Manual for routine age estimation of Patagonian toothfish (*Dissostichus eleginoides*)

Diverse • Professional • Resilient • Resourceful

Otolith selection, preparation, interpretation, and precision analysis.

FEBRUARY 2026, VERSION 2

Le Luherne E.

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VERSION 2

Participating Scientific Staff

Emilie Le Luherne (FIFD, Fisheries Scientist Toothfish) – Writing - Original Draft Preparation, Pictures

Comments provided by

Andreas Winter (FIFD, Head of Science)

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Andreas Winter [signature] Date: 04 February 2026
Head of science

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The following protocol is in used at the Falkland Islands Fisheries Department (FIFD) and describes with details the different steps from otolith sampling onboard fishing vessel to age estimation.

1. Introduction

The Patagonian toothfish *Dissostichus eleginoides* Smitt 1898 (family Nototheniidae) is a large (>200 cm TL, >150 kg weight) slow-growing benthopelagic predatory fish. Toothfish have a wide geographic distribution in the Southern Ocean, occurring across the continental shelf, slope, and deep-sea plains of southern Patagonia and Chile; on plateaus surrounding sub-Antarctic islands (e.g. South Georgia, Kerguelen, Heard and McDonald Islands), banks and seamounts (Eastman, 1993; Collins et al., 2010).

The Falkland Islands Patagonian toothfish longline fishery began in 1992 as an exploratory fishery and became an established fishery in 1994 (Laptikhovsky and Brickle, 2005). This fishery is managed by the Falkland Islands Fisheries Department (FIFD) according to international boundaries and its first stock assessment was held in 2005. In the longlining fishery, Patagonian toothfish are targeted in depths greater than 600 m all year around with a closing period between the 1st of June and the 31st of August at Burdwood Bank (Saulnier and Skeljo, 2024). The regulation of the fishing on Burdwood Bank, the main spawning area within the Falkland Islands waters, was established in 2006 to protect the stock from overfishing during the spawning season (Saulnier and Skeljo, 2024). Besides targeted longline fishery, toothfish are bycatch in the shelf-based (<400 m depth) finfish and calamari trawler fisheries. In the finfish fishery, toothfish is a commercially valuable bycatch, while they are typically discarded due to the small size of the specimens in the calamari fishery (20-30 cm; Skeljo and Winter, 2024). These fisheries exploit different parts of the toothfish population in distinct areas: longlining occurs on the deep-water slope, while finfish trawling occurs on the shelf primarily north and west of the Falkland Islands, and calamari trawling on the shelf south and east of the Falkland Islands (Skeljo and Winter, 2024). These fisheries are two components of the Patagonian toothfish stock around the Falkland Islands, and are thus considered in the stock assessment modelling framework (Skeljo and Winter, 2024). The Patagonian toothfish stock assessment has been undertaken using an integrated age-structured model implemented in CASAL (Bull et al., 2012; Skeljo and Winter, 2024). Management advice is thus based on statistical catch-at-age models which are used to estimate relative biomass (Skeljo and Winter, 2024).

The validity of catch-at-age models relies on a robust estimation of the population age distribution. The age estimation of Patagonian toothfish, a slow-growing species, is thus critical to a robust stock assessment. From 2014, the FIFD has been in charge of the annual age estimation of its Patagonian toothfish population. No protocol including all the steps from the collection of the otoliths onboard the fishing vessels to the age estimation precision analysis has yet been written at the FIFD. This protocol thus gathers the FIFD protocols for otoliths collection (Lee, 2019) and laboratory processing (Le Luherne and Peruzzo, 2024), and describes the other steps based on the FIFD procedures not yet described in FIFD protocols, but described in protocols internationally recognised for toothfish otoliths processing (Sutton et al., 2012; Nowara et al., 2014, 2024; CCALMR, 2025).

2. Collection of otolith onboard fishing vessels

2.1. Otolith collection rules

Patagonian toothfish otoliths have been collected since the establishment of the Falkland Islands Fisheries in 1987. They are routinely sampled by Fisheries Observers and Scientists aboard commercial fishing vessels and during dedicated research surveys. Otolith sampling is conducted quarterly (Table 1), with the schedule designed to reflect the temporal and spatial distribution of fishing activity throughout the year.

Table 1. Description of the quarterly collection time period.

Collection	Period covered
A	January-March
B	April-June
C	July-September
D	October-December

For each quarterly collection, otoliths from Patagonian toothfish are collected to reflect the length distribution of sampled individuals. For this purpose, we apply a fixed otolith sampling strategy (FOS; Chang et al., 2019), collecting a specified number of otoliths per sex (juveniles, males and females) and per 1 cm length interval for each quarter (Table 1). These sampling targets have evolved over time to accommodate the requirements of the archive collection and the objectives of specific research projects (Table 2).

Table 2. Number of Patagonian toothfish otoliths collected per sex (juveniles, males and females) and 1 cm length interval for each quarter (Table 1), and by fishery since the inception of the collection.

Dates	Trawler and surveys (per sex/cm/quarter)	Longliner (per sex/cm/quarter)
1987-2016	5	5
2017-2018	5	2
2019-2025	5	3
2026 onwards	3	3

Onboard the trawlers, we were collecting five otoliths per sex (juveniles, males and females) for each 1 cm length interval of each quarter (Table 2). However, since 2022 only around 15% of the otoliths collected on trawlers were selected for routine age estimation, whereas the proportion aged from longliner collections ranged between 25% and 60%. Consequently, we have recommended reducing the sampling effort from five to three otoliths per sex and length interval per quarter from 2026 onwards. Onboard the longliner, we are collecting three otoliths by sex and 1 cm length interval for each quarter (Table 2).

Every week, the Observers Manager compiles all the data from the Fisheries Observers working onboard a vessel, updates the ongoing quarterly collection, and provides an update on the otolith collection status to the Fisheries Observers.

During the bi-annual Groundfish and Pre-season Assessment Falkland calamari (*Doryteuthis gahi)* surveys, additional otolith collections are carried out, which are then linked to the ongoing collection. In the Groundfish surveys, otoliths are sampled using a combination of fixed (FOS) and random (ROS) otolith sampling strategies (Chang et al., 2019). For the FOS, we collect five otoliths per sex (juveniles, males, and females) for each 1 cm length interval, reduced to three from 2026 onwards (Table 2). Additionally, two otoliths from randomly selected individuals are extracted per station as part of the ROS strategy to enhance the spatial coverage of the otolith collection (Ramos et al., 2025). In the Pre-season Assessment Survey for Falkland calamari (*Doryteuthis gahi)*, we also collect five otoliths per sex for each 1 cm length interval, reduced to three from 2026 onwards (Table 2). Furthermore, supplementary non-random otoliths are collected whenever juvenile toothfish (less than 20 cm) are caught (Chemshirova et al., 2025).

2.2. Otolith extraction and storage

The list of materials needed for this step is specified in Annex 1.

We use three different techniques for extracting otoliths from Patagonian toothfish, depending on the fishery being sampled (Fig. 1).

Onboard the trawlers, otoliths are extracted by positioning a sharp knife with the blade just behind the pre-opercula, slightly more than halfway between the eyes and the corner of the gill cover. A vertical cut is then made through the fish's head (Fig. 1.a). An alternative method is to hold the knife horizontally, making a lateral cut from the base of the skull, aiming for slightly above the mid-point of the eyes (Fig. 1.b). Once the knife breaks through the skull, the head is carefully cracked open to expose the brain (Fig. 1.b).

Onboard the longliner, otoliths are extracted using the keyhole method to prevent damaging the cheeks, which are a by-product of the longlining toothfish fishery. A shape knife is used to create a rectangular opening on the top of the fish's head (Fig. 1.c).

The otoliths are located on the ventral side on each side of the brain tissue. They are carefully removed using tweezers (Fig. 1), cleaned of any remaining organic material, and dried before being stored in otolith paper envelopes. For individuals smaller than 80 cm, the otoliths are placed in an Eppendorf tube filled with 96% ethanol which is then stored in otolith paper envelope to prevent breakage during transportation.

The following information is recorded on the otolith paper envelopes, including callsign, station, serial number, species, length, weight, sex, and maturity.

Otolith paper envelopes are sorted by sex, length, callsign, and station for each quarter and are stored in the archive otolith collection. The Toothfish Fisheries Scientist is notified once the otoliths from each quarterly collection are sorted, checked, and ready for extraction and processing.

a
b
c

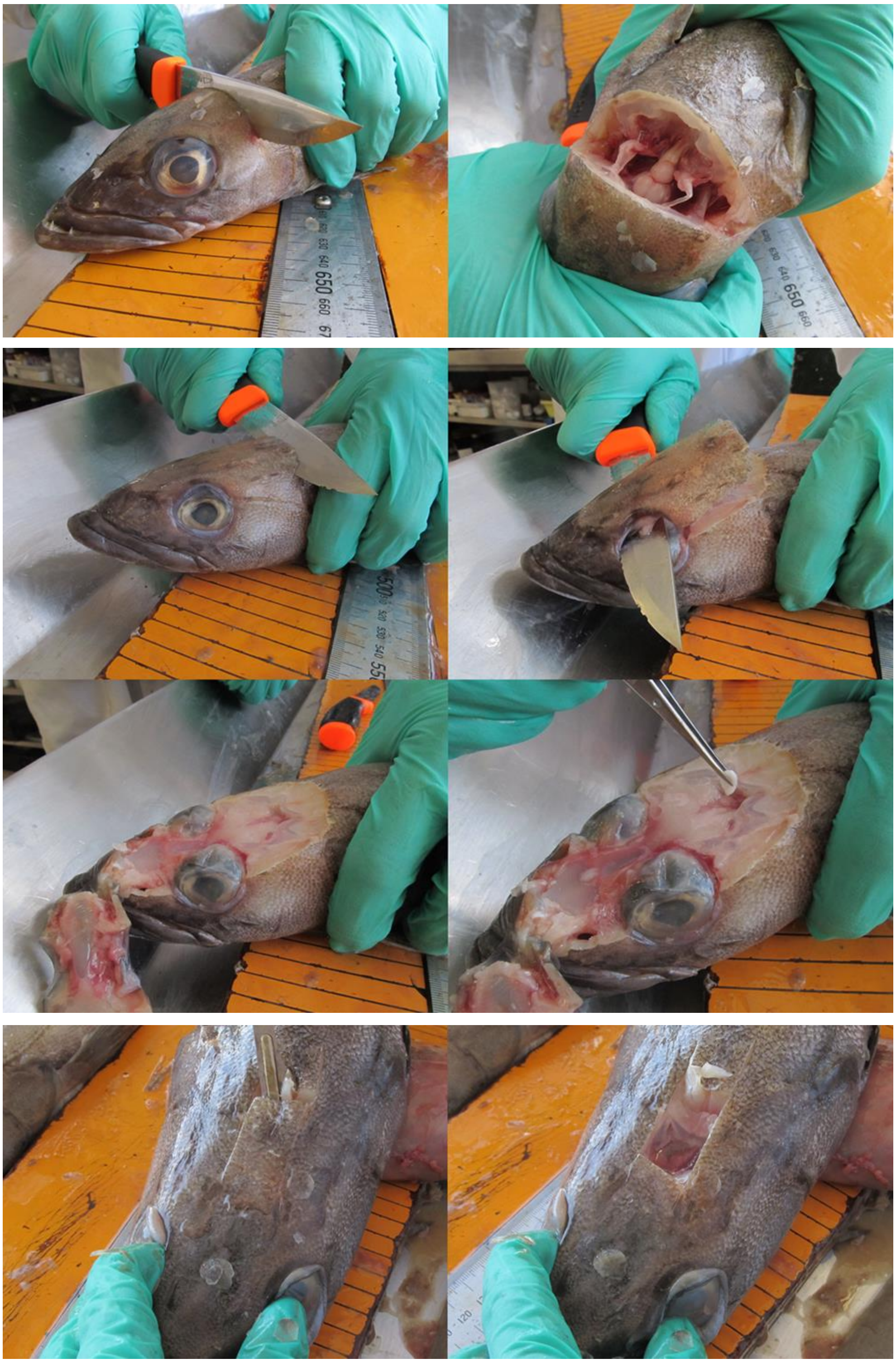

Figure 1. Patagonian toothfish head cutting and otoliths extraction using a (a) vertical cut and a (b) diagonal cut for the individuals sampled onboard the trawlers, and (c) the keyhole method for the individuals sampled onboard the longliner.

3. Selection of otolith for age estimation

Between 2014 and 2021, we aimed to select 1000 otoliths annually for age estimation. From 2022 onwards, this target number has been lowered to 500 otoliths to strike a balance between the minimum age estimations needed each year and the time-consuming nature of the age estimation process. This reduction was recommended by Lee B., who was responsible for Patagonian toothfish age estimation at the time, and was endorsed by the Head of Science.

Between 2014 and 2021, otoliths selected for age estimation were chosen to reflect the length distribution of the sampled fish. The selection process involved manually reviewing the otolith collection and choosing otoliths by sex and quarterly distribution to cover the length frequency for the analysed year. We also ensured that this selection overlaps with the trawling and longlining fisheries for the lengths common to both fisheries. This method ensures that a sufficient number of otoliths are aged for all lengths on both a temporal and spatial basis.

From 2022 onwards, the otoliths selection for age estimation has been modified to the following procedure. A minimum of 60 otoliths per sex and quarterly collection is selected to cover the length distribution of sampled fish for age estimation (n = 480). Using an R script, we select 20 otoliths/sex/quarterly (25 in 2022 and 2023) for trawlers and 40 otoliths/sex/quarterly (37 in 2022 and 2023) for the longliner to cover the length frequency of the analysed year. We also ensure that this selection overlaps with the trawling and longlining fisheries for the lengths common to both fisheries. We select otoliths to have at least one individual per 5 cm length class for every sex/activity/quarterly collection. This ensures that sufficient otoliths are aged for all lengths on a temporal and spatial basis. To meet the target of 500 otoliths, we additionally selected large individuals with a total length greater than 120 cm to account for variability in the age distribution of larger specimens.

It should be noted that we do not actively select otoliths from different spatial areas. We believe that the spatial distribution is adequately represented by selecting otoliths from each quarterly collection, as fishing vessels target different areas on a weekly or monthly basis. The spatial coverage of the selected otoliths is yet checked to validate that it is representative of the annual spatial distribution of the Patagonian toothfish captures.

4. Laboratory processing of otolith for age estimation

The list of materials needed for this step is specified in Annex 1.

Otolith laboratory processing is performed in the Fisheries Department wet laboratory. To work in the wet laboratory, appropriate Personal Protective Equipment (PPE) must be worn, including laboratory coat, closed shoes, and gloves (when needed).

You must work under the fume cupboard to prepare West System® Epoxy Resin and to clean the grinding disks with acetone.

4.1. Collection of the selected otoliths

The selected otoliths are picked in the archived collection, and the information written on the envelopes is cross-checked with the printed list to ensure their validity. The suitability of the otoliths for age estimation analysis is checked, i.e. at least one otolith is not vateritic and not broken close to the nucleus. If no otolith is suitable, we exchange it for another individual with the same or similar size, sex, maturity and area. Once the final list of individuals is produced, we assign a unique serial number to each individual and write it down on the Excel sheet and on the envelope with a marker.

4.2. Otolith embedding

Otoliths are embedded between two layers of resin. The first layer has to be made at least a day before to let it cure, and should reach a thickness of 2–3 mm (Fig. 2.b and Annex 2). Make sure that this first layer is as smooth as possible (i.e., flat and regular surface) and try to avoid bubbles at its surface because it will affect the future positioning of the otoliths.

Before placing the otolith in the well, remove as much organic material as possible that is sticking to the otolith. Bubbles often adhere to any leftover organic material on the otoliths, which can weaken the resin embedding. If the material can’t be removed with the preparation needle, soak the otolith in tap water and remove the organic material. Please note that after soaking, the otolith needs to dry for at least 24-48 h before being embedded in resin, as the embedding process will not work on wet material.

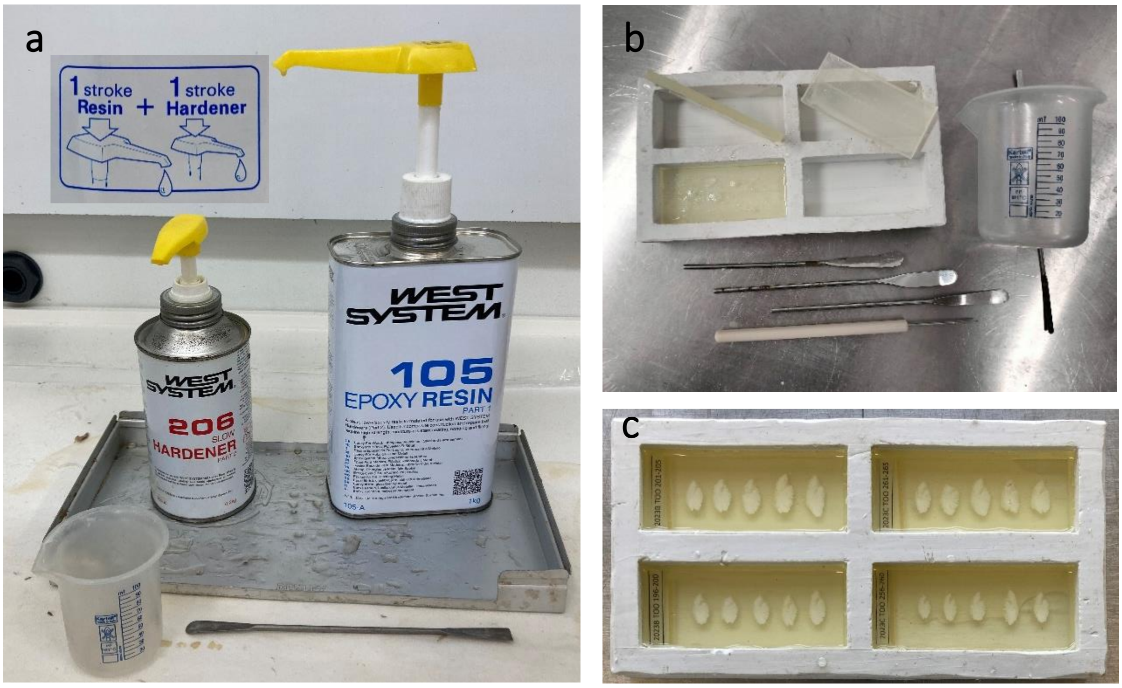

Figure 2. (a) West System® Epoxy Resin components: 105 Epoxy Resin and 206 Slow Hardener. (b) Material for otolith embedding: silicon mould, spatulas, beaker, preparation needle, and resin base examples. (c) Otoliths embedded with label and spaghetti.

Place in the well the label and a piece of spaghetti, then place the first clean and dry otolith close to the spaghetti and continue in ascending order of serial number (Fig. 2.c). Place otoliths with rostrum part up and sulcus face down towards the first layer of resin (Fig. 2.c), checking every time that the number on the envelope and on label are matching. To facilitate otolith reading, choose consistently the same otoliths (left or right) of each pair and always place the otoliths in the same orientation (Fig. 3). To prevent trapping air inside the sulcus, and potentially affects the quality of the sections, place otoliths sulcus side facing you and turn them around (i.e., sulcus face down towards the first layer of resin) with the preparation needle and tweezers after pouring the second layer of resin. Using a preparation needle, place the otoliths in the same orientation perpendicular to the middle line of the well with their nucleus in line in the middle of the block (it will allow cutting less sections to reach the nuclei of the 5 embedded otoliths, Fig. 2.c and 3). To prevent otoliths from slightly changing their alignment while the resin is still fluid, they must be monitored constantly until the resin becomes very sticky (~ 15 min). Let the resin cure for at least 24h.

If no otolith remains in the envelope after taking one for age estimation, cross out the envelope. This will inform the next scientist using the otolith collection that just one otolith was collected and used for age estimation. Seal all the otolith envelopes with Sellotape and store them back in the archived otolith collection.

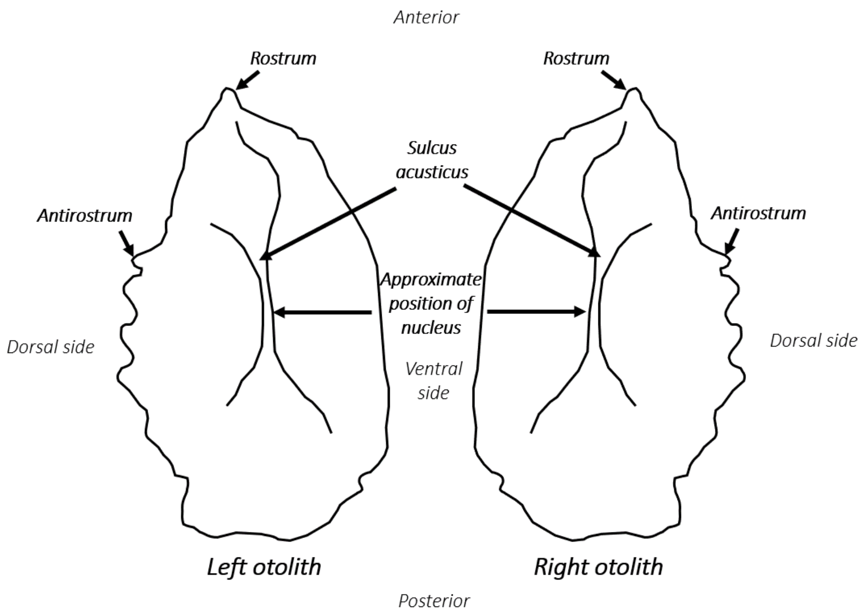

Figure 3. Illustration of left and right otoliths of Patagonian toothfish (*Dissostichus eleginoides*) with the defining features.

