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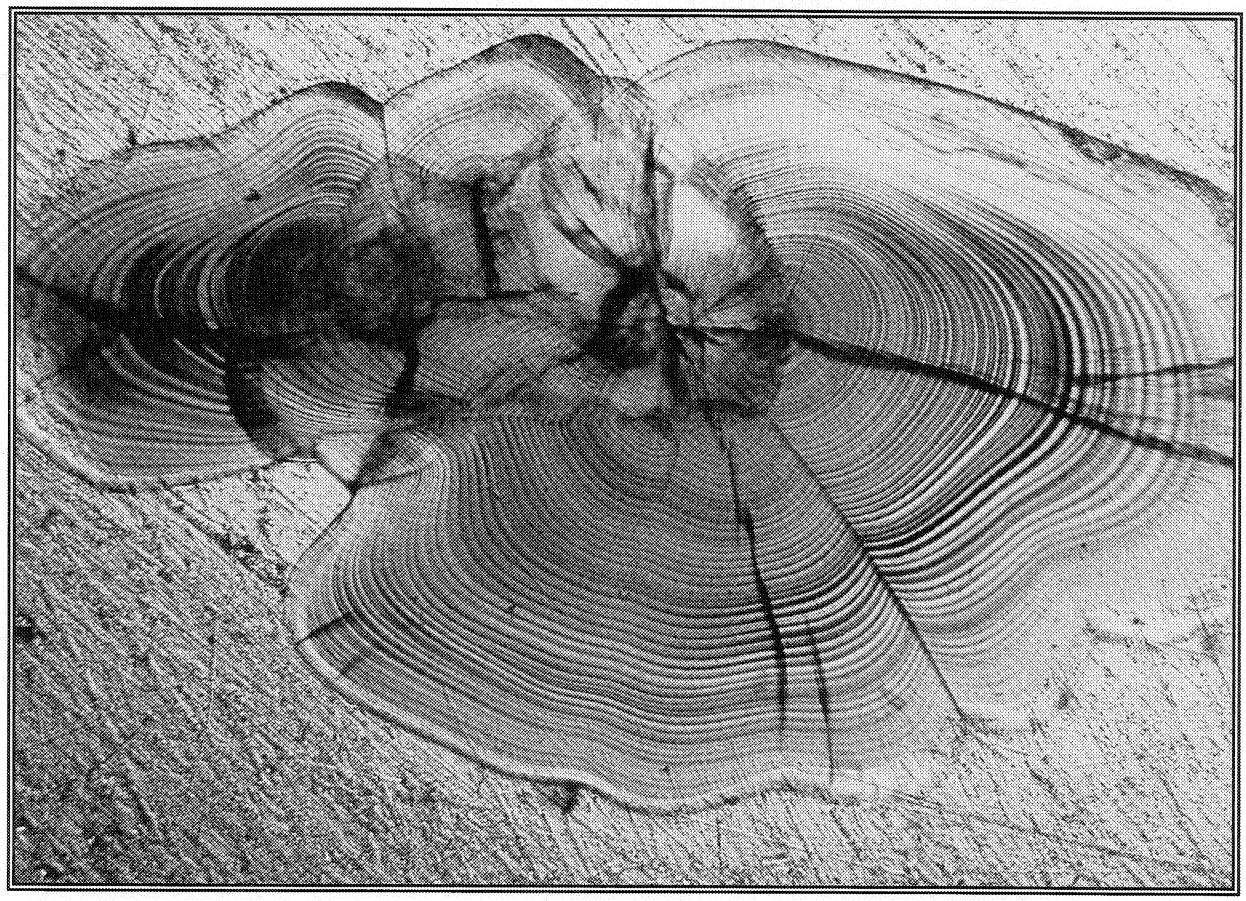
Otolith Microstructure Examination and Analysis

Edited by

David K. Stevenson • Steven E. Campana



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Contents

Abstract

STEVENSON, D. K., AND S.E. CAMPANA [ED.]. 1992. Otolith microstructure examination and analysis. Can. Spec. Publ. Fish. Aquat. Sci. 117: 126 p.

The field of otolith microstructure research has experienced phenomenal growth since the early 1970's and now forms the basis for hundreds of studies of early life history, age, growth, recruitment, migration, mortality, and stock structure. While the field continues to grow and evolve, there is no question that otolith microstructure examination is now an important and accepted technology in fisheries biology.

This book represents the first effort to compile and summarize the many techniques and procedures associated with studies of otolith microstructure. The complete sequence of events, from sample collection to data analysis, is covered comprehensively, so as to be applicable to most species and situations. The various chapters include both published and unpublished procedures, making the book valuable to beginning and experienced investigators alike.

Résumé

STEVENSON, D. K., AND S.E. CAMPANA [ED.]. 1992. Otolith microstructure examination and analysis. Can. Spec. Publ. Fish. Aquat. Sci. 117: 126 p.

L'étude de la microstructure des otolithes a fait d'immenses progrès depuis de début des années 1970 et est maintenant appliquée à des centaines de travaux de recherche portant sur le développement, l'âge, la croissance, le recrutement, la migration, la mortalité ou la structure des stocks. Ce domaine de la recherche continue de s'élargir et d'évoluer, mais il est maintenant acquis que l'examen de la microstructure des otolithes est devenu une technique importante et reconnue en biologie des pêches.

Ce livre est la première tentative visant à compiler et résumer le grand nombre de techniques et procédures appliquées à l'étude de la microstructure des otolithes. Le déroulement complet d'une étude, du prélèvement des échantillons à l'analyse des données, y est présenté de façon détaillée afin qu'il soit possible de l'appliquer à la plupart des espèces ou des situations. Les divers chapitres traitent de procédures connues ou inédites, ce qui rend le livre utile à tous les chercheurs, qu'ils soient débutants ou expérimentés.

Preface

This publication represents the first effort to compile and summarize the many techniques and procedures associated with studies of otolith microstructure. Reviews to date have focused on the theoretical basis for the formation of periodic features in the otolith. While a handful of earlier studies have provided technical protocols for particular species or procedures, none have attempted to describe the complete sequence of events, from sample collection to data analysis, in a form sufficiently comprehensive to be applicable to most species and situations. The primary objectives of this book are to provide detailed descriptions of otolith microstructure techniques and procedures and to recommend preferred approaches to all stages of otolith microstructure research.

The genesis of this publication dates back to February 1988 when the Maine Department of Marine Resources convened an otolith microstructure workshop in Boothbay Harbor, Maine. Cynthia Jones was one of the participants in that workshop. Given the rapid proliferation of otolith microstructure studies that was evident even then, she felt that it was time to provide some advice to new investigators that would assist them in selecting appropriate techniques and applying them correctly. The rest of the group agreed this was a good idea and plans were made to collaborate in writing a paper to meet this objective. David Stevenson, the organizer of the workshop, agreed to coordinate the preparation of the paper.

As time passed the original idea evolved into a more ambitious one, i.e., to compile a manual with chapters containing detailed explanations of how to collect samples, how to remove and prepare otoliths from larval and juvenile fish, what techniques to use in examining them, what are the preferred methods for data analysis, how to validate daily ring deposition, and what sources of error to expect and how to correct for them. At the same time, two of the original group of authors dropped out of the project and three new ones were recruited. First drafts were written and distributed among authors for review. As revisions were made ready for editing, it became clear that a second editor was needed to further review the technical content of each chapter. Steve Campana, who was already heavily involved as an author and in coordinating the review process, agreed to act as the technical editor.

Each contribution to this publication has been reviewed according to the same high standards expected of the better scientific journals. Since the authors involved in the preparation of this book were among the most knowledgeable in the field, each chapter was reviewed by the other authors in the group. The editors insured that all manuscripts received at least two thorough reviews and that the review process was both critical and objective. In the case of Steve Campana's contributions, David Stevenson was responsible for the editorial process.

In its final incarnation, this publication is not, strictly speaking, a manual. Each of the seven chapters in this volume stands on its own as a discrete contribution, but the logical progression and cross-referencing of chapters has produced, in our judgement, a detailed and complete source of technical information that is of value to beginning and experienced investigators alike. Beginning investigators who are designing new research that requires use of the otolith increment technique would be well advised to read all the chapters in the order in which they are presented. The more experienced investigator may want to use this book to review specific topics or use the bibliographies at the end of each chapter to obtain more specific information on topics of interest. Even though much of the material presented will be familiar to the experienced investigator, new information and procedures which have not been published elsewhere are presented in several of the chapters in this publication. This is particularly true of the chapter on data analysis. Note that we have not included a chapter on elemental analysis techniques in recognition of the rapid advances now being made in this discipline.

The first chapter in this monograph, by Cynthia Jones, summarizes the development of the otolith increment technique and presents an overview of current and future applications of the technique in fisheries science. It also outlines some important issues that should be considered in planning and executing otolith microstructure studies. This chapter serves as a valuable introduction to the subject matter covered by the rest of the chapters in this monograph. The second chapter, by John Butler, describes some of the troublesome aspects of sampling and the collection and preservation of larval and juvenile fish. In the next chapter, David Secor, John Dean and Elisabeth Laban give detailed information on various techniques used to remove otoliths of different types and sizes from larval and juvenile fish and how to prepare them for microscopic examination, either with a light microscope or scanning electron microscope. The fourth chapter, by Steve Campana, provides valuable guide-

lines for light microscopic examination and interpretation of otoliths, including a discussion of image analysis techniques. The fifth chapter, by Steve Campana and Cynthia Jones, presents an overview of statistical and data analysis techniques most appropriate to otolith microstructure data, particularly those recommended for estimating growth rates, mortality rates, and hatch date distributions. In the next chapter, Audrey Geffen reviews the various methods which are used to validate daily otolith increment deposition. Finally, in the last chapter, John Neilson provides some insight into the various sources of error that are inherent in otolith microstructure studies and suggests some ways to avoid them.

The field of otolith microstructure research has experienced phenomenal growth since the early 1970's and now forms the basis for hundreds of studies of early life history, age, growth, recruitment, migration, mortality, and stock structure. While the field continues to grow and evolve, there is no question that otolith microstructure examination is now an important and accepted technology in fisheries biology.

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CHAPTER 1
Development and Application of the Otolith Increment Technique

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History of the Otolith Increment Technique

Growth rings have been used to age fish for a long time. Annual rings in vertebrae were used to age eels as long ago as 1759 (Hederstrom 1959). Scales were first used to age fish in 1888 (Carlander 1987). Otoliths have been used to age fish since Reibisch first observed annular ring formation in *Pleuronectes platessa* in 1899 (as reported in Ricker 1975). However, counting annuli was not useful in estimating the age of young fish which haven't yet formed their first annulus or for tropical or deep-sea adult fish for whom growth is more constant and annulus formation less certain. The daily increment technique solved these problems by permitting the estimation of daily age.

The daily increment technique was developed in the early 1970's and has gained wide acceptance during the last twenty years. Pannella (1971) observed approximately 360 fine increments between the otolith annuli of temperate water fish. These increments were postulated to be daily changes in the microstructure of the otolith. Pannella (1974) observed what seemed to be daily increments in adult tropical fish and also observed that these patterns followed a larger, 14-day cycle that coincided with lunar behavior patterns. It did not take scientists long to see the value of this new technique. Within five years, Struhsaker and Uchiyama (1976) validated daily increment formation in young Hawaiian nehu by holding field-captured larvae and juveniles in the laboratory, developing growth curves and using this information to show growth rate differences between reef areas. During the same time, Brothers et al. (1976) realized the usefulness of this technique to determine age and timing of life history events in larval and juvenile fish. Their enthusiasm for the technique brought it to the attention of other investigators. Application of the otolith increment technique increased rapidly in the 1980's when published studies using the daily ageing method increased almost exponentially (Fig. 1).

Many of the currently employed applications of the technique were discovered in these early years, as were some of the difficulties. Taubert and Coble (1977) applied the technique to fresh water fish and found that shortened day length and low temperatures resulted in the apparent cessation of daily increment formation in sunfishes. In 1978, investigators reported that two important estuarine fish, Atlantic silversides and mummichogs, also formed daily increments (Barkman 1978; Radtke 1978). Barkman, comparing different otolith types, found that both the sagitta and lappillus could be used in ageing, but that the asteriscus was unreliable. This is not surprising, for in many species it is formed later in development. By 1979, Methot and Kramer had fit Gompertz growth curves to field-caught anchovy to obtain *in-situ* growth rates. Many of the issues that are covered in this monograph (reliability, choice of otolith, application in the field, formation of subdaily increments, lack of resolution) were encountered in these early studies. Although Pannella began using the technique to define annuli in adults, most investigators have since used the technique for the early life stages which previously could not be reliably aged.

Overview of Age and Growth Techniques

The knowledge of age and growth is fundamental to fishery science. In the early life stages, information on age structure can be used to clarify the effects of changes in the environment on growth and survival, and can result in an improved understanding of factors affecting recruitment success. In adults, knowledge of age and growth is used to determine the effect of fishing on the stocks, the efficacy of management policies, to understand life history events, and to maximize yield while still ensuring the future of the resource.

Age and growth can be determined by various methods: growing fish in confinement, raising fish from birth, examining hard parts which encode age information, and through biochemical tests. The usefulness of these methods depends of the habitat and the life history stage of the fish. In most instances, we need to determine the age of wild fish and to do so we

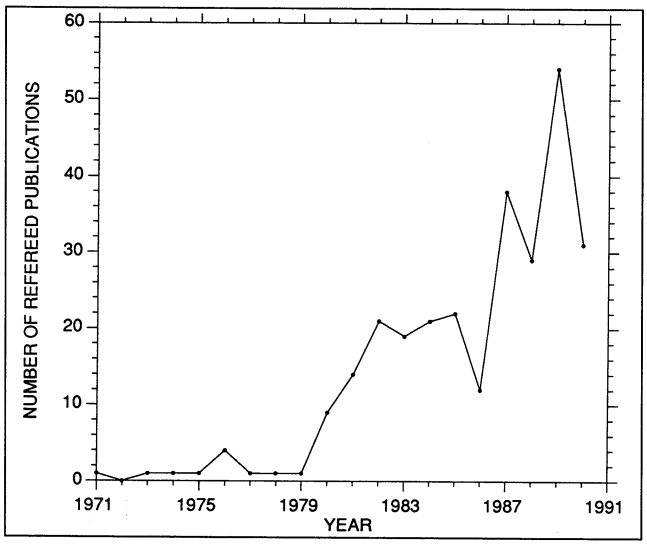

FIG. 1. The number of papers which use the daily ageing technique has risen dramatically in the past 11 years.

must examine calcified structures which contain age information. The structures which encode age information are bones (fin rays, vertebrae, cleithra, opercular bones), scales and otoliths. For most fish, otoliths have been the most reliable indicators of age. Otoliths show annual and, for younger fish, daily patterns and therefore form a permanent record of life history events. Otoliths also have an added advantage: experimental evidence shows no resorption of otoliths under stress conditions. This is not true of other hard parts. Scales have not proven to be as reliable because they can be lost and regenerated and because deposition ceases at older ages, thereby giving false age readings. In most instances, scales don't record daily growth patterns and can't be used to age fish under one year of age (for an exception see Szedlmayer et al. 1991). The prime advantage of using scales for ageing is that their removal doesn't cause death of the fish and extraction is quick. Other bones can sometimes be as accurate as otoliths, but their reliability is species specific and their use for daily age estimation has not been documented. It is apparent from the literature that ageing based on otoliths is often more reliable than other techniques. The difficulty in ageing young fish by any means other than daily otolith increments demonstrates why this technique is so widely used.

There are many important reasons to choose otolith microstructure to determine age and growth. The otolith is the only structure that consistently records daily events in the early life stages and annular events throughout life. With the advent of new computer image analysis systems, the task of increment identification, daily and annular counts and increment width calculations has been made quicker and more precise. With computer systems, data can be downloaded or plotted almost instantaneously. However, there are also drawbacks to otolith microstructure analysis. The fish must be killed to extract the otoliths. Also, otoliths can be difficult to read during certain life phases, such as at metamorphosis when accessory primordia may be laid down on the periphery of the otolith, and in older fish when increments are incomplete or compressed at the outer edges. Even with automation, the technique is time consuming, may call for specialized facilities (availability of computer-image systems and at times a scanning electron microscope) and does require training.

The daily increment technique is not limited to bony fishes. Volk (1986) has used the technique on statoliths of sea lampreys to determine annular age. Sea lampreys are agnathans and as such lack scales, bones and spines. Statoliths, which are analogous to otoliths, appear to be usable for age determination, and may prove useful for other invertebrates if daily ring deposition can be demonstrated. Hurley et al. (1985) have validated daily increment formation in the statoliths of short-finned squid and Jackson (1989) has used statolith microstructure to analyze the life-history events of a tropical cephalopod.

Information Obtained from the Otolith Increment Technique

The purpose of this section is to acquaint investigators using otolith microstructure with recent research results, beginning in 1985. For reviews of work accomplished prior to 1985, the reader is referred to Campana and Neilson (1985) and Jones (1986).

Age Determination

To estimate age from field-captured young fish, two pieces of information must be known: the age at first increment formation and the accuracy of increment counts. The age at first increment deposition is best determined through laboratory rearing. Alternatively, the age of increment initiation can be assumed to occur at hatch, at yolk-sac absorption, or at the stage at which a similar laboratory-reared species deposited its first increment. However, such an assumption may not be valid (Jones, 1986). If not, age estimates will be biased accordingly. The actual formation of daily increments has also been questioned. Several investigators have reported that increments were not formed daily or that deposition rates were related to growth rates (Geffen 1982; Lough et al. 1982; McGurk 1984). Geffen (1982) reported that increments were daily

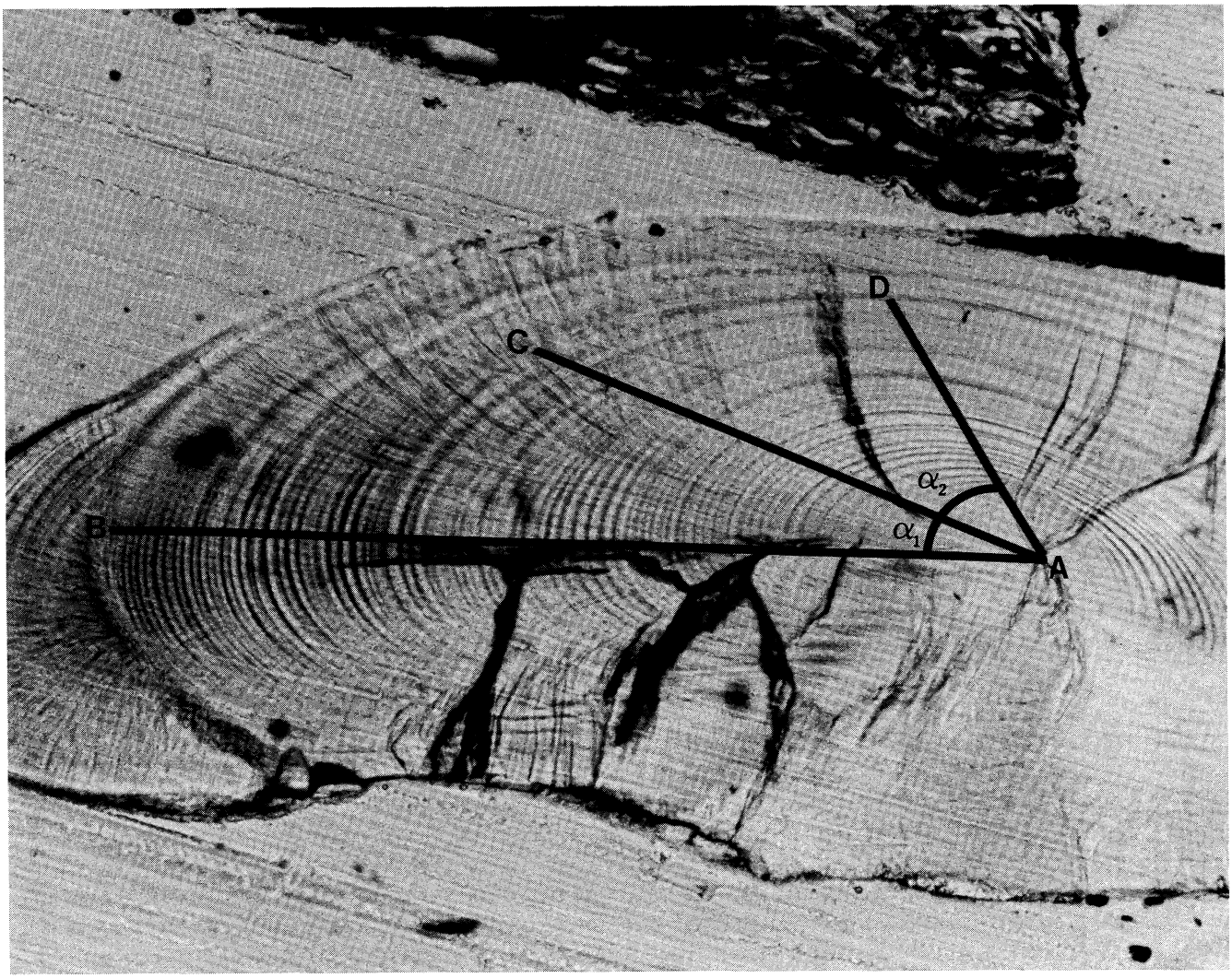

FIG. 3. Relationship between the width of a growth increment and the angle of the pathway along which the increment width is measured. Because $\alpha_1 \neq \alpha_2$ one increment width from line segment A–C is not equal to the increment's width from line segment A–D. Line segment A–B is the radius of maximum length.

cease at this stage. Often, this leveling off of growth can be an artifact of sampling bias due to escapement from nets of fast growing larvae or juveniles (see Butler, this volume). Differences in growth rate over the entire life history of the fish can theoretically be correlated with environmental parameters. In practice, this can be difficult to do, as there is intrinsic heterogeneity within populations and subtle environmental changes can have cumulative effects on growth. At various life stages, young fish are differentially sensitive to changes in their environment. Environmental changes which may have profound effects on the larvae may be better tolerated by juveniles.

Traditionally, growth has been estimated by following the peaks in length-frequency data through time. This is difficult to do for individual cohorts because of the continuous production of eggs within the spawning season, confounded by heterogeneity of variance in growth. McGurk (1987) found that in fortnightly hatching of Pacific herring, length frequency modes can be followed when daily age information derived from otoliths is also available.

In recent years, more sophisticated techniques have been applied to growth estimation analysis. These techniques have included time series analysis, development of an age-temperature growth model (Campana and Hurley 1989), and cohort tracking techniques, among others. For example, Gutiérrez and Morales-Nin (1986) used time series analysis to estimate and forecast growth of sea bass in the laboratory. Their results showed that the growth response changed over different life stanzas. Juvenile sea bass were able to integrate their growth response to temperature events over previous days, whereas larvae did not, but rather experienced an imme-

only when the growth rate of larval Atlantic herring was ≥0.4 mm/day. McGurk (1987) also observed this problem when fitting growth curves to data derived from field-captured Pacific herring larvae. Nondaily (less than daily) ring deposition has been reported in several other species (Methot and Kramer 1979; LaRoche et al. 1982; Campana 1984). In these studies, use of scanning electron microscopy (SEM) provided no confirmation of nondaily deposition. However, Campana et al. (1987) reported that growth rates of 0.4 mm/day would still lead to the formation of increment widths that would be below the resolving power of the light microscope. Indeed, Jones and Brothers (1987) showed that narrow daily increments, deposited in the otoliths of starved striped bass could not be seen under the light microscope but were observed under SEM. Accordingly, deposition of daily increments appears to be a universal phenomenon under perhaps all but the most severe conditions. (For more complete discussions of sub-daily increments and the limits of light microscopy, see papers by Campana and Neilson, this volume).

Growth

Daily otolith growth, as measured with the width of each growth band, can be used to infer daily somatic growth. Two important relationships are used in fisheries: (1) the allometric relationship (an allometric relationship can be either isometric (proportional) or curvilinear) between the size of the otolith and the size of the fish, and (2) the growth rate of the individual fish or population expressed as length or weight increase per day. If the investigator intends to use retrospective growth analysis he must first establish the allometric relationship between otolith growth and fish growth. The allometric relationship can be a simple linear relationship (isometric) or a more complex curvilinear relationship depending on the species and the life history stage of the fish. In theory, once the allometric relationship between otolith size and fish size is known, then size at prior age can be back-calculated from the otolith alone (Fig. 2). In reality, such back-calculations are more complex to execute. First, the proper back-calculation algorithm must be used (see Campana and Jones, this volume, for further discussion) to insure reliable estimation of retrospective lengths. Second, when the relationship between otolith growth and fish growth is decoupled for individuals experiencing poor growth (Secor and Dean 1989), a single allometric relationship can no longer be used. Third, in asymmetric otoliths, the relationship between otolith size and fish size must be related to a standardized transect on the otolith along which increment widths can be measured. However,

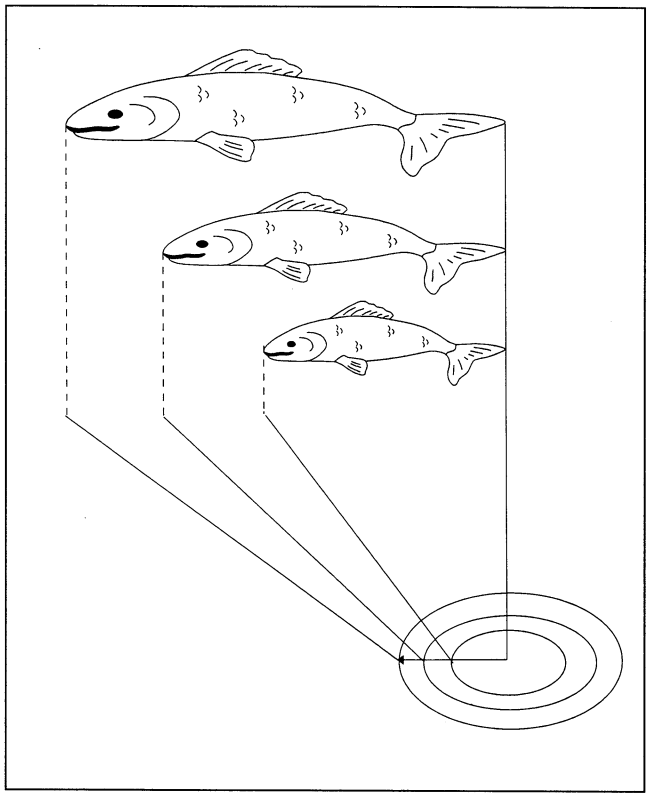

FIG. 2. Figure redrawn from Bagenal and Tesch (1978). A simple example using otolith back-calculations to estimate retrospective growth histories of fish.

the usual case is that no single transect will be readable throughout the entire length of the otolith section (see Campana, this volume). By choosing a series of alternate paths, increment widths become a function both of the allometry, and more importantly, the changing angles at which the increment width is measured (Fig. 3). It would then be necessary to standardize these widths using their geometric properties. Such approaches have not yet been used, and until these problems are solved, use of increment widths are problematic in some situations.

Population (somatic) growth curves can be developed once individual ages and lengths are known. Growth data for the early life stages are commonly fit with a greater variety of curves than adult growth data (which are usually fit using the von Bertalanffy growth curve). During the larval and juvenile stages, population growth rate can be expressed using exponential (Struhsaker and Uchiyama 1976), logistic or Gompertz (Brothers et al. 1976; Lough et al. 1982; Laroche et al. 1982), or linear (Townsend and Graham 1981; Jones 1986) models. It is important to be wary of fitting curves that indicate an upper asymptote during the early life stages as it is obvious that growth does not

diate change in their growth rate. An application of hatch date groupings into within-season cohorts allowed Crecco and Savoy (1985, 1987) to show the significance of short-term hydrographic and meteorologic events on growth and survival of American shad. Savoy and Crecco (1988) were able to separate density-dependent and density-independent growth, and thus determine that year-class strength for these fish was established in the egg and larval stages. Their analysis demonstrates the power of the otolith increment technique for examining stock-recruit relationships.

Stock-specific differences in growth rates are important in the study of population dynamics. Watanabe et al. (1988), with the use of daily increment ageing, showed geographic differences in the growth of Pacific saury. Previous studies had estimated sauries could reach five years of age. Watanabe et al. presented the first clear evidence that this species is short-lived, a fact which was not clear from examination of length-frequency data. Additionally, western stocks were shown to grow significantly faster than eastern stocks. This paper is a clear example of the benefit of using daily ageing techniques to refine growth estimates in short-lived or tropical fish.

Knowledge of growth can also be used in aquaculture. Knowledge of growth rates of cultured versus wild fish are useful to the mariculturist who uses this information to determine the feasibility and, potentially, profitability of rearing fish in captivity. It is particularly important to determine whether growth can be accelerated within the first year of life and which conditions enhance growth. The application of the daily increment technique is not absolutely necessary for these estimates, but can be used to refine them.

Growth increment widths are used to determine somatic response to habitat change. Campana and Neilson (1985) point out the difficulties in estimating individual increment widths and daily instantaneous growth. It is quite difficult to accurately measure narrow increments. As stated previously, larvae and juveniles have different capacities to integrate environmental change and their physiological response, hence sudden changes in increment widths are less understandable in juveniles than in larvae. Before predictions of body growth can be drawn from otolith growth data, the nature of the relationship between the two must be known for the entire life time over which inferences are to be drawn. With metamorphosis, for example, the otolith may change its axis of growth. This will result in a change in the relationship between otolith size and fish size. See Campana and Neilson (1985) for further discussion.

Life History Events

Because the otolith records daily events, many life history events such as metamorphosis or a change in habitat will be reflected by changes in the width of increments and in the elemental composition of otoliths. Several investigators have realized that the timing of these events and associated growth changes can be estimated by examining otoliths for check marks and changes in composition. One early example is the study of recruitment from the pelagic larval to the demersal adult reef habitat of the bluehead wrasse (Victor 1982). Prior to this study, it had been hypothesized that recruitment was limited by the rate of adult mortality. Victor, however, demonstrated that recruitment was sporadic and related to environmental events. He was able to show this by back-calculating hatch dates for juveniles and for adults one year later. Similarly, the timing of outmigration of anadromous fish can be determined by observing the change in otolith increment width, leading to an evaluation of enhanced growth and survival resulting from this habitat change. Neilson et al. (1985a) were able to discern the timing of migration of chinook salmon from river to estuary using this technique and to determine the resultant growth enhancement. They were able to support the hypothesis that growth rates were reduced by density-dependent factors which occurred in the estuary, a hypothesis that was otherwise only testable with extensive mark-recapture efforts. Examination of daily increments permitted them to back-calculate the growth of individuals and to correlate this with population size and environmental factors. This study extended the use of otolith microstructure beyond simple assessment of age and growth to the testing of hypotheses of population structure, migration and behavior.

Recent studies have used the elemental composition of otoliths to infer the timing of day-to-day environmental changes or changes of physical habitat. For example, the change in the calcium/strontium ratio can be used in combination with increment number to estimate the dates of migration of anadromous and catadromous species. Radtke (1984) and Mulligan (1987) used stable-isotope and elemental composition techniques in an effort to enhance the understanding of stock identification. Mulligan (1987) and Mulligan et al. (1987) used the elemental composition of otoliths to discriminate river of origin in young-of-the-year striped bass and white perch stocks. Recently, Kalish (1989) has demonstrated that the wave-dispersive electron microprobe can provide accurate measures of five elements (Ca, Sr, Na, K and S) and is superior to the energy-dispersive microprobe

for elemental analysis. Even though this procedure seems straightforward, it is as yet not fully tested for application to otoliths. For example, Radtke (1989) reported that a change in physiology could influence the chemical composition of otoliths. In cases where the elemental composition is both a function of the environment and the individual fish's physiology, this technique will have limited utility. As yet not enough work has been done to evaluate this technique.

Recruitment

With the advent of the daily ageing technique investigators can now estimate individual birthdates (data necessary in recruitment calculations) and survival-at-age. The work of Savoy and Crecco (1988) and Crecco and Savoy (1987) is an example of the sophistication and relevance of such analyses. Natural mortality was calculated and partitioned into density-dependent and density-independent components. In these papers and in earlier ones, the ability to age fish with the daily increment technique, and the ability to then assign these fish to within year-class cohorts, led directly to the calculation of mortality (decrease in numbers-at-age) and to the definition of a quantitative relationship between mortality and environmental events with cohort abundance. These studies were done under especially amenable conditions; anadromous species are easier to assess than oceanic pelagics. Houde (1987), in his synthesis of recruitment variability, states that accurate calculations of mortality not only demand knowledge of age, but also demand unbiased and representative sampling of the life stages that are being studied. Savoy and Crecco (1988) worked in a river system which was amenable to such sampling. The marine and estuarine environments tend to be more heterogeneous and because fish change physical habitat as they mature, representative sampling can be difficult. Houde (1987) suggested that growth is intimately related to mortality and that growth estimates, which rely only on a knowledge of age and do not require estimates of abundance, can be used as surrogates for mortality estimates. However, this hypothesis has not yet been tested in a detailed manner.

Stock Identification

It is possible that otolith microstructure may be a useful tool for separating sympatric populations of juvenile fish. Neilson et al. (1985b) used the number and position of primordia and the nuclear core size at first increment formation as criteria to separate stocks of juvenile steelhead and rainbow trout. Their results showed that temperature had a greater effect than stock origin. Although these results are discouraging with this species, the technique may be useful for stocks which develop under similar temperature regimes. For example, Campana et al. (1989 a,b) used otolith microstructure to monitor larval drift and thus infer stock structure.

Technical Considerations

This section will provide a brief overview of some important technical topics and issues which should be considered in preparing and executing studies using the otolith increment technique, many of which are covered in greater detail in the other papers in this monograph. Researchers who incorporate this information into their work will be rewarded by improved results.

Importance of Establishing Initial Objectives

A clear expression of objectives is fundamental to the design of any study involving otolith microstructure examination and analysis. The objectives should be specific and detailed. Once objectives are stated, the desired level of precision and the required sample size necessary to achieve it will be calculable. This prior planning is obviously valuable, but not always done. In age and growth studies, for example, the nature of the inquiry will affect the methods that are used and the number of samples that are needed. In regards to accuracy, mortality can be calculated with knowledge of relative age, without knowing absolute age (Essig and Cole 1986). In this case, if the estimate of age-at-first increment formation is wrong, the error will be constant for all calculations and will not alter the relative change in numbers-at-age. However, the timing of life history events relies on accurate estimation of hatch age (Brothers and McFarland 1981) and the age of first increment deposition must be known accurately. The need for a specified level of precision will dictate the number of samples that need to be taken, and the degree of replication of measurements that are necessary. The intrinsic variability in age and growth characteristics of the population to be studied, along with the magnitude of the differences that must be detected, will dictate the number of samples that are necessary. Careful specification of experimental design will allow calculation of the power of the test (Rice 1987; Peterman 1990). Developing hypotheses, setting objectives, and following through with careful experimental design are all quite time consuming. However, such an approach will maximize the chance of success in the research endeavor.

Importance of Setting Uniform Standards

There are advantages in standardizing research procedures. The importance of establishing defined stan-

dards has been discussed by several authors (Beamish and McFarlane 1983; Campana and Neilson 1985; Jones 1986; Campana et al. 1987). The advantages of standardized methodology are: potential increase in precision, ease of intercomparison and comparability of results. As an example, there are several published papers which postulate non-daily deposition of otolith increments (McGurk 1987; Geffen 1982; Lough et al. 1982; Laroche et al. 1982). McGurk (1984) found that the rate of ring deposition was correlated to growth rate in larval Pacific herring and that daily increment deposition occurred at growth rates ≥ 0.36 mm per day. This was a carefully done study, but was limited to the resolving power of light microscopy. In reading the primary literature, one is left with some confusion concerning daily versus non-daily increment formation in this species. If it were common practice to use higher resolution techniques (such as SEM) to confirm the presence of apparently non-daily increments, this confusion could have been avoided. Several authors (Jones and Brothers 1987; Campana et al. 1987) have recognized the potential for scanning electron microscope validation when apparently non-daily increments are noted with the light microscope. SEM may be feasible for many species and if so should be used in these cases to verify apparent non-daily deposition. The standard use of SEM when conclusions of non-daily deposition are reached with light microscopy would minimize confusion. However, SEM is not feasible for all species and life history stages.

Collection of Materials

The collection methods and sampling procedures used in an otolith study profoundly influence the representativeness of the sample and the validity of conclusions which can be drawn. For example, the otolith increment technique is often used to estimate growth and mortality. When these are the aims of a study, then the age range over which the samples are obtained restricts inferences to these age groups. If the objective of the collection is to assess mortality or growth of the population, then the sample will have to be random and unbiased, including individuals in the same proportion of abundance as they occur in the population. If the collection is intended to produce an age-length key, then age groups will have to be represented in sufficient abundance to produce reliable results, not necessarily in proportion to their abundance. Preservation will affect length measurements and different preservation techniques will affect these measurements differently. Mesh size and gear type will differentially select certain lengths over others and different gears will sample the populations differently. These topics are covered in the paper by Butler in this volume.

Preparation of Otoliths

Proper otolith preparation will enhance the investigators ability to see relevant microstructure. Selection of techniques used in preparing an otolith for microstructural examination is dependent, in part, on its size and on the type of analysis which is intended. It often comes as a surprise to the neophyte that otolith size can be species dependent as well as age dependent. Big fish do not necessarily have big otoliths. Once fish have been collected, the investigator needs to match the removal technique with otolith size. A beginning investigator, having read a paper which describes the apparent ease of handling otoliths 200 μm in diameter, can be unprepared to handle larvae such as a day old striped bass and remove otoliths which are only 10 μm in diameter. Additionally, samples destined for SEM examination will be handled differently than those to be prepared for light microscopy only. The investigator needs to be prepared for these contingencies. Methods of preparing otoliths are covered in detail in the paper by Secor et al. in this volume.

Interpretation of Otolith Microstructure

Estimations of increment count and increment width, measurements fundamental for modeling growth, rely on the investigator's ability to interpret otolith microstructure. Important considerations include the choice of the "right" counting and measuring axis, selection of criteria for defining a daily increment, and image optimization through choice of a microscope and image analysis system.

Difficulty can sometimes be encountered in trying to differentiate daily increments from other structures. There are specific criteria that the "increment" must meet. The literature presents the simple case of opaque/translucent zones. Increments, however, do not always conform to the simple light band, dark band model, but can have varying dark and gray areas in the middle. For a comprehensive summary of otolith microstructure, the reader is referred to Campana and Neilson (1985) and to the papers by Secor et al. and Campana in this volume.

Another difficulty in interpreting otolith microstructure is the presence of "subdaily" increments, which are formed at a greater than daily rate. Campana (1984) studied the effect of manipulated environments (constant light; fluctuating light; fluctuating temperature) on the formation of increments in

larval and juvenile plainfin midshipmen and found that subdailies occurred throughout both stages, but were more likely to occur in young larvae. This lability in entraining a diel rhythm was corroborated by studies of other vertebrates. Campana offers an excellent suggestion of using known-age/otolith size data to help forecast the likely width of increments, then using this information in determining the probable width of increments when it is hard to discern daily from subdaily increments. In addition to Campana's studies (1983, 1984), several other authors have tried to deal with this problem (Pannella 1980; Neilson and Geen 1982). Uchiyama et al. (1986) were able to differentiate subdailies in dolphins by changing the optical focus. Eckmann and Rey (1987) have demonstrated that subdaily increments in coregonids can be produced by manipulating environmental conditions in the laboratory. Research to date has not shown whether this is a problem for fish of all ages or only during certain life history stages.

It is usually easier to discern increments in field-captured larvae and juveniles. The constancy of the laboratory environment tends to produce fainter increments. However, even the clarity of field-captured young fish otoliths depends on how well they are prepared (see Haake et al. 1982 and Secor et al., this volume). Staining techniques have been developed to enhance annuli (Bouain and Siau 1988). Such techniques have not yet proven useful in enhancing microstructural increments. There are also limits to the resolving power of the light microscope, even with the best preparations (clean otoliths, ground to the core, well etched, blue light source), because the light microscope is limited by the size of the smallest wavelength of the visible spectrum (see Campana, this volume). Very fine increments, smaller than 1μm, are usually only a problem in slow growing fish. However, if fine increments are not recognized, this will lead to the incorrect conclusion that increments are non-daily, underestimate age, and overestimate growth rate. These fine increments are visible with SEM, but very few studies (Jones and Brothers 1987; Castonguay 1987) have used SEM when increments appear to be non-daily or growth too fast.

Validation of Daily Increment Formation

Beamish and McFarlane (1983) emphasized the need to validate age in studies of adult fish which require age-based growth and mortality estimation. This requirement also applies to the use of daily increments, but with the added difficulty of validation in the first months of life. Some of the standard techniques used for adults, such as marginal increment analysis, are rarely used with daily increments because of the difficulty of resolving marginal increments, while other techniques such as mark and recapture are made far more difficult by the high natural mortality which occurs during the larval stage in many species. Chemical marking techniques can be used effectively in both lab and field situations. The paper by Geffen (this volume) discusses the techniques which are used to validate daily increment formation in the early life history stages.

Sources of Error

An important consideration in any carefully designed otolith study is the evaluation of potential sources of error that have been introduced during collection, preparation, or interpretation of material. Under certain circumstances bias can be corrected (see Campana et al. 1987 for a discussion of how to correct for unresolvable increments), but in other circumstances the magnitude and direction of the bias may not be estimable and may cause fallacious conclusions to be reached. It is a good practice to first be aware of potential errors which can occur and guard against their introduction. The paper by Neilson (this volume) discusses these sources of error and recommends procedures for avoiding them.

Statistics and Data Analysis

Statistical analysis is often conducted after completion of the research study, with no prior planning. Yet, to insure success, experimental design and analysis should be considered carefully as a first step, as the foundation of the research. Proper preparation entails appropriate random or systematic sampling, estimation of sample size, and selection of the appropriate analytical tests prior to implementation of research.

Selection of the appropriate statistical test is important. For example, precision and accuracy of counts and increment widths need to be tested. When comparing the precision among counters and accuracy of increment counts between counters, it is not appropriate to use simple *t*-tests. A multiple series of *t*-tests will yield significant results in 5% of the studies (at an α level of 0.05) by random chance alone, when no real biological differences exist. Additionally, since variances are usually related to the magnitude of the mean age, real differences can be obscured when sample sizes are small. Analysis of variance (ANOVA) or analysis of covariance (ANCOVA) are far more powerful and appropriate tests. One approach to testing for differences in precision has been presented by Chang (1982).

Proper definition of the sampling frame and the sampling unit for otolith studies will help avoid incorrect

inference and pseudoreplication (*sensu* Hurlbert 1984). The sampling frame can be defined as the entire population of interest. Say we are interested in understanding the growth of bay anchovy in Chesapeake Bay. The spatial limits of the sampling frame would be the entire bay and the temporal aspect would be the time period over which the age group occurred in the bay. If we are interested in the earliest life period the sampling frame would consist of all bay anchovy of, say, up to one month of age. Theoretically, the entire area of the bay where these fish occur must be sampled, unless a smaller region can be demonstrated to represent the entire bay. The elucidation of the sampling unit is more complex. If one could choose any individual fish with equal probability to any other fish, then the sampling unit is the individual fish. If not, then the sampling station or perhaps the neuston tow might be the sampling unit, depending on the sampling design. Determination of the sampling unit is not a trivial exercise because the sampling unit determines the degrees of freedom used in statistical tests. For example, all otoliths from one fish are related to each other. When two otoliths are examined from the same fish, in statistical terms, only one observation has been made. The average of the two otolith counts would be the observation. By understanding the sampling unit we also avoid the problem of pseudoreplication (Hurlbert 1984). Pseudoreplication occurs when the observations or measurements are not independent from each other and are not true replicates.

Several categories of analysis are common to otolith daily increment analysis: modeling of growth, back-calculation of hatch dates, and estimation of mortality. These analytical methods are covered in detail by Campana and Jones, in this volume.

Looking Ahead

Over the past 10 years, the otolith increment technique has been used with increasing sophistication not only to determine age and growth, but to unravel conditions affecting recruitment, the timing of life history events and even stock differentiation. Recruitment has historically been examined from the standpoint of mortality rate in relationship to the ambient environment and, recently, the relationship between growth, mortality and year class strength (Houde 1987). While the second approach has been widely discussed, it has not been well tested. Otolith analysis provides the means by which this approach can be tested. The otolith increment technique has already been applied to the issue of larval drift and stock identification (Campana et al. 1989 a,b; Thresher et al. 1989), but these are only the first of many studies to be expected. The limitations on such studies are defined not so much by the difficulty in obtaining age-structured data, but by its incorporation into an appropriate spatial analysis. An increasingly popular use of the otolith increment technique is the analysis of hatch date distributions in relation to dates of egg production; results of this type of study provide a means for evaluating which factors influence the survival of young fish. With the recent discovery of growth rate effects on the fish:otolith relationship (Secor and Dean 1989), quantification of increment width autocorrelation (Gutiérrez and Morales Nin 1986), and a new approach to growth back-calculations (Campana 1990), there is a reasonable potential for vastly improved back-calculations at the daily level.

Recent technological developments promise major advances in the interpretation of otolith microstructure. One such recent development is the use of the electron microprobe to quantify the elemental composition of otoliths. Elemental analysis may be used in the future to link the larval fish to its natal water mass, and to define environmental conditions during previous growth phases (Radtke 1984; Kalish 1990). Elemental analysis can be used for stock identification, while isotope analysis can be used in temperature and food web studies. These techniques rely on the assumption that the otolith records conditions under which the fish grew, perhaps even on a day-to-day basis. However, this technique has yet to be refined sufficiently for routine reliable use (Kalish 1989). New advances in underwater acoustic methods may one day permit the fishery scientist to accurately estimate the size and extent of larval fish aggregations, which, when combined with estimates of age, could improve our knowledge of mortality during the early life history stages. In the interim, chemical mass-marking of the otoliths of hatchery-reared fish is providing new opportunities for determining short-term in-situ mortality rates. On a more speculative front, recent advances in instruments such as scanning tunneling microscopes and NMR scanners could conceivably be used to map (in 3-D) the microstructure of an otolith without any prior sample preparation.

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CHAPTER 2
Collection and Preservation of Material for Otolith Analysis

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Introduction

The methods used for collecting, handling and preserving fish for otolith analysis vary, depending on the nature of the material that is required and the objectives of the study. Larval and juvenile fish are collected using different methods than those used to collect adult fish; handling and preservation techniques are similar. Problems associated with gear selectivity and shrinkage are common to all life stages. Improper preservation techniques can cause otolith damage.

Sampling and Gear Selectivity

The methods used to collect adult fish are as diverse as the methods of fishing. These methods include spearing, traps and weirs, hook and line, trolling, night lighting, bottom trawls, pelagic purse seines, gill nets, lift nets and pumping as well as nonconventional methods such as poisons and electrofishing. These methods may be employed by the investigator or the investigator may simply sample the catch from fishermen who use these techniques. All of these methods are to some degree selective in terms of the species and size of fishes collected. Spear fishing and baited traps tend to select for large individuals. Hook and line and set lines select large individuals (Klein 1986), but selectivity is also a function of hook size (Ralston 1982, 1990) and bait size (Løkkeborg 1990). The selectivity of nets is well known and is often used as a management strategy to regulate the harvest of fish. The size of the mesh opening largely determines the size of fish collected. Gill nets, for example, are more size selective than other nets such as tangle nets. The mesh size of bottom trawls determines the minimum size of fish collected while avoidance determines the maximum size (Hemmings 1973). Other techniques such as poisons (Weinstein and Davis 1980) and electrofishing are not selective if all of the stunned fish are collected.

Sampling gear used to collect larval and juvenile fishes are also size selective. The minimum size of larvae collected in plankton nets is determined by mesh size and extrusion (Lenarz 1972; Colton et al. 1980; Lo et al. 1989). The maximum size is determined by net avoidance (Lenarz 1973; De Ciechomski and Sanchez 1983; Somerton and Kobayashi 1989) and time of day (Bridger 1956). Large-volume plankton pumps and plankton nets sample small fish larvae equally efficiently (Taggart and Leggett 1984).

The catchability of larval fishes affects apparent growth and mortality rates estimated from individuals collected in plankton nets (Morse 1989). Estimated growth rates of larvae collected in plankton nets are strongly influenced by the size and age of the largest individuals. Because avoidance is a function of size and condition, there is a strong possibility that captured individuals will be the slowest growing members of their cohort. More research needs to be done comparing the estimated growth rates of large larvae collected in plankton nets and similar sized larvae collected by small mesh midwater trawls.

The degree of size selection is important in age and growth studies because growth varies among individuals of a cohort. Collecting samples using highly selective sampling gear will reduce the observed variability in age and growth. This is particularly troublesome if the objective of the study is to analyze the effect of environmental variability on growth or to back-calculate dates of hatching. As an example, consider a study designed to monitor changes in the hatch date distribution of a cohort as it progresses from the pelagic egg stage to that of a bottom-dwelling juvenile. Representative sampling of the eggs and early larvae is relatively straight forward with a gear such as a bongo net. However, once the fish reach the late larval stage, a severe sampling problem exists; how can all members of a cohort be representatively sampled when: (1) the slow-growing fish are still small pelagic larvae and can be captured only with small-mesh gear, (2) the intermediate-growth fish are too large for bongo nets, but just right for pelagic trawls such as Tucker trawls, and (3) the very fastest-growing fish have metamorphosed to a bottom-dwelling form and can be captured only with a bottom trawl? While different gear types can be used to representa-

tively sample each size range, how can these samples subsequently be recombined to form one sample from which the hatch date distribution for the entire cohort can be determined? Unless the catchabilities of each gear type are identical (or known) and the numbers at each size and in each habitat can be calculated, data derived from the various samples cannot just be combined. Yet, if hatch dates from only one gear type are examined, the hatch date distribution will be skewed either to slow-growing (early hatch date) or fast-growing (late hatch date) fish. There is no easy answer to this problem. However, the problem must be recognized before interpretation of the data is begun, and preferably at the sampling design stage.

Preservation and Shrinkage

Sample preservation techniques differ among life stages. Adult and juvenile fishes may be frozen for later analysis, but if freezer space is limited otoliths may be removed at sea. Larvae collected with plankton nets are usually preserved in the field. Fish which are not processed immediately or which are preserved or frozen can be expected to shrink before measurement and otolith removal.

Plankton samples are usually preserved at sea and larval fish removed at a later time. Plankton samples may be preserved with 5% formalin buffered by marble chips, sodium borate or sodium carbonate or preserved with 95% ethanol. Although otolith dissolution is a problem with formalin preserved samples (see below), Ré (1983) reports obtaining intact otoliths from plankton samples preserved with formalin. Brothers et al. (1976), Methot and Kramer (1979), and others recommend alcohol (ethanol) preservation. The ethanol used to initially preserve samples should be changed within 24 hours because water in the tissues of gelatinous plankton or fish flesh quickly dilutes the preservative. Ethanol solutions less than 80% are too acidic for safe larval otolith preservation. In addition, ethanol produced from petrochemicals may be contaminated with sulfuric acid, and denatured ethanol has been altered to make it unpotable. For these reasons the pH of the preservative should be checked and, if necessary, the ethanol should be buffered with 20 nM tris(hydroxymethyl)aminoethane (such as Sigma 7-9). Regardless of what type of preservative is used, the pH of samples kept for long periods of time should be monitored. It is also advisable to avoid placing too many specimens in the same sample container since they may not be properly preserved (this is especially true of ethanol). Another problem to avoid is the evaporation of preservative from sample containers which are not completely sealed.

Otolith dissolution is a problem with samples preserved in either alcohol or formalin. Nothing is more frustrating than to spend a month at sea making collections and to find later that all of the otoliths are dissolved, etched, pitted or discoloured. Damaged otoliths may provide some information after etching, but seldom a complete record. Although buffering helps, preserved samples may still become acidic with time. Therefore it is strongly recommended that larval fish destined for otolith analysis be separated from the rest of the sample as soon as possible after collection. Since small otoliths have a higher surface area: volume ratio than large otoliths, larval otoliths will dissolve more rapidly than juvenile otoliths. Removing the otoliths from the larvae as soon as possible and storing them, either dry or mounted on microscope slides, eliminates the possibility of lost time and effort due to otolith damage.

Preservation, whether with chemicals or freezing, and handling affect fish size. Upon death, osmoregulatory functions cease; fish in seawater begin to lose water to the medium, while fish in freshwater begin to absorb water. Consequently, marine species shrink after death; preservation and freezing cause additional shrinkage. The degree of shrinkage varies with species, type and strength of preservative, the time between death and preservation, and the size of the fish (Table 1). Fish larvae with unossified skeletons shrink most. Handling and the time from death to preservation affect shrinkage; the degree of shrinkage itself is a function of fish size (Theilacker 1980). Because autolysis and shrinkage begin as soon as larval fish die, it is recommended that the duration of plankton-net tows used to collect larval fish for otolith analysis be limited. It is also important to preserve the sample as soon as possible after it is collected. Use of standardized collections procedures (e.g., duration of tow, time between end of tow and preservation) is also recommended.

Leak (1986) used the relationship of otolith size to live fish length measured in the laboratory to correct for shrinkage of field collected larvae. This approach has promise but must be used with caution because Reznick et al. (1989) found larger otoliths relative to standard length in slower growing guppies in the laboratory. Some have suggested that stunted fish have larger heads (and therefore probably larger otoliths) relative to body length than faster growing fish. Larval herring from the same eggs reared in a mesocosm, but experiencing different growth rates (Wespestad and Moksness 1989), had otoliths of different relative sizes (Erland Moksness, pers. comm.). Butler (1989) found different allometric relationships of otolith size and fish length from juvenile anchovies

TABLE 1. Shrinkage correction factors for different species of fish.

Species	Preservative	Treatment	Percent shrinkage
Anchoa mitchilli[1]	95% ethanol	Bongo net	22–30
Catostomus commersoni[2]	Davidson's B		3.2
Clupea harengus[3]	2% formalin	15 ppt SW	11–12
	2% formalin	34 ppt SW	14–16
	4% formalin	15 ppt SW	11–14
	4% formalin	34 ppt SW	9–13
	10% formalin	15 ppt SW	5–7
Clupea pallasi[4]	4% formalin	—	8
	4% formalin	Net	13–43
Dicentrarchus labrax[5]	4% formalin		5.3
	70% ethanol		5.6
	4% formalin	Net 5 min	12.4
	70% ethanol	Net 5 min	18.9
	4% formalin	Net 10 min	15.8
	70% ethanol	Net 10 min	23.1
	4% formalin	Net 15 min	19.8
	70% ethanol	Net 15 min	24.2
	4% formalin	Net 20 min	22.4
	70% ethanol	Net 20 min	26.0
Engraulis mordax[6]	5% formalin		8
	80% ethanol		0
	Bouin's		8
	—	Net	8–19
Esox americanus[2]	Davidson's B		0
E. lucius[2]	Davidson's B		3.5
E. lucius[7]	Freezing		5.4
Etheostoma nigrum[2]	Davidson's B		0
Gadus morhua[8]	95% ethanol	—	0
	—	Death	30–40
Limanda ferruginea[9]	Ice	Death	1.5
Merluccius bilinearis[10]	4% SW formalin		3.4–4.3
	95% ethanol		4.8–7.0
	Freezing		1.4
M. productus[11]	3% formalin		4.5–4.6
	80% ethanol		4.4
Onchorhynchus nerka[12]	10% formalin		4.6–6.8
O. gorbuscha[12]	3.8% formalin		2.3–4.1
O. keta[12]	3.8% formalin		1.8–4.8
O. nerka[12]	3.8% formalin		2.0–4.3
Paralichthys lethostigma[14]	4% FW formalin	1 h–6 yr	0–6.6
	4% SW formalin	1 h–6 yr	5.2–9.4
Parophrys vetulus[15]	80% ethanol		3.2
	10% formalin		5.1
Perca fluviatilis[7]	Freezing		1.7

Continued

TABLE 1. Shrinkage correction factors for different species of fish. *(Cont'd)*

Species	Preservative	Treatment	Percent shrinkage
Pimephales notatus[2]	Davidson's B		4.3
Pleuronectes platessa[16]	4% formalin		2.4–2.7
Psuedopleuronectes americanus[17]	4% formalin		3.7
Rhinichthys atratulus[2]	Davidson's B		3.1
Trachurus symmetricus[6]	Bouin's		8
Thunnus albacares[18]	Freezing brine		0.9–2.6
Siganus caniculatus[19]	4% SW formalin		0
S. guttatus[19]	4% SW formalin		0
S. vermiculatus[19]	4% SW formalin		0
Sphyraena argentea[6]	Bouin's		8

[1]Leak (1986); [2]Leslie and Moore (1986); [3]Blaxter (1971); [4]Hay (1981); [5]Jennings (1991); [6]Theilacker (1980); [7]Treasurer (1990); [8]Radtke (1989); [9]Lux (1960); [10]Fowler and Smith (1983); [11]Bailey (1982); [12]Burgner (1962); [13]Parker (1963); [14]Tucker and Chester (1984); [15]Laroche et al. (1982); [16]Lockwood (1973); [17]Pearcy (1962); [18]Anonymous (1974); [19]Rosenthal and Westernhagen (1976).

collected during EL Niño years and normal years. If otolith size is used to calibrate shrinkage of larval fish, it is recommended that the allometric relationship be determined from live larvae.

Shrinkage is rarely documented but will affect growth rates calculated from otolith information. Before combining data from fish preserved by different methods, corrections must be made for shrinkage (Watanabe et al. 1988). Owen et al. (1989) found that systematic variation in handling time affected growth rates calculated from daily increments in the otoliths of larval northern anchovy collected at two different localities. These results indicate that as much care must be devoted to measuring or estimating live fish size as is devoted to accurately determining the number and, in the case of back-calculated growth, the width of daily otolith increments.

Otolith Storage

Otolith storage procedures vary with life stage and the method of analysis. Adult otoliths are often stored dry in numbered trays, in labeled vials, envelopes or capsules. Adult and juvenile otoliths may also be stored in liquid filled vials. The liquid may be water, ethanol, or glycerin and water. Thymol is added to water or glycerin solutions to prevent the growth of mold (Chilton and Beamish 1982).

Otoliths for daily increment analysis may be stored dry or mounted on labeled microscope slides for later analysis. Mounting media should be clear and have optical properties near that of glass. Mounting media that harden completely allow polishing of the otoliths. Small otoliths may be stored dry in covered trays used to store foraminifera and coccoliths. Some investigators store otoliths in oil on microscope slides (Brothers 1987), but the slides must be stored in a horizontal position.

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CHAPTER 3
Otolith Removal and Preparation for Microstructural Examination

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Introduction

Over the past 20 years, the number of published otolith microstructure studies has grown exponentially (see Jones, this volume). In a literature review, we found that otolith studies exist for 50 families and over 300 species of fishes and squid (Table 1). Over 100 different approaches to examining otolith microstructure have been described for these species. Despite routine use of otolith microstructure in fishery research, the diverse methods used to remove and prepare otoliths have received little attention, although several authors have provided detailed description of specific techniques (Pannella 1974; Wild and Foreman 1980; Neilson and Geen 1981; Haake et al. 1982; Brothers 1987; Secor et al. 1991). In this chapter, we categorize basic approaches to otolith removal and preparation for microstructural examination.

Preparation of otoliths is labor intensive craft-work. The methods used are closely allied to those in metallurgy. Preparation can be tedious, and requires persistence and determination in addition to creativity. The aims of this paper are: (1) to introduce the many methods researchers have used, (2) to emphasize the importance of planning protocol to specific applications, and (3) to suggest some methodological standards. Detailed methods are provided on otolith removal, cleaning, storage, sectioning, polishing, and etching. Otolith position and morphology are also introduced to aid in otolith removal and choices of otolith type and polishing plane.

We want to emphasize that there are many alternative methods to those highlighted in this chapter (see Pannella 1980b; Neilson and Geen 1981; Wild 1982; and Brothers 1987). To facilitate a review of other laboratories' techniques, tables are provided listing 171 published methods, by author and species (Tables 1 and 2). In several sections of this paper we have found it useful to apply technical descriptions to striped bass, *Morone saxatilis*. Other fishes will have different methodological requirements and we give several examples of these.

Position and Morphology of Otoliths in Fishes

Three pairs of otoliths occur in teleosts and they each differ in location, function, size, shape, and microstructure. These differences influence protocol decisions. A knowledge of vestibular anatomy and otolith morphology is important in removing otoliths efficiently, choosing otolith type and polishing plane, and accurately documenting methodology.

Position

The three pairs of otoliths are most commonly termed the lapilli, sagittae, and asterisci (Table 3, Fig. 1). Many synonyms have been used to describe each pair (Table 3) but we recommend that otoliths be termed as listed above, taking care to determine and then communicate clearly which otolith is used. In studies which use the generic term "otolith" without precise definition (9% of reviewed reports, Table 1), it is difficult to evaluate and replicate protocols.

The anatomy of the vestibular apparatus, and the otoliths which it contains, shows bilateral symmetry, except in some flatfishes (Nolf 1985; Sogard 1991). The vestibular apparatus is divided into dorsal sacs (pars superior) and ventral sacs (pars inferior). The lapilli are located most anteriorly in the pars superior. The sagittae and asterisci are typically located in close proximity within the pars inferior and are medial and ventral to the lapilli (Fig. 1). The individual sacs (vestibules) which contain the three pairs of otoliths are termed the utriculus, sacculus, and lagenus for the lapillus, sagitta, and asteriscus, respectively (Fig. 1, Table 3).

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TABLE 1. Review of otolith preparation procedures from otolith microstructural studies. Literature selected includes peer-reviewed papers from primary journals and proceedings of age and growth, early life history, and biomineralization symposia. Review articles were not included. Procedures are listed by author since most variation in technique can be ascribed to individual investigators.Several authors use different techniques for different size otoliths. These techniques are differentiated by "small" and "large," refering to small (eg. larval) and large (eg. juvenile) otoliths, respectively. Symbols: S = sagitta; L = lapillus; A = astericus; Stat. = statolith (squid); - = procedure not used; + (?) = procedure used but not documented; ? = no documentation on procedure; s = seconds; m = minutes; h = hours. Underlined otoliths indicate which otolith was predominately used for analysis.

Author(s)	Species	Otolith	Medium	Sectioning, Polishing	Clearing Cmpd	Etching
Alhossaini and Pitcher 1988	*Pleuronectes platessa*	S	epoxy	800, 1200 grit paper, carborundum aluminum oxide slurry	immers. oil	—
Bailey 1982	*Merluccius productus*	?	Protex or Euparal	—	—	—
Barkman 1978 Barkman and Bengston 1987	*Menidia menidia*	SA	Euparal (small) Flotex (large)	see Taubert and Coble 1977	?	1% HCl (20 s)
Beckman and Dean 1984	*Leiostomus xanthurus*	S	Spurr	see Haake et al. 1982	—	EDTA-GA (5 m)
Boehlert and Yoklavich 1985	*Anoplopoma fimbria*	S	histol. mounting medium	diamond saw section, ground, double polish (large)	—	—
Bolz and Lough 1983[1] 1988[2]	*Gadus morhua* *Melanogrammus aeglefinus*	SLA	Permount (small) epoxy resin (large)	1. 1 µm diamond cmpd 2. 600 grit carborundum paper (large)	Permount	1. 10% HCl (5–15 s) 2. 6% EDTA, pH 7.0 (? m)
Bradford and Geen 1987	*Oncorhynchus tshawytscha*	S	crystal bond	see Neilson and Geen 1982	—	
Brothers et al. 1976	*Engraulis mordax* *Leuresthes tenuis* *Morone saxatilis* *Clevelandia ios* *Ilypnus gilberti* *Ouietula y-cauda* *Merluccius angustimanus* *Merluccius bilinearis*	?	polyester resin	400, 600, 900 grit silicon carbide or aluminum oxide, polish with diamond paste (1 µm) (small) glass plate with silicon carbide slurry (large)	immers. oil	0.1 N HCl (? m)
Brothers and McFarland 1981	*Haemulon flavolineatum*	L		See Brothers et al. 1976		—
Brothers et al. 1983a	*Thunnus thynnus*	SL		See Brothers et al. 1976		—
Brothers et al. 1983b	12 tropical families, including over 38 spp., see their index.	SL		See Brothers et al. 1976		?
Butler 1989	*Engraulis mordax*	?	Eukitt	15 to 0.3 µm lapping film	—	—
Campana and Neilson 1982	*Platichthys stellatus*[1]	S	instant glue	30 to 0.3 µm lapping film with aluminum oxide on rotator, jig assisted (Neilson and Geen 1981)[2]	—	1. 1% HCl (90 s) 2. 2% HCl (16 m) 0.1 M EDTA (10 m) 3. 2% HCl (2 m) 0.1 M EDTA (4 m) 4. 2% HCl (2–4 m) 0.1 M EDTA (3–5 m) 5. 0.1 M EDTA (2–4 m)
Campana 1983a	*Oncorhynchus kisutch*[1]	S				
1983b	*Platichthys stellatus*[2]	S				
	Salmo gairdneri[3]	S				
1984a	*Platichthys stellatus*	S				
1984b	*Platichthys stellatus*[4]					
1984c	*Porichthys notatus*	S				
Campana et al. 1987	*Clupea harengus*[5]	S				
Campana and Hurley 1989	*Gadus morhua* *Melanogrammus aeglefinus*	S S	Krazy glue	lapping film (3–30 µm)	—	—
Campana et al. 1989	*Gadus morhua*	L				
Castonguay 1987	*Anguilla rostrata* *Anguilla anguilla*	S S	epoxy	hand-grinding on very fine sandpaper	—	5% EDTA (2–3 m)
Comyns et al. 1989	*Sciaenops ocellatus*	S		see Haake et al. 1982	—	1% EDTA (2 m)
Crecco et al. 1983, 1986 Crecco and Savoy 1985, 1987	*Alosa sapidissima*	S	—	—	immers. oil	1% HCl (1 m)

Continued

TABLE 1. Review of otolith preparation procedures from otolith microstructural studies. Literature selected includes peer-reviewed papers from primary journals and proceedings of age and growth, early life history, and biomineralization symposia. Review articles were not included. Procedures are listed by author since most variation in technique can be ascribed to individual investigators.Several authors use different techniques for different size otoliths. These techniques are differentiated by "small" and "large," refering to small (eg. larval) and large (eg. juvenile) otoliths, respectively. Symbols: S = sagitta; L = lapillus; A = astericus; Stat. = statolith (squid); - = procedure not used; + (?) = procedure used but not documented; ? = no documentation on procedure; s = seconds; m = minutes; h = hours. Underlined otoliths indicate which otolith was predominately used for analysis. *(Cont'd)*

Author(s)	Species	Otolith	Medium	Sectioning, Polishing	Clearing Cmpd	Etching
Currens et al. 1988	*Salmo gairdneri*	S	epoxy	600 grit wet sandpaper	—	rinsed in 5% HCl for several seconds
Davies et al. 1988	*Pseudocyttus maculatus* *Allocyttus* sp.	S S	epoxy resin	petrographic grinder, 2000 grit wet/dry paper	—	0.1 M EDTA (15–20 m) 1% HCl (20–30 s) 2% HistoLab RDO (HCl-EDTA) (5m) acetate peel, fracture
Davis et al. 1985	*Dorosoma cepedianum*	S	thermoplastic cement	—		
Dean et al. 1983	*Luciana goodei* *Fundulus heteroclitus* *Heterandia formosa* *Lepomis macrochirus* *Leiostomus xanthurus* *Coryphaena hippurus* *Xiphias gladius* *Makaira nigracans*	S	Spurr	see Haake et al. 1982	—	+ (?)
Deegan and Thompson 1987	*Brevoortia patronus*	S	Permount	(large) 600 grit wet-dry silicon carbide paper, alumina on felt disk	glycerine	—
Dunkelberger et al. 1980	*Fundulus heteroclitus*	S	Spurr	TEM: Ultramicrotome SEM: fractured	—	—
Eckmann and Rey 1987	*Coregonus* spp	S	Epon (large)	+ (?)	cedarwood oil (small)	—
Eckmann and Pusch 1989	*Coregonus lavaretus*	S	Epoxy	ground on abrasive wheels, turned over and ground again	immers. oil	—
Essig and Cole 1986	*Alosa pseudoharengus*	S	Canada balsam	—	Canada balsam	—
Fagade 1980	*Chrysichthys nigroditatus*	S	DPX	glass plate with carborundum powder, 400, 500, 800 grit	xylene	10 N HCl (6–10 M) (? m)
Fowler 1989	*Chaetodon rainfordi* *Chaetodon plebius* *Chelmon rostratus*	SL	Euparal (L) Spurr (S)	dry lapping film (3–10 µm), grinding jig on grinding wheel with ebony paper	Euparal (LS)	—
Fives et al. 1986	*Anchoa mitchelli*	S	Flo-Texx	—	—	—
Gauldie 1987	*Hoplostethus atlanticus*	?	Epoxy resin	Struers pedemax grinder	—	0.1 M EDTA (? m)
Gauldie and Nelson 1988	*Hoplostethus atlanticus* *Macruonus novaezelandiae*	? ?				1% trypsin acetate peel
Geen et al. 1985	*Oncorhynchus tshawytscha*	S		see Neilson and Geen 1981	—	
Geffen 1982 1983 1986	*Clupea harengus* *Scopthalmus maximus* *Salmo salar* *Clupea harengus*	S S S S	epoxy cement (large)	600 grit wet carborundum paper, ground-glass plate; metal polish	immers. oil	—
Graham and Orth 1987	*Micropterus dolomieui*	S	thermoplastic cement	600 grit wet sandpaper	—	—
Graham and Townsend 1985	*Clupea harengus*	S	Permount	—	—	—
Haake et al. 1982	*Lepomis macrochirus* *Lepomis gulosus*	S S	Spurr	isomet saw used to cut Spurr block; 600 grit wet-dry paper, 0.3 µm alumina slurry	—	pH 3 HCl (1–10 m) 5% EDTA (1–5 m) 2% GA (1–5 h)
Haldorson et al. 1989	*Hippoglossoides elassodon* *Theragra chalcogramma*	LS	fingernail polish medium	—	—	—

Continued

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Author(s)	Species	Otolith	Medium	Sectioning, Polishing	Clearing Cmpd	Etching
Hayashi et al. 1989	*Sardinops melanostictus*	S	Euparal epoxy resin	+ (?)	—	1% HCl (5 s)
Heath 1989	*Clupea harengus*	LS	polyester resin	see Brothers et al. 1976	immers. oil	—
Holland-Bawtels and Duval 1988	*Ictalurus punctatus*	S(?)	epoxy resin	—	—	—
Hovenkamp 1989	*Pleuronectes platessa*	S	finger nail polish	ground and polished with 800 grain sandpaper and polished with 1200 grain powder	—	—
Hurley et al. 1985	*Illex illecebrosus*	Stat	Protexx	fine grit carborundum paper polishing paper, diamond (1 μm) slurry	—	—
Isely and Noble 1987a,b Isely et al. 1987	Micropterus salmoides	S		see Miller and Storck 1982	immers. oil (small)	—
Jackson 1989	*Idiosepius pygmaeus*	Stat	plastic mountant	—	D.P.X.	—
Jenkins 1987	*Rhombosolea tapirina* *Ammotretis rostratus*	SL SL	Gurr's neutral mounting medium	—	—	—
Jones and Brothers 1987	*Morone saxatilis*	?	Euparal (small) Flowtex (large) Spurr (SEM)	Beuhler lapidary wheels, 180 grit grinding, 0.25 μm diamond paste polish	—	0.02 N HCl (? m)
Karakiri and von Westernhagen 1988, 1989	*Limanda limanda* *Pleuronectes platessa*	?	—	modified record player with glass disc, plexiglass fitting SEM stub, and 2–6 μm aluminum carbide slurry	—	0.1 M EDTA
Karakiri et al. 1989	*Pleuronectes platessa*	S	mounting medium		Euparal	0.1 M EDTA (2–5 m)
Karakiri and Hammer 1989	*Oreochromis aureus*	S	shellac		—	5% acetic acid (5–7 m)
Keener et al. 1988	*Mycteroperca micolepsis*	L	polyester resin	see Brothers and McFarland 1981	—	—
Kendall et al. 1987	*Theragra chalcogramma*	S	histol. mounting medium	—	—	—
Kingsford and Milicich 1987	*Parika scaber*	SL	—	—	immers. oil	—
Koutsikopoulos et al. 1989	*Solea solea*	S	—	see Karakiri and von Westernhagen 1988	—	0.1 M EDTA (4–6 m)
Lagardere 1989	*Solea solea*	SLA	Permount	—	—	—
Laroche et al. 1982	*Parophrys vetulus*	S	Protexx	—	—	—
Laurs et al. 1985	*Thunnus alalunga*	S	Plasticene	—	—	—
Leak and Houde 1987	*Anchoa mitchilli*	S	coverslip mounting medium	—	—	—
Lecomte-Finiger and Yahyaoui 1989	*Anguilla anguilla*	S	plastic resin	polishing on 600, 1000, 1500 grit paper	—	5% EDTA (1 m)
Lough et al. 1982	*Clupea harengus*	SA	Permount	—	Canada balsam	—
McFarland et al. 1985	*Haemulon flavolineatum*	L	immers. oil		immers. oil	—
McGurk 1984, 1987	*Clupea pallasi*	S	cyanoacrylate glue	metallic lapping paper	immers. oil	—
McMichael and Peters 1989	*Cynoscion nebulosus*	S	Spurr	see Haake et al. 1982	glycerine (1–4 wks)	—

Continued

TABLE 1. Review of otolith preparation procedures from otolith microstructural studies. Literature selected includes peer-reviewed papers from primary journals and proceedings of age and growth, early life history, and biomineralization symposia. Review articles were not included. Procedures are listed by author since most variation in technique can be ascribed to individual investigators.Several authors use different techniques for different size otoliths. These techniques are differentiated by "small" and "large," refering to small (eg. larval) and large (eg. juvenile) otoliths, respectively. Symbols: S = sagitta; L = lapillus; A = astericus; Stat. = statolith (squid); - = procedure not used; + (?) = procedure used but not documented; ? = no documentation on procedure; s = seconds; m = minutes; h = hours. Underlined otoliths indicate which otolith was predominately used for analysis. *(Cont'd)*

Author(s)	Species	Otolith	Medium	Sectioning, Polishing	Clearing Cmpd	Etching
Marshall and Parker 1982	*Oncorhynchus nerka*	S	Euparal, Canada balsam	600 grit wet sandpaper	Canada balsam	10% HCl (5–10 s)
Messieh et al. 1987	*Clupea harengus*	S	immers. oil	—	immers. oil	—
Methot 1983 Methot and Kramer 1979	*Engraulis mordax*	S	Pro-texx	—	—	—
Michaud et al. 1988	*Anguilla rostrata*	?	epoxy resin	wet grinding machine, metall. lapping film (5–3 μm)	—	?
Miller and Storck 1982	*Micropterus salmoides*	S	thermoplastic cement (large)	see Taubert and Coble 1977	immers. oil (small)	1% HCl (30–45 s)
Mitani 1988	*Engraulis japonica*	S	Euparal	—	—	—
Moksness et al. 1987	*Clupea harengus*	S	Protexx	—	—	—
Morales-Nin 1987	*Merluccius capensis* *Merluccius paradoxus* *Genypterus capensis*	?	plastic resin	fracture, rotating wheel with 400, 600, 900 grit aluminum oxide, 0.3 μm diamond paste, fracture	—	0.1N HCl (2 m) 0.2M EDTA (2 m)
Mosegaard et al. 1988	*Salvelinus alpinus*	S	thermoplastic resin	aluminoxide paste	—	—
Mugiya 1987b	*Salmo gairdneri*	S	glycerine	—	glycerine	—
Mugiya and Uchimura 1989	*Carassius auratus*	L	epoxy resin	+(?)	xylene	0.5% HCl (60–90 s)
Mulligan et al. 1987	*Morone saxatilis*	S	LR White resin in silicon flat molds	#200, #400 grain B carbide hand lapstones, 0.25 diamond paste.	—	25% acetic acid (100 s)
Natsukari et al. 1988	*Photololigo edulis*	Stat.	orthodontic acrylic resin	fine sandpaper	Canada balsam	—
Neilson and Geen 1981[1] 1982[1],1985[1],1986[2] Neilson et al. 1985a[1] 1985b[1]	*Oncorhynchus tshawytscha*	S	1. crystal bond 2. thermosetting glue on roofing nail head	1. metallurgic lapping film (0.3–30 μm) with jig (Neilson and Geen 1981) 2. grooved cast iron wheel with 240 grit silicon carbide slurry, 100 grit slurry on glass plate; Beuhler Ecomet II polisher with 0.3 μm alumina	— —	1. 1% HCl (90 s) —
Nishimura and Yamada 1984	*Theragra chalcogramma*	S	epoxy	whetstone	—	0.2 M EDTA
Ntiba and Jaccarini 1988	*Siganus sutor*	SL	glycerine and immers. oil	—	glycerine, immers. oil	—
Nyman and Conover 1988	*Pomatomus saltatrix*	S	instant glue	polished on wet-dry sandpaper and wet felt, masking tape guides used to contol section thickness and plane	—	—
Owen et al. 1989	*Engraulis mordax*	?	?	?	?	—
Pannella 1971	*Merluccius bilinearis* *Urophycis chuss* *Gadus morhua*	S	epoxy and Epon	grinding before and after mounting on 2600 carborundum,	—	1% HCl (45–180 s) acetate replicates
Peebles and Tolley 1988	*Cynoscion nebulosus*	S	?	—	—	—
Penney and Evans 1985	*Sebastes* spp.	S	Epon	—	glycerine (1–2 d)	—

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Author(s)	Species	Otolith	Medium	Sectioning, Polishing	Clearing Cmpd	Etching
Peters and McMichael 1987	*Sciaenops ocellatus*	S	Spurr	see Haake et al. 1982	glycerine (1–4 wks)	—
Polunin and Brothers 1989	*Plectroglyphidodon lacrymatus*	SL	Spurr	diamond saw, polished on 9, 3, 1, 0.25 µm diamond cmpd	—	0.1 N HCl (20 s)
Post and Prankevicius 1987	*Perca flavescens*	S	crystal bond	aluminum oxide lapping film (0.3–9 µm)	70% glycerol	—
Powles and Warlen 1988	*Perca flavescens*	S	Flo-texx (small) cyanocrylate glue (large)	600 grit sandpaper (large)	immers. oil	—
Radtke and Dean 1982	*Fundulus heteroclitus*[1]	S	1. Euparal 2–7. 5-min. epoxy	1–7. 600 grit sandpaper and 0.3 µm alumina polish	—	1. 7% EDTA (1–5 m) 2. 7% EDTA (1 m)
Radtke 1983	*Euthynnus pelamis*[2]	S		3. low speed rock saw		3. 7% EDTA (duration differing over section)
Radtke and Hurley 1983	*Xiphias gladius*[3]	S		7. sharpening stone		
Radtke 1984	*Mugil cephalus*[2]	SL				4. 7% EDTA (10–15 m)
Radtke et al. 1988	*Stenogobius genivittatus*[4]	S				5. 7% EDTA (5–15 m)
	Awaous stamineus[4]	S				6. 8% EDTA (10–20 m)
Radtke 1989	*Gadus morhua*[5]	S				7. 6% EDTA (1–20 m)
Radtke and Morales-Nin 1989	*Thunnus thynnus thynnus*[6]	S				
Radtke et al. 1989	*Trematomus newnesi*[7]	S				
Ralston and Miyamoto 1983	*Pristipomoides filamentosus*	S	Euparal (?)	thin-sectioning (?)	?	+ (?)
Ralston and Williams 1988	*Pristipomoides zonatus*	S	casting resin	isomet sectioning, Beuhler Ecomet polisher/grinder with 180 and 600 grit disks	Euparal Flotexx	1% HCl (5–30 s)
Re et al. 1986	*Dicentrarchus labrax*	S	quick drying medium, DePeX	—	—	—
Rice et al. 1985, 1987	*Coregonus hoyi*	S	thermoplastic glue	400 grit wet-dry paper (large)	immers. oil (small)	—
Rosenberg 1982	*Parophrys vetulus*	S	Protexx	600 grit carborundum paper	—	—
Rosenberg and Haugen 1982	*Scophthalmus maximus*	?	Protexx	—	—	—
Savoy and Crecco 1987, 1988	*Alosa sapidissima*	S	Histoclad	—	—	—
Secor and Dean 1989	*Morone saxatilis*	S	Spurr	see Haake et al. 1982	—	2% EDTA (4–10 m) 2% GA (3–4 h)
Secor et al. 1989	*Morone saxatilis*	S				
	Pagrus major	SL				
	Leiostomus xanthurus	S				
Simoneaux and Warlen 1987	*Brevoortia tyrannus*	S	acrylic adhesive	—	—	—
Sogard 1991	*Pseudopleuronectes americanus*	S	Spurr	400–1500 grit sandpaper with 0.3 µm alumina	immers. oil	2% EDTA (1–5m)
Solomon et al. 1985	*Rhodeus ocellatus ocellatus*	L	epoxy resin	carborundum stone # 2000	—	1% HCl (5 m)
Stevenson et al. 1989	*Clupea harengus*	S	Permount	—	immers. oil	—
Struhsaker and Uchiyama 1976	*Stolephorus purpureus*	S	Euparal	rough sandpaper, 400 grit ilicon carbide paper, alumina oxide slurry	glycerine	1% HCl (up to 3 m) (small)
Suthers et al. 1989	*Gadus morhua*	L	?	lapping film, 3 µm	—	—
Tabeta et al. 1987	*Anguilla japonica*	S	epoxy resin	whetstone	—	0.5% HCl (? m)

Continued

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Author(s)	Species	Otolith	Medium	Sectioning, Polishing	Clearing Cmpd	Etching
Tanaka et al. 1981	*Tilapia nolitica*	S	epoxy resin	fractured, whetstone with whetting compound	—	0.5% HCl (20 s)
1987	*Conger myriaster*	S				
Taubert and Coble 1977	*Lepomis gibbosus* *Lepomis cyanellus* *Lepomis macrochirus* *Tilapia mossambica*	S	Canada balsam	600 grit carborundum slurry on glass plate, rotating polishing wheel with aluminum oxide on wet felt	glycerine, water, ethanol	1% HCl (15–45 s)
Thorrold 1989 Thorrold and Williams 1989	*Herklotsichthys castelnaui*	S	immers. oil	—	—	—
Thresher et al. 1989	*Macruronus novaezelandiae*	S	polyester resin	see Brothers et al. 1976	immers. oil	0.1 N HCl (? m)
Townsend and Graham 1981	*Clupea harengus*	S	Permount	—	—	—
Townsend and Shaw 1982	*Micromesistius poutassou*	S	—	fractured with a scalpel	—	0.1 M HCl (4 m)
Townsend et al. 1989	*Clupea harengus*	?	epoxy resin	polishing with 3 μm diamond paste and 0.3 μm alumina	—	0.1 N HCl (? m) 7% EDTA (? m)
Tsuji and Aoyama 1982, 1984	*Pagrus major*	S	epoxy resin	grindstone (large)	glycerine	0.1% HCl (30 s)
	Engraulis japonica	S				
Tucker and Warlen 1986	*Centropomus undecimalis*	S	clear acrylic medium	—	—	—
Tsukamoto and Kajihara 1987	*Plecoglossus altivelis*	S	Euparal	—	Euparal	—
Tsukamoto 1989	*Anguilla japonica*	S	epoxy resin thermoplastic	polished with #1200 and #12000 emory paper	—	1% HCl (1–3 s)
Tsukamoto et al. 1989			Euparal	—	—	—
Tzeng and Yu 1988,1989	*Chanos chanos*	S	Permount	—	—	—
Uchida et al. 1989	*Plecoglossus altivelis*	S	Euparal	—	Euparal	—
Uchiyama and Struhsaker 1981	*Katsuwanus pelamis* *Thunnus albacares*	S S	Euparal	—	Euparal (1 month)	1% HCl (3–5 m)
Uchiyama et al. 1986	*Coryphaena hippurus* *Coryphaena equiselis*	S S	Euparal	—	Euparal (1 month)	—
Umezawa et al. 1989	*Anguilla japonica*	S	Euparal epoxy resin	electric grinder with rubber stone, 2000 and 8000 grit emory paper	Euparal	0.1 N HCl (10–20 s)
Vero et al. 1986	*Anguilla anguilla*	S	thermoplastic cement Canada balsam	grinding assembly with rotary mechanism, otolith-holding assembly, and grinding or polishing discs (0.3–30 μm aluminum oxide)	xylol, cresol	—
Victor 1982	*Thalassoma bifasciatum*	SL	—	—	immers. oil	—
	Halichoeres bivittatus	SL				
1986a	*Thalassoma bifasciatum*	SL				
1986b	*Thalassoma bifasciatum*	SL				
1986c	100 species of Labridae	SL	?	(large) 600 grit immers. oil colloid on glass plate	immers. oil	—
Victor and Brothers 1982	*Semotolis corporalis*	L		see Brothers et al. 1976	—	—

Continued

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Author(s)	Species	Otolith	Medium	Sectioning, Polishing	Clearing Cmpd	Etching
Volk et al. 1984	*Oncorhynchuy keta*	S	?	315, 600 grit carborundum and immers. oil colloid, 1 μm diamond slurry	immers. oil (whole)	—
Walline 1985	*Theragra chalcogramma*	S	Protexx (small) epoxy resin (large)	(large) fine sandpaper and 0.3 μm alumina	—	0.1 N HCl (? m)
Warlen 1988	*Brevoortia patronus*	S	Flo-texx	—	—	—
Warlen and Chester 1985	*Leiostomus xanthurus*	S	Flo-texx	—	—	—
Watabe et al. 1982	*Fundulus heteroclitus* *Tilapia nilotica*	S	epoxy resin	fractured, whetstone, diamond paste	—	0.2 M EDTA (5 m)
Wellington and Victor 1989	100 species of Pomacentridae	L	—	see Victor 1982	immers. oil	—
Wild and Foreman 1980	*Thunnus albacares* *Katsuwanus pelamis*	S S	Plasticene	cellulose acetate replication	—	5N HCl applied with brush to portions of otolith or otolith immersed in 0.5N HCl (6s)
Wilson and Dean 1983	*Xiphias gladius*	S		see Haake et al. 1982	—	5% EDTA (pH 7.5)(? m)
Wilson and Larkin 1980[1], 1982[2]	*Oncorhynchus nerka*	S	acrylic glue	1. (large) sintered glass plate with aluminum oxide 2. see Neilson and Geen 1981	glycerine	—
Wilson 1988	*Coryphaenoides armatus* *Coryphaenoides yaquinae*	S S	epoxide resin	epoxy block trimmed with free-abrasive lapping (400 silicon carbide), and Beuhler low speed diamond saw, ground on Logitech LP sectioning system with 600 silicon carbide, polished with 30 μm diamond paste on rotating wheel	—	—
Yoklavich and Boehlert 1987	*Sebastes melanops*	S	histological mounting medium	600 grit carborundum on rotating wheel; polished with jewelers rouge (3 μm)	—	—

Morphology

The most obvious systematic difference in otolith morphologies occurs between ostariophysan fishes and other bony fishes. The proximity of the asteriscus to the Weberian apparatus in ostariophysean fishes suggests that this otolith has an important role in hearing. In ostariophysan groups the asterisci are much larger than the sagittae (Popper 1983). The asterisci are usually round or oval, and the sagittae are sickle or needle shaped (Jenkins 1979a,b). In bony fishes other than ostariophysans, asterisci are reduced in size and the sagitta is usually the most conspicuous otolith (Nolf 1985). Tremendous variation in otolith morphology exists within these two major fish groups as well. However, most of the shapes and relative size differences among species occur within the juvenile and adult life history stages.

Otolith type is less easily distinguished during embryonic and larval stages. Sagittae and lapilli are commonly disk-shaped and have similar sizes early in the fish's development. The asteriscus typically forms later in development but is also initially disk-shaped. Because shape and size can be similar among otolith types, care should be taken to observe the relative position of otoliths in the embryo or larva's head (Fig. 2). The sagittae are located ventral, medial, and slightly caudal to the lapilli. The asteriscus in nonostariopysans can usually be distinguished by their relatively small size compared to the other otolith types in embryos and larvae.

Which Otolith?

The first decision facing an investigator is which otolith is best for microstructural analysis: sagitta,

TABLE 2. Species listed in Table 1, sorted according to family. Note that families and species are listed in alphabetical order, not according to systematic relationships. Within each species, authors are sorted by date.

Species	Reference
Anguillidae	
Anguilla anguilla	Vero et al. 1986
Anguilla anguilla	Castonguay 1987
Anguilla anguilla	Lecomte-Finiger and Yahyaoui 1989
Anguilla japonica	Tabeta et al. 1987
Anguilla japonica	Tsukamoto 1989
Anguilla japonica	Tsukamoto et al. 1989
Anguilla japonica	Umezawa et al. 1989
Anguilla rostrata	Castonguay 1987
Anguilla rostrata	Michaud et al. 1988
Anoplopomatidae	
Anoplopoma fimbria	Boehlert and Yoklavich 1985
Atherinidae	
Leuresthes tenius	Brothers et al. 1976
Menidia menidia	Barkman 1978
Menidia menidia	Barkman and Bengston 1987
Bagridae	
Chrysichthys nigrodigitatus	Fagade 1980
Batrachoididae	
Porichthys notatus	Campana 1984c
Bothidae	
Scophthalmus maximus	Geffen 1982
Scophthalmus maximus	Rosenberg and Haugen 1982
Centrarchidae	
Lepomis cyanellus	Taubert and Coble 1977
Lepomis gibosus	Taubert and Coble 1977
Lepomis gulosus	Haake et al. 1982
Lepomis macrochirus	Taubert and Coble 1977
Lepomis macrochirus	Haake et al. 1982
Lepomis macrochirus	Dean et al. 1983
Micropterus dolomieui	Graham and Orth 1987
Micropterus salmoides	Miller and Storck 1982
Micropterus salmoides	Isely and Noble 1987a,b
Micropterus salmoides	Isely et al. 1987
Cephalopoda ((Mollusca)	
Idiosepius pygmaeus	Jackson 1989
Ilex illecebrosus	Hurley et al. 1985
Photololigo edulis	Natsukari et al. 1988
Chaetodontidae	
Chaetodon rainfordi	Fowler 1989
Chanidae	
Chanos chanos	Tzeng and Yu 1988, 1989
Cichlidae	
Oreochromis aureus	Karakiri and Hammer 1989
Tilapia mossambica	Taubert and Coble 1977
Tilapia nilotica	Watabe et al. 1982
Tilapia nilotica	Tanaka et al. 1981
Clupeidae	
Alosa pseudoharengus	Essig and Cole 1986
Alosa sapidissima	Crecco et al. 1983, 1986

Continued

TABLE 2. Species listed in Table 1, sorted according to family. Note that families and species are listed in alphabetical order, not according to systematic relationships. Within each species, authors are sorted by date. *(Cont'd)*

Species	Reference
Alosa sapidissima	Crecco and Savoy 1985, 1987
Alosa sapidissima	Crecco et al. 1986
Alosa sapidissima	Savoy and Crecco 1987, 1988
Brevoortia patronus	Deegan and Thompson 1987
Brevoortia patronus	Warlen 1988
Brevoortia tyrannus	Simoneaux and Warlen 1987
Clupea harengus	Townsend and Graham 1981
Clupea harengus	Geffen 1982, 1986
Clupea harengus	Lough et al. 1982
Clupea harengus	Graham and Townsend 1985
Clupea harengus	Campana et al. 1987
Clupea harengus	Messieh et al. 1987
Clupea harengus	Moksness et al. 1987
Clupea harengus	Heath 1989
Clupea harengus	Stevenson et al. 1989
Clupea harengus	Townsend et al. 1989
Clupea harengus pallasi	McGurk 1984, 1987
Dorosoma cepedianum	Davis et al. 1985
Herklotsichthys castelnaui	Thorrold 1989
Herklotsichthys castelnaui	Thorrold and Williams 1989
Sardinops melanostictus	Hayashi et al. 1989
Congridae	
Conger myriaster	Tanaka et al. 1987
Coryphaenidae	
Coryphaena equiselis	Uchiyama et al. 1986
Coryphaena hippurus	Uchiyama et al. 1986
Coryphaenoides armatus	Wilson 1988
Coryphaenoides yaquinae	Wilson 1988
Cyprinidae	
Carassius auratus	Mugiya and Uchimura 1989
Rhodeus ocellatus	Solomon et al. 1985
Semotolis corporalis	Victor and Brothers 1982
Cyprinodontidae	
Fundulus heteroclitus	Dunkelberger et al. 1980
Fundulus heteroclitus	Radtke and Dean 1982
Fundulus heteroclitus	Watabe et al. 1982
Fundulus heteroclitus	Dean et al. 1983
Luciana goodei	Dean et al. 1983
Elopidae	
Centropomus undecimalis	Tucker and Warlen 1986
Engraulidae	
Anchoa mitchilli	Fives et al. 1986
Anchoa mitchilli	Leak and Houde 1987
Engraulis japonica	Tsuji and Aoyama 1984
Engraulis japonica	Mitani 1988
Engraulis mordax	Brothers et al. 1976
Engraulis mordax	Methot and Kramer 1979
Engraulis mordax	Methot 1983
Engraulis mordax	Butler 1989
Engraulis mordax	Owens et al. 1989
Stolephorus purpureus	Struhsaker and Uchiyama 1976

Continued

TABLE 2. Species listed in Table 1, sorted according to family. Note that families and species are listed in alphabetical order, not according to systematic relationships. Within each species, authors are sorted by date. *(Cont'd)*

Species	Reference
Gadidae	
Gadus morhua	Pannella 1971
Gadus morhua	Bolz and Lough 1983
Gadus morhua	Campana and Hurley 1989
Gadus morhua	Campana et al. 1989
Gadus morhua	Radtke 1989
Gadus morhua	Suthers et al. 1989
Melanogrammus aeglefinus	Bolz and Lough 1988
Melanogrammus aeglefinus	Campana and Hurley 1989
Micromesistius poutassou	Townsend and Shaw 1982
Theragra chalcogramma	Nishimura and Yamada 1984
Theragra chalcogramma	Walline 1985
Theragra chalcogramma	Kendall et al. 1987
Theragra chalcogramma	Haldorson et al. 1989
Urophycis chuss	Pannella 1971
Gobiidae	
Awaous stamineus	Radtke et al. 1988
Clevelandia ios	Brothers et al. 1976
Ilypnus gilberti	Brothers et al. 1976
Quietula y-cauda	Brothers et al. 1976
Stenogobius geniyittatus	Radtke et al. 1988
Haemulidae	
Haemulon flavolineatum	Brothers and McFarland 1981
Haemulon flavolineatum	McFarland et al. 1985
Ictaluridae	
Ictalurus punctatus	Holland-Bartels and Duval 1988
Istiophoridae	
Makaira nigracans	Dean et al. 1983
Labridae	
Halichoeres bivittatus	Victor 1982
Thalassama bifasciatum	Victor 1982, 1986a,b
100 species (see ref.)	Victor 1986c
Lutjanidae	
Pristipomoides filamentosus	Ralston and Miyamoto 1983
Pristipomoides zonatus	Ralston and Williams 1989
Merlucciidae	
Macruonus novaezelandiae	Gauldie and Nelson 1987
Macruonus novaezelandiae	Thresher et al. 1989
Merluccius angustimanus	Brothers et al. 1976
Merluccius bilinearis	Pannella 1971
Merluccius bilinearis	Brothers et al. 1976
Merluccius capensis	Morales-Nin 1987
Merluccius paradoxus	Morales-Nin 1987
Merluccius productus	Bailey 1982
Monacanthidae	
Parika scaber	Kingsford and Milicich 1987
Moronidae	
Morone saxatilis	Brothers et al. 1976

Continued

TABLE 2. Species listed in Table 1, sorted according to family. Note that families and species are listed in alphabetical order, not according to systematic relationships. Within each species, authors are sorted by date. *(Cont'd)*

Species	Reference
Morone saxatilis	Jones and Brothers 1987
Morone saxatilis	Mulligan et al. 1987
Morone saxatilis	Secor and Dean 1989
Morone saxatilis	Secor et al. 1989
Mugilidae	
Mugil cephalus	Radtke 1984
Ophidiidae	
Genypterus capensis	Morales-Nin 1987
Oreosomatidae	
Allocyttus sp.	Davies et al. 1988
Pseudocyttus maculatus	Davies et al. 1988
Percichthyidae	
Dicentrarchus labrax	Ré 1986
Percidae	
Perca flavescens	Post and Prankevicius 1987
Perca flavescens	Powles and Warlen 1988
Plecoglossidae	
Plecoglossus altivelis	Tsukamoto and Kajihara 1987
Plecoglossus altivelis	Uchida et al. 1989
Pleuronectidae	
Ammotretis rostratus	Jenkins 1987
Hippoglossoides classodon	Haldorson et al. 1989
Limanda limanda	Karakiri and von Westernhagen 1988
Parophrys vetulus	Laroche et al. 1982
Parophrys vetulus	Rosenberg 1982
Rhombosolea tapirina	Jenkins 1987
Pleuronectes platessa	Alhossaini and Pitcher 1988
Pleuronectes platessa	Karakiri and von Westernhagen 1988
Pleuronectes platessa	Hovenkamp 1989
Pleuronectes platessa	Karakiri and von Westernhagen 1989
Pleuronectes platessa	Karakiri et al. 1989
Platichthys stellatus	Campana and Neilson 1982
Platichthys stellatus	Campana 1983b,1984a,b
Pseudopleuronectes americanus	Sogard 1991
Poeciliidae	
Heterandia formosa	Dean et al. 1983
Pomacentridae	
Plectroglyphidodon lacrymatus	Polunin and Brothers 1989
100 species (see ref.)	Wellington and Victor 1989
Pomatomidae	
Pomatomus saltatrix	Nyman and Conover 1988
Salmonidae	
Coregonus hoyi	Rice et al. 1985, 1987
Coregonus laveratus	Eckman and Pusch 1989
Coregonus peled	Dabrowski and Tsukamoto 1986
Coregonus spp.	Eckman and Rey 1987
Oncorhynchus keta	Volk et al. 1984
Oncorhynchus kisutch	Campana 1983a
Oncorhynchus nerka	Wilson and Larkin 1980, 1982
Oncorhynchus nerka	Marshall and Parker 1982
Oncorhynchus tshawytscha	Neilson and Geen 1981-82,85,86
Oncorhynchus tshawytscha	Geen et al. 1985

Continued

TABLE 2. Species listed in Table 1, sorted according to family. Note that families and species are listed in alphabetical order, not according to systematic relationships. Within each species, authors are sorted by date. *(Cont'd)*

Species	Reference
Oncorhynchus tshawytscha	Neilson et al. 1985a,b
Oncorhynchus tshawytscha	Bradford and Geen 1987
Salmo gairdneri	Campana 1983b
Salmo gairdneri	Mugiya 1987b
Salmo gairdneri	Currens et al. 1988
Salmo salar	Geffen 1983
Salvelinus alpinus	Mosegaard et al. 1988
Scaridae	
Coryphaena hippurus	Dean et al. 1983
Sciaenidae	
Leiostomus xanthurus	Dean et al. 1983
Leiostomus xanthurus	Beckman and Dean 1984
Leiostomus xanthurus	Warlen and Chester 1985
Leiostomus xanthurus	Secor et al. 1989
Cynoscion nebulosus	Peebles and Tolley 1988
Cynoscion nebulosus	McMichael and Peters 1989
Sciaenops ocellatus	Peters and McMicheal 1987
Sciaenops ocellatus	Comyns et al. 1989
Scombridae	
Euthynnus pelamis	Radtke 1983
Katsuwonus pelamis	Wild and Foreman 1980
Katsuwonus pelamis	Uchiyama and Struhsaker 1981
Thunnus alalunga	Laurs et al. 1985
Thunnus albacores	Wild and Foreman 1980
Thunnus albacores	Uchiyama and Struhsaker 1981
Thunnus thynnus	Brothers et al. 1983a
Thunnus thynnus thynnus	Radtke and Morales-Nin 1989
Scorpaenidae	
Sebastes melanops	Yoklavich and Boehlert 1987
Sebastes spp.	Penney and Evans 1985
Scytalinidae	
Trematomus newnesi	Radtke et al. 1989
Serranidae	
Mycteroperca micolepsis	Keener et al. 1988
Siganidae	
Siganus sutor	Ntiba and Jaccarini 1988
Soleidae	
Solea solea	Koutsikopoulos et al. 1989
Solea solea	Lagardere 1989
Sparidae	
Pagrus major	Tsuji and Aoyama 1982
Pagrus major	Secor et al. 1989
Trachichthyidae	
Hoplostethus atlanticus	Gauldie 1988
Hoplostethus atlanticus	Gauldie and Nelson 1988
Xiphiidae	
Xiphias gladius	Dean et al. 1983
Xiphias gladius	Radtke and Hurley 1983
Xiphias gladius	Wilson and Dean 1983

TABLE 3. Terms used for otoliths.

Otolith	Description	Synonyms
Lapillus Lapilli (pl.)	Occupies utricular vestibule of pars superior, lateral and dorsal to the sagitta.	Utricular otolith "Otolith" Utriculith
Sagitta Sagittae (pl.)	Occupies saccular vestibule of pars inferior; largest otolith in non-ostariophysans.	Saccular otolith Sagittal otolith "Otolith" Sacculith
Asteriscus Asterisci, Asteriscuses (pl.)	Occupies lagenar vestibule of pars inferior, caudal to the sagitta; largest otolith in ostariophysans.	Lagenar otolith "Otolith" Lagenalith

lapillus, or asteriscus (Table 3). "Best" will be determined by resolution qualities and regularity of microstructural patterns of the prepared section. As a general rule, the largest otolith will be the easiest to remove and handle. Investigators may also assume that the sagittae will contain the widest increments for clearest resolution of microstructural features. For these reasons, researchers have chosen to use sagittae in 60% of their decisions (statistic includes 221 decisions made on individual species, Table 1). However, resolution and differentiation of daily increments from subdaily features can be difficult in faster growing otoliths, and shifts in growth axes which commonly occur in sagittae can also cause problems. A recent trend in the literature has been the choice of the lapillus in microstructural studies (Table 1; S.E. Campana pers. comm.). The lapillus was chosen as the only otolith, and used together with the sagittae in 5% and 25% of the reviewed reports, respectively (Table 1). However, since the lapilli are generally much smaller than the sagittae, increments will be narrower in the former, and may become unresolvable in fishes with slow rates of otolith growth.

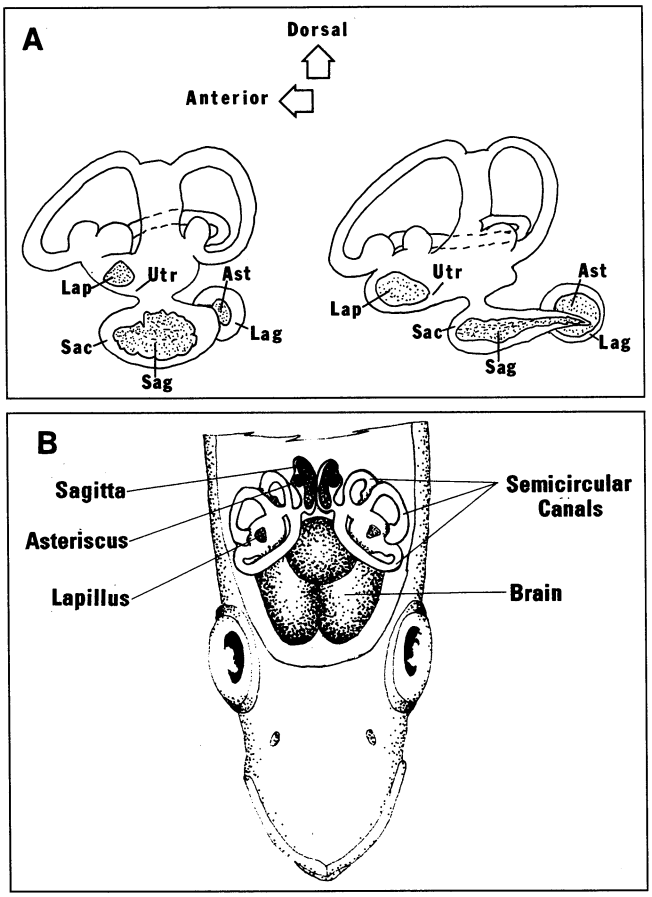

FIG. 1. Anatomy of vestibular apparatus. (A) Otoliths within the labyrinth systems of representative teleost and ostariophsyan (cyprinoid) fishes (modified from Lowenstein 1971). (B) Dorsal view of the vestibular apparatus as it sits in a typical teleost. Top of head is cut away. Ast=asteriscus; Lag=lagenar vestibule; Lap=lapillus; Sac=saccular vestibule; Sag=sagitta; sc=semicircular canals; utr=utricular vestibule (Secor et al. 1991).

We expected that studies on ostariophysan fishes (5% of researched species) would use the asteriscus. However, most studies used the lapillus. No investigations reported using the asteriscus by itself. The sagitta was used in species from the families Chanidae, Ictaluridae, and Bagridae (Table 1). While we have had no experience with chanid fishes, our experiences with catfish strongly suggest that lapilli or asterisci were misidentified as sagittae in those studies. Because the asteriscus forms late in larval development, care should be taken in validating the formation of the first increment or cross-validating asteriscus microstructure with those of other otoliths.

Which Section?

The decision on otolith type should be coordinated with sectioning/polishing plane (sagittal, frontal, trans-

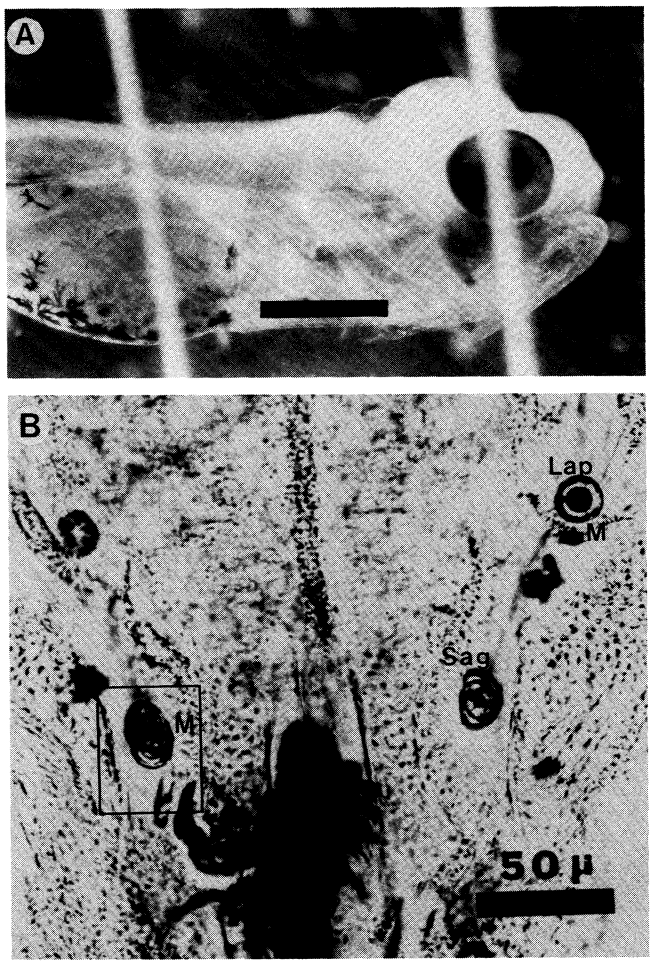

FIG. 2. Morphology and position of sagittae and lapilli in a small fish larva. (A) Embedded 6 day old striped bass larva. Otoliths are birefringent under polarized light and are located ~300 µm behind the eye. Bar=500 µm. (B) Oblique transverse section through embedded larva showing two pairs of otoliths. Note that the lapilli are lateral and anterior with respect to the sagittae. Bordered sagitta contains a double primordia and two apparent discontinuous zones. Lap=lapillus; M=macula; Sag=Sagitta.

verse, or oblique) (Fig. 3). The best section will depend on how the otolith grows. As they grow, the sagittae of many perciform fish project away from the sagittal plane. Therefore, a sagittal section will not contain increments in all the peripheral areas. Either the transverse or the frontal planes will contain all the increments in peripheral regions. The transverse section contains narrower increments than the frontal plane. Pannella (1980a), in his representation of a generalized sagitta, suggested an optimum sectioning plane would occur along the anterio-caudal growth axis.

It is extremely important to investigate different section planes. Section planes containing the greatest area may have increments which are obscured due to apparent subdaily features, shifts in growth axes, or secondary growth centers. Some otoliths have no single plane that contains all increments. In these instances, try a section through only a portion of the otolith (e.g., the rostrum). Alternatively, try serial or oblique sectioning techniques (Wild and Foreman 1980, Natsukari et al. 1988), or use another type of otolith.

Techniques for Otolith Removal

Anatomy of the Auditory Capsule

In adults, five otic bones (sphenotic, pterotic, prootic, epiotic, and opisthotic) comprise the auditory capsule. These bones are fused about the sagittae and asterisci (Harrington 1955; Mujib 1967; Cailliet et al. 1986). In many species of fishes, the sagitta is recessed in the floor of the cranial cavity (comprised mainly of the prootic bulla) and its posterior end is completely encapsulated. In other species, such as some salmonids, perch, and billfishes, sagittae are not so tightly confined within a bony chamber. A common feature in teleosts is that the anterior portion of the sagitta projects (curves) laterally within the capsule. The sulcus and macula occur on the medial (convex) face of the sagitta.

The lapillus occurs near the confluence of the three semicircular canals and is rarely encapsulated in bone. In juvenile striped bass, the lapillus occurs above the pterotic bone. The asteriscus is usually in close association with the sagitta. In non-ostariophysan fishes, it occurs within the same cavity as the sagitta. In ostariophysan fishes, the asteriscus is typically contained within its own cavity located caudal and slightly dorsal to the sagitta.

Which Removal Technique?

The best method for otolith removal will depend on the morphology of the otolith and auditory capsule, fish size, how the specimen was preserved, and individual preference. Therefore, in this section we will outline general procedures and make specific applications to larval and juvenile striped bass.

The following techniques are presented according to the size of the otolith, defined by the greatest otolith length. In sagittae, otolith length is measured from the rostrum to postrostrum (Fig. 4). Otoliths less than 300 µm in length are difficult to see and handle. Although this size is somewhat arbitrary, it denotes our distinction between microscopic and macroscopic techniques. Obviously otolith size has little to do with fish size among species: a 300 µm sagitta can correspond to a 10 mm larval striped bass or a 1 kg swordfish (*Xiphias gladius*). Therefore, we find it

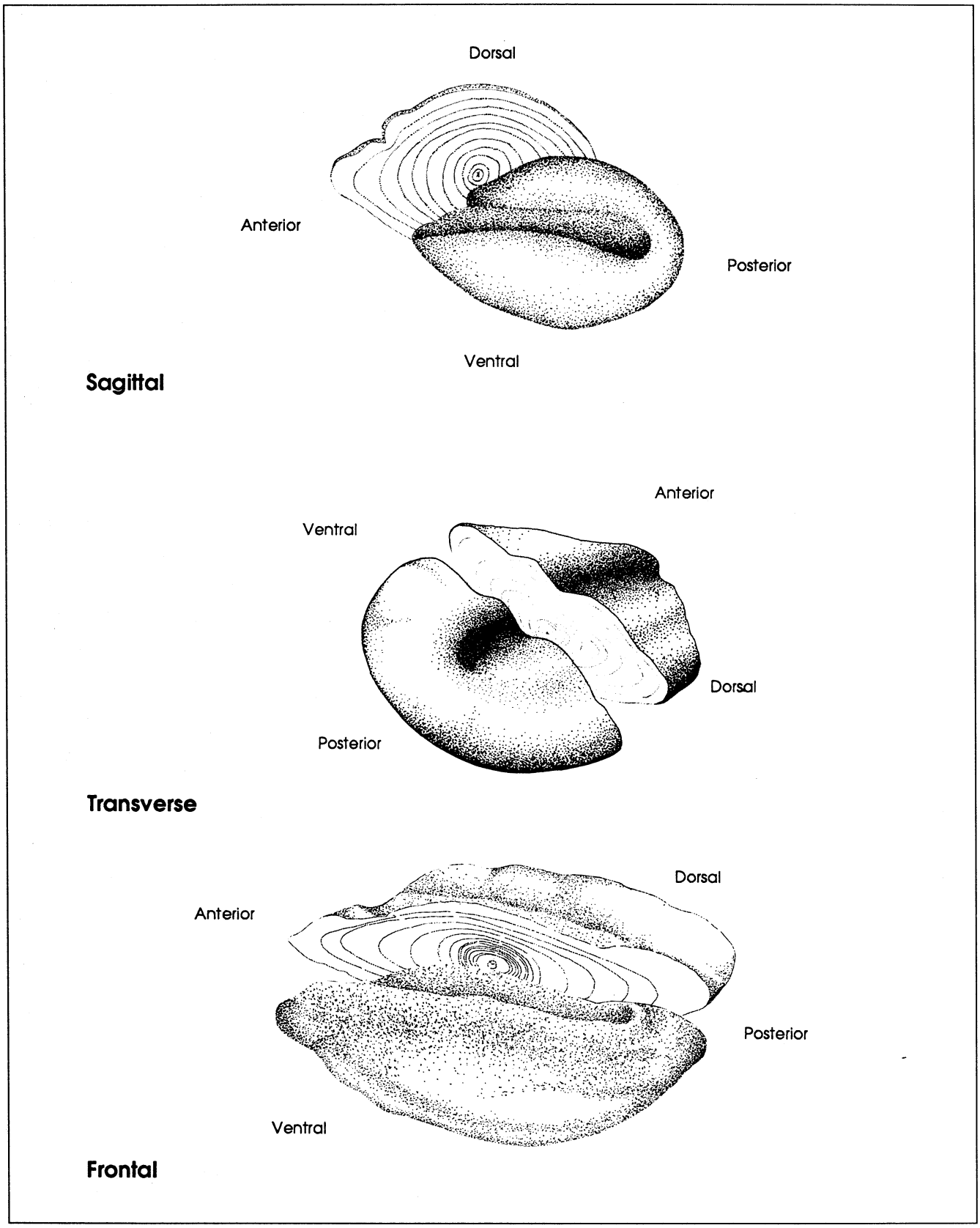

FIG. 3. Typical sagitta showing sectioning planes. (A) Sagittal plane; (B) Frontal Plane; (C) Transverse plane. Note how polishing plane influences the appearance of the otolith microstructure (Secor et al. 1991).

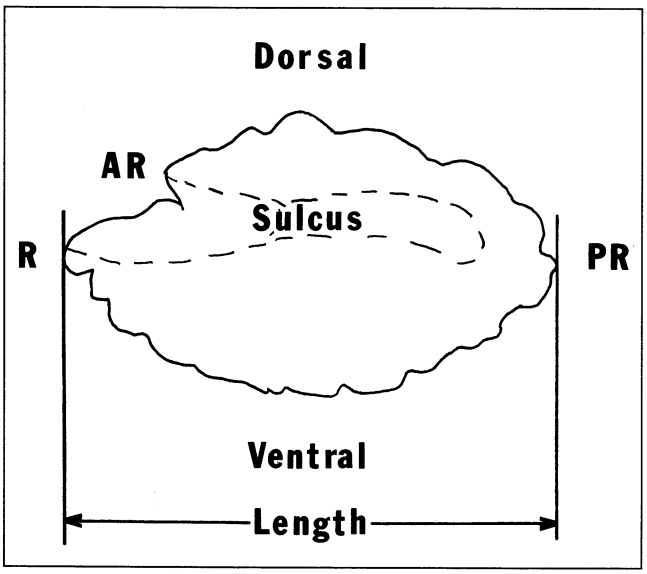

FIG. 4. Medial view of right sagitta of typical teleost. In all sagittae, the sulcus faces medially and the rostrum defines the anterior margin of the sagitta. Recognition of the sulcus and rostrum will allow determination of right or left sagitta. AR=antirostrum; PR=postrostrum; R=rostrum.

more convenient to apply techniques based on otolith size rather than fish size.

Removal of Otoliths Greater Than 300 μm: Macroscopic Techniques

We apply the following techniques to fish with otoliths over 300 μm in length. Depending on the size of the head and the amount of bone, cutting tools range from razor blades to buck knives and meat saws. Given below are summaries of techniques appropriate to fish sizes and types (given in parentheses).

Open-the-hatch method (Fig. 5A) (laterally compressed fishes, flatfishes, fishes with relatively large otoliths, fishes with sagittae located close to the midsagittal plane)

Make a dorso-ventral transverse cut just posterior to the occipital or just dorsal to the opercular margin using a sharp instrument (filet knife, scalpel, razor blade, or sharpened dissecting needle). Continue the cut ventrally to a position parallel to the dorsal margin of the orbit. Then make a cranio-caudal frontal cut along the dorsal margin of the orbit. Continue the cut to the initial transverse cut, thus exposing the brain. Carefully remove the brain. For fish with smaller or less conspicuous otoliths, use a dissecting microscope. Through careful observation, locate the semicircular canals along the lateral walls of the brain cavity (Fig. 1B). Locate the lapillus, which occurs at the confluence of the canals. With forceps, remove the lapilli with portions of the utricular vestibule and semicircular canals. Locate the sagittae; they usually occur ventral and caudal to the midbrain. Grasp the sagittae with forceps and remove. In fishes with partially encapsulated sagittae, gently shift the otolith back and forth within the capsule and gradually angle it out of the capsule. If done too hastily, portions of the sagitta (e.g., the rostrum) can be lost. If the sagitta is entirely encapsulated, chip or cut away portions of the auditory capsule. The asterisci in non-ostariophysans occur in close proximity to the sagittae. Carefully remove the vestibule of the sagitta (sacculus) with the sagitta so that the vestibule of the asteriscus (lagenus) and the asteriscus will remain intact and attached to the sacculus.

Guillotine method (Fig. 5B) (sagittae removal from large fishes [juvenile and adult: SL >100 mm] and flatfishes)

Make a transverse cut from the top of the head through the preopercle. It is not always necessary to continue the cut through the gill arches. Hinge the fish's head away from the body. Locate the exposed postrostrum of the sagitta within the butterfly-shaped capsules that house the sagittae. If the capsules are not exposed, make an additional cut just anterior to the initial cut. Grasp the otolith and work it back and forth so that the otolith is "backed out" of the fish. This method requires practice because the cut is sometimes not exactly on the preopercle and there is also a risk of cutting through the sagittae. For some species, the postrostrum does not align itself with the preopercle. However, with practice, we have found this technique to be a quick method for removing large sagittae (>3 mm) from big, adult fish (>100 mm). The method may also have applications for asteriscus removal in ostariophysan fishes but we do not recommend it for otoliths other than the sagittae in non-ostariophysan fishes. Workers with some expertise can expose the sagittae using oblique cuts to the transverse plane through the cranium.

Right between the eyes method (Fig. 5C) (sagittae removal in deep bodied fishes, asterisci removal in ostariophysans, first time removal of any otolith, lapilli removal)

Make a mid-sagittal cut from the snout to a position posterior to the occipital (operculum). It is often convenient to sever the head before or after this cut. Remove the brain halves to locate the entire exposed labyrinth lateral to the brain (Fig. 1B). The bones of the neurocranium surround much of the semicircular canals. Tease the utricular vestibule and lapillus away from the rest of the labyrinth. Due to the awkward

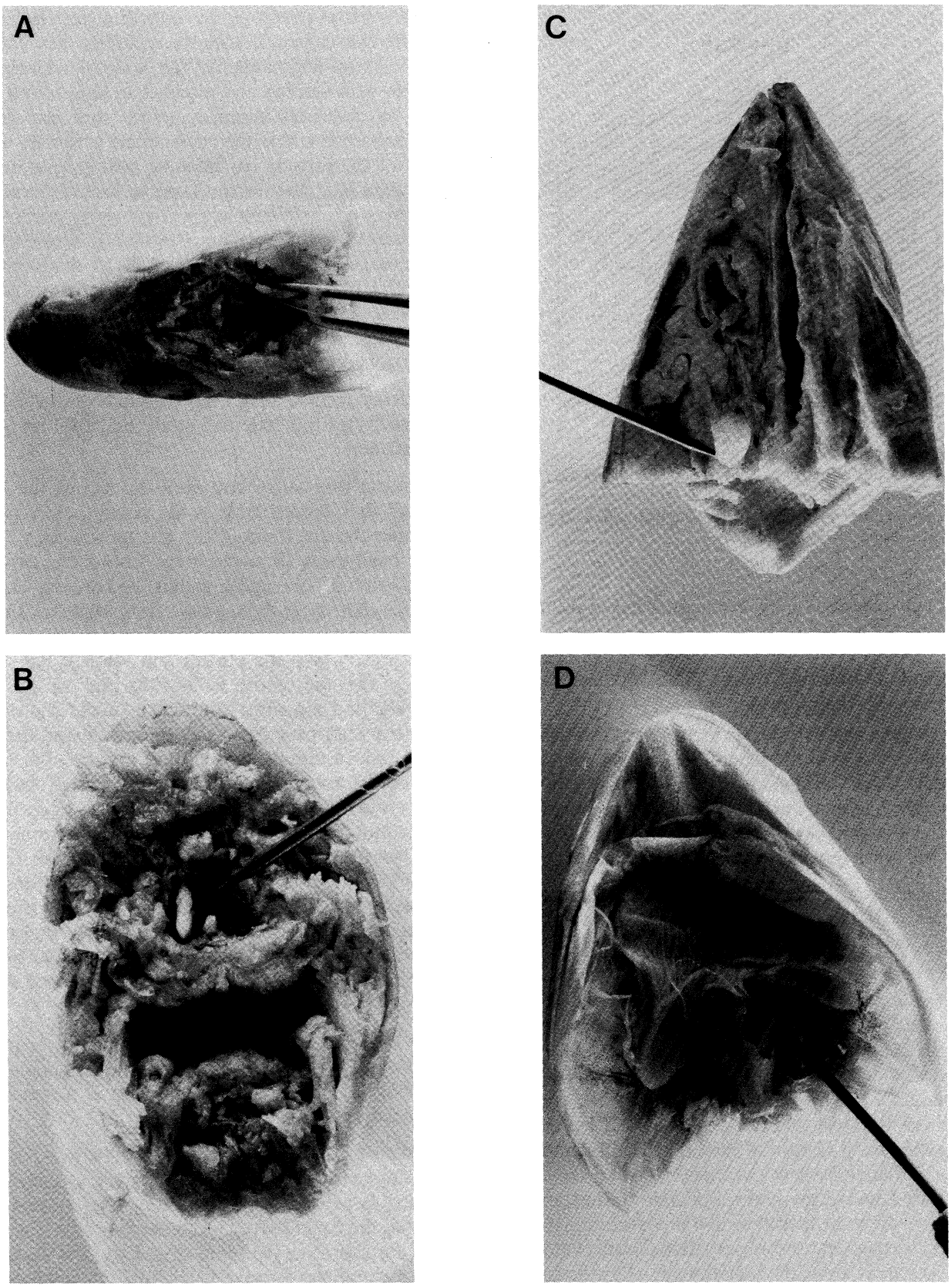

FIG. 5. Macroscopic dissection techniques used on young striped bass: otolith removal. (A) Open the hatch method; (B) Guillotine method; (C) Between the eyes method; (D) Up through the gills method.

medial location of the sagittae, it is important to bisect the cranium precisely. In larger fish, first cut away the head from the rest of the body (posterior to the operculum) and remove the lower jaw. Position the head squarely and securely on the cutting surface to make the midsagittal cut.

This method works well for some ostariophysans that have asterisci slightly lateral to the mid-sagittal plane. We use this method for otolith removal on a species with which we have had no previous experience. It permits a view of the anatomical position of all the otoliths within the vestibular apparatus. However, it is difficult to remove otoliths by this method in laterally compressed fishes and fishes in which the sagittae occur very close to the midline (e.g., flounders). Conversely, the method works well for deep bodied and large (TL >200 mm) fishes. In very wide bodied fishes, such as puffers (Tetradontidae), burrfish (Diodontidae), and monkfish (Lophiidae), we use parasagittal cuts to expose the otoliths along a sagittal plane.

Up through the gills method (Fig. 5D) (sagittae removal in juvenile and young adults [15–200 mm SL], flatfishes)

Cut or rip through the gill isthmus and bend the head back away from the gills and the rest of the body. In smaller fishes (TL <100 mm), locate the exposed bulla portion of the prootic. In larger fishes, it might be necessary to cut away gill arches and then strip away epidermal, connective, and muscular tissue from inferior portions of the neurocranium before exposing the prootic bullae. Carefully crack or chip through the bulla with forceps and grasp the sagitta. By working the otolith back and forth, remove it through the opening made in the bulla. Take special care not to push the sagitta into the brain cavity because it is very difficult to then retrieve the otolith.

Like the guillotine method, this method is appropriate for removal of only the sagittae (in non-ostariophysans). Other dissection techniques are better for removal of the lapilli or asterisci. With practice, this technique can be a rapid technique, especially for juveniles and young adults (15 mm to 200 mm SL). A variation of this technique is to expose each prootic bulla by cutting or lifting away the operculum and gill arches on each side. This technique has been used for large tuna (Thorogood 1986), salmon (McKern and Horton 1970), flounder (Jearld 1983), and tarpon (Cyr 1991).

Any combination of the above methods

None of the above mentioned methods are mutually exclusive. For instance, if one starts with the *hatch method*, but is unable to locate the sagittae after removing the brain, then a midsagittal cut through the rest of the head can be done (*between the eyes method*). Or, if the *guillotine method* fails to expose the postrostrum of the sagittae, an inferior view up "*through the gills*" can locate the otolith's position relative to the initial transverse cut. Likewise, sagittal cuts through the head can localize the sagittae.

Dry, "crunch, and crumble" method (in-laboratory removal of all otoliths from large samples of smaller fish [SL <100 mm])

Dry fish in an oven at 40–60°C until completely desiccated (1–3 days depending on fish size). Break, "crunch, or crumble" the fish. Next, distinguish the calcified otoliths from the rest of the dried fish. Smaller otoliths (<1 mm) will be more difficult to separate. They can be readily located under a dissecting microscope with cross-polarized light. This can be an extremely efficient method, especially in experimental protocols that require dry weight measures. However, it should not be applied for species with hard-to-find or fragile otoliths.

Removal of Otoliths Less Than 300 μm: Microscopic Techniques

The following methods are appropriate for otoliths smaller than 300 μm. Handling is difficult because otoliths much less than 300 μm cannot be easily handled or observed without a microscope. Therefore, we advise that otolith removal, handling, and storage occur simultaneously. To facilitate a review of the following techniques, appropriate applications for each technique are given in parentheses.

Teasing method (large samples of larvae [otolith length 50–300 μm])

On a normal or well-glass slide, immerse small fish (<15 mm) in a few drops of water, ethanol (>70%), immersion oil, glycerine, xylene, or some other clearing medium. Under a dissecting microscope, locate and identify the otoliths with cross-polarized light, which causes the otoliths to become birefringent. Tease them from the head using small dissecting needles. It is sometimes convenient to cut the head away from the rest of the larvae before removing the otoliths. Manipulation of otoliths during dissection requires steady hands, appropriate tools, practice, and patience. After removing the otoliths from the head, gingerly scrape away or jostle loose any adhering tissue from the otoliths. Keep the otoliths as separate from the tissues of the dissected fish as possible. It is useful to provide a mark or draw a circle on the

underside of the slide near or around the otolith to facilitate future manipulation and analysis.

Bleaching method (very small otoliths [<100 μm], large fish with small otoliths [marlin, tuna, burrfish], fail-safe method for obtaining all otoliths from small fishes)

Under a dissecting microscope, place small fish (SL <20 mm) on glass slides, and immerse them in a few drops of bleach (sodium hypochlorite). Keep track of the position of the otoliths in the clearing fish as they fall away from the lysed head (<3 min). If the bleach dries about the otolith, crystals will form and adhere to the otolith and slide. Therefore, before the bleach dries, slowly flood the entire slide with distilled water (done one drop at a time with a pipette). Then air-dry the slide or draw off the water and bleach with a Kimwipe (holding the Kimwipe at the slide's edge).

Otoliths from larger fish (10–100 mm) can also be removed using bleach. Place whole fish, fish heads, or parts of heads in beakers of bleach. After the tissue is digested (5 min to several hours), locate the otoliths in the bottom of the beaker. With larger otoliths, bleach-removal does not require frequent checks on otolith position. We have found this method to be fail-safe for obtaining all three pairs of otoliths. The technique does not appear to result in any manifest dissolution or degradation of the otoliths, although it has not been tested extensively with larval otoliths.

Embedding method (very small otoliths [<100 μm], embryos, SEM examination of small otoliths)

The embedding method, like the bleaching method, also works well for very small larvae (Haake et al. 1982). Completely dehydrate larval samples with 100% ethanol (from 95% ethanol preservative, we run two changes in 100% ethanol). Then work samples through graded mixtures of 100% ethanol and embedding medium (Spurr or Epon; e.g., 1:1, 1:3, 1:9, 0:1), without the catalyst added, until they are completely infiltrated with the medium. Larvae can be embedded using techniques similar to those for embedding otoliths (Fig. 6). After embedding larvae, polish the blocks of Spurr or Epon to the plane of the otoliths. (See later sections on embedding and polishing methods for details). This method requires time and practice, but has several advantages in working with small larvae. By embedding the larvae and surrounding the otoliths with a block of plastic, it becomes possible to handle them for analysis and storage. Also, the otoliths maintain their position in the cleared larvae. This permits accurate recognition of otolith types (lapillus, sagitta, asteriscus) by their anatomic position in the fish (Fig. 2). Finally, embedding is necessary for SEM examination.

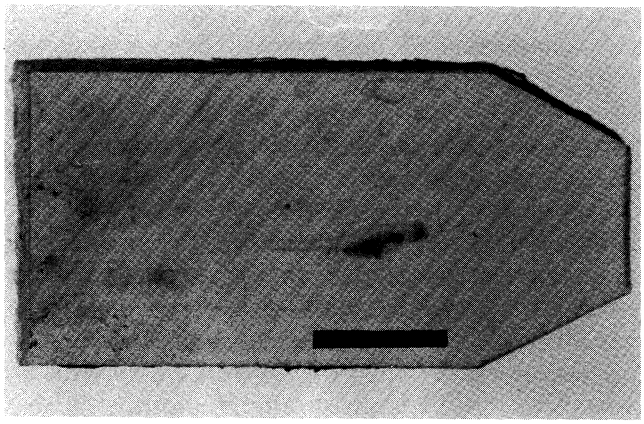

FIG. 6. Embedding method for handling small otoliths (<100 μm): Larval striped bass oriented in sagittal view embedded in Spurr low viscosity resin. Bar=5 mm.

Otolith Cleaning and Handling

Cleaning

Fibrous tissue, composed of the macula, otolithic membrane, and vestibule, adheres to otoliths. Cleaning otoliths of this tissue will permit better observation of the microstructure in whole otoliths. Also, mounting and embedding media will more completely penetrate the otolith. Clearing oils, which are used to improve resolution of microstructure in whole otoliths (Table 1), will also clear adhering tissue. However, these tissues can interfere with light optics and we recommend that otoliths be cleaned mechanically or with bleach prior to preparation and observation.

Mechanical cleaning is accomplished by teasing away the vestibule, macula, and otolithic membrane with fine tools such as forceps and dissecting needles while the otoliths remain in some aqueous medium (e.g., water, ethanol). Bleach-clean otoliths by immersing them in dilute (10%) bleach (sodium hypochlorite) for a few minutes to several hours depending on the size of the otolith and the amount of adhering tissue. Follow with a water or ethanol rinse so bleach crystals do not form on the surface of the otolith. Following cleaning, completely dry otoliths in a low temperature oven or by exposure to air.

Handling

Large otoliths (>300 μm)

Use forceps for routine handling of larger otoliths. Following removal, clean otoliths and store them dry, or mount or embed them. Store dry otoliths in vials or tissue culture plates ("otolith trays"), not in paper envelopes, because curved otoliths can easily fracture when envelopes are wrapped with rubberbands, slipped into pockets, or placed in notebooks. We use

culture plates for safe and convenient storage. Another advantage of culture plates is that otoliths can be cleaned and rinsed in their own well without handling. Information can be written on the tray for each individual well (otolith or otolith pair). Dry otoliths can be stored indefinitely.

Mounting and embedding procedures for large otoliths follow the same techniques described under "otolith preparation." We find it useful to embed very large otoliths (>10 mm) directly, and store them in blocks of Spurr or Epon marked for later identification.

Small otoliths (<300 μm)

Otoliths less than 300 μm cannot be easily manipulated with forceps. With too little pressure, the otolith may be dropped. Too much pressure might cause it to be crushed, especially if there are any flanges or irregular surface features. Also, if the otolith is removed from an aqueous solution, it may be difficult to overcome the surface tension of the liquid. We recommend that investigators treat otoliths less than 300 μm with special care. We also recommend setting up procedures that reduce otolith handling as much as possible.

In some instances, it may not be necessary to transfer small otoliths. If otoliths are to be viewed whole, then they can be removed in oils (e.g., immersion oil, glycerine) or other clearing compounds and left "mounted" to the slide. Otoliths can also be removed in aqueous solutions (e.g., water or ethanol), separated from the rest of the fish, dried, and mounted on the same slide. In small embryos and larvae with no hardparts, a "squash" method can be employed by simply placing a cover slip over the fish (Uchiyama et al. 1986). However, this technique only allows one observation of the otoliths because fish tissue will quickly dry and adhere to the otoliths rendering them opaque.

There are several methods available for transferring small otoliths. They all require some practice and patience.

1. Using a micropipet, remove otoliths with a small amount of the dissection medium. Under a dissecting microscope, transfer the otoliths to a clean slide or storage container. A small mouth pipette can increase control.
2. Shunt the otoliths over to the side of the media with a dissecting needle, thus separating them from the tissue of the fish. Once the dissecting media has dried (if water or ethanol), press a finger down on top of the otolith. Those less than 300 μm will fit nicely into the epidermal ridges of a (clean) finger. Scrape the otoliths from the ridges using a dissecting needle.
3. Pick up otoliths with a wetted dissecting needle. This works well for picking up dry otoliths and placing them in aqueous media.
4. Use small brushes, bacterial loops, attenuated micropipets, or invertebrate forceps to transfer small otoliths.

Small otoliths are difficult to handle; hence advance consideration should be given to the protocol which will be adopted. Considerations include the following: How were otoliths removed? Are otolith weights desired? Will sectioning and polishing be necessary? How many times will each otolith be examined? How will otoliths be randomized between increment counts? Is any SEM work going to be done? One important decision is which dissecting medium to use. Use bleach, water, or 95% ethanol if otoliths will be weighed. Otherwise, otoliths can be removed in oils. If sectioning and polishing are necessary (e.g., for SEM work or more detailed microstructural study), then use bleach, water, or 95% ethanol as dissecting media. Alternatively, the entire larvae can be embedded (see Figs. 2, 6 and description of embedding techniques under "otolith preparation").

We have observed that long term storage in immersion oil or glycerine (and presumably other oils) can cause clearing and degradation of otoliths. The rate of degradation may be significant over weeks, months, or years. Therefore, promptly examine and process otoliths mounted in these media.

Otolith Preparation

Most otoliths need some form of preparation before their microstructure can be viewed. Often (36% of reviewed reports), simply mounting and/or clearing smaller otoliths will permit sufficient resolution of microstructure. Larger otoliths have too much three dimensional depth and irregularity to permit external observation of the otolith's microstructural features. Otoliths requiring polishing may vary between a minimum of 50–300 μm in diameter depending on their type and morphology. Therefore, they are sectioned and polished to remove material and expose the core and all increments (see Table 4 for definition of microstructural terms). Otoliths which contain increments less than 1 μm may have to be prepared for SEM or acetate replication protocols (see description of section preparation techniques).

Mounting otoliths

Mounting media refer to media that simply affix the otolith, usually to a slide. Some mounting media can serve a dual purpose in affixing an otolith and also permitting polishing procedures (see section on pol-

TABLE 4. Glossary of otolith and microstructure terms.

Accretion zone: Component zone of daily increment comprised predominantly of aragonitic calcium carbonate as demonstrated by Dunkelberger et al. (1980), Watabe et al. (1982), and Mugiya (1987a). Ultrastructural examination has shown elongate crystals in this zone which are perpendicular to the periphery of the otolith. Intralamellar matrix also occurs in this zone (Dunkelberger et al. 1980, Watabe et al. 1982). Also termed "incremental zone" (Wilson et al. 1987).

Antirostrum: Anterior "thumb-like" projection of the sagitta. Its location is dorsal to the rostrum.

Core: Calcified area occurring within the earliest deposited **increment** (area contained within the first **discontinuous zone**). Related terms, "nucleus" and "kernal" are ambiguous and not commonly used in microstructure studies (Wilson et al. 1987).

Discontinuous zone: Component zone of daily increment comprised predominately of organic matrix as demonstrated by Mugiya (1987a). Zone preferentially dissolves when weak acids (e.g., low concentration HCl or EDTA) are applied resulting in narrow grooves which are observed in SEM examination.

Growth axes: Axes within the otolith along which proportionately rapid rates of deposition occur (Pannella 1980a). Axes within the microstructure where increment widths are greatest. Otoliths can have more than one growth axis in which case axes are sometimes referred to as major and minor. Growth axes have been demonstrated by calcium-45 incorporation (Irie 1960; Mugiya 1974).

Increment: Bipartite concentric ring comprised of alternating zones of the predominately calcium carbonate **accretion zone** and predominately organic **discontinuous zones. Daily increments** are **increments** which have been validated to occur at a daily rate. Mugiya (1987a) has verified the antiphasic deposition of calcium carbonate and organic matrix over a daily period.

Increment width: Linear measure of increment, comprised of one **accretion zone** + one **discontinuous zone**. Usually measured along a major growth axis.

Macula: Sensory epithelium composed of sensory hair cells and supporting cells. Cilia bundles of the macula serve as mechanoreceptors in hearing. In the **saccular vestibule** it is located along the **sulcus** of the sagitta.

Ostariopyhsan: Teleost families (eg. Chanidae, Characidae, Cyprinidae, and Siluridae) which contain a Weberian apparatus.

Otolithic membrane: Noncellular membrane which adheres to portions of the otolith (see Dunkelberger et al. 1980).

Primordia: Initial deposition sites of organic matrix and calcium carbonate. Usually located in the core, primordia may fuse or remain separate, forming multiple cores. Peripheral or accessory primordia (areas beyond the core) have been described for the juvenile transition of pleuronectids (Campana 1983a).

Postrostrum: Posterior most projection of the sagitta. Can also refer to the entire posterior margin of the sagitta.

Rostrum: Anterior most projection of the sagitta.

Sulcus: Sculptured groove along the medial face of the sagitta (Fig. 4). Sulcus rests on the **macula**.

Vestibule: Sac structure which contains the otolith. Composed of epithelial tissue. The **lagenar vestibule** contains the lapillus, the **saccular vestibule** contains the sagitta, and the **utricular vestibule** contains the astericus.

ishing procedures). Media used in otolith protocols are listed in Table 1. When mounting is used without subsequent polishing, two desirable outcomes are (1) strong adhesion between the otolith and the glass slide to which it is affixed (affixing media) and (2) clearing of the otolith to enhance resolution of microstructural features (clearing media). Most affixing media clear otoliths to some extent.

Affixing media

If otoliths are dissected in an aqueous media, then it is possible to mount small otoliths in a variety of affixing media including Permount, Canada balsam, Euparal, Spurr, Epon, LR White, household epoxy resins, cyanoacrylate glues, thermoplastic glue (CrystalBond), and clear fingernail polish. No single mounting medium predominates in the literature, and

very often the reports only describe using a generic "mounting medium" (28% of methods). Specific mounting media have various advantages and disadvantages largely due to their viscosities and rates of hardening. Canada balsam, Spurr, Epon, Euparal, and household epoxy resin all require hours to harden whereas LR White, cyanoacrylate and thermoplastic glues, and fingernail polish harden in minutes. Some of these media will clear young fish and can be used as dissecting media. However, if the dissection is to be carried out in the mounting medium, only those media which require longer periods of time (e.g., hours) to polymerize should be used (e.g., Canada balsam and Epon) to allow time for dissection.

Canada balsam, epoxy resins, and thermoplastic glues are very viscous before hardening so spreading or mixing them can cause bubbles to form. Heating these media at 40–60°C can reduce the number of bubbles. Viscous media also tend to form a dome over the otolith which can limit working distance in later microscopic examination. Take care to use only enough of these media to contain the otolith. For instance, small amounts of melted thermoplastic glue can be manipulated with a dissecting needle so that the otolith is affixed with a minimal amount of glue. Media which are less viscous prior to hardening like Spurr, Epon, fingernail polish, and cyanoacrylate glues tend to spread across the slide and can cause portions of the otolith to be exposed, resulting in lost otoliths or inadequate clearing. This is usually not a problem in small otoliths (<100 μm) and, for larger otoliths, a probe can be used to draw the medium up and over the otolith, taking advantage of the surface tension between the probe and the medium. Alternatively, a well can be constructed to contain the media so that it completely surrounds the otolith. Wells can be constructed from layered rings of cyanoacrylate glue or fingernail polish, gummed notebook rings (Marshall and Parker 1982), monofilament, or hair. Where further otolith preparation is not required, coverslips can then be placed on top of these wells to further protect the otolith and aid in later microstructural examinations. However, do not place coverslips directly on top of mounting media and otoliths without some form of support. Hardening of the media can bring the coverslip down on top of the otolith and cause the otolith to crack.

There are difficulties associated with specific mounting media. Epoxy resins and Canada balsam can form a translucent surface obscuring otolith microstructure. Cyanoacrylate glue is very hard and can sometimes cause otoliths to crack. Occasionally, affixed otoliths and glue can peel off glass slides after prolonged storage or after use with immersion oil. It will also loosen in water.

We recommend affixing otoliths with thermoplastic glue. The glue is melted on a slide on a hot plate at 40–60°C. Its viscosity can be adjusted by temperature so that it can be "gathered up" to completely surround the otolith. The glue hardens within minutes at room temperature, does not require mixing and adheres well to glass slides for many years. Polished preparations are prepared for photography by briefly heating the glue to remove surface scratches.

Non-hardening clearing media

Several types of viscous non-hardening media can clear otoliths including immersion oil, cedar oil, clove oil, xylene, and glycerine. As mentioned in the previous section, these media can also serve as dissecting media since they will clear the larva. However, when otoliths are left in viscous dissecting media, fish tissue and adhering tissue should be carefully removed from the slide or displaced from the otolith. Our experience is that these media clear at similar rates but care should be exercised that otoliths do not over-clear. There are disadvantages to long-term storage in immersion oil or glycerine (see description of section preparation techniques). These media can erode the otolith (Struhsaker and Uchiyama 1976), or result in the "over-clearing" of increments. Oils can be solubilized with 100% ethanol or acetone.

Viscous media should not be left uncovered since dust will quickly collect in the media. Construct a well around the media as described above and place a cover slip on top of the well supports, without resting it on the otolith itself. Store otoliths mounted with viscous media on a level surface.

Polishing Procedures

The overall aim of polishing is to prepare a section through the otolith in a consistent anatomical plane which contains all the increments within the otolith's microstructure (Fig. 7). Sometimes sufficient resolution of otolith microstructure can be obtained with a single polishing procedure (removing calcareous overburden from a only single face). A single polish will also have advantages in SEM protocols (see otolith preparation for SEM examination: polishing). A double polishing procedure is used to obtain a thin section (<50 μm) through the otolith. This requires that the otolith be turned over after an initial polish to polish opposite faces (Fig. 7).

Two general classes of polishing procedures exist in the literature. One class of procedures embeds otoliths within molds to form plastic blocks about them to facilitate later handling and polishing (Fig. 8).

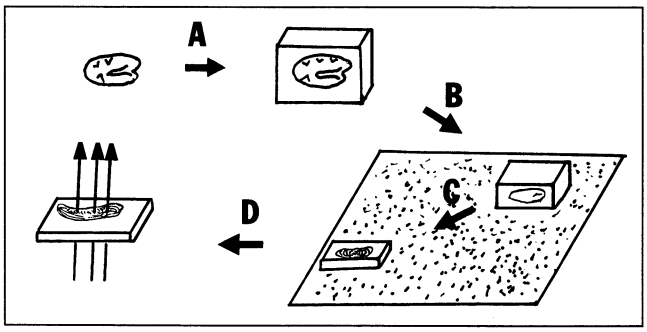

FIG. 7. Simplified diagram of embed and polish technique. (A) Otolith is embedded in plastic resin. (B) Embedded otolith is polished against various grit wet sandpaper or lapping wheels until the core is reached. (C) Embedded otolith is turned over and polished from the side opposite the originally polished side. (D) When sufficiently thin (<40 μm) the otolith section is polished until smooth and viewed with high intensity light under a compound microscope. The diagram does not include steps for gluing the embedded otolith to a glass slide-prop which facilitates polishing.

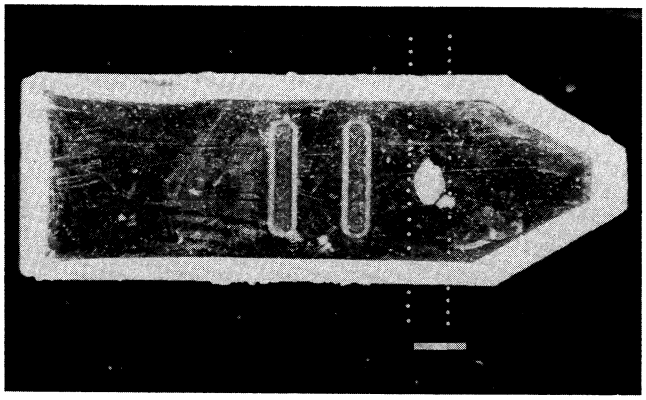

FIG. 8. Sagitta embedded in block of polymerized (hardened) Spurr. Orientation of sagitta was maintained by using a small amount of cyanoacrylate glue to affix the sagitta to the half-filled block of Spurr. The block will be sectioned along the dotted lines to remove excess Spurr. Note that 1 mm of excess material is left to one side of the otolith's center to ease later handling. Bar=1.25 mm.

The other class affixes and contains otoliths on glass slides (see "mounting otoliths: affixing media"). Several media work well for either procedure but, in general, Euparal, Epon, Spurr, LR White, and other resins are used to embed otoliths within blocks, and fingernail polish, cyanoacrylate glue, and thermoplastic glues are used to affix otoliths to glass slides. For convenience the two polishing procedures are termed according to whether otoliths are embedded or glued. The *embed and polish method* refers to polishing otoliths contained in blocks of various plastic resins (Haake et al. 1982). The *glue and polish method* refers to polishing procedures which affix otoliths directly to slides.

Embed and polish method

1) Embedding — In our laboratory's procedure we have routinely used Spurr, a low viscosity embedding medium (Spurr 1969), Epon and now use Embed 812. Euparal and LR White have similar qualities to these media. They all have viscosities which can be adjusted, infiltrate the otolith well, and are transparent. LR White is advantageous because if used with a catalyst, it polymerizes within minutes. Canada balsam and household epoxy resins are often problematic due to their low hardness. Embedding media are usually purchased in a kit with instructions for mixing component ingredients and adjusting for hardness.

The orientation of the otolith within the block of plastic will depend on the desired section plane. Blocks of resin are commonly much larger than the otoliths they contain. To avoid extra work polishing, the blocks are sectioned with an Isomet saw (see next section). Cuts are typically perpendicular to the long axis of the mold (Fig. 8). For transverse sections, we recommend orienting the long axis of the otolith parallel to the long axis of the mold. For frontal sections, orient the otolith perpendicular to the long axis of the mold. Either orientation will work for sagittal sections. Positioning the otolith at one end of the mold will allow excess plastic to be removed with a single cut.

Fill the embedding molds about half way with embedding medium and polymerize (usually in an oven). Place otoliths on a small amount of cyanoacrylate glue on the half-blocks of plastic in an appropriate orientation for later sectioning and polishing. Alternatively, otoliths can be oriented in a partially polymerized layer of plastic resin. Add enough liquid resin to cover the otolith and polymerize the plastic. Remove the block from the embedding mold, and place it in a demarcated otolith tray well (see section on handling), vial, or affix it to a glass slide.

It is important that the plastic resin infiltrate the otolith since it will "hold" the otolith in place during polishing. Infiltration is also critical to SEM work because "empty" spaces in the microstructure can cause channeling of etching agents and unevenly etched or over-etched preparations. Infiltration can be promoted by running otoliths through an ethanol-embedding media series, but this is time-consuming. Otoliths that are completely dry usually show sufficient infiltration of Spurr and other resins. To insure that otoliths are dry, a common practice is to place otoliths in drying ovens (40–60°C) for 24 h.

2) Sectioning — The goals of sectioning are to remove excess plastic, gain proximity to the core (Table 4), and obtain a flat surface parallel to the desired section plane (i.e., sagittal, frontal, or transverse plane) of the otolith. At the same time, enough plastic must remain on one side of the desired polishing plane

to allow it to be turned over and polished from the opposite side (see below). In general, we leave 1 mm of material to one side of the core's plane. Sometimes it is not necessary to section a block, depending on the orientation and size of the otolith within the embedding block (Fig. 8). However, in these cases more time is spent polishing excess plastic.

Cut away peripheral areas of larger otoliths (length >3 mm) with an Isomet saw. On smaller otoliths, we recommend sectioning through only the plastic and then polishing through peripheral areas of the otolith. It is useful, as a guide for sectioning, to place scratches on the block. Another method is to place scratches on glass slides upon which the block has been affixed (S. Epperly, NMFS, Beaufort, NC, pers. comm.). This method permits easier handling of the otolith and block in the Isomet's chuck. Techniques for sectioning many otoliths simultaneously are also available (McCurdy 1985). When sectioning, take care to use low weights and speeds on the Isomet saw. Store blocks and sectioned blocks in otolith trays, vials, or affix them directly to slides with thermoplastic glue.

3) Polishing

a) Attaching the embedded otolith to a glass slide — Thermoplastic glue (CrystalBond), which softens when heated, allows the embedded otolith to be glued down to a slide, polished on one surface, and then turned over. Then the block's polished surface is affixed to a slide and the otolith is polished on its opposite surface (Fig. 7). The "retrievability" of the sectioned block allows the use of a glass slide for both the first and second polishing steps, resulting in better control in the polishing procedure.

Heat a slide on a hot plate to a temperature between 40 and 60°C (the range between the softening and the flow point of CrystalBond). Liberally apply thermoplastic glue on to the slide (Fig. 9A). Remove the slide from the heat and place the sectioned plastic block onto the glue, making certain to glue the proper surface of the block down. This should be the face which is at least 1 mm distant from the core (see previous description of sectioning techniques). Also be sure that the desired polishing plane is parallel to the surface of the slide. Hold the block firmly down with forceps until the glue hardens (<1 min) (Fig. 9B). Mark the slide with the proper information.

b) General polishing — The most exacting part of otolith preparation is polishing. In order to minimize the tedious and time consuming aspects of polishing, the otoliths should be properly aligned and the block sectioned to reduce the time necessary to adjust the polishing plane and to polish through the plastic (see previous descriptions of embedding and sectioning techniques). The slide provides reference on the orientation of the block relative to the polishing surface (Fig. 9C). Its large size permits small adjustments to be made in the polishing plane.

Depending on individual need or preference, otoliths are polished on grinding stones, wet-dry sandpaper, lapping wheels, polishing cloths, and various other abrasives. We prefer hand polishing techniques because they afford us greater control over preparation quality. However, automated techniques using polishing wheels are commonly used (16% of reviewed methods). Wet/dry sandpaper, with grit sizes ranging from 220 to 2000 (100–1 μm), is kept wet for coarse polishing, and a metalographic polishing cloth with an alumina slurry (0.3 μm) is used for fine polishing. Metallurgical lapping films can be used dry at grit sizes from 0.3–30 μm.

Grasp the slide on its edges and surface. Alternatively, apply light pressure with the index and middle fingers to the back of the slide, one finger on each side of the otolith. Coarse polish the affixed block using wetted 220 grit (100 μm) paper (Fig. 9C) with a smooth and consistent circular motion to polish evenly all parts of the block and otolith. Monitor the position of the slide relative to the polishing board to insure a parallel polish, and polish in circles of about 3–15 cm diameter. Periodically check the progress of the polish by viewing the block from the side and noting the position of the otolith relative to the polished block's surface.

After reaching the peripheral regions of the otolith, it is prudent for less experienced polishers to move to finer grit paper. More experienced polishers can continue with coarser grit paper until reaching some landmark feature of the otolith (e.g., the sulcus or a plane just prior to the rostrum of the sagitta). The goal of the first polish is to reach the plane containing the core. To check progress through the internal structure of the otolith, polish the surface of the section smooth by polishing on finer grit paper (e.g., 600 [4 μm] or 1200 [2 μm) grit] and the polishing cloth and alumina slurry. Again, this polishing is done with an even circular motion with the slide maintained in a position parallel to the polishing board (Fig. 9D). Rinse the polished block with water, dry it, and inspect the polished surface under a compound microscope (Fig. 10). Under the microscope at low power (100–300×), adjust the focal plane to observe polishing scratches occurring on the surface of the sample. Then focus "into" the specimen to gauge how much further polishing is necessary (see below, "locating the core"). Since the width of the block can significantly reduce transmitted light intensity, use a microscope equipped with a powerful light source.

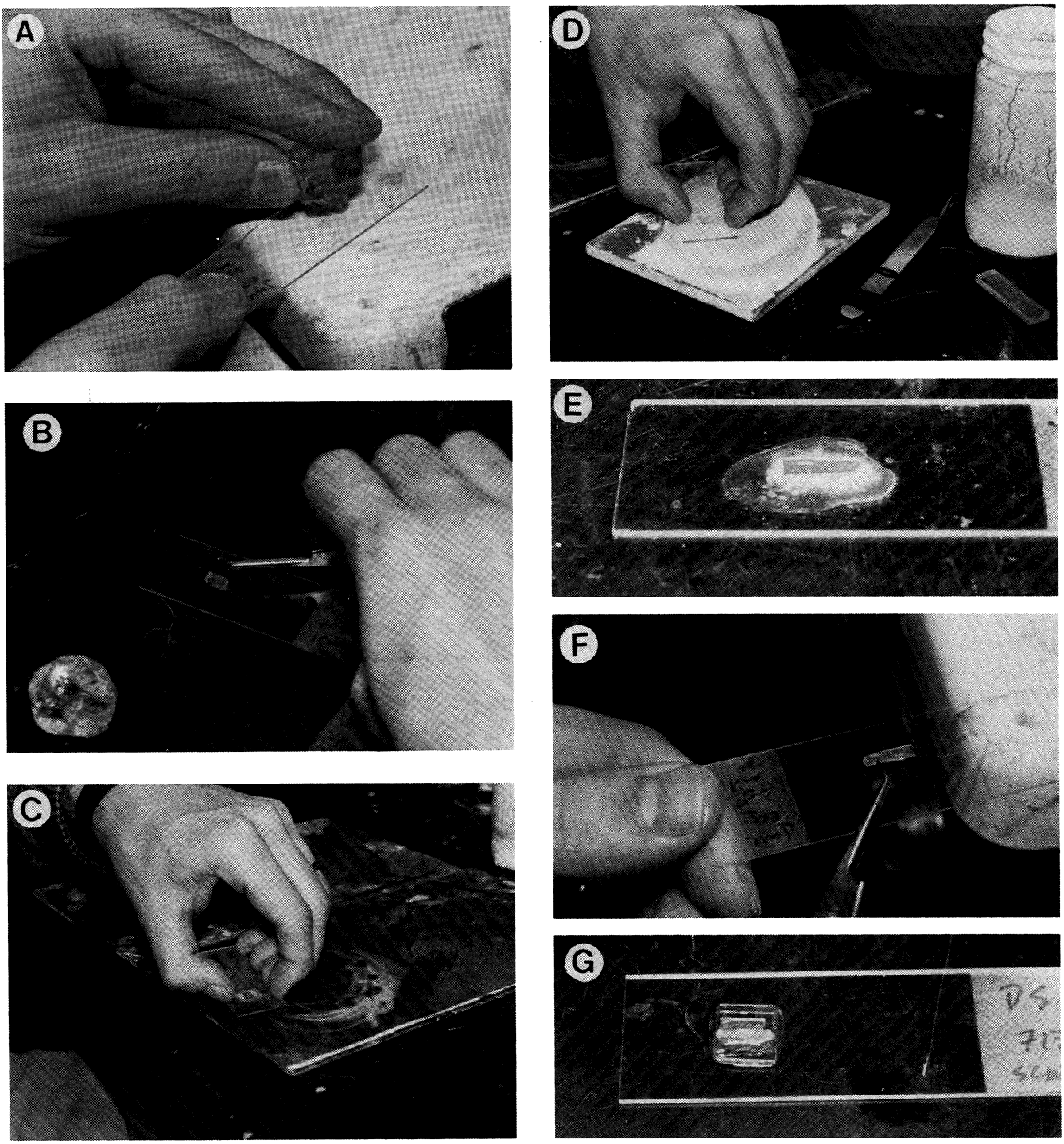

FIG. 9. Polishing procedure. (A) Thermoplastic glue is applied liberally to a glass slide after the slide has been heated on a hot plate. (B) The block of Spurr containing the otolith is held down on the slide for a moment to make sure bubbles do not form under the block. (C) The glass slide is grasped by its edges and the block of Spurr and otolith are polished on various grit wet sandpaper. (D) Before checking the progress of polishing under a microscope, the section is polished until smooth with fine grit sandpaper and polishing cloth with a 0.3 μm alumina slurry. (E) When the core is observed the first polish is complete (see Fig. 11). (F) The slide is gently heated on a hot plate so that the thermoplastic glue melts and the section can be removed. (G) A small piece of glass (1 square cm) is glued to the slide with thermoplastic glue and the section, polished face down, is glued onto the piece of glass. The otolith is polished as described for (C) and (D) until the core is observed.

FIG. 10. Typical polishing work station. From left to right: Olympus compound microscope with high intensity light attachment; hot-plate kept at 40–60°C; box of cut pieces of glass for the second polish; polishing board with 220, 400, and 600 grit sandpaper; thermoplastic glue; tissue well tray for otolith storage; box of glass slides; Kimwipes; polishing slurry (water and 0.3 μm alumina); polishing cloth; bowl of water.

c) Locating the core — The core is an area of non-incremental growth surrounded by concentric increments (Table 4). There is no easy, clear way to describe how to find the core (an area typically less than 30 μm) in a vast array of microstructural features. We advise inexperienced workers to polish a little bit at a time with frequent checks on position. This will help them gain an understanding of what features to look for as they approach the core.

For instance, in juvenile striped bass sagittae, we polish through the entire antirostrum before observing the core. Under a microscope, we check the progress frequently while polishing through the antirostrum (dorsal surface). The overall size of the antirostrum and the widths of the increments it contains will increase in the focal (surface) plane. Polishing further into the antirostrum, the increment widths and antirostrum decrease in size. At this point, we focus into the section. As the increments of the antirostrum move out of focus, we can observe the increments of the rostrum and postrostrum (Fig. 11). Just beyond the antirostrum, as the peripheral region of the rostrum is reached, the core is located by focusing into the section (Figs. 11A,B). In some sections, the core can be located as a dark spot, which is the primordium (or primordia) (Figs. 11C,D). In other sections, only the first increment surrounding the core is visible (Fig. 11).

Polish cautiously when approaching the core. Slow the rate of polishing and use finer grit paper and the polishing cloth. Periodically check the remaining distance to the core. First focus on surface scratches; then focus into the section until the core is in focal plane (e.g., Figs. 11A vs. 11B). Calibrate the focal distance between these two planes (e.g., half a turn on the fine focus dial) with polishing effort (e.g., 40 seconds on the 600 [4 μm] grit paper). The core need not be exactly traversed to obtain sufficient resolution for later microstructure examination under light microscopy. Indeed several experienced polishers agree that resolution is lost if the core is exactly in the focal plane (see discussion of "further polishing" below). We typically strive to get within 10 to 20 μm of the core's plane without going past it. Determining the best stopping point for the first polish requires trial and error, patience, and experience.

With experience, greater speed in sample preparation is obtained. For instance, a polisher who has polished hundreds of otoliths can rapidly determine where the core occurs based on subtle internal and external landmarks. Experience in examining polished sections under a compound microscope will increase efficiency and precision. Careful adjustments in the light intensity, condenser, and diaphragms can also help polishers find their way through the otolith's microstructure.

d) Turning the section over and further polishing — After reaching the core, polish the block smooth on the polishing cloth and alumina slurry. Clean it in water, or an ultrasonic bath, and allow it to dry (Fig. 9E). Place the slide on a hot plate until the thermoplastic glue softens and remove the block with forceps (Fig. 9F). Be careful not to leave the block on the hot plate too long because most plastic resins will soften with heat. This is especially true with sections less than 1 to 2 mm in thickness. Turn the block over so the polished surface faces down. (To avoid problems turning sections over, see discussion on otolith sectioning techniques). Glue the block to a small piece of glass (1 cm squares cut from microscope slides) and glue this assembly to a glass slide (Fig. 9G). The piece of glass permits some working distance between the polishing surface and the polisher's fingers. This significantly reduces the abrasion of the polisher's fingertips, a hazard of the profession.

The second polish is easier because the location of the core is known. Visually inspect polishing progress by viewing the width of the remaining block. When the block becomes paper-thin (ca. 100 μm), check polishing progress frequently under a compound microscope. For finished preparations of juvenile striped bass, the thickness of the section ranges between 10 and 50 μm and averages about 25 μm. Adjust the section thickness, based on the position of the core and contrast qualities of the increments (Fig. 11E). There are also technical constraints. In most cases, the first polish has not sectioned exactly through the core. The core might remain 30 μm away from the surface of the section.

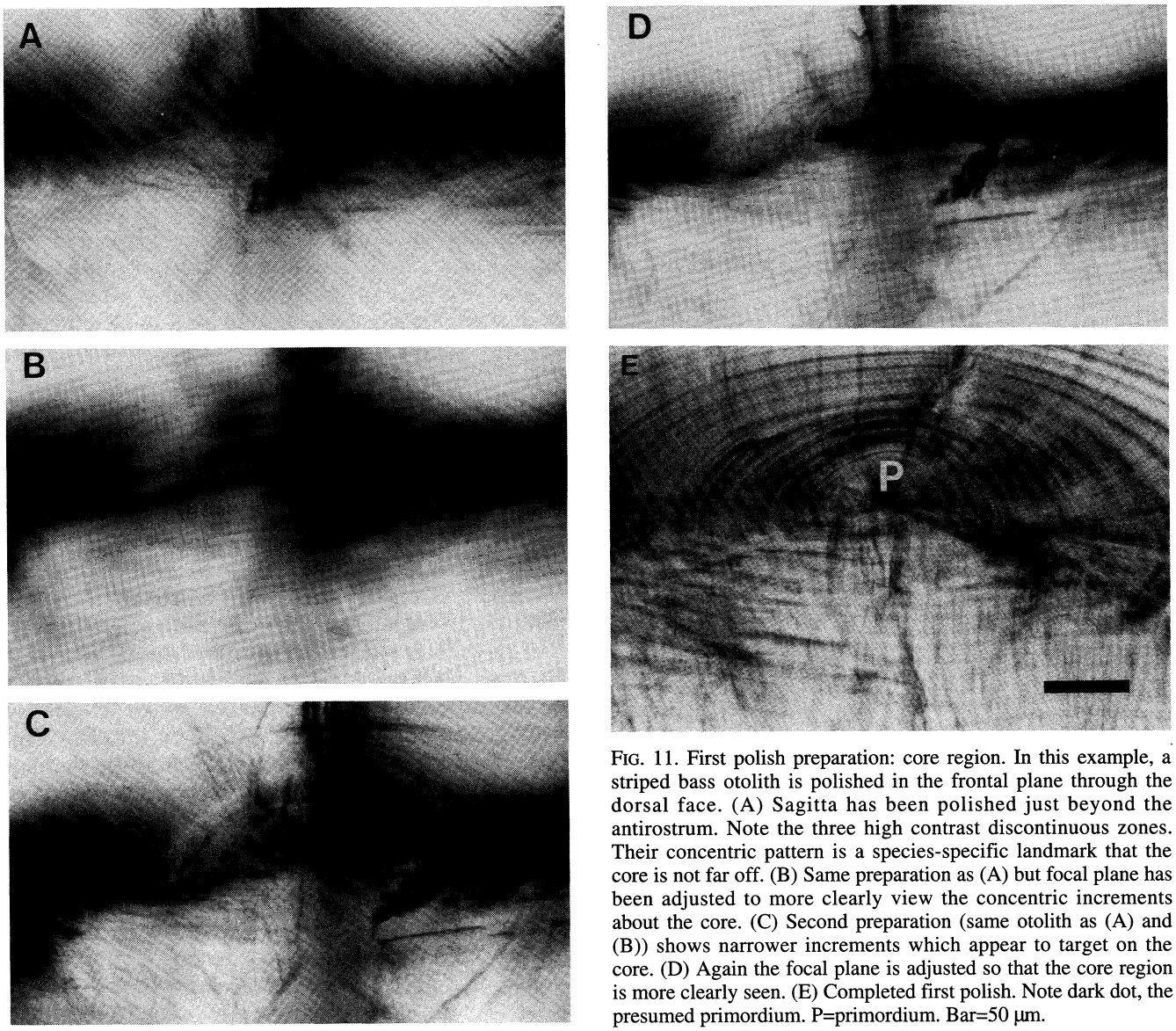

FIG. 11. First polish preparation: core region. In this example, a striped bass otolith is polished in the frontal plane through the dorsal face. (A) Sagitta has been polished just beyond the antirostrum. Note the three high contrast discontinuous zones. Their concentric pattern is a species-specific landmark that the core is not far off. (B) Same preparation as (A) but focal plane has been adjusted to more clearly view the concentric increments about the core. (C) Second preparation (same otolith as (A) and (B)) shows narrower increments which appear to target on the core. (D) Again the focal plane is adjusted so that the core region is more clearly seen. (E) Completed first polish. Note dark dot, the presumed primordium. P=primordium. Bar=50 μm.

The second polish is then limited to a thickness of 30 μm. Avoid extremely thin sections (less than 10 μm) because contrast between the discontinuous and the accretion zones of the increments will be diminished. Also avoid sections (thicker than 40 μm), since increments are obscured.

After the core is plainly visible and the increments are of sufficient contrast, polish the section on the polishing cloth until completely smooth. Clean it in water, dry it, and store the labeled polished section in a slide box.

Glue and polish method

Krazy glue (cyanoacrylate glue) and other resins are commonly used to mount otoliths on slides for immediate polishing. Because the otolith is not surrounded by a block of plastic, sectioning is usually not necessary. Polishing by this method follows many of the same procedures described for the *embed and polish method* above. Methods unique to the *glue and polish method* are given below.

1) Mounting — Krazy glue, thermoplastic glue, clear fingernail polish, and other mounting media can be used to mount otoliths on slides for subsequent polishing. Krazy glue and thermoplastic glue are the most popular of these media because they harden within minutes, penetrate the otolith, clear it to varying extents, and have suitable hardness to withstand polishing pressure. They also permit the mounted

otolith to be turned over and polished from an opposite face (see next section).

When mounting otoliths on slides, it is important to carefully position them on the slide to insure a polishing plane which is parallel to the otolith growth axis. This means that in most cases a sagittal polishing plane is chosen. However, an advantage of thermoplastic glue is that it can be "gathered up" about the otolith as it hardens and the otolith can be reoriented within the glue to polish other planes of the otolith. While props can be constructed to provide working distance between the polishing surface and the polisher's fingers (Neilson and Geen 1981), such are not necessary if the slide is polished using light pressure from the back of the slide (see previous description of polishing techniques).

2) Polishing — Polishing procedures are similar in principle to those described previously under the *embed and polish method.* Because cyanoacrylate glue and fingernail polish are harder than most other media, they do not "give" as much during polishing and care should be taken not to apply excessive pressure. However, the absence of plastic around the otolith results in considerably more rapid removal of otolith material. Otoliths which are about 300 μm in diameter can be rough polished in less than a minute with a 30 μm grit size. Smaller otoliths require even less polishing effort; it would be unwise to apply grit sizes of >5 μm to an otolith of 100 μm diameter, even in the preliminary polishing stages. Since the adhesion of cyanoacrylate glues to the slide is weakened by water, polishing is best conducted on dry, adhesive-backed metallurgical lapping films. Use of such films eliminates the need for a slide cleaning step in between polishing and microscopic examination. However, the dry papers have to be replaced more frequently than do wet papers.

After polishing to a point just above the core, the mounting medium and otolith can be turned over to expose the other surface by several methods. Immerse otoliths mounted in Krazy glue in distilled water for several minutes to loosen the glue's adhesion to the slide. Then cut around the otolith with a scalpel, using a rocking motion on the curved blade to ensure that contact between the glue and the slide is broken. Leave a minimum of 1–2 mm of glue between the cut and the otolith on each side. After the slide and the section containing the otolith are dry, carefully turn over the section of glue containing the otolith and reglue it to a slide. We have found that making scratches on the slide will cause better adhesion between the Krazy glue and the slide. Thermoplastic glue-mounted otoliths can be carefully turned over after heating the slide. Other mounting media like fingernail polish and Epon can be softened with acetone before turning over the section.

Which Polishing Technique?

The choice of polishing technique will depend on the objectives of the microstructural examination, the laboratory resources available, precedence, individual preference, and microstructural differences due to species and life stages. The *glue and polish method* is faster and requires less material than the *embed and polish method* since the embedding and sectioning steps are eliminated. The method permits rapid access to otolith microstructure; a researcher can obtain microstructural information from a fish within minutes to hours, whereas the *embed and polish method* typically requires a day before information can be obtained from a given otolith.

Our laboratory has emphasized the development of the *embed and polish method* (Haake et al. 1982). Although greater time is required per sample, we think that the quality of sample preparation and the precision of later microstructural examination is enhanced over other methods. The *embed and polish method* permits precise otolith positioning so that any plane can be polished, whereas the majority of *glue and polish method* applications are appropriate primarily for the sagittal polishing plane. With the *embed and polish method*, the sectioned blocks provide a flat surface which makes it easier to gauge the rate and consistency of the polish (i.e., how level the polishing plane is). Also, the greater amount of material about the otolith "cushions" it from excess pressure which can cause the otolith to crack.

Special Applications of Polishing Methods

For small otoliths (<100 μm), whole larvae can be embedded and sectioned (see "handling: small otoliths") (Figs. 2, 6), just like otoliths. Depending on the polishing plane used, it is possible to obtain a section which includes more than one pair of otoliths (Fig. 2B). The otoliths can be located during polishing by identifying anatomical features in the section, such as eyes, midbrain and gut. This method eliminates time consuming dissection and handling methods. It also permits the identification of the otolith based on anatomical position. We have used this technique to examine increment formation in embryos and prolarvae.

Otolith morphology can become much more complex as fish age. For instance, in otoliths of flatfish, there is a distinct transition in the microstructure of the otolith which relates to the juvenile metamorphic transition (Campana 1984b). Increments can be diffi-

cult to observe in certain planes as a result of these growth transitions. Therefore, the choice of plane becomes critical (see "position and morphology: which section?"). Some otoliths undergo shifts in growth axes which will cross over several planes so that a single plane containing the core and all the increments does not occur. For example, the statoliths of the squid *Photololigo nolitica* grow in a helical manner so that the growth axes are not simultaneously visible (Y. Natsukari, Faculty of Fisheries, Nagasaki University, Nagasaki, Japan, pers. comm.). The statoliths must be polished and photographed alternately. Thus, to observe entire microstructures, a montage of images reconstructs the core and all increments contained along the major growth axis of the statolith. Wild and Foreman (1980) used an acetate replicate technique (see "automation: section preparation techniques") on scombrid otoliths to obtain microstructural information from otoliths with complex morphology.

Efficiency and Precision

Efficiency and precision can be conflicting goals. When otoliths (sections) are processed more rapidly, the rate of technical error is likely to increase. Polishing is the limiting step in otolith preparation. An experienced polisher can spend one hour polishing and will produce a beautiful section. Another polisher can polish ten otoliths an hour, but three-fourths of them might be marginal preparations and not yield information that can be interpreted. What one should strive for is a compromise, for example, polishing five otoliths per hour that are of sufficient quality for further microstructural analysis.

Both efficiency and precision will improve greatly with experience. Inexperienced workers should not over-commit themselves on sample processing. It is necessary to allow plenty of room for error. When techniques are first learned, workers should practice on otoliths which have no direct bearing on any planned investigation. Precision should be emphasized first; then, over time, efficiency can be improved.

Automation

Polishing Wheels

Automated polishing wheels can increase the efficiency of otolith processing. Devices include: metallurgic lapidary wheels (Jones and Brothers 1987), rotating wet stones (Radtke and Hurley 1983), sanding drill attachments (Maceina 1988), and even modified record turntables (Karakiri and von Westernhagen 1988). Polishing wheels require a prop or holder which holds the block in one position while it is polished. Fingers cannot be relied upon to hold blocks in the same orientation. (Props are discussed below). The advantage of polishing wheels is that a lot of the tedious work is done by a mechanical device. Wheels rapidly polish away excess embedding medium and peripheral portions of the otolith. The core is approached by changing grit size on the polishing surface. As with hand-polishing techniques, the progress of the section needs to be checked frequently. In a survey of otolith polishing techniques, a significant number of investigators (16%) used some form of polishing wheel.

However, polishing wheels can have some disadvantages. Moving to a finer grit can be time consuming. Also, as described above, there is an efficiency/precision trade-off. Automation can affect the precision of the polishing techniques because the prop used to hold the block can prove cumbersome when checking the section frequently under a light microscope. This discourages frequent inspection of microstructure and increases the chance of polishing through the core or obtaining a section which is thicker than desired. For this reason, polishing wheels are best used on otoliths of adults, where it is not critical to gain proximity to the core.

For application to fine-scale microstructure work, we need further advancement in polishing automation. Such developments may occur with further metallurgical applications. For example, Karakiri and von Westernhagen's (1988) modified record turntable has produced preparations with sufficient precision for high quality SEM work.

Section holders, props, and mounting devices

Props maintain the section (block) in a specific orientation during polishing. In essence, the embedding or mounting medium itself function as a prop. The choice of props centers on their level of elaboration. Microscope slides can serve as simple props and assist the polisher in maintaining the appropriate polishing plane. More elaborate props are used in both hand-polishing and polishing wheel procedures and comprised 20% of the procedures we reviewed. Karakiri and von Westernhagen (1988) used a modified stylus head to hold the otolith's position against their adapted "long-playing" polishing wheel. Neilson and Geen (1986) glued whole otoliths from adult chinook salmon on nail heads to gain leverage on the polishing surface. One of the more elaborate props is the metallurgic jig adapted by Neilson and Geen (1981). The jig rests on the polishing surface on three aluminum legs, which facilitates polishing. The section holder itself is spring loaded to encourage fre-

quent inspection of the section, and can be adjusted to apply more or less pressure to the section.

The inconvenient feature of props is that the same device which holds the section in place must be dismantled to allow viewing of the section under a microscope. There are some exceptions. Some props (e.g., slides) may be small and transparent enough to permit microscopic examination of the section. In general though, devices which allow easy removal of the section will also be more likely to mechanically release the section during polishing, and devices which hold the section more firmly make retrieval of the section for frequent inspection more difficult.

Section Preparation Techniques for Light Microscopy: Oiling, Etching, Burning, and Acetate Replication

Often investigators wish to enhance the contrast of microstructural features in a finished section. In order to increase contrast between accretion and discontinuous zones, either or both zones can be chemically or physically altered. This is accomplished by using oils, etching, burning, or acetate replication. In adults, these techniques are commonly used to distinguish annuli in whole or sectioned otoliths. They have also been applied to most microstructure investigations (65%) (Table 1).

In the literature we reviewed, the most popular technique was oiling (see discussion of non-hardening clearing media). Etching, either for light microscopy or SEM (see next section), was also frequently used (30%). We found no reports of burning otoliths to enhance contrast of daily increments, but burning has been used extensively to increase contrast of annuli. Acetate replication was employed by Pannella (1971, 1980b) in his initial observations of otolith microstructure and has also been applied by Wild and Foreman (1980). Because acetate replication requires careful etching, it is as reasonable to do SEM examination on the otolith section itself as on the acetate peel. Therefore, its application should be limited to otoliths for which there is no single plane which includes the core and all increments (see "special applications of polishing methods").

Etching Otolith Sections

Etching refers to the preferential dissolution of either organic matrix or calcium carbonate. The report by Haake et al. (1982) describing these procedures is still the most comprehensive source of information available and is replete with figures showing the effects of different decalcification agents. We recommend its use along with this chapter.

The aim of etching is to provide three-dimensional relief of the polished otolith surface. This effect can enhance contrast between discontinuous zones and accretion zones for light microscopy, and is also necessary for SEM examination.

Otolith Preparation for SEM Examination

In scanning electron microscopy, a narrow beam of electrons bombards a specimen's surface. Secondary electrons emitted from the bombardment are collected, and their signal is amplified and spread over a cathode ray tube. The quality and resolution of the image depends on beam size (voltage level and condenser adjustment), orientation of the specimen to the electron beam, and the surface quality of the specimen. Smooth-polished otolith sections will have little contrast (Haake et al. 1982). The goal of etching is to provide consistent relief patterns throughout the section, while at the same time limiting preparation artifacts.

Polishing

Perform the first polish as described in the section on "otolith preparation: polishing procedures." However, be sure to expose the core at the surface, rather than leave the excess material which is necessary for microstructural resolution using light microscopy. Check the core's position relative to the surface by focusing on surface scratches, gauging the focal distance relative to the core. Do any further polishing with very fine grit (>1200) (2 μm) wet/dry paper and polishing alumina or 3 μm lapping wheels. If sections are not polished until they are smooth, channeling of etching agents and uneven etching will occur. Do not do a second polish on the opposite side unless it is required to resolve the core. This will permit easier handling of the section. Clean the section in deionized water in an ultrasonicator. However, do not ultrasonicate Krazy glue mounted otoliths since the otolith can shatter or be displaced from the slide (pers. obs.; J. Neilson, pers. comm.). Leave the final preparation on the slide for etching.

Etching

Without experience, it is difficult to predict which of several etching techniques will provide good results. Therefore, try an array of etching agents and exposure times (Haake et. al. 1982). We principally use three agents: dilute (0.1–2%) HCl (pH=2.0–5.0), 5–7% tri-sodium ethylenediaminetetraacetate (EDTA) (pH=7.2–7.6), and 2% aqueous EM grade glutaraldehyde (GA) (pH=7.2–7.6). Additionally, the combination EDTA and GA (EDTA-GA) can be effective.

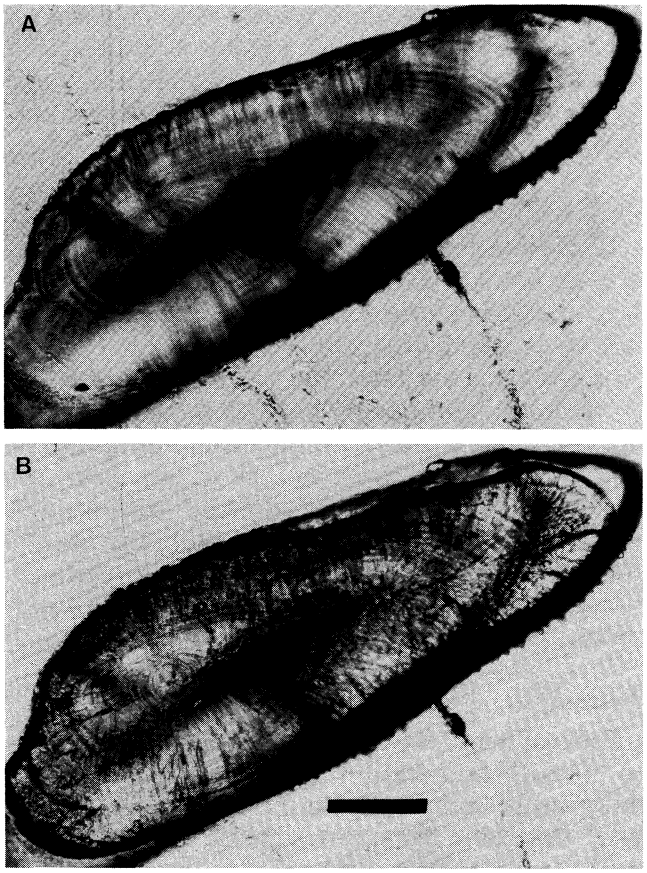

FIG. 12. Sectioned lapillus of juvenile striped bass under different types of examination. (A) "Smooth" section under light microscopy; (B) Etched section under light microscope. Etching was performed with 2% EDTA (5 min). Bar=50 μm.

Occasionally, we use a stronger concentration of HCl (2–10%) on larger (adult) otoliths to expose annuli. The pH ranges given for each agent are not the only acceptable ranges but rather the range in which pH is likely to occur for specific applications (e.g., species, developmental stage [larvae, juvenile, adult] otolith type, section type, otolith, etc.). Therefore, the optimal level of pH and time of exposure will need to be determined by trial and error.

Apply EDTA and HCl etching solutions gently to the section with a micropipet. After a carefully measured length of time, which is experimentally determined (usually 1–10 min), remove the solution by placing the slide and affixed section in a beaker of deionized water. Gently dab the slide and section dry with a kimwipe, carefully avoiding the otolith itself, or allow the section to air dry. Examine the progress of etching under a light microscope (Figs. 12A,B). Etching causes the discontinuous zones to appear irregular; occasional canals traverse the accretion zones. Re-apply the etching agent if necessary.

The handling of sections is a major concern when using GA or EDTA-GA agents because the preserved organic matrix is quite fragile and is easily dislodged from the section. If the matrix is to be examined, do not clean in an ultrasonic bath. Carefully immerse the section in a beaker of GA or EDTA-GA. Place it in a refrigerator for hours (up to 2 days). Gently rinse the section in deionized water briefly and allow it to air dry. Limit handling or any mechanical disturbance as much as possible.

After etching, scratch each section's sample identity into the embedding or mounting media with a dissecting needle. For small otoliths (<300 μm), carefully trace a circle around the otolith so that it can be later identified under SEM. Carefully remove the section from the slide (see previous descriptions of polishing techniques) and attach the section to a SEM stub with thermoplastic glue or carborundum paint. Double sided tape can also be used, but at high magnifications sections can become unstable and actually move at speeds of μm per minute, resulting in poor micrographs. Sputter coat the sections with gold (100 Angstroms) and examine with SEM at 10 to 25 kV. Time can be saved on sputter coating and evacuating the air from the SEM if more than one section is mounted onto the same stub.

Choice of Etching Agents

The method chosen for etching will depend on the purpose of SEM examination. For enumeration purposes, etching with a weak acid or EDTA may be preferable (Fig. 13). In other cases, GA or EDTA-GA etching might provide useful microstructural information on the distribution of organic matrix. In published methods, most investigations use HCl (59%), followed by EDTA (23%), HCl or EDTA (13%), EDTA-GA (2.6%), and acetic acid (2.6%) (Table 1). Other investigators fracture otoliths for SEM preparation. However, it is difficult to fracture otoliths consistently through the core. For smaller otoliths, it is advisable to use weaker acids and shorter exposure periods.

Recommendations

The type of microstructural analysis which is to be undertaken should be reviewed before deciding upon a laboratory protocol. An important consideration is the quantity and quality of preparations needed for an investigation. Before investing time and effort in the craft of preparing otolith sections, it would be wise to anticipate the problems inherent in the analysis of otolith microstructure. Important questions to consider are:

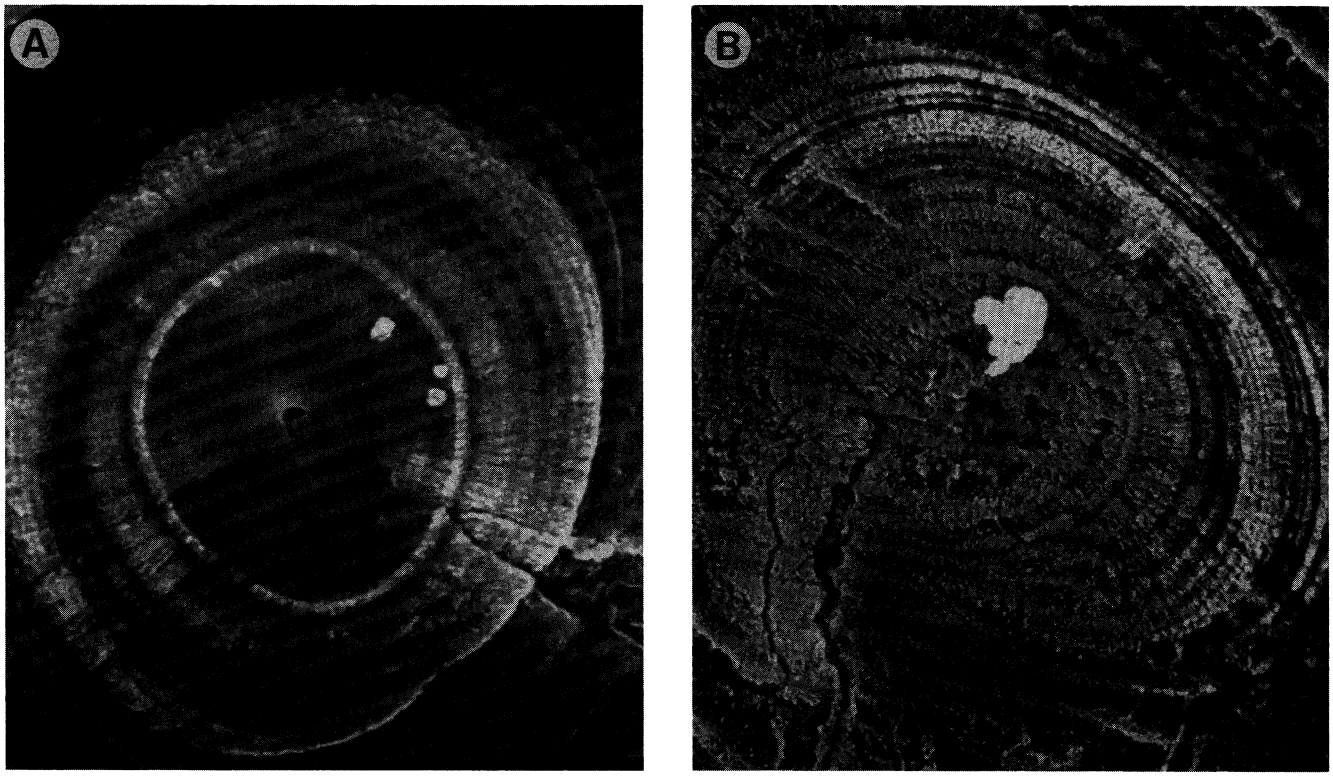

FIG. 13. Core region of sagitta from starved larval striped bass etched with HCL and EDTA. (A) Otolith section etched with 0.1 N HCl (pH=2.5). Note rounded appearance of crystals. (B) same otolith section etched with 5% EDTA (pH=7.5). Note rugose appearance of crystals. Samples from Sacramento population larvae provided by Dr. Maxwell Eldridge (NMFS, Tiburon, CA).

1. What potential information is available in the otolith's microstructure?
2. What equipment and skills are needed in microstructure analysis?
3. What precision and error criteria are associated with the analysis? and,
4. What current technical constraints exist?

Many techniques have been applied to a wide variety of fishes (Tables 1 and 2), and technical precedence should be considered. Although the general polishing techniques described here can be applied to most fishes, the specific techniques described by other authors can allow better comparison of microstructural analyses in certain cases. For instance, if an investigation on a salmonid is planned, Neilson and Geen's papers (Neilson and Geen 1981, 1982, 1985, 1986) should certainly be reviewed. Since methodology is often incompletely reported in publications, considerable time and expense can be saved by consulting with investigators who have had previous experience with a particular species. We would like to see further consultation and collaboration in otolith studies, which would contribute to the standardizing of the technical aspects of otolith preparation and analysis.

In conclusion, we would like to urge the development of standardized practices or procedures such as those listed in the text for otolith preparation. This is essential if we are to compare data or calibrate readers between laboratories. However, this is a young technology, and advances will be made. As new techniques are developed, it is essential that changes in procedures be carefully explained and documented so that others can fully interpret the results and duplicate the methods.

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CHAPTER 4
Measurement and Interpretation of the Microstructure of Fish Otoliths

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Introduction

A typical look at an otolith microstructure preparation reveals many ring-like structures, only a fraction of which are daily growth increments. While the interpretation of daily increments is more firmly based now than was the case even 6 years ago, an undeniable and substantial element of subjectivity yet remains. This point was underlined in a recent study, in which large numbers of known-age, mesocosm-reared herring (*Clupea harengus*) larvae of various ages were distributed for age estimation to experienced otolith readers representing 12 different countries (Campana and Moksness 1991). Larval ages and sampling frequencies were completely unknown to the otolith readers. On average, inter-sample age differences were accurately estimated in the study. However, there were significant (and occasionally large) differences among the readers, most of which could be ascribed to differences in training, technique and increment interpretation. An important conclusion of the study was that certain practices, both technical and interpretational, were superior to others, and that accuracy and precision, both among and within otolith readers, could be improved through reference to a standard protocol. This paper will attempt to provide some recommendations concerning otolith microstructure interpretation in order to meet the above goal.

In the following sections, a number of guidelines will be offered as aids to the successful interpretation and quantification of otolith microstructure preparations. Most of the guidelines have a theoretical basis, but are allied with solid empirical support. Emphasis will be directed towards light microscopic images, since this is the medium most commonly used in microstructural examinations. For similar reasons, the discussion of quantification techniques will focus on daily increment counts and measurements. Other forms of measurement, such as isotope analyses, will not be discussed here. The reader is referred to Neilson (this volume) for additional information on sources of error associated with light microscope examination and interpretation of otolith increments.

Increment Counts

Currently accepted concepts of daily increment formation state that daily increments form as a result of an endogenous circadian rhythm (Mugiya et al. 1981; Campana and Neilson 1985). While environmental masking by fluctuating variables such as temperature and feeding may occur, such environmental cues tend to produce increments in addition to those produced as a result of the endogenous rhythm. Thus, the otolith microstructure can be expected to appear as a regularly-recurring daily increment sequence, occasionally overlain by or interspersed with subdaily increments of environmental origin. A useful analogy might be that of a regularly-recurring pattern of waves on the ocean (the endogenous circadian rhythm), overlain by waves and ripples resulting from passing boats (the environmental cues). The phase and amplitude of the waves due to boats would, of course, vary with their size and time of passing, resulting in boat-induced waves which could be either smaller or larger than the oceanic waves. In addition, the apparently-random phases and wavelengths of the boat waves could amplify, negate, or intersperse with the oceanic waves, resulting in an overall wave pattern which may or may not appear regular. Subdaily increments form a similar pattern, in that they may be of variable intensities, widths and phases within a daily increment. However, the underlying daily increment pattern is usually smooth and regular. These observations, which are consistent with the underlying conceptual basis for increment formation, simplify the interpretation of previously-unexamined otoliths since microstructural growth patterns appear to be ubiquitous among all species (Campana and Neilson 1985; Jones 1986).

Selection of Counting Axis

Selection of an appropriate counting path or axis is a mandatory step prior to further examination. Two criteria should be considered in the selection process: axis length and increment clarity. Axis length is a key fac-

FIG. 1. Selection of an increment counting path (solid line) in a polished juvenile cod lapillus. The path begins at the hatch check, proceeding distally to a check which serves as a landmark when the counting axis is shifted. While there are a number of possible counting paths, all would avoid an extended region of confluent increments (C) and a second region of unclear increments (U). Bar = 20 μm.

tor, since not all otolith radii exhibit a complete increment sequence, particularly along the shorter axes (Fig. 1). Sectors containing incomplete sequences can be recognized by the presence of confluent daily increments and/or checks, and should be studiously avoided. While in theory it would be preferable to select the longest axis for examination, potentially-confusing subdaily increments will often be most prevalent along this axis. A practical compromise generally involves the shortest radius consistent with a complete increment sequence (Fig. 1). Selection of the counting path should then be tempered by the second criterion, that of increment clarity. Variations in increment clarity along a counting axis are the norm, and may be due either to inconsistencies in preparation or to uncontrolled factors associated with otolith growth. Since increment counts need not be carried out along a straight line, it is often helpful to shift the counting axis where required to avoid regions of ambiguity or poor clarity. Of course, increment continuity must be maintained at all times. When examination is to be made with a light microscope, counting paths are best first mapped out in the mind using magnifications that are lower than required for the subsequent increment counts.

Image Optimization

Microstructural features of most otoliths are minute; thus, subtle refinements in observational technique can prove to be of substantial value. Careful preparation of the sample, particularly through polishing, is generally most influential in enhancing visibility of an increment sequence (see Secor et al., this volume). However, the influence of proper microscopic technique, and more recently, image analysis systems, cannot be overstated. In what follows, emphasis will be directed towards optimization of light microscopic images, since this is the medium most commonly used in microstructural examinations. Optimization of SEM images is addressed in most SEM (scanning electron microscope) operating manuals, and will not be discussed here.

Microstructural examinations are best made with a compound microscope with the following features (at a minimum): binocular eyepieces, at least one of which can be focused; objective lenses with nominal magnifications of 16, 40 and 100×, at least one of which is designed for oil immersion; a moveable specimen stage; a substage condenser lens; an aperture diaphragm; and a variable-intensity illuminator with its own focusing/condensor lens. Use of an inferior microscope will, at best, introduce eyestrain in the observer, and at worst, introduce substantial error into the increment count. The most serious risk involved in the use of a microscope with inadequate or poorly-aligned optics is the failure to recognize the presence of narrow increments (<1 μm in width), such as would occur in temperate fishes with a pelagic larva stage (Campana et al. 1987; Jones and Brothers 1987) and subadult/adult fishes.

Image quality in general, and resolution in particular, can be influenced as much by microscopic technique as by hardware. For this reason, and given the sensitivity of many otolith interpretations to the quality of the image, some discussion of the factors influencing resolution are warranted. Resolution is defined here as the minimum distance between two structures consistent with the two structures remaining visually discernable. For a more complete discussion of microscopic principles and techniques, the reader is referred to one of the many excellent texts on the subject (eg. Eastman Kodak Co. 1980).

The objective lens is probably the single most influential factor in modifying resolution. Of the three major types, the achromat lens is the most popular and the least expensive. Such a lens provides partial correction

for colour and spherical aberration, as well as a low but serviceable numerical aperture (NA). (The lens type, objective magnification, and NA are invariably etched into the body of the objective). Users of an achromat lens will often note that the image appears to improve when the aperture diaphragm is closed down, and when light of a single colour (e.g., green light) is used. The change in image quality can be attributed to the fact that the lens is not fully corrected for all wavelengths of light or for the entire field of view. The semi-apochromat lens, generally made of fluorite, is more completely corrected for aberrations, while the apochromat lens is almost fully corrected. The latter provides both the best image quality and resolution (Table 1), although all three types can be used for routine examination of otolith microstructure. All lens types can be purchased as flat-field objectives (e.g., planoachromat), which improves the image towards the edge of the field of view.

The numerical aperture (NA) of the objective ultimately controls both the magnification and the resolution that are obtained. Resolution (R) increases with NA as in:

$$R = \text{lambda}/(2\ NA)$$

where lambda is the wavelength of light that is used. Thus, the highest resolution is possible with objectives of the greatest NA (Table 1). Note, however, that the NA of the microscope is limited by the refractive index of all media between the condenser and the objective, as well as by the NA of the objective lens. The presence of air along the light path limits the effective NA to 1.0, no matter how large the NA of the objective being used. Therefore, an oil immersion objective must be used if an NA > 1.0 is to be achieved. What is not as widely known is that immersion oil must also be used between the condenser lens and the bottom of the microscope slide for an overall NA > 1.0 to be reached. Few microscope users (in the field of otolith microstructure) appear to be aware of this constraint, making the useful resolution limit of a perfectly-aligned light microscope close to 0.27 μm (Table 1). The use of a blue filter over the light source can improve this limit by 15–20%.

While resolution has a well-defined limit in light microscopy, magnification can be increased almost endlessly. Thus, it is fairly easy to set up a microscope with a 100× objective lens, 25× eyepieces and a 2× body tube to yield a magnification of 5000×. However, most of the magnification is "empty"; that is, the image is large, but reveals no extra detail beyond that visible at around 1250×. The maximum useful magnification for most microscopes is 1000–1250× (Table 1).

Aside from the objective, an optimized source of illumination will have the greatest influence on image quality. Kohler illumination is the most common means of optimizing a light source, and will result in bright and even illumination over the entire sample, with good depth of field and resolution. The steps involved in adjusting for Kohler illumination are discussed elsewhere (Eastman Kodak Co. 1980), but revolve around centering the light source in the image, focusing the light on the plane of the stage (by focusing the condenser), and adjusting the aperture diaphragm. A properly focused condenser will generally be near the top of its travel range. Once in place for a given microscope, few extra adjustments are needed as the specimen or magnification are changed. While it is often used (incorrectly) to compensate for changes in illumination, a properly adjusted aperture diaphragm will balance contrast, depth of field and resolution.

TABLE 1. Limiting characteristics of a compound microscope using each of the major objective lens types. All numbers assume a perfectly aligned and optimized optical system. Adapted from Eastman Kodak Co. (1980). NA = numerical aperture.

	Typical NA of objective	Overall NA of microscope	Resolution under green light (μm)	Maximum useful magnification	Depth of field under green light (μm)
Achromat					
10×	0.25	0.25	1.10	250×	8.52
45× (dry)	0.65	0.65	0.42	650×	0.99
100× (oil)[a]	1.25	1.00	0.27	1000×	0.30
100× (oil)[b]	1.25	1.25	0.22	1250×	0.30
Apochromat					
10×	0.32	0.32	0.86	320×	5.83
45× (dry)	0.95	0.95	0.29	1000×	0.19
100× (oil)[a]	1.40	1.00	0.27	1000×	0.16
100× (oil)[b]	1.40	1.40	0.20	1400×	0.16

[a]Using immersion oil between specimen and objective, but not between condenser and slide.
[b]Using immersion oil both between specimen and objective and between condenser and slide.

Once the optics of the microscope have been aligned and optimized, the otolith reader must select the magnification which will be used to examine the sample. The intent should be to balance the apparent clarity of the increment sequence with ease of counting. Daily increments invariably appear most distinct at lower magnifications, in part because visual artifacts and subdaily increments (and any narrow daily increments) are less prominent. Aside from the reduced resolution of adjacent increments, it is intrinsically difficult to count sequences of growth increments at low magnification; the human eye tends to wander involuntarily when large numbers of structures with similar appearances are visible in the same field of view. At the other extreme, high-magnification examination of broad increments can be very confusing; the internal structure of both the discontinuous and incremental zones can be surprisingly complex. As a general rule of thumb, a magnification of 400× is often appropriate for the examination of rapidly-growing otoliths, while 1000–1250× will be mandatory where increments are less than 1–2 μm in width. In both instances, a useful endpoint is a field of view where about 20 increments are visible at one time. Fortuitously, the use of immersion oil with high magnification objectives (those above 40×) tends to smooth out the image by obscuring surface imperfections in the sample.

While not necessarily useful in other applications of microscopy, frequent focal adjustments during the scanning and/or counting of growth increments are almost mandatory. Focal adjustments not only simplify the differentiation of daily and subdaily increments, but they compensate for intrinsic variations in the focal plane of the increments themselves. Such variations, whether due to alignment errors during

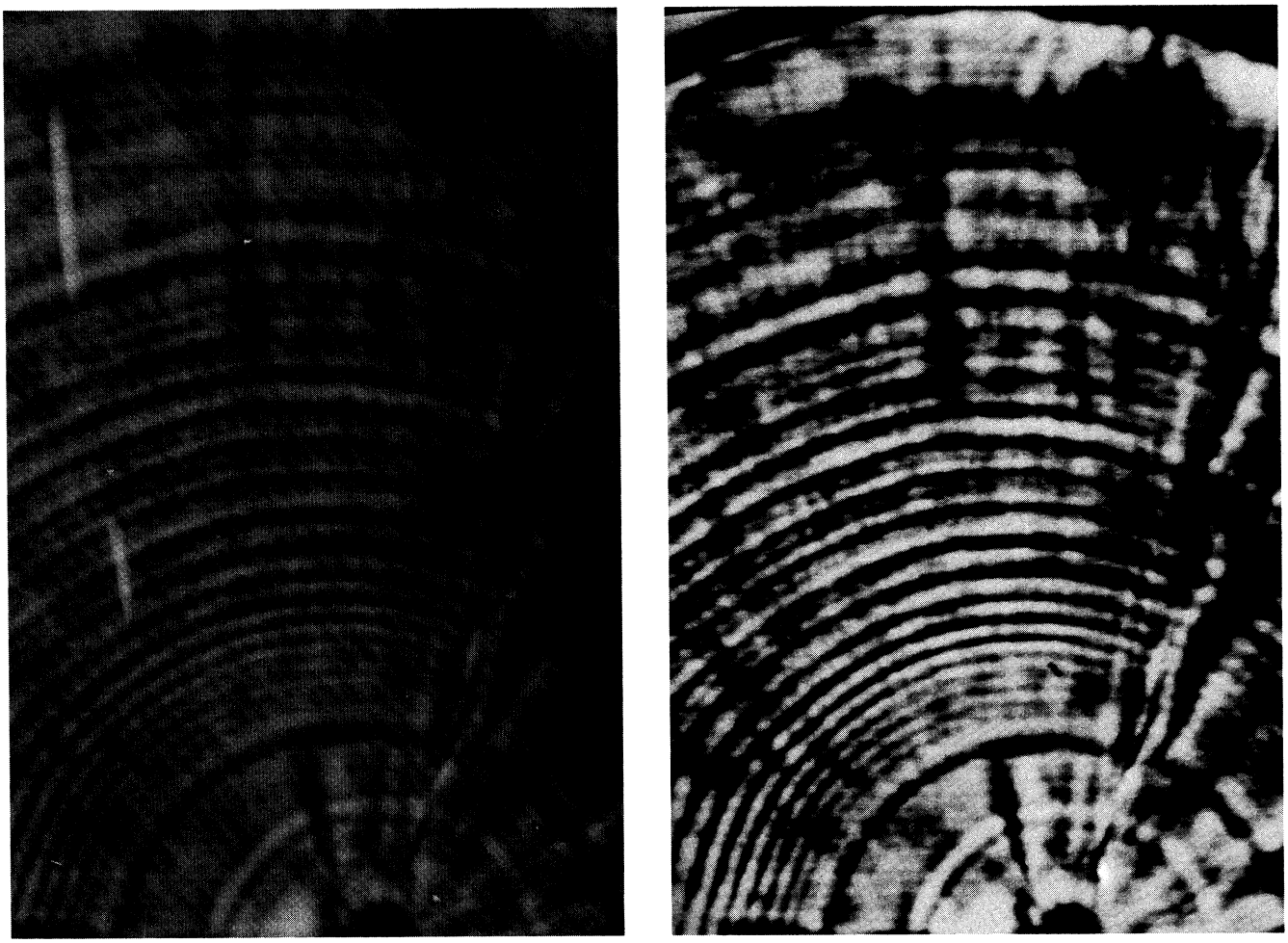

FIG. 2. Grey level expansion (*right*), with an image analysis system, of a poorly-contrasted light-microscopic view (*left*) of an otolith growth sequence, as photographed directly off of the video monitor. Both photographs were taken, developed and printed under identical conditions. Preparation of the enhanced video image took approximately 2 seconds. While grey level expansion is an effective means of enhancing contrast, other image analysis procedures can be used to further sharpen or filter an image.

mounting or to nonplanar otolith growth, account for the difficulties and/or inaccuracies that many workers experience in counting daily increments from photographs. The photographic depth of field is also very small (Table 1). For this reason, direct microscopic examination is generally preferred over photomicrography for counting increments.

Image Analysis Systems

An image analysis system can be a powerful tool to those working with otolith preparations. Thus, a brief description of the capabilities and applications of image analysis is warranted. Image analysis is a generic term used to refer to the digitization and manipulation of visual images. In its simplest form, an image analysis system can store a picture in memory and and reproduce it, unaltered, upon command. In practise however, images entered into an image analysis system are enhanced and/or quantified before re-display; therein lies their advantage over visual examination. The end product is an image (or data) which can be more easily interpreted than the original. Image analysis systems should not be confused with simple video-microscope display units. While the latter have been used to advantage in studies requiring precise otolith measurements (i.e., Methot and Kramer 1979; Bolz and Lough 1983), such units are capable of neither image enhancement nor image manipulation.

Recent technological advances have brought microcomputer-based image analysis systems within the financial grasp of an individual researcher. Standard systems now consist of a video camera, a digitizer board mounted within a microcomputer, and a monitor (for an example, see Campana 1987). The video camera would be mounted on a microscope for otolith-based research. Most of the digitizer boards available today are "framegrabbers", capable of digitizing and storing 30 images per second. Thus, real-time viewing and image manipulation is not only possible, but the norm.

The basis of operation for all image analysis systems is the conversion of an image into an array of numbers — in other words, image digitization. Each position in the array represents a pixel (grid square) in the image, and each numerical value represents a gray level (measure of light intensity) for that pixel. Thereafter, anything that can be done to an array of numbers can be done to an image. For example, image contrast can be doubled by doubling each pixel's gray level. Since the results of an array manipulation can be seen immediately on the monitor, image manipulation can be as interactive or as automated as desired.

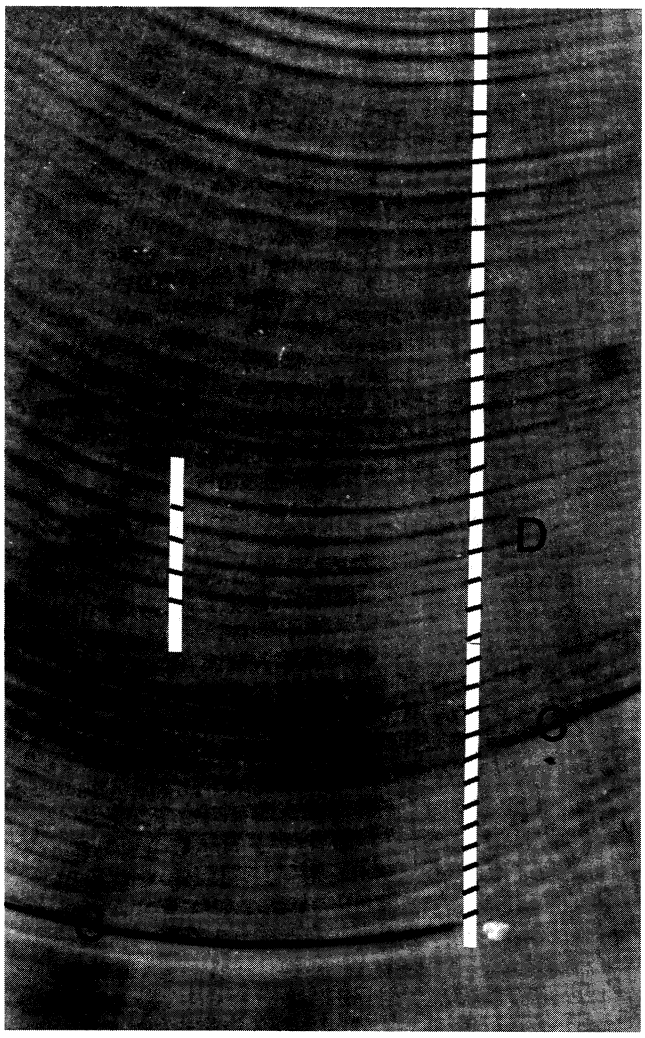

FIG. 3. A growth increment sequence characterized by daily (D) and subdaily (S) increments, as well as by checks (C). The daily increment sequence is smooth and regular in its appearance; changes in increment width and contrast are often gradual.

In the context of microscopic observations, image analysis systems provide three major advantages: image enhancement, manipulation, and quantification. Image enhancement is one of the most important and widely-used features. Simple procedures allow the operator to subtract an image background from the entire image, average several noisy images, or use high or low-frequency filters to add or remove detail. Gray level expansion, whereby the gray levels in a poorly-contrasted image are spread out over all 128 (or more) levels, can bring out detail that is totally invisible to the unaided eye (Fig. 2). All of these enhancement procedures are effective because of the limited capability of the human eye — differentiation of 128 gray levels is well beyond our visual capacities.

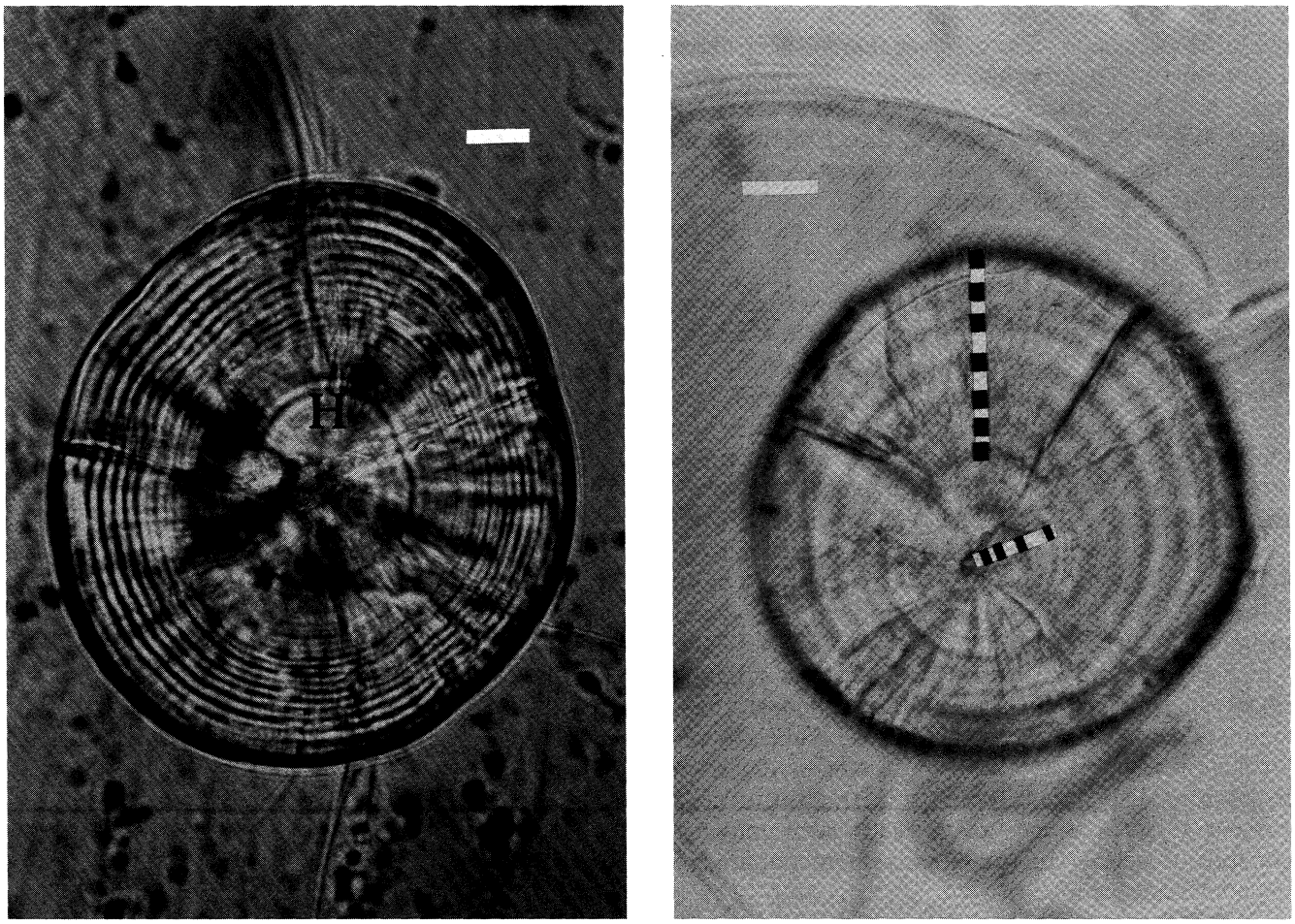

FIG. 4. Daily increments can often be differentiated from similar-appearing structures by their location on the otolith and by species-specific characteristics. (*left*) Daily increments encircling the hatch check (H) of a polished haddock sagitta are narrow and weakly expressed for the first 10–20 d after hatch, but broaden as the postlarval and juvenile stage is entered. Many of the perinuclear daily increments visible under greater magnification with the light microscope are not evident in this photograph, which shows only 24 of the total of 39 increments from a 8.35 mm larva. Bar = 10 μm. (*right*) In contrast to the haddock sagitta, the lapillus from an 11-d old walleye larva has broad daily increments almost from the date of hatch. Broad increments such as these are also characteristic of many tropical fish otoliths. In such cases, low magnifications (300–500×) may be most appropriate for the examination. Bar = 20 μm.

Increased ease of visual interpretation is the primary advantage of an image analysis system in counting growth increments. When measurements are being made, a variety of other features become evident. Foremost of these is ease of measurement. Not only is a video monitor target easier to position than an ocular micrometer, but distances approaching the theoretical resolution limit of light microscopy (0.20 μm) can be measured. Of course, all measurements can also be stored directly in computer memory, eliminating the error potential of handwritten transcription. These and many other applications are detailed elsewhere (Gonzalez and Wintz 1977; Hall 1979; Ballard and Brown 1982; Campana 1987).

Despite the undeniable benefits of image analysis systems to otolith microstructure examination, it is important to recognize their limitations as well. Use of such systems does not improve resolution; while visual contrast can be enhanced considerably, the resolution limit of light microscopy is inviolable. Secondly, the automatic-count capabilities of many systems are not yet appropriate for studies of otolith microstructure. And finally, image storage presently requires too much memory (256K) for the creation of large image archives. Since image analysis technology is now progressing rapidly, these latter two constraints may well disappear in the near future.

Increment Interpretation

In a typical otolith preparation, numerous ring-like structures are evident, only a fraction of which are daily

growth increments. With familiarity, the distinction between "real" daily increments and most other features is a routine procedure. However, unpractised otolith readers can introduce enormous errors into an increment count, while even practised workers can differ (sometimes substantially) in their interpretation of a given increment sequence. This element of subjectivity is one of the most significant sources of error in otolith microstructure examination, and largely explains the current absence of automated counting instruments. It also explains why validation (discussed in Geffen, this volume) is more important as a check on the interpretive skill of the worker, than as a check on the true frequency of increment formation. In this section, a number of guidelines will be offered as an aid to more informed interpretation of microstructural features. Not surprisingly, ease of interpretation improves with experience and the degree of sample preparation.

The three light-microscopic features most commonly confused with daily growth increments are, in order of importance: subdaily increments, visual artifacts, and checks. All except visual artifacts serve to confound SEM interpretations as well. Criteria for the differentiation of the three features are largely based upon visual appearance; in particular, contrast and relative width (Fig. 3). However, location on the otolith may also provide clues as to the identity of a particular structure. For instance, daily increment widths are often narrow in the region encircling the hatch check, while broad, clearly-defined increments characterize growth during the juvenile stage. As age increases beyond this point, increments tend to narrow, and may become vanishingly small, or even intermittant in their formation. Knowledge of this general growth pattern is often helpful in interpreting an increment sequence (Fig. 4).

Daily and subdaily increments are morphologically similar, making differentiation of these two types of structures difficult. Criteria for their differentiation take advantage of the differing factors behind their formation. Daily increments form at a constant frequency, and due to the conservative nature of otolith growth, often appear as a regular sequence with smooth transitions in both increment width and increment contrast. Subdaily increments on the other hand, may form at any date or time of day, rendering their widths less regular. The visual prominence of subdaily increments is usually less than that of adjacent daily increments (Fig. 3), but will vary with the strength of the masking agent responsible for their formation. In practice, it is

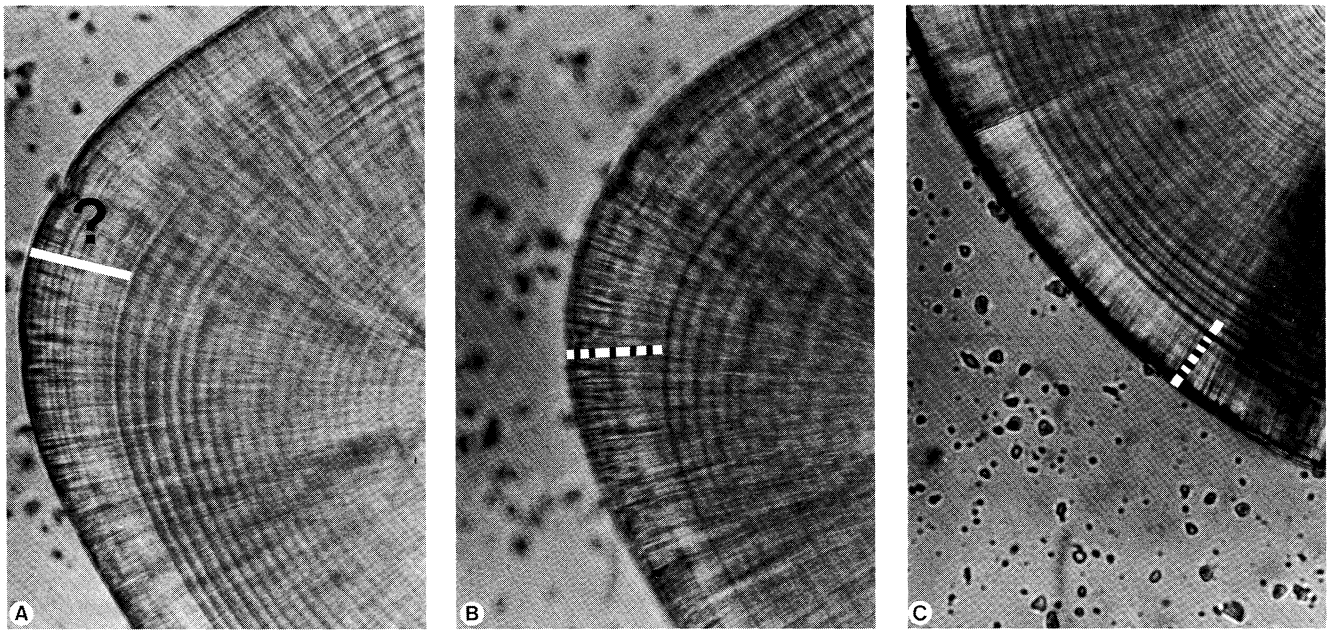

FIG. 5. Tactics for dealing with "split" or subdaily increments. (A) A properly-focused view of the edge of a polished herring sagitta. Whether due to preparation difficulties or to the presence of subdaily increments, an unambiguous sequence of daily increments from nucleus to edge suddenly shifts to "daily" increments (11 or more) of much narrower width. The sudden shift in increment widths, along with the apparent splitting of the most medial increment of the marked zone, indicates that the zone should be interpreted carefully. (B) The same view as in (A), intentionally made out of focus. The periodicity of the broad, underlying pattern in the suspicious zone is similar to that of the unambiguous daily increment sequence, suggesting that the zone actually represents 4 or 5 daily increments. (C) A different region of the edge of the same otolith visible in (A) and (B). This region of the otolith confirms the increment interpretation derived from the out-of-focus examination in (B).

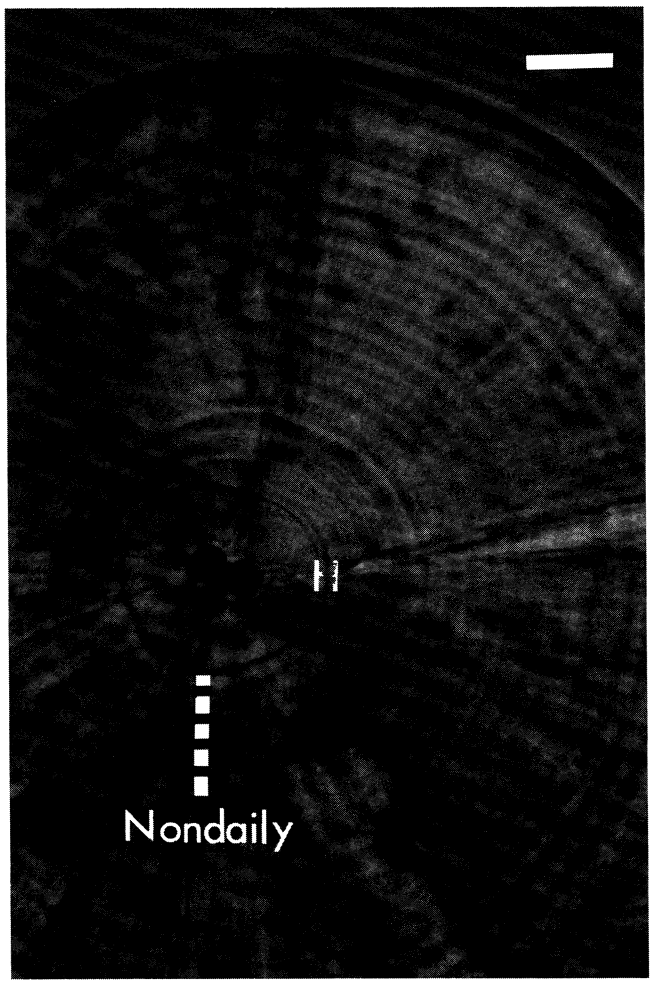

FIG. 6. Aggregates of daily increments may sometimes look more convincing than do the actual daily increments. The 4 false increments marked on this photograph of a known-age herring larva sagitta actually correspond to a period of about 20 d. Some of the true daily increments are visible to the upper right of the hatch check (H). Careful and frequent adjustments of the microscope focus are required to correctly interpret this type of increment sequence. Familiarity with the overall growth pattern of herring otoliths would also prove useful here. Bar = 10 μm.

often best to locate a region of unequivocal daily increments along the intended counting path, and then proceed outwards (or inwards) from that point, using the regularity/continuity criteria in interpretation. Where adjustment of the microscope focus appears to "split" increments, the broader of the two patterns can often be assumed to be daily. Indeed, in cases where otolith growth has been rapid and subdaily increments are numerous, a slightly out-of-focus examination may aid in eliminating subdaily increments from the field of view (Fig. 5). This approach is not appropriate where increments are narrow, such as around the hatch check, since aggregates of daily increments may then become evident (Fig. 6). Where ambiguity between daily and

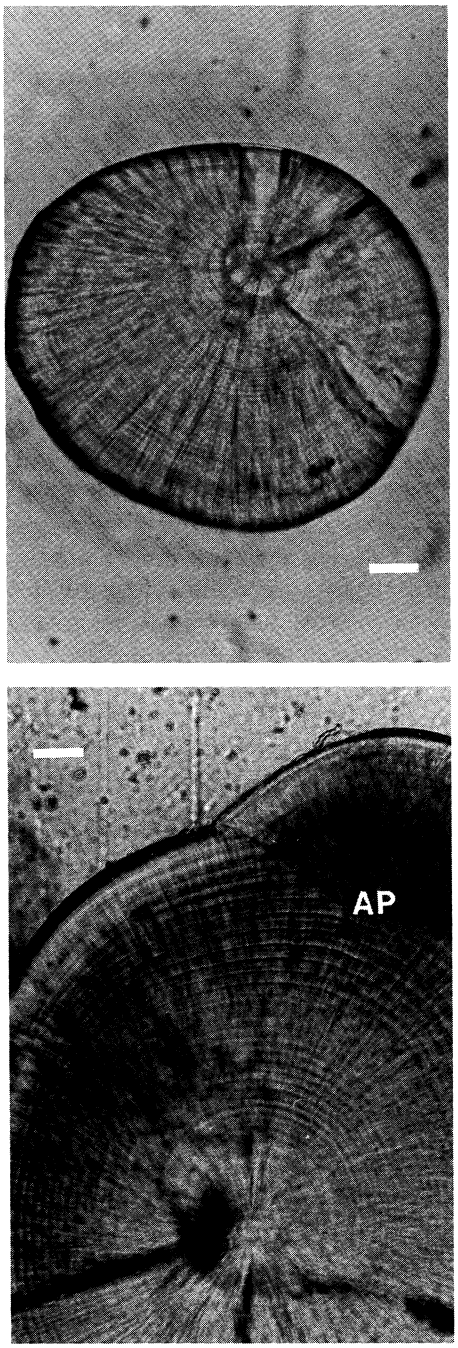

FIG. 7. A comparison of the microstructure of lapilli and sagittae from the same, 20 mm cod. Both otoliths have been polished, and reproduced at the same scale. Bar = 20 μm. The growth sequence in the lapillus (*top*) has well-defined and spatially-uniform increments, although the latter would become increasingly narrow and difficult to interpret in older juveniles. In contrast, the daily increments in the sagitta (*bottom*) are narrower than those of the lapillus for the first 5–15 d after hatch (not visible at this magnification), but become increasingly broad with age. Increments towards the edge of the sagitta are more than 3 times as broad as those at equivalent ages in the lapillus; the sagitta also shows evidence of splitting and/or subdaily increments in the outermost 15 d. Daily increments are broader yet, but indistinct, around the newly-formed accessory primordium (AP) at upper right.

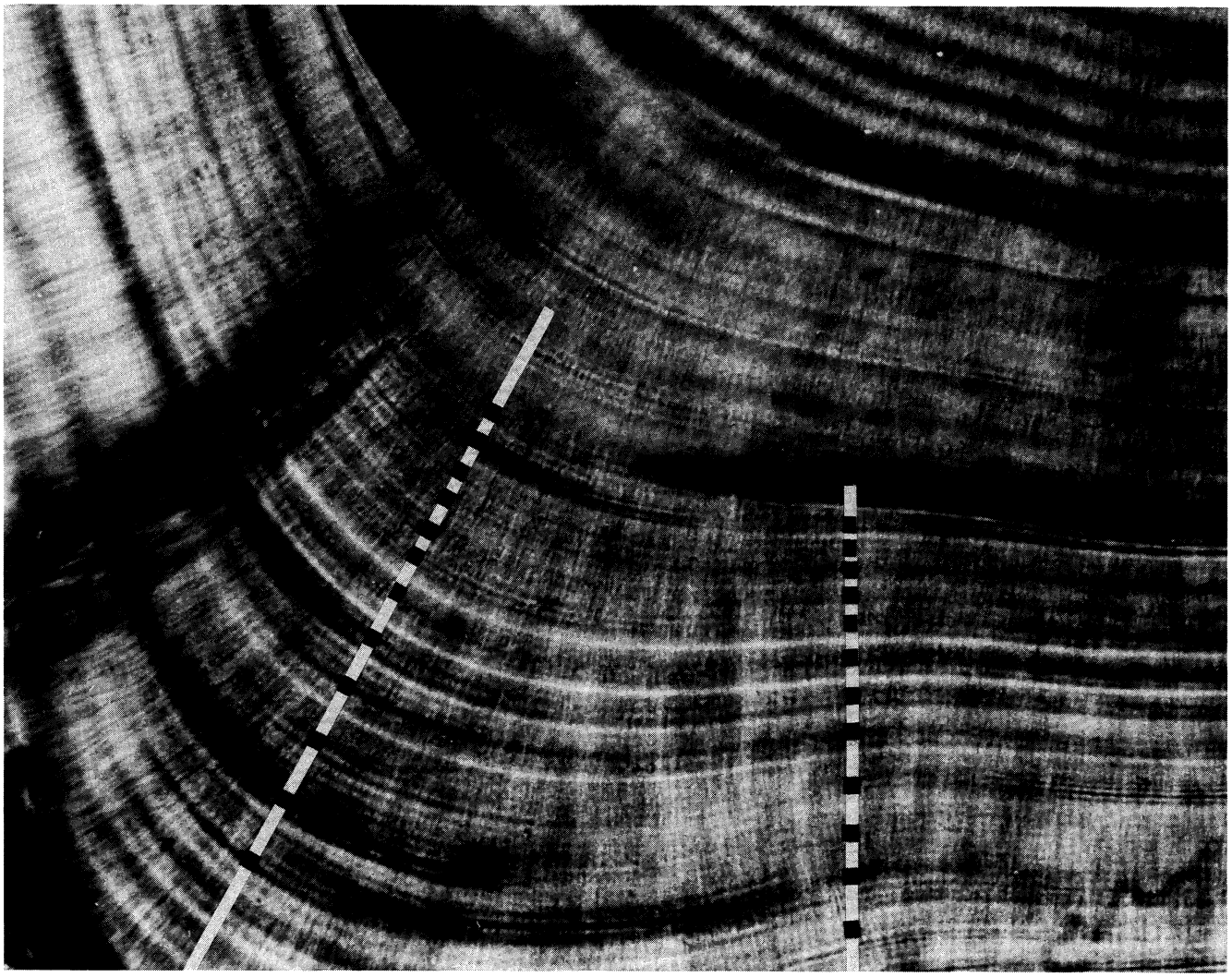

FIG. 8. Overgrinding can make subdaily increments appear more prominent than they would be otherwise. Subdaily increments are prominent, and daily increments indistinct, along two marked growth axes of an overground starry flounder (*Platichthys stellatus*) sagitta. Daily increments have been indicated in black; the daily pattern is also more apparent in the better-prepared section to the lower right.

subdaily increments persists, the best tactic is to avoid that region of the otolith. Counting paths need not be linear, and interpretive ease of a given sequence often varies among the potential counting axes.

Subdaily increments tend to be most prevalent in two situations. The first is in regions in which the otolith has grown rapidly, resulting in very broad daily increments. The broader the incremental zone of a given increment, the greater the potential for subdaily increments to have formed, and more importantly, the easier it is to see them. This is one reason why the microstructure of a juvenile fish sagitta can be difficult to interpret — the increments tend to be very broad (Fig. 7). Since the lapillus has a lower and more spatially uniform specific growth rate, increments are narrower, and as a result, better defined (Fig. 7) (see Secor et al., this volume, for further detail). Of course, this inter-otolith growth difference can be counterproductive in lapilli of old fish, since increments there can be so narrow so as to be difficult to resolve.

The second situation promoting visibility of subdaily increments is an artifact of preparation difficulties. For various reasons, overgrinding can make subdaily increments appear more prominent than daily increments. Indeed, the former can appear both regular and well-defined in overground preparations, making this a particularly dangerous sequence to interpret (Fig. 8). Proper recognition of overground regions can minimize counting inaccuracies due to this effect.

Visual artifacts take several forms, some of which may mimic daily increments. Refraction of light through and around the curved edge of the otolith can

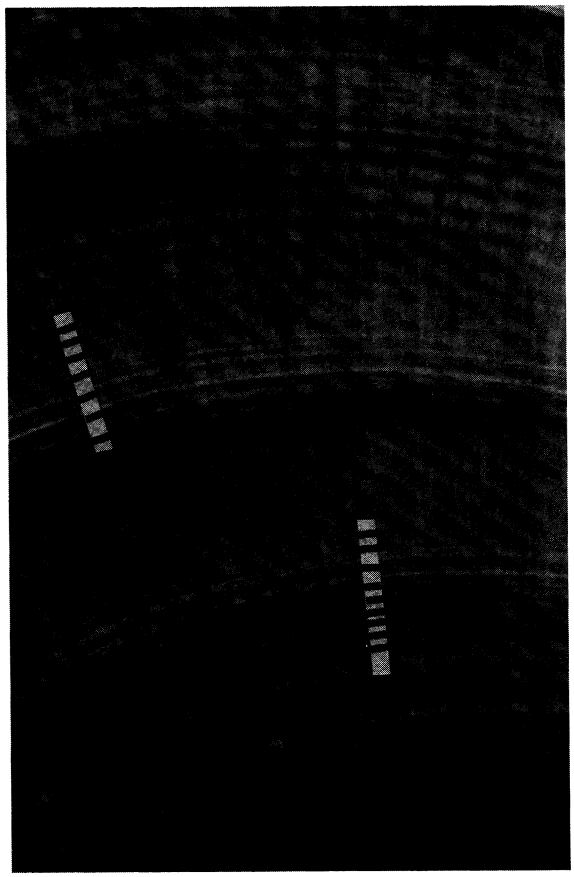

FIG. 9. While the fine lines between the marked daily increments in this photograph of a starry flounder sagitta were originally termed subdaily increments (Campana and Neilson 1982), their appearance under a light microscope and their absence under a scanning electron microscope indicates that they are actually visual artifacts associated with prominent increments and/or checks. Bar = 10 μm.

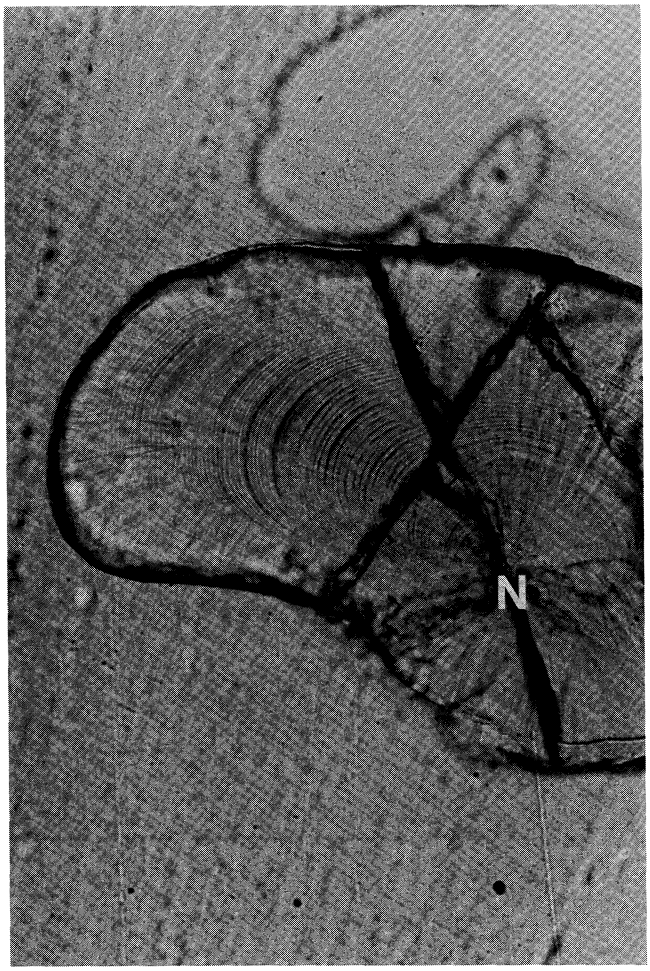

FIG. 10. Curvilinear growth axis in the lapillus of a juvenile flyingfish, *Paraexocoetus brachypterus*. There is no single straight line which can be drawn from the nucleus (N) to the otolith edge which will intersect a complete sequence of growth increments at right angles. However, all standard growth backcalculation procedures assume a linear backcalculation trajectory.

distort the width and/or number of increments visible, making accurate interpretation difficult. However, edge effects usually compromise the appearance of only a few increments, thus allowing interpolation if necessary. Artifacts resulting in increment "reflections" are most visible just outside the perimeter of the otolith, but are also associated with checks and prominent increments. Differentiation is on the basis of the appearance of the adjacent structures; artifacts appear as exact reflections, sometimes in multiple copies, of the nearest increment, but are usually more sharply defined than the reflected structure (Fig. 9). Focal adjustments can serve to minimize the number of visual artifacts, but since they may also influence the interpretation of true daily increments, should not be used as the sole defining criterion.

Checks have never been adequately defined, due in part to the variety of agents attributed to their formation (Pannella 1980; Campana 1983, 1984; Gauldie 1988). Where they appear as particularly prominent increments, perhaps in response to short-term stress (Campana 1983), interpretation is not a problem. Checks associated with the lunar cycle (Campana 1984) or interrupted otolith growth (Pannella 1980) are easily confused with cracks or fissures, and may appear to reside on a different focal plane than that of the surrounding increments (Fig. 3). Such checks are not daily increments, and should not be counted as such, although they may overlay true increments. Regions of interrupted otolith growth, perhaps characterized by confluent or rapidly-narrowing increments, should be avoided during increment counts. If a com-

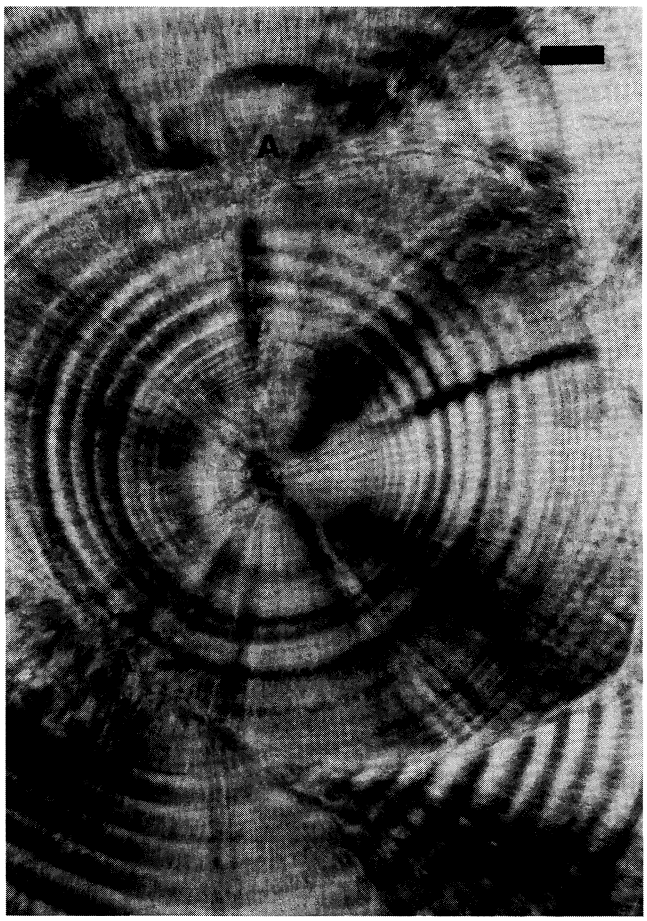

FIG. 11. Central (C) and accessory (A) primordia in the sagitta of a starry flounder. Individual increments are contiguous as they pass from one primordial growth field to another, but their width changes substantially. Bar = 20 μm.

plete sequence around the problem area cannot be found, the otolith must be discarded; after all, there is no way to assess the duration of the otolith growth interruption. Note, however, that checks that overlay an apparently normal increment sequence seldom signify the presence of interrupted growth (e.g., Fig. 3).

While the presence of accessory primordia can complicate measurements of increment width (as discussed in a later section), they should have little effect upon increment counts. Increments are almost invariably contiguous across the growth zones corresponding to different points of nucleation (e.g., Fig. 7B, 11).

Optimization of Counts

The practice of providing a single best increment count for a given otolith can be unexpectedly difficult. Most scientists are capable of counting up to 100, or even beyond. However, increment counts are invariably complicated by interpretive difficulties, variations in preparation quality along the counting path, nonlinear counting paths, and the limitations of the human eye. In this section, we provide some empirically-derived suggestions for minimizing these problems.

The human eye tends to wander involuntarily when counting extended sequences of tightly-packed, repeated structures such as growth increments. Distinct otolith features such as checks, scratches, or prominent increments should thus be used to advantage as stopping points or landmarks for the eye. Counting bursts of 10–15 increments between landmarks are ideal, since they allow the examiner to scan the region ahead for interpretive difficulties without losing track of the last counted increment. Such landmarks are also useful when lateral shifts of the counting path are to be made. Note also that constant adjustment of the focus while scanning not only simplifies the differentiation of daily and subdaily increments, but compensates for variations in the focal plane of the increments.

Preparation quality is seldom uniform along the counting path. In most cases, a complete count will be possible. However, there may be instances where cracks, overgrinding or undergrinding occlude short sections of the increment sequence. Since daily increment widths tend to be autocorrelated, interpolation may be justified if the interpolated increment number is very small relative to the overall count. Age estimates based on 5% interpolation are probably acceptable, although the exact percentage is arbitrary. Percentages as high as 20% have been reported (Methot 1983), but are not recommended. Interpolation is often appropriate at the otolith edge; however, it is not appropriate where increment widths are changing (i.e., around the nucleus).

A minimum of two complete counts (and preferably more) should be obtained for each otolith. Counting errors will normally be minor compared to differences in interpretation. Since interpretation may differ with the point of origin for the counting path, one count should originate at the otolith periphery, while the other should begin at the hatch check (or designated first increment). There is no strict convention concerning the inclusion of the hatch check and the otolith periphery in the increment count; however, the method that is eventually adopted should always be reported. Calculation of a single "best" increment count for a given otolith is discussed in a later chapter (Campana and Jones, this volume).

Increment Measurements

Accurate measurements of daily increment widths are intrinsically more difficult than simple counts of the same increments. In addition, interpretation of the mea-

surements in terms of otolith or fish growth is not necessarily straightforward (see Campana and Jones, this volume). In what follows, guidelines for the selection of a measurement axis and the collection of accurate measurement data will be presented. Details of image optimization and interpretation are similar to those discussed earlier, and will not be addressed further.

Selection of Measurement Axis

Selection of a suitable measurement axis requires the same axis length and increment clarity criteria as those associated with increment counts. However, there are two additional constraints put upon the selection procedure: the measurement path must be linear, and otolith growth should be roughly symmetrical. These constraints are based upon the eventual application of increment width measurements to calculations of otolith or fish growth. To be interpreted, increment widths must be put into the context of overall otolith size and/or growth rate. Yet virtually all otoliths have eccentrically positioned nuclei, implying that the width of a given increment can be expected to change with the length and orientation of the otolith radius under examination. Thus, a single, linear axis must be used for all increment width measurements within a given otolith. When the increments of more than one otolith are to be measured, the orientation of the measurement axis should be standardized to minimize among-fish variation. Note that the above requirements are far more constraining than those associated with increment counts, since regions of difficult interpretation or poor preparation quality cannot be avoided through lateral shifts of the field of view. There is also an additional complication. In instances where the axis of otolith growth is curvilinear, it is impossible to measure maximum increment width (parallel to the growth axis) while maintaining linearity between the nucleus and otolith edge (Fig. 10). Since oblique measurements of increment width are meaningless, growth backcalculations of such an otolith would have to be based upon the length of the curvilinear growth axis. Although theoretically possible, curvilinear growth axes have seldom been measured, presumably due to the difficulty of defining a curved line which intersects all growth increments at right angles.

The second constraint, that of growth symmetry, is associated with measurement axes that pass through regions where the axis or rate of growth has shifted. For example, the width of a given increment may change substantially with proximity to an accessory primordium (Fig. 11). Accessory primordia are seldom observed in lapilli, unlike the situation in sagittae, making the former a more suitable choice for increment width measurements. However, shifts in growth axes may occur in the absence of accessory primordia. For instance, many otoliths are nearly circular in larval fishes, but become increasingly asymmetrical through the juvenile stage. Thus, what may be a perfectly suitable measurement axis in a larval fish may be completely unsuitable in a juvenile. Such an effect can be controlled by ensuring that the measurement axis used for the juveniles is the same as that measured for the larvae. Since the two axes will not necessarily correspond with the longest axis of the otolith (at either life history stage), some care must be taken to ensure that the appropriate axes are used. The ubiquity of asymmetric otoliths also indicates that increment widths, by themselves (without an accompanying fish-otolith relationship) are poor indicators of growth rate.

Optimization of Measurements

Increment widths may be measured from SEM micrographs, light micrographs, digitized images, video displayed images, or using a light microscope with an ocular micrometer. Irrespective of the method used, individual increment measurements are predicated upon orientation parallel to the axis of growth at that increment, not just parallel to the overall growth axis. All techniques suffer from potential sources of bias or difficulty in preparation, although some problems are more acute than others. The selection of measurement technique should therefore be based on the requirements for accuracy and precision, as well as access to specialized equipment.

The exact procedure by which individual increments are measured is probably less important than is the consistency in procedure across increments. That is, there is little reason to believe that the measurement of an increment from the medial side of one discontinuous zone to the medial side of the next discontinuous zone will be any more (or less) accurate than, say, from the center of one incremental zone to the center of the next. However, whichever protocol is applied, it is very important that it be applied consistently to all of the increments under study.

The major source of bias in the measurement of increment width is the effect of the focal plane upon the image magnification and the resulting increment width. In light microscopy, adjustment of the focal plane is critical to obtaining an undistorted image of the increment sequence. Yet focal adjustments also induce shifts in the apparent width and position of each increment. Accuracy is maximized when the increment being measured is in optimum focus; since the optimum focal plane for one increment is not necessarily the same as that for the adjacent increment,

compensations must be made for the consequent shift in increment position whenever the focus is adjusted. However, it is more difficult to compensate for the accompanying shifts in magnification. This problem applies as much to light micrographs as to visual microscopy. The best solution reported to date is to minimize or avoid the problem; the otolith should be mounted so that the incremental plane is as close to horizontal as possible. Small shifts in focus are unlikely to result in major changes in apparent increment width. However, the apparent lateral shift in increment position can be more substantial, and can result in significant measurement error if the new measurement start point is not used after refocusing.

Light-microscopic increment width measurements are best measured with an image analysis system (Campana 1987) or video-microscope system (Methot and Kramer 1979; Bolz and Lough 1983). While the former provides the added advantage of image enhancement capabilities, both provide the operator with a target on a large video screen, as well as the flexibility to make continual focal plane adjustments. Such measurements are much more precise than those obtained with an ocular micrometer, and reduce the potential for error by transmitting the data directly to a computer file. Video measurements are also much more rapidly obtained than those from an ocular micrometer or photographs.

The most accurate increment width measurements are derived from SEM micrographs. Such measurements are not subject to the refractive effects that can shift or distort an image under a light microscope. Accuracy and precision is then limited only by the clarity of the photograph and the means by which the increments are digitized. Aside from accessibility, the major constraint of SEM measurements is sample etching. Etching of a full increment sequence can be difficult to achieve (Blacker 1975; Campana and Neilson 1985). For this reason, SEM measurements are more appropriate for discrete regions of the otolith rather than complete radii.

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CHAPTER 5
Analysis of Otolith Microstructure Data

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Introduction

The preparation and interpretation of an otolith is only the first step in the extraction of useful information about a fish. It is understandable that the technical difficulties associated with otolith microstructure examination can occupy much of a researcher's time. However, the analysis of the resulting data is often given so little attention that much of the information acquired so painstakingly is effectively lost. Indeed, many publications reporting otolith data contain only the size-at-age data, perhaps in the form of a scatterplot, thus ignoring what is often more interesting and useful information. Of course, most analyses, even those as simple as growth rate calculations, require complete and representative sampling of all of the relevant cohorts/life history stages (see Butler, this volume). The intent of this chapter is to highlight the most useful and powerful applications of otolith microstructure examination, and in so doing, attempt to encourage a more complete analysis of otolith-based data on a routine basis. Many of the analyses are not particularly difficult to undertake, but merely require some forethought as to the best way to proceed. Thus we shall also offer our views on the most appropriate way to approach and complete each analysis. Examples are given wherever possible.

Many of the applications which make use of otolith microstructure examination have analogs in other areas of fisheries science. Obvious examples include the estimation of age and growth rate, both of which have long been studied at the yearly level. However, a typical sequence of daily growth increments lends itself to most applications much better than those at the yearly level, largely because of the longer and temporally more exact sequence of marks in each otolith. In addition, applications such as hatch date analysis are almost unique to otolith microstructure studies. In this chapter, we shall review all of the major applications of otolith microstructure data, but focus our discussion on the analyses not generally found in other fields of research. The simulations and discussion of hatch date analysis are new, reflecting the still-evolving nature of this type of analysis. However, much of the remaining information has been presented elsewhere; this chapter simply serves to bring it all together in a coherent form, much of it for the first time.

Age Estimation

Conversion of Increment Counts to Age Estimates

Given a life history stage in a species in which daily increment formation has been validated (see Geffen, this volume), the number of daily increments must be proportional to, but not necessarily equal to, the age of the fish. Since the inner-most increment does not necessarily form at hatch, experimentation or observation is required to determine the age at which the first increment is formed. Of course, increment counts can be initiated at any otolith landmark to which an age can be reliably and consistently assigned; neither the inner-most increment nor the hatch check need be used. One example of an alternate landmark is that of a check formed at mouth-opening (Lagardère and Chaumillon 1988). Irrespective of the landmark used, age is then calculated as the sum of the age at landmark formation and increment count distal to that landmark. While fish age is the usual objective of otolith microstructure examination, individual increments can also be interpreted in terms of date of formation, through knowledge of the date of sampling (=date of formation of

the marginal, or last-formed, increment). Dated increments are proving to be of increasing value to analyses cross-correlating environmental factors to the otolith growth sequence (e.g., Methot 1981; Campana and Hurley 1989; Suthers et al. 1989).

The estimation of age from daily increment counts is simple in principle, but the practice is confounded by the errors and uncertainties associated with microstructural examinations (see Neilson, this volume, and Campana, this volume). To some extent, the ageing uncertainties can be reduced through examination of multiple otoliths. All teleosts have three pairs of otoliths, of which two pairs are often interpretable. Since increment counting error is at least partially due to preparation artifacts, examination of both otoliths from a given pair can aid in reducing the variance (increasing the precision) of each age estimate. Where it can be demonstrated that other otolith types contain the same age information (or can be calibrated to the same age), more than one otolith pair can be read to further increase precision (Campana and Hurley 1989). Of course, peculiarities in the otolith microstructure attributable to fish growth will be reflected in all of the otoliths, and that source of error is unlikely to be reduced by multiple readings. It should also be noted that readings of multiple otoliths from a single fish are not equivalent to the same number of readings from a single otolith; the latter will reduce the variance attributable to counting error while the former will reduce the variance due to both counting and preparation error.

A single best estimate of age from a given fish will result from multiple readings of each of several otoliths, at least where possible. However, an overall average of all of the readings will seldom be appropriate. A more appropriate age estimation procedure involves: (1) the determination of the single "best" estimate of increment count for each otolith, (2) pooling of the increment counts from a given otolith type, (3) converting increment counts from each otolith type to age estimates, and (4) pooling the results from the otolith types. At least, this is the most appropriate procedure in theory. In practise, the time and effort involved in reading multiple otoliths from a single fish must be balanced against the benefits of increasing the number of fish which are examined. As a general rule of thumb, the examination of two otoliths (usually of a single otolith type) from each fish appears to be a useful compromise between within-fish precision and overall sample size.

The single "best" estimate of increment count from a given otolith may be the mean of multiple counts if all counts were considered equally reliable. Use of a median, rather than a mean, reduces the influence of single, aberrant counts. However, since otolith readers often give higher credence to certain readings, some form of weighting in terms of reliability is often preferred. Weighting can be as described below, or can be slightly more subjective through selection of the single count with which the most confidence is associated. In most cases, the difference between the two weighting procedures will be minimal. After assigning a single increment count to each otolith, a single value is calculated for each otolith type, either by averaging or through weighted averaging. Increment counts from each otolith type are then converted to age estimates using the appropriate conversion (e.g., age = lapillar count + 2). The final stage is the averaging (weighted or unweighted) across otolith types. The mathematical algorithm for the single best increment count (C_{tj}) from the jth (first or second) otolith of the tth otolith type (eg. sagitta, lapillus, or asteriscus) is:

$$C_{tj} = \frac{(\sum_{1}^{R} X_i \times W_i)}{\sum_{1}^{R} W_i} \quad (1)$$

where R represents the number of times that the otolith was counted, and W_i is the weight given to each count (X_i). Although statistical weights are often calculated as the inverse of the variance, there is no variance associated with a single count. A more useful approach here is to weight on the basis of perceived confidence in the otolith reading; an arbitrary scale from 1 (little confidence) to 5 (unambiguous count) for each otolith is one such approach. Calculation of the mean increment count for a given otolith type is a simple variant of Equation (1), whereby weights are either assigned to each otolith based on confidence, or the weights are assumed to be equal, resulting in a simple mean. After converting each of the otolith type increment counts to age estimates, the final (weighted) mean is then calculated across otolith types. It is important to note that the above procedure, whereby (weighted) means are calculated at each stage, is not equivalent to a (weighted) mean of all of the readings combined. As for the use of weighted means, weighting appears to be most important in the first two stages (calculation of the best increment count for each otolith and otolith type); unless one otolith type is clearly superior to the other, a simple mean across otolith types is probably sufficient.

Consider the following example, based on three readings each of two sagittae and one lapillus (one lapillus was lost during preparation). Confidence ranks were assigned on a scale of 1 (low confidence) to 5 (high confidence). Assume that increment counts were initiated at a check which formed one day after hatch:

	Count	Rank	Count	Rank	Count	Rank	Weighted mean
Sag 1	20	5	18	4	28	1	20.0
Sag 2	22	4	23	3	19	4	21.2
Lap 1	19	4	20	4	21	4	20.0

Assuming that Sag 1 and Sag 2 were comparable in ease of interpretation, the mean Sag count would be 20.6. Converting the Sag and the Lap counts to ages (count + 1), and taking the mean, results in an age estimate of 21.3 d. Note the difference between this estimate and the simple mean of all of the above readings (=21.1 + 1 = 22.1 d). Note also that the otolith types were equally weighted in the calculation of the final age estimate, despite the fact that there were two sagittae and only one lapillus. Equal weighting is appropriate if the two otolith types differ in their ease of preparation and/or interpretation, yet there is no basis for considering one otolith type more reliable than the other. Where one otolith type is considered to be more reliable than the other, it is probably best to age only the reliable pair.

Accuracy and Precision

Age estimates are most valuable when they are both accurate and precise. However, accurate estimates need not be precise, and vice versa (Campana and Moksness 1991). Accuracy refers to the proximity of the estimate to the "true" value, while precision refers to the reproducibility of the individual measurements. Thus a mean age can be accurate (close to the truth) while the individual observations are imprecise (vary widely). Conversely, and this is often the case in ageing studies, age estimates can be precise (highly reproducible, either within or among readers) but not necessarily accurate. Tests of accuracy require an independent and absolute means of age determination (see Geffen, this volume); for instance, accuracy has not been demonstrated if age estimates from otoliths and vertebrae concur. However, indices of precision are easily generated, and they can provide useful information concerning sources of error in an ageing study. Common applications include comparisons among age readers and ageing methodologies (Secor and Dean 1989). They can also be used to judge the relative difficulty of ageing different species, and to reject samples of questionable reliability (Secor and Dean 1989; Schultz 1990).

Traditional indices of precision are of little value to otolith microstructure studies, and in any event, have also fallen out of favour in ageing studies at the annular level. Specifically, measures of percent agreement vary substantially both among species and among ages within a species. Beamish and Fournier (1981) illustrated this point by noting that 95% agreement to within one year between two age readers of Pacific cod (*Gadus macrocephalus*) constituted poor precision, given the few year classes in the fishery. On the other hand, 95% agreement to within 5 years would constitute good precision for spiny dogfish (*Squalus acanthias*), given its 60-yr longevity. Thus, Beamish and Fournier (1981) recommended the use of average percent error (APE), defined as:

$$(2) \qquad 100\% \times \frac{1}{R}\sum_{i=1}^{R} \frac{|X_{ij} - X_j|}{X_j}$$

where X_{ij} is the ith age determination of the jth fish, X_j is the mean age of the jth fish, and R is the number of times each fish is aged. When averaged across many fish, it becomes an index of average percent error. Chang (1982) agreed that APE was a substantial improvement over percent agreement, but suggested that the standard deviation be used in Equation (2) rather than the absolute deviation from the mean age. The resulting equation produces an estimate of the coefficient of variation (CV), and unlike Equation (2), does not assume that the standard deviation is proportional to the mean. The CV is expressed as the ratio of the standard deviation over the mean, and can be written as:

$$(3) \qquad 100\% \times \frac{\sqrt{\sum_{i=1}^{R} \frac{(X_{ij} - X_j)^2}{R-1}}}{X_j}$$

Equation (3) is the CV of the age estimate for a single fish (jth fish). As with Equation (2), it can be averaged across fish to produce a mean CV. Both Equations (2) and (3) produce similar values for precision (Chang 1982); however, because of the absence of an assumed proportionality between the standard deviation and the mean, the latter is statistically more rigorous and thus is more flexible. The index of variation proposed by Lai et al. (1987) is probably the same as Equation (3), although there appears to have been a typographical error in its presentation. In some species, both the APE and the CV will decrease with age until the juvenile stage, reflecting the relative difficulty of precisely ageing very young larvae (e.g., Savoy and Crecco 1987; Campana and Moksness 1991). However, it is important to note that both APE and CV will decrease with age, even if the absolute counting error remains constant. For instance, counting variability of ±1 in a 10-d old larva corresponds to a CV of about 9%, while the same variability in a 1-d old larva will result in a CV close to 90%. Therefore, comparisons of age precision between two groups will not be comparable if they contain substantially different age distributions.

Age–Length Keys

The age determination of large numbers of fish, whether at the daily or the annual level, almost invari-

ably requires some form of subsampling. Since fish lengths are far easier to measure than are ages, subsampling can be used to estimate the age of a large number of fish for which only length is known, based on a smaller sample for which both age and length are known. Mean age-at-length can be calculated through inverse regression of a linear growth curve, and then applied to a sample of known length (Bolz and Lough 1988). However, such an approach ignores the inherent variability in size-at-age, and can be used for only the most general of applications. Age–length keys, which are essentially contingency tables of age categories by length categories, use more of the age–length information, and are commonly applied in commercial fisheries situations. There is a large literature on the use and abuse of age–length keys (Kimura 1977; Westrheim and Ricker 1978; Doubleday and Rivard 1983), which will not be reviewed here. An important assumption underlying the appropriate use of age–length keys is that they are drawn from the same population, at the same time and place, as the larger length–frequency samples. Since serious error can arise if this assumption is ignored, age–length keys will generally not be transferable across seasons, years, populations, or environments.

Age–length keys are most commonly prepared in one of two ways. Both approaches are based on two-stage sampling (Cochran 1963) in which a large length–frequency sample is subsampled for age determination. Subsampling can either be based on a random sample of the length–frequency sample, or stratified on the basis of length category (e.g., a random sample is aged from each length category). Length-based stratification is generally preferred since it avoids the problem of underrepresentation of the oldest, least abundant fish (Fournier 1983). Subsample sizes within each length category can either be fixed, or proportional to the number of fish in that length category (Kimura 1977). Whichever approach is adopted, it is important that the range of length categories in the key span the same range as that observed in the length sample.

Consider the following simple example, whereby an age–length key derived from a small subsample is used to prorate a larger length–frequency sample (LF):

Length	Age Category							
Category	5	6	7	8	9	10	Sum	LF
10	2	—	—	—	—	—	2	20
12	1	3	2	—	—	—	6	30
14	—	2	7	5	1	—	15	50
16	—	—	2	4	3	1	10	40
Sum	3	5	11	9	4	1	33	140

The vector LF is multiplied by the proportion at age in each key length category, resulting in:

Length	Age Category						
Category	5	6	7	8	9	10	Sum
10	20	—	—	—	—	—	20
12	5	15.0	10.0	—	—	—	30
14	—	6.7	23.3	16.7	3.3	—	50
16	—	—	8.0	16.0	12.0	4	40
Sum	25	21.7	42.3	32.7	15.3	4	140

Length at age comparisons are most commonly made with parametric tests, although there are non-parametric equivalents for most of the two-sample tests. If the relationship between length and age is linear (or can be so transformed), and given the other assumptions of an ANOVA, an analysis of covariance (ANOCOVA) can be a powerful test of differences among samples (e.g., Secor and Dean 1989; Thorrold and Williams 1989). Note that a two-sample ANOCOVA is not necessarily equivalent to a *t*-test of the regression slopes of the two samples. A comparison between regression slopes assumes similar intercepts; if the latter are dissimilar, interpretation of slope differences can be difficult. ANOCOVA is better suited to dealing with this type of problem.

In all statistical analyses, but particularly those mentioned above, it is important to consider both significance and power before reaching a conclusion. Statistical significance, the probability of rejecting the null hypothesis (of no difference) when it is in fact true, is rather widely understood. Thus, statistically significant differences among samples are usually easy to interpret. However, non-significant differences may either be due to an actual similarity between the samples, or to low statistical power. The latter may arise from low sample size or high variability in the data, among other things, which can serve to hide a real difference between the samples. Thus, it is not appropriate to conclude, or even suggest, that there are no differences between the samples unless the statistical power can be demonstrated to be high. Analyses with low statistical power are widespread, and inferences drawn from them have often obscured the truth (Rice 1987; Peterman 1990).

Age Estimation by Numerical Integration of Daily Increment Widths

To this point, the discussion has been focused on the estimation of age in young fish, primarily larvae and juveniles. While some workers have attempted, with varying degrees of success, to age adult fish through daily increment counts (Pannella 1971; Brothers et al.

1976; Radtke 1984), adult fish otoliths are generally conceded as being both difficult and tedious to prepare and interpret. In addition to the possibility that daily increment formation becomes intermittent in old fish as somatic growth slows (Campana and Neilson 1985), the logistical problems of preparing a large otolith for microstructural examination can leave extended sequences of daily increments uninterpretable. Where a presumed annular pattern is present, daily increment counts between the nucleus and first annulus have been successfully used to verify the nature of the first annulus (Victor and Brothers 1982; Morales-Nin 1988). The nature of the subsequent annuli remains problematic. Despite problems with the interpretation of the microstructure of adult fish otoliths, in cases where otolith annuli are ambiguous or absent (e.g., in many tropical species), and particularly if done in conjunction with an alternate age determination technique (such as length frequency analysis), some form of otolith ageing can be of substantial benefit. With these caveats in mind, Ralston and Miyamoto (1983) developed an approach whereby the daily increment widths in an adult fish otolith were subsampled and measured across the interpretable sections of the otolith radius. When put into the context of a relationship between increment width, section width, and distance from the nucleus, the integrated data could be interpreted in terms of daily age at specific otolith sizes. Use of a predictive relationship between otolith size and fish size then allowed estimates of fish size at age to be derived. While still sensitive to extended interruptions in otolith growth, this approach successfully circumvented problems associated with sequences of poorly defined increments, and enhanced efficiency and productivity relative to a complete enumeration of increments.

A complete description of the numerical integration approach is provided elsewhere (Ralston and Williams 1989). Basically, it begins with scanning the prepared otolith section along some predefined axis between the nucleus and the otolith margin in a search for unambiguous daily increment sequences. At frequent but arbitrary intervals, the average width of the daily increments is determined by measuring the axial length of a small number of increments (~10–20) in a sequence and dividing by the number of daily increments contained therein. In conjunction with the measurement of the distance from the midpoint of the sequence to the nucleus, an estimate of mean increment width at some otolith radius can be calculated. This can then be used to calculate the instantaneous growth rate of the otolith.

To estimate age, Ralston and Williams (1989) subdivided the data into 500 μm intervals of otolith length, beginning at the nucleus. The selection of a 500 μm interval was arbitrary and could be varied to suit the species under study. Mean otolith growth rate within each 500 μm interval was then calculated, based on the number of increment sequences which were present within that interval. Each within-interval otolith growth rate (in μm units) was next divided into 500 μm to estimate the number of days needed to complete growth through that interval. When the sum of the interval calculations (days) for each fish was divided by 365, an age estimate, in years, resulted. The age estimate and observed fish length for each fish were then entered into one of the standard growth models. In general, unbiased growth estimates are best provided by entering only one age–length estimate per fish. However, where data are limited, the overall fish-otolith length relationship can be used to backcalculate fish length at the otolith size corresponding to the completion of growth through each 500 μm interval. Fish ages at those same points are available as described above. Thus several estimates of size at age are available for each fish, which can then all be entered into a growth model. However, multiple observations from a single fish are not independent.

The numerical integration method for annular age estimation assumes that daily increment formation is continuous throughout the lifetime of the fish. However, short periods of interrupted otolith growth are unlikely to result in a noticeable reduction in accuracy. Of more concern is the possibility that daily increment widths become so attenuated with age that they become unresolvable. If this were to occur, none of the increments produced in older fish would be measurable and the corresponding otolith growth rates would be based on previous periods of faster growth. The resulting age calculations would underestimate the actual age of the fish. While increment widths in adult fish otoliths have seldom been measured, it is disturbing to note that Ralston and Williams (1989) encountered this very problem when examining gindai (*Pristipomoides zonatus*) otoliths. As a result, they were unable to measure increment widths at otolith diameters exceeding 7500 μm, although most of the fish present in the fishery had otoliths exceeding this size. Accordingly, Ralston and Williams (1989) expressed the greatest confidence in their age estimates of smaller fish; the age estimation error associated with the larger fish could not be estimated.

A second assumption underlying the numerical integration technique is that the measured increment sequences are unbiased representatives of the corresponding otolith interval. Where preparation artifacts have obscured increments in a particular section, there

should be no problem. However, Ralston and Williams (1989) caution that some care should be taken to ensure that increment sequences are selected as objectively as possible, and should not be selected on the basis of increment width and the associated ease of interpretation.

Growth Models

The preparation of a parameterized growth model is often considered to be a standard product of otolith microstructure examination. Growth models may vary in complexity from that of a simple linear regression of fish size on age/increment count to sophisticated maximum likelihood estimates of size at age. In most instances, the rationale for model preparation is to allow prediction of an expected mean size or growth rate at some age and/or to facilitate comparisons of estimated growth with other published estimates. Common to many models is the removal of information concerning the observed variance in size at age. For this reason, a simple scatterplot of fish size versus age is a useful starting point for any analysis of growth.

In principle, calculations of growth rate should be based upon the growth trajectories of individual fish; in practise, population trajectories are often taken to represent individual growth, despite potential biases introduced through size-selective mortality and gear avoidance (Ricker 1975). Any measure of fish size may be used in the calculations, although we will only refer to length in our discussion. There are also several measures of growth rate available, with the most familiar being "absolute growth rate", defined as the change in fish length (or weight) per time interval, and the "instantaneous growth rate", where the time interval is reduced to near-zero and the growth rate is calculated as a proportion of the initial fish size (Ricker 1979). It is important to note that the absolute growth rate will vary with the time interval that is selected if growth is nonlinear. For this reason, the instantaneous absolute growth rate, or the tangent to the slope of the length at age curve at the desired age, can sometimes be a more meaningful measure than the absolute growth rate.

Calculations of growth rate may be based upon equations derived from either empirically-fitted curves or some of the generally accepted growth models; in actual fact, the distinction between the two is somewhat arbitrary. An advantage of the more commonly-used growth models (e.g., linear regression, Gompertz, logistic and von Bertalanffy models) is that the associated parameter estimates are often readily interpretable by other workers. However, when the above models cannot be fitted, the utility of empirically-fitted curves should not be overlooked.

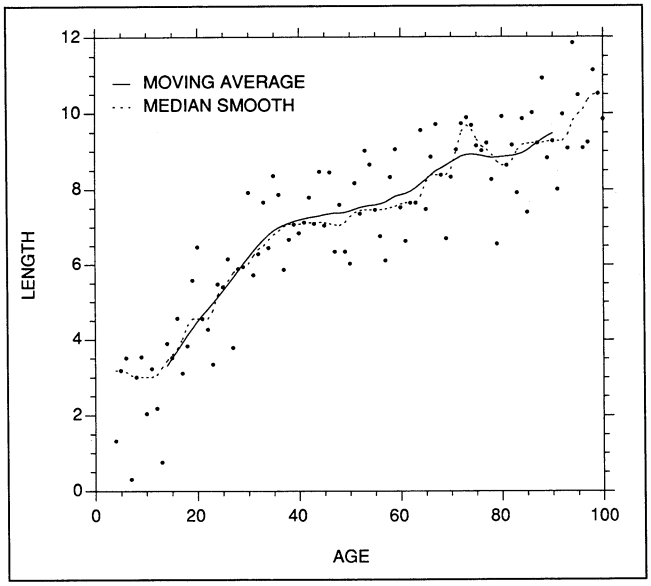

FIG. 1. Examples of parametric and nonparametric smoothing techniques applied to a set of simulated length at age data. Both the parametric (10-term moving average, resmoothed) and the nonparametric (5RSSH median smooth) techniques fit the data well, but neither were accompanied by descriptive equations.

Empirical Models

There are a large number of empirical curve-fitting procedures available for use with growth data (Lancaster and Salkauskas 1986). Smoothing techniques generally associated with time series analysis can provide useful measures of central tendency, but not all are suited to calculations of growth rate. Resistant nonlinear smoothing (more commonly referred to as median smoothing) is a nonparametric technique, and thus is relatively insensitive to outliers in the data. The parametric analog is a moving average. Both techniques calculate the median (or average) of a selected number of points on either side of a target point. If desired, the points can be weighted on the basis of their proximity to the target. Both the median smooth and the moving average curves provided reasonable fits to a set of simulated length–age data (Fig. 1), and thus were suitable for summarizing trends in length at age. Note however, that neither approach resulted in an equation from which growth rate could be calculated. Where necessary, growth rate at age could be approximated by calculating the slope of the tangent to the curve at the desired age. The application of moving averages to growth data is exemplified by the study of Brothers and McFarland (1981).

Polynomial regressions can be an effective means of summarizing length at age data, especially since

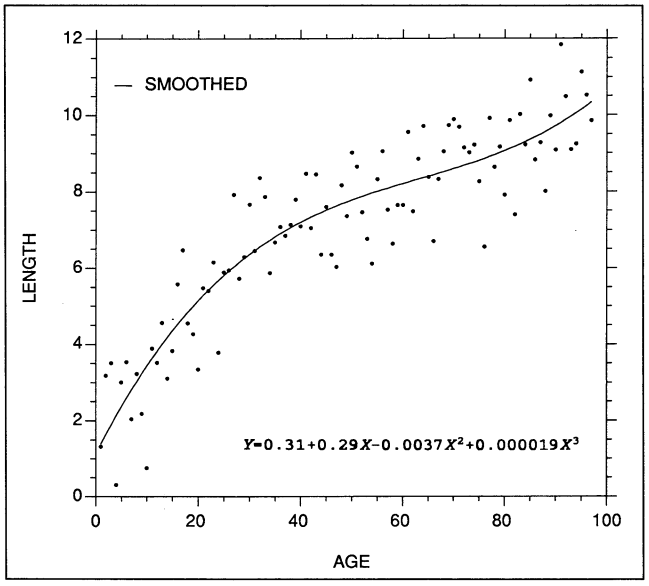

FIG. 2. Example of a polynomial regression fitted to the same length at age data as that of Fig. 1. A third order regression was fitted, resulting in two inflection points in the fitted curve. While a polynomial regression is often considered to be an empirically-fit curve, the accompanying regression equation can be used for predictive purposes.

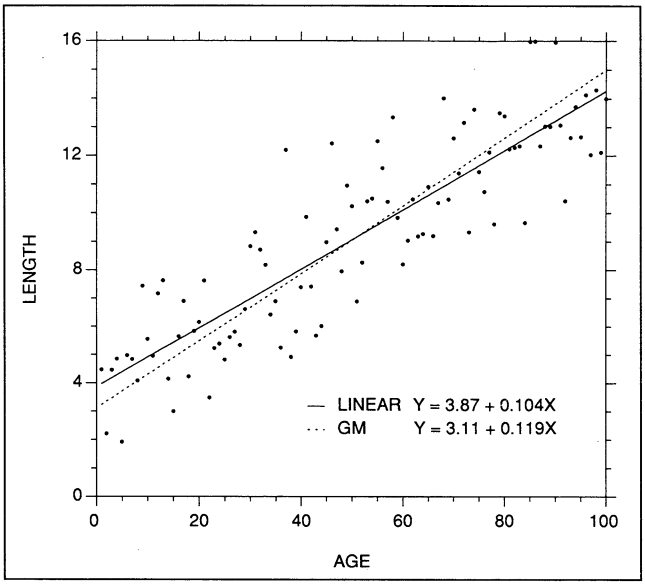

FIG. 3. Examples of simple linear and geometric mean regression fits to a set of simulated length at age data. The slopes of the two regressions become increasingly similar as the correlation between length and age increases.

they incorporate a descriptive equation which can provide the basis for calculations of instantaneous growth rate. Polynomial regressions are based on the general formula:

$$L = a + b_1X + b_2X^2 + b_3X^3 + \ldots + b_nX^n \quad (4)$$

where a and $b_1 \ldots b_n$ are regression parameters to be estimated (generally through least squares), L is fish length (or weight), and X is age or increment count. The number of terms (n) that are introduced should be one more than the number of inflection points in the curve, but in most growth curves, seldom exceeds four. As an example of polynomial smoothing, Fig. 2 presents a third order polynomial regression fitted to the simulated data of Fig. 1. Polynomial regressions have been applied to otolith data by Wilson and Larkin (1982), West and Larkin (1987), and McMichael and Peters (1989).

Simple Linear Regression Models

While the distinction between empirical length-at-age curves and growth models is somewhat arbitrary, simple linear regressions are the most commonly applied of what are generally termed growth models (e.g., Geffen 1982; Walline 1985; Leak and Houde 1987; Victor 1987), and are of the form:

$$L = a + bX \quad (5)$$

Linear regressions (Fig. 3) are easily fit, easily interpreted, and are amenable to confidence interval calculations both around the slope b (growth rate) and around point values. While they are usually fitted to relatively short growth intervals, in which even intrinsically curvilinear growth patterns can appear linear, they can be applied over any interval in which growth rate has remained constant.

Where a straight line fit is desired, an alternative to the linear regression is the functional regression or geometric mean (GM) regression (Ricker 1973; Ricker 1984), where

$$Y = u + vX \quad (6)$$

and the slope (v) is the ratio of the standard deviations (s) or the square root of the sum of squared deviations (SS) of Y and X, as in:

$$v = \pm\frac{S_y}{S_x} = \sqrt{\frac{SS_y}{SS_x}} \quad (7)$$

Ricker (1973, 1984) suggested that the GM regression be applied in instances where inherent (non-measurement) variability was associated with both the X and the Y variables, or when the variables were non-normally distributed. While he presented examples in which the regression was used for predictive purposes, the primary application was intended to be that of description, in which neither of the variables was

clearly causal (e.g., body length versus body weight). A full description of the advantages and disadvantages of functional regressions is beyond the scope of this chapter. Suffice to say, there is some controversy over the relative value of GM regressions to fisheries research (Sprent and Dolby 1980; Jensen 1986). The major disadvantages appear to be those associated with the error distribution assumptions and the absence of significance statistics for the slope estimate. However, the GM slope appears to provide as good a measure of central tendency (functional relation) as any other measure, and perhaps better than that of predictive regressions. In the context of otolith growth models, GM regressions appear to have limited utility, since most growth models are fit in order to predict length from age, and predictive regressions are best suited to this task (Jensen 1986). Further, the daily increment count data generally entered as the independent variable in an age–length regression can incorporate a substantial amount of measurement error, and Ricker (1973, 1984) cautions against the use of a functional regression when measurement error exists in the independent variable. While some workers have fit GM regressions to otolith-fish length data (Gjosaeter 1987; Watanabe et al. 1988), we are not aware of anyone who has done so with age–length data. In any event, GM regression fits become increasingly similar to those of simple regression as the correlation between the X and Y variables increases. A comparison of the two fits, using simulated data, is presented in Fig. 3.

Curvilinear Growth Models

Curvilinear growth models tend to be well suited to the description of young fish growth, particularly that of larvae. There are a large number of potential choices, although none can be used to fit all life history stages in all species (Ricker 1979). The major advantage of this class of model is that of flexibility, a feature which is required to deal with the S-shaped growth curves that are characteristic of most young fish. While a number of the growth models were initially developed on the basis of perceived growth processes, the latter have never been firmly substantiated. Therefore, selection of an appropriate curvilinear growth model is generally based on goodness of fit and convenience (Ricker 1979). On the basis of the above criteria, as well as familiarity and general acceptance, the exponential, Gompertz, logistic, and von Bertalanffy models will be briefly discussed here. For a more complete description of these and other growth models, the reader is referred to the excellent reviews of Ricker (1979) and Brett (1979).

Exponential curves are the curvilinear analogs of the simple linear regression discussed earlier, where

$$L = ae^{GX} = a \exp[GX] \quad (8)$$

or equivalently

$$L = \exp[a' + GX] \quad (9)$$

where a and $\exp(a')$ are the size of the fish at age 0, and G is the instantaneous growth rate. The absolute growth rate (g) at any given age is the derivative of Equation (8):

$$g_X = aG \exp[GX] \quad (10)$$

Since an exponential curve can be fitted with a simple linear regression after log transformation of the length data, the two model types share the same statistical advantages. A somewhat less flexible alternative, due to its fixed intercept through 0, is the power curve:

$$L = aX^b \quad (11)$$

with the absolute growth rate at age described by:

$$g_X = abX^b \quad (12)$$

The family of exponential and power curves can be used to fit virtually any monotonically increasing growth curve which does not contain an inflection point. Since they are not suited to S-shaped growth curves, they have been used most effectively in describing short growth intervals, particularly in the larval stage (Beckman and Dean 1984; Gjosaeter 1987; Tzeng and Yu 1988; Campana and Hurley 1989). With the degree of curvature being controlled by the value of the exponent, exponential and power curves can be used to fit straight-line sequences as well as curves, and are thus considered to be easily-fit but powerful descriptors of short growth sequences (Ricker 1979). Examples of exponential and power models are presented in Fig. 4.

The Gompertz, or Laird-Gompertz, model (Gompertz 1825; Laird et al. 1965) has become the most frequently fitted of the young fish growth models, particularly with respect to larvae (e.g., Methot and Kramer 1979; Lough et al. 1982; Warlen and Chester 1985; McGurk 1987). Like the logistic and von Bertalanffy models, the Gompertz model is well suited to descriptions of sigmoidal growth (Fig. 5). Some supporters of the model have suggested that it become the preferred choice for modelling fish growth (Zweifel and Lasker 1976). However, like other models, the Gompertz model can seldom be used to describe all life history stages in a species (Ricker 1979). Ricker (1979) presents three alternative forms for the same model:

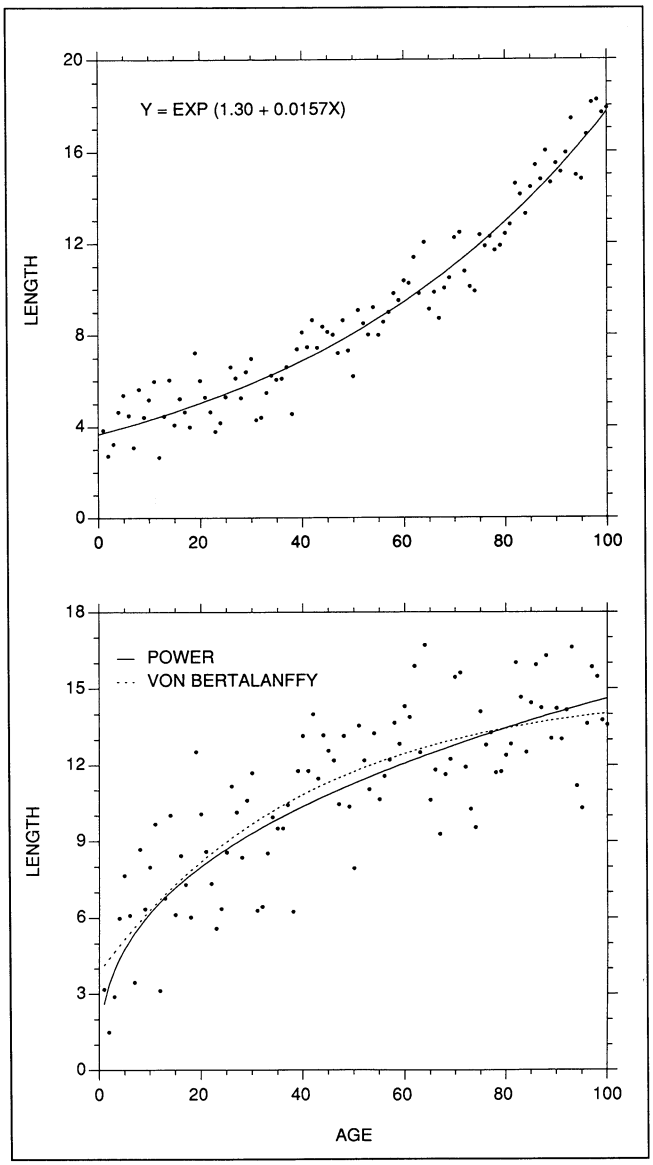

FIG. 4. Examples of models which can be fit to curvilinear data with no inflection points. (Top) The exponential model is often fit to short growth sequences, since the degree of curvature is controlled by the value of the exponent. (Bottom) The curvature of the fitted power curve ($2.61\ Age^{0.37}$) is also controlled by the value of the exponent, but this form of model is constrained through the origin. If necessary, an intercept parameter could be added to the model (eg. Y=Intercept + aX^b) to remove this constraint. While the length-based version of the von Bertalanffy model ($Y = 14.98(1-\exp(-0.0247(X+12.10)))$) is not constrained through the origin, it cannot be fitted to sigmoidal data as can the weight-based version.

(13) $L = L_0 \exp[k(1 - \exp\{- GX\})]$

(14) $L = L_\infty \exp[- k \exp(- GX)]$

(15) $L = L_\infty \exp[-\exp(-G\{X - X_0\})]$

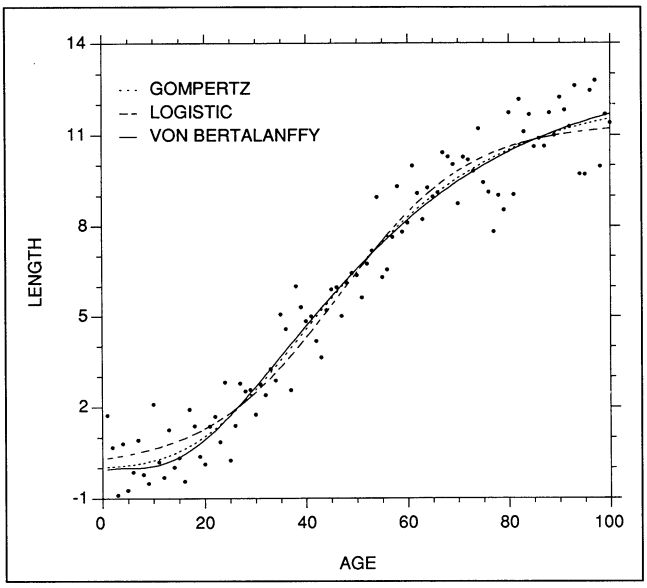

FIG. 5. Examples of the Gompertz, logistic, and von Bertalanffy (weight) growth models fit to a set of simulated sigmoidal length at age data. The fitted models are: Length = $12.29 \exp(-\exp(-0.0459(X-39.70)))$ – Gompertz; Length = $11.39/(1+\exp(-0.0777(X-46.40)))$ – Logistic; Length = $13.00(1-\exp(-0.0353(X-4.754)))^3$ – von Bertalanffy.

where L_0 is the length at age $X = 0$, L_∞ is the asymptotic length, G is the instantaneous rate of growth at age X_0, X_0 is the inflection point of the curve and the age at which absolute growth rate begins to decline, and k is a dimensionless parameter. The absolute growth rate (g) at age X is calculated as:

(16) $g_X = GL_X(\ln L_\infty - \ln L_X)$

The logistic growth model will often result in a growth curve fit which is very similar to that of the Gompertz model (Fig. 5). However, the former differs in that the regions above and below the inflection point are symmetrical, while those of the Gompertz curve are not. The effect of this difference is difficult to see except where the data extend well beyond the inflection point on each side. Two forms of the logistic curve are:

(17) $L = L_\infty(1 + \exp[-G(X-X_0)])^{-1}$

(18) $L = L_\infty(1 + c \exp[-GX])^{-1}$

where G is the instantaneous growth rate at the origin of the curve, X_0 is the age at the inflection point of the curve and the age of maximum absolute growth rate, and c is a parameter to be estimated. The absolute growth rate (g) of the logistic curve at age X is:

(19) $g_X = GL_X(L_\infty - L_X)\ (L_\infty)^{-1}$

The logistic curve has traditionally been used to describe the growth of populations, and forms the

basis for surplus production models in fisheries. However, it has also been used to model the growth of individual fish (Nishimura and Yamada 1984; Campana and Hurley 1989).

The von Bertalanffy growth model (von Bertalanffy 1938) has long been used to describe the growth of adult fish (Ricker 1979), but has also seen application to the early life history stage (Ralston 1976; Wild and Foreman 1980; Laroche et al. 1982; Young et al. 1988). The standard length-based model can be used to fit most growth data lacking an inflection point (Fig. 4b), but it is not suitable for a sigmoidal growth pattern. It has the form:

$$L = L_\infty(1 - \exp[-K(X - X_0)]) \quad (20)$$

and an absolute growth rate at age described by:

$$g_X = K(L_\infty - L_X) \quad (21)$$

where K is the von Bertalanffy (or Brody or Putter) growth coefficient, and X_0 is the predicted age at which fish length is zero. Some care is required in the interpretation of the von Bertalanffy parameters, since the nomenclature is somewhat misleading. The growth coefficient K is a measure of the rate at which the growth rate declines, not a measure of growth rate itself. Of greater consequence for those studying the growth of young fish, X_0 is a statistical parameter only, and seldom corresponds with the age of the fish at hatch. As with the other growth models, selection of the von Bertalanffy model should be based upon goodness of fit and convenience. However in general, we have found it to have fewer applications to larval growth than some of the other models, largely because of its inapplicability to sigmoidal growth data. Generality is enhanced through use of the cubic version of Equation (20), designed for modelling growth in weight, which can be used to fit either length or weight data containing a growth inflection (Fig. 5):

$$W = W_\infty(1 - \exp[-k(t - t_0)])^3 \quad (22)$$

Age–Temperature Growth Models

The growth models presented to this point are considered to be among the best available for prediction of length and growth rate when only age data are available. Age, of course, is a useful predictor of fish size. However, both food and temperature are strong modifiers of growth rate in fish (Brett 1979), and both variables may differ markedly between populations, sampling dates and environments. Accordingly, age–structured growth models may have limited utility when the objective is to compare the growth of fish among different environments.

To our knowledge, there are no age–structured growth models available which include both food and temperature terms and which can be easily parameterized in field situations. However, where temperature data are available, the use of an age- and temperature-mediated growth model can be of substantial value in predicting the growth of young fish in different environments (Campana and Hurley 1989). The model is of greatest value when the contrast in the temperature data is high, or alternatively, when the growth of the target species is particularly sensitive to small temperature gradients. These conditions are most likely to be met when multiple fish samples have been collected from a heterogeneous environment, or when samples have been collected at different times in the year.

The basis for the age–temperature growth model is the logistic growth model described earlier (Equation 17) (Campana and Hurley 1989). It has been clearly established that temperature influences the absolute growth rate of fish, with a temperature optimum beyond which growth rate decreases (Brett 1979; Ricker 1979). The absolute growth rate in the logistic model varies with age. Therefore, the age–temperature model incorporates a parabolic temperature term which serves to modify the absolute growth rate on a daily basis. The general form of the model is:

$$L_{age} = L_{hatch} + \sum_{t=0}^{age} (\text{Absolute growth rate} \times \text{Temperature term})\, dt \quad (23)$$

Using Equation (19) for the absolute growth rate of the logistic curve, and the equation describing a parabola for temperature, and assuming that the model will be fit on a daily basis, the result is:

$$L_{age} = L_{hatch} + K \times \sum_{t=0}^{age} (Gl_t - Gl_t^2 L_\infty^{-1}) \times (c - (T_t - T_{opt})^2) \quad (24)$$

where $l_t = L_\infty(1 + \exp[-G(t - t_0)])^{-1}$; G, L_∞, t_0, c, and T_{opt} (= temperature optimum) are model parameters; and L_{hatch} and K are fixed parameters to be determined independently. At first glance, Equation (24) may appear somewhat daunting. However, the data requirements are modest, consisting only of the ages and the daily temperatures to which each larva was exposed. Once the data are prepared, the model can be fit with any of the available nonlinear regression procedures. Figure 6 presents an example of the fitted model taken from Campana and Hurley (1989). The input data were derived from five independent cruises, made at monthly intervals.

Two points deserve amplification. First of all, the age–temperature model can and should be fit using the pooled inventory of samples (rather than one sample at a time). Since the model was designed to test

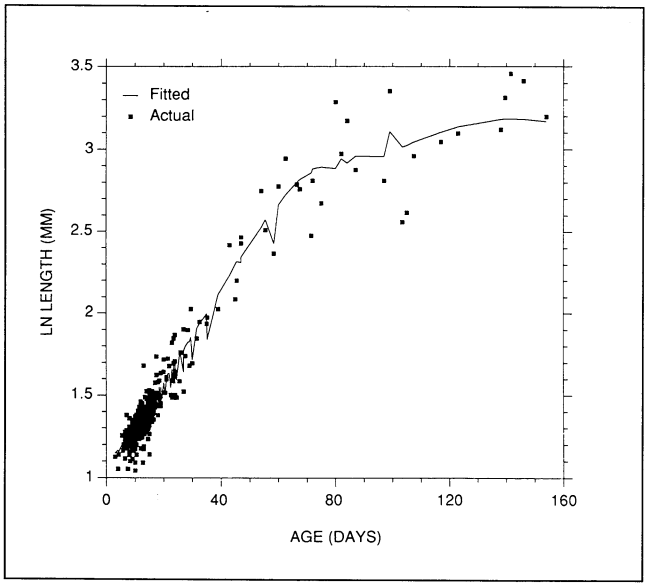

FIG. 6. The age-temperature growth model combines a logistic growth equation with a parabolic temperature term which modifies absolute growth rate on a daily basis. In the example here, taken from Campana and Hurley (1989), the equation is of the form of Equation 24, with G=0.0502, L_∞=59.18, t_0=60.57, c=22.77, T_{opt}=5.925, L_{hatch}=3.0, and K=0.2. Lengths were ln-transformed to stabilize the variance. The fitted line appears irregular since only one of the two independent variables is plotted.

for temperature effects on growth, sample pooling increases the contrast in the data, and thus improves the model's discrimination of those effects. Secondly, examination of the model residuals is a mandatory part of any analysis (see later), but is particularly important with respect to this model. Residuals should be random across predicted values, sizes, ages, and temperatures, both within and among cruises, before the model should be considered satisfactory.

Since the age–temperature model integrates the effect of temperature on growth rate for each day of a young fish's life, a daily temperature series, rather than a point estimate, is required for each fish. Normally, all fish within a given sample will be assumed to have experienced the same temperature on a given date. However, daily temperature records will not always be available for each sample. Reasonable approximations of the daily temperature series can be made through fitting a curve to periodic (e.g., monthly) measurements. Campana and Hurley (1989) provide an example of this approach, in which a sinusoidal curve was fit to each monthly mean temperature record, and the resulting equation used to predict the temperature on each day.

Common Model-Fitting Errors

In any fitted model, care should be taken to ensure

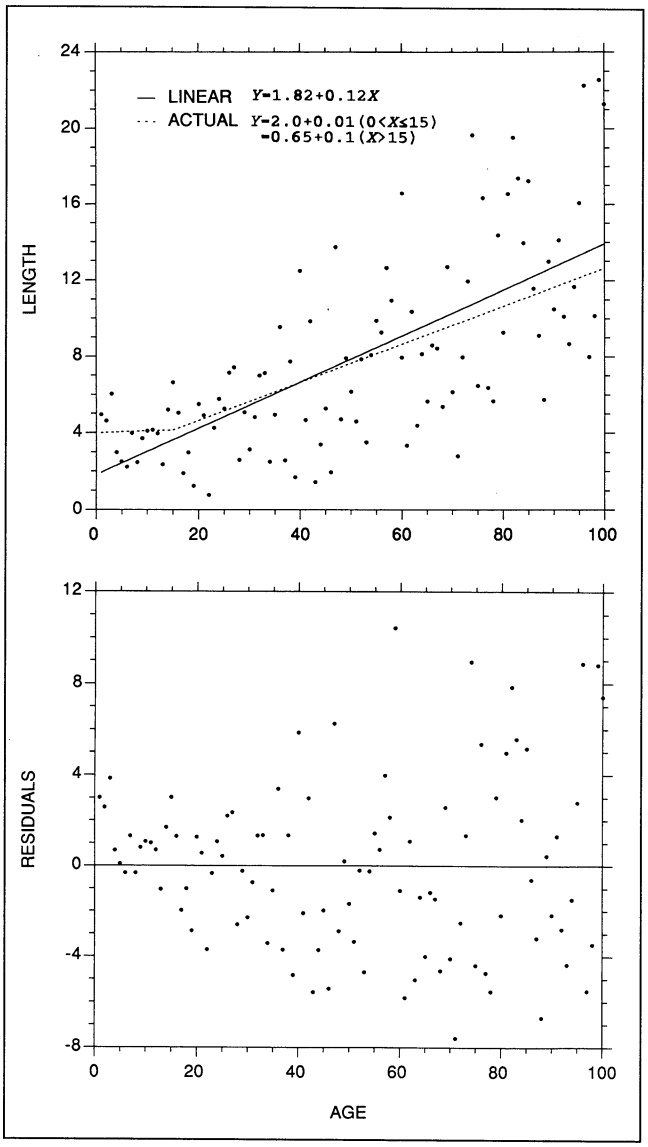

FIG. 7. Example of some common errors which can be made in fitting a growth model. (Top) The actual underlying relationship is a two-stage linear process under which the slope (growth rate) increases by a factor of 10 after age 15 (dotted line). A normally-distributed error term which is proportional to the mean has been added to the underlying relationship. On first glance, a linear regression (solid line) appears to fit the data well. (Bottom) Examination of the residuals indicates that the fit of a single linear regression to the data is inappropriate; the residuals are not randomly distributed around the regression, particularly at young ages, and the variance increases with age (heteroscedastic), making individual observations of older fish more influential than those of younger fish. Use of the fitted regression to estimate growth rate would overestimate the growth of young fish by a factor of 12.

that the residuals are randomly distributed and that the variance is constant across the entire data range. Failure to test these latter two assumptions can result in estimates of growth rate which are inaccurate, biased at certain ages, or unduly influenced by out-

liers. In the example of Fig. 7, the fitted linear regression appears to be well suited to most of the simulated data. However, the residuals are not randomly distributed at the younger ages, indicating that the model should not be fit to the young fish data. Growth rate calculations based on the entire data set would overestimate the growth rate of the young fish (<15 d) by more than an order of magnitude. A similar effect can result from inclusion of data with high leverage, wherein a regression can be forced through, or near to, isolated data points at very high (or low) X values at the expense of goodness of fit of the remaining data. This effect should be evident as a pattern in the residuals, or equivalently, a substantial shift in the regression parameters after removal of the high-leverage data. The influence of increased variance with age (heteroscedasticity) (Fig. 7) is reflected in undue influence on the regression slope by the older fish data. Removal of an outlier among the older fish resulted in a change of slope that was twice as large as the removal of a proportionally-equivalent outlier among the young fish. To provide a robust and accurate estimate of the growth rate of the fish in Fig. 7, a linear regression would have to be fit only to the data corresponding to fish older than 15 d, after transformation to stabilize the variance.

Growth Backcalculation

Growth backcalculations derived from a series of daily growth increments represent what is conceivably the most powerful application of otolith microstructure examination. Theoretically, it is possible to use the measured widths of a daily increment time series, in conjunction with a fish length:otolith length relationship, to determine both the size and the growth rate of an individual fish for each day of its life. In practise, such calculations suffer from a number of logistical and theoretical constraints (Campana and Neilson 1985; Bradford and Geen 1987; Secor and Dean 1989; Neilson, this volume), all of which would have to be addressed prior to use of any of the backcalculation procedures presented here.

Problems with Traditional Growth Backcalculations

Virtually all growth backcalculation procedures are based upon the presumption of proportionality (a linear relationship) between the size of the otolith (or scale or other bony structure) and the size of the fish (Carlander 1981; Bartlett et al. 1984; Weisberg 1986; Smale and Taylor 1987; Campana 1990). Irrespective of whether the backcalculations are being made from annuli or daily growth increments, two underlying assumptions exist: (a) the frequency of formation of the periodic feature (e.g., daily increment) is constant, and (b) the distance between consecutive features is proportional to fish growth. Validation of the frequency of increment formation is a mandatory component of otolith microstructure examination, and is covered in detail elsewhere (Geffen, this volume). While a complete validated sequence of daily increments is to be preferred, backcalculations are possible even when early otolith growth appears to be characterized by nondaily increment formation (e.g., in herring [Campana et al. 1987]). In such cases, backcalculations would be restricted to the contiguous region between the date of sampling (otolith edge) and the initiation of uninterrupted daily increment formation. Clearly, such calculations would have to be presented as a function of size at date, rather than at age. As for the assumption concerning proportionality between fish growth and otolith growth, justification has generally been based on empirical correlations between fish and otolith size. These correlations and various experimental studies (Wilson and Larkin 1982; Volk et al. 1984) certainly indicate a general correspondence between fish and otolith growth, but the correspondence need not, and often does not, apply on an individual or detailed level (Gutiérrez and Morales Nin 1986; Bradford and Geen 1987). To some extent, the apparent breakdown between fish and otolith growth is a function of a recently-demonstrated correlation between growth rate and the fish:otolith relationship (Mosegaard et al. 1988; Reznick et al. 1989; Secor and Dean 1989). However, there are a number of species in which the fish-otolith length relationship is inherently nonlinear. Backcalculation in these species is difficult unless the relationship can be described mathematically (e.g., Butler 1989). When backcalculating from a curvilinear fish–otolith relationship, there is an implicit assumption that the inflection point of the curve occurs at the same fish–otolith size in each fish. This assumption is unlikely to be met in most cases, but the implications of such are not yet known.

The traditional regression and Fraser–Lee (Carlander 1981) procedures are capable of introducing bias into otolith microstructure backcalculations, so they should be used with caution, if at all (Campana 1990). As is the case with most of the backcalculation methods, they assume a linear relationship between fish and otolith length. The regression method estimates fish length (L) at some previous age (a) through insertion of the measured size of the otolith (O) at age a into a fish length–otolith length regression derived from samples of the population,

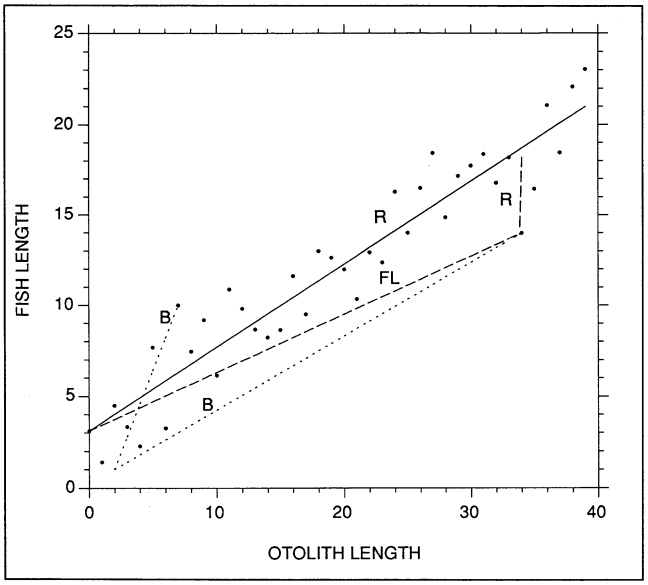

FIG. 8. An example of growth backcalculations from individual fish using the regression (*R*), Fraser–Lee (FL), and biological intercept (B) procedures. Regression-based backcalculations assume no deviation from the overall regression, while Fraser–Lee backcalculations assume that individual fish–otolith deviations are maintained proportionally throughout the backcalculation. Both procedures result in mean backcalculated lengths which are equal to the overall fitted regression (solid line). In contrast, the biological intercept procedure (Equation 27) is in no way influenced by the overall fitted regression; the slope of each fish–otolith trajectory is independent of all others in the sample. In this example, independent observations would have been used to determine that fish and otolith growth were proportional after the biological intercept, which in this example occurred at an otolith length of 2.0 and a fish length of 1.0.

$$(25) \quad L_a = bO_a + d$$

where b and d are the slope and intercept of the regression, respectively. Since this procedure assumes no deviation of individual fish and otolith measurements from the overall regression, it has generally been applied when mean backcalculated lengths, rather than individual values, are of importance. In contrast, the Fraser–Lee (or Lee) procedure assumes that any deviation of an individual measurement from the overall fish–otolith regression will be observed proportionally at backcalculated lengths, as in

$$(26) \quad L_a = d + (L_c - d)\, O_c^{-1}\, O_a$$

where L_c and O_c are the fish length and otolith size at capture, respectively. While the Fraser–Lee approach does not incorporate the regression slope directly, the value of the regression intercept is, of course, influenced by the slope. Indeed, the regression and Fraser–Lee procedures differ algebraically only in that the latter is intercept-corrected. As a result, the two procedures produce identical mean backcalculated lengths, although backcalculations at the individual level may differ (Fig. 8). Both the regression and the Fraser–Lee procedures are sensitive to the value of the intercept that is employed; as a result, more sophisticated linear and maximum likelihood models have been developed to account for age- and sample-dependent variations in the fish-length relationship (Bartlett et al. 1984; Weisberg 1986; Smith 1987). However, common to all of the procedures is the assumption that the fish–otolith length relationship does not vary in a systematic fashion with growth rate, and further, that one or both of the regression parameters can be accurately estimated from the population. It has now been convincingly demonstrated that the fish–otolith relationship does vary systematically with the growth rate of the fish: otoliths from slow-growing fish are larger and heavier than those from fast-growing fish of the same size (Templeman and Squires 1956; Boehlert 1985; Mosegaard et al. 1988; Reznick et al. 1989; Secor and Dean 1989). Further, a recent study indicates that individual variations in growth rate result in a population-wide fish-otolith regression which differs significantly from that of the mean of the individual fish (Campana 1990). The net result is that traditional growth backcalculations can underestimate previous lengths at age, a finding which appears to account for the apparent ubiquity of Lee's phenomenon.

Backcalculation with the Biological Intercept Algorithm

The "biological intercept" backcalculation algorithm is a modification of the Fraser–Lee equation which employs a biologically determined, rather than a statistically estimated, intercept value (Campana 1990). Like the Fraser–Lee method, the biological intercept procedure assumes proportionality between fish and otolith growth within an individual. However, unlike the former, the value of the biological intercept is determined by the mean size of the fish and otolith at the initiation of proportionality, and thus is insensitive to sample to sample variations in regression parameters. Indeed, the biological intercept procedure doesn't require any samples from the population, other than those used to verify proportionality between fish and otolith growth after the biological intercept. In many cases, the biological intercept can be determined by simple measurements of fish and otolith size in newly-hatched larvae in the laboratory. The procedure is also insensitive to the growth rate effect described earlier, since the fish–otolith slope is calculated independently for each fish. And finally, backcalculation accuracy is relatively insensitive to normal variation around the intercept value, largely

because of the small values involved. The equation is:

$$(27) \qquad L_a = L_c + (O - O_c)\,(L_c - L_i)\,(O_c - O_i)^{-1}$$

where L_i and O_i are the size of the fish and otolith at the biological intercept, respectively. An example of its use is presented in Fig. 8. Note that the slope and intercept of the fish in the sample are not used in the backcalculations. Assuming that an independent study has determined that fish and otolith growth are proportional within individuals from the time of hatch, growth backcalculations back to the time of hatch may be warranted. In contrast, regression or Fraser–Lee backcalculations would require that backcalculations be restricted to the range of fish and otolith lengths evident in the sample.

In some situations, the differences between growth backcalculations made with traditional methods and those made with the biological intercept procedure will be relatively small. This will be particularly true when the statistical and biological intercepts are collinear, such as when samples of very young fish (near the size of the biological intercept) have been collected. However, the biological intercept procedure will always be at least as accurate, if not more so, than the traditional methods. On the other hand, it should be clearly recognized that all of the above methods are based on the assumption of a constant linear relationship between fish and otolith length within an individual. Neither the traditional nor the biological intercept methods will provide accurate backcalculations in the presence of nonlinear fish–otolith relationships (Campana 1990; Secor and Dean 1992).

Backcalculation with Multivariate Algorithms

Where there is an intrinsically curvilinear relationship between fish and otolith length, transformation of the data to a linear form will allow the use of Equation (27). However, where time-varying growth rates have been in effect, use of any of the linear backcalculation procedures described in the previous section will result in at least some error. There is now increasing evidence that the width of a given daily increment is linked more closely to metabolic rate and/or temperature than to somatic growth (Mosegaard et al. 1988; Wright 1991; Secor and Dean 1992). If true, reliable growth backcalculation procedures will almost certainly have to incorporate a chronological history of either metabolic rate or temperature. No such procedure yet exists. However, there are two multivariate algorithms, both very experimental, which use proxies for the metabolic/temperature term. Secor and Dean (1989, 1992) argued that age affects the relationship between otolith size and fish size in a cumulative manner, resulting in different-sized otoliths in fast- and slow-growing fish of the same size. Growth backcalculations made with their model accurately predicted the growth history of laboratory-reared fish, but performed poorly when applied to pond-reared fish (Secor and Dean 1992). Using a different rationale, Campana (1990) suggested that previous lengths at age could be estimated using a measured series of daily increment widths and an estimate of the magnitude of the growth rate effect on the fish–otolith relationship. An algorithm was presented, but was not tested. Therefore, at present, there exist no backcalculation algorithms which can provide accurate estimates of past growth under all conditions. In addition, none of the available backcalculation procedures was designed to deal with the observation that otolith growth tends to be smoothed relative to fish growth (Campana and Neilson 1985). Time series models are necessary when account is to be taken of autocorrelated increment widths (Gutiérrez and Morales Nin 1986). Indeed, time series models appear to be well suited to the analysis of these types of data.

Backcalculation of Recent Growth

Given exact proportionality between fish and otolith growth, the width of the most recently formed daily increments should provide a measure of recent growth. Such measures are difficult to obtain through other means, thus explaining the widespread interest in this approach by workers studying the environmental conditions which promote the survival of young fish (Methot 1981; Thomas 1986; Bailey 1989; Suthers et al. 1989; Powell et al. 1990; Hovenkamp and Witte 1991). The assumptions underlying the use of increment width measurements as a proxy for instantaneous growth rate are the same as those presented earlier for general growth backcalculation. However, the scale of the analysis makes the resulting inferences considerably more sensitive to deviations from the assumptions. In particular, any short term deviations from a linear fish–otolith size relationship will be much more evident at the daily level than when averaged across the entire life history. For this reason, most workers have employed aggregates of increments, such as those corresponding to the outermost 7–30 days, as their index of recent growth. Use of aggregated increment widths reduces, but does not eliminate, the influence of autocorrelated otolith growth and short-term curvilinearity in the fish–otolith relationship. However, we are not aware of any studies which have quantified the level of aggregation which is required.

There are three basic steps involved in the estimation of recent growth rates based on otolith growth: measurement, preparation of a quantitative (usually, but not necessarily, linear) relationship between fish and otolith growth, and conversion of otolith growth to fish growth. Measurement of the outermost daily growth increments along a pre-defined radius, either individually or in aggregate, has been discussed elsewhere (Campana, this volume). Preparation of a fish–otolith relationship may be as simple as the regression of fish length on otolith length, if fish and otolith growth are proportional. If the latter, the residuals from the regression will be randomly distributed around zero with respect to otolith size. Note that fish length is best considered as the dependent variable, since it (rather than otolith length) is the variable to be predicted. In instances where otolith length increases curvilinearly with fish length, log transformation of the otolith measurements is often sufficient to induce linearity, although this should be checked. The importance of inducing a linear fish–otolith relationship cannot be overemphasized, since increment widths can increase with otolith size, even under constant (or in some cases, decreasing) fish growth rates, if the fish–otolith relationship is nonlinear. Finally, the (transformed) otolith measurements are converted to fish measurements through the use of Equation 27, and interpreted in terms of daily growth rates after dividing the net change in fish length by the number of daily increments used in the aggregate increment measurement. Note that Equation 27 incorporates an inherent adjustment for individual variations in otolith size among fish of the same length; the size correction used by Methot (1981) is not necessary.

Backcalculation of recent growth patterns suffers from the same constraints as those described in the last two sections. Specifically, nonlinearities in the fish–otolith relationship due to growth, metabolic rate and/or temperature will introduce error into the resulting backcalculations. Indeed, these errors can be more pronounced when backcalculating recent growth than when estimating the growth of an earlier life history stage, due to the strong influence of a recent shift in the slope of the fish–otolith relationship on the backcalculated lengths. There are as yet no published procedures which have dealt successfully with this problem. However, it may be avoidable if it can be demonstrated that the fish–otolith slope connecting samples collected just before and just after the growth period of interest is similar to the slope being used for backcalculation.

Growth and the Environment

Analyses designed to link the growth chronology evident in the otolith to associated environmental observations constitute one of the most promising, and complex, applications of otolith microstructure examination. In theory, such analyses can be used to test many of the current hypotheses concerning growth, survival, and recruitment. However, a meaningful test of an environment-growth relationship is anything but straight forward: a simple correlation or regression between a growth index and an environmental variable(s) can be grossly misleading. Valid statistical approaches to the analysis of otolith–environment data are still being developed. To this end, the parallel field of dendrochronology (tree ring chronologies) is much more developed than is our own. Investigators wishing to pursue otolith–environment analyses are urged to review the tree ring literature, and note its reliance on time series analysis and general linear models (Fritts 1976; Hughes et al. 1984; Stahle et al. 1988).

The growth indices available for analysis in relation to the environment can be classified into three broad categories: recent growth, mean growth, and individual growth rate time series. All are valid growth indices, but the means by which they can be interpreted differ widely. For instance, indices of recent growth have often been related to environmental variables (e.g., Methot 1981; Thomas 1986; Bailey 1989; Karakiri et al. 1989; Suthers et al. 1989; Hovenkamp and Witte 1991), either in a relative sense or through correlation (e.g., both temperature and recent backcalculated growth, as indicated by the mean 10-d outer increment width, at Site A was larger than that of Site B). The advantage of this approach is associated with the independence of the observations; that is, each fish provides a single estimate of recent growth rate, thus avoiding the statistical problems of autocorrelated otolith growth. The danger of this approach becomes evident if the analysis does not test explicitly for the possibility of a faster growth rate in larger individuals. Since larger fish often experience greater absolute growth rates than smaller fish, and given differences in mean size between samples, inter-sample differences in indices of recent growth may well result from size differences between samples, and be falsely attributed to environmental sources. Suthers et al. (1989) applied a simple analysis of covariance approach to overcome this problem in the search for environmental correlates of enhanced growth in Atlantic cod (*Gadus morhua*).

A second approach is to relate mean growth rate, rather than recent growth rate, to some combination of environmental variables. This approach is recommended only for very young fish, if only because environmental fluctuations during an extended period

can confuse any interpretation of the corresponding growth data. Cohort-specific growth rates of young larvae have been successfully related to temperature and other variables by several workers (Methot and Kramer 1979; Crecco and Savoy 1985).

While potentially the most powerful of the growth indices, analysis of the entire sequence of daily increment widths within each otolith is complicated by the inherent autocorrelation of otolith growth. As a result, the backcalculated growth observations are not independent of each other, and thus are difficult to relate statistically to any other time series of variables. This problem may account for the unexpected results of workers who have regressed environmental time series on sequences of backcalculated growth rates (e.g., Barkman and Bengtson 1987). In an innovative and statistically rigorous approach, Thorrold and Williams (1989) applied a repeated-measures ANOVA, followed by polynomial contrasts with time, to test for growth sequence differences among cohorts. Observed differences were then interpreted qualitatively with respect to the environment. However, the most powerful approach, and the almost universal choice of dendrochronologists, is that of time series analysis. Time series analysis, particularly of long growth sequences, takes full advantage of the available information, takes explicit account of any inherent autocorrelation, and is well suited to testing a broad range of hypotheses concerning environmental influences on growth. While appropriate for detecting cycles in growth data (e.g., lunar cycles; Campana 1984), its most powerful applications have been directed towards determining the influence of environmental variables on growth (e.g., Gutiérrez and Morales-Nin 1986; Thorrold and Williams 1989).

An understated danger with respect to the search for growth-environment relationships is that of spurious correlation. Spurious correlations occur most often when two variables, each characterized by a trend through time, are correlated or regressed against each other. A relevant example is that of a declining trend in a sequence of daily increment widths and a declining trend in temperature. While the two sequences will be very strongly correlated, the high correlation will be largely due to the coincident trends, and not to any inherent relationship between the two. For instance, the declining increment widths may be due solely to the reduced growth rates characteristic of older fish. Since regression analysis assumes that each of the observations are independent of each other, and since trended observations are not independent, a more appropriate regression analysis would require that the two time series first be detrended through one of the available techniques (e.g., first differencing; the reader is referred to the time series literature for further information). Note also that spurious correlation can obscure underlying relationships as much as it can enhance nonexisting ones. Detrending is a universal precursor of any time series analysis, and should also be implemented prior to regression of an environmental sequence on a growth sequence. An unfortunate byproduct of its use is that it can also remove real as well as spurious correlations, resulting in a loss of power. A good example of detrending was presented in the environment-recruitment sequence analysis of Thompson and Page (1989).

Hatch Date Analysis

Hatch date analysis, also known as birthdate analysis, is one of the more promising tools for the study of recruitment processes. The underlying principle is simple; given a random sample of fish collected on a known date, and through examination of the otolith microstructure to determine the age of each fish, the frequency distribution of hatch dates for the survivors in the population (the random sample) can be calculated. The resulting hatch date distribution is, of course, a transposed (mirror) image of the age–frequency distribution. The hatch date distribution can then be compared with the observed production schedule of newly-hatched larvae (or late-stage eggs). In the

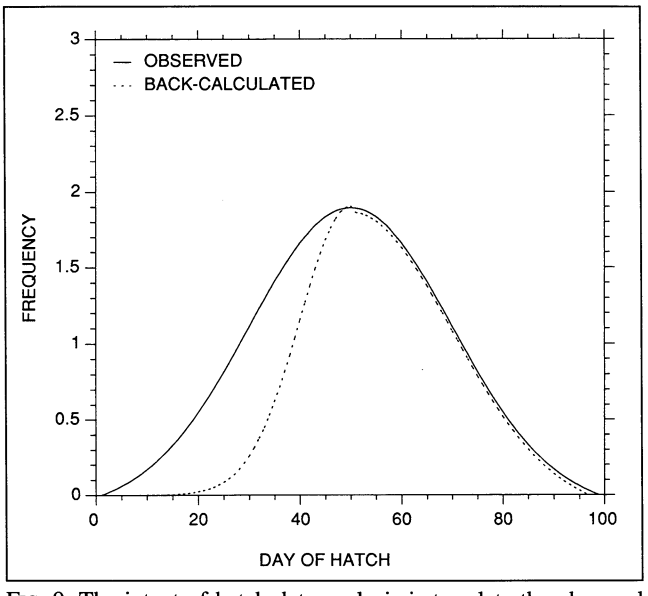

FIG. 9. The intent of hatch date analysis is to relate the observed frequency distribution of hatch dates (or egg or larval production) to those of the survivors. In principle, differences between the observed and backcalculated distributions would indicate that the survival of larvae hatched on certain dates was enhanced relative to those hatched on other dates. In this example, larvae hatched in the first half of the spawning season survived poorly relative to those hatched later in the season.

absence of selective mortality, the shapes of the back-calculated hatch date distributions and the observed larval production distributions should be identical. However, if differences between the two distributions exist (Fig. 9), such would suggest that the survival of larvae hatched on certain dates was enhanced relative to those hatched on alternate dates. The subsequent challenge is to relate the relative survival of the daily cohorts to likely environmental sources, and thus identify potential modifiers of recruitment success.

One of the most useful features of hatch date analysis is the fact that it focuses attention on the characteristics of the survivors, rather than on the population at large. There are many potential sources of young fish mortality, only some of which may be important in determining year–class strength. However, where certain daily cohorts contribute disproportionately to the abundance of the survivors, one may be certain that critical factors influencing recruitment have been involved. In his pioneering work with hatch date analysis, Methot (1983) related monthly differences in the relative survival of larvae to various environmental signals, as well as to the overall effect on year–class strength. Analogous studies are now underway around the world, indicating the value which is attributed to this type of study. There is no question that hatch date analysis is a potentially powerful application of otolith microstructure examination. However, it should not be viewed as a panacea; there are certain species and life history stages for which hatch date analysis will not be appropriate for anything more than a general description of hatching dates. Indeed, without proper caution, hatch date analysis can be more misleading than instructive. Gear selectivity and the difficulty of adequately sampling each of the relevant life history stages further complicates the issue (see Butler, this issue). The remainder of this section illustrates some of the properties and caveats associated with hatch date analysis, and provides some recommendations as to its use.

The most serious problem associated with hatch date distributions is with respect to their instability. While the dates of production of the newly-hatched larvae would normally be determined through frequent sampling throughout the hatching period, backcalculated hatch date distributions are normally determined from samples collected during a much shorter range of dates. Given natural mortality, representatives of the larvae hatched earliest in the season will inevitably experience greater cumulative mortality than those hatched late in the season. Accordingly, early season larvae will be underrepresented in the backcalculated hatch date distribution relative to late season larvae. Thus, the hatch date distribution will be skewed, and will be unrepresentative of the true numbers of the survivors at a given age. Consider the example of Fig. 10. In this simple case, the hatch date distribution of a sample of postlarvae has been simulated assuming a constant, post-hatch instantaneous mortality rate of 0.1 d^{-1}. The hatching period was taken to extend over 30 d, and the collection was made 50 d after the end of the hatching period. Clearly, the backcalculated hatch date distribution is skewed relative to the initial hatching distribution. Yet at a given age (not date), the survivors of each daily cohort make up the same proportion of the original production as do all of the other daily cohorts. The distributional skew is due solely to the differential in cumulative mortality between the youngest and oldest larvae. In this example, the oldest larvae will have experienced 30 d more mortality on a given date than the youngest larvae, resulting in an abundance of the former which is a mere 5% of that of the youngest larvae on any given date. This conclusion holds irrespective of the mortality rate, length of hatching period, and interval to collec-

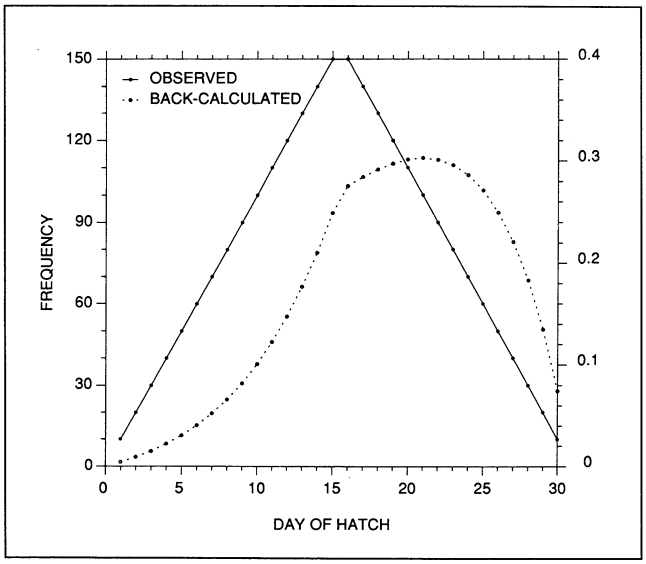

FIG. 10. Example of a skewed hatch date distribution due solely to cumulative mortality differences among members of the cohort. In this example, larval production was assumed to extend symmetrically over a 30-d period (Observed). All larvae experienced an instantaneous mortality rate of 0.1 d^{-1}. Although all daily cohorts survived equally successfully to any given age, the mortality rate was sufficiently high that the first-hatched larvae were less abundant in any given sample solely because they were older. The skew in the hatch date distribution does not disappear with time; that is, as long as the mortality rate remains constant, the same distributional pattern will be observed at any collection date after completion of the hatching season. Thus, the back-calculated hatch date distribution does not accurately represent the hatch dates of the survivors at age, and could be used to mistakenly infer that early-season larvae survived relatively poorly. The left hand axis label refers to the observed hatch date frequencies, while that on the right refers to the backcalculated frequencies.

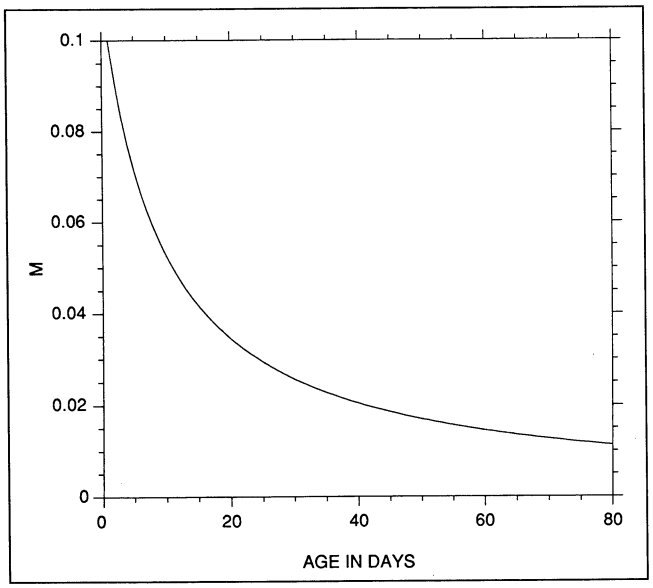

FIG. 11. Inverse relationship between instantaneous mortality rate (M in units of d^{-1}) and daily age which was used in the simulation of Fig. 12.

tion that is assumed; in all cases, the differential in cumulative mortality between the youngest and oldest larvae will control the shape of the hatch date distribution. This is best seen if the mortality rate in the above example is assumed to drop to zero at some given age. Once all of the daily cohorts have reached that age, the cumulative mortality differential between youngest and oldest becomes zero, and the backcalculated hatch date distribution becomes identical to that of initial production. In other words, if hatch dates are being determined from a life history stage with a low mortality rate, the resulting hatch date distribution will be relatively stable.

The constant mortality rate assumed in the example of Fig. 10 is clearly unrealistic. More probable is some form of age- or size-selective mortality, whereby the mortality rate on the youngest/smallest larvae is greater than that on the older/larger individuals. Any number of age–mortality functions can be envisioned. However, one possible relationship is an inverse relationship between instantaneous mortality rate and age (Fig. 11). Figure 12 demonstrates the resulting evolution of the shape of the hatch date distribution as the time interval after hatching is increased. Note the initial skew in the distribution immediately after the end of the hatching period, due to the large cumulative mortality differential between youngest and oldest larvae. However, as the mortality rate reaches a low level (Fig. 11), the mortality differential between youngest and oldest larvae is greatly reduced, resulting in a hatch date distribution which very nearly mirrors that of the initial production. Simulations using size-selective mortality, rather than age-selective mortality, produced similar results, although the variance in age at size resulted in effects which spread across multiple daily cohorts. It is also important to note that the shape of the age–mortality curve itself is irrelevant. Rather, what is important is the cumulative mortality differential between the youngest and oldest larvae in the sample. In the absence of other mortality sources, the age-specific mortality rates which occur at ages prior to the youngest age in the sample have absolutely no effect on the shape of the hatch date distribution.

There are several implications of the simulation results presented in Fig. 12. First, it would appear that hatch date distributions will be least stable, and most unreliable, when the mortality rate at age is high at the time of collection, since the cumulative mortality differential between youngest and oldest larvae will also be large. Conversely, the hatch date distribution will be most stable when two conditions are met: (a) the fish are relatively old at the time of collection, with an accompanying mortality rate which is stable and low, and (b) the duration of the spawning (hatching) period is short, resulting in a minimal differential in cumulative mortality between the youngest and oldest larvae in the cohort. As a rough rule of thumb, the relative stability of a hatch date distribution can be approximated by examining the abundance ratio of the oldest to youngest fish in the sample, which is in turn an approximation of the cumulative mortality difference between the two ages, as in:

$$\frac{N_{\text{old}}}{N_{\text{young}}} = e^{-\left(\sum_{i=\text{young}}^{\text{old}} M_i\right)}$$

where N is the relative abundance in the population, young and old are the youngest and oldest daily ages (i) in the sample respectively, and M is the instantaneous mortality rate (d^{-1}). Where the abundance ratio is very high (e.g., 0.9), a mortality correction will not make any significant difference to the hatch date distribution. On the other hand, a low ratio (e.g., 0.05) would indicate that a mortality correction is mandatory, since the distribution is unstable. Note that this ratio will only be useful in cases where the representative sampling of the two age categories has not been confounded by gear selectivity or patchiness of the fish.

In theory, an unstable hatch date distribution can be rendered stable through correction for the differential in cumulative mortality rates within the cohort. Interpretation of the hatch date distribution without first correcting for the cumulative mortality differential will result in incorrect inferences: the larger the mortality

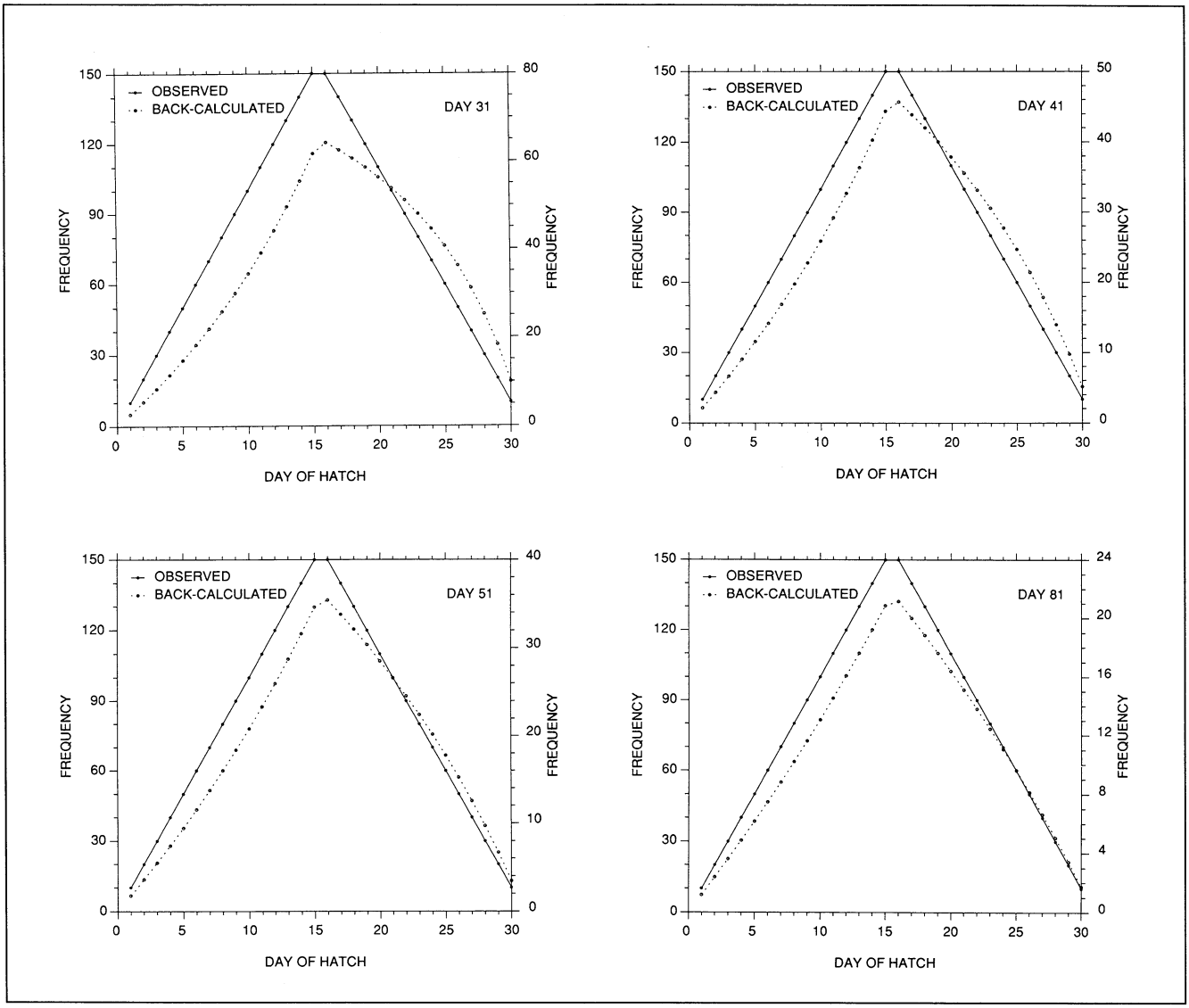

FIG. 12. Evolution of a backcalculated hatch date distribution as the time interval between hatching and collection is increased. In this example, hatching extended symmetrically over 30 d (Observed) and instantaneous mortality rate (M) was assumed to decrease inversely with age (Fig. 11). Linear and exponential declines in M produced similar hatch date distributions to those presented here. Note the skew in the backcalculated hatch date distribution immediately after completion of hatching (Day 31). As the daily cohorts age, and as the M on the youngest larvae declines, the cumulative mortality difference between youngest and oldest larvae decreases, resulting in an increasingly symmetrical backcalculated hatch date distribution. The left hand axis label refers to the observed hatch date frequencies, while that on the right refers to the backcalculated frequencies.

differential, the larger the error that will result if the hatch date distribution is not first corrected accordingly. Note, however, that the mortality correction has nothing to do with the interpretation of the hatch dates; the mortality correction is an age-specific one across all cohorts, used simply to put the calculated hatch date distributions on the same scale as that of the observed production. The corrected hatch dates can then be interpreted in terms of date- and cohort-specific mortality/survival processes which have changed the original production date distribution.

Methot (1983) corrected for the mortality differential in his samples of juvenile fish through multiplication of the numbers at age by the inverse of the survival rate between the age at capture and the age of the youngest fish in the sample. The survival rate estimates he used were derived independently. Yoklavich and Bailey (1990) made similar corrections to their larval hatch date distributions, although they were forced to correct using an assumed mortality rate during the larval stage. The effect of the mortality corrections differed substantially between the two studies,

clearly demonstrating the difficulties of analyzing hatch date distributions in larvae with a high mortality rate. Mortality through the juvenile stage in Methot's (1983) study was relatively low, and the mortality correction resulted in only minor differences between the shapes of the corrected and uncorrected hatch date distributions. In contrast, the mortality differential between young and old larvae in the samples of Yoklavich and Bailey (1990) was substantial, and the corrected distribution differed markedly from that of the uncorrected. Use of an alternate mortality curve could have changed the hatch date distribution in a different manner.

It will seldom be possible to correct unstable hatch date distributions without ambiguity. Independently-derived survival estimates, such as those of Methot (1983), will not normally be available. And in the presence of high and unpredictable mortality rates, such as those of many pelagic larvae, mortality correction based on average or assumed mortality curves may well result in hatch date distributions which do not represent reality. Accordingly, hatch date analysis is best carried out on a life history stage characterized by a low and stable mortality rate, in which a mortality correction makes little difference to the shape of the distribution. In cases where an influential mortality correction must be applied, the shape of the correction should be carefully justified.

While mortality correction is one means by which an unstable hatch date distribution can be corrected, there may be an approach (as yet untried) by which the instability can be avoided altogether. As mentioned previously, the production of newly-hatched larvae (or eggs) is generally determined by sequential sampling throughout the hatching period. In principle then, it should be possible to monitor the abundance of a given cohort, or many cohorts, through sequential sampling over a time interval equal in length to that of the production period. The hatch date distributions for each sample for a given range of ages could then be summed across all dates to produce a single distribution in which each daily cohort was sampled at the same range of ages. Thus, there would be no cumulative mortality differential between daily cohorts, and hence no need for mortality correction. As an example, consider the production of newly-hatched larvae in a small lake. Assume that the production was monitored daily throughout the hatching period of 30 d (Day 0–30). If the lake was re-sampled for juveniles some 50 d later (Day 80), the calculated hatch date distribution would be skewed by any cumulative mortality differential between the 50-d old and the 80-d old fish. However, if all of the juveniles were sampled daily between Day 50 and Day 80, the summed hatch date distributions of the 50–80 d old fish in each daily sample should accurately represent the hatch date distribution of all cohorts after 50 d. Note that this does not represent a simple summation of all hatch date distributions during the collection period, since fish less than 50 d of age would not be included in the calculations (e.g., only the 50-d old fish would be included from the juvenile collection at Day 50, despite the fact that the sample would include fish of age 20–50 d). Key assumptions of this procedure are that there is no age- or size-specific gear selectivity, and no age- or size-related immigration or emigration out of the sampling area. As well, the periodicity of sampling will determine the resolution with which the final hatch date distribution can be interpreted.

It is probably possible to combine sequential sampling on a nondaily schedule (e.g., weekly) with some form of mortality correction in order to produce a corrected hatch date distribution. Presumably, such a procedure would minimize the mortality correction required of a single sample, yet would be logistically easier than a daily sampling schedule. However, to our knowledge, no one has yet generated a hatch date distribution through daily sequential sampling, let alone through sequential sampling combined with a mortality correction.

The most common application of hatch date analysis involves the identification of enhanced or depleted portions of a year–class, followed by correlation of the perturbed portions with prominent environmental signals. Examples of the latter might include periods of storm-enhanced mixing, upwelling, advection out of the survey area, high or low food availability, and high or low predator abundance, as well as others. Most of these correlations are best made with a specific life history stage (e.g., first-feeding larvae). Yet it is important to note that hatch date analysis cannot be used to determine the date or age at which the hatch date distribution was perturbed from its initial state. That is, the forces which enhanced/depleted a portion of the cohort could have been active just after hatching, or weeks later, just prior to collection; hatch date analysis cannot be used to differentiate between these two possibilities. Accordingly, it is difficult to unambiguously relate a particular environmental cue to a change in the hatch date distribution, since the latter could have occurred anytime between hatching and collection. In principle, sequential sampling could be used to bound the possible dates during which the change in hatch date distribution occurred. However, as noted earlier, hatch date analysis is of questionable value when mortality rates are high, which unfortunately, may well correspond to the life history stage of interest. Of course, certain sources of mortality are

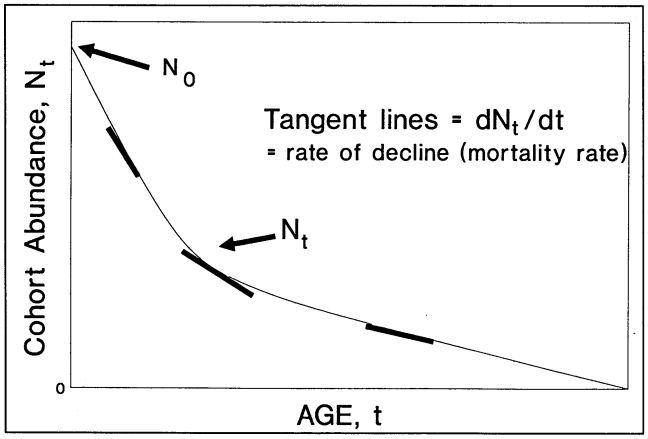

FIG. 13. Theoretical representation of cohort abundance over its lifetime. The three bold tangent lines illustrate the decline in the slope, the absolute mortality rate at time *t*, as the fish age. In this model, *Z* (the instantaneous mortality rate) is constant, although this need not be the case.

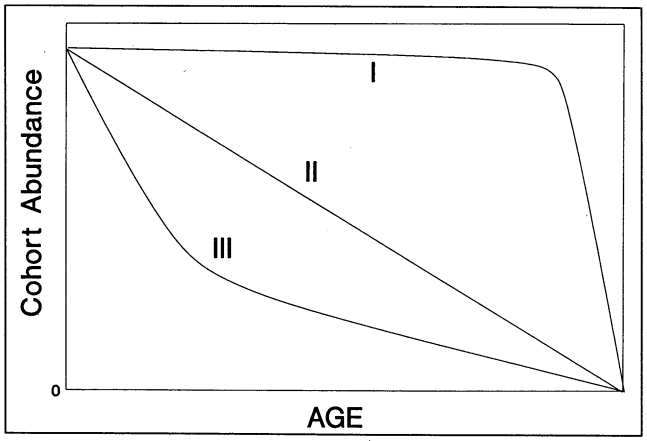

FIG. 14. Hypothetical survivorship curves (modified from Slobodkin 1961). A type I curve has little mortality until senescence; the Type II curve has an equal absolute amount of mortality in all life stages; Type III is frequently used to model fish mortality since it indicates that a constant proportion of the cohort dies through time.

more likely to occur at specific life history stages than others; for instance, advection out of a favourable area is more likely to kill very young larvae, with poorly-developed locomotory skills, than older juveniles. On the other hand, the absence of large prey items may result in high mortality of juveniles with no net effect on young larvae. Therefore, while a stable hatch date distribution may provide strong evidence of enhanced survival by certain daily cohorts, it will not necessarily be a trivial problem to identify either the sources of the enhanced survival, or the age of the larvae which were affected.

Mortality Estimation

Within the past few years the daily ageing technique has been increasingly used to investigate survival for fish younger than one year of age. The ability to measure age-specific abundance and survival is a significant improvement over mortality estimation based on size alone. Size in young fish is not a good measure of age and a given size category often contains a wide range of ages. Age-specific measures of abundance may offer the possibility to investigate subtle causes which affect the survival of young fish. Some of the recent applications that rely on daily ageing include: Houde (1989), Owen et al. (1989), Alhossaini et al. (1989), Fortier and Gagné (1990) and Pepin (1991). These recent studies were largely concerned with partitioning mortality to various causes within the early life stages. This list is only a small sample of the types of studies that are now being undertaken.

Fundamental Concepts of Mortality Estimation

Mortality is estimated by measuring the decline in abundance of a cohort over a specific period of time. A cohort is a closed or limited group of individuals which, once born, can only decline in abundance. A graph (Fig. 13) of cohort abundance shows that initial abundance (N_0) is highest at hatch then decreases as members are lost through starvation, predation, disease, and advection to areas where survival is diminished, among other causes. Before the development of the daily increment ageing method, mortality was estimated by enclosure experiments or field observations of the decline in abundance of progressively larger size classes. The difficulty with this length-based approach is that sources of mortality are often time-specific (for example, advection events, timing of food availability, presence of predators) and cannot be discerned with length-based measures. Because size, in general, is not a particularly good indicator of age, length-based methods don't track well-defined cohorts, and, therefore, yield results which are often too crude to be useful.

We can illustrate the patterns of mortality with theoretical survivorship curves (see Fig. 14, after Slobodkin 1961). Such survivorship curves model the shape of decline in abundance of a given cohort through the cohort's lifetime. The early life stages of fishes are usually best modeled by type III curves. In general natural mortality is extremely high during egg and larval stages (for example, 2–10% per day in plaice and clupeoids — Cushing 1975; Smith 1985), decreases quickly during the juvenile period (Dahlberg 1979; Crecco et al. 1983), becomes rela-

tively stable during adulthood, and then may increase again in senescence (Vetter 1988). The most familiar curve, type III, is represented by the often used equation for the negative exponential function:

(28) $N_t = N_0 e^{-Zt}$

By rearranging Equation 28 and taking logarithms, we can solve for Z, effective total instantaneous mortality between time 0 and t. Note that Z is, by convention, a positive number.

$$Z = \frac{-\ln(\frac{N_t}{N_0})}{t}$$

The relationship that we represent here is based on the calculation of the instantaneous rate. This rate is not as intuitive to understand as the actual survival rate, S, where $S = \frac{N_t}{N_0}$, or the actual mortality rate, A, where $A = 1 - S = 1 - \frac{N_t}{N_0}$. Such finite rates, often used to express larval survival or mortality, do not lend themselves to partitioning the components of mortality, since they are not readily compared across different units of time (e.g., a 10% per year mortality rate is not the same as two periods of 5% mortality per 6-months). In contrast, instantaneous rates are readily additive.

The instantaneous mortality rate varies with the ratio of abundances, where the ratio is simply the proportion surviving. Hence, the above equation indicates that mortality affects a constant proportion of the population over time, say 10% of the remaining fish die each day. Because this constant percentage is taken from an ever-decreasing abundance, the absolute numbers that die in each time period actually decrease over time. Ten percent of 100 fish is 10 deaths, while 10% of the remaining 90 is 9, and so on.

Total mortality (Z) is usually modeled as $Z = M + F$, where M is instantaneous natural mortality and F is the instantaneous fishing mortality. Even in species which are subject to commercial or recreational fishing, fishing doesn't usually occur in the first year of life. Therefore, total mortality in the first year of life is equal to natural mortality; $Z = M$. Actually M probably changes over the various life stages, and is best represented as $M = \sum_{i=1}^{n} M_i$, where n = the number of life stages included in the analysis. In most cases, M is assumed to be an average of the M_i's.

The instantaneous mortality rate is often very high initially, decreasing over time, and can be best represented by a curve in which mortality changes between

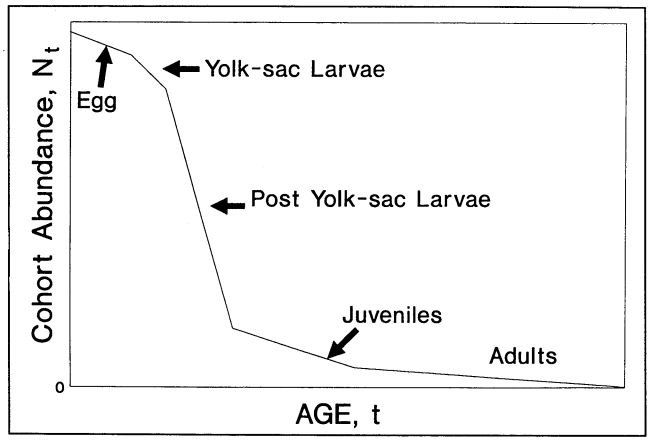

FIG. 15. Hypothetical survivorship curve when mortality changes between life history stages.

developmental stages (Fig. 15). When abundance drops off this quickly, the negative exponential equation will not match real-world data very well. One alternative approach is to estimate mortality over only a part of the lifetime. If the negative exponential still gives a poor fit, more complex and less well-known mathematical functions can be fitted to the data. The Weibull function, used by engineers to predict equipment failure, has been used to model adult mortality data (Neilson et al. 1989). Lo et al. (1989) used the Pareto function to simulate mortality of larval anchovy. The goodness of fit of all of these functions can be assessed with a Chi-Square test (see for example Zar 1984, page 40). However, it is often preferable to use the more familiar exponential function for modelling mortality rates. The disadvantage in using other functions is their lack of familiarity, lack of comparability to other work and in the added complexity in confidence interval estimation.

Conceptually, the estimation of mortality is very simple. Cohort abundance is measured at some initial period (N_0) and again at some later time (N_t), providing all the information required to solve Equation 28. In actuality, obtaining these abundance estimates with precision can be quite difficult, particularly in the larval and juvenile stages. When sampling is limited, as is often the case, the variance will be large and the confidence intervals wide. With limited sampling the estimate of abundance can be far from the true value. Gear avoidance by larger fish is a particular problem with mortality calculations (see Butler, this volume). Additionally, larval distribution is patchy, especially for marine species, and may therefore cause nonsensical estimates of Z where, due to sampling variability, estimates of abundance are greater later in life than in the beginning ($N_t > N_0$).

Methods of Calculating Mortality

Traditionally, cohorts have been defined as all the fish produced from the eggs spawned by a population in a year. With the capability to age at the daily level, the term may now be assigned to weekly or daily "cohorts" produced within a spawning season. Previously, within-season cohorts were identified by methods based on length alone. The assignment of age classes by following the progression in length modes over time is often impossible due to the lack of differentiated spawning pulses for many species (no distinct modes are produced). Even if spawning pulses occur, the variability in growth results in blended mixtures of several age (daily) classes in a length category soon after hatching. However, direct estimates of age-specific mortality are possible with the daily increment method.

While some investigators have simply compared early versus late spawned larvae when looking for evidence of within-year mortality, other time groupings are possible (e.g., weekly, biweekly or even daily). One source of variance in these groupings is the measurement error in reading and assigning age (see Neilson, this volume); this assigned age is actually an estimate which can vary by several days, even if it is unbiased. The variability of the age estimate can be reduced by grouping young fish into multi-day cohorts within the spawning season (see Crecco and Savoy 1985, for their technique of cohort grouping).

Methods for estimating mortality can be divided into direct and indirect approaches (Krebs 1972). The direct approach is to mark and recapture cohort members, following the decline in the numbers of marked individuals over time. Indirect measures include (1) analysis of catch-curve data, (2) correlations of natural mortality with other life-history parameters and (3) estimation of death due to predation (Vetter 1988). Of all methods, the catch curve method is most frequently used for larvae and juveniles. The other two indirect methods are rarely, if ever, used for young fish. After a brief description of mark and recapture techniques, the catch curve methods will be discussed in more detail.

Mark and recapture

Mark–recapture techniques are commonly used on adult fish to determine the sources of mortality (Brownie et al. 1985; Burnham et al. 1987), but have been infrequently used to estimate mortality in very young fishes. In young fish, marking can be done en masse (the marks are the same on all individuals) or with tags which specifically identify each marked individual. Small and very young fish are delicate and difficult to handle, hence the otoliths are usually batch marked with chemicals (Hettler 1984; Schmidt 1984; Tsukamoto 1985). Batch marking precludes tracking individual fish, but is well suited to measuring changes in abundance due to the large numbers which can be marked. Juveniles are larger, easier to handle and resilient enough to carry individual tags, such as binary coded wire. Individual marking can be used to test for more subtle differences in mortality between groups (either within or between cohorts), and for interactions with growth and/or location, although it is usually more difficult to mark large numbers of fish.

Several important assumptions must be met before using mark-recapture methods to estimate population abundance and mortality. These assumptions include: (1) the tagged fish are representative of the population from which mortality information is sought, (2) there is no emigration of tagged fish, (3) the number of tagged fish that are released is known exactly, (4) there are no tag losses and no misread tags, (5) survival rates are not affected by tagging, and (6) that the fate of each individual tagged fish is independent of other tagged individuals, among other assumptions (Brownie et al. 1985; Burnham et al. 1987). None of these assumptions is specific to otolith-tagged fish, although tetracycline has been reported to both enhance (Tsukamoto 1985) and reduce (McFarlane and Beamish 1987) the survival of tagged fish relative to control fish.

There are four major potential obstacles to the use of otolith tags in estimating the mortality of larval fishes: (1) mortality from handling and marking, (2) intrinsically high mortality rates, implying that very large numbers must be marked to get any returns, (3) the lack of commercial and sport fisheries on the young fish, requiring that the investigator recapture the marked fish, and (4) biases introduced from net avoidance and gear changes. As was the case with the underlying assumptions, these limitations are not specific to otolith-tagged fish, and are often present in other mark–recapture studies of young fish.

The mortality calculation from a mark recapture study is simply the decline in the number of recaptures over time. Equation 28 can be rewritten to reflect mortality estimation from tagging studies:

$$N_{rt} = N_{r0}\, e^{-Zt}$$

where N_{rt} is the number of recaptures at time t and N_{r0} is the number of fish initially tagged and released. Since there may be variability in the numbers recaptured, it is best to sample over several dates in order to stabilize the estimate of mortality (see Gulland 1983, p. 110–115 for a description of this approach).

Catch curve analysis

The most frequently used indirect method of mortality estimation is catch curve analysis. Although used mainly for adult fishes, catch curve analysis is also useful during the early life stages (Crecco et al. 1983; Essig and Cole 1986). The estimation of mortality in larval fishes is based, almost exclusively, on catch curve analysis, even though the methods section of papers may not explicitly state this. Krebs (1972) warns that indirect methods are based on the acceptance of certain assumptions and that these assumptions must be valid for these methods to be used correctly. This is especially true in catch curve analysis. Catch-curves plot the frequency of fish grouped by either size or age (Fig. 16). Because size is often a poor indicator of cohort membership (May 1974; Warlen 1981), age estimated from the daily increment ageing technique is preferred. Abundance-at-age usually decreases exponentially, making the slope, Z_t (expressed as a positive number by convention), the time-specific rate of mortality. The value for Z can be estimated with either nonlinear regression of the untransformed data or by converting abundance to log of abundance (Ricker 1975). Conversion to log of abundance will usually result in a more or less straight line with a negative slope which can then be fit through ordinary least squares regression (see Robson and Chapman 1961; Ricker 1975; Draper and Smith 1981). Often the abundance in the youngest age categories (Fig. 16) will be less than the peak abundance due to incomplete capture by the sampling gear, resulting in an ascending left limb. This ascending limb is ignored when fitting the regression; only the data with descending abundances are used in data analysis. The absolute value of the slope of the fitted regression is an estimate of Z, which in the case of unexploited early life stages is equal to natural mortality, M.

There are two types of catch curves: time-specific and cohort-specific. The time-specific catch curve is often used with adults, and involves taking a single sample at only one point in time. This method is not applicable to within-season estimation of cohort mortality of the early life stages. The extremely restrictive underlying assumption for this catch curve is "that the groups from which the data were collected must be in steady state relative to each other" (Vetter 1988); this means that the abundances of each class (i) at the beginning of its life, N_{oi}, must be equal to each other. However, for larvae and juveniles the relative abundance at one point in time is also a function of the time-dependent intensity of spawning. The abundance of eggs (larvae) produced during the spawning season often follows a normal distribution or perhaps even a polymodal distribution, hence violating the assumption of uniform and equal starting abundance for each cohort within the spawning season.

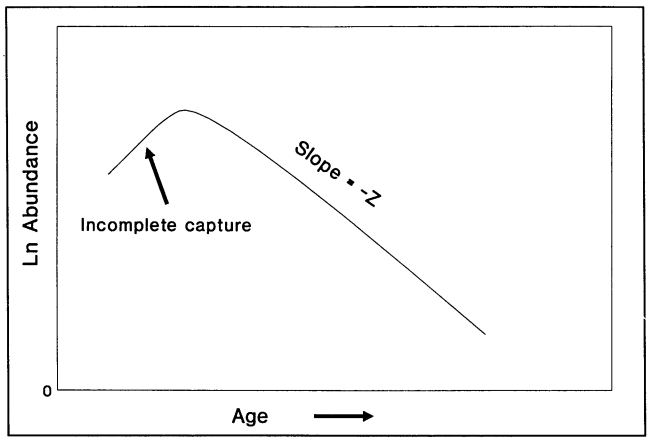

FIG. 16. Simplified representation of a catch curve. Estimated abundance is transformed to a logarithm. Incomplete capture of the youngest fish produces an ascending limb on the left-hand side of the graph. The right-hand side alone is used in mortality estimation.

The cohort-specific catch curve method, which is similar to life-table analysis, is the method of choice for within-season estimation of mortality in the early life stages of fishes. The cohort-specific catch curve is based on sequential sampling of the abundance of a single identifiable group through the life stage of interest (Fig. 16). For larvae this could be a single week's production sampled in subsequent weeks (see Crecco and Savoy 1985), or more simply, a two point estimate of mortality based on the abundance of a discrete cohort at two different ages. If mortality is not constant between sampled ages, a curved catch curve will result. If this happens, mortality can be estimated for portions of the cohort's life, but there will be no single number which describes mortality.

The cohort-specific method is subject to several assumptions which must be met: (1) the samples are representative of the entire population (usually that they are randomly drawn), (2) the population is not subject to migration or differential gear selection with age (constant catchability, q), and (3) cohorts must be identifiable and reliably defined (Vetter 1988). Often, several of these assumptions are violated in early life stages because of changes in behavior, habitat and gear used for capture. For example, larger larvae are more capable of avoiding the net (assumption 2 of constant catchability) and a decline in abundance can be due both to mortality and net avoidance. Net selectivity may also result from changes in diurnal behavior (May 1974), such as the initiation of diel migrations as the larvae mature. This

change in habitat, e.g. planktonic to benthic, can also coincide with the onset of schooling or territoriality, which in turn can alter the density of fish and their catchability. Density of larvae therefore is not directly comparable to density of juveniles. In this case, the survivorship curves must be segmented by age/size stanzas, and mortality obtained separately for each stanza. The slope of the catch curves, Z, can then be compared among stanzas.

In normal practice, the actual abundance, N_t, is not incorporated into catch curves, but rather the surrogate measurement of catch-per-unit effort, C_t/f, is estimated. The equation for catch is

$$C_t = qfN_t$$

where f is sampling effort, and q is the catchability coefficient. It can be rewritten to give catch-per-unit effort:

$$C_t/f = qN_t.$$

In other words, catch per unit effort is assumed proportional to abundance. The equation for mortality can be rewritten, $\ln\{N_t/N_0\} = -Z_t$, in catch-per-unit effort terms:

$$\frac{\frac{C_t}{f_t}}{\frac{C_0}{f_0}} = \frac{q_t N_t}{q_0 N_0} = \frac{q_t f_t}{q_0 f_0} e^{-Zt} = \frac{q_t}{q_0} e^{-Zt}$$

It is relatively easy to standardize sampling effort and thus set f_0 and f_t equal to each other. It is often assumed that q_0 and q_t are also equal, or that any differences in q are attributed to net avoidance and extrusion. Correction factors are added in an attempt to equalize abundance frequencies. If the assumption of equal catchability is not met, mortality estimates derived from catch curves will not reflect true rates.

Because of size-related changes in catchability, determination of absolute mortality rates can be difficult for early life history stages, even with the advantage of daily ageing. Accordingly, Hoenig et al. (1990) introduced a technique to compare the relative survival of two cohorts of fish spawned in the same season (for example early versus late spawned). This method can be used when catchability, q, is not equal between the two groups. It can also be used when patchiness is great and when the estimated ratio of absolute abundances, N_t/N_0, is nonsensical (>1). Hoenig et al. (1990) used the ratio of catches of early to late spawned larvae which are sampled at a point in time, t. The equation for the ratio is, $R_t = \frac{C_{Lt}}{C_{Et}}$, where C_{Lt} and C_{Et} are the catches of late and early spawned cohorts, respectively. Fish are sampled for two, or preferably more, times and the log of R_t is plotted on t. The slope of this straight line is an estimate of the difference in instantaneous mortality rates between the two groups of fish. This ratio estimator rests on the assumptions that (1) there is no immigration or emigration from the study area, and (2) the ratio of catchability coefficients, $\frac{q_t}{q_0}$, of the groups does not change over the time period studied. This method can be used to compare the relative survival of groups such as early and late spawned larvae even though the numbers spawned in each group, N_{0i}, are unequal. This method may be useful when assumptions of equal catchability can't be proven. However, it should be noted that the second assumption, that of a constant ratio of catchability coefficients, is difficult to test.

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CHAPTER 6
Validation of Otolith Increment Deposition Rate

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Introduction

Wilson et al. (1983) defined validation as "the confirmation of the temporal meaning of an increment. It is used to determine the accuracy of an age determination. This term is frequently confused with verification, i.e., the repeatability of a numerical interpretation that may be independent of age. For example, if two readers agree on the number of zones present in a hardpart, or if two different age determination structures are interpreted as having the same number of zones, verification has been accomplished." Thus, validation of daily ring deposition may be accomplished with or without the verification of the increment counts. Conversely, counts may be verified without validating their rate of formation. Precision in otolith studies refers to the measure of repeatability when verifying counts and accuracy is defined in this context as the closeness of the age estimate to true age (see Campana and Jones, this volume).

Four separate topics in otolith growth are addressed in this chapter, each of which has appropriate validation techniques and requirements:

1. Deposition rate, i.e., how ring structure can be used as a chronological record at the daily and annual scale and how well the information applies at the individual as well as at the population level;
2. Time of initial increment formation, i.e., the age of larvae when regular ring deposition begins and how well ring structure relates to spawning, incubation, hatching, endogenous and exogenous feeding;
3. Relationship of somatic to otolith growth for back-calculation (this topic is discussed in more detail in Neilson and in Campana and Jones in this volume, but as evidence accumulates about the uncoupling of otolith and somatic growth in some situations, it is clear that validation is necessary before back-calculating length or growth rate at earlier ages); and
4. Physiological mechanisms of deposition, i.e., the physiological and biochemical processes of increment formation and the effects of environmental changes on those processes.

This chapter describes different validation methods which apply to each of the preceding topics. Whenever possible, examples from the literature have been tabulated for each topic. These tables do not represent complete reference lists for each method. Rather, the entries were chosen to represent different species, approaches and levels of success. Likewise, references within the text are representative and were selected to identify a range of authors and species.

Some species produce readily validated daily increments, often from hatching or first feeding (e.g., Miller and Storck 1982; Barkman and Bengtson 1987; Parsons and Peters 1989; Karakiri and Westernhagen 1989). We may consider these species to be easily entrained. In other species, the rate of increment deposition has not yet been validated conclusively. Collins et al. (1989) examined the otoliths of fast growing king mackerel larvae, but did not detect a diurnal periodicity of marginal increment formation. Failures to validate daily ring deposition are often attributable to poor sample preparation and the reliance on light microscopy (Campana and Neilson 1985; Karakiri et al. 1991). However, in some species the entrained diurnal rhythms which result in regular increment formation may not develop until later in larval life and may be preceded by a period of free-running increment deposition, regulated by metabolic rate (Geffen 1982; Ré et al. 1985; Mosegaard et al. 1988; Maillet and Checkley 1990). Therefore, until the expression of the physiological process for each species is known, it is necessary to validate the increment formation rate in order, for example, to use increment number to estimate larval age.

What are the requirements of a good validation procedure? Ideally, validation should entail the monitoring of a known-age population of tagged and marked individuals from hatching through metamorphosis (or whatever endpoint will be studied in the field). Thus, information about both the population and the individual would be obtained simultaneously. The fish should be reared under conditions which contribute to good growth, feeding activity, and swimming behavior (including vertical migrations).

In general, methods which release fish to the wild or where fish are maintained under laboratory condi-

tions which provide for good growth and natural behavior (i.e., mesocosms) are preferable for validation studies. This is because validation experiments should be designed to avoid the effects of captivity. Laboratory conditions often affect the growth of larval, juvenile and adult fish, and differences have been observed in the optical nature of otolith increments produced by larvae reared in the laboratory from those captured in the wild (Campana and Neilson 1985; Rice et al. 1985; Hovenkamp 1990). For these reasons, deviations from daily rates of ring deposition are usually attributable to laboratory artifacts, even when not produced in a laboratory setting (Collins et al. 1989). The quality of laboratory conditions is not usually questioned if evidence of daily increment deposition can be produced (Uchiyama et al. 1986).

Probably the optimum environment for validation studies is in large outdoor enclosures where photoperiod and temperature cycles reflect natural conditions. On the other hand, ring deposition cycles observed under manipulated conditions of light, temperature, feeding, etc. reveal a great deal about the sensitivity of otolith growth to environmental change and also about how likely the ring deposition rate is to be modified in the wild. It is also useful to look at the effects of various stresses on increment formation patterns. Will periods of starvation or lowered temperature alter the relation between ring number and age? Will identifying marks be left on the otolith that can be used to identify individuals which have undergone stress?

There are several publications which address the problems of validation. Beamish and McFarlane (1983) called for more rigorous attention to validation of assumed annual deposition patterns in calcareous structures used to age adult fish. Both Jones (1986) and Geffen (1987) reviewed and discussed validation methods and evaluated the literature in relation to the requirements of validation. In the time since these publications appeared there has been a greater awareness of the importance of validation. The importance of checking light microscope counts and increment width measurements using scanning electron microscopy (SEM) has been stressed (Campana and Neilson 1985; Jones and Brothers 1987; Karakiri et al. 1991). Although most investigators now include some sort of validation, SEM examination of a representative proportion of samples is still not widespread.

Recent publications have addressed the possibility of identifying periods of larval stress by the patterns of increment deposition (Campana 1983a; Eckmann and Rey 1987; Berghahn and Karakiri 1990). Thin, often poorly-defined, increments can be related to episodes of starvation (Rice et al. 1987; Maillet and Checkley 1990), temperature or salinity stress (Karakiri and Westernhagen 1989) and handling (Volk et al. 1984). Such markers can be used to separate stressed from non-stressed individuals in the field (Rice et al. 1987), thus providing a basis for excluding individuals with abnormal or unknown ring deposition rates from ageing analysis. While these markers do not in themselves validate increment deposition rates, their presence and identification may increase the applicability of the technique in field studies of larval ecology.

It is of critical importance to validate the rate of increment formation in order to age fish larvae. This is especially true since we have only a rudimentary understanding of the mechanisms which give rise to ring structure. Mugiya (1987) proposed a model for calcium-calmodulin interaction to explain how the differences between the incremental and discontinuous zones are formed. The biochemical changes within the otolithic membrane, and their relationship to environmental rhythms have been studied (Mugiya and Oka 1991). The mechanisms by which diurnal environmental factors might trigger these changes and produce particular increment growth patterns are still not clear. Until a general model is developed which successfully predicts which increment formation patterns are produced from given environmental stimuli, rigorous validation will remain as the only means for applying otolith increment counts to field studies. Even after such a model is developed, however, the need for validation may remain; given the difficulty of differentiating daily increments from other structures in some species, validation provides a useful check of the interpretive skill of the otolith reader.

Methods

Validation methods can be organized according to the type of otolith study which is implemented.

Deposition Rate

Several techniques can be used to validate ring deposition rate. Validation in these studies is taken to mean establishing the temporal relationship between ring number and age. The most common hypothesis is that there is a 1:1 relationship between primary increment number and age, or between annular otolith structure and age. Validation of ring deposition rate is a prerequisite for otolith studies for any species and it is sometimes necessary for different spawning stocks or geographic locations within the range of an individual species.

Validation techniques include the monitoring of known-age larvae, marking otoliths, statistical inferences (back-calculating to events, analysis of incre-

ment widths, calculating hatch dates, etc.), inference from older fish, and marginal increment analysis. Examinations of otoliths from known-age or from marked individuals are probably the most rigorous and reliable methods for validating daily ring deposition. Statistical inferences suffer from many shortcomings, only one of which is the problem of generalizing from populations to individuals. Marginal analysis is used more often to validate annual rings in adults, but is increasingly used for field captured larvae of certain fast-growing species.

Marking Otoliths

The only direct method available for validating the deposition rate of an individual is by counting the rings laid down by individuals whose otoliths have been marked at the beginning of an experimental period, and perhaps again at intervals during the time period. The data generated by this method include the number of rings deposited between known dates for each individual.

Marking otoliths is probably the best method for validation in species which cannot be reared in the laboratory. However, the effort required to capture, mark and recapture a statistically reliable number of individuals may be very high. Marking is also the best validation method for the purposes of back-calculation (see discussion re. the relationship of otolith to somatic growth). If the fish are measured before marking and can be identified individually when sacrificed, as in Alhossaini and Pitcher (1988) and Wright et al. (1990), it is possible to determine exactly the relationship between otolith growth and fish growth on an individual basis. Volk et al. (1984) used patterns induced by handling and transfer stress as temporal markers in laboratory experiments, and others report natural markers which are linked with physiological or developmental events (Marshall and Parker 1982, Geffen 1983, Fowler 1989).

The requirements to be met for marking include:

1. Testing of mark incorporation and retention;
2. Holding conditions which provide for optimal growth, and reflect natural conditions (unless testing for the specific effects of environmental manipulations);
3. Monitoring the effects of the marking treatment on feeding, growth and survival using a control group of fish; and
4. Storing fluorescent-marked material in the dark to preserve the visible mark.

1) *Marking with chemical compounds (Table 1)* — Within the last decade, and especially within the last five years, techniques for marking otoliths have become very sophisticated. Large numbers of embryos, larvae, juveniles or adults can be marked with confidence that survival, mark incorporation and retention will be high. Major restocking programs can routinely include the marking of hatchery produced larvae or juveniles at the time of release to evaluate long-term hatchery success (Tsukamoto et al. 1989, Secor et al. 1991).

Marking otoliths with tetracycline or other fluorescent compounds is the best method for validating increment deposition rate in larvae or juveniles of unknown age. These individuals may be captured from the wild, marked, and retained for the duration of the experiment (Simoneaux and Warlen 1987, Alhossaini and Pitcher 1988, Parsons and Peters 1989). Siegfried and Weinstein (1989) successfully captured and marked 16mm larval spot (*Leiostomus xanthurus*) from the wild. Those retained in field conditions grew better than those retained in the laboratory. Marked individuals may be released for recapture, depending on the behavior of the species or life stage (Fowler 1989; Tsukamoto et al. 1989; Fowler 1990). Validation of annual increments in adults which have been marked and released is a well established technique (Fargo and Chilton 1987; Bumguardner 1991). Mark and recapture is probably preferable to mark and retention because the conditions experienced during the course of the experiment are by definition those experienced in the wild. Otolith marking may also be used for larvae where it is not possible to work with groups hatched within one day, for instance where the supply of larvae is limited and it is necessary to mix batches.

Small larvae are best marked by immersion as described by Hettler (1984). Improvements to the basic technique have been tested for marking large numbers of individuals by Secor et al. (1991), Tsukamoto et al. (1989), and Dabrowski and Tsukamoto (1986). Juveniles may be immersed, injected, or presented with food containing tetracycline (often available as commercially produced medicated food). After marking, the fish should be sampled periodically to determine the number of increments deposited since the marking date. Methods of marking vary and the mark is not always confined to a single increment. It is therefore desirable to sample daily from the time of marking and examine the otolith for the fluorescing band, until it can be determined how many increments are included in the mark.

Three compounds are recommended for marking otoliths, either for validating ring deposition rates, for studying the relationship between otolith growth and fish growth, or for marking the individuals produced from certain hatcheries or in certain years. These

TABLE 1. Examples of primary increment studies which utilize marking with chemical compounds.

Species (age)	Conditions	Method	Dose	Reference
Alosa sapidissima, American shad (larvae)*	laboratory	OTC imm	50mg/L 12 h/d for 4 d	Lorson and Mudrak 1987
Ambassis vachelli, glass fish*	laboratory	OTC imm	250mg/L 40 h	Molony and Choat 1990
Anguilla japonica, Japanese eel (elvers)	laboratory	TC imm	300mg/L 24 h	Umezawa and Tsukamoto 1991
Archosargus probatocephalus, sheepshead (larvae 7–10mm)*	laboratory	OTC imm	10–15mg/L 7 h	Parsons and Peters 1989
Carrasius auratus, goldfish	laboratory	ATZ		Mugiya and Muramatsu 1982
Chaetodontidae, butterflyfish (juveniles)*	field	OTC imm or inj	50mg/kg	Fowler 1989, 1990
Chanos chanos, milkfish (larvae 11–14mm)*	enclosures	OTC imm	400–500mg/L 24 h	Tzeng and Yu 1989
Coregonus peled, peled (embryos, larvae)	laboratory	TC imm	600mg/L 3–12 h	Dabrowski and Tsukamoto 1986
(juveniles)	laboratory	TC imm	300mg/L 35 h	
Cynoscion nebulosus, spotted seatrout (larvae 7–10mm)	laboratory	TC imm	10–15mg/L 6 h	McMichael and Peters 1989
(juveniles 50–156mm)	laboratory	TC inj	0.1mg/g	
Leiostomus xanthurus, spot (juveniles 71–114mm)	enclosures	calcein imm	125mg/L 2 h	Wilson et al. 1987
Leiostomus xanthurus, spot (larvae 16mm)*	enclosures/lab	TC imm	400mg/L 24 h	Siegfried and Weinstein 1989
Leiostomus xanthurus, spot (larvae)	laboratory	TC imm	100–500mg/L 0.5–1.5 h	Hettler 1984
Micrometrus minimus (embryos)*	laboratory	OTC inj ovarian	0.2–0.4mL	Schulz 1990
Micropogonias undulatus, croaker (juveniles 62–85mm)	enclosures	calcein imm	125mg/L 2 h	Wilson et al. 1987
Morone saxatilis, striped bass (larvae, juveniles)*	enclosures	OTC imm	250–350mg/L 2–3 h	Secor et al. 1991
*Nototheniops nudifrons**	laboratory	OTC inj ATZ inj	0.025mg/g	Radtke and Hourigan 1990
Pagrus major, red sea bream (juveniles)*	field	ALC imm	50–200mg/L 24 h	Tsukamoto et al. 1989
Parika scaber (juveniles)*	laboratory	tetralysal (TC) imm	300mg/L 12 h	Kingsford and Milicich 1987
Petromyzon marinus, lampreys*	enclosures	OTC inj	35mg/kg	Beamish and Medland 1988
Platichthys stellatus, starry flounder	laboratory	OTC inj	100 mg/kg	Campana and Neilson 1982
Pleuronectes platessa, plaice (juveniles)*	laboratory	OTC inj	100mg/kg (0.025mL/g)	Alhossaini and Pitcher 1988
Pseudopleuronectes americanus, winter flounder (juveniles)*	enclosures	OTC imm	500mg/L 24 h	Sogard 1991
Sciaenops occelatus, red drum (juveniles 61–80mm)	enclosures	calcein imm	125mg/L 2 h	Wilson et al. 1987
Sebastes melanops, black rockfish (juveniles)*	laboratory	OTC inj		Yoklavich and Boehlert 1987
Thunnus alalunga, Albacore tuna (adults)*	field	OTC inj	1.5ml of 100mg/L	Laurs et al. 1985

*Indicates validation studies.
OTC = oxytetracycline, TC = tetracycline, ATZ = acetazolimide, ALC = alizarin complexone, imm = immersion, inj = injection

compounds are the tetracycline antibiotics (including tetracycline (TC) and oxytetracycline (OTC)), calcein fluorescent green (calcein fluorexon: 2,4-bis-[N,N'-di(carbomethyl)-aminomethyl], and alizarin complexone (alizarin fluorine blue: alizarin-3-methylamine-N,N-diacetic acid). All are fluorescent compounds which are incorporated into the structure of the otolith, usually within one day of contact. The marks on the otolith are visible using ultraviolet (UV) light. The easiest arrangement is to use a microscope with epifluorescent attachments (mercury vapor lamp, filters, etc). The wave length necessary to produce fluorescence differs for each compound. Tetracycline fluoresces at 400 nm, calcein at 499 nm, and alizarin at 427 nm. Beamish and Medland (1988) used a portable UV source giving a 253.7–375 nm excitation range to allow screening and detection of marks with a dissecting microscope. Other authors use UV sources at 355–425 or 400–425 nm (Kalish 1989; Tzeng and Yu 1989). Some authors advise that marked material should be stored in the dark since UV florescent compounds are often light-sensitive and will degrade with time if exposed to light (Fargo and Chilton 1987).

Some studies have used multiple exposure treatments to produce codes on the otolith (Dabrowski and Tsukamoto 1986; Tsukamoto et al. 1989). Tetracycline is the most common, but also the most problematic treatment. Mortality rates are sometimes higher and the degree of incorporation lower than for calcein or alizarin. OTC marks have been retained and identified on otoliths at least three years (Fargo and Chilton 1987) or four years (Leaman and Nagtegaal 1987) after marking. Alizarin retention in the otolith lasts at least two years (Tsukamoto et al. 1989). Calcein has been tested against tetracycline and resulted in lower mortalities, and required smaller doses (Wilson et al. 1987). Tetracycline has been faulted for toxic effects in several studies (Lorson and

TABLE 2. Examples of primary increment studies which utilize marking with environmental manipulation, stress, or natural tags.

Species (age)	Conditions	Method	Resulting Mark	Reference
Anoplopoma fimbria, sablefish (juveniles)*	laboratory	capture stress	check	Boehlert and Yoklavich 1985
Halichoeres bivittatus, wrasse*	field	isolation/dark or supplemental feeding	large incr. zone wide increments	Victor 1982
Oncorhynchus kisutch, coho salmon	laboratory	handling stress	check	Campana 1983a
Oncorhynchus nerka, sockeye salmon (juveniles)	laboratory	developmental	changing widths	Marshall and Parker 1982
Oncorhynchus tshawytscha, chinook salmon (juveniles)*	laboratory			Neilson and Geen 1985
Salmo salar, Atlantic salmon (parr)*	laboratory	developmental temp fluctuations	first feeding ring narrow, indistinct rings	Wright et al. 1991
Salvelinus alpinus, Arctic char (juveniles)	laboratory	temp fluctuations	check	Mosegaard et al. 1988
Salvelinus namaycush, lake trout (embryos, fry)	laboratory	temp fluctuations	large discont. zone	Brothers 1985
Solea solea, Dover sole*	laboratory	developmental	large discont. zone	Lagardère 1989
Thalassoma bifasciatum, wrasse	field	isolation/dark or supplemental feeding	large incr. zone wide increments	Victor 1982
Theragra chalcogramma, walleye pollack (juveniles)*	laboratory	temp fluctuations	large disc. zone	Nishimura and Yamada 1984

* Indicates validation studies.
temp fluctuations = controlled changes in temperature

Mudrak 1987). Secor et al. (1991) found no significant increase in mortality associated with mass OTC marking of striped bass (*Morone saxatilis*) larvae.

a) *Embryos* — The otoliths of embryos can be marked with tetracycline (in the form of oxytetracycline, OTC) either by immersion of the eggs (Dabrowski and Tsukamoto 1986) or in the special case of viviparous fish, by injection into the cloaca of the gestating females (Schultz 1990). The information obtained from these studies includes the number of increments formed before hatching, the length of embryonic life, embryonic growth of the otolith, etc.

b) *Larvae* — Larvae (3–30mm) are most easily marked by immersion in a solution containing a fluorescent compound. Oxytetracycline hydrochloride (OTC HCl) is usually dissolved in saline 16–20 ppt water. The doses and immersion times range from 25–400 mg/L OTC HCl for 2–40 h. Lorson and Mudrak (1987) used 50 mg/L OTC buffered with KPO_4 (32 mg/L) and $NaPO_4$ (67 mg/L). Tzeng and Yu (1989) recommend using sodium chloride solution rather than sea water because the tetracycline combines with the calcium and magnesium salts in the seawater before getting to the otolith. Other tetracycline compounds have delivered lower incorporation and higher mortality rates. Tsukamoto et al. (1989) described alizarin immersion methods and Wilson et al. (1987) evaluated the application of calcein.

c) *Juveniles* — Juveniles can be marked either by immersion or injection. Incorporation rates for OTC marking by immersion are lower for juveniles than for larvae (Secor et al. 1991). Immersion times must be increased (up to 40 h), and usually require special tanks with adequate aeration and insulation such as transportation tanks. The advantage of immersion, although requiring more chemicals, is that handling of the fish is reduced. Because the treatment time is increased, however, it is debatable whether or not the total stress experienced by the fish is less for immersion or injection. Concentrations for injection range from 5 to 40 mg/kg fish for OTC HCl dissolved in a saline solution of about 16 ppt. Alhossaini and Pitcher (1988) administered intraperitoneal (ip) injections at 100 mg/kg, corresponding to 0.025 mL/g fish weight. Some species, notably coral reef fish, have been injected in situ, the dose calculated based on a previously developed weight-length key (Fowler 1990). Yoklavich and Boehlert (1987) marked juvenile black rockfish otoliths with OTC injected intramuscularly (im). They also tried labelling otoliths with $^{45}CaCl_2$ and were able to detect the mark by autoradiography, although this is a more involved and less accurate technique.

Rapid injection guns which are used widely for mass inoculation programs are not reported to be in use for fish marking. Similar methods, using microwire injecting guns, should be tried to improve the speed and safety of injection procedures for juvenile fish.

d) *Adults* — Adult fish are marked almost always via injection, using concentrations of 25–100 mg/kg. Both OTC and calcein have been recommended (Wilson et al. 1987). It is possible to introduce marking compounds into food, but this entails the capture and holding of adult fish in conditions conducive to the establishment of feeding. Such conditions are not available to every project. It may be difficult to gauge the correct dosage to be administered orally. One way to do this is to consult the fish health literature and to compare the recommended oral vs injected dosage for antibiotics. Fortunately OTC is frequently used for antibiotic treatments and the dosage comparisons are readily available and may be used as a guide for other compounds. Fluorescent marks corresponding to a

medicated food treatment were visible in recaptured red drum (*Sciaenops ocellatus*) nearly two years after treatment (Bumguardner 1991).

2) *Marking with stress (Table 2)* — In addition to the introduction of marking compounds, otolith growth can be manipulated by external factors to produce permanent identifiable marks (Boehlert and Yoklavich 1985, Volk et al. 1984).

a) *Larvae* — The otoliths of larvae and embryos of some species have been marked successfully by manipulating environmental conditions. Frequent temperature fluctuations were successful for marking lake trout (Brothers 1985). Wright (1991) used short photoperiod cycles, 2–4 days of 6L6D, to mark salmon and stickleback otoliths.

b) *Juveniles* — Marking the otoliths of juveniles by manipulating conditions is an attractive option. It is usually possible to handle large numbers of individuals and it avoids the use of chemicals. Temperature fluctuations and photoperiod changes must be done with fish in controlled laboratory tanks. Other methods may be more adaptable to marking recently captured fish in the field. Distinctive checks have been produced on otoliths by exposing fish to short temperature increases (Mosegaard et al. 1988), increases in UV radiation (Berghahn and Karakiri 1990) and anaerobic stress (Mugiya and Uchimura 1989). The stress of capture and transport can often induce an otolith check which can then be used as a temporal marker (Boehlert and Yoklavich 1985), although this approach has not been tested systematically.

Monitoring Known Age Larvae (Table 3)

The following requirements should be met.

1. Larvae should come from a single batch of eggs, hatched within one 24 h period.
2. Embryonic otoliths should be checked for rings.
3. Larvae should be reared under conditions which resemble natural conditions of photoperiod and temperature. It is also important to provide conditions which assure the development of normal behavior, especially diurnal behavior such as vertical migration.
4. Samples should be taken daily after hatching to establish the timing of first ring deposition.

TABLE 3. Examples of primary increment studies which utilize known-age larvae.

Species (age)	Conditions	Duration	Validation Criteria	Reference
Anchoa mitchilli, bay anchovy (larvae)*	laboratory		incr count vs age regression slope = 1	Fives et al. 1986
	laboratory	0–18 days	incr count vs age regression slope = 1	Leak and Houde 1987
Brevoortia patronus, Gulf menhaden (larvae)	laboratory	0–32 days	incr count vs age regression slope = 1	Warlen 1988
Clupea harengus, Atlantic herring (larvae)	enclosures/lab	0–120 days	incr count vs age regression slope = 1	Geffen 1982
(larvae)	laboratory	0–80 days	incr count vs age regression slope = 1 and resolution model	Campana et al. 1987
Clupea pallasi, Pacific herring (larvae)*	enclosures	to metamorphosis	good fit for incr count vs age regression	Moksness and Wespestad 1989
(larvae)	laboratory		incr count vs age regression slope = 1	McGurk 1984
Coregonus hoyi, bloater (larvae)*	laboratory	to metamorphosis	incr count vs age regression slope = 1	Rice et al. 1985
Coryphaena hippurus, dolphin (larvae)*	laboratory	0–121 days	good fit for incr count vs age regression	Uchiyama et al. 1986
Fundulus heteroclitus, mummichog	laboratory	prehatch-juveniles		Radtke and Dean 1982
Gadus morhua, cod (larvae)	enclosures	0–140 days	incr counts = age (t-test)	Bergstad 1984
(juveniles)			incr count vs age regression slope = 1	
Lepomis spp., (larvae, juveniles)*	laboratory	>125 days	incr count = age	Taubert and Coble 1977
Menidia menidia, Atlantic silverside (larvae)*	laboratory		good fit for incr count vs age regression	Barkman and Bengtson 1987
Micropterus salmoides, largemouth bass	laboratory			Miller and Storck 1982
Morone saxatilis, striped bass (larvae)*	laboratory	0–97 days	incr count vs age regression slope = 1	Jones and Brothers 1987
Parika scaber, (larvae)*	laboratory	0–8 days	incr count = age	Kingsford and Milicich 1987
Platichthys stellatus, starry flounder (larvae)	laboratory	0–54 days	incr count vs age regression slope = 1	Campana 1984
Pleuronectes platessa, plaice (larvae)	laboratory		incr counts = age	Karakiri and Westernhagen 1989
Sciaenops ocellatus, red drum (larvae)	laboratory		incr count vs age regression slope = 1	Peters and McMichael 1987
Solea solea, Dover sole (larvae)	laboratory			Lagardere 1989
Theragra chalcogramma, walleye pollack (larvae)*	laboratory	0–38 days	incr count vs age regression slope = 1	Bailey and Stehr 1988
Tilapia mariae (juveniles)	laboratory	0–33 days	incr counts = time elapsed	Rosa and Ré 1985

*Indicates authors' validation criteria met.

5. Subsequent samples should be taken at frequent intervals (3–5 days), through metamorphosis or beyond if the intention is to age wild juveniles.
6. Sample size should be determined on the basis of 95% confidence limits around estimated individual age values (Rice et al. 1985). If it is intended to use increment counts as a population statistic, then sample sizes could be based on 95% confidence limits around length-at-increment-number data.

A description of larval rearing techniques is beyond the scope of this manual, but it is important to remember that the results of validation are only as good as the rearing conditions used. Large enclosures seem to give the best larval growth and development for pelagic larvae, especially outdoor enclosures with natural plankton as the food base. This approach is not always practical, however, but one should be wary of using results when rearing conditions do not produce good survival or growth. It may be worthwhile noting the common aquaculture practice of improving growth and survival by increasing the day length, or even working with 24 h light. Although the larvae produced are in very good condition, the results should be viewed critically for otolith validation studies.

In a laboratory environment it is often convenient to measure larvae immediately after sampling. Again, based on the intended use, it may be more advisable to preserve the larvae in the same manner that wild larvae would be preserved, so that length comparisons could be made directly. In any case, it is important to keep in mind the differences that occur with death and preservation, which may make laboratory to field extensions less accurate (see Butler, this volume).

One final point worth considering is the evaluation of how accurate laboratory results will be for field-caught larvae. So far, very few researchers have provided any information on the accuracy of their field age determinations. Work by Barkman and Bengtson (1987) and Rice et al. (1987) are good examples of a rigorous approach to actually applying laboratory validation to field use.

Statistical Inference

Daily increment formation may be difficult to validate in some species by the usual, more rigorous methods. The requirements for validating daily ring deposition indirectly by statistical inference include:

1. Large numbers of larvae should be examined;
2. Larvae should come from discrete populations or spawning sites and should all be subject to the same environmental fluctuations or events, and there must be no age or size-dependent migration into or out of the study area during the period in question;
3. Extrapolation from the results in a single year or for a single population should be avoided; and
4. Extrapolation from population statistics to individuals should be avoided.

Into this category of validation methods should be placed several ways of analyzing increment count data. The underlying assumptions and calculation techniques are described in more detail elsewhere in this volume (Campana and Jones). Readers are strongly advised to consider the limitations and appropriate applications given in that chapter.

1) *Regressions of length-at-capture on increment counts* — The relationship between length and ring number is sometimes used directly as a population growth curve. Although extremely limited as a validation technique, this method is often used to infer daily increment deposition if the growth rate produced "looks right" (Cowan 1988, Ntiba and Jaccarini 1988, De Vries et al. 1990). Much of the justification for this approach depends on the assumption that larvae which are not growing fast enough to produce clearly defined daily increments are not likely to survive in the population (Graham and Townsend 1985, Jones and Brothers 1987). Rice et al. (1985) present a method of checking for minimal growth rate in field samples.

2) *Counting back to specific events* — Prominent marks on the otolith are assumed to be related to specific developmental events or environmental phenomena. The number of increments between the time of capture and the mark, or between the core region and a mark identified with an event at a known date are used to infer the age of the larvae. For many flatfish, the association between accessory primordia and metamorphosis or settling is used as a natural marker (Alhossaini et al. 1989; Karakiri et al. 1991; Sogard 1991). However, the date of hatching or settlement should be known with some accuracy. Counting back can provide field validation in certain circumstances to support validation experiments. The distribution of hatching dates estimated from ring counts may be compared to known hatching dates (Brothers et al. 1983; Rice et al. 1987; De Vries et al. 1990). A disparity between increment counts and known hatching dates would be a valuable indication that rigorous laboratory validation is required. This method is best confined to populations with restricted hatching periods and known hatching dates. Even then, various studies have documented differential survival of early versus late-hatched larvae (e.g., Methot 1983), suggesting that known hatch dates, by themselves, do not make this a rigorous validation technique.

3) *Analysis of increment widths* — Ralston and Miyamoto (1983) used the average increment width to

calculate the number of increments in unreadable parts of dolphin otoliths, (see Campana and Jones this volume). Campana et al. (1987) presented a model for identifying cases where increment counts would misrepresent larval age.

4) *Ring number equals time elapsed* — Some studies validate ring deposition rates by testing the relationship between the change in mean increment count and the time elapsed between successive sample dates (Essig and Cole 1986). Testing that the slope of the regression of increment count for pooled samples vs calendar day is not significantly different from one has also been employed (Taubert and Tranquilli 1982; Post and Prankevicius 1987). Post and Prankevicius (1987) used the relationship between ring number for all samples taken throughout the growth season and calendar (julian) date. They considered the daily increment hypothesis to be validated since the slope did not differ significantly from one. Taubert and Tranquilli (1982) compared the number of primary increments with the number of potential growing days based on temperature in order to validate annular bands in largemouth bass otoliths.

Inference from Older Fish

It is possible to exploit improved techniques for otolith preparation to examine the core area of fish caught as adults (see Secor et al., this volume). Counts of increments from SEM micrographs can be used to show that increment numbers between annuli equal or approach the number of days in a year (Taubert and Tranquilli 1982; Wenner et al. 1986; Hill and Radtke 1988; Wilson 1988). Radtke and Targett (1984) made use of this method for polar fish species. Counts of primary increments between annuli should be checked by scanning electron microscopy (Morales-Nin 1988). The primary increments in older fish are often very thin and light microscope examination can lead to erroneous counts, and therefore poor validation. The problem with this technique is that it takes a good deal of time to prepare and examine larger otoliths, so that sample sizes are often small. The technique also assumes that increment formation continues uninterrupted through the adult stage, an assumption which may or may not be warranted (Ralston and Williams 1989). It should also be kept in mind that the fish examined are those that have survived the larval and juvenile stages, and thus it may be difficult to generalize from these fish to the larval population as a whole. In general, this technique is not a substitute for direct validation on young fish, especially where investigators are interested in ageing larvae to study recruitment processes. The application of this technique is best limited to new species whose larvae cannot be obtained. It could be useful in examining within year growth processes.

Marginal Increment Analysis

Marginal increment analysis is a widespread technique used to determine the timing of annual increment formation in adult fish (Wenner et al. 1986; Maceina et al. 1987), though it is not always successful (Withell and Wankowski 1988). If it is impossible to do anything else with a group of larvae, it may be useful to examine the state of completion of the last (marginal) increment in samples taken over several 24 hr periods (Tanaka et al. 1981; Ré et al. 1985; Geffen 1987). If the width of the marginal increment increases throughout the day and is completed by the next day, daily increment formation may be inferred. Ré (1984) describes marginal increment analysis as a way of corroborating daily increment formation from field-caught larvae. Jenkins and Davis (1990) followed the state of completion of the final (marginal) increment in day and night samples taken over six consecutive days to infer daily ring formation in southern bluefin tuna (*Thunnus maccoyii*) larvae which could not otherwise be validated in a laboratory setting. However, marginal increment analysis is not always conclusive even when otoliths are examined using SEM (Collins et al. 1989). Given the difficulties in discerning increments near the otolith margin, this technique should not be contemplated unless otolith growth is rapid and increment widths are broad. Furthermore, marginal increments should be examined only under the most stringent and reproducible microscopic conditions.

Detailed physiological studies of the rhythmic nature of increment deposition also provide support in validating daily increment formation in some species (Mugiya 1987). Mugiya and Oka (1991) looked at the diurnal rhythm of otolith growth by measuring ^{45}Ca uptake in incubated intact sacculi. Although these studies have demonstrated the diel physiological process, they do not guarantee that increments counts will always be a reliable indicator of larval age, nor do they indicate that the increments can be unambiguously interpreted.

Timing of Initial Increment Formation

Validation techniques are used to determine when daily ring deposition begins, and the relationship of the first otolith increments to early life history events such as hatching or first feeding. There are differences between species as to when rings are first deposited. Several methods can be used to validate the timing of

initial ring deposition. This validation is critical to determining the relationship between ring number and true larval age.

Presence of Embryonic Rings

Rings formed before hatching or before exogenous feeding may be different in character to those normally referred to as primary increments. In some species embryonic rings are absent or occluded at hatching, in others they are clear and distinct, and it is important to be able to identify them as pre-hatch rings (Geffen 1983; Dabrowski and Tsukamoto 1986; Bailey and Stehr 1988; Karakiri et al. 1991).

The number of embryonic rings is best determined by examining otoliths from individuals before and at hatching. The different pairs of otoliths develop at different stages and it is important to determine the age at otolith formation for each pair when more than one type of otolith is used for ageing.

Initial Ring Formation

By sampling daily between the beginning of hatching and the establishment of exogenous feeding, it is possible to construct a histogram or cumulative frequency graph which will describe the number of rings at hatching or at first feeding. An intensive sampling schedule like this will also help to identify whether or not a distinct band is deposited in association with hatching, first feeding, or any developmental event in early larval life. If possible, maintaining a group of larvae without food past the time of first feeding, but not past the time of irreversible starvation, will validate the coincidence of any distinct band with exogenous feeding or with development (e.g., yolk sac absorption) (Bailey and Stehr 1988; Maillet and Checkley 1990). Validation with known age larvae can help to resolve any uncertainty regarding whether the innermost increments coincide with yolk sac absorption (a developmental event) or first feeding (a behavioral response).

Histograms showing the percentage of larvae that form their first increment as a function of time since hatching should be constructed and the mode used to determine the timing of first ring deposition (Fives et al. 1986). An alternative method is to use the y-intercept from the regression of otolith ring number on fish age. The latter method suffers from the disadvantage of giving a population statistic only. If the regression is based primarily on older larvae, the regression should not be used to predict beyond the data points. Also, data derived from older larvae will only reflect the increment number versus age of survivors, and may not truly represent the distribution of increment numbers in very young larvae which may be present in the population soon after hatching or at first feeding. The objectives of each study must be considered when evaluating the importance of precision in estimating the timing of initial ring formation. In some cases, where field studies of juveniles are contemplated, the variation around the mean in the timing of first ring deposition is not important. In studies of newly hatched larvae, however, it is very important.

The Relationship of Otolith to Somatic Growth and Manipulation of Ring Deposition

Manipulation experiments give insight into the increment formation process and also provide clues as to what types of conditions may produce visible effects in the otoliths of wild larvae (Rice et al. 1985; Lagardere and Chaumillon 1988; Mosegaard et al. 1988; Koutsikopolous et al. 1989). Some manipulations, such as photoperiod, are mainly relevant to examining the question of rhythmicity (Karakiri and Westernhagen 1989). Other manipulations, especially variations in temperature and feeding regimes, are probably more relevant to likely events which occur in the field (Volk et al. 1984; Neilson and Geen 1985; Eckmann and Rey 1987; Jones and Brothers 1987; Molony and Choat 1990). When individuals with marked otoliths are subjected to experimental manipulation of food ration or temperature, the relationship between growth and increment width can be determined directly (Alhossaini and Pitcher 1988; Mosegaard et al. 1988; Molony and Choat 1990; Wright et al. 1990). Changes in otolith growth rate may lag behind changes in somatic growth rate. This uncoupling means that the otolith daily increment width does not always reflect the daily length or weight increment (Secor and Dean 1989; Mosegaard et al. 1988; Moksness and Wespestad 1989; Wright et al. 1990). The validation of a close relationship between somatic growth rate and otolith growth rate is necessary before back-calculating the size of larvae at earlier ages. The methods commonly used to back-calculate size-at-age for adult fish have been critically reviewed by Francis (1990). He proposes more biologically sound models, as do Campana (1990) and Campana and Jones (this volume).

Physiological and Biochemical Mechanisms of Deposition

Biochemical studies of the otolith growth process provide a fundamental means for validating the rate of deposition. Mugiya initiated much of the work in this area (Mugiya 1987; Mugiya et al. 1981; Mugiya and Oka 1991), examining the uptake of ^{45}Ca into otoliths

in response to environmental factors. Serum and labyrinth Ca levels can be measured over diurnal cycles as well as changes in calcitonin, calcium inhibiting enzymes, or calcium binding properties of the otolith protein matrix (Wright 1991). SEM examination of otolith ultrastructure also shows variations in response to environmental change (Gauldie and Nelson 1988). Unfortunately because of the need to separate the intact otolith sac, or to obtain blood samples, or to collect sufficient material many of these methods are practical only for juveniles and adults.

Summary

Since the mid 1980's the application of larval otolith techniques has been well supported by validation studies. Although not all rigorous to the same extent, most studies involving new species now include an examination of reared or captive fish and are backed up with SEM examination to determine the reliability of light microscope ring counts. There are two procedures which are still missing from most current work: an analysis of the variation in increment number at age or at length, and an evaluation of the reliability of the intended applications.

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CHAPTER 7
Sources of Error in Otolith Microstructure Examination

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Introduction

The purpose of this chapter is to review all aspects of fisheries studies related to otolith microstructure and to identify potential sources of error. This review includes collection, preparation, examination, analysis and validation phases, in the order which they appear in the rest of this volume. While an overview of sources of error is provided here, the actual quantification of error is described elsewhere in this volume.

Collection and Handling

Size selection during the collection phase can cause obvious biases. A review of such biases is beyond the scope of this paper. The interested reader is referred to Butler (this volume) and to papers such as McGurk (1985) and Munk (1988), who reviewed the problem for herring *Clupea harengus*.

The most obvious source of error with regard to the collection phase of operations is an inappropriate preservation technique. When whole fish are preserved, use of an acidic preservative such as unbuffered formalin or ethanol in concentrations less than 95% (Radtke and Waiwood 1980) may result in erosion of otolith material. Such erosion will likely result in an underestimation of fish age. However, in my experience with otoliths of juvenile salmonids, it is relatively easy to detect problems with formalin preservation, as material stored in formalin for more than about one week becomes discoloured and the edges of the otolith appear "scalloped". Smaller otoliths, such as those of pelagic larvae, may erode considerably more quickly. Such material is also difficult to grind and polish adequately, as it is prone to breakage.

Butler notes (this volume) that freezing can cause fine cracks in the otolith. Such cracks will likely result in increased breakage during preparation, perhaps with disproportional loss of smaller material. I have also heard reports, as yet unconfirmed, that lysis of tissues in the otic capsule in some species may result in an acidic environment in a matter of hours, resulting in possible damage or loss to the otoliths. In view of such concerns, the prudent investigator may wish to remove otoliths promptly.

Preparation

The three pairs of otoliths present in teleosts do not always form at the same time. Examples of this phenomenon are found in the Salmonidae (Neilson and Geen 1986), Chanidae (Secor et al., this volume) and the Atherinidae (Barkman 1978). Under such circumstances, the investigator must select the otolith pair to be investigated with caution, since the timing of first increment formation will also vary, resulting in apparent age differences among otolith pairs.

Although perhaps self-evident, methodological problems associated with preparation should be carefully quantified and critically examined in terms of their potential for causing biases. For example, under certain circumstances, it might be expected that a greater proportion of smaller otoliths break or are lost during the process of grinding and polishing, thus leading to biased estimates of age and growth.

An example of possible biases which can result when different regions of the otolith can not be interpreted with the same ease is provided by Ralston and Williams (1989). Those authors studied sagittae of the gindai (*Pristipomoides zonatus*), and measured increment widths at certain locations along a radius where otolith increments were clearly visible using a light microscope. They noted that the possibility of introducing systematic biases existed if the regions of the otolith selected for increment width measurement were characteristic of either periods of fast or slow growth. Under such circumstances, estimates of mean growth rate might be biased. No authors to my knowledge have circumvented this problem, at least not without extensive use of scanning electron microscopy. However, workers would do well to follow the example of Ralston and Williams in carefully and frankly documenting possible limitations of their analyses.

With small otoliths, overgrinding may obscure entire portions of the otolith. An example of this is given in Fig. 1, which shows the effects of overgrinding in the sagitta of a 15 mm haddock *Melanogrammus aeglefinus*. The top micrograph shows an underground preparation, with relatively few areas where growth increments are apparent. The middle micrograph indicates a preparation near the optimal plane of grinding. Increments are visible in most areas, including very narrow ones formed early in the fish's life. In contrast, such increments are obliterated in an overground preparation, shown in the bottom micrograph. The obvious implication of using either over- or under-ground preparations include underestimates of age, and overestimates of growth rate.

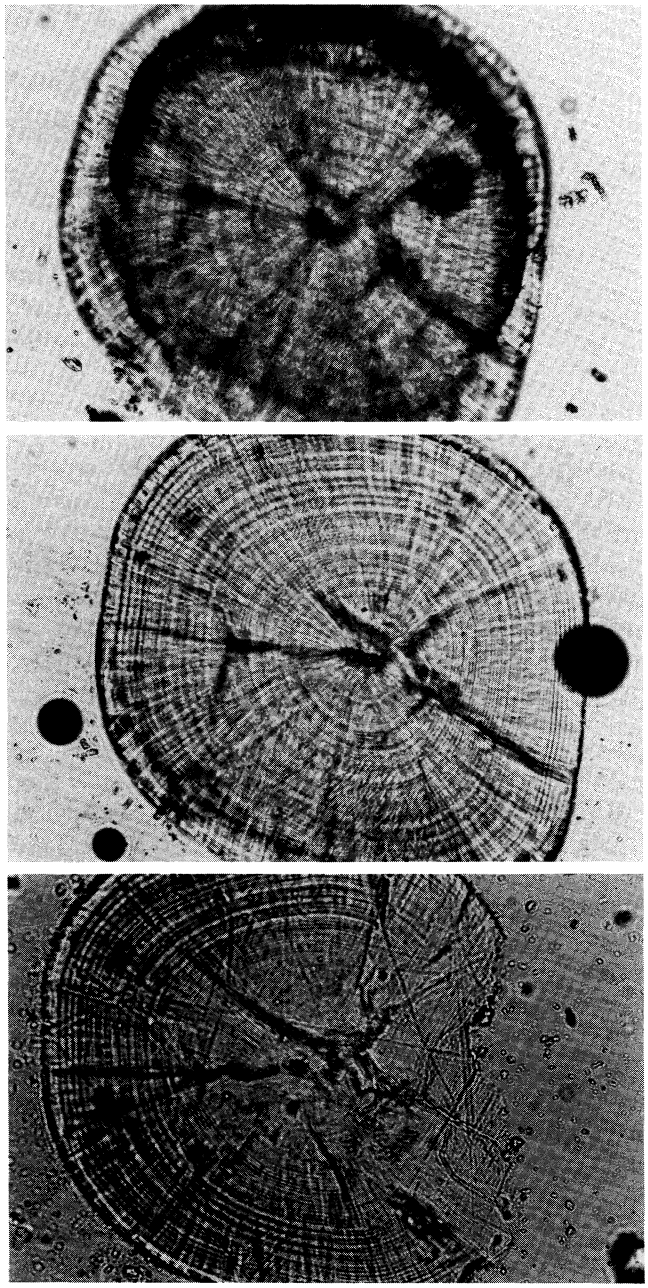


FIG. 1. The effects of over- and under-grinding in the sagitta of a haddock (*Melanogrammus aeglefinus*). The top and bottom micrographs are under- and over-ground, respectively, while the middle preparation is near the optimal plane.

Examination and Analysis

Protocols for Examination

Use of the so-called "blind" technique is strongly recommended, whereby coded material is read twice, preferably by different readers. It is important that all information which could influence the interpretation of the preparation, including date of capture, length, sex, etc., be unavailable to the reader. Some investigators also elect to discard material if the counts or measurements obtained by two independent readers exceed some predetermined value, say 10%. However, the possibility of inadvertent biases being introduced by the removal of certain material from the sample is introduced by such a practice.

Differences between left and right otoliths have also been documented, particularly in the Salmonidae (Neilson and Geen 1982). Investigators are advised to check for systematic differences in dimensions between left and right otoliths. If significant differences are found, then investigators should choose to work with otoliths from either the left or right side. It is also advisable to use the largest of the three pairs of otoliths (not always the sagittae) since growth increments can be expected to be largest in that instance. The obvious exception to such a rule is when patterns of development of the largest otolith pair renders them less suitable for microstructural examination (see Secor et al. and Campana, this volume, for examples). Where possible, it is important to select the largest of the three pairs even if the intention is to count otolith increments only, as resolution-related effects (see below) may result in underestimated counts from the smaller of the two pairs.

Limits Associated with Light Microscopy

In my view, errors associated with light microscopy constitute the most frequent and often the most serious source of error in any examination of otolith microstructure. Current applications of otolith microstructure examination place demands on light microscopy which sometimes exceed its capability. For example, the theoretical resolution of a light microscope is 0.20 μm, yet for most practical applications, it is really closer to about 1.0 μm. However, there are numerous circumstances where deposition of

increments less than 1.0 μm in width have been documented. Morales-Nin (1988) gives an interesting account of how improper use of light microscopy can lead to incorrect results. She showed that in *Sardinella longiceps*, the estimates of age and the high growth parameters (L_∞ = 14.5, K = 5.62) obtained by Dayaratne and Gjøsaeter (1986) using light microscopy may have been in error. When Morales-Nin examined otoliths from the the same species with a scanning electron microscope, considerably different von Bertalanffy parameters were obtained, indicative of a lower growth rate (L_∞ = 19.3, K = 1.22). Similar discrepancies between counts of otolith increments obtained using light microscopy and scanning electron microscopy have been noted by Nishimura and Yamada (1984), Jones and Brothers (1987) and Morales-Nin and Ralston (1990). Campana et al. (1987) examined otoliths of larval herring and concluded that due to the limited resolution of light microscopy, the hypothesis proposed by Geffen (1982) who stated that rates of increment production significantly less than one/24 h might be expected under conditions of slow growth, should be modified to reflect the relationship between *otolith* growth and apparent increment count, not *somatic* growth. However, such a change does not really address the problem of accurately enumerating and measuring increments whose widths approach the limits of the capabilities of light microscopy.

Campana et al. (1987) do offer some practical guidelines regarding species where increment width might be expected to cause problems when counting increments using a light microscope. They suggest that when left and right side otoliths differ in size and significantly greater counts are associated with the larger of the two, the results be viewed with caution, as resolution-related effects might be present. Campana and Neilson (1985) also have noted that increment counts may be problematic near the edge of otoliths. Such edge effects are due to the refraction of transmitted light through the curved surface of the otolith edge. As noted by these authors, increments near the edge can appear laterally compressed or disappear from view altogether.

FIG. 2. Effect of slight changes of focal plane on appearance of growth increments of larval cod *Gadus morhua*. The otolith was polished on both sides. Note how the relatively narrow increments are in sharp focus in the bottom micrograph but the high-contrast outermost increments are ill-defined. Slight adjustment of focus (middle, then top) brings the outermost increments in better focus but obscures the innermost fine structure. The white bar in the top right corner is 50 μm in length.

If resolution-related effects cause difficulties in enumeration of increments, they are likely to pose even more substantial problems with the accurate measurement of width. An often observed and disconcerting phenomenon is the apparent "movement" of microstructural features when adjustments of fine focus are made at high magnification. Associated with this, increments which are clear at one plane of focus may virtually disappear at another, while others come into prominence. Such problems are often related to incorrect grinding (including the failure to grind and polish both sides of the otolith) and insufficiently thin sections, although I have noted such effects even in very carefully prepared material. Needless to say, measurements of increment width under such circumstances become more an exercise in faith rather than quantitative science. An example of such problems is provided in Fig. 2, which shows the effects of slight changes in focal plane on the appearance of increments in a sagitta of a larval cod.

To avoid problems associated with individual increment width measurement, some authors have measured a series of widths along a certain radius. Wilson and Larkin (1982) back-calculated growth of sockeye salmon fry *Oncorhynchus nerka* over 28 d, and noted a measurement error of about 5% associated with the radius of back-calculation along the otolith. Much of the error was associated with difficulty in determining the exact location of the nucleus. A more detailed discussion of this source of error is given elsewhere in this paper. Bradford and Geen (1987) collected similar data for *O. tshawytscha* and found errors in back-calculated size similar to those observed by Wilson and Larkin. In discussing the use of otolith radii, Methot (1981) identified two limitations associated with such an approach. Firstly, data on size at age integrate growth rate over the lifetime of each larva and may have little meaning for examining environmental effects since such conditions are typically measured only at the time of capture. Secondly, the morphology of the central portion of otoliths of larger larvae can usually only be examined through the relatively time-consuming process of grinding and polishing.

Interpretation of Subdaily Increments

While the occurrence of subdaily growth increments has been questioned by a minority of workers, it seems clear that subdaily increments are to be expected under certain circumstances, particularly in some families. For example, several workers have documented the occurrence of subdaily increments in the Salmonidae (Neilson and Geen 1982, 1986; Campana 1983b; Marshall and Parker 1982). It may be that this family is one where the occurrence of subdaily increments is more common than in others. Further examples of species where subdaily increments are thought to occur include king mackerel, *Scomberomorus cavalla,* and Spanish mackerel, *Scomberomorus maculatus* (De Vries et al. 1990), Japanese anchovy, *Engraulis japonica* (Tsuji and Aoyama 1984), and largemouth bass, *Micropterus salmoides* (Miller and Storck 1982).

Subdaily increments, when misidentified as daily increments, can result in overestimates of age and underestimates of somatic growth rate. To resolve this problem, a means by which sub-daily increments can be objectively identified is required. The few approaches which exist are often somewhat *ad hoc*, and based on subjective interpretations of the appearance of otolith microstructure rather than a clear understanding of the growth processes. An example is the study of Wild (1986) who developed such criteria for the otoliths of yellowfin tuna *Thunnus albacares*. He concluded that if a periodic structure when compared with adjacent, sharply-defined increments (acetate replicas were examined), had diffuse edges, was lower in height, or merged with another increment, it was a subdaily increment. Criteria suggested for the identification of subdaily increments in other species by Brothers (1978) (relative thickness and protein content) and Taubert and Coble (1977) (visual definition when examined with a light microscope) did not prove to be useful for yellowfin tuna. The experience of Wild (1986) underscores the need to develop species-specific criteria for the identification of subdaily growth increments. To obtain such criteria, laboratory investigations are often required.

Age-related effects further complicate the identification of subdaily increments. Campana (1984a) showed that in plainfin midshipmen *Porichthys notatus*, age may influence the production of daily and subdaily increments, as do some exogenous factors and the circadian rhythm. In Campana's study, a greater number of subdaily increments was associated with younger fish. However, Jones (pers. comm.) has noted an opposite tendency: in the species she has examined, subdaily increments are usually found in older larvae, and along the axis of maximum growth.

Unfortunately, there are no easy answers to the problem of identifying possible sub-daily increments, apart from careful and thorough validation using the methods suggested earlier.

Effect of Growth Rate

The extent to which increment width and somatic growth rate are correlated received close scrutiny in a

paper by Bradford and Geen (1987). In studying the response of otoliths of chinook salmon, they found no correlation between increment width and growth rates of individual fry over relatively short-term (7–15 d) intervals, although significant correlations over a longer period (51 d) were found. Those authors point to the relatively conservative character of otolith growth, as did Gutiérrez and Morales-Nin (1986) and Ralston and Williams (1989), as being responsible for the lack of relationship between somatic and otolith growth rates. Careful note should be made of the study by Bradford and Geen. In my view, too many workers attempt to correlate fish growth with increment widths on too fine a scale. However, as noted by Bradford and Geen (1987) and others, the closeness of the coupling between fish growth and otolith growth will be species and fish-size specific. Smaller fish species than the chinook salmon fry used by Bradford and Geen may exhibit a more rapid response of otolith growth to somatic growth.

In some instances, authors have found delayed effects of environmental conditions on otolith growth. For example, Gutiérrez and Morales-Nin (1986) used time series analysis to show that otolith growth on any given day depended upon somatic growth and the temperature disturbances occurring on the day of increment deposition and the day before. The effect of water temperature decreased exponentially over three days. Neilson and Geen (1986) found a lag period of about 3 weeks before otolith increment width changes were recorded in juvenile chinook salmon. Similarly, Molony and Choat (1990) concluded that otolith increment widths in the estuarine glass fish *Ambassis vachelli* changed approximately 10–15 d after somatic growth rate had changed.

Reznick et al. (1989) showed that slowly-growing guppies *Poecilia reticulata* have larger otoliths than equal-sized, rapidly-growing guppies, even when the genetic background, food quality and other aspects of the aquatic environment were controlled. Similarly, somatic growth rate of pond-reared larval and juvenile striped bass *Morone saxatilis* influenced the relationship between otolith size and fish size (Secor and Dean 1989). A similar finding has been made for Atlantic salmon *Salmo salar* by Wright et al. (1990).

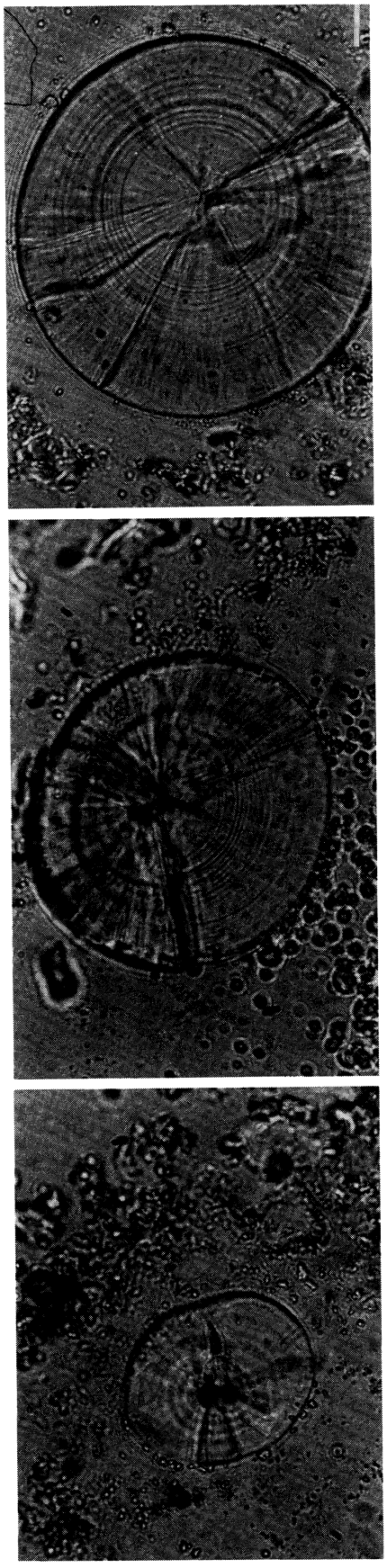
Mugiya and Oka (1991) offer an alternative view, stating that in rainbow trout *Oncorhynchus mykiss*, otolith growth ordinarily reflects somatic growth rates on a daily basis. These workers appear to be suggesting that the uncoupling of otolith and somatic growth generally only occurs under suboptimal conditions for fish growth.

An example of how growth rate can influence the interpretation of otolith microstructure is given in Fig. 3. That figure shows light micrographs of sagittae of young (51 d old) winter flounder *Pseudopleuronectes americanus* from the same parents and of the same age. Larvae of this species appear to be relatively slow-growing. Moreover, growth rate among members of the cohort differed so much that it was possible to simultaneously collect pre-metamorphic, metamorphic and post-metamorphic fish. Clear differences in the appearance of the otoliths are apparent, with considerably fewer growth increments apparent in the pre-metamorphic fish. However, even in the relatively fast-growing post-metamorphic individual, it is dubious whether 51 growth increments could be enumerated on the light micrograph.

Complex Otolith Growth Patterns

It is well known that otoliths change shape during development. At the earliest stages of development, variation in nucleus dimension and its impact on otolith microstructure have been documented by Neilson et al. (1985). They described how sagittal otoliths in rainbow trout *Salmo mykiss* and chinook salmon *Oncorhynchus tshawtyscha* arise by fusion of otolith precursors called primordia. The number of primordia fusing to form the otolith nucleus in the salmonid species examined was variable, even within the progeny of a single female. Those authors determined the effect of nucleus size variation on otolith size by examining correlations between nucleus area and otolith area in steelhead trout and chinook salmon of similar size at several stages of development. The best correlations were noted in relatively small otoliths of recently hatched alevins. The greatest degree of variability in otolith area occurred up to 15 d after nucleus formation. Hence, in otoliths formed from multiple primordia, daily increment widths associated with early stages of development may reflect nucleus dimension to some extent. Examples of species studied by Neilson et al. (1985) where multiple primordia have been noted include all five species of Pacific salmon and Pacific herring *Clupea harengus pallasi*. Radtke and Dean (1982) noted multiple primordia in masou salmon, *O. masou*; Arctic char, *Salvelinus alpinus*; brook trout, *S. fontinalis*; and the sculpin, *Cottus nozawa*.

The coupling between otolith growth and somatic growth can also be affected by post-embryonic developmental events. For example, Burchett (1984) noted considerable change in the external shape and morphological structure of otoliths of *Notothenia rossii* during the first 5 years of development, but once sexual maturity was reached, further changes were minimal and growth became more allometric with respect



to somatic growth. An alternative and perhaps more common pattern of development was demonstrated by Nishimura and Yamada (1984) who presented a series of four micrographs showing stages in the development of otoliths of walleye pollock *Theragra chalcogramma*. These micrographs revealed increasing complex otolith structure with age. Among the more interesting features were zones of overgrowth which displayed a completely different pattern of growth than did other parts of the otolith. Thomas (1983) showed that the relationship between otolith radius and fish length in the pilchard *Sardinops ocellata* off southwest Africa varied seasonally. Similarly, Marshall and Parker (1982) demonstrated that the otolith diameter-body length relation varied significantly between fed and starved experimental groups of sockeye salmon *Oncorhynchus nerka* fry. Other ontogenetic effects have been noted, such as the work of Morales-Nin (1986), cited earlier.

Flatfish otoliths cause special problems which appear to be associated with metamorphosis. For example, Jenkins (1987) found an allometry between otolith growth and somatic growth at the beginning of metamorphosis which caused a significant alteration in the morphology of growth increments and eventually led to a cessation in the production of visible increments. He further noted that increment formation may resume upon settlement. In contrast, Campana (1984b) showed that in lab-reared starry flounder *Platichthys stellatus*, no evidence of metamorphosis was apparent in the sagittae of postmetamorphic fish, and that neither increment width nor appearance changed substantially through metamorphosis. As shown in Fig. 3, changes associated with metamorphosis in winter flounder also appear more subtle than the effects described by Jenkins (1987), and more consistent with those described by Campana for starry flounder. A further complication of otolith microstructure studies of flatfish is the morphological contrast between the left and right sagittae. Such variation dictates that investigators use either the right or left otolith in a consistent fashion, not interchangeably.

Changes in otolith microstructure associated with life history transitions are not limited to metamorpho-

FIG. 3. Changes in otolith growth patterns associated with metamorphosis and differences in growth rate in the sagitta of a winter flounder (*Pseudopleuronectes americanus*). The bottom micrograph is of a premetamorphic 51 d old larvae, the middle is a sagitta from an individual undergoing metamorphosis and the top micrograph is an otolith from a postmetamorphic juvenile. All individuals were of the same parents, and were 51 d old. The white bar in the top right corner is 50 μm in length.

sis in flounder larvae. For example, changes in increment appearance at the end of the pelagic phase of larval life have been documented by Bailey et al. (1977), Brothers and McFarland (1981), Victor (1982), and Nishimura and Yamada (1984). Such changes may cause errors in enumeration of growth increments or, if the relationship between otolith and body growth is changed, require adjustment of estimates of growth rate obtained from otoliths.

A further source of error associated with complex growth patterns is the formation of accessory primordia. Such structures represent new foci for the formation of otolith growth increments which are formed at later points of otolith development. An example of such a structure and the way in which it complicates the interpretation of microstructure is provided by Campana and Neilson (1985), in a figure (Fig. 3) which illustrates the change in otolith increment width as it passes from one field of view to another.

Is the uncoupling of otolith growth to somatic growth somehow related to resorption? There is some evidence of such a phenomenon available from the amphibian literature. For example, within aragonitic otoliths of the frog *Rana esculenta*, zones of erosion have been noted. Such zones coincide with metamorphosis and can be related to a process of calcium carbonate ($CaCO_3$) mobilization (Marmo et al. 1983). There is no evidence that otoliths of teleosts are affected by resorption. However, reduction in pH has been observed to result in the formation of so-called "false annuli" (Hultberg 1977) in sea trout (*Salmo trutta*) where it was stated that no accretion of $CaCO_3$ was occurring. However, the evidence offered for the cessation of $CaCO_3$ accretion was largely conjectural and based on gross examination of otolith macrostructure. Nevertheless, if the observation is correct, then reductions in pH might have serious ramifications for the unbiased interpretation of otolith microstructure. Subsequent more detailed studies have yet to corroborate Hultberg's findings. Geen et al. (1985) found that chinook salmon *Oncorhynchus tshawytscha* alevins and fry produced one otolith growth increment per 24 h under pH regimes of 5.0, 5.5, 6.2 and 7.0. Those workers also found that otolith formation was not impaired at low pH, nor did otolith size significantly differ between fish exposed to pH 5.0 and 7.0. These results suggest that mobilization of otolith calcium, if it occurred at all, was not significant. The results of Geen et al. (1985) do not support the suggestion of other workers, such as Beamish et al. (1975) that since plasma calcium concentrations in fish exposed to low pH are maintained by bone demineralization, smaller otoliths or those with visible differences in microstructure might have been expected. Campana (1983a) also noted that stress (periodic 60 s air exposure) did not result in resorption of otolith calcium, but it did result in a "check" or discontinuity which interrupted the normal sequence of otolith increment deposition.

Reay (1972) studied the seasonal pattern of otolith growth and its application to growth studies of the sandeel *Ammodytes tobianus*, and made an unusual observation. He found that linear growth of the otoliths occurred mainly from April to August, with the deposition of opaque material occurring in April and May. During the initial period of opaque zone deposition, however, there was little or no otolith growth, and from measurements of the zones in successive samples, Reay concluded that the initial deposition of opaque material is by "ingrowth" into the hyaline material present at the edge. Although Reay's work was with otolith annuli, it has important ramifications for microstructure examination, if this phenomenon is found to be widespread. However, to my knowledge, no other workers have reported such results for any type of periodic structure occurring in otoliths.

A further major potential source of error results from changes in the type of crystal morph deposited. As reviewed by Strong et al. (1986), otoliths of teleosts increase in size by the accretion of calcium carbonate secreted by macular cells located on the inner surface of the membrane enveloping the otolith. The calcium carbonate normally consists of aragonite, one of the three naturally occurring crystal polymorphs. The other two polymorphs, calcite and vaterite, are found relatively infrequently and are often associated with otoliths which have aberrant characteristics. While infrequent, they are of consequence in certain cases. For example, Campana (1983a) found that 27% of the otoliths of juvenile steelhead trout *Oncorhynchus mykiss* were of a non-aragonitic morph.

The external morphology of non-aragonitic otoliths was described by Mugiya (1972) for many species. However, detailed investigations of the nature of daily growth increments are not available. While detailed study of daily growth increments has not yet been made in aberrant otoliths, it is known that such otoliths often depart substantially from the usual species-specific shape (see, for example, Fig. 5 from Strong et al. 1986). Moreover, those workers have suggested that given the highly translucent nature of calcitic crystallization found in the otoliths of pollock *Pollachius virens*, the organic matrix may be either absent or much reduced. Under such circumstances, it is reasonable to expect that both the ease of interpretation of daily growth increments and the usual relationships with somatic growth will be affected. Fortunately, such changes in crystal morph also affect

the appearance of the otolith, so that abnormal otoliths can usually be quickly detected and screened.

Environmental Masking Effects

Campana and Neilson (1985) first suggested that environmental masking effects could overlay or modify a fundamental 24-h periodicity in otolith increment formation. Since then, there have been some data presented which are consistent with this hypothesis. For example, Berghahn (1989) found hyaline zones in the otoliths of age-0 plaice *Pleuronectes platessa*. When the times of formation of the hyaline zones were determined through otolith microstructure examination, it was found that the zones corresponded with hot, cloudless days on the tidal flats which are nursery grounds for this species. Similarly, Koutsikopoulos et al. (1989) suggested that bands of very narrow growth increments in sagittae of sole *Solea solea* corresponded with oxygen deficiencies in the Bay of Vilaine. Morales-Nin (1987) speculated that periodic production of narrow increments in the hakes *Merluccius capensis* and *M. paradoxus* was related to environmental perturbations which were in turn related to changes in upwelling intensity. Such narrow increments may cause problems in assessing either age or growth.

As noted in several papers by Neilson and Geen, temperature cycles, feedings and induced activity of a non-24 h periodicity may result in the formation of increments of corresponding frequency. Given the number and apparent ease with which the 24-h frequency of otolith increment formation can be disrupted, workers are advised to pay special attention to cyclic environmental factors. When undetected, such agents can cause problems in the interpretation of age and growth from otolith microstructure.

Industrial pollution has also been documented to affect otolith growth. For example, in the frog *Limnodynastes tasmaniensis*, exposure to pesticides such as dieldrin resulted in developmental abnormalities of both the otoliths and the otic capsules (Brooks 1981). In fourhorn sculpin *Myoxocephalus quadricornis* exposed for 1 year to a heavy metal containing effluent from a sulphide ore smelter, weight differences between right and left otoliths were accentuated relative to the control fish (Bengtsson and Larson 1986). Although otolith growth increments were not examined in the latter instance, it seems likely that the observed gross abnormalities would also be manifested at the microstructural level.

Timing of First Increment Production

As noted by Campana and Neilson (1985), the date of first increment formation is a species-specific phenomenon and may vary from before hatching in the case of several salmonid species to the time of first feeding (for examples, anchovies *Engraulis* sp. (Brothers et al. 1976, Tsuji and Aoyama 1984), pleuronectid flounder *Rhombosolea tapirina* (Jenkins 1987) and milkfish *Chanos chanos* (Tzeng and Yu 1988)). Without knowledge of when increments start forming, estimates of age are of reduced value. An example of this type of problem is the study of Wilson and Larkin (1982) who assumed that increment formation in sockeye salmon *Oncorhynchus nerka* commenced at the time of emergence of alevins from the gravel of the redd. However, a subsequent study by Marshall and Parker (1982) found that increment production commenced well before hatching.

Errors Associated with SEM Preparation and Examination

In my own view, examination using SEM is advised for increments less than 2 μm apart, and obligatory for those less than 1 μm, although others may view these limits as overly conservative. The most commonly encountered problem in examination of otolith microstructure with SEM is failure to obtain uniform etching across the desired portion of the otolith. This difficulty is often related to the complex manner with which otoliths grow. Ideally, after grinding and polishing otoliths to the midplane, all increments should intersect the plane of polishing at 90°. However, this is rarely the case, particularly in older fish (see, for example, Neilson and Geen 1986). In cases where the investigator is unable to obtain a surface across which all growth increments intersect at right angles, uneven etching results will be obtained. Moreover, estimates of increment width will be biased in the affected area. To avoid such problems, the investigator should consider carefully what the pattern of otolith growth is for the species under consideration, possibly through study of a developmental series. If otolith growth is irregular to the extent that obtaining a useful midplane for grinding would be difficult, the options are to consider a shorter series of increments across a region of otolith growth where consistent grinding results are expected, or to consider the use of light microscopy. Another way to deal with this problem is to employ a variable etching time which is contingent on the nature of the otolith microstructure, such as done by Radtke and Hourigan (1990).

Choice of etching agent and duration of etching have considerable influence on results. Good practice demands that once a choice has been made regarding etching agent and duration, the same technique should be employed throughout. Although such advice might

seem to be common sense, there are some complications. For example, it is possible that the investigator might find that different etching times are required for fish of different ages, particularly if a wide range of ages are included in the material to be examined. Recent investigations have shown that the chemical composition of daily growth increments varies with age. For example, Morales-Nin (1986) made a study of the structure and chemical composition of Cape hake *Merluccius capensis* and found that the organic component, known as otolin, seems to change with age. The percent composition of acidic and hydrophobic amino acids increases, whereas the basic and polar amino acids and glycine remain constant. Given such changes, it is unclear how the effects of etching might vary with the age of the fish. If etching is incomplete, age will be underestimated and growth rate overestimated through the affected series of growth increments.

Errors may be introduced by inconsistent use of the scanning electron microscope. Modern equipment presents the novice operator with a considerable array of options, some of which may affect the type of results obtained. For example, the mechanical stage upon which the SEM stub is mounted can be adjusted with regard to the angle of the impinging electron beam. Such an adjustment might also affect measurements of increment width taken directly from a micrograph (Fig. 4) . I advise that the stage always be positioned such that the beam impinge on the sample at 90°. Figure 4 also shows the subtle effect of "shadowing" which occurs when the electron beam does not impinge on the preparation at 90°. For example, note how the scratch on the left series becomes more prominent as the stage is tilted. It is also important to understand how the angle of tilt usually only affects appearance, not the interpretation of size (or increment width), when the angle of tilt is parallel with the growth increment field (left series of micrographs). However, when the angle of tilting is at 90° to the increment field, measurement errors can occur. For example, the white line on Fig. 4 is superimposed on the same place on the otolith. The apparent length of the line on the left micrographs ranges from 38 to 34 μm, representing a 12% error.

Usual practice also dictates careful calibration of the SEM using known size objects. Many operators also elect to exclude critical measurements of object size in the outermost 20% of the rastered area, where it is felt that measurement errors due to beam imperfections may occur.

Varying the accelerating voltage (typically 10–20 kV) has less impact on interpretation, although higher accelerating voltages assist viewing at very high magnifications. Some scanning electron microscopes are equipped with the more commonly-used secondary electron image (SEI) as well as backscattered electron image (BEI) collectors. As shown by Jones and Brothers (1987), use of different collectors can give different visual impressions of otolith microstructure (see their Fig. 2), but in their case, no significant differences were found between counts obtained through use of either the BEI or SEI collectors.

An additional concern is the obscuring of increments near the otolith periphery by the mounting media or glue. Campana (1984b) noted such an effect, adding that SEM counts probably underestimated the true increment number. However, instances where glue obscures the outermost increments should be easily detectable and such otoliths routinely screened, or re-prepared.

Validation

As demonstrated elsewhere in this volume, (see Geffen), validation is an essential aspect of any otolith microstructure investigation. There are, however, several pitfalls associated with validation which investigators must be aware of. For example, one common method used to convert otolith increment counts to age estimates is to identify a mark or check in the series of increments and then relate this mark to the timing of the presumed event which caused it. In this context, terms such as "hatching checks" or "metamorphic checks" are often used rather loosely, without any demonstration of correspondence between the event and the mark on the otolith. For an example of a critique of a paper which employed such an approach, see Neilson and Campana (1990).

The nature of the validation study may also introduce errors. For example, Radtke (1989) has asserted that moribund Atlantic cod *Gadus morhua* do not form otolith increments every 24 h. If Radtke's assertion is correct, inclusion of moribund animals may bias validation studies, and may indeed be responsible for the inference that reduced growth rate is correlated with frequency of increment production.

Marginal increment analysis has also been suggested as a technique for validation. However, given the problems associated with edge effects using either light or scanning electron microscopy, this technique would appear to be of limited value and indeed could lead to erroneous conclusions regarding the rate of increment formation.

As noted by Geffen (this volume) there are dangers in assuming that the results of a validation study in the lab could be extended to the field. Similarly, otolith growth responses may vary throughout fishes' life histories, thereby meaning that validation studies

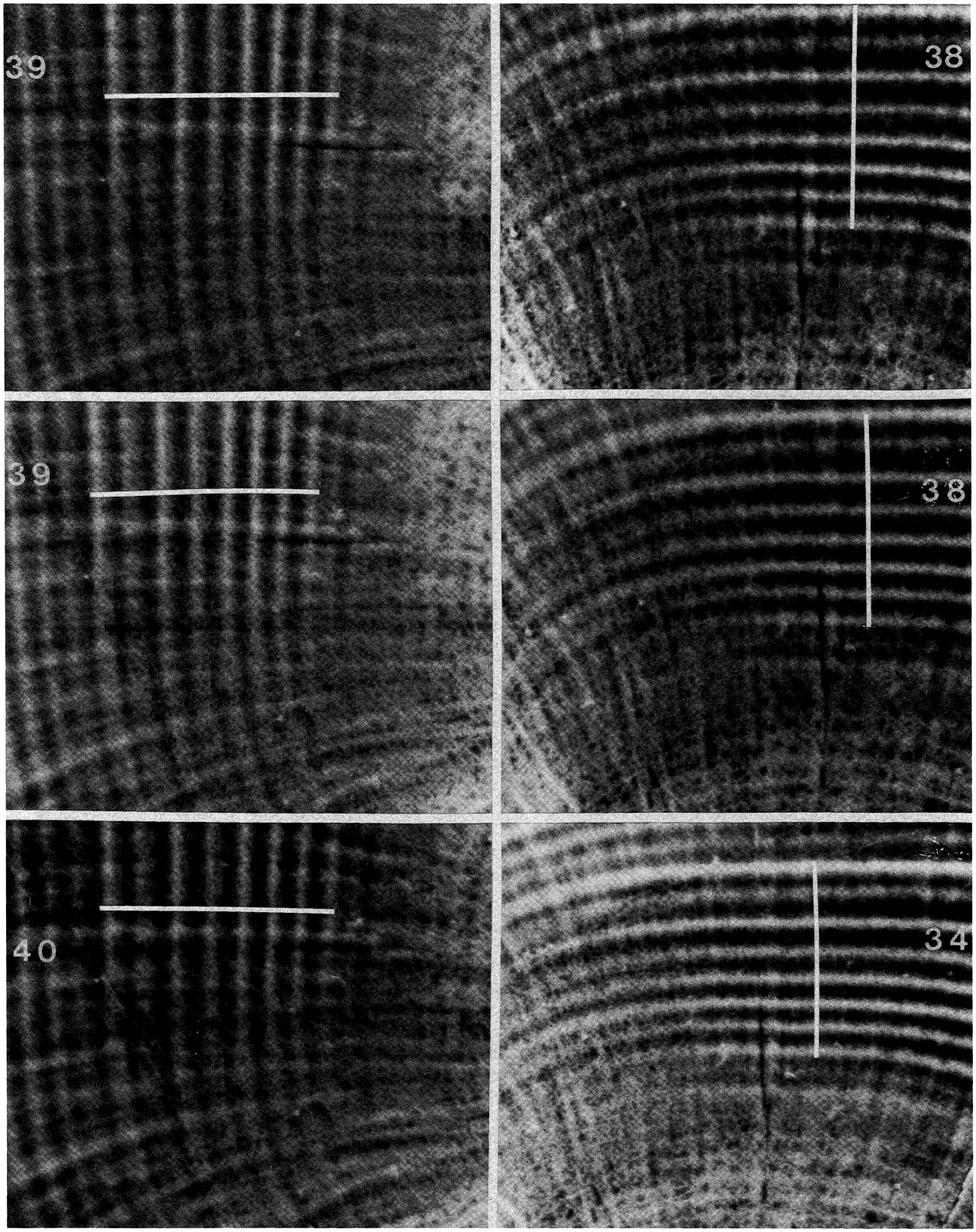


FIG. 4. Effect of tilting SEM stage through 0, 15 and 30 degrees from top to bottom, respectively, on the appearance of a sagitta of a juvenile cod *Gadus morhua*. The left series of micrographs represents the view when the axis of tilt is parallel with the growth increments, and the right series when the axis of tilt is at 90 degrees relative to growth increments. The white bar which appears on each micrograph is superimposed on the same microstructural features, and the length in microns is shown.

cannot necessarily be extrapolated beyond the range of ages included in the experiment.

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