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A cost-effective method of preparing larval fish otoliths for reading using enzyme digestion and staining

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A fast and cost-effective method for examining otoliths in fish larvae was developed whereby the otolith remains *in situ*. Whole fish of the clownfish *Amphiprion melanopus* were enzyme-cleared using a laundry pre-soak and then stained using the Von Kossa silver staining method for calcium. The otolith nucleus, daily rings and the otolith edge were all clearly visible and were suitable for a variety of age and growth analyses. The total ' hands on ' time required to process these otoliths was *c.* 3 min, and multiple samples could be processed simultaneously. The reduction in labour of this method to produce clear daily rings in the otolith lends itself to broad use in fish biology where large quantities of otoliths need to be examined in a cost- and time-efficient manner.

Key words: otoliths; daily increments; fish larvae; *Amphiprion melanopus*.

INTRODUCTION

Since the discovery of daily growth increments in the microstructure of otoliths (Panella, 1971), there have been major advances in the understanding of the early life history of fishes. The use of otolith microstructure to provide age and growth estimates has revolutionized the interpretation of ontogeny and early life history traits (Fuiman & Higgs, 1996). Recent analysis of the international otolith processing industry have estimated that over one million otoliths are aged annually (Campana & Thorrold, 2001). This represents a large investment in both facilities and labour in removing, grinding, polishing and then reading otoliths to retrieve the information carried within the micro-structure of the otolith. This problem is inflated when processing the otoliths of marine fish larvae. The current procedure is generally similar to the methods used for processing adult otoliths with a scaling down of the tools involved. Fish larvae are, however, orders of magnitude smaller than adults, with tropical marine larvae ranging in size at hatching from 1–20 mm. There is a hundred-fold difference in the size of larval and adult otoliths, for example, the sagitta of a 5 day-old *Amphiprion melanopus* Bleeker (used in this study) is *c.* 150 µm in diameter, compared with the otoliths from an adult serranid e.g. *Epinephelus quernus* Seale at 12–16·5 mm (Williams & Lowe, 1997). Using the current processing methods, otoliths of larvae are difficult to handle in the absence of

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specialized tools. This leads to an increase in the removal and processing time compared to otoliths from adults, thereby, inflating the labour requirements for analysis of larval otoliths.

A faster, more reliable procedure would encourage broader examination of larval otoliths and increase the understanding of processes affecting the early life stage of reef fishes. In a review on ageing fish larvae, Jones (1986) concluded that cost effectiveness was one of the most important issues concerning the use of otoliths for ageing fishes. The present paper describes a technique that not only eliminates most of the labour and technically demanding aspects of larval otolith examination, but also enables large numbers of samples to be processed simultaneously. This rapid and efficient method of preparing otoliths for increment count and measurement *in situ* has the potential to significantly reduce the cost of processing otoliths from larval fishes.

MATERIALS AND METHODS

SAMPLES

The larvae of the clownfish *A. melanopus* were used in trials of the ' clear and silver stain ' method. Larvae were obtained from a breeding programme established at the James Cook University Aquarium Facility as outlined in Green & McCormick (1999). Fish were sampled in the morning every day from hatching until metamorphosis which occurs 8 days after hatching. All samples were euthanased on ice and preserved in borax buffered formalin.

CLEARING WHOLE FISH

Fish were rinsed thoroughly in distilled water and then placed in a 5% solution of enzyme-based laundry pre-soak Neon™ for digestion of muscle tissue. This was employed as a cheaper alternative to clearing (enzyme digestion) using pancreatic enzymes such as trypsin (Gosztonyi, 1984; Green & McCormick, 2001). Fish were kept in this solution for 3 weeks or until the tissue was transparent, leaving only the articulated skeleton and connective tissues. The solution was changed every week. In larvae >3 days, pigment in the skin and along some muscles obscured the view of the bones. These samples were placed in 3% hydrogen peroxide for 24–48 h to bleach the melanin.

HANDLING AND STAINING SAMPLES

Once the fish were cleared they were prepared for staining to highlight different densities of calcium. Samples were placed into biopsy cassettes (Tissue Tek® III cat. no. 4086) with perforated bases so they could be exposed to solutions in bulk without handling the individual fish. A series of 14 petri dishes were used for the staining procedure, consisting of four baths of distilled water, one bath of 0·5% aqueous silver nitrate solution, four baths of distilled water, one bath of 0·5% sodium thiosulphate followed by four more baths of distilled water.

Samples were moved through the first distilled water series then placed into a petri dish of 0·5% aqueous silver nitrate solution for 2 min. They were rinsed through a series of distilled water again and placed in a solution of 0·5% sodium thiosulphate and viewed under a dissecting microscope to monitor the staining. They were left in this solution for 30 s or until some brown staining was visible along the fins. To ensure the reaction was quenched and the staining complete, samples were then rinsed in a series of distilled water and left to soak in the final dish of water for at least 30 min. This system should be scaled

to accommodate for the fish being processed. Alternative holding vessels would be required for processing larger amounts or size of larvae, and the size of the baths could be variable depending on the size and number of larvae to be processed.

STAINING AND REMOVING OTOLITHS FROM OLDER FISH

Otoliths were removed from the cranium of fish older than 3 days after the staining procedure. These otoliths were mounted in the same manner as whole specimens.

MOUNTING

Samples were placed onto concave slides on the edge of a hotplate at low temperature. A small piece of Crystalbond™ thermoplastic resin was placed into the cavity next to the sample. The hotplate dried the sample and melted the resin around the sample. Coverslips were placed over the sample and the slide was taken off the heat so the resin could harden.

RESULTS AND DISCUSSION

The ‘ clear and silver stain ’ method described takes advantage of the otoliths’ chemical structure to cost-effectively enhance the visibility of the otolith structure and increments. Calcium is bound inside living organism in bones where it is combined with phosphates, carbonates and other anions. In the von Kossa silver reduction technique (von Kossa, 1901), these anions react to the silver stain, highlighting areas of denser calcium deposition as the bone mineral is blackened by the deposition of silver (Page, 1977). The calcium-dominated bands within the organic-matrix of the otolith (Watabe *et al.*, 1982) are clarified using the von Kossa silver reduction technique by darkening the bone mineral as described above. The ‘ clear and silver stain ’ method revealed visible rings in both the sagitta and lapillus, corresponding in number directly to the number of nights since the fish had hatched (Fig. 1).

The greatest advantages the ‘ clear and silver stain ’ method offers for examining age and growth in fish larvae are time-efficiency, capacity to process samples in bulk and reduced risk of breaking or losing otoliths. The total ‘ hands-on ’ time for processing and ring clarification for multiple samples is *c.* 3 min compared with up to 30 min per individual using removal and polishing techniques, based on tropical reef fish larvae (unpubl. data). Alternative methods for processing small otoliths include whole-fish embedding and grinding (Secor *et al.*, 1991; Bishop *et al.*, 2000), bleaching method and teasing method (Secor *et al.*, 1991). All three methods are time-consuming as they require that fishes are processed individually, and the latter two methods require handling of individual otoliths, increasing the risk of loss or damage. Further, embedding whole fish followed by grinding exposes the processor to harmful chemicals and dusts.

The ‘ clear and silver stain ’ method proffers a safety advantage over methods that employ grinding and polishing as it avoids exposure to hazardous substances. The safety labels on resins and catalysts conventionally used for embedding fishes or otoliths describe the products as carcinogenic, poisonous, mutagenic, irritant and corrosive (eg. West systems™ Epoxy or Synthetic resins™ MEKP catalyst). These resins and catalysts require extensive safety equipment such as fume hoods and facemasks to reduce the risk to the user. The ‘ clear and silver stain ’ method employs domestic laundry pre-soak and low

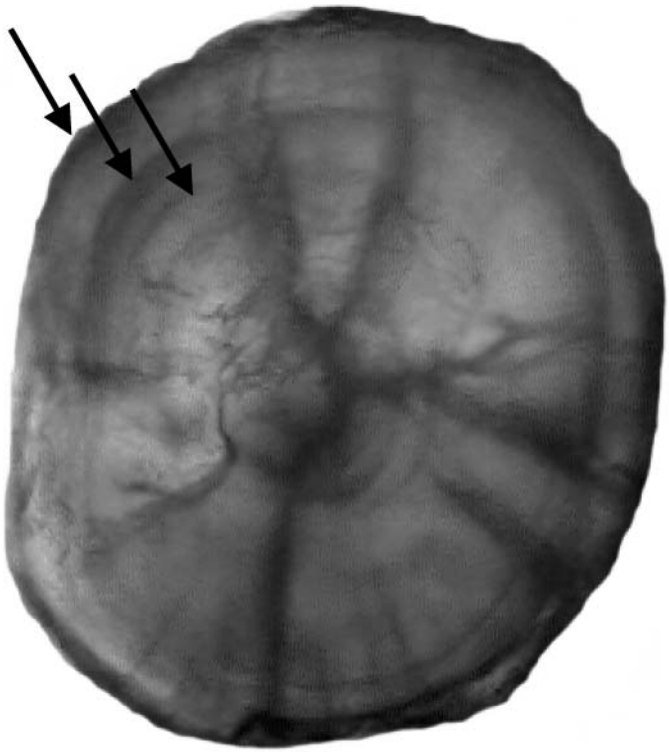

FIG. 1. Sagittal otolith from a 3 day-old *Amphiprion melanopus*, inside the fish's skull prepared using the ' clear and silver stain ' method, stained with von Kossa silver stain. →, daily increments, × 400 magnification.

concentration solutions of silver nitrate and sodium thiosulphate, avoiding the safety risks encountered using resin embedding and subsequent grinding (Secor *et al.*, 1991).

The ' clear and silver stain ' method was useful in whole specimens until the point of development when the otic complex ossified. The uptake of the von Kossa stain by the newly calcified otic complex obscured the view of the otoliths encased within the head. To circumvent this obstruction the otolith was removed from the cranium and mounted. This ' silver staining ' method clearly defined the daily increments and nucleus in late-stage larval otoliths (Fig. 2). In the species studied, ossification of the otic complex occurs at *c.* 3 days after hatching (Green & McCormick, 2001), however, this fish species is one of the fastest developing marine fish yet identified (Green & McCormick, 2001; Bellwood & Fisher, 2001). For comparison, a tropical serranid, *Plectropomus leopardus* (Walbaum), undergoes cranium ossification 13 days after hatching (Masuma *et al.*, 1993) and Atlantic cod *Gadus morhua* L. at 60–80 days after hatching (Hunt von Herbing, 2001). Therefore the ' clear and silver stain ' method for examining otoliths of fish larvae *in situ* would be effective for more than the first days of the larval duration.

It is recommend that a pilot study for each species is carried out to: determine the maximum useful age; test the recommended staining and development times

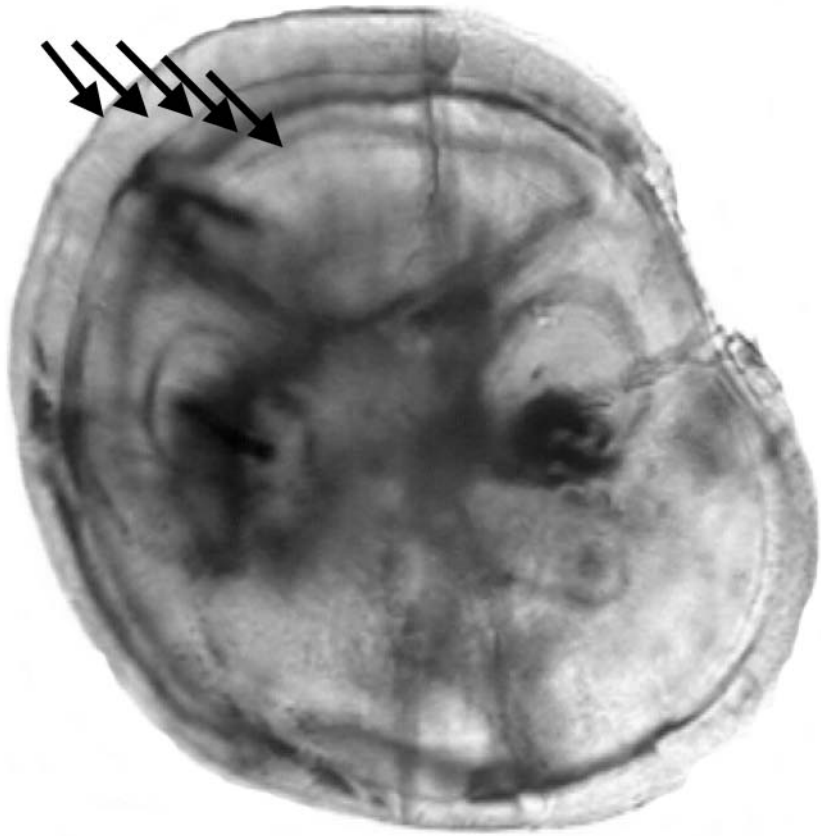

FIG. 2. Sagittal otolith from 5 day-old *Amphiprion melanopus* stained while *in situ* and then removed from the fish and mounted. Stained with von Kossa silver stain. →, daily rings. × 400 magnification.

as these may vary with the size and bulk of the fish and calcium density; maximize the size of the processing unit. The unit in this study was suited to a sample size of *c.* 10 fish per sample, but larger containers will permit simultaneous processing of many samples, while the order of chemical treatments remains the same. Once the set-up is optimized, processing otoliths of fish larvae using the ' clear and silver stain ' method proffers to be a safe and efficient method of estimating biological parameters relating to age and growth in fish larvae. It has potential to be a valuable tool in fisheries monitoring where large quantities of otoliths need to be examined in a cost- and time-efficient manner (Jones, 1986).

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