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Early development and growth of the eastern rainbowfish, *Melanotaenia splendida splendida* (Peters) II. Otolith development, increment validation and larval growth

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Abstract. A method of preparing and interpreting the microstructure of otoliths of the eastern rainbowfish, *Melanotaenia splendida splendida*, was developed and used to validate the periodicity of increment formation. Otoliths were collected from laboratory-reared *M. s. splendida* of known age. The sagittal otolith was preferred for ageing because resolving the earliest increments was easier than in the lapillus during an early slow-growth period, up to 15 days after hatching. Increments formed in the sagittae before hatching, and a distinct discontinuity was visible in the otolith sections at a time corresponding to hatching. Another discontinuity occurred at the time of yolk-sac absorption, when larvae became completely reliant on exogenous feeding. After this, increments were clear, regularly spaced and easily resolved. Linear relationships were found between fish size and sagittal length, breadth and perimeter. Observations of the otolith sections confirmed that the increments in sagittae of *M. s. splendida* were laid down daily. The information provided here enables growth and mortality rates of *M. s. splendida* to be measured, providing a useful tool for monitoring the impacts of contaminants in tropical Australian waters.

Extra keywords: ageing, sagittal otolith, yolk sac.

Introduction

The formation of daily growth rings in fish otoliths was first described by Pannella (1971), opening the way for techniques to assess age and growth of fish with a greater degree of accuracy and precision than was previously possible. Brothers *et al.* (1976) applied this knowledge when they verified daily increment deposition in larval fish reared from eggs in the laboratory. Daily growth increments in otoliths have been observed in numerous teleosts, including freshwater (e.g. Admassu and Ahlgren 2000; Panfili and Tomas 2001) and marine species (e.g. Geffen 1995; Newman *et al.* 2000), distributed across the world from polar regions (e.g. Shafer 1989) to the tropics (e.g. Fowler 1989; Shafer 2000). Otolith microstructure analysis has proven to be valuable in relating biotic and abiotic factors to life-history traits in fishes.

A prerequisite for any study of otolith microstructure is validation of the periodicity of increment formation (Beamish and McFarlane 1983, 1995; Beamish 1992; Fowler and Short 1998; Hernaman *et al.* 2000; Campana 2001). The rate of increment formation in otoliths can be precisely determined from fish of known age (Neilson and Geen 1982; Oxenford *et al.* 1994; Powell *et al.* 2000) or from the incorporation of chemical markers such as tetracycline, calcein or alizarin complexone in the otolith (Campana and Neilson 1982; Neilson and Geen 1985; Vigliola 1997; Hernaman *et al.* 2000). Determination of the age of first increment formation and periodicity of increment formation should also be an integral component of validation studies (Campana 2001). Without validation, serious errors can result in estimates of growth rates, mortality rates, hatch date distributions and analysis of cohorts.

Melanotaenia splendida splendida might be an excellent species for use in laboratory bioassays and for monitoring the health of Australian tropical waters. Already several other *Melanotaenia* species have been used in laboratory tests assessing the toxicity of chemicals and pollutants (e.g. Holdway *et al.* 1994; Barry *et al.* 1995*a*, 1995*b*; Kumar and Chapman 1998; Williams and Holdway 2000) and for the monitoring of wastes in the field (e.g. Neilsen and King 1995). We have found that the growth rate of *M. s. splendida* exposed to contaminants in the laboratory is an extremely sensitive indicator (C. Humphrey and D. W. Klumpp, unpublished data). However, for this technique to be used in the detection and monitoring of contaminants in the field, validated estimates of daily growth rates are required.

The objectives of this study were: (*1*) to determine whether the sagitta or lapillus is the most useful otolith for ageing larval and juvenile *M. s. splendida*; (*2*) to validate the period of increment formation; (*3*) to identify the age when the first increment is formed; (*4*) to determine the daily ages of fish;

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(*5*) to describe the relationship between otolith and fish size; and (*6*) to describe the growth rates of a sample of fish.

Materials and methods

Otolith collection and examination

We (Humphrey *et al.* 2003) have provided detailed information on breeding, maintenance, feeding and collection of *M. s. splendida*. Briefly, 36-L glass flow-through aquaria were set up, each with filtered water, spawning substrate and two males and three females of *M. s. splendida*. Eggs were attached to the spawning substrate by means of sticky filamentous threads. Spawning substrates, with eggs attached, were collected from the breeding tanks and placed into 25-L glass flow-through aquaria where the eggs were allowed to develop. Water temperature in breeding and rearing tanks was maintained at 28 ± 1°C.

We sampled 25 embryos at the beginning of otolith development and on each subsequent day until hatching. Hatching success was greater than 93%. Larvae hatched within a 12-h period, were sampled daily for the first 10 days after hatching, then every 2 days for 10 days and then every 10 days until the completion of the study. The standard length (SL) of tricaine-anaesthetized larvae was measured to the nearest 0.01 mm with a calibrated graticule eyepiece fitted to a stereo dissecting microscope. Larvae were then preserved in >70% buffered ethanol until their otoliths were removed, which was within 3 months of sampling.

All otoliths (asteriscus, sagitta and lapillus) were collected from each fish. Larvae were placed on a well-glass slide and immersed in a few drops of water. The otoliths were teased from the head with fine dissecting needles under a stereo dissecting microscope with cross-polarized light. After the otoliths were removed from the head, any adhering tissue was scraped away, and the otolith was allowed to dry. Otoliths were transferred on the end of a wet dissecting needle to a clean glass slide and were mounted in thermoplastic glue, where they remained until required for analysis.

Comparison between sagittae and lapilli

The asteriscus was not considered because it did not form until about 15 days after hatching. Sagittae and lapilli from the smallest fish did not require any preparation; however, larger otoliths required grinding and polishing to remove extraneous material. Initially, both otoliths were flat and round and the lateral surface could be ground and polished with four grades of lapping film (1–12 μm) to produce a thin sagittal section (orientation follows Secor *et al.* 1992). Sagittae from larger fish had a more convex–concave section that made sagittal sections inappropriate, so they were sectioned transversely. Sagittae embedded in thermoplastic glue were moved to the edge of a glass slide by making the glue viscous with gentle heating. The otolith was positioned transversely so that the core was aligned with the edge of the glass slide. It was gently ground to the level of the core and then polished with four grades of lapping film as described above. The otolith was glued again, polished side down, in the centre of the glass slide and the process repeated from the other side. Grinding from both directions produced a transverse section that was 0.05–0.10 mm thick.

To determine the most useful otolith (sagitta or lapillus), preparations from each of 50 fish, of a range of ages, were examined and increments counted. The two otoliths were compared quantitatively by comparing counts of increments, and qualitatively by considering the time required for their preparation and the clarity of the increments.

To determine the date of first increment formation, 45 otoliths were collected, 15 each from embryos, newly hatched larvae and 64-day-old larvae. Otoliths were examined for pre-hatching increments and the presence of a hatch mark.

Otolith size was determined for 238 specimens up to 55 mm SL by three methods. Before being ground, each otolith was measured along its longest and shortest axis and perimeter by a computer-based image-analysis system (OPTIMAS). Owing to the large range in otolith sizes they were examined at magnifications of 50–1000×.

Counts of increments were made on 126 otoliths, from fish of a range of ages, viewed under a compound microscope at magnifications of 400–1000× under transmitted light. The microscope was linked by a high-resolution video camera to a video monitor and computer. Increments were counted in one of the pair of otoliths with the aid of OPTIMAS. Because interpretation could differ with the point of origin for the counting path, one count originated at the otolith periphery, while the other originated at the hatch check. Two counts were made blind, with a 3-month period between each reading. The mean of these independent replicates was used as the estimated increment number.

Results

Morphological development of otoliths

The sagitta and lapillus were first observed in embryos as small, round calcified concretions, 2 days after fertilization. The asteriscus was not present at this time. The lapillal and sagittal otoliths were distinguishable by their position in the head, with the sagitta posterior to the lapillus and closer to the mid-lateral line. At this stage the two otoliths were about the same size. The sagittal otolith, which grew to be the largest, attained the distinctive adult form at about 35 mm SL (Fig. 1). The lapillus grew more slowly. The asteriscus, found just posterior to the sagitta, was first observed when larvae reached a size of 8.2 mm SL. This otolith grew relatively quickly, eventually outgrowing the lapillus at about 15 mm SL. The contours of the three otoliths 87 days after hatching were analogous to those of adults (Fig. 1).

Comparison between sagitta and lapillus

The lapillus required polishing only on the sagittal plane, making preparation and handling much easier and faster. The core was offset to the posterior end of the otolith, so could

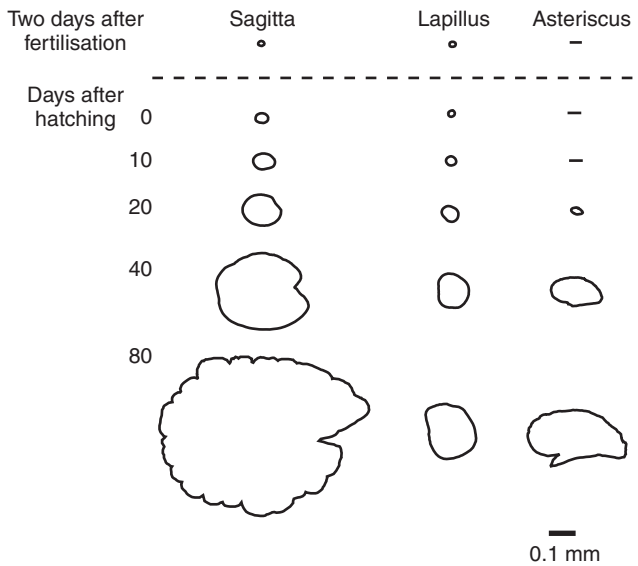

Fig. 1. Morphological development of otoliths in embryonic, larval and juvenile *M. s. splendida.*

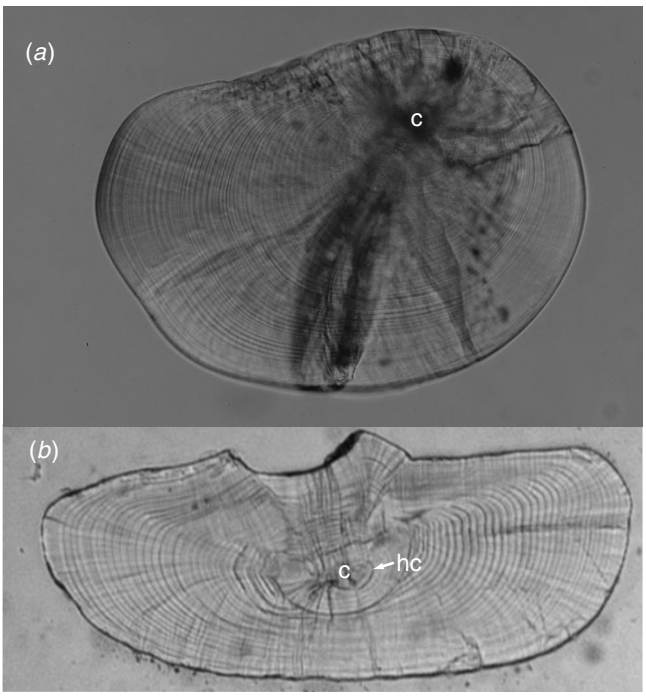

Fig. 2. Otoliths from *M. s. splendida*. (*a*) Lapillus of a 67-day-old larva showing offset location of the core (c). (*b*) Transverse section of a sagitta from a 49-day-old larva showing location of core (c) and hatching check (hc).

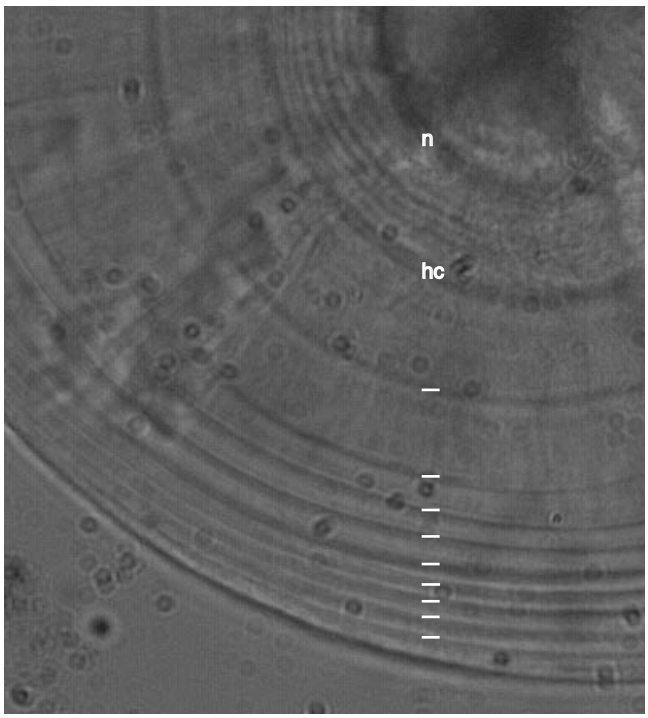

Fig. 3. Section of sagitta from a 10-day-old larva showing fine irregular increments in the core and clearer regular increments in peripheral area. n, Nucleus; hc, hatching check.

be conducted only along one growth axis (Fig. 2*a*). Because of the extremely slow growth of the lapillus, it was initially impossible to get an accurate count of increments. However, after 15 days it was relatively easy to count increments, and counts from the lapillus and sagitta from the same fish were comparable. Because resolving early increments in the lapillus was difficult, counts resulted in an underestimation of age by about 7–12 days.

The sagittae were used for all further work because of this difficulty with the lapillus. Up to an age of about 50 days a sagittal section could be used, after which transverse sectioning was required. The latter procedure was more time consuming, but it provided a more readable section. A transverse section of a sagitta consisted of a core around which concentric increments were laid towards a short dorsal axis and a long ventral axis. Two obvious checks divided the transverse section into a pre-hatching region or core, a section representing the slow growth up to about 15 days, and a peripheral region (Fig. 2*b*). Increments were difficult to resolve in the area of slow growth, although it was possible to get an accurate age. The resolution of increments in the peripheral region was quite good.

Date of first increment formation

A thin, pronounced discontinuity that delineated the core, a darker area, from a clearer peripheral area (Fig. 3) was obvious in sagittae from 31 of 37 fish. This discontinuity corresponded to the known hatching date. In the core, 5–7 fine, closely spaced, irregular increments were found (Fig. 3). The number of these increments did not correspond to the number of days before hatching. Increments outside this discontinuity were clearer and more regularly spaced (Fig. 3). For the sagittae of the other six fish, no such discontinuity could be discerned.

Relationships between standard length and age and otolith size

Larvae grew slowly during the first 12 days after hatching, then grew more rapidly (Fig. 4). The Logistic and Gompertz models were fitted to the data. Akaike's information criterion was used to assess each model; it gave values of 1721 and 1674, respectively. The Gompertz model,

$$L = 67.9\, e^{-e[-0.02(t-48.8)]}, \qquad (1)$$

was the best descriptor of early growth. Variation in body length at a particular age increased with increasing size. The coefficient of variation increased from around 3 to 6% at 12 days after hatching, to around 15% at 87 days after hatching. Fish size and sagittal length, breadth and perimeter (Fig. 5) were linearly related, indicating that isometric growth of the otolith occurred during this early life-history stage.

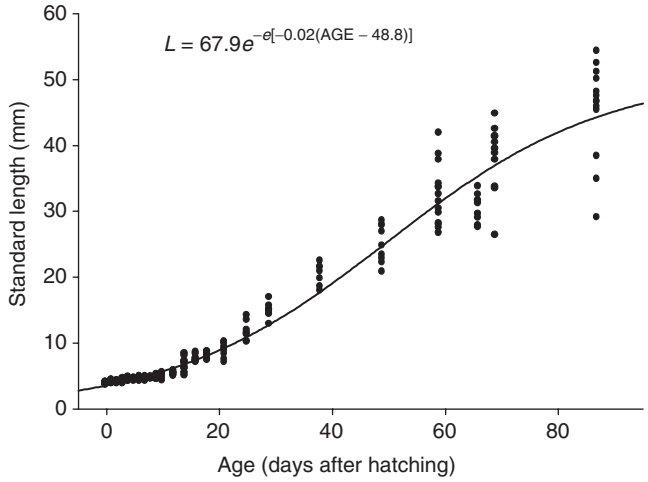

Fig. 4. Variations in body length (L) in *M. s. splendida*.

Increment periodicity

The number of sagittal rings was linearly related to daily growth. Estimates of age were obtained from 123 of the 132 sagittae. Estimated ages ranged from 3 to 101 days (Fig. 6). A linear relationship of the form $y = ax + b$ was fitted to these data. The best fit of the equation was:

$$\text{SAGITTAL INCREMENT COUNT} = 1.00 \times \text{AGE} + 0.258$$

($r^2 = 0.980$). The slope of the regression, 1.00 ± 0.0131 (s.e.) increments per day, was not significantly different from 1, so it was assumed that increments were formed daily. The precision of ageing within each age class was assessed with the coefficient of variation (c.v.) as defined by Chang (1982) and is shown in Fig. 7. The precision was poor (c.v. > 15%) for larvae younger than 25 days; however, for older larvae the c.v. was normally between 5 and 10%.

Discussion

Otolith microstructure analysis of known-age fish is a valuable technique for validating daily increment formation. Our findings support those of Barkman (1978) in that the sagitta and lapillus could be used for ageing *Melanotaenia splendida splendida*, but the asteriscus was unreliable as it did not form until the larva was roughly 8 mm SL or about 15 days old.

The sagitta is the most commonly used otolith in microstructure analysis (Campana and Neilson 1985; Secor *et al.* 1992). It is the largest otolith, the easiest to remove and handle, and generally contains the widest increments for clearest resolution of microstructural features. However, in faster-growing otoliths the formation of sub-daily increments is more likely and differentiation of these and daily increments can be a problem (Secor *et al.* 1992). This is less likely in the slower-growing lapilli. However, for *M. s. splendida* the sagitta was preferred because increments were clearer than in the lapillus, especially during the first two weeks after hatching. Despite this, there were still difficulties in interpreting increments on many otoliths. It has been suggested that the constant conditions encountered in the laboratory tend to produce fainter increments, making interpretation more difficult (Laroche *et al.* 1982; Lough *et al.* 1982; Campana 1984; Jones 1986).

The sagittae of *M. s. splendida* consisted of a core with the nucleus and a region of increments formed before hatching that differed in optical density. Geffen (1983) has suggested

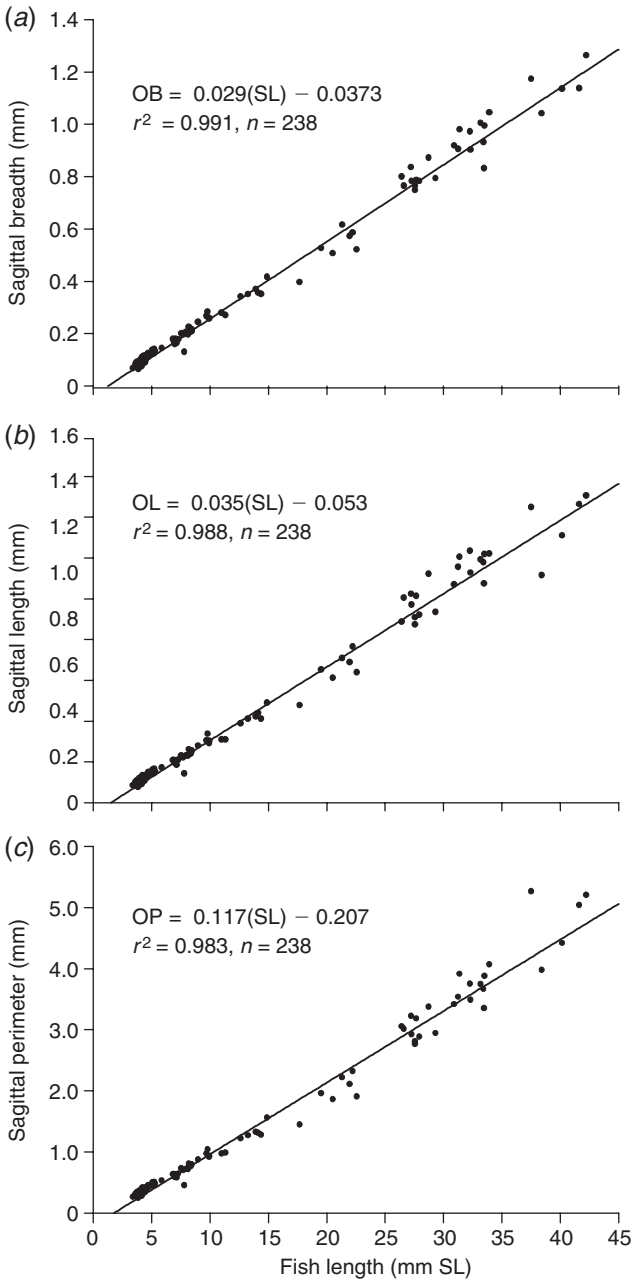

Fig. 5. Relationships between fish length and (*a*) sagittal breadth (OB), (*b*) sagittal length (OL) and (*c*) sagittal perimeter (OP).

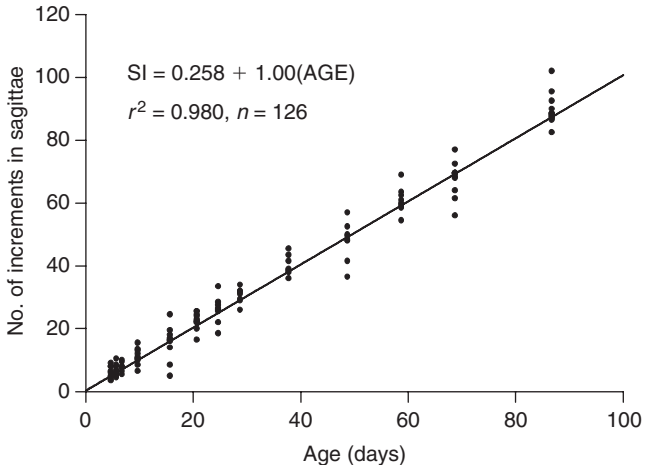

Fig. 6. Relationship between otolith increment count and age in sagittae of *M. s. splendida*.

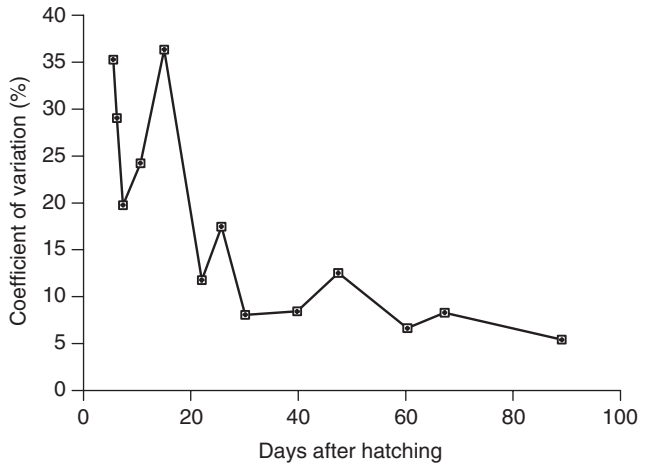

Fig. 7. Percentage coefficient of variation (c.v.%) of age estimated by counting sagittal increments, as a function of true age (days after hatching).

that the deposition of rings in embryonic otoliths may be more closely associated with developmental events, such as vascularization, eye pigmentation and development of other structures, than to environmental factors such as temperature and photoperiod. Radtke (1984) speculated that in species having slowly developing embryos, initial increment deposition occurs at or before hatching, while in species having rapidly developing embryos, initial increment deposition does not occur until yolk-sac absorption or first feeding. This speculation is not substantiated here, where increments were formed before hatching.

The formation of discontinuities or checks in otoliths as we observed at the time of hatching is a common phenomenon, although not always related to hatching. In many instances a discontinuity is laid down at important life-history events such as first feeding, settlement or metamorphosis (Marshall and Parker 1982) or may be induced during periods of stress to the fish (Campana 1983). A region from hatching up to about 15 days was distinguished by several extremely fine increments that corresponded to a period of slow fish growth. A distinct discontinuity separated this region from the peripheral region, which marked a significant change in the microstructure of the otolith. Such substantial changes have been interpreted as significant transitions in life history, reflecting changes in physiology and morphology (Brothers and MacFarland 1981) or osteological development (Brown *et al.* 2001). This discontinuity in the sagitta of *M. s. splendida* corresponded with the complete absorption of the yolk sac and transition to sole reliance on exogenous feeding.

This distinct hatching check in *M. s. splendida* coincided with first increment formation and thus can be used as the benchmark for ageing studies. However, the hatching check and first feeding check could not be recognized in all sagittae. This is probably because the discontinuity may need to be some distance away from the margin of the otolith before it can be resolved optically, making it difficult to detect in newly hatched fish or fish that have recently undergone the transition to exogenous feeding (Brothers *et al.* 1983). Yet there were fish in the present study that were obviously old enough for this discontinuity to be readily visible. Brothers and MacFarland (1981) have suggested that since many check marks are thought to be associated with an 'eco-behavioural' change, the clarity of such marks may reflect the ease with which a fish copes with the transition. It is possible that individuals of *M. s. splendida* that failed to show distinct discontinuities had readily made the transition from reliance on yolk reserves to exogenous feeding. Rainbowfish begin feeding within hours of hatching (e.g. Ivantsoff *et al.* 1988; Reid and Holdway 1995), and those fish that grow more quickly and are able to handle prey items more competently may be less reliant on yolk reserves and thus find the transition to exogenous feeding not physiologically challenging.

Fish reared under laboratory conditions generally produce increments that are fainter and more difficult to interpret (Laroche *et al.* 1982; Lough *et al.* 1982; Campana 1984; Jones 1986; Campana 2001). Many of the problems encountered in interpreting the increments in this study may not be encountered in field-caught fish with clearer increments. However, since the error in increment count was relatively small for most of the fish, the appropriate model for increment formation is one of daily formation. Greater accuracy might be attained by determining the number of increments formed between hatching and yolk-sac absorption. If the number of increments formed during this period is relatively constant, then counts could begin from the mark laid down at yolk-sac absorption and age adjusted accordingly. This could improve the accuracy of ageing and simplify the preparation of the otoliths. In this way, the relative age of the fish will be known, which may be just as important or informative as absolute age (Campana 2001).

This study presented an analysis of the microstructure of otoliths of juvenile and larval *M. s. splendida*. Techniques for preparation and ageing as outlined in this study are now sufficiently advanced to begin looking at important questions on the ecology of early *M. s. splendida*. This, together with our related work on early development and growth and laboratory exposure to contaminants (C. Humphrey and D. W. Klumpp, unpublished data; Humphrey *et al.* 2003) provide a useful basis for further research, for example on the effects of contaminants on growth and mortality in wild *M. s. splendida*.

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