Dune Lake Galaxiid otolith preparation methodology

Prepared for Northland Regional Council

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1 Background

Dune Lake Galaxiids (*Galaxias sp.)* are a freshwater fish with a highly restricted distribution being largely confined to dune lake habitats in the Northland region of New Zealand. These dune lake populations have been recognised as an evolutionarily significant unit of the *Galaxias maculatus* species complex, and work is currently underway to investigate if they are a separate species. According to the Department of Conservations freshwater fish threat ranking system, Dune Lake Galaxiids (DLGs) have a threat status of "At-risk" and there are growing concerns about the conservation and management of DLGs in Northland (Dunn et al 2018).

An Envirolink advice report commissioned in 2017 examined the effects of continued trout stocking in Lakes Waikare and Taharoa on DLG populations and an extensive monitoring design for DLG populations was proposed (Gee and Franklin 2017). Key information gaps regarding DLG biology and ecology were identified in particular the extent of DLG reproduction timing (Pingram, 2005) and the location of DLG spawning habitats. The DLG Science Working Party was formed to make the monitoring advice provided by Gee and Franklin (2017) operational. The DLG science strategy is implemented by a joint working group called "The Kai Iwi Lakes Dune Lake Galaxias Working Group". This working group consists of representatives from Northland Regional Council, the Department of Conservation, Kaipara District Council, Te Roroa/Te Kuihi iwi, Northland Fish and Game Council and NorthTec. This working group has identified seven research priorities for DLG management and conservation in Northland. One of these priorities is to gain a better understanding of the spawning ecology of DLGs with the overarching goal of gaining a better understanding of pressures from predators such as rainbow trout (*Oncorhynchus mykiss*) and gambusia (*Gambusia affinis)* in the Kai Iwi Lakes. The spawning habitats of DLGs are presently unknown but it is hoped that an analysis of the larval hatching dates of DLGs from otoliths will narrow the reproductive timing of DLGs and based on the results, targeted searches for eggs and spawning habitat can be done in the future.

To support the working groups research priority, an Envirolink Medium Advice Grant was applied for to provide training for the aging of DLGs using otoliths (calcium carbonate structures akin to ear bones) and to back-calculate DLG larval hatching dates from daily growth rings. The skills acquired by Northland Regional Council will be used in future monitoring and research programmes on DLG populations. Developing capacity and capability among stakeholders in the Northland Region is key to future work on DLG populations. This training can be shared further with other parties managing freshwater native fish recovery, especially the galaxiids.

A workshop was hosted at NorthTec in Whangarei (16th – 20th November 2018) and was attended by Northland Regional Council and NorthTec staff and students. Otolith training was provided by Dr Eimear Egan (NIWA Hamilton). This document summarises the methodology and key steps in the preparation of DLG otoliths for ageing and larval hatch date estimation.

1.1 What are otoliths

Otoliths are calcium carbonate structures that are used by fish to maintain balance and are used for hearing. Otoliths are regarded as one of the most informative tools for understanding the biology and ecology of fish because a vast amount of information about an individual is recorded in the otolith via daily ring deposition (Campana and Neilson, 1985). Daily ring counts can be used to estimate age at time of capture and hatching dates of DLG larvae (date at capture – age = larval hatch date). Although the exact date of spawning is difficult to determine, an assumed egg development duration of 3-4 weeks means that spawning dates can be estimated (larval hatch date – 21/28 days = spawning date). Furthermore, dark features/check marks and abrupt changes in the otolith coincide with important life history events such as larval hatching, metamorphosis and sexual maturity and can be used to identify the timing of these critical events (Campana and Neilson, 1985).

Otolith size and fish size are often proportional meaning that the distance between daily rings can be used to examine growth rates (Campana and Neilson, 1985). From the daily increment pattern, a time series of an individual's growth history can be derived, providing insights into how DLGs grow from the larval through to juvenile and adult stages. The growth of DLG can be compared among fish hatched at different times of the year. This would give an indication of how growth and potentially survival varies between larval hatch dates and associated seasonal changes in environmental conditions (Egan et al. 2019).

2 Methodology

2.1 Otolith extraction, preparation, polishing and increment counts

A random subset of DLGs were used in the otolith workshop including fish that were preserved in ethanol, fish that were frozen immediately following sampling and DLGs that were extracted from the stomachs of trout. The deposition of daily rings on the otoliths has not been validated for DLGs, however daily ring deposition has been validated for a closely related species īnanga (*Galaxias maculatus*; McDowall 1994) and was assumed here for DLGs. A checklist of equipment needed for otolith extraction and preparation is given in Appendix A.

The sagittal otoliths lie inside the otic capsule located toward the posterior end of the ventral surface of the skull (Figure 2-1). DLG sagittal otoliths were extracted under a dissecting microscope by making an incision in the head with a scalpel and peeling apart the tissue so that the otic capsule could be identified. Fine-tipped forceps were then used to extract both the left and right sagittal otoliths which were cleaned from adhering tissue using a fine paint brush and deionised water (Figure 2-2). The otoliths were stored in 96 well trays to dry for 24 hours before polishing.

