveal evidence of emigration of fish from the upper Missouri River to the middle Mississippi River and to the lower Missouri River.

11.4 CONCLUSION

Specific elements and isotopes incorporated into the calcified structures of fish reflect the physical and chemical characteristics of the ambient water, although not necessarily in a simplistic manner. Fish that spend at least part of their lives in different water masses often exhibit differences in the chemical composition of calcified structures; therefore, the chemical signature of the whole or core region of fish structures can serve as an environmentally induced tag of groups of fish. The utility of chemical analysis of otoliths for stock identification has been relatively well studied in fresh, estuarine, and marine systems. However, further work is needed to fully understand the sources and pathways of elements, metabolic stability, and influential factors on chemical composition on fish spines, rays, scales, and vertebrae.

Chemical analysis of calcified structures is one in a suite of effective tools for the identification of stock structure (Cadrin, 2005; Campana, 2005; Sturrock et al., 2012). Because of the ability to detect phenotypic differences, this tool can permit observation of higher spatial complexity compared to molecular studies, such as identification of differences in life history types within a population (i.e., resident versus migratory forms, Secor et al., 2001; Kerr et al., 2009). Ideally, a comprehensive study would employ several complementary approaches, combining information on life history traits, genetics, behavior, and biological tags, in a holistic approach to stock identification (Cadrin, 2010). Together, these techniques can provide stronger evidence for population structure, as well as insight into the mechanisms that maintain population structure. The application of structural chemistry can be particularly important in cases with subtle population structures that may not be detected by genetic applications alone.

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