4.3. Otolith block cutting

3.1. Block grinding

Before cutting otolith sections, we must grind the surplus of resin from the top side of the resin block (Fig. 4.a), making sure that bottom and top sides of the resin block are flat and parallel (Fig. 4.b). This step is very important to ensure the good quality of the following cutting.

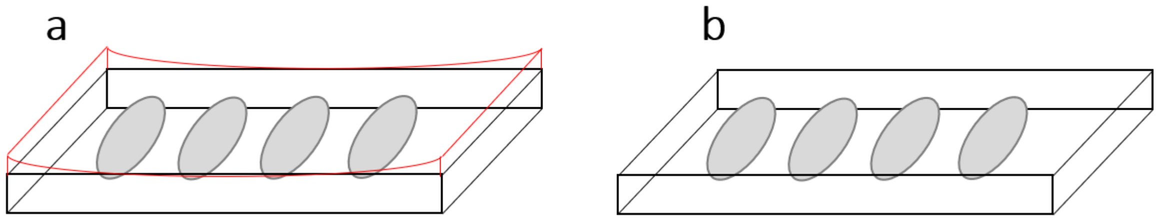

Figure 4. Resin blocks (a) before grinding with this surplus of resin in red and (b) after grinding.

The surplus of resin is ground using CarbiMet™ SiC Abrasive Paper of grain size P120 on the BUEHLER Metaserv 2000 Twin Grinder/Polisher (Fig. 5.a). For beginners, a rotation speed ≤ 150–200 rpm is recommended. Experienced scientists can increase the rotation speed.

a

b

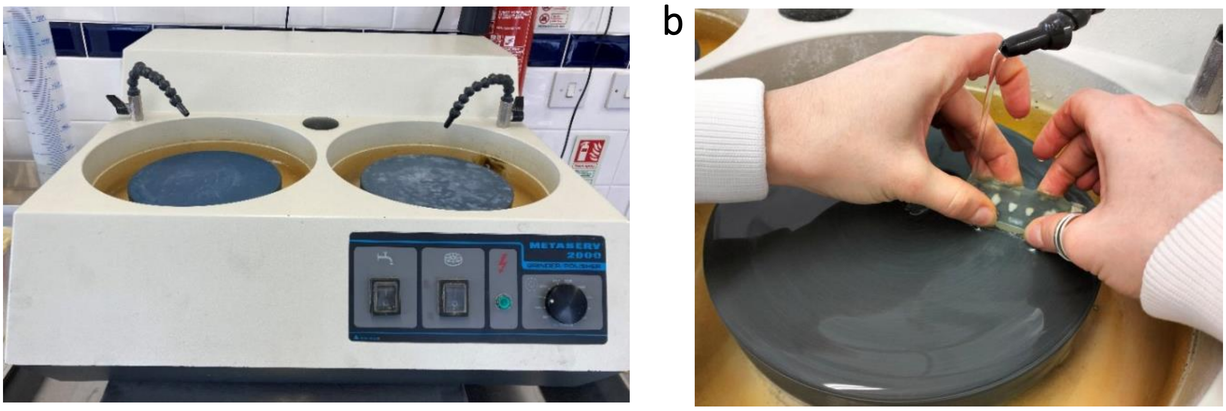

Figure 5. (a) BUEHLER Metaserv 2000 Twin Grinder/Polisher. (b) Otolith resin block grinding.

Gently but firmly support with two hands the resin block on the grinding disk and grind the excess of resin (Fig. 5.b). Level both sides of the resin block, being careful not to grind it excessively. Check the block from time to time to avoid giving an unwanted angle to the block and to reach the otoliths. Depending on the dimension of the otolith, try to obtain a thin block with a thickness of 4-6 mm. Stop grinding before reaching and exposing the otoliths. Exposing otoliths will weaken the resin embedding and may cause otolith cracking during the cutting. If you expose the otoliths, let the block dry for at least 2 days (remember that you can't embed wet material) and put a third layer of resin to re-embed it. Repeat the grinding process.

When the block is flat and both faces are parallel, dry it and write its label (i.e., collection, species, and otolith serial number) on its back with a marker. Clean the grinding machine after use.

3.2. Otolith section cutting

Circle the nucleus location with a pencil, and draw two lines with a ruler to delineate the section to cut according to the uppermost and the lowermost edges of the otolith nucleus delineation (Fig. 6).

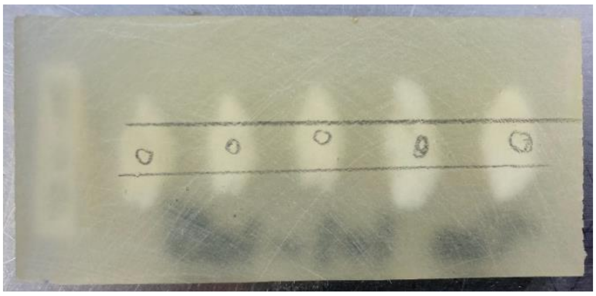

Figure 6. Identification of the nucleus and delineation of the cutting area.

The sections are cut using the BUEHLER IsoMet® Low Speed Saw with BUEHLER® Cool 3 Cutting fluid lubricant (Fig. 7). This lubricant is essential for facilitating the cutting process and preventing the saw from overheating. It is recommended to change the lubricant solution every 6-7 blocks.

Figure 7. Set up of the cutting area with BUEHLER IsoMet® Low Speed Saw, dissecting microscope and all the needed materials.

Before starting a new cutting session, the blade needs to be dressed. For this purpose, cut 6 sections of the dressing stick and replace the lubricant solution after dressing the blade.

Insert the resin block perpendicularly into the chuck (the block holder component), ensuring that the cutting delineation marks is facing the blade. Position the support arm next to the blade and align the outermost line to the blade. Set the saw speed to 10. The speed can be adapted according to the size of the otolith. The bigger the otoliths, the more sensitive they are and so the lower speed is recommended (e.g. between 7 and 5).

We use a section thickness of 80 increment (i.e. 400 µm), having experimentally found this as the right compromise between the thickness of the section and avoiding otolith cracking during the cutting. The number of sections is determined by the width from this first cut and the line delineating the end of the cutting section (Fig. 6).

Rinse the section in two different glasses of tap water and gently dab it on two layers of paper towel (Fig. 7). Cut the two edges of the section with scissors, keeping the spaghetti marker on the section (Fig. 8.a) and taking care to firmly hold the section between two fingers to avoid its division between the two layers of resin.

Check the section under the dissection microscope and decide to keep it or not according to the visible structures of the otoliths; i.e., if it is too far from the nucleus with no clear annual rings don't keep the section.