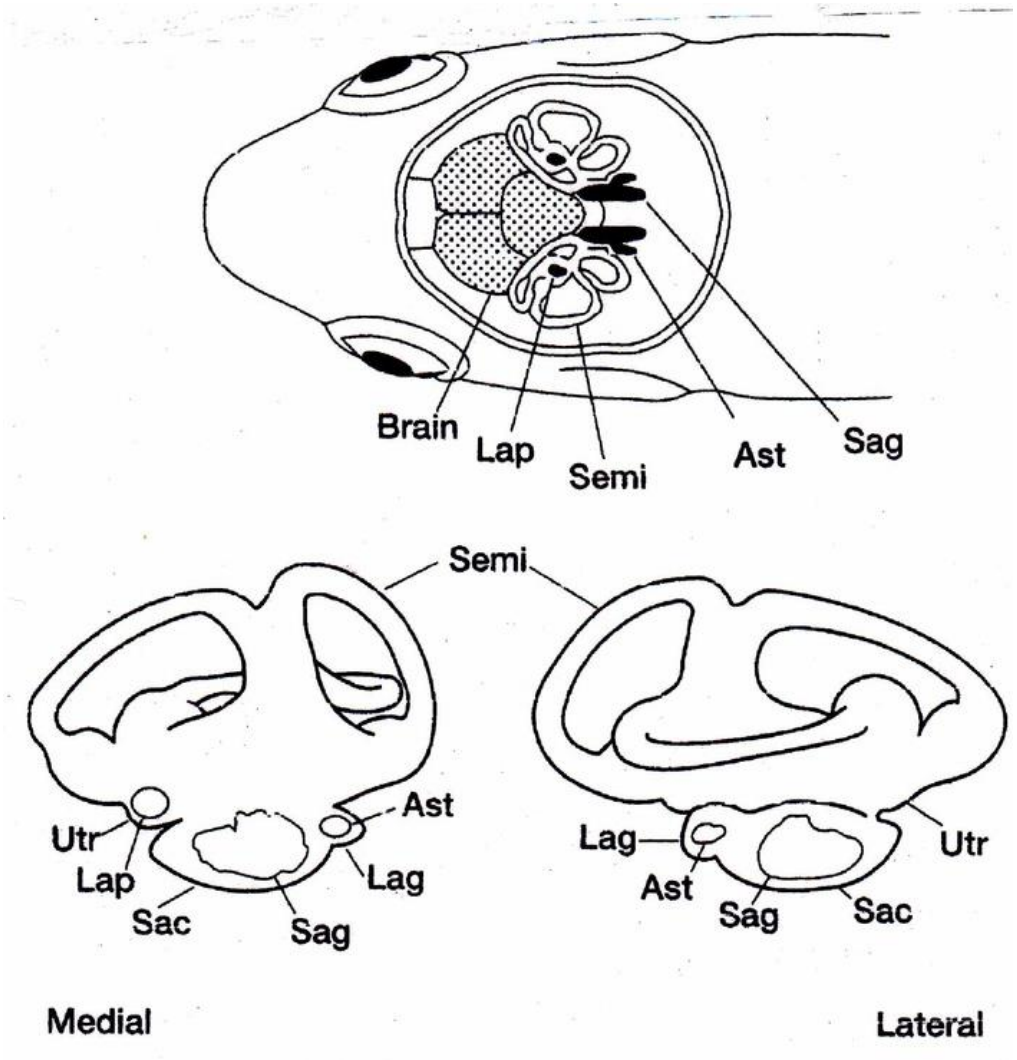

Figure 2-1: Diagram showing location of otoliths from Campana (2004).

Figure 2-2: Adult Dune Lake Galaxiid and extracted sagittal otolith with sulcus side facing up.

The sagittal otoliths were mounted sulcus side up onto glass microscope slides using CrystalBond™ 509 adhesive. Sagittae were polished longitudinally using South Bay Technology aluminium oxide 12µm and 0.3µm lapping film[1] (Figure 2-3). Polishing was done using the "wet" method where a few drops of water were added to the polishing film to increase contact with the otolith. Firm but even pressure was place on the microscope slide and otoliths were polished in a clockwise direction. Where necessary, sagittae were remounted and polished sulcal side down until a complete daily increment sequence from core to post-rostral edge was visible. Remounting was done by placing the microscope slide on a hot plate at approximately 180°C until the CrystalBond™ was melted sufficiently to allow the otolith to be reoriented. This process was done multiple times because of the thickness of the otoliths especially for larger DLGs.

The otolith polishing process was inspected continually to ensure over polishing does not occur (Figure 2-4). Examples of otoliths that were discarded from subsequent analysis are shown in Figure 2-5. These include otoliths that cracked during polishing from excess pressure, otoliths that were not cleaned of adhering tissue which resulted in black "spots" and an over polished otolith showing poorly resolved rings at the otolith edge (Figure 2-5). Polished DLG sagittae were wiped with tissue and then soaked in a drop of immersion oil. A coverslip was placed over the otolith and sealed with clear nail varnish. A unique identifier for each otolith was inscribed on the glass slides using a

[1] http://www.southbaytech.com/consum.shtml

diamond pen. The otoliths were then soaked overnight to increase clarity before photomicrographs were captured using 10x, 20x and 40x objective lenses.

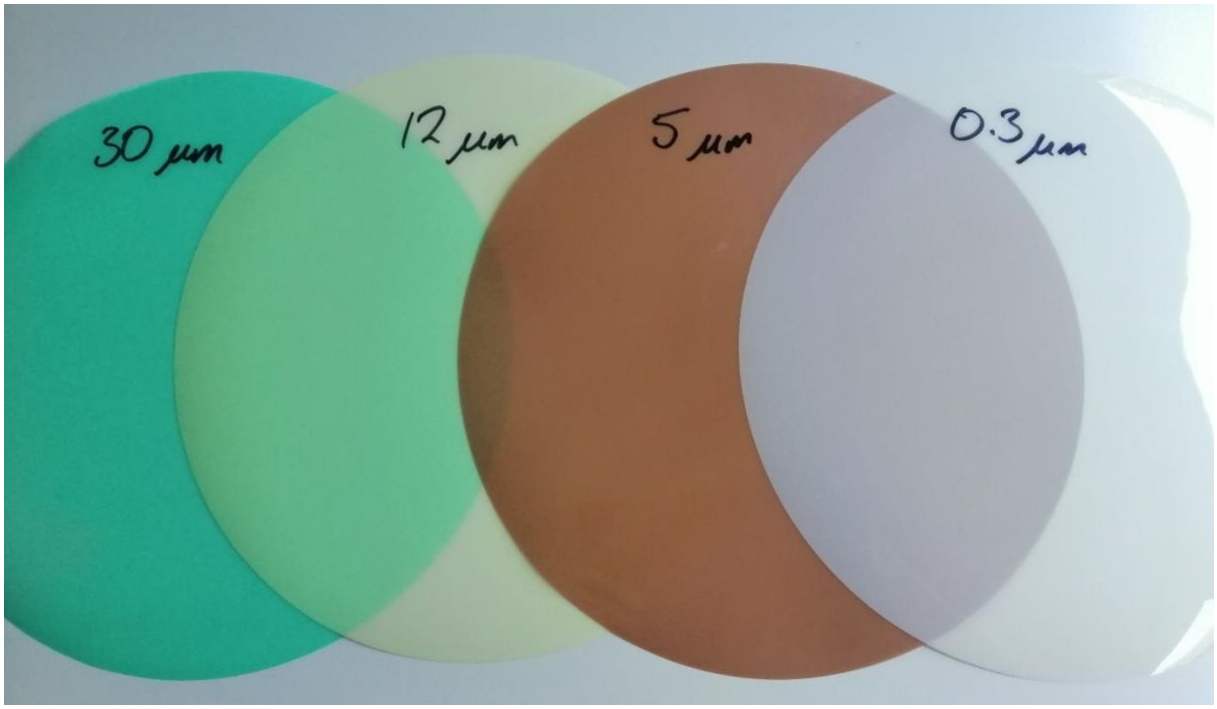

Figure 2-3: 30, 12, 5 and 0.3 µm film that can be used to polish Dune Lake Galaxiid otoliths.

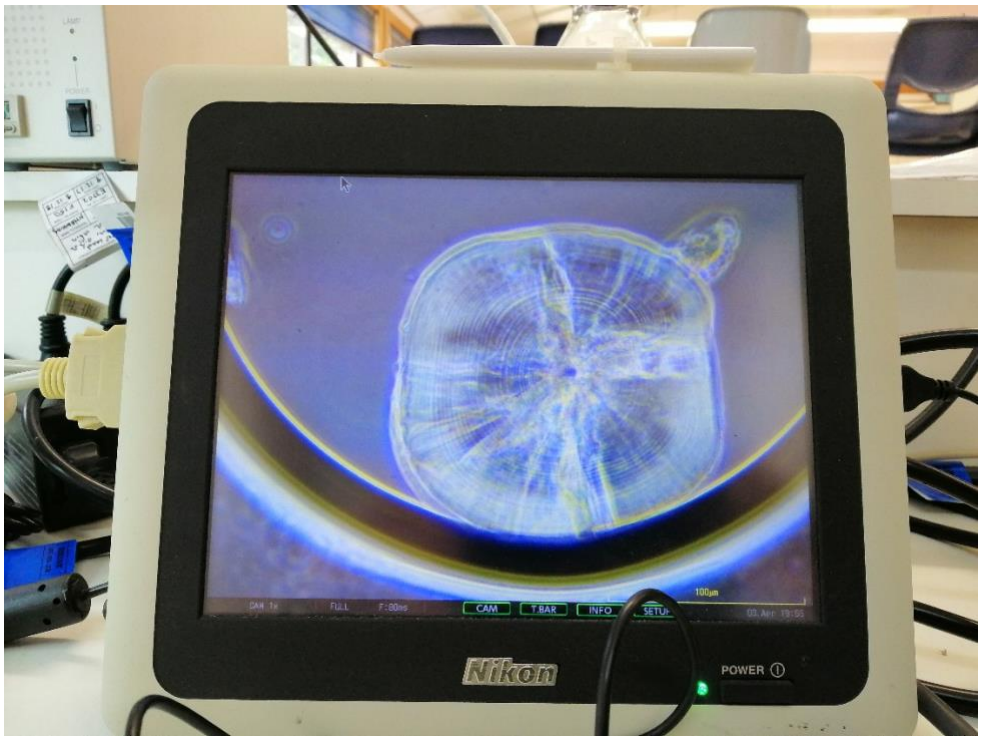

Figure 2-4: Otoliths are continually checked during the polishing process to ensure an even polishing axis is obtained and that over polishing does not occur.

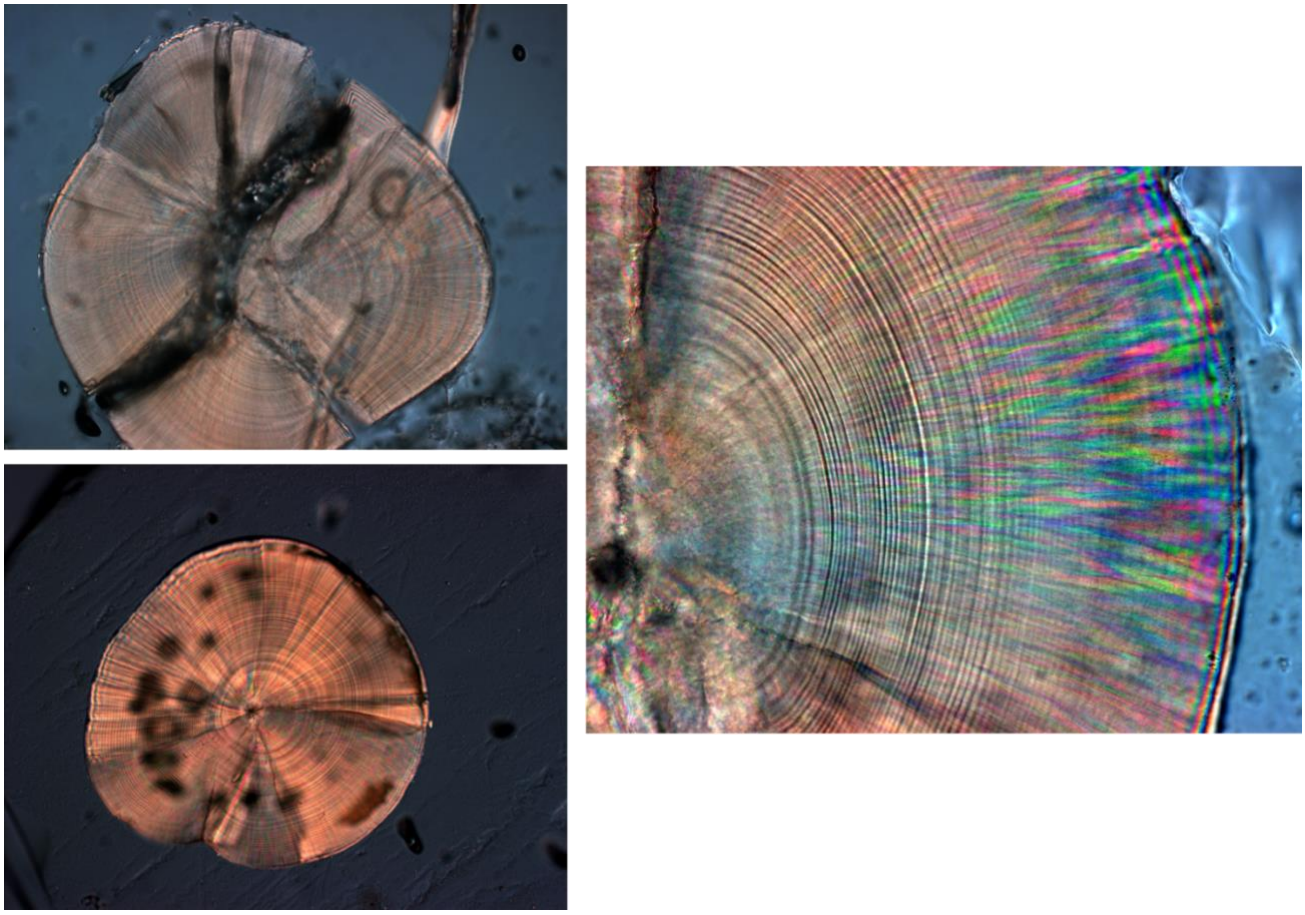

Figure 2-5: Examples of poor Dune Lake Galaxiid otolith preparation including cracked otoliths, burned organic matter which appears as dark splotches because of poor cleaning and over polishing giving a rainbow effect.

Examples of good otolith preparations are shown in Figure 2-6. A clear sequence of daily increments from the otolith core to edge was obtained. Furthermore, the larval hatching mark is clearly defined, and the daily increments are clear. Increment deposition from the hatch mark was naturally directed towards the post-rostral axis and the presence of confounding checkmarks and convergent/divergent rings were generally less apparent here (Figure 2-6). The relationship between fish size (total length (mm)) and otolith size (width (mm)) can be derived using linear regression (Figure 2-7) to meet the assumption that otolith growth is linear and proportional to somatic growth if growth reconstructions are required (Campana and Neilson 1985). Prepared otoliths should be stored in a slide tray or slide box which is clearly labelled.

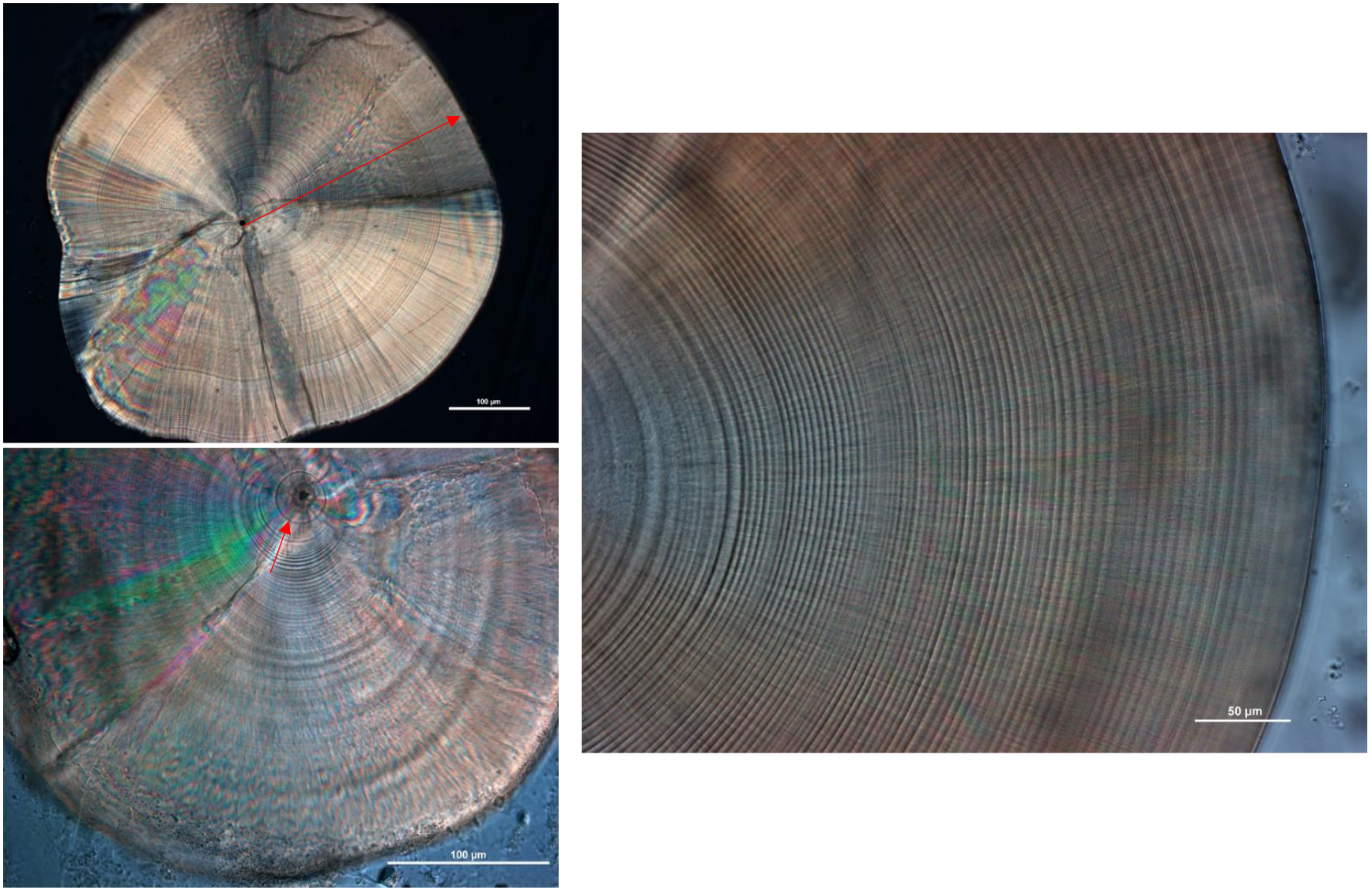

Figure 2-6: Examples of good otolith preparation showing a clear transect from otolith core to post-rostral edge (red arrow), a clearly demarcated hatch mark at the center of the otolith (red arrow) and clear daily increment sequences. Taken from Egan et al. 2019.

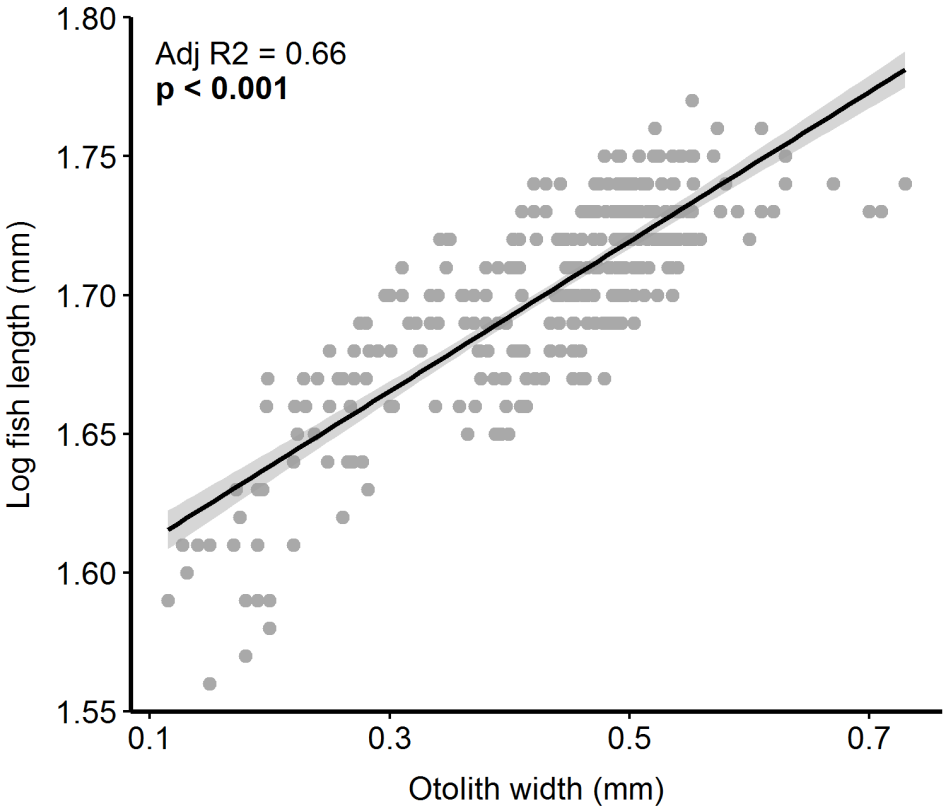

Figure 2-7: Relationship between otolith width (mm) and fish length (mm) for a closely related species īnanga (*Galaxias maculatus*). Taken from Egan et al. 2019.

Image J[2] is a freely downloadable software with a plugin called Object J[3] that can be used for counting otolith increments and to measure the distance between increments. Excellent tutorials exist for the analysis of otoliths using Object J (see https://sils.fnwi.uva.nl/bcb/objectj/examples/otoliths/MD/otoliths.html). The Object J manual for counting and measuring otolith growth rings is given in Appendix B. The hatch-mark is well defined in DLG otoliths and was clearly identified in all otoliths examined in the workshop (see example in Figure 2-6). Increment counts should be made from the hatch mark to the otolith edge and the data for each individual fish stored in an excel sheet.

[2] https://imagej.nih.gov/ij/download.html
[3] https://sils.fnwi.uva.nl/bcb/objectj/examples/otoliths/MD/Tutorial_Otoliths_ObjectJ.pdf

3 References

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Appendix A Equipment needed for otolith preparation

- Compound microscope
- Dissecting microscope
- Camera to take photomicrographs
- 5x, 10x, 20x, 40x objective lenses
- Microscope slides
- Cover slips
- Nail varnish
- Immersion oil
- Hotplate
- CrystalBond™
- Fine forceps (several)
- Small scalpel and spare blades
- Polishing film (12, 9, 5, 0.3 µm)
- 96 well extraction plates for otolith storage
- Diamond pen
- Slide holders/slide box
- Fine tipped permanent marker
- Paintbrush
- Petri dish
- Software for doing otolith increment measurements (Image J and Object J plugin)

Appendix B Object J manual

ObjectJ: Measuring Growth Rings in Fish Otoliths

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Description

This tutorial is destined to help using ImageJ with plugin ObjectJ for "Measuring growth increments in fish otoliths". Ageing and growth of fish based on otoliths has been adapted from the study of tree rings and is now widely used and developed in fisheries science. By measuring otolith increments, one can estimate past growth and correlate it with different environmental or population variables.

This plugin is designed to measure these increments in a standardized and straightforward way, as well as to provide a comprehensive output directly transferable for further use. Note that while it is flexible, the plugin was primarily developed to measure growth patterns along the distal axis. Consequently, it might require a few changes to be adapted for ventral or dorsal axis measurements, since standard methodologies often require break points and multiple lines from the core to the edge of the otolith.

Setting up a project

- Loading data in ObjectJ

To start a new project, download the project file (currently "Otoliths_1.8.ojj") provided on the website and move it into the directory that contains your otolith images. The ".ojj" project file contains the proper setup for measuring increments, as well as the necessary embedded macros. The first step is to link the images to the project, either one by one or by directly linking all images from a folder.

Just choose ObjectJ>Show Project Window > Linked Images > choose images one by one or choose a location folder to link all images from. Images can also be dragged directly from Finder/Explorer onto the "linked images" logo.

ObjectJ stores the overlay consisting of colored markings in the project file. Neither the images nor their own overlays are not modified. Additionally, all the measurements will be saved inside the project file without creating additional files.

- Scaling pictures (pixels/unit)

All linked images should now appear in the project window. If the pictures aren't scaled or if they are in a format that doesn't contain metadata (such as .JPG), they can easily be scaled in ObjectJ. In the project window, right click on the "px/unit" column and enter a scale and a unit. If multiple pictures were taken in a batch, the scale can be propagated to all of them by clicking the corresponding checkbox.

If the scale is not known, it can be measured using a calibration picture. In base ImageJ, measure the scale with the line tool and extract the corresponding value. It can then be used as a scale for the images, granted they were taken under the same conditions than the calibration picture. It is possible to scale the linked images afterwards, which will recalculate the results.

- Project window

With a quick look at the rest of the project window, two more tabs can be noticed: "Objects" and "Columns".

- "Objects" shows the "items" that will be created during the measuring process. The sequence is made of two lines called "core" and "vector" and finishes with a series of points called "increments". The column "clones" indicates the number of items from one type: in this project, there will always be only one core line, one vector line, and up to 99 increment points. One can easily change these values but it is recommended to leave them as it is, since the number of increments can easily range from a few to several dozen. These items are essential to the measuring process and their names are tied to the macros coding: it is thus also recommended to leave them, as any change will require to manually edit the macros correspondingly. The item colors (currently green core, red vector and blue ingrement) can be changed without problem.

- "Columns" shows various values that will be displayed with "ObjectJ results". However, the ring measurements won't be displayed here but in a macro output. Because these columns names are also tied to the functioning of the macros, it is recommended to leave them untouched.

Measuring sequence

- Annotating images

Once the images are linked to the project and scaled, the measuring sequence can start. Just open any image with a Double Click. The sequence can be started by clicking in ObjectJ > Start a sequence, or by simply pressing F1, which is the designed shortcut of this first macro.

This starts the sequence with the first item, a segment called "Core". Simply place the two extremities on the core edges so that it crosses the theoretical center. Determining with precision the core origin can be difficult and highly subjective, and therefore the first increment is often removed from chronologies. Drawing this arbitrary line will give a standardized distance that can then be used as an additional proxy for comparing first years of growth.

The sequence then automatically switches to a second item, another segment called "Vector". This segment is central for further processing. It will act as both an axis and a measuring tool. Simply place two points, one at the theoretical core center and one at the otolith edge, to draw the corresponding axis.

It will then move on to the final item, a series of points called "Increment". Just place each point on its corresponding growth ring to mark it, and progressively move from one extremity

to the other. If a point is misplaced, simply delete it by pressing Backspace. This will remove points by chronological order, starting from the most recent.

To delete specific items, go in the ObjectJ Tools window and select the Pistol tool. Keep Alt key down and click on one point to delete it, or keep Shift down and click on a point to delete the entire associated item. Then, to resume annotating, select the Finger tool and double click on any point, which "opens" the selected object so it can receive more markings. The sequence will resume at the selected item type. To delete an entire object (i.e all three items), simply use the Pistol tool without pressing any action key.

While it is important to be precise when annotating growth increments, keep in mind that this macro also has a built-in function to pin dots on the measuring axis (see below). Consequently, it is not problematic if the points aren't exactly aligned, as long as the deviation isn't significant. Similarly, the points can be clicked in any order without affecting the results.

- Inputs for known year and quality

Once every item has been placed, the sequence can be ended by clicking on ObjectJ > Year and quality, or by pressing the F2 shortcut. This macro opens a dialog box with two fields.

- First, it requires a known year. A positive value assigns subsequent years from the core to the last increment, thus corresponding to a known cohort. A negative value can instead be used if only the fishing date of the individual is known. The given year will here be attributed to the outermost increment and the ageing will go backwards down from the edge to the core center. Use the macro F4 to toggle on and off these values for reading purposes.

- Then, it asks for a quality value. This is an arbitrary scale of the sample quality, defined directly by the user. A basic scale goes as follows. If the increments are clear, well contrasted and easy to read, mark the samples with quality "1" (Very Good). If the sample is readable as a whole but small issues hinder the measuring process, a quality "2" (Good) can be chosen. On the other hand, if the sample is degraded or broken, if the picture is of poor quality, or if the increments are split and significantly harder to read, assign a quality 3 (Poor). This stamp makes it easy to manipulate the measurement data, for example to subset difficult otoliths from the dataset to remove uncertainties.

As mentioned before, this macro also pins (projects) the increment (blue dots) onto the "Vector" line. This way the displacement will be minimal and the results won't be impacted, as long as the points were placed close to the axis.

After an otolith is finished via "Enter year and quality", simply move on to the next image and repeat the sequence to annotate it.

Getting the output results

Once every sample in the project has been annotated, the output results can be generated by clicking on ObjectJ > Create output table, or once more by simply pressing the shortcut F3. This macro does several things:

1) It arranges vector direction and ring sequence to facilitate increments measurement. First, distances between increments will always be calculated as an ascending path from the inner to outer end of the Vector, no matter which way the axis was drawn. Second, if the increments weren't put in order or if some were noticed and added later, it will correct their sequence so that they always rank from the core to the edge.

2) It measures the distance between each increment along the Vector. The first measurement is the distance from the Vector start to the first Increment point. The following ones will be between consecutive increments. The final measurement will be the remaining distance between the last increment and the edge of the otolith (end of the Vector). This measure will correspond to the most recent growth over a period inferior to a year.

3) Finally, it groups the results in an output table organized in several columns:
   a. Sample: name of the image, minus the file extension
   b. Quality: quality stamp assigned with the second macro
   c. Age: age of the fish calculated with the number of increments measured
   d. Cohort: cohort of the fish (birth year), calculated from the known year and the number of increments
   e. Core: arbitrary core diameter
   f. I: sequence of the increments. Will always start with "core" and end with "edge", with any number of increments in between.
   g. Year: year assigned to each increment based on the year provided in macro "Year and Quality..."
   h. Increment: measured width of each increment

This table displays each sample one under another in alphabetical order, and each

Sample 49, with each increment on an individual row

Otolith Parameters.txt

File Edit Font

Sample	Quality	Age	Cohort	Core	I	Year	Increment
N-2005_49	1	8	1997	1927.8	core		
N-2005_49	1	8	1997	1927.8	1	1998	406.1
N-2005_49	1	8	1997	1927.8	2	1999	250.4
N-2005_49	1	8	1997	1927.8	3	2000	267.2
N-2005_49	1	8	1997	1927.8	4	2001	232.8
N-2005_49	1	8	1997	1927.8	5	2002	168.4
N-2005_49	1	8	1997	1927.8	6	2003	149.8
N-2005_49	1	8	1997	1927.8	7	2004	139.5
N-2005_49	1	8	1997	1927.8	8	2005	139.2
N-2005_49	1	8	1997	1927.8	edge		49.6
N-2005_50	1	8	1997	1902.1	core		
N-2005_50	1	8	1997	1902.1	1	1998	314.6
N-2005_50	1	8	1997	1902.1	2	1999	279.0
N-2005_50	1	8	1997	1902.1	3	2000	276.5
N-2005_50	1	8	1997	1902.1	4	2001	228.2
N-2005_50	1	8	1997	1902.1	5	2002	171.8
N-2005_50	1	8	1997	1902.1	6	2003	188.1
N-2005_50	1	8	1997	1902.1	7	2004	163.6
N-2005_50	1	8	1997	1902.1	8	2005	142.2

measurement as an individual row. See figure below for illustration.

Because this table will display any number of measurements from all the images linked to the project file, is it recommended to first annotate every sample and then print out the results as a single, comprehensive table. Nonetheless, the table can be re-generated every time a new image has been annotated or corrected: the output table will always display the samples in alphabetical order, and any image left unannotated simply won't appear in the measuring table.

Another great functionality of this plugin is its ability to produce measurements that are easy to export and use. With "Otolith Parameters.txt" in front, simply save the table as a .csv document that can be effortlessly imported in Excel/R/Matlab or examined in a standard growth chronology model.

Final words

This ObjectJ project works as a simple and intuitive extension to annotate and measure growth from fish otoliths in a non-destructive way. Its ability to annotate increments in a few clicks and to deliver result tables that are both comprehensive and simple to export makes it a useful tool for any level, without requiring any advanced knowledge of image editing software. ObjectJ's overlay also allows to annotate and later review JPG images without having to store them as TIF. Besides, it can be used on a wide range of fish species, and can even be adapted to measure daily growth in juveniles' hard structures with some slight changes to the embedded macros.