Can otolith microchemistry reveal whether the blind cave gudgeon, *Milyeringa veritas* (Eleotridae), is diadromous within a subterranean estuary?

W.F. Humphreys[a], Jen-Chieh Shiao[b], Yoshiyuki Iizuka[c] & Wann-Nian Tzeng[d]
[a]*Western Australian Museum, Locked Bag 49, WA 6986, Welshpool DC, Australia*
(e-mail: bill.humphreys@museum.wa.gov.au)
[b]*Institute of Cellular and Organismic Biology, Academia Sinica, Nankang, 115, Taipei, Taiwan, Republic of China*
[c]*Institute of Earth Sciences, Academia Sinica, Nankang, 115, Taipei, Taiwan, Republic of China*
[d]*Institute of Fisheries Science, College of Life Science, National Taiwan University, Taipei, Taiwan, Republic of China*

Synopsis

The blind cavefish, *Milyeringa veritas*, inhabits an anchialine system, effectively a groundwater estuary, in which salinity varies between fresh and seawater at different locations and depths. Owing to the inaccessible habitat and the threatened status of the cavefish it is hard to obtain the biological information needed for their management. This paper explores the utility of otolith Sr:Ca ratio in elucidating cavefish biology. The mean Sr:Ca ratio of the water inhabited by the cavefish is correlated with both the TDS (total dissolved solids) of the habitat and with the Sr:Ca ratio of the sagittal otolith of the cavefish inhabiting that site. Mean values of Sr:Ca in otoliths suggest some cavefish inhabit sea or brackish waters while others remain in freshwater. Some individuals appear to move between waters of very different TDS at various stages but there is no consistency in the direction or apparent TDS range of the water bodies inhabited which indicates that the cavefish utilise the different water bodies opportunistically. Residual analyses indicate clear and routine changes in the TDS of the water occupied at various phases of growth, irrespective of the TDS at which the cavefish were sampled. Annular markings are present in some otoliths but they cannot be related to likely periodicities in the subterranean environment.

Introduction

The biological information required for the management of fish populations is lacking for the two species of Australian cavefish. The blind cave gudgeon, *Milyeringa veritas* Whitley 1945 (Eleotridae), a monotypic genus, and the Blind Cave Eel, *Ophisternon candidum* (Mees 1962) (Synbranchidae) occur sympatrically in the Cape Range karst of northwestern Australia. Only two specimens of *O. candidum* are in collections and both species are listed under both Commonwealth of Australia and Western Australian threatened species legislation. Few specimens are available for study on account of the inaccessibility of the populations and their limited distribution (Humphreys 2001), and sufficient samples cannot acceptably be taken to establish life history parameters owing to their threatened status, an issue raised by Radtke et al. (1988) in relation to

Hawaiian freshwater gobies. Thus, there is a need to explore alternative methods of deducing significant life history events.

Milyeringa is an obligate inhabitant of subterranean wetlands (Humphreys 2000b) which occurs exclusively in karst landforms adjacent to the North West Shelf of Western Australia where it is found within 150 m of the coast and up to 4.3 km inland (Humphreys 2001). It inhabits the lower foothills and the coastal plain of Cape Range peninsula, and the freshwater lens on Barrow Island. The species is versatile and may variously be found swimming in dim sunlight at karst windows (Humphreys 2001) and in groundwater commencing 68 m below the ground surface. It occurs in freshwater caves and may be found in full seawater in the lower part of anchialine caves (Humphreys 1999) and several kilometres removed from natural openings. Through most of the distribution of *Milyeringa* its habitat is affected by marine tides (Humphreys et al. 1999, figure 1; Humphreys 2002) and comprise anchialine systems. Such ecosystems are the focus of considerable endemic biodiversity, both in the areas inhabited by *Milyeringa* (Humphreys 2000a, 2001, 2002) and more widely (Sket 1996, Humphreys 1999, Iliffe 2000, Jaume et al. 2001). Within this context *Milyeringa* occurs in a wide range of water chemistries encompassing freshwater and seawater and, in some cases, with complex physico-chemical profiles (Table 1) (Humphreys & Adams 1991, Humphreys 1994, 1999, Yager and Humphreys 1996, Humphreys et al. 1999). Populations are known from freshwater caves (300 mg l^{-1} TDS (total dissolved solids): Poore and Humphreys 1992) and from the seawater part of the anchialine system (34, 000 mg l^{-1} TDS: Yager and Humphreys 1996, Humphreys 1999). The cavefish occur in hyperoxic surface water and in the suboxic (see Sket 1996) waters found below the pycnocline (Humphreys 1999) where they have been seen foraging immediately above sediments covered by sulphur bacteria (Humphreys 1999). The best studied part of the anchialine system in Australia is Bundera Sinkhole (Australian Karst Index number C-28, a water filled sinkhole or cenote) which lies 1.7 km from the ocean and exhibits a tidal range about 10% of that at the ocean shore. The halocline is associated with a complex physico-chemical profile involving a cascade of nitrogen

species, and complex redox, dissolved oxygen and hydrogen sulphide profiles (Humphreys 1999, unpublished data).

In general, fish may move from seawater to freshwater through estuaries, usually associated with the search for food and/or refuge rather than the need to reproduce. Only about 1% of fishes are diadromous but of these about a fifth are amphidromous and they can spawn in either freshwater or in a marine environment. The waters available in the subterranean estuary (in the sense of Moore 1999) encompass the range from freshwater to seawater. Consequently, within the confines of the subterranean estuary there is the potential for the full range of diadromous life histories in *Milyeringa*; analagous to anadromy, catadromy and amphidromy. Although the general range of water chemistry inhabited by *Milyeringa veritas* is known (Humphreys 2001), this information comes from different individuals and so there is no direct evidence to show that they can move freely, periodically or occasionally, among habitats of different salinity. Since otolith microchemistry is a proxy for salinity, although not in simple relationship, it is possible to understand the movement of *Milyeringa* among these habitats of different salinity. This is important because the cavernous karst would permit individual *Milyeringa* in some populations to move between fresh water and seawater salinities, either vertically within the water column, or laterally through karst conduits, thus exposing them to quite different water chemistries. It is not known, for example, whether individuals or cohorts move between these different water masses, or whether different subpopulations permanently inhabit the different water masses. However, there is evidence of genetically distinct subpopulations along the coastal plain of the Cape Range peninsula (Humphreys & Adams 1991, Humphreys 2001). Further, it is not known whether the same individuals move regularly or rarely between the water types, or whether there are life stage movements either at random or sequentially. Knowledge of the range of salinity (or a surrogate index such as specific conductivity or total dissolved solids: TDS) occupied by an individual would provide a measure of such movements and thus enhance understanding of their biology.

In surface estuaries, the regions adjacent to salt fronts are essential nursery habitats for some fish

Table 1. Otolith specimen related habitat information for *Milyeringa veritas*.

Specimen	Name[a]	SBL mm	Habitat	Notes	TDS mg l^{-1}	Source
1151B	Kubura Well C-27	43.6	Cave	11 m water ~3% ocean tide	3431	Humphreys unpublished data
1151C	Kubura Well C-27	35	Cave	11 m water ~3% ocean tide	3431	Humphreys unpublished data
1182	Kubura Well C-27	37.3	Cave	Surface water	2800	Humphreys 1994
1182A	Kubura Well C-27	34.8	Cave	Surface water	2800	Humphreys 1994
3194	C-215	–	Cave	Freshwater ~6% ocean tide	1100	Humphreys 1999 and unpublished data
3943	Bundera Sinkhole C-28	44.5	Flooded sinkhole at 8.5 m	Anchialine ~10% ocean tide 18 000–36 000 TDS	25600	Humphreys 1999 and unpublished data
3949	Bundera Sinkhole C-28	48.4	Flooded sinkhole at 8.5 m	Anchialine ~10% ocean tide 18 000–36 000 TDS	25600	Humphreys 1999 and unpublished data
4281	Bundera Sinkhole C-28	16.2	Flooded sinkhole at > 8 m	Anchialine ~10% ocean tide 18 000–36 000 TDS	25600	Humphreys 1999 and unpublished data
4622	5 Mile Well C-273	26.5	Concrete well 9.4 m deep, water depth c. 0.5 m.	4350	4350	Humphreys 1994
4664	Ampolex AB5	10.7	Borehole	24 700	–	Humphreys, unpublished report
4677	Ampolex Site D	13.3	Borehole	15 100	–	Humphreys, unpublished report
5231	C-215	46.3	Cave	Freshwater ~6% ocean tide	1250	Humphreys 1994
5232	Tulki Well C-149	41.5	Concrete well ~ 5m deep to 0.2 m water	0.2 m water tidal	4600	Humphreys 1999 and unpublished data
9915A	Exmouth borefield MB 18	23.5	Deep borehole	2500 – 5500	3000	Water Corporation, 2001
9915B	Exmouth borefield MB 18	23.5	Deep borehole	2500 – 5500	3000	Water Corporation, 2001
9916A	Exmouth borefield DSO 4/96	9.4	Deep borehole	300 – 7000	1000	Water Corporation, 2001
9916B	Exmouth borefield DSO 4/96	9.4	Deep borehole	300 – 7000	1000	Water Corporation, 2001
Sea	Wapet Jetty	–	–	–	36500	Humphreys 1994
Fresh rainfall	Exmouth	–	–	8	15	Humphreys 1994

[a]Numbers with C-prefix are Australian Karst Index numbers; other numbers refer to bores.

(Secor 2002) but other species also make use of estuarine waters. For example, sea bass, *Morone labrax*, young-of-the-year may concentrate adjacent to and down-estuary of the saltfront (Secor 2002), whereas a proportion only of newly transformed juveniles may move to the freshwater side of the salt-front, thus exhibiting facultative amