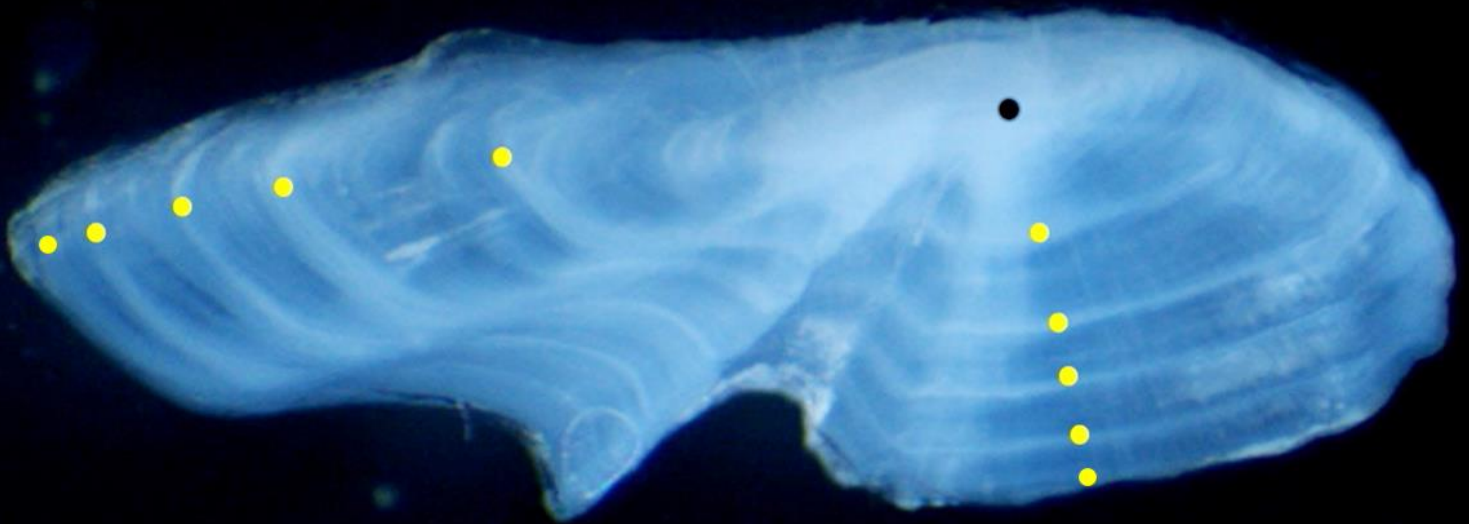


AN INTRODUCTORY MANUAL TO FISH AGEING USING OTOLITHS



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**Crawford
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FISH AGEING SERVICES PTY LTD



Australian Centre
for International
Agricultural Research



Preparation of this manual commenced as an extension resource following the Fish Ageing Training Workshop, delivered to Indonesian fisheries scientists during 23 – 27 April 2012, at Research Institute for Marine Fisheries (RIMF), Jakarta. It was progressed under ACIAR Project FIS/2009/059 and completed as an output of ACIAR Project FIS/2016/116.

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Sectioned sagittal otolith of redfin perch, *Perca fluviatilis*, prepared and photographed by Irwan Jatmiko. Position of primordium and annual growth bands marked by black spot and yellow spots respectively. This section would be scored as a 5N.

Preface

In April 2012, with funding support from The Crawford Fund, the Australian Centre for International Agricultural Research (ACIAR), CSIRO, and Fish Ageing Services Pty Ltd, and in collaboration with Research Institute for Marine Fisheries (Indonesia), a *Fish Ageing Training Workshop* was delivered to 17 fisheries scientists from research institutes within Indonesia's Agency of Marine and Fisheries Research and Development¹ (AMAFRAD), Ministry of Marine Affairs and Fisheries. The workshop was a basic introduction to the theory and practice underlying the ageing of fish using otoliths, providing the participants with sufficient skills and understanding that they could build on and hopefully progress to becoming experts in ageing their fish species of interest. This manual is a compilation of the information provided to the workshop participants. It is not intended as a step by step guide to becoming a fish ageing expert, but more as an introduction to the field for those wishing to learn the 'art' of fish ageing.

Like otoliths, this manual has grown incrementally. Its preparation commenced as a direct output from the abovementioned training workshop, as an extension resource of ACIAR Project FIS/2002/074 *Capacity development to monitor, analyse and report on Indonesian tuna fisheries*. It progressed under ACIAR Project FIS/2009/059 and was finally completed as an output of current ACIAR Project FIS/2016/116 *Harvest strategies for Indonesian tropical tuna fisheries to increase sustainable benefits*. The manual's completion, after its long evolution, is timely, as this latter project includes a component on examining age and growth of three tuna species. Hopefully the manual is a useful training resource for the scientists involved and to others more broadly.

Craig Proctor, recently retired, was a fisheries scientist at CSIRO Marine Laboratories in Hobart for 33 years and has experience in preparation of otolith sections for analysis of chemical micro-constituents, as a tool for examining stock structures. **Simon Robertson** is one of the founding partners of Fish Ageing Services (Portarlington, Victoria) and has more than 30 years of experience in preparation and reading of otolith sections for estimation of age of a large range of demersal and pelagic fish species. **Irwan Jatmiko** is a scientist of Research Institute for Tuna Fisheries in Bali (Indonesia) and completed a Masters of Applied Sciences research project at University of Tasmania, characterising age-growth dynamics of freshwater redfin perch by examining otolith sections. **Naomi Clear** is a fisheries scientist at the CSIRO Marine Laboratories and has 25 years of experience in preparation and analysis of otoliths from tuna and billfish species for age estimation, age validation and otolith microchemistry.

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The Crawford Fund, ACIAR, CSIRO, Fish Ageing Services Pty Ltd, and AMAFRAD are thanked for providing the support funding for the Fish Ageing Training Workshop in 2012, and the Research Institute for Marine Fisheries (Jakarta) is thanked for hosting and assisting with organisation of the workshop. Research Institute for Tuna Fisheries (Bali) is thanked for providing Irwan Jatmiko with the time to contribute as a co-author of this manual. Jessica Farley and Scott Cooper (both scientists of CSIRO Marine Laboratories in Hobart) are thanked for their respective review comments and contributions to this manual. This manual is an output of ACIAR Project FIS/2016/116: *Harvest strategies for Indonesian tropical tuna fisheries to increase sustainable benefits*.

¹ Now "Agency for Marine and Fisheries Research and Human Resources".

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Photos

The photos used in this manual were taken by the authors, except those acknowledged in Figure captions to other persons/sources.

1. Why do we need to age fish?

Knowing the age of fish² is essential in the understanding of the dynamics of fish stocks (Cooper 2006; Haddon 2011, Maunder and Piner 2014). Fish ages are routinely used, together with measurements of length and/or weight, in determining population/stock³ composition (in respect of size and age), age at maturity, maximum life span (longevity), mortality, and production. Performing an age structure analysis is also important for examining variations in growth rate within a population and across a species' range, and as a key parameter in population dynamics estimates. With age analysis studies we can better model the current and future impacts of fishing pressures and environmental stresses on the species' different life stages; the impacts on adult spawning populations and those on new recruits in the stocks being of particular importance. The availability of accurate information on the age structure of fish populations empowers those tasked with managing that species as a sustainable fisheries resource.

The longevity of fish species varies enormously. Some fish, such as many small pelagic species (e.g. sardines, *Sardinella spp.*), are very fast growing, mature early and only reach a maximum age of 4 – 10 years. Of the large tuna species, yellowfin tuna (*Thunnus albacares*) and bigeye tuna (*T. obesus*) are examples of species that grow fast and mature relatively early at 2 – 4 years of age (Sun et al. 2005; Murua et al. 2017). By contrast, ageing studies have revealed that southern bluefin tuna (*T. maccoyii*) also grow fast and to a large size but do not mature and start their migrations from temperate to tropical waters to spawn until at least 8 – 9 years of age (Farley et al. 2014). Some deep-water fish species are very slow growing, take a long time to reach maturity, and have an extremely long life-span. As example, ageing studies revealed that orange roughy, *Hoplostethus atlanticus*, do not mature until age of at least 25 years and can reach a maximum age of more than 200 years (Fenton et al. 1991, Smith et al. 1995). Species such as these that mature relatively late in age have populations that are at high risk from collapse due to overfishing; the risk of too many individuals being removed from the population that have not had opportunity to contribute to recruitment. Knowing the age of the fish is essential to the scientific modelling and decision making to mitigate such risks.

2. How do we age fish?

2.1 Structures for ageing

The structures most commonly used to age fish are otoliths, vertebrae, scales and spines (also called "fin rays") (Figure 1). Otoliths are the most widely used structure for ageing fish, primarily because, in general, the growth zones are more clearly defined and hence more easily 'read' than those in vertebrae, scales and spines. Some studies have shown that ageing by scales or spines can yield under-estimates in age for older fish compared to ages obtained from otoliths. Otoliths are generally more

² Unless otherwise stated, the use of the word "fish" in this manual refers to bony fish (Osteichthyes).

³ For the purposes of this manual "population" is used to refer to a group of individuals of the same species that are spatially, genetically, or demographically separated from other groups (Wells and Richmond 1995) and includes "stocks", as commonly used in reference to fisheries resources.

easily removed from the fish and easier to prepare for reading than are vertebrae. Scales, and perhaps to a lesser extent spines, have a significant disadvantage of being a structure that can be lost or damaged and replaced during the lifetime of a fish and therefore ages determined from scales may not reflect the full age of the fish. In contrast, otoliths are an internal organ and are not lost and replaced at any stage during the fish's lifetime. The one key advantage that scales and spines have over otoliths and vertebrae is that they can be sampled without the need to sacrifice the fish i.e. the fish can be returned to the water alive after samples are taken.

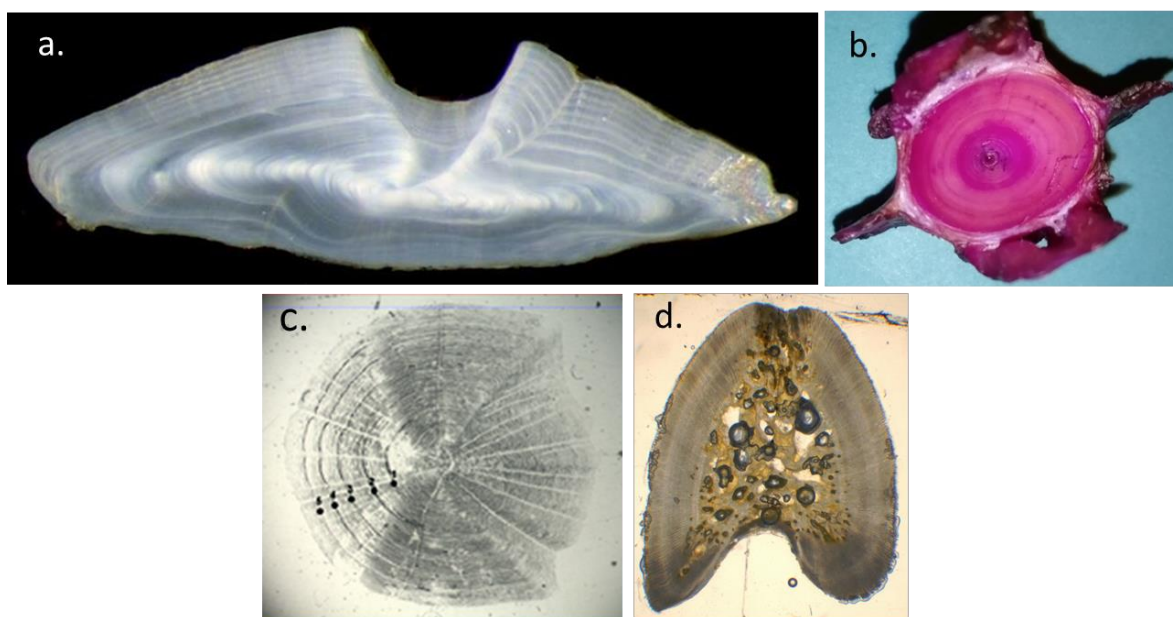


Figure 1. Images to illustrate the structures that have been used in age determination of fish: (a) otolith from an 8 year old golden perch (Source: J. Morrongiello), (b) vertebra (stained with alizarin red) from a 3 year old southern bluefin tuna of 108 cm FL. (Source: N. Clear), (c) scale from a 5 year old roach (Source: Laboratory of Fish Age Analysis, Institute of Freshwater Research, Swedish University of Agricultural Sciences), and (d) spine (sectioned) from a 3 year old yellowfin tuna of 109 cm FL (Source: J. Farley).

2.2 Otolith types and morphology

Otoliths (*Oto* = ear, *lithos* = stone) are commonly called “ear-bones” because they are a sense organ in the fish's head that provides sensory information to the fish's brain about orientation, movement, and sound vibrations. Most fish (Teleosts and Cyprinoids) have three pairs of otoliths – the *sagittae* (singular *sagitta*), *lapilli* (singular *lapillus*), and *asterisci* (singular *asteriscus*).

The sagittae are the largest of the three types (Figure 2) and are the otoliths most commonly used for fish ageing. Otoliths are composed largely of calcium carbonate crystals - most often the aragonite form (orthorhombic crystals), but sometimes vaterite (hexagonal crystals), combined with a proteinaceous matrix.

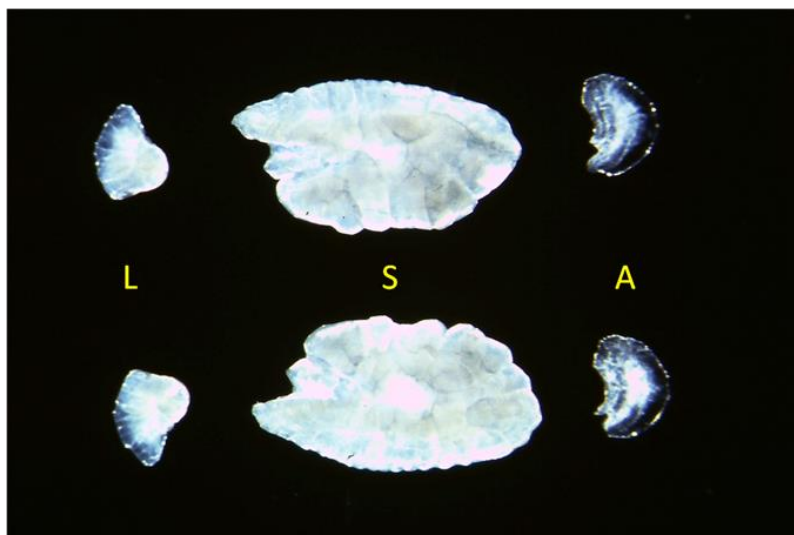


Figure 2. An otolith 'set' from a sub-adult jackass morwong (*Nemadactylus macropterus*, Cheilodactylidae), viewed under transmitted light, illustrating the relative size of the three types of otolith: L – Lapilli, S – Sagittae, A – Asterisci..

All three pairs of otoliths are located within the organ known as the vestibular apparatus. The vestibules within the semi-circular canals that contain the otoliths are called the *sacculus*, the *lagena*, and the *utricle*, for the sagittae, asterisci and the lapilli respectively (Figure 3).

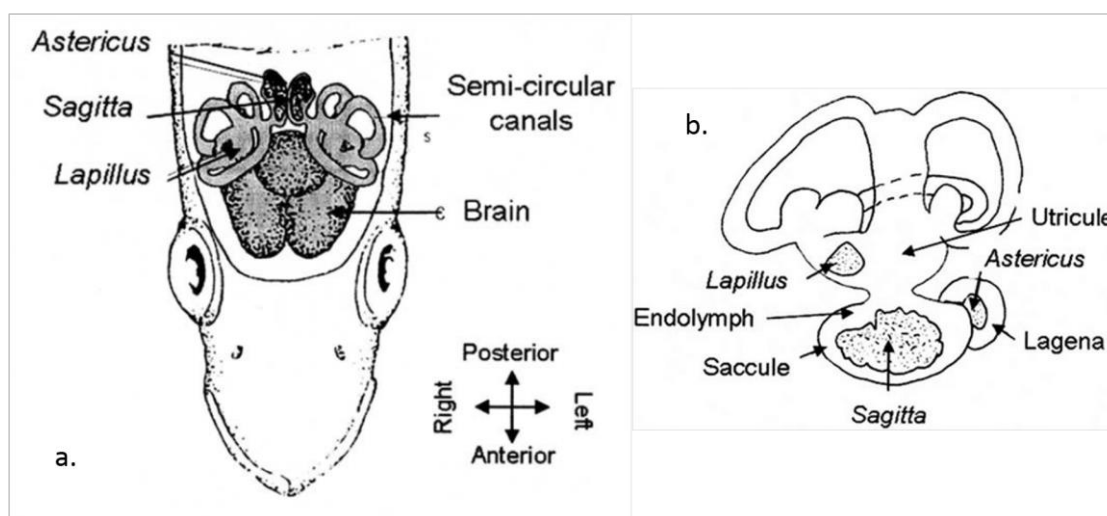


Figure 3. Generalised diagram to illustrate the position of the three pairs of otoliths in the head of Teleost fish: (a) Top of head cut away to expose the brain region, and (b) otoliths within the endolymph filled labyrinth system. (Modified from Payan et al. 2004).

Otolith-like sense organs are also present in molluscs and are called *statoliths*, and *statocysts* in crustaceans. These structures have also been used for ageing. We humans, and other mammals, also possess otoliths as sense organs for balance. Our otoliths are in the form of microscopic granules in our inner-ear canals.

The size and shape of otoliths in fish vary greatly from species to species. In general, demersal, bottom-living species (e.g. snappers, groupers, croakers) have larger sagittal otoliths than those of pelagic, highly migratory species (e.g. mackerels, tunas,

swordfish, marlins). However, the factors underlying otolith shape and size are still largely unclear.

If you are to work with otoliths, it is important you become familiar with terms used to describe otolith morphology. Figure 4 illustrates the general terms, but we must emphasise that the prominence of some of these features varies considerably between species. For example, many otoliths have a prominent, deep sulcus, whereas others have a shallow almost unrecognisable sulcus. Similarly, some otoliths have a pronounced rostrum (e.g. sagittae of many Scombridae – tunas and mackerels), whereas others have a much less pronounced rostrum (e.g. sagittae of snappers and other Sparidae, and catfish of family Aridae).

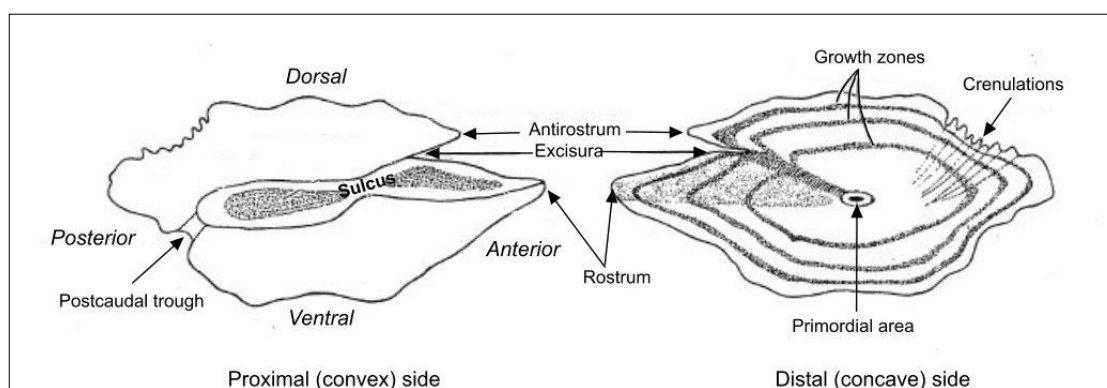


Figure 4. A diagram illustrating the features, on the proximal and distal sides, of sagittal otoliths. We emphasise that this is a **generalized diagram** and that the size and shape of otoliths varies greatly from species to species. (Modified from Pannella 1980).

2.3 Determining age from otoliths

The content of this manual, from this point on, is **confined to age determination using annual growth zones (often referred to as growth “bands” or “rings”) in otoliths**. Age determination of larval and juvenile fish is possible for many species, using counts of daily growth increments. Some of the principles of counting these increments are the same as those for counting annual growth bands.

In general, the estimate of age of a fish is determined by reading (i.e. counting) the growth bands in their otoliths, which are laid down as zones of opaque and translucent material in the otolith over the full life history of the fish. For fish with short life-spans (< 10 years), the age estimate can often be obtained by examining the whole (i.e. unsectioned) otolith with a stereo microscope under reflected light. Using this light source, the opaque zones appear white and the translucent zones appear dark. One year is shown by a combination of an opaque and a translucent zone. With fish older than 10 years (and even in younger fish of some species e.g. large tuna), attempting age estimates by reading whole otoliths can lead to errors, and most often will produce under-estimates of age. As the fish reaches the asymptotic length, the overall growth of the otolith slows and the growth the zones become compacted towards to margin of the otolith, making them difficult to distinguish (Figure 5). Sectioning the otolith is necessary to reveal these narrow growth bands. The sections are viewed with a stereo or compound microscope under transmitted light; the opaque zones appear dark and the translucent zones appear light (the opposite to reflected light).

Otolith samples should always be aged 'blind', with the reader not having reference to the fish's length, sex, date-of-capture and area-of-capture, as knowledge of these data may influence the reader's estimate of age. In general, the reader should also make his/her estimate of age without knowledge of the age estimates by other readers for the same fish.



Figure 5. Sectioned sagitta otolith from *Macquaria ambigua* (golden perch), as example for showing a narrowing and compaction of growth zones (marked with "+") towards the otolith margin. The zones closest to the margin would not be visible if viewing a whole otolith. (Source⁴: DELWP, Victoria).

After counting the growth zones (the number of opaque zones between the otolith core and the margin; many studies do not count an opaque zone forming on the otolith edge unless it is completely formed) we need to classify the edge type of the sectioned otolith. This is called the "marginal state of the otolith". The marginal states are often classified as "Narrow" (N), "Intermediate" (I) and "Wide" (W). This is determined by how much of the last visible growth zone has been formed. Table 1 shows the % of zone formed that can be used for each marginal state.

The estimation of "% formed" for determining marginal state is made by assessing the width of the marginal zone (translucent zone plus opaque zone, if present) relative to the width of the preceding growth zone (i.e. the last full growth zone). With the combination of the marginal state and the number of zones counted prior to the marginal zone it is possible to assign a sample of fish to a cohort (Figures 6 and 7).

⁴ Courtesy of the Arthur Rylah Institute, Department of Environment, Land, Water and Planning, Victoria, Australia.

Table 1. The marginal states in otolith growth.

Marginal State	Abbreviation	% formed
Narrow	N	< 20
Intermediate	I	20 - 70
Wide	W	> 70

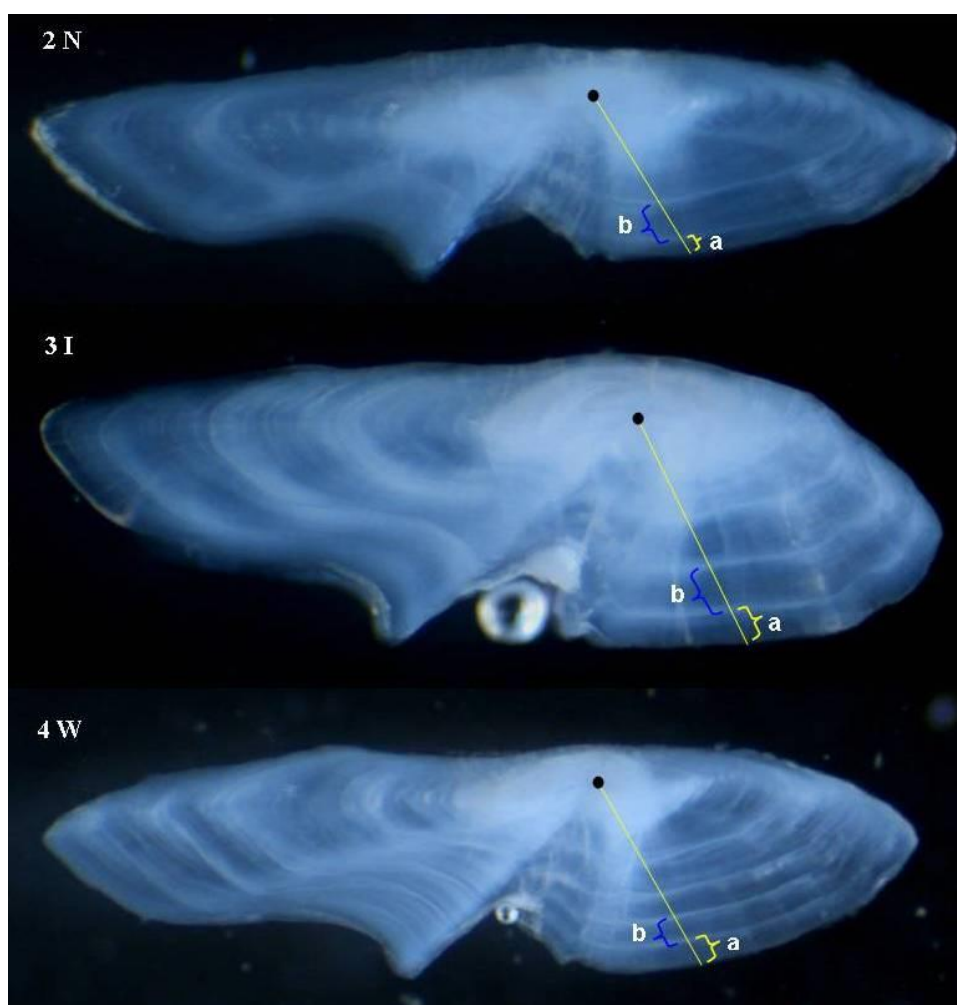


Figure 6. Sections of sagittal otoliths of redfin perch, *Perca fluviatilis*, showing width of marginal zone (a) and last full growth zone (b), and corresponding age estimate: 2N = 2 Narrow, 3I = 3 Intermediate, and 4W = 4 Wide. (Photo from Jatmiko 2012).

2.4 Fish birthdays and biological age

Fish have ‘birthdays’ for the purpose of ageing, which are usually an average for a population based on the peak in the spawning season. If the spawning season is unknown, or fish spawn year-round, a standard birth date of January 1 can be used in the northern hemisphere and July 1 in the southern hemisphere (Morales-Nin 1992). We use the assigned birthday to place fish that are spawned in the same cohort into

the correct year-class or age-class. It is important to understand that not all fish in the one cohort will form their growth zones in synchrony. For example, some fish from a particular cohort may display a 2W configuration in their otolith sections, whereas others in the same cohort and caught on the same day, may show a 3N configuration. Even though the configuration is different, the age estimates for both should be the same.

We use the marginal state information to adjust the zone count to an age-class (e.g., see Figure 7). The zone count is adjusted within an “adjustment period”, which is the period when the zones counted are completed.

Age estimates at a finer scale than whole years may be required and may be obtained from the otoliths of some species, where the reading of daily growth increments is possible. A “fractional age” can be calculated by adding the number of days between the last birthday and capture date, to the age class.

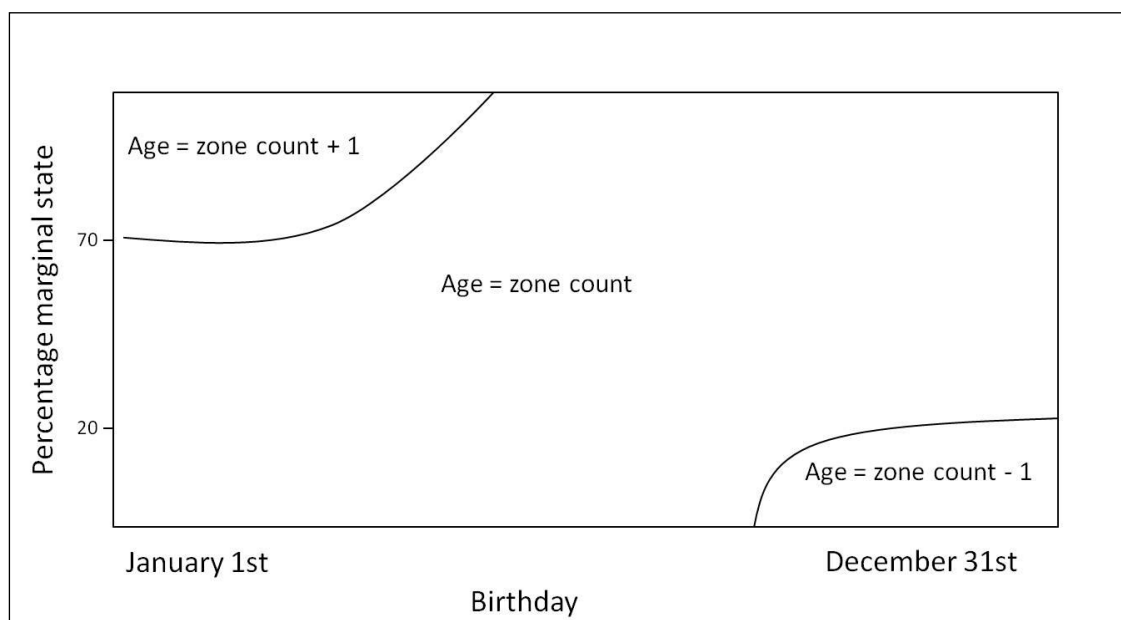


Figure 7. Diagram to illustrate the process of assigning an age, adjusted according to the percentage marginal state

2.5 Readability, precision and bias

When ageing a new species, the age readers should familiarise themselves with the growth patterns in the otoliths they are studying and establish counting criteria that can be interpreted and used by all readers. A reference set of otoliths of different ages can act as a training set and should be used regularly to maintain the established reading methods and prevent interpretation of the growth patterns from changing over time.

Two areas that can be particularly difficult to interpret are the first annual increment and the margin. The distance between the primordium and the first increment will vary because not all individuals are spawned at the same time, so a range is normally stated. The appearance of the margin has been discussed above but interpretation criteria should be set and discussed between readers.

A word of caution that repeatable age estimates do not necessarily mean that they are accurate, i.e. the age estimates may be consistent but not necessarily be the true age of the fish, unless those ages are validated. Only with validation can the temporal meaning of the structures being counted be confirmed.

Ageing confidence

The ability to read or interpret growth patterns on otoliths varies as some otoliths have very clear patterns and others do not. Readability is an indication of both the clarity of the growth patterns in an otolith and the confidence the reader has in the count. It is useful to assess and record the readability of otoliths in order to discuss the most difficult samples with other readers and to possibly eliminate those readings assigned with very low readability.

Many labs use a readability scale to identify how clear the otoliths are and the readers' confidence. These scales can be simple, such as: 1 easily readable, 2 moderately readable, or 3 unreadable. Fish Ageing Services (FAS) uses a readability scale from 1 to 5 to indicate confidence in the age reading (5 being highest confidence). Their scale includes a readability of 0 to indicate otoliths that are either missing or were too broken to prepare (Table 2). A readability of 1 indicates the otolith was intact and the preparation was successful, but no reading could be obtained.

Table 2. Readability Index of Fish Ageing Services (Victoria, Australia)

Score	Interpretation
5	Sample is exceptionally clear with unambiguous increments
4	Sample is clear and confident with estimate.
3	Sample may be \pm one year from determined age
2	Sample is difficult to interpret and subject to multiple interpretations
1	Sample is unreadable due to structure with no meaning
0	Unable to prepare sample/missing sample

Ageing precision

Ageing precision is the level of reproducibility of age estimates. The consistency of how counting criteria are interpreted, i.e. the ability of readers to identify the same structures consistently and hence produce repeatable age estimates, should be tested. Intra- and inter-reader variability (consistency) between otolith readings can be quantified using several methods.

If there are systematic differences between readers' interpretations of the growth patterns in the same otoliths, a bias may be detected using age-bias graphs, where the age estimates of one reader are plotted against the age estimates of another reader and can be interpreted using an equivalence line (Figure 8). When Age estimate 1 = Age estimate 2 there is perfect agreement. Age-bias plots can also be useful detecting drift in one reader's estimates over time.

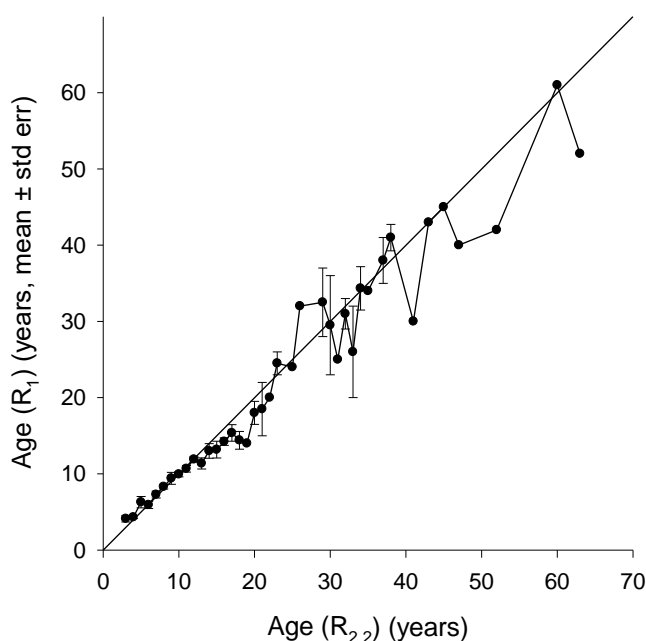


Figure 8. Comparison of hapuku (*Polyprion oxygeneios*) age estimates between two readers. Diagonal line indicates equality of age estimates. Adapted from Francis *et al.* (1999).

A statistical method for determining ageing precision is the calculation of the average percent error (APE). Beamish & Fournier (1981) recommended the use of APE, defined as:

$$APE_j = 100 * \frac{1}{R} \sum_{i=1}^R \frac{|X_{ij} - X_j|}{X_j} ,$$

where X_{ij} is the i th age determination of the j th fish, X_j is the mean age estimate of the j th 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.

The acceptable level of APE varies between species, influenced by factors such as longevity, the structure being read (e.g. whole otoliths, sectioned otoliths or vertebrae) and the readability of the structure. A maximum level of 10% is adopted in many studies, beyond which age estimates should be treated with caution. Different accepted levels of APE may be appropriate for otoliths from younger and older fish of your species of interest. For example, if your species prove more difficult to age at >5 years, you may decide to set your APE at somewhere between 5 – 10%, whereas perhaps at somewhere between 3 – 5% for younger fish.

Summary

In summary, and in simplest terms, the key steps to ageing fish from whole or sectioned otoliths are:

1. Counting the completed zones on the sections;
2. Determining the marginal state of the sample;

3. Using the zone count and the marginal state to determine the age-class.

To determine the year of spawning, subtract the age from the year-of-capture. To determine the biological age, add the difference in days between the last birthday and capture date, to the age-class.

Three things make a good fish ager: Practice, Practice, and Practice! There is no substitute for practise. Practice = Experience, and Experience is a prerequisite for acquiring the necessary expertise (i.e. Capability).

3. Dissecting otoliths from fish

There are several ways otoliths can be removed from fish and the best way will be determined by the size and type of fish that you are working on. The method you choose will also depend on your personal preference i.e. what method you find to be most efficient time-wise and the method that results in minimal loss or damage of the otoliths.

For small to medium size fish, the three most commonly used methods for removal of the otoliths are:

1. Removing the top of the head to expose the top of the brain-case - sometimes referred to as “lifting-the-lid” technique or “open-the-hatch” technique;
2. A vertical, dorsal to ventral, cut posterior to the eye to expose the rear of the brain-case and the rear of the auditory capsules that house the vestibular labyrinths containing the otoliths (referred to below as “vertical cut method”);
3. The underside approach method, sometimes called the “Up-through-the-gills” technique.

3.1 Lifting the lid

The ‘lifting-the-lid’ technique is as follows:

1. Position the fish on its ventral side and, using the sharp knife, make a vertical cut (dorsal – ventral) at a point one eye-diameter distance from the posterior edge of the eye, down to the level past an imaginary horizontal line across from the top of the eyes (Figure 9). It is critically important that this vertical cut is no closer to the eyes than the one eye-diameter distance. If you cut too close, you will cut right through the otoliths!;

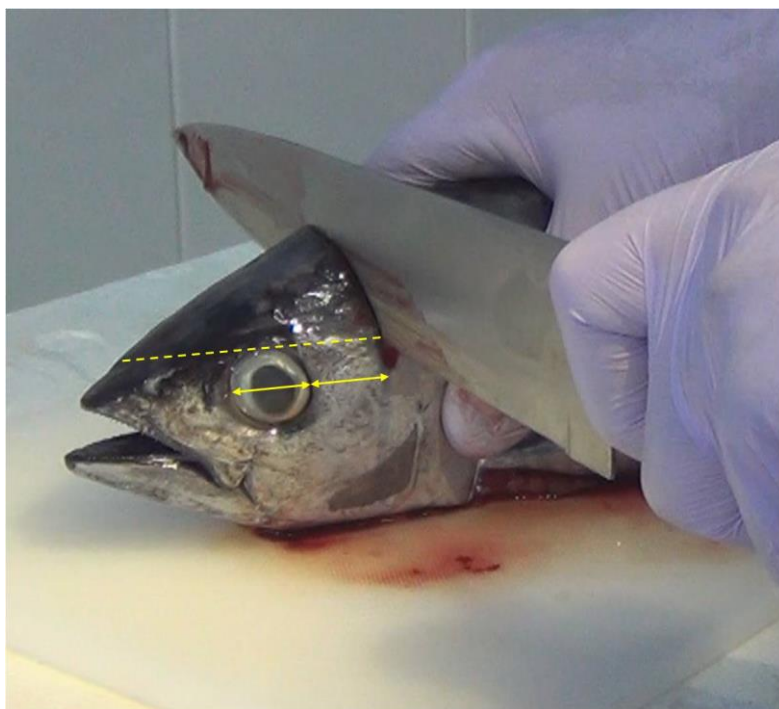


Figure 9. Making the first cut for lifting-the-lid technique, at one eye diameter to rear of the posterior margin of the eye. The cut should go slightly further than the level of top margin of the eyes. The line of the second cut is shown by dashed line. The fish is a small (~ 40 cm FL) yellowfin tuna, *Thunnus albacares*.

2. Then make a second cut (Figure 9); an almost horizontal cut at the level of the top of the eyes, but with a slight angle down (no more than 10° from horizontal) towards the tail, to intersect with your first cut. The amount of downward angle on the cut will vary from species to species. For some species you may find a horizontal cut, with no angle, is best. Trial and error is the best way of perfecting your technique;
3. You can now raise the top of the head, to expose the brain (Figure 10a);

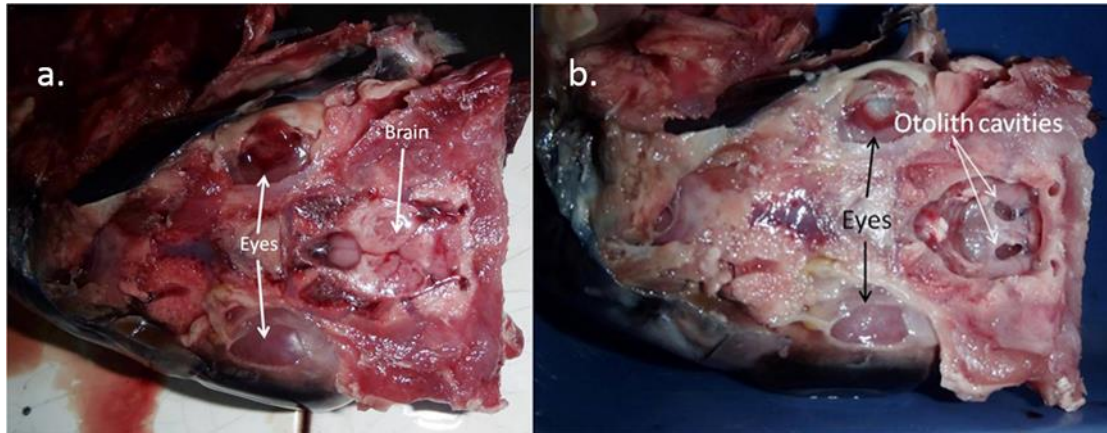


Figure 10. (a) Dorsal view of the brain case, exposed by 'lifting-the-lid', and (b) otolith cavities visible after removal of the brain. Again, the fish is a small (~ 40 cm FL) yellowfin tuna, *Thunnus albacares*.

4. Carefully move aside the brain tissue with your tweezers from the posterior area of the brain region (Figure 10b). Do not discard the brain tissue, just in case you inadvertently entrap an otolith among that tissue while moving it. You may need to go through it later if you can't locate one or both of the otoliths. The brain tissue can either be removed to expose the cavities in which the otoliths are located, or the brain tissue can be left in place and forceps used to locate the otoliths more by feeling 'blind' rather than by visual cues.

This technique is not recommended for species that have thin, fragile otoliths, as feeling around under the brain-tissue can lead to broken otoliths.

3.2 Vertical cut method

Using a sharp knife, make a dorsal to ventral cut along a line approximately the distance of one eye diameter posterior to the posterior margin of the eye (Figure 11a). If the cut is made true vertical, from dorsal to ventral, the rear of the auditory capsules should be exposed and the sagittal otoliths clearly visible for removal with forceps (Figure 11 b & c).

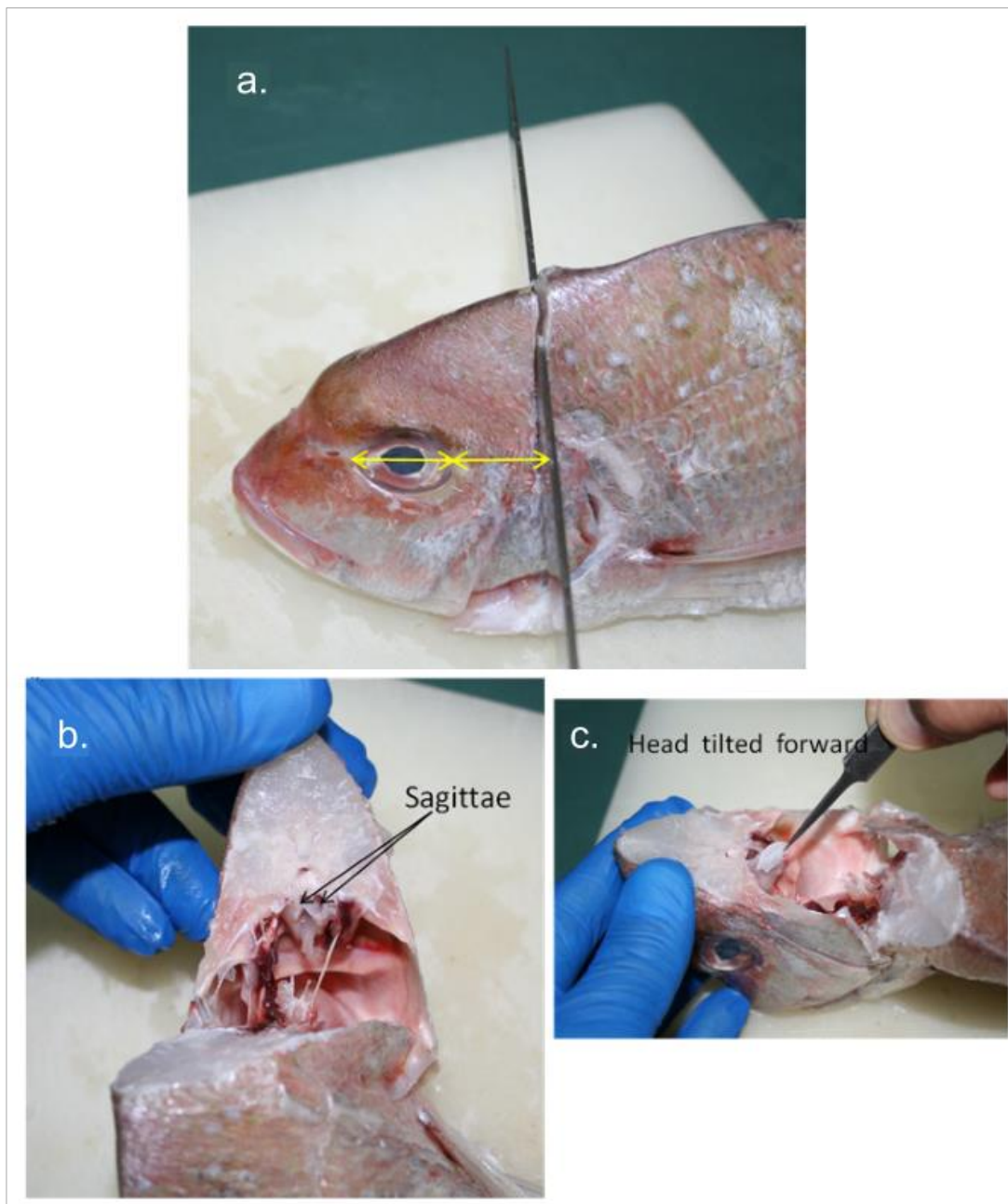


Figure 11. **a.** Dorsal to ventral cut through the head of an Australasian snapper, *Pagrus auratus*, along a line approximately one eye diameter posterior to the posterior margin of the eye. **b.** The cut exposes the posterior side of the otolith cavities, and **c.** The head can be tilted forward and the otoliths removed with forceps.

If the cut is not true vertical and is inadvertently angled slightly forwards towards the mouth, the knife may pass through the otolith cavities and damage the otoliths, so care must be taken. Similarly, if the cut is inadvertently angled slightly backwards

towards the tail, the otolith cavities will not be exposed and a second cut then becomes necessary but can make finding the otoliths difficult.

3.3 Underside approach method

The underside approach method is preferred by many for small fish and/or where otoliths are small and fragile. Of the three methods explained here, this method carries the least risk of damaging or losing the otoliths during the removal process. A negative aspect of this method is that it can be more time consuming. However, with practice and increased familiarity with the position of the bony capsules, the method can be relatively quick. The steps are as follows:

1. Using both a sharp knife and your hands, fully remove the gills from the head region or at least move them to one side;
2. With the knife, scrape away the tissue that overlies the junction point of the spinal column to the rear of the skull. This will expose the ventral surface of the bony capsules that house the otoliths;
3. With the knife and using great care, slice open the top surface of the bony capsules, being careful not to cut into the otoliths;
4. Using fine forceps, gently remove the otoliths through the openings you have made in the bony capsules (Figure 12). It is important to create openings that are larger than the otoliths so that the otoliths can be removed without using force.

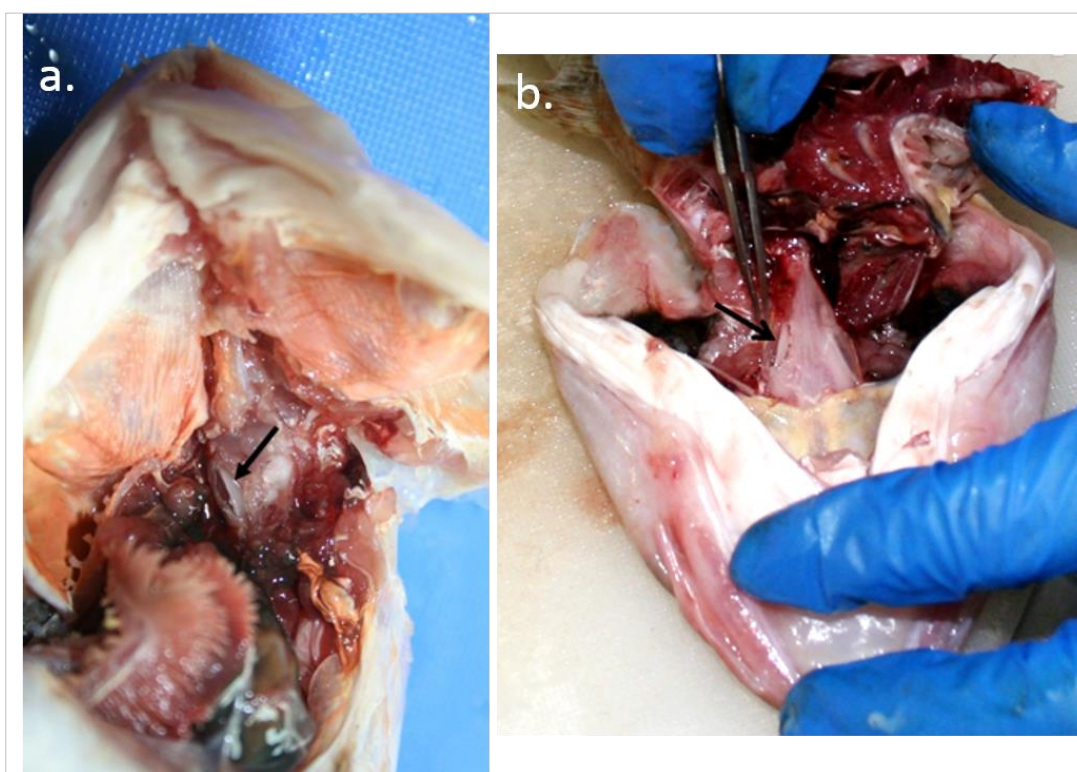


Figure 12. Accessing the otoliths in (a) an Australasian snapper, *Pagrus auratus* (Sparidae), and (b) a sand flathead, *Platycephalus bassensis* (Platycephalidae), by removing the gills, then exposing the bony capsules at the point where the spinal column joins to the skull, and opening the capsules with a sharp knife or scalpel. Arrows point to the position of the exposed sagittae.

Note: In some species of fish, including many demersal species, the bony capsules are very prominent, as well-rounded ‘bulges’, and easily located after removing the overlying tissues. In other species, including many pelagic fish, the bony capsules are much less rounded and more difficult to locate. If you can locate the first vertebrae and its junction with the skull, you should be able to find the capsules. Again, as you become more familiar with the anatomy of your species of interest, the task of removing the otoliths will become easier!

Safety: When using a sharp knife or scalpel to remove otoliths, always remember to cut in the direction away from yourself and always be aware of where your hands and fingers are. If the knife slips during the cutting of fish’s tissues and bone, you can sustain very serious injuries if you are cutting towards yourself or if one of your hands is in the way of the knife.

3.4 Drilling otoliths

A technique has been developed to remove otoliths from tuna which does not affect the external condition or quality of the fish. The method uses a cordless electric drill and hole-saw to remove a core of material from the region around the brain containing the otoliths. This leaves the fish essentially intact, except for the core hole. We have found a 38 mm hole-saw to be adequate for coring small fish (<120 cm FL) but a 44 mm hole-saw⁵ is preferable for larger fish (Figure 13). Sampling takes only 1 - 2 minutes per fish.

1. Lay the fish on its back and make sure all gill-filaments and surrounding tissue is removed as the core sample is drilled from inside the gill cavity;
2. The location for drilling are the bony plates (basioccipital plates) which are visible, on both sides, immediately in front (anterior) of the first vertebra (Figure 14). The location of the plates is one eye-diameter measurement behind the level of the eye. Sometimes residual tissue may obscure the plates; this should be scraped away with a knife or with the hole-saw itself prior to drilling. The line of coring is through the plate, with the drill initially directed slightly down and forward (i.e. on an angle roughly towards the eye on the opposite side of the head). Drill in as far as the hole-saw will allow (Figure 15). This may require the drill to be moved in/out a little so that it does not get jammed. Then remove the drill and switch to the other side. Drill onto the opposite basioccipital plate so that the two drill cuts intersect. This will usually detach the core from the surrounding bones;
3. Some force may be required to free the core. Care must be taken at this point not to break the vertebral column as this can reduce the market price of the fish. If lucky, the core (Figure 16) will come out still inside the hole-saw and can be easily pushed out with a pair of tweezers. If the core does not come out of the fish easily, use your fingers to push it out. Occasionally, the core may break into a few pieces, but the otoliths are often still present and intact;
4. Remove the otoliths carefully with tweezers from the cavities (Figure 16). Initially, it may take some time to familiarise yourself with the orientation of the core and

⁵ If you have access to workshop/engineering services, you could consider having the length of your hole-saws extended by addition of metal tubing of same diameter. This allows you to drill right through from one side to take the bone core. See hole-saws in left-hand image of Figure 12.

the location of the cavities. Look for the brain cavity and the semi-circular canals. To reduce sampling time, the core can be placed in a labelled bag and the otoliths removed at a later time. However, it is recommended that you try and find the otoliths in the first 10 - 20 cores and adjust your drilling angle if you are unsuccessful.

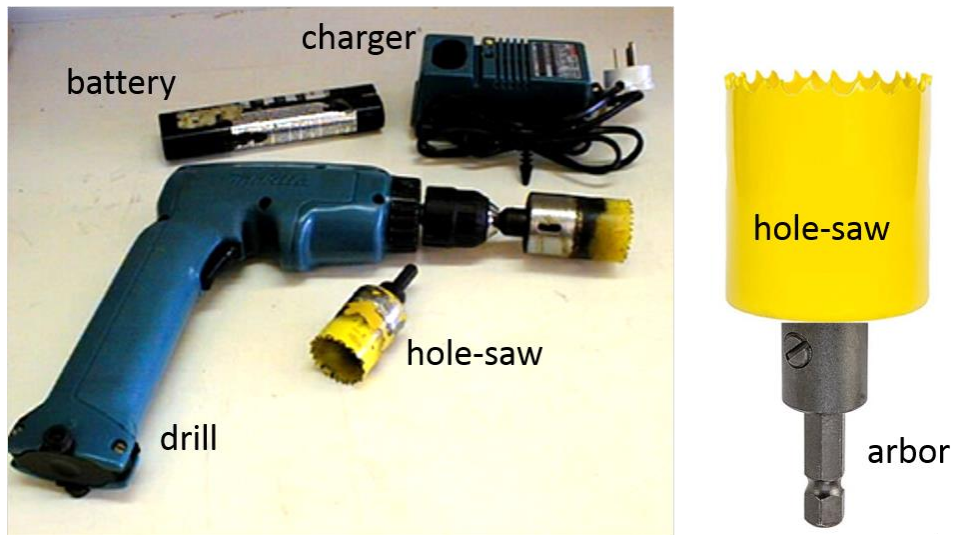


Figure 13. Cordless drill and hole saws used to remove otoliths. Right-hand image shows the hole-saw mounted on a metal arbor.

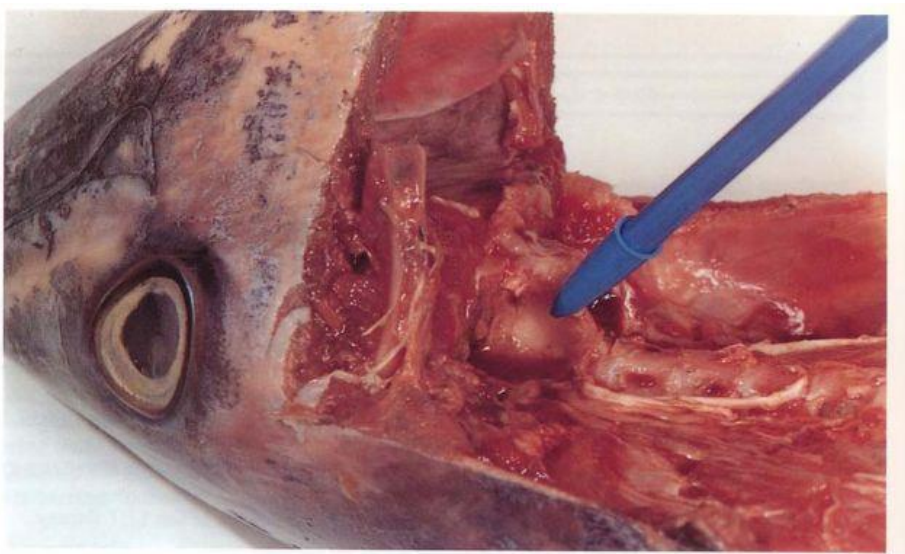


Figure 14. Image showing a cut away section of a head on a southern bluefin tuna (*Thunnus maccoyii*) to illustrate the location of the basioccipital plate (where the hole-saw is placed to take the core for removal of the otoliths).



Figure 15. Drilling with hole-saw⁶ to remove the bone core from a large southern bluefin tuna. Note that the angle of drilling is pointing slightly to anterior.

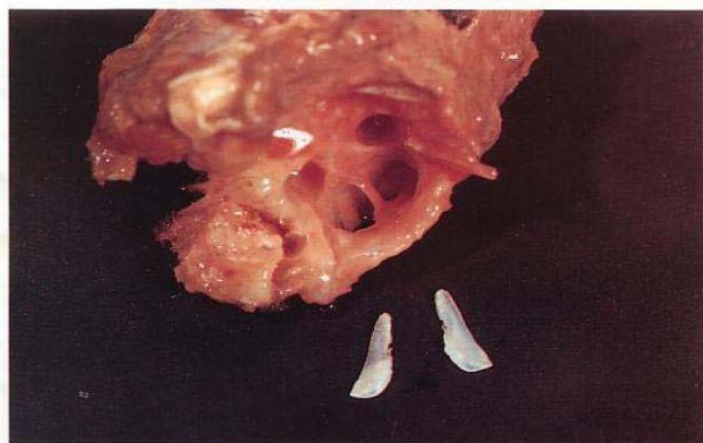


Figure 16. Image showing the bone core after removal by hole-saw from head of a southern bluefin tuna, showing the otolith cavities and the otoliths.

The above four methods are not the only methods of otolith removal, but most fisheries scientists or others (e.g. fisheries observers) who are tasked with the sampling of otoliths, will use a version of one of these techniques. The choice of method will be largely determined by the size and species of the fish being sampled, the size and fragility of the otoliths, and the time available for dissection of each fish.

⁶ The person doing the drilling in this photo is Mr Kiroan Siregar, scientist of RITF. Mr Siregar has been sampling otoliths from southern bluefin tuna with this method for the past 25 years.

4. Archiving and processing of otoliths prior to use

4.1 Otolith storage

Immediately after you have removed otoliths from a fish, your highest priorities are to clean the otoliths, place them into safe storage, and ensure that all necessary information is recorded for that sample (i.e. sample registration). The first step of the registration process should always be the recording of the information by the otolith sampler onto a data record sheet (Figure 17) with links to the sample vials or sample envelopes, and if the latter is used, also onto the envelopes (Figure 18).



Figure 17. Recording of complete and accurate information to accompany your sampled otoliths is essential.

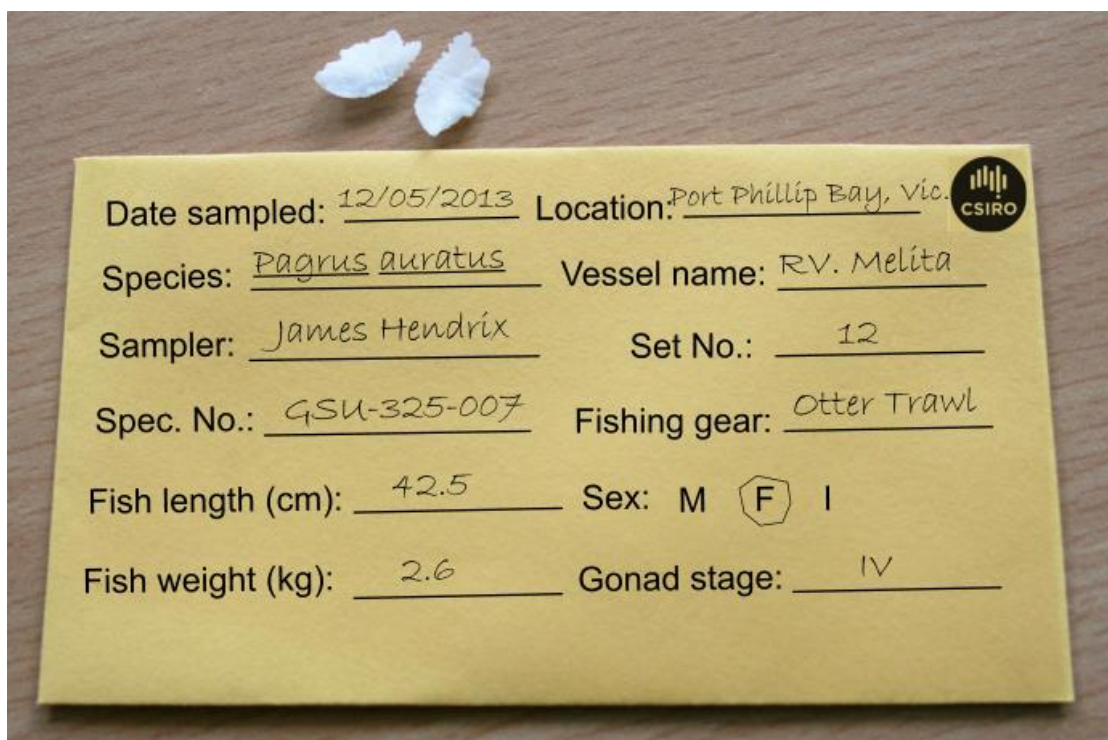
Storage methods

Paper envelopes (Figure 18) are the preferred method of otolith storage for many fisheries scientists because:

- (i) more information can be recorded onto the envelopes than can be recorded onto a vial, and therefore provide the most direct possible link between the otoliths from a fish and the associated information for that fish;
- (ii) the envelopes allow the otoliths to air-dry while safely contained;
- (iii) envelopes are relatively inexpensive.

Efficiency of information recording can be increased by having the sample envelopes pre-printed with all the necessary data entry requirements, but if this is not possible, plain envelopes will suffice if labelled clearly and in a standardised format (i.e. the

layout of information is the same on each envelope). Ideally, the envelope should be labelled prior to the otoliths being placed inside, as this reduces the risk of damage to the otoliths through pressure on the envelope with pen or pencil.



Two small white otoliths are placed above the envelope. The envelope is yellow with a CSIRO logo in the top right corner. The following information is handwritten on the envelope:

Date sampled:	12/05/2013	Location:	Port Phillip Bay, Vic.
Species:	<i>Pagrus auratus</i>	Vessel name:	RV. Melita
Sampler:	James Hendrix	Set No.:	12
Spec. No.:	GSU-325-007	Fishing gear:	Otter Trawl
Fish length (cm):	42.5	Sex:	M <input checked="" type="radio"/> F <input type="radio"/> I
Fish weight (kg):	2.6	Gonad stage:	IV

Figure 18. An otolith storage envelope, showing examples of the types of information that can be added to the envelope exterior.

Plastic vials (Figures 19 & 20) are the other commonly-used method of storing otoliths and have three key advantages over envelopes:

- (i) the vials provide more protection against damage, as otoliths are, in general, fragile and prone to breakage;
- (ii) even the smallest of otoliths can be stored in vials, whereas there is a risk of small otoliths becoming 'lost' in envelopes or at least difficult to extract from them;
- (iii) large numbers of small vials can be stored in custom-made cabinets for easy reference (Figures 21 & 22). The storage capacity, in terms of number of vials that can be accommodated in one cabinet, can be increased significantly by 'off-setting' the position of the holes in the shelves (Figure 23).



Figure 19. Labelled otolith sample vials. Otoliths that are particularly fragile can be wrapped in a small piece of plastic foam or tissue, to protect them from breakage. (Photos from Siregar 2008).

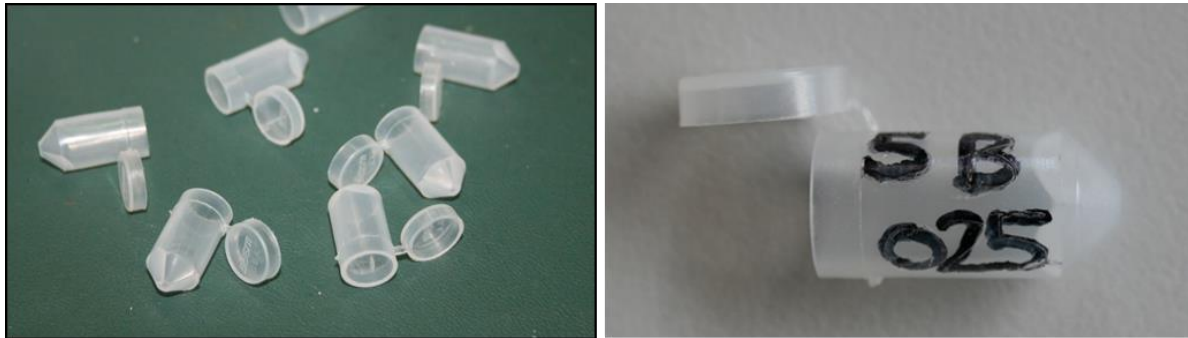


Figure 20. Polyethylene embedding capsules are a relatively inexpensive method of storing small to medium-size otoliths. These capsules are best labelled by engraving a registration number with a sharp implement and highlighting with a permanent marker.



Figure 21. Small vials and custom-made cabinets provide a relatively space efficient method of storing a large number of otoliths. Such a storage system is only suitable for small to medium-size otoliths.



Figure 22. The cabinets shown are used by CSIRO in Hobart for storing tuna otoliths. Each cabinet, of dimensions 40cm x 40cm x 40 cm, has capacity for 13 shelves of otolith sample vials, with approximately 306 vials per shelf if the holes in the shelves are in straight columns. This provides a total storage capacity of 3,978 vials per cabinet.

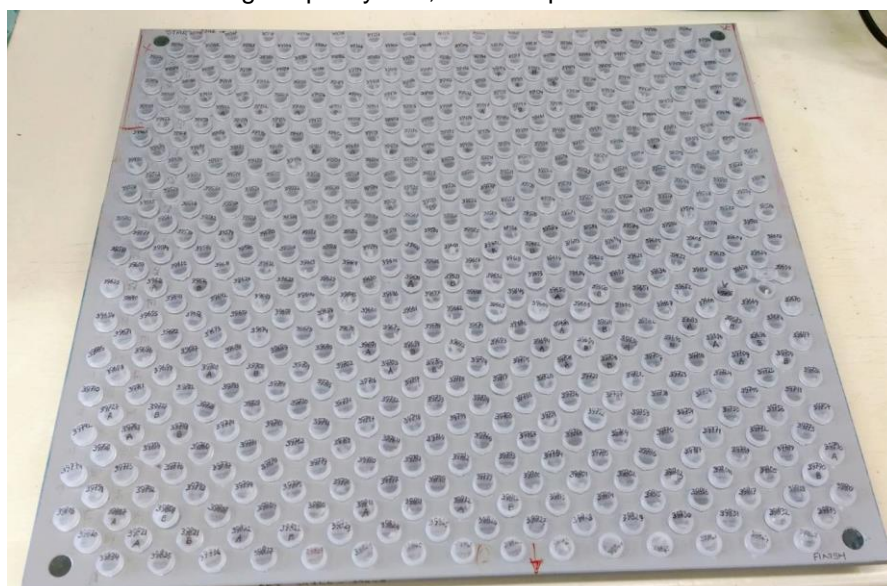


Figure 23. By 'off-setting' the columns of holes in the shelves, the storage capacity of each shelf can be increased to 496 vials and achieve a cabinet's total storage capacity of 6,448 vials.

The **disadvantages** of vials are:

- (i) it is difficult to record much information onto the vial and therefore a linked datasheet is necessary;
- (ii) writing details onto vials is, in general, more time consuming than for envelopes;
- (iii) the otoliths of many species of fish are too large to fit into small plastic vials. Using large plastic vials for storing large numbers of otoliths is impractical; and
- (iv) mould will grow on otoliths if they are placed moist into capped vials. The vials should initially be left uncapped to allow the otoliths to adequately air dry.

As with any recording of information in fisheries science, it is essential that the person recording information on envelopes and vials writes clearly and at a size that

is legible and can be easily read by all other persons. **Many difficulties and errors can be generated by poorly recorded information on envelopes and vials!**

4.2 Registration and databases

The collation and storage of information associated with an otolith collection are critically important and, in many ways, more important than the otoliths themselves. Without complete and error-free information, the otoliths may be rendered useless for your research. All the information linked to otoliths that you have sampled yourself from fish in the field or in the laboratory, or that you have received from other sources, should be entered into a database as the second, high priority, step of the registration process.

The types of information that are most commonly recorded for an otolith sample include:

- (i) date of capture or date of sampling or both;
- (ii) vessel name or name of fisher;
- (iii) catch or sampling location (including sufficient detail to ensure no uncertainty if the place name is shared by more than one location);
- (iv) species name and/or species code (see notes below on types of species codes commonly used in fisheries research);
- (v) shot/set number;
- (vi) type of fishing gear;
- (vii) fish length (Fork Length and/or Standard Length and/or Total Length, including measure mm, cm, or m);
- (viii) fish condition (e.g. whole, gilled, gilled & gutted, fresh, frozen, brined, poor condition, damaged);
- (ix) fish weight (including measure, g or kg);
- (x) sex;
- (xi) batch number;
- (xii) number of samples in the batch;
- (xiii) name or code of sampler/recorder.

Other information associated with otolith samples that are commonly included in databases:

- (i) depth of water where fish was caught/sample was taken;
- (ii) temperature of water where fish was caught/sample was taken;
- (iii) other environmental variables e.g. salinity, turbidity, pH, dissolved oxygen;
- (iv) fish stomach weight and/or level (%) of fullness;
- (v) fish stomach contents – weight and/or descriptors;
- (vi) gonad weight;
- (vii) gonad stage/maturity status;
- (viii) method used to measure length of the fish e.g. callipers, measuring board, deck tape, straight-length tape, over-the-body tape (least preferred);
- (ix) method used for weighing e.g. electric balance, beam balance.

Choosing and establishing an otolith collection database

As mentioned above, the collecting of otoliths for ageing purposes will usually be accompanied by a large amount of recorded information (referred to as “data” from this point on) of different types. All that data needs to be stored in a safe (i.e. secure) and well organised repository. Obviously, the data needs to be readily accessible by you, but most likely, also by others. Therefore, the way the repository is structured needs careful consideration.

A **relational database** is the recommended form of electronic repository. In its simplest terms, a relational database is a database structured to recognise relations between stored tables of information. Your first thoughts might be to use one or more spreadsheets (e.g. MS Excel) as repository for your data. That temptation is often there because, in general, spreadsheets are relatively easy to establish and use. However, **a spreadsheet is not a database** and there are several reasons why a relational database is a far better choice than a spreadsheet(s) for storing and managing your data:

1. A relational database allows data from multiple sources and types to be integrated into one database. The data is entered into fields (generally as columns) so that all data in all fields from all sources is organised and fully searchable. This can be accomplished with a spreadsheet, but it requires other applications or custom programming to achieve it;
2. As data is entered into a spreadsheet the overall size (i.e. file size) of the spreadsheet increases much faster than it does in a comparable database. As more and more information are entered the large spreadsheet becomes unwieldy. For example, if a fishing operation catches 100 fish then the date, vessel, location etc. is repeated 100 times for each fish in a spreadsheet. A relational database will store only one record for the fishing operation and join

- the 100 fish to this single record. Also, if an update is required then only this single record needs to be changed, not all 100 records as in a spreadsheet;
3. The time it takes to do operations (e.g. generating reports, graphs and data manipulations) increases substantially for a spreadsheet as compared to a database;
 4. Almost all relational databases (e.g. MS Access) use Structured Query Language (SQL) to manipulate and query the database. Queries can be established relatively easily for 'pulling out', filtering, aggregating and sorting the specific information you wish to work on, whilst still having the links to all data in the database. The queries can be saved for occasional or regular interrogations of your data;
 5. Once you become familiar with your relational database, you can generally work more efficiently than working with a large spreadsheet(s). The time savings with increased efficiency can be considerable.

Details on designing your database, for storing and managing your otolith collection data, are beyond the scope of this manual. If you don't have the knowledge yourself to design a relational database, we recommend you seek advice and assistance from a database expert. Taking the time to achieve a well-structured, highly secure (**including a 'fail-safe' system of back-up**) and user-friendly database from the beginning will be of benefit to you and to all who need to use the data.

4.3 Adopting standard labelling systems

Establishing and using a standard, systematic, labelling system will greatly reduce the likelihood of errors caused by mix-up of samples. This applies for your sample envelopes and/or vials, for the otolith sections that you produce, and for the digital images that you produce from those sections for your age readings. Efficient labelling systems also allow you to record, systematically, several key pieces of information in one label and provide a permanent reference for that sample, and a systematic link between envelope or vial (i.e. otolith sample), section, and image.

As an example, at Fish Ageing Services (FAS), the labelling systems used are of the following formats. You may use different formats to best suit your research purposes:

1. For otolith sample envelopes

{Species code, 3 digits}_ {Batch number, 3 digits}_ {Fish number, 3 digits}

As example, an envelope label "453_786_001" would mean:
Species code 453, Batch no. 786, and fish no. 1 within that batch.

2. For otolith preparation methods

The same identification method is used regardless of the sample preparation technique. The method used to prepare the technique for ageing is noted when the estimate is obtained. Most samples are aged as a transverse section, in this case the image name would default to a standard nomenclature. When the sample is read whole, a 'w' is appended to the image name. This is FAS internal procedure. The method chosen by the individual researcher or institute would be dependent on personal choice or institute procedure.

3. For digital images

{Client code, 3 digits} {Job number, 3 digits} {Species code, 3 digits} {Batch number, 3 digits} {Fish number, 3 digits} _Reading/image number_Reader code.

As example, a digital image label “034001478188001_1_1” would mean: Client code 034, Job no. 001, Species code 478, Batch no. 188, Fish no. 001, and it was the first image/first reading by the Reader whose code no. is “1”.

There is no international standard format for labeling and there is nothing to prevent you from creating your own labeling systems. The FAS formats were provided just as example. However, it is critically important that you document the meaning of whatever formats you use, so that others may understand your labels. The point of an efficient and easy-to-understand system for assigning a code and labelling each sample is that a sample will be able to be identified and all the associated metadata pertaining to sample is available in minutes. **A labeling system must be simple to understand and be unambiguous.**

Species codes

Fish Ageing Services uses an in-house three-digit code for identifying species. A common code used by researchers elsewhere is the FAO_3-Alpha code. This code has worldwide recognition and can be accessed in the link below. Whichever code is chosen, a relational table between the codes is recommend so data can be formatted with either the internal or external species codes.

<https://www.afma.gov.au/sites/default/files/uploads/2014/11/fao-species-codes.xls>

4.4 Weighing and measuring otoliths prior to embedding

Weighing otoliths prior to embedding and sectioning (and for otoliths that are read unsectioned) is important because you may at some stage wish to examine the relationship between otolith weight and fish length and/or otolith weight and estimated age. Otoliths should be weighed to at least 3 decimal places (but 4 decimal places for small otoliths), on an electrical balance with closed cabinet (Figure 24). Either the left or right otolith from a fish can be weighed, as there is generally no significant difference between the weights of both.



Figure 24. Obtaining an accurate weight for your otoliths, prior to embedding, is important. Handle the otoliths with great care, using fine forceps, and always over the lab bench and not over the floor!

Otoliths that are broken during handling or storage can often still be used for ageing, if the otolith's primordium and the growth axis of interest are still present together in one of the pieces. Only whole otoliths should be weighed. If the otolith is broken and all the pieces are present, then the pieces of the otolith can be weighed. As a general rule, an otolith broken into more than three pieces is recorded as a null record.

If you have access to image analysis software and the facility to capture digital images of your otoliths under a stereomicroscope, you might consider taking various morphological measurements (Figure 25) prior to embedding. These measures may not initially appear necessary or useful, but at a later stage may prove useful for understanding the dynamics of otolith growth in relation to fish growth. Otolith morphology has also been found to be useful as a tool in some stock discrimination studies (Campana and Casselman 1993, Stransky *et al.* 2008, Tracey *et al.* 2006).

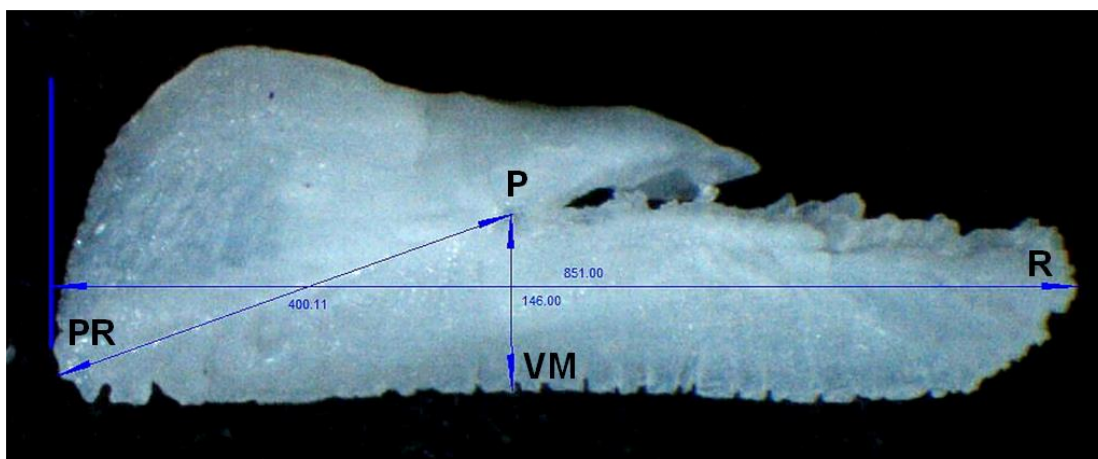


Figure 25. Image of a sagitta from a sub-adult yellowfin tuna (*Thunnus albacares*) showing some of the commonly used otolith morphological measures: primordium (P) to ventral margin (VM); post-rostral distance = primordium to post-rostrum (PR); and otolith length = maximum horizontal distance between rostrum and post-rostrum. The lengths shown are in pixels (140 pixels = 1 mm).

The length measures that you choose to make on your otoliths will largely be determined by the otolith shape for that species. Commonly used measures include primordium to post-rostrum, primordium to ventral margin, primordium to dorsal margin, and otolith length (maximum distance between rostrum and post-rostrum). It is very important that you calibrate your image capture equipment and software against a known, standard length (e.g. a graticule) before commencing each session of otolith measurements, particularly if the equipment is used by other users.

5. Embedding the otoliths

5.1 Safety

Safety considerations are critically important in setting up the area of your laboratory for embedding otoliths in resin and in the embedding operations. The resin compounds contain toxic substances that are potentially dangerous for your health and that of others working in your laboratory. Your resin embedding area needs to be well ventilated and ideally the mixing of the resin and the embedding should be done in a fume-cupboard or in a room with a strong exhaust fan. You should also wear a lab coat, gloves, and a protective mask and protective eye-wear. First preference is a full-face mask with gas vapour filters, covering mouth, nose and eyes, but second-best option is a particulate dust mask if the embedding area is well ventilated. If you are not using a full-face mask, you need to wear safety glasses or safety-goggles.

The resin and catalyst may cause PERMANENT BLINDNESS if it comes in contact with your eyes!

Pregnant women should not be exposed to the resin vapours and therefore should not participate in the embedding operations.

This is an appropriate place in the manual to mention two other IMPORTANT SAFETY RULES FOR YOUR FISH AGEING LABORATORY (but also for laboratories in general):

- **No eating, drinking or smoking in the laboratory,** and
- **No wearing of bare-feet or sandals.** Fully enclosed footwear should always be worn.

5.2 Resin

The best type of resin for making your otolith ‘blocks’ is a clear casting resin, such as “Polyplex Clear Cast”. When mixed with a small amount of MEKP catalyst, the resin sets hard and clear. The amount of catalyst that is required may vary, depending on the type and brand of the resin that you purchase, and you should strictly adhere to the recommended quantities in the instructions and safety directions that accompany the resin and catalyst. **Adding too much catalyst is EXTREMELY DANGEROUS** as the reaction between resin and catalyst is exothermic and generates heat. If you add too much catalyst your resin-catalyst mix may generate intense heat and burst into flames. When you purchase a new batch of resin, you should experiment with a very small amount of catalyst and gradually increase the volume to determine the optimal quantity (i.e. optimal % mix, resin: catalyst). The optimal % mix will vary depending on the temperature and humidity in your laboratory.

You **MUST NEVER STORE THE RESIN AND CATALYST CONTAINERS TOGETHER**. If the containers were to accidentally spill or leak and the contents mixed, an explosion and/or fire are likely to result.

ALL RESIN RESIDUES AND ASSOCIATED RUBBISH SHOULD BE DISPOSED OF PROPERLY.

We thoroughly recommend that you cover your lab benchtops in your embedding room with protective ‘bench-roll’. Bench-roll is generally a 4 ply absorbent tissue, with a polyethylene waterproof backing and can be easily cut to size with scissors. If the bench-roll is fixed with tape onto your bench-tops with the polyethylene surface uppermost, it will provide good protection against the inevitable resin droplets and spills. If you do not have protection the resin will remain as a sticky mess on your lab benches and be extremely hard to remove. If you do need to clean up spilt resin, drops of resin on your bench, or resin on any instruments, use tissues and 100% Acetone, (CH₃)₂CO. However, **BE CAREFUL as Acetone is highly flammable**.

5.3 Embedding process

Depending on the size of your otoliths, prior to embedding it is often useful to first make a small graphite pencil mark (Figure 26) on the *distal* surface of the otolith, at the point of the primordium. Remembering from the earlier generalised description for otoliths (see Section 2, Figure 4), the *distal* side of the otolith is the one that faces to the exterior of the fish and the *proximal* side of the otolith faces to the interior of the fish (Figure 4). Obviously, pencil marks cannot be made on very small otoliths, but with care and experience they can be made, by hand, while viewing under a stereo-microscope, on medium to large size otoliths. These marks will assist you in the lining-up the primordia of your otoliths in the embedding mould (as detailed below).

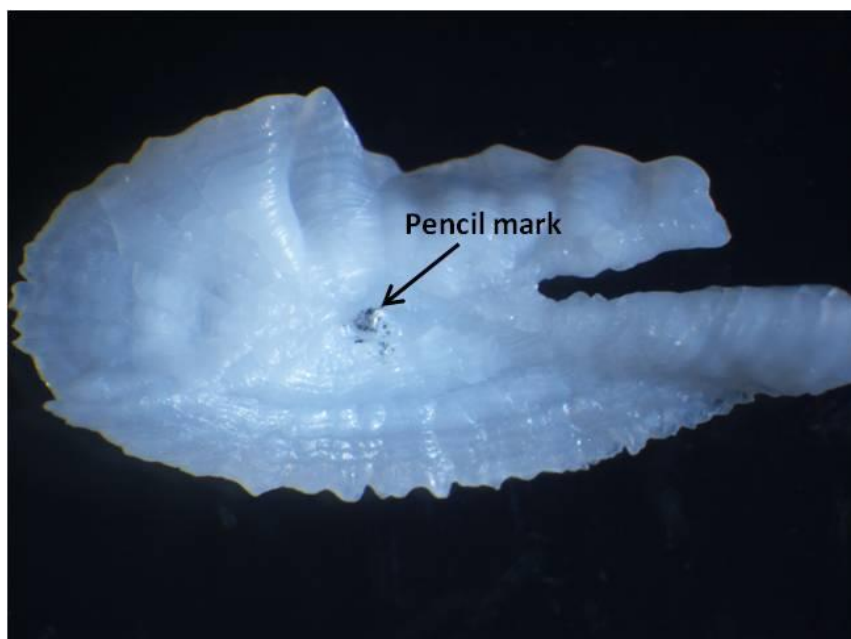


Figure 26. Distal side of a jackass morwong (*Nemadactylus macropterus*) sagittal otolith marked with a graphite pencil mark on the primordial area.

The recommended steps for embedding are as follows:

1. Mix your polymer casting resin with the predetermined correct amount of catalyst (MEKP). A good mixing container is a disposable, waxed paper cup and using a clean 'ice-cream stick' as the mixing tool. The resin mix will generally be workable for 20 mins, before it starts to become too viscous and begins to set;
2. Pour enough resin into the mould compartments to form a 1 - 2 mm base (Figure 27). Spread the resin with plastic spreaders to cover the bottom of each mould. Plastic spreaders can be made from the lids and sides of take-away food containers;
3. Organise your otoliths into the resin base in your mould compartments, in 2 columns, with the primordia aligned in each column (Figure 28). Place the label at the top of the mould compartment, running left to right. Then gently top up with resin-mix from the cup to cover the otoliths, using a stick to wipe excess resin from the lip of the cup to prevent messy drips. The label is placed on an angle in the block. This can be extremely helpful to sort out issues if a mix-up of sections occurs;
4. If you find during the embedding process that you have a missing otolith and therefore a gap in your samples relative to your block label, you can put a piece of coloured paper as a substitute otolith (Figure 28). This helps you to keep your otolith sequence complete. It is best to use coloured paper as that will be easier to see in the section;
5. After the resin has set hard in the mould, preferably after several hours on the lab bench (e.g. overnight), place the mould on an oven tray and into an oven at 55°C for 3 hours.



Figure 27. Pouring resin over otoliths that have already been organised in the compartments of the mould, and using a stick to wipe excess resin from the lip of the cup to prevent messy drips.

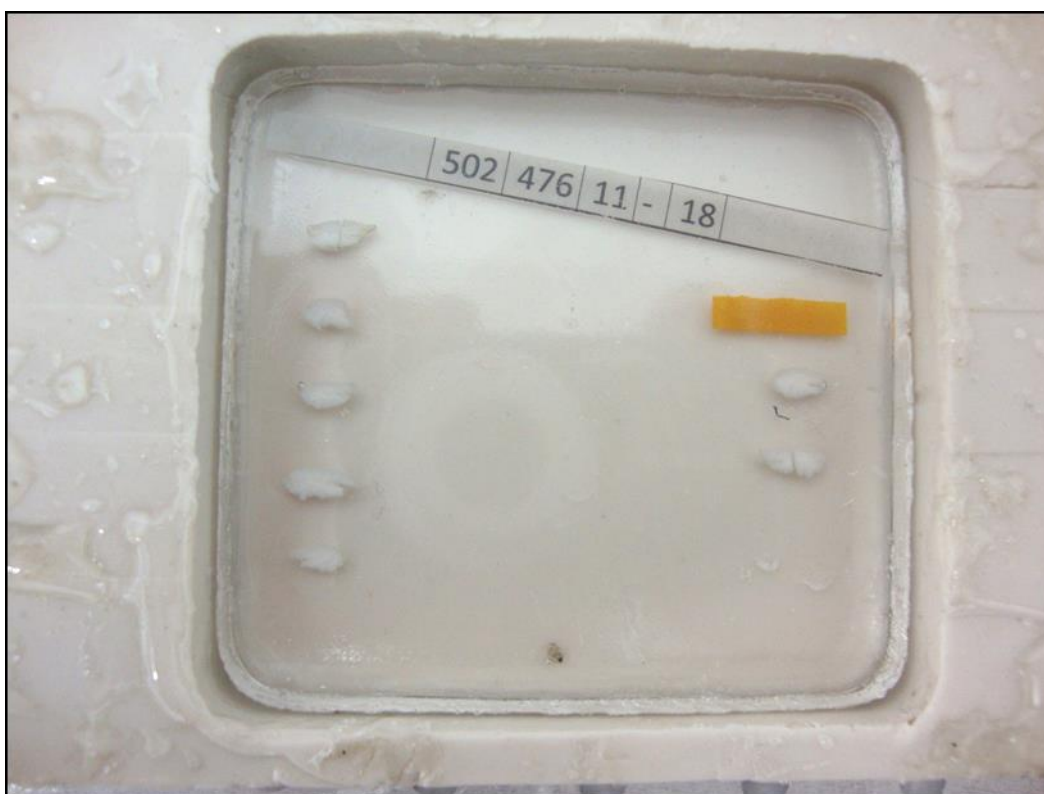


Figure 28. Otoliths embedded in a resin block with their primordia aligned. Note the block label at the top and the coloured paper in place of a missing otolith.

Tips to make embedding easier

If possible, it is best to have all your otoliths in the same orientation (e.g. all the rostrums pointing to the left) in your blocks as this will make the reading process much easier. If you can move from one section to the next under the stereo-microscope without need to reorientate it will save you a lot of time.

Silicone rubber moulds with multiple compartments are an excellent investment for your embedding lab. Depending on the size of your otoliths, each compartment (from this point on referred to as “block”) of the mould can accommodate up to 6 otoliths per column and therefore 12 otoliths per block. With generally 4 blocks per mould, that equates to a maximum capacity of 48 otoliths per mould.

The catalyst can be dispensed to the resin with a plastic pipette but a safer, more effective method is using a pump-action, adjustable volumetric ‘bottle-top’ dispenser. A similar style dispenser (but for much larger volume) can also be used for adding the resin to the mixing cup from your resin storage container. Using such dispensers takes all the guesswork out of the mixing process, and greatly reduces likelihood of mistakes being made in the adding of catalyst to the resin. It also reduces spillages and makes for a much cleaner and more efficient lab.

6. Sectioning the otoliths

6.1 Choosing plane of section

The decision on which plane of section you choose for your otoliths will largely depend on three factors:

1. The size, shape, and ‘topography’ (e.g. depth and length of the sulcus) of the otoliths of your fish species of interest;
2. The definition and clarity in the growth banding in the different planes, as this can vary markedly between planes, and between species;
3. The methods and equipment that you have available for achieving your otolith sections.

If you are beginning to work with otoliths of a fish species that is new to you, ideally you would first research what plane(s) of section has proved best for ageing purposes, in studies of other fisheries scientists on otoliths of similar species (e.g. fish of the same Family or Genus). If little information is available, you may have to experiment to determine that best plane of section; most commonly a transverse plane (i.e. dorsal to ventral, sometimes referred to as “lateral”) or a longitudinal plane (i.e. anterior to posterior) (Figure 29). Comparing growth band counts in sections of more than one plane is an interesting and useful exercise in itself. Making such comparisons can assist you in validating that what you perceive to be annual growth bands are as you think they are.

In some cases, particularly with large otoliths, you may be constrained to a transverse section just through the difficulty of achieving an effective section longitudinally. However, for large otoliths, you can consider cutting the otoliths into smaller pieces, to achieve a size that is more manageable for embedding and sectioning, whilst retaining the full growth history (i.e. primordium to margin).

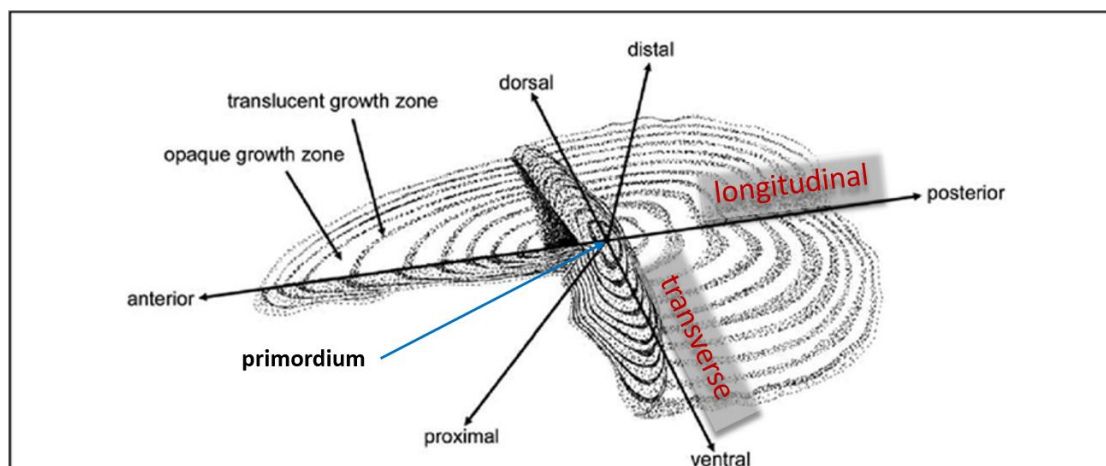


Figure 29. Generalised diagram illustrating transverse and longitudinal planes of section for a sagittal otolith. (Image: Modified from Pannella 1980).

Commonly you are faced with a 'trade-off' in using a transverse plane of section. The transverse section is generally easier to obtain than a full longitudinal section, particularly in otoliths such as those from tuna that are elongated rather than those more circular in shape e.g. those of many tropical reef fish. A negative aspect of using a transverse section is that the spacing between the growth bands may (but not always) be 'tighter' than with a longitudinal section, and more of a challenge to count with confidence.

6.2 Equipment and set-up

The following notes are written with particular reference to the GemMasta GS6D lapidary cutting machine that is used at FAS and also at RITF. However, many of the following notes are equally applicable to other types of cutting machine.

FOR SAFETY, the electricity supply to your cutting machine should always be equipped with a safety-switch. A powerboard with an inbuilt RCD (Residual Current Device) is recommended as this device will interrupt the electric current in milliseconds in event of a short-circuit or other electrical fault, greatly reducing the chances of a dangerous electric shock being received. This safety consideration is critically important given the use of water in the sectioning process. It is important to test the RCD at the beginning of each cutting session.

A protective apron, mask and boots are recommended for when you are sectioning otoliths. The apron and boots prevent you from getting wet, and the mask stops you breathing in particulate resin (Figure 30).



Figure 30. Protective apron, mask and boots are recommended for when you are sectioning otoliths. The apron and boots prevent you from getting wet, and the mask stops you breathing in particulate resin.

The blade commonly used to section the otolith blocks is a diamond edged blade of thickness 0.004 inches, and there must be constant dripping of water onto the saw-blade during the sectioning for cooling. Two jets provide water to both sides of the saw-blade, and another jet provides water from above the saw-blade (Figure 31).

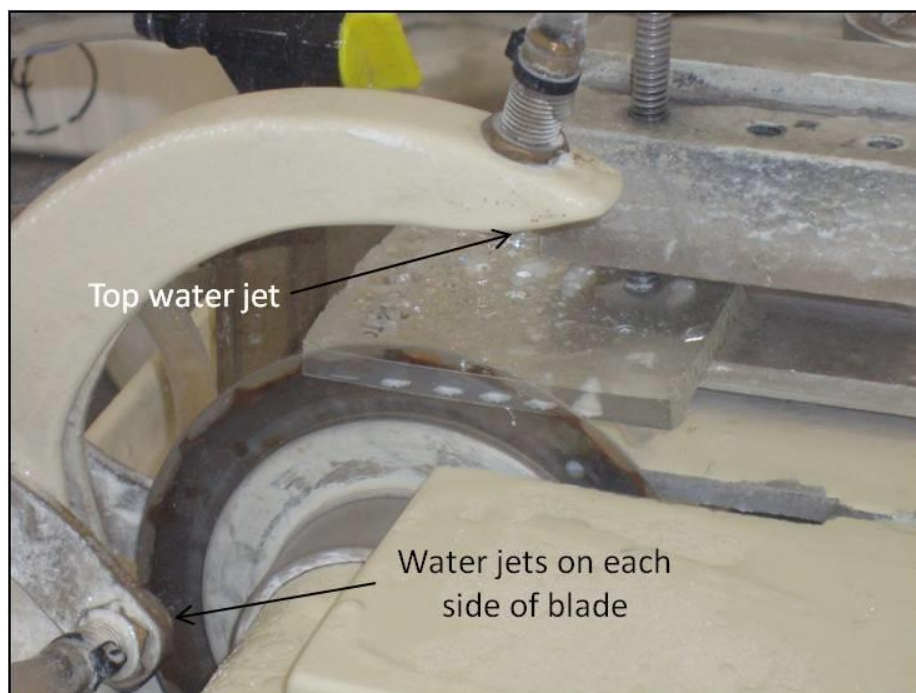


Figure 31. Sectioning of otoliths in progress, showing position of water jets.

6.3 Steps for sectioning

1. Mount the block in the jig on the saw using the front screw only. The line of primordia in the column of otoliths should be to the right of the saw blade by about 2 mm;
2. If using the GemMasta GS6D, the indicator on the position-adjustment-knob ('winder') should be on a whole number (0, 2, 4, 6, 8);
3. Holding the table-slide (as shown in Figure 32), slowly move the jig towards the blade and begin the cut;



Figure 32. Sectioning otoliths on the GemMasta GS6D.

4. For the first cut, continue the cut completely through the block;
 5. Pull the table-slide back and then SWITCH THE MACHINE OFF before advancing the winder. And, as example, advancing the winder by 6 units will provide a section 280 to 320 μm Sections thinner than this are likely to be too translucent for reading;
- Note:** Be careful to only turn the winder to advance the block, and the machine SHOULD ALWAYS BE SWITCHED OFF whenever making position adjustments;
6. When set in the new position, switch on, and again move the table-slide to make the second cut, but this time stop the cut by slowing and stopping approximately 1 mm from the edge of the block (Figure 33). This allows the section to be removed by hand before it drops off the block;

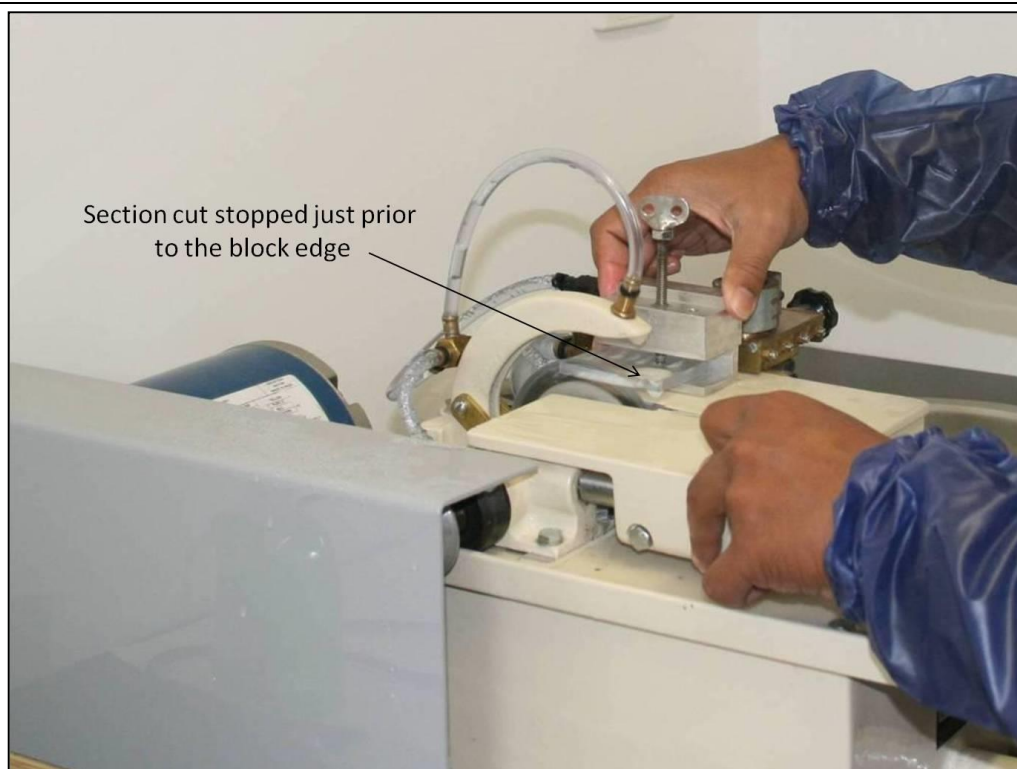


Figure 33. Cutting through the otolith block but stopping just before the edge so that the section does not drop off and can be carefully removed by hand, AFTER THE MACHINE IS TURNED OFF.

7. Break the section off with your fingers and place it on the table;
8. Repeat these steps until you have taken your desired number of sections through the block (commonly 4 – 6 sections);
9. The sections are then washed in water to remove particulate resin (Figure 34);

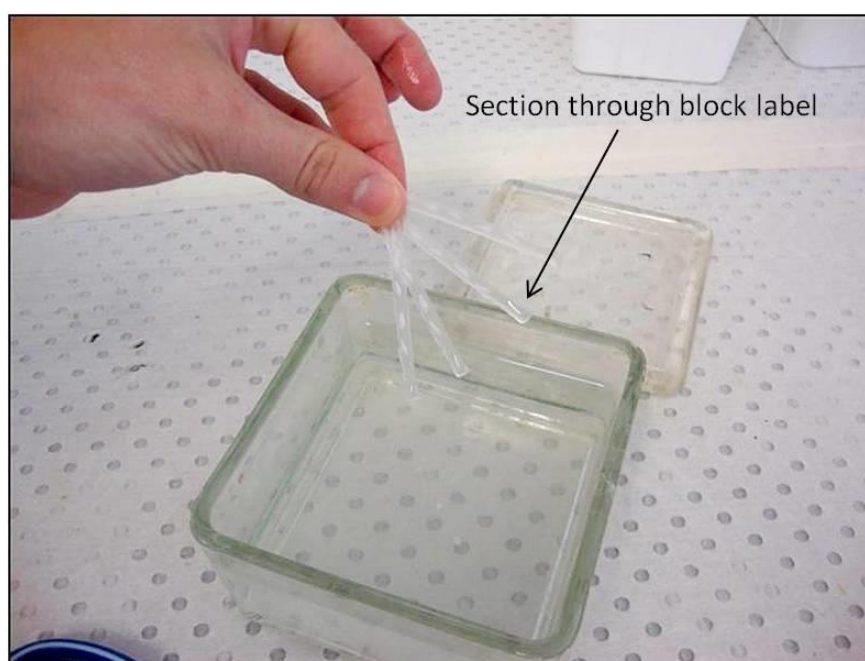


Figure 34. Sections being washed to remove particulate resin. Care must be taken as the sections are delicate. The sectioned block label is visible as a short straight line at the end of the section.

10. CAREFULLY dry the sections by laying them on absorbent tissue and place them in a glass or plastic vial (Figure 35). The vials can be stored in a tray to keep things in order. Make sure the slide label in the vial matches the sections you are placing in the vial. The sections should be placed into the vials with the label-end to the top. The sectioned label will appear as a short vertical line at the end of your section (Figure 34).

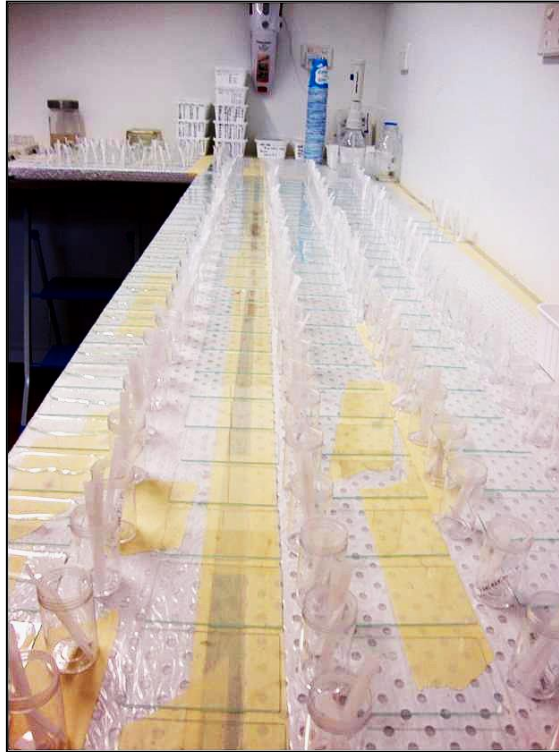


Figure 35. Multiple sections from blocks, placed into separate vials together with a slide label and placed next to large glass slides.

6.4 Mounting sections onto slides

1. Lay slides out on the bench in a row, with the number of slides equal to the number of vials. Use two or more rows if necessary;
2. Place a vial containing the sections above each slide;
3. Take a plastic pipette and cut the end off, as shown in Figure 36. Doing this is necessary as the resin is viscous and will not flow through an uncut pipette;



Figure 36. Plastic pipette with tip cut off. Note the angle of the cut.

4. Using the pipette, place about 2 – 2.5 ml of freshly mixed resin along the left-hand side of the slide;
5. Spread the resin evenly across the slide with a plastic spreader. A good spreader can be made by trimming the edges off the lid of a clear plastic takeaway food container and cutting it into thirds;
6. Take a section from your vial and place it on the left-hand side of the slide;
7. The sections must be placed on the slide with the paper cross section (from the block label) at the top and the otolith sulcus on the right-hand side. Continue until four sections are on the slide;
8. Gently press each section into the resin layer on the slide;
9. Leave the slides on the lab bench for the resin layer to set. Do not put the slides into an oven before the coverslips have been added;
10. Place another 2 ml of resin on the slide in a line on the left-hand side of the slide and use the plastic spreader to spread the resin over the slide;
11. Place a coverslip with the long edge in the middle of the slide;
12. Carefully lower the coverslip onto the resin being careful not to trap bubbles of air underneath;
13. Place a cover slip on the other side, starting from the middle of the slide. Carefully lower to complete the cover of the whole slide;
14. Again leave the slides on the lab bench for the resin to set;
15. Finish the slide making by placing your slides on a tray and 'cooking' in the oven at 55° for 3 hours.

7. Setting up your microscope and your ergonomic position

7.1 Ensuring parfocal set-up of microscope optics

BEFORE you commence a session of otolith reading, **it is essential that you have the optics of your microscope adjusted to suit your eyes**. Everyone's eyes are different, and if you shared a microscope with other users, you should not assume that the microscope has been left by the previous user with the eyepieces set to suit the focal distances of your eyes. If you do not have the microscope eyepieces set to suit your eyes you are likely to experience bad headaches and tiredness.

Are you left or right eye dominant?

Before you can set the microscope eyepieces to suit your eyes, you need to know whether you are left or right eye dominant. Approximately two-thirds of people are

right-eye dominant and one-third left-eye dominant. However, in a small portion of the population neither eye is dominant.

The following is a relatively easy way to determine whether one of your eyes is more dominant than the other:

1. WITH BOTH EYES OPEN, fully extend one arm (left or right) with your forefinger upright and align your finger to the edge of an object or other vertical line at least several metres away (e.g. edge of a door or bookshelf on the other side of the room if you are inside, or a the edge of a building across the street if you are looking outside);
2. Now repeatedly close and open your left eye, using your free hand as the cover if you need to, and note how much the position of your forefinger appears to move relative to the object you chose, when the eye is closed;
3. Keeping your extended arm and forefinger locked on the same object, now open your left eye and repeatedly close and open your right eye in the same manner. Again, note how much your forefinger appears to move relative to your chosen object;
4. If your forefinger moves significantly more when your left eye is closed and right eye is open, that means you are left eye dominant. If your forefinger moves significantly more when your right eye is closed and your left eye is open, that means you are right eye dominant. Your dominant eye is the one that provides your brain with the most accurate positional information.

If there was no significant difference in the distance moved by your forefinger during the opening and closing of your left and right eyes, then you are one of the minority of people who do not have a dominance of one eye over the other.

Adjusting your microscope eye-pieces - see Figure 37 for the steps, illustrated with photos.

Once you have determined whether you are left or right eye dominant, follow these steps to set your microscope eyepieces to the best position for your eyes:

1. Place something onto the microscope stage onto which you can easily adjust focus. A business card with some small text or other flat object with typed letters on it is good for this purpose;
2. Adjust the inter-eyepiece distance to best suit the distance between your eyes. There is often a large difference in this inter-ocular distance, from person to person;
3. Focus the microscope with main focus control to the point of sharpest image possible;
4. Set the adjustable eyepiece focus-ring to "0" for the eyepiece on your dominant side;
5. With your dominant eye closed, set the adjustable eyepiece focus-ring for the eyepiece on your non-dominant side to sharpest focus;

6. Open your dominant eye, close your non-dominant eye, and set the adjustable eyepiece focus-ring for the eyepiece on your dominant side to sharpest focus;
7. Now view your object with both eyes open. Hopefully it will appear in sharp focus for both your eyes at the same time **i.e. the eyepieces are parfocal.**

Note: Not all stereo and compound microscopes will have adjustable eyepiece focus-rings with a “+/-” scale marked on both eye-pieces. If absent from one or both eyepieces, the underlying principles of eyepiece adjustment remain the same: adjust each eyepiece to sharpest focus independently, first for your non-dominant eye and then for your dominant eye. Some older microscopes may only have one adjustable eyepiece, the other being non-adjustable. In such cases, first achieve a sharp image looking down the non-adjustable eyepiece by adjusting focus with the microscope’s main focus knob, and then achieve sharp focus for the adjustable eyepiece by adjusting its focus-ring. This is done with the relevant eye open for each side, and the other eye closed.



Figure 37. Steps in the set-up of microscope to ensure eyepieces are parfocal, prior to commencing otolith readings.

Note: The ergonomics of the work set-up shown are not ideal. The microscope is too low (or person is sitting too high) and the user has to 'hunch' over to see down the eyepieces.

7.2 Ergonomics

Equally important as having your microscope eyepieces set to the most comfortable focus positions for your eyes is doing a good ergonomic⁷ assessment of your work station before you commence an otolith reading session. If your work station is poorly set up for body posture, the subsequent stresses and strains on your body will most likely lead to aches and pains in your shoulders, lower and upper back, neck and arms, and possibly headaches. If you repeatedly do long sessions of microscope work with bad body posture you may end up with a serious Repetitive Strain Injury and a bad back!

The following points relate to achieving a good work station set-up (Figure 38) for your otolith reading at the microscope:

1. The bench height is extremely important. Ideally the bench height would be adjustable so that eye-piece height is adjustable, but this is often not practicable. The best alternative is to adjust the height of the microscope and your chair to achieve a good posture;
2. The back of your chair should ideally be high enough to support the shoulder blades and be adjustable for height and angle, with the most prominent part being the lumbar support. The seat should be low enough to have your back straight and allow you to see through the microscope eye-pieces without having to tilt your head downward. The eyepieces should rest just below the eyes with your eyes looking downward at an angle 30 to 45 degrees above the horizontal;
3. If needed, the height of the microscope can be adjusted by placing books or other stable support underneath to achieve the position mentioned above;
4. The most common mistake is to have the seat of your chair too high, and/or the microscope too low which results in 'hunching'. Sitting for long periods in the hunched position places strain on the lower back and shoulders (Figure 38);
5. Your neck and head should bend as little as possible, preferably no more than 10 - 15 degrees from vertically straight;
6. Your upper arms should be perpendicular to the floor, elbows close to the body (not winged or sticking out), forearms parallel to the floor; wrists should be straight;
7. Consider using supports under your forearms so that you do not constantly keep lifting your arms off the bench to adjust the microscope. These supports may be best if they are padded and are sloping;
8. Your feet should rest firmly on the floor or a footrest, and not tucked underneath the chair. You should adjust the position of the feet from time to time, to spread the load on the back and leg muscles;

9. TAKE REGULAR REST BREAKS (every 20 – 30 mins) AND DO STRETCHES.

⁷ "Ergonomics" is sometimes defined as the science of fitting the work to the user instead of forcing the user to fit the work. Physical ergonomics is most often related to body posture in the work situation.

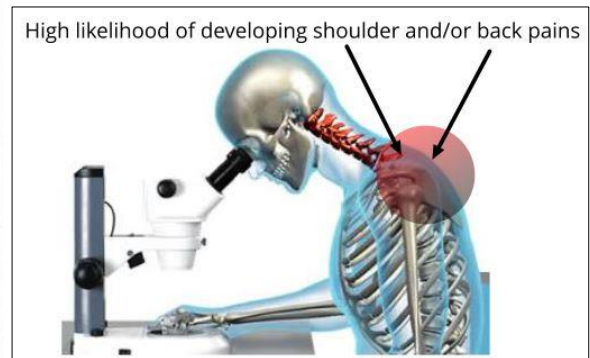
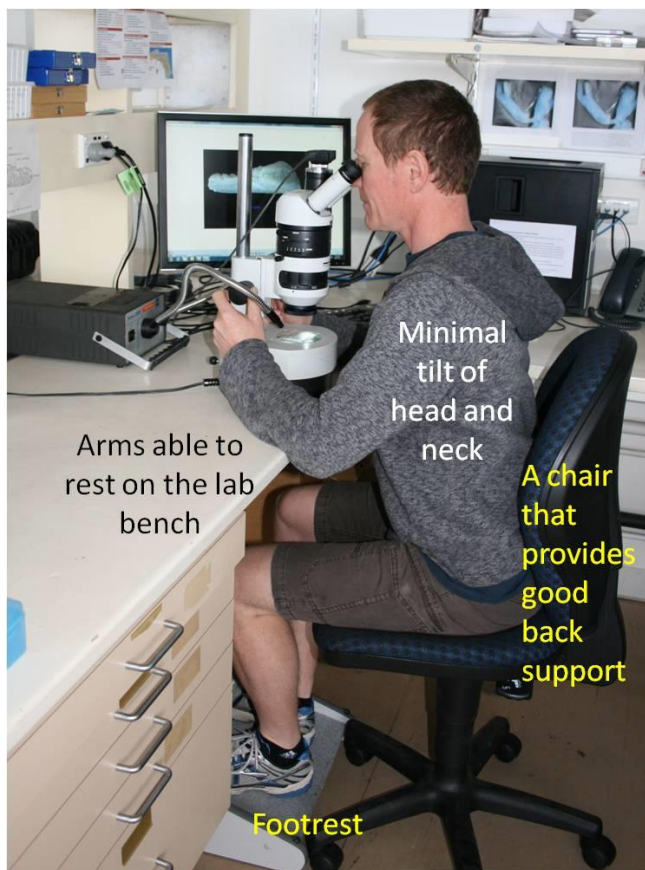


Figure 38. Demonstrating (left) the features of a good ergonomic set-up, and emphasizing (right) that a microscope and chair set-up that requires significant neck and head tilt has a high chance of causing shoulder and/or back pains. (Right image – Modified image of Vision Engineering).

8. Useful resources

This is not intended as a comprehensive list of otolith related resources. The scientific literature on use of otoliths for ageing fish is vast. The following are various resources that you may find useful. The website links provided below were correct at time of writing this manual.

Other fish ageing manuals

Anon (2002) A manual for age determination of Southern Bluefin Tuna, *Thunnus maccoyii*: Otolith sampling, preparation and interpretation. A product of The Direct Age Estimation Workshop of the Commission for the Conservation of Southern Bluefin Tuna, 11 – 14 June 2002, Queenscliff, Victoria, Australia, 36 pp.

https://www.ccsbt.org/sites/ccsbt.org/files/userfiles/file/docs_english/operational_resolutions/age_determination_manual.pdf

Dwyer, K. (2004) Yellowtail Flounder (*Limanda ferruginea*) ageing manual. Submitted to Scientific Council Meeting of Northwest Atlantic Fisheries Organization, June 2004. NAFO SCR Doc. 04/5, Serial No. N4947. 27 pp.

<http://archive.nafo.int/open/sc/2004/scr04-005.pdf>

Matta, M.E., and Kimura, D.K. (eds). (2012). Age determination manual of the Alaska Fisheries Science Center Age and Growth Program. NOAA Professional Paper NMFS 13, 97 pp. <https://repository.library.noaa.gov/view/noaa/4149>

Nowara, G., Verdouw, J. and Hutchins, J. (2008) Otolith preparation and ageing of Patagonian Toothfish, *Dissostichus eleginoides*, at the Australian Antarctic Division. In: Evaluating gear and season specific age-length keys to improve the precision of stock assessments for Patagonian Toothfish at Heard Island and McDonald Islands. Editors: D.C. Welsford, G.B. Nowara, S.G. Candy, J.P. McKinlay, J.J. Verdouw and J. J Hutchins. FRDC Tactical Research Fund Project 2008/046 Final Report. 78 - 101.

See: <http://frdc.com.au/Archived-Reports/FRDC%20Projects/2008-046-DLD.PDF> and for methods of otolith preparation refer to the appendices.

Secor, D.H., Dean, J.M. and Laban, E.H. (1991) Manual for otolith removal and preparation for microstructural examination: A users manual. 85 pp.

Otolith identification guides (atlases)

Campana, S.E. (2004) Photographic atlas of fish otoliths of the Northwest Atlantic Ocean. NRC Research Press, Ottawa, Ontario. 284 pp.

Furlani, D., Gales, R. And Pemberton, D. (2007) Otoliths of common Australian temperate fish: A photographic guide. CSIRO Publishing, Australia. 216 pp.

Smale, M.J., Watson, G., and Hecht, T. (1995) Otolith atlas of southern African marine fishes. J.L.B. Smith Institute of Ichthyology, 253 pp.

Lin C-H. and Chang C-W. (2012). Otolith Atlas of Taiwan Fishes. National Museum of Marine Biology and Aquarium. NMMBA Atlas Series 12 (ISBN: 978-986-03-5356-3). 45pp.

Fish ageing/otolith reading practical exercises

Age reading demonstration and interactive tests on NOAA website:

https://www.afsc.noaa.gov/refm/age/ageinginteractive/atka_easy.htm

(long time for page to upload)

9. References

Beamish R. J. and Fournier D. A. (1981). A method for comparing the precision of a set of age determinations. Canadian Journal of Fisheries and Aquatic Sciences 38, 982–983.

Campana S. E. (2001). Accuracy, precision and quality control in age determination, including a review of the use and abuse of age validation methods. Journal of Fish Biology, 59, 197-242.

Campana S. E. and Casselman J. M. (1993) Stock discrimination using otolith shape analysis. Can. J. Fish. Aquat. Sci. 50, 1062 – 1083.

Cooper A. B. (2006). A Guide to Fisheries Stock Assessment From Data to Recommendations. University of New Hampshire. 44 pp.

Farley J. H., Eveson J. P., Davis T. L. O., Andamari R., Proctor C. H., Nugraha B. and Davies C. R. (2014). Demographic structure, sex ratio and growth rates of Southern Bluefin Tuna (*Thunnus maccoyii*) on the spawning ground. PLoS ONE 9(5): e96392.

<https://doi.org/10.1371/journal.pone.0096392>

Fenton G. E., Short S. A. and Ritz, D. A. (1991). Age determination of orange roughy, *Hoplostethus atlanticus* (Pisces: Trachichthyidae) using ²¹⁰Pb:²²⁶Ra disequilibria. Mar. Biol. 109, 197 – 202.

Francis M. P.; Mulligan K. P.; Davies N. M. and Beentjes, M. P. (1999). Age and growth estimates for New Zealand hapuku, *Polyprion oxygeneios*. Fishery bulletin 97(2), 227-242.

Haddon M. (2011). Modelling and Quantitative Methods in Fisheries. 2nd edition. Publisher Chapman and Hall, CRC Press. 471 pp.

Jatmiko I. (2012). Investigating secondary growth increases of Redfin Perch, *Perca fluviatilis*, in Tasmania. Masters of Applied Sciences Theses. Australian Maritime College, University of Tasmania, Australia. 43 pp.

Maunder M. N. and Piner K. R. (2014). Contemporary fisheries stock assessment: many issues still remain. ICES Journal of Marine Science, 72, 7–18.

Morales-Nin B. (1992). Determination of growth in bony fishes from otolith microstructure. FAO Fisheries Technical Paper No. 322, Rome, FAO, 51 pp.

Murua H., Rodriguez-Marin E., Neilson J. D., Farley J. H. and Juan-Jorda M. J. (2017). Fast versus slow growing tuna species: age, growth, and implications for population dynamics and fisheries management. Rev. Fish Biol. Fisheries. 41 pp.

Pannella G. (1980). Methods of preparing fish sagittae for the study of growth patterns. In, Skeletal growth of aquatic organisms, edited by D. C. Rhoads & R. A. Lutz, Plenum Press, New York, 750 pp.

Payan P., De Pontual H., Boeuf G. and Mayer-Gostan N. (2004). Endolymph chemistry and otolith growth in fish. C. R. Palevol 3, 535 – 547.

Siregar K. (2008). Teknik pengambilan otolith southern bluefin tuna (*Thunnus maccoyii*). A manual as output of the Indonesia-Australia Southern Bluefin Tuna sampling program, Benoa (Bali). 18 pp.

Smith D. C., Robertson S. G., Fenton G. E. and Short S. A. (1995). Age determination and growth of orange roughy (*Hoplostethus atlanticus*): a comparison of annulus counts with radiometric ageing. Canadian Journal of Fisheries and Aquatic Sciences, 52(2), 391-401.

Stransky C., Murta A. G., Schlickeisen J. and Zimmermann C. (2008) Otolith shape analysis as a tool for stock separation of Horse Mackerel (*Trachurus trachurus*) in the Northeast Atlantic and Mediterranean. Fisheries Research, 89, 159-166.

Sun, C-L., Wang W-J., and Yeh S-Z. (2005). Reproductive biology of the female Yellowfin Tuna *Thunnus albacares* in the western Pacific Ocean. Paper submitted to 1st Meeting of the Scientific Committee of the Western and Central Pacific Fisheries Commission, Noumea, New Caledonia, 8 – 19 August 2005. WCPFC–SC1-BI WP–1. 14 pp.

Tracey S. R., Lyle J. M., and Duhamel G. (2006) Application of elliptical Fourier analysis of otolith form as a tool for stock identification. *Fisheries Research* 77(2), 138-147.

Wells J. V. and Richmond M. E. (1995). Populations, metapopulations, and species populations: What are they and who should care? Wildlife Society Bulletin. 23 (3): 458–462.

10. Further information

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