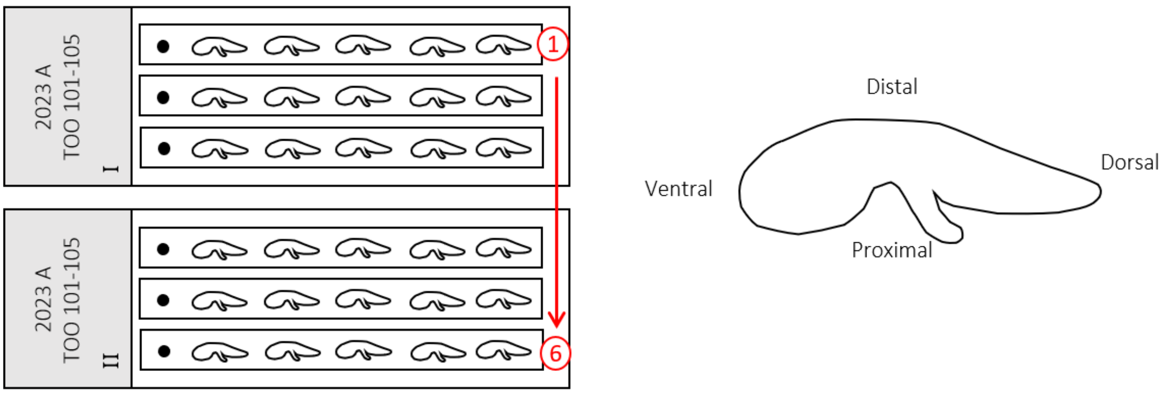

Figure 8. (a) Disposition and ordering (from 1 to 6) of otolith's sections of microscope slide and labelling example of microscope slide. (b) Features of otolith transverse section.

Place the cut section on a microscope slide with its corresponding label (Fig. 8.a). Previously clean microscope slides with 96% ethanol and a clean towel. Write down the collection, species code, and otoliths serial number on the frosted part of the microscope slide with a pencil (Fig. 8). If more than one microscope slide is needed for the otolith's sections, continue with successive slides and number them with roman numbers (i.e. I, II, etc.), to maintain the continuity of the sections and to avoid confusion with the otolith serial number (Fig. 8.a).

Arrange the otolith sections from top to bottom, starting with the first section at the top and the last section at the bottom. Place them in the same orientation with the spaghetti marker close to the frosted edge, otolith distal side facing the top of the slide, and otolith proximal side facing the bottom part of the slide (Fig. 8).

Let the sections dry for at least 24h before embedding them. It is very important to let the sections dry properly, otherwise, the second embedding becomes opaque and otolith sections are unreadable.

4.4. Otolith section mounting

Prepare a small amount of West System® Epoxy Resin under the fume cupboard (Annex 2), i.e. one pump stroke of 105 Epoxy Resin and one of 206 Slow Hardener.

Take all the otolith sections from the microscope slide and keep them in order between your fingers. Using the spatula, spread a thin layer of resin on the microscopic slide and then put back the otolith sections in the same order (Fig. 9). Leave an empty space without resin and otolith sections of at least 4 mm at the end of the microscope slide (side opposite to the frosted one) to enable the storage of the slide in the microscope slide storage box (Fig. 8.a and 9).

Figure 9. Embedded sections on a microscope slide (on the left) and sections waiting to be embedded (on the right).

With the preparation needle, remove bubbles under the otolith sections by gently squeezing and pressing them on the microscope slide. Align otolith sections of each otolith (it will help the reading under microscope; Fig. 8.a and 9). The sections can slide during the resin curing; reposition them using the preparation needle if needed.

After 24h, prepare a second small amount of West System® Epoxy Resin under the fume cupboard (Annex 2). Spread a thin layer of resin on the otolith sections and put a cover glass on the top of them covering all the otolith sections. Remove the bubbles formed on the top of otolith sections that can prevent good readability of the section. Leave an empty space with the cover glass of at least 4 mm at the end of the microscope slide (side opposite to the frosted one) to enable the storage of the slide in the microscope slide storage box. Let the resin cure for 24h and then store the slides in order in the microscope slide storage box.

5. Otolith section photography

The list of materials needed for this step is specified in Annex 1.

This step of the protocol was introduced in 2023. Before 2023, otolith age estimation was conducted under a microscope.

For optimal image quality, it is recommended to work in a dark environment to enhance contrast. Otolith section images are captured using an OLYMPUS BX51 microscope paired with an OLYMPUS DP74 camera (with OLYMPUS U-TV1X-2 & U-CMAD3 Camera Adapter). The system is connected to a computer running the CellSens software. The images are taken under transmitted light at a 20x magnification to capture the entire otolith section (i.e. objective x2 and eyepiece x10), and at a 40x magnification to focus on the ventral and dorsal parts of the otolith section (i.e. objective x4 and eyepiece x10). It is important to select the correct magnification in CellSens to ensure the scale is accurately represented in the images.

When preparing to capture images of each otolith, examine all sections to identify the best one for photography. Be sure to inspect both sides of the sections, as the optimal view may be facing the microscope slide. The ideal section is characterised by a clearly identifiable nucleus, and distinct increments and edges. It may be beneficial to photograph several sections to ensure all relevant features are documented. Save images in PNG format to preserve the embedded scale, and label each file using the following ID format: TOOyear_otolith number_magnification_picture number. Accurately saving the image with the scale is crucial, as otolith size is essential for age estimation.

6. *Otolith interpretation and age estimation*

Age estimates are made on otolith section images by counting opaque zones or increments in the otolith structure which have been shown to correspond to annual increases in growth (Horn, 2002). It is crucial for the quality of the data that all counts of annuli are made without prior knowledge of fish size or previous age estimates. Important definitions of terminology can be found in Annex 3.

Estimates of otolith age are entered first into an Excel sheet (Table 3) and when all the data are collected they are download into the FIFD database.

Table 3. Age estimation table.

callsign	station	collection	serial nb	species	sex	maturity	length	weight	oto age serial nb	readability 1	nb ring rpv	nb ring rpd	reader 1	reading date1	remark
ZDLS3	359	OT2022A	2	TOO	F	1	27	140	1						
ZDLS3	372	OT2022A	10	TOO	F	1	36	460	2						
ZDLT1	3462	OT2022A	12	TOO	F	1	40	694	3						
ZDLS3	352	OT2022A	1	TOO	F	1	50	1240	4						

Age estimation is performed using the ImageJ software. Select the image with the best resolution and open it in ImageJ. For each reading, record the otolith's readability (see section 6.1.), the number of annual increments estimated on reading path ventral (rpv) and reading path dorsal (rpd), the reader name, and the reading date.

The first step is to identify the initial increment, which can be aided by measuring the distance between the primordium and the first increment (section 6.2.2). Then, use the multipoints function in ImageJ to annotate each identified annual increment. To embed the annotation on the image, press Ctrl+B. For each otolith, perform the age estimation on the ventral (rpv) and dorsal (rpd) axis. Save the annotated image in PNG.

6.1. *Readability*

We used a five-point readability score used by Sutton et al. (2012) and validated by the CCAMLR Age Determination Workshop (WS-ADM3-2025, CCAMLR, 2025) (Table 4). When annual rings cannot be clearly delineated or counted, otoliths are considered unreadable and eliminated from the age estimation process.

Table 4. Five-point otolith readability scores used to characterize otolith's reading (Sutton et al., 2012).

Readability	Synthetic description	Description
1	Very easy	Otolith very easy to read; excellent contrast between successive opaque and translucent zones.
2	Easy/Good	Otolith easy to read; good contrast between successive opaque and translucent zones, but not as marked as in 1; potential error ± 1 opaque zone.
3	Readable/Fair	Otolith readable; less contrast between successive opaque and translucent zones than in 2, but alternating zones still apparent; potential error ± 2 opaque zones.
4	Readable with difficulty/Poor	Otolith readable with difficulty; poor contrast between successive opaque and translucent zones; potential error ± 3 opaque zones.
5	Unreadable	Otolith unreadable.

6.2. Growth zone interpretation

Most of the Patagonian toothfish otoliths exhibit a clear zonal structure, characterised by a dark region near the core, a transition zone, and a more translucent area extending to the edge of the otolith. Annuli near the core tend to be wider and gradually become narrower with age. Typically, the inner dark zone contains between three to five annuli and often includes macrostructures known as false checks (Horn, 2002).

6.2.1. Reading axis

In accordance with the CCAMLR Age Determination Workshop guidelines (WS-ADM3-2025, CCAMLR, 2025), annual increments are estimated in the proximal area on both the ventral and dorsal side (reading path ventral = rpv and reading path dorsal = rpd, Fig. 10). However, if one side is unclear, age estimation could be done only on one side. Note that the shape of the otolith can also be a useful rough guide to the age of the otolith.

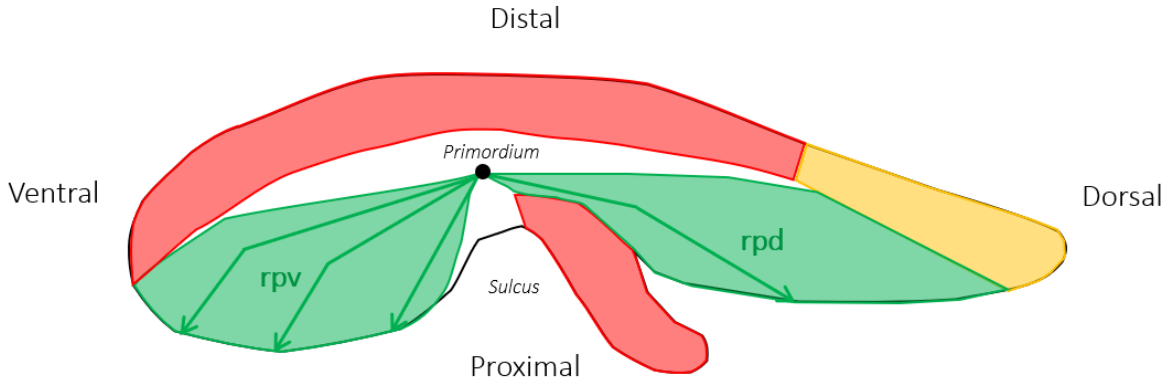

Figure 10. Localisation of the preferred zone (in green) for the reading of Patagonian toothfish otolith for age estimation, reading path ventral (rpv) and reading path dorsal (rpd). Red areas are unsuitable for age estimation and orange area may be helpful to confirm annuli in some specimens, but this area is prone to split and create double bands.

6.2.2. Identification of the first increment

The first and most crucial step is to identify the first increment. In Patagonian toothfish, this can be challenging due to a large dark region at the center of the otolith. The first opaque zone is most easily located by identifying the first translucent zone outside of the dark core, followed by the adjacent dark (opaque) band. In sections that include the primordium, the sulcus acusticus usually extends to the edge of the first increment (Kalish and Timmiss, 2000). Starting from the nucleus, each annulus comprises one opaque and the next adjacent translucent zone.

It is useful to make measurements of the distance from the primordium to the outer proximal edge, and from the primordium to the proximal edge of the first increment using the image measurement tool in ImageJ.

Measurements taken on the juvenile Patagonian toothfish used in Lee et al. (2024), indicated that the distance from the primordium to the first opaque zone ranged from 0.28 to 0.32 mm when measured proximally (P1; Table 5 and Fig. 11) and from 0.72 to 0.95 mm when measured ventrally (V1; Table 5 and Fig. 11). The distance between the edges of the first opaque zone measured between 0.66 and 0.77 mm along a disto-proximal axis (DP1; Table 5 and Fig. 11) and between 2.19 and 2.73 mm along a ventro-dorsal axis (VD1; Table 5 and Fig. 11). Ventral and proximal measurements of the distance between the primordium and the first opaque zone were consistent with the findings of other studies (Table 5). These known distances can be a useful reference when identifying the first increment. Using a measuring tool also assists with consistency in reader ages and reader training.

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VERSION 2

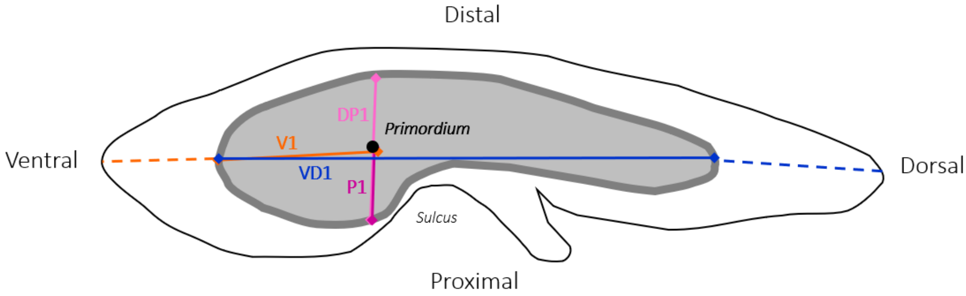

Figure 11. Distances measured to ease the identification of the first and second increments. The distances were measured from the primordium to the first opaque zone on the ventral axis (V1) and the proximal axis (P1); and between the edges of the first opaque zone along a disto-proximal axis (DP1) and a ventro-dorsal axis (VD1).

Table 5. Measured distances of the first and second increments to ease their identification.

Increment	Measurement	Distance in mm	Source
First increment	Ventral (V1)	[0.72 – 0.95]	FIFD
		0.64	Krusic-Golub et al., 2005
		[0.6- 0.9]	Nowara et al., 2008
		[0.62- 0.86]	Nowara et al., 2024
	Proximal (P1)	[0.28 – 0.32]	FIFD
		[0.22- 0.31]	Nowara et al., 2024
	Disto-proximal (DP1)	[0.66 – 0.77]	FIFD
	Ventro-dorsal (VD1)	[2.19 – 2.73]	FIFD
Second increment	Ventral (V2)	[1.09– 1.38]	FIFD
	Proximal (P2)	[0.40– 0.48]	FIFD

6.2.3. False checks

Otoliths may exhibit false checks, particularly in the first three to six annual increments. False checks are translucent zones that can be confused as annuli but are inconsistent in appearance. They are typically narrower and/or lighter in colouration than “true” annual increments, they are not continuous over the section, and may be less regularly spaced than “true” annual increments. These “false” bands should not be counted.

6.2.4. Split zones

Sometimes it will be noticeable that two bands appear very close together and it is unclear whether these represent one or two opaque zones. The CCAMLR Age Determination Workshop (WS-ADM3-2025, CCAMLR, 2025) recommended that where the split zones are within the first eight years of life that they be considered a split annulus, but if they occurred after eight years it should be counted as two annuli. This concept can be best understood by examining the annuli in the sectioned otolith of an older fish, paying particular attention to those zones closer to the nucleus.

6.3. Birth date and calculation of biological age

By convention, the birth date of Patagonian toothfish is the 1st July (Fig. 12). This date was chosen and agreed by the CCAMLR workshop on estimating age in Patagonian toothfish (SC-CAMLR, 2001) because it (1) conforms with the best knowledge of the timing of spawning (Kock and Kellerman, 1991; Lee et al., 2024), and (2) is congruent with the best available knowledge of the time of formation of the translucent zone (Horn, 2002; Horn et al., 2003; Fig. 12).

Estimated ages are thus determined based on the number of ring estimates and the month at which the individual was captured. For individuals caught between July and December, the age is calculated as the estimated number of rings plus one (La Mesa, 2007; Sutton et al., 2012; Fig. 12). This adjustment accounts for the timing of ring deposition (La Mesa, 2007).

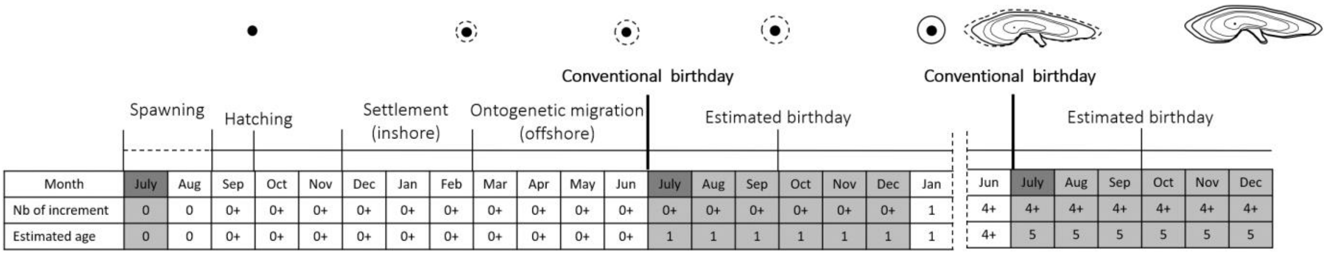

Figure 12. Timing of otolith growth and deposition of translucent zone, and the estimated age. Solid circles represent annuli and dashed circles represent plus growth (based on Sutton et al., 2012 and Lee et al., 2024). The estimated age between July and December is calculated as the estimated number of rings plus one (La Mesa, 2007; Sutton et al., 2012).

6.4. Age estimation precision

Currently, only one scientist is conducting the routine annual age estimation at FIFD. To improve the accuracy of age estimation, it is forward recommended to have two readers. This section will thus be update when two readers will be available to perform the age estimation in routine, and when the reference collection will be finalised. When the reference collection will be is use, (1) if we have one reader, its intra-reader variability will be measured with the reference collection and no second read of the routine collection will be needed, and (2) if we have two readers, their intra-reader variability will be measure with the reference collection, and the inter-reader variability will be measured by comparing their age estimations on the ongoing routine collection.

Repeated readings of the same otoliths provide a measure of intra-reader or inter-reader variability. While these readings do not validate the assigned ages, they provide an indication of the expected error associated with a set of age estimates due to variations in the interpretation of an otolith.

After the first reading of each otolith, we randomly sampled 30% of the aged otoliths for a second reading by the primary reader. This subsample is used to calculate the reading precision (Morison et al., 1998; Campana, 2001). Although the literature suggests that 25% of the otoliths could be used for a second reading (Morison et al., 1998; Campana, 2001), we opted to increase this sample size to 30% due to the recent training of the primary reader.

To examine the precision among age estimations, we calculated the four following indices across all age estimations using ageBias function of the FSA package (Ogle, 2016): Average Standard Deviation (ASD), Average Absolute Deviation (AAD), Average Coefficient of Variation (ACV), and Average Percent Error (APE) (Ogle, 2016). The ASD is the average (across all fish) of standard deviation of ages within a fish (Ogle, 2016). The AAD is the average (across all fish) absolute deviation of ages within a fish (Ogle, 2016). The APE is the average (across all fish) percent error of ages within a fish using the mean as the divisor (Beamish and Fournier, 1981). The APE was calculated as:

$$APE = 100 * \left[\frac{1}{n}\sum_{j=1}^{n}\left(\frac{1}{R}\sum_{i=1}^{R}\frac{|X_{ij} - X_j|}{X_j}\right)\right]$$

where *n* is the number of fish aged, *R* is the number of times fish are aged, X_{ij} is the i^{th} determination for the j^{th} fish, and X_j is the average estimated age of the j^{th} fish. APE was calculated for all repeated readings undertaken by the primary reader.

To avoid the inherent assumption in the APE that the standard deviation of age is proportional to the mean age for individual fish, the ACV should be measured Chang (1982). The ACV was defined as:

$$ACV = 100 * \frac{1}{n}\left(\sum_{j=1}^{n}\frac{s_j}{\bar{x}_j}\right)$$

where *sj* is the standard deviation of the *R* age estimates for the *j*th fish.

When the calculated Average Percent Error (APE) and Average Coefficient of Variation (ACV) are close to the 5% threshold, it indicates that the intra-reader precision in age estimation is relatively good (Morison et al., 1998; Campana, 2001). In such cases, the first reading is retained for age estimation.

From 2025, we also use the method used by the Australian Antarctic Division (Nowara et al., 2024) to set the acceptable level of discrepancy between the two independent reads. This level of acceptable discrepancy is based on stepwise fashion from no age difference for fish aged between zero and three years old, up to five years differences for fish over 21 years old (Fig. 13). This conservative upper limit of age discrepancy has been set based on a bomb radiocarbon study of toothfish otoliths by Kalish and Timmiss (2000), in which the authors suggest that ages obtained by reading otolith thin sections are likely to be within five years of true fish age.

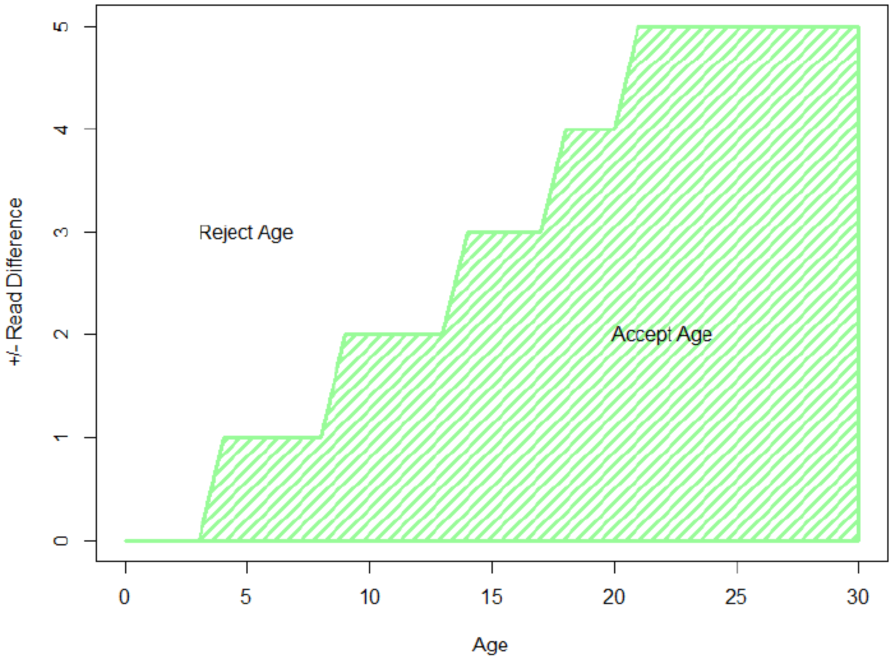

Figure 13. Plot showing limits for acceptable age discrepancies between two independent readers at the Australian Antarctic Division (Nowara et al., 2024).

For otoliths where the two readings differ by more than the acceptable level of discrepancy between the two independent reads (Fig. 13), a third reading is conducted with the information of the first and second readings, and this reading is used as the estimated age.

If the calculated APE and ACV exceed 7% (Morison et al., 1998; Campana, 2001), the primary reader should train on the reference collection before to read the routine collection.

7. *Otolith reference collection*

Otolith reference collection for Patagonian toothfish at FIFD was developed in 2025 and consists of a set of annotated digital images. Based on Campana (2001) recommendations, 216 otoliths belonging to the archived collection aged between 2013 and 2024 were selected to start the reference collection. This reference collection will increase with time to account for any annual variation.

Otoliths were selected according to the following criteria:

- 50% each sex
- normal distribution of otolith readability
- equal distribution of sampling months to consider the annual timing of the last increment deposition
- 6 otoliths/ 5cm length class
- spread among the studied years to consider any difference in growth rate
- spread among the fishing area and fisheries (trawl and longline) to consider any difference in a geographical pattern of growth rate.

As such, the collection includes otoliths of varying ages and readability, and provides a realistic snapshot of what readers may encounter in a given batch of toothfish otoliths.

The reference collection was read by the Emilie Le Luherne (FIFD Toothfish Fisheries Scientist) in charge of the routine annual age estimation for Patagonian toothfish and two external readers, Andy Nicholls and Evan Leonard from the Australian Antarctic Division. Andy Nicholls and Evan Leonard are experts in Patagonian toothfish age estimation, and belong to the CCAMLR group of experts in charge of building a Patagonian toothfish reference set for CCAMLR with the FIFD Toothfish scientist.

The reference set is used to train new readers and to measure the long-term drift in the age estimation precision and bias of experienced readers against an FIFD accepted age.

New readers are reading the entire reference collection, while experienced readers will read a random sub-set of 100 otoliths at least two times per year. A value of APE of less than 5% (Morison et al., 1998), and a CV of less than 10% (SC-CAMLR, 2012) are considered acceptable to start reading new otoliths.

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Annex 1. List of materials by step

Step	Material
Otolith extraction and storage	- Sharp dissecting knife - Tweezers - Otolith board (or ice cube tray used as a storage board) - Otolith envelopes - Eppendorfs filled with 96% ethanol - Measuring board or tape - Balance - Pencil and scribing board - Kitchen towels - PPE to work onboard a fishing vessel
Collection of the selected otoliths	- Printed list of the selected individuals - Otolith envelopes storage cardboard box - Pencil and ruler
Otolith embedding	- Epoxy Resin components: West System® 105 Epoxy Resin and 206 Slow Hardener - 100 mL beaker - Spatula, preparation needles and tweezers - Silicon moulds with 4 wells. There are 3 sizes. Choose the size adapted to your otoliths size (e.g. for Toothfish, use the small and medium moulds). The silicon moulds are made by Stephen Massam in the Falkland Islands. They are made with Tiranti RTV128 Silicone Mould Rubber and Standard Catalyst Kit. - Small pieces of spaghetti (used as a marker of otoliths order) - Labels printed or hand-written for each otolith's block, including the following information: Collection (YEAR A, B, C or D), species, and otolith serial number. Otoliths are mainly embedded by batch of 5. For the continuity of otolith embedding process, serial number on the labels followed the sequence 1-5, 6-10, 11-15, 16-20, etc. If for any reason, one otolith is missing, put a piece of paper or cardboard with its serial number instead. - Sellotape - Paper towel
Block grinding	- BUEHLER Metaserv 2000 Twin Grinder/Polisher - CarbiMet™ SiC Abrasive Paper of grain size P120 - Marker - Acetone and paper towel (to use under the fume cupboard with all the PPE needed)
Otolith section cutting	- BUEHLER IsoMet® Low Speed Saw - BUEHLER® Cool 3 Cutting fluid - Grey dressing stick - Dissecting microscope - Pencil, ruler and scissors

	- 2 jars filled with tap water - Kitchen towel and paper towel - Microscope slides with frosted edge (frosted 76 x 26 mm microscope slide) and cardboard microscope slide tray
Otolith section mounting	- West System® Epoxy Resin components: 105 Epoxy Resin and 206 Slow Hardener - 100 mL beaker - Spatula and preparation needle - Microscope cover glasses 24 x 60 mm and 22 x 50 mm - Paper towel - Microscope slide storage box
Otolith section photography	- OLYMPUS BX51 microscope with an OLYMPUS DP74 camera (with OLYMPUS U-TV1X-2 & U-CMAD3 Camera Adapter) - CellSens software - Microscope slide storage box

Annex 2. West System® Epoxy Resin preparation

West System® Epoxy Resin (https://www.westsystem.com/) is made by mixing 105 Epoxy Resin with 206 Slow Hardener. The cured resin mixture yields a rigid, high-strength, moisture-resistant solid bond with bonding and barrier coating properties. West System® Epoxy Resin is made by a mix of 5:1 ratio of Epoxy Resin to Hardener. The pumps adapted to the 105 Epoxy Resin with 206 Slow Hardener deliver different amounts of liquid which dispense the 5:1 ratio with the same number of pump strokes (Fig. 2.a).

The cure times of the mixed components are the following:

Working time (thin film): At 22°C: 90 to 110 minutes

Solid state: At 22°C: 10 to 15 hours

Prepare the resin under the fume cupboard. To embed a batch of otoliths, prepare a full beaker (~ 90 mL) of resin. For that purpose, pump 4 strokes of 105 Epoxy Resin and then 4 strokes of 206 Slow Hardener into the beaker (Fig. 2.a). Make circular and vertical movements with the spatula during 3-4 min to ensure the good mixing of the resin.

Clean the spatula and the beaker straight away after use with paper towel.

Annex 3. Terminology

Useful definitions for Patagonian toothfish age estimation process (SC_CAMLR, 2001).

Opaque zone: a zone that inhibits the passage of light. Summer growth zones are normally composed of opaque material. This zone appears dark under transmitted light and bright under reflected light.

Translucent zone: also named hyaline zone. A zone that allows the passage of the light. Winter zones are normally composed of hyaline material. This zone appears bright under transmitted light and dark under reflected light.

Annulus or annual increment: a combination of an opaque zone and the next adjacent translucent zone.

Primordium: the point from which all growth in the otolith originates, formed when fish are still embryonic.

Nucleus: includes the primordium and extends outwards to the inside edge of the first translucent zone.

Year 1: the part of the otolith from the nucleus extending out to the outer edge of the first translucent zone.

Year 2: the part of the otolith that extends from the inner edge of the first opaque zone after the nucleus to the outer edge of the second translucent zone.

False check: translucent zone that can be confused with annuli but lacks consistency in its appearance. False checks should not be counted as annulus. False checks are often encountered between three and six years old.

Plus-growth: opaque zone on the edge of the otolith. Plus-growth zones are not counted in age estimation.

Transition zone: a region of change in the form (e.g. width or contrast) of the increments. The change can be abrupt or gradual. Transition zones are often formed in otoliths during significant changes in habitat or lifestyle, such as the transition from a pelagic to a demersal habitat and at the onset of first sexual maturity.

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Version Number	Date	Author Title	Approval Status	Comment/Reason for Issue
1.0	30.09.2025	Le Luherne E.	Approved	
2.0	04.02.2025	Le Luherne E.	Approved	Block cutting – specify the section width (µm) to allow repeatability across equipment.
3.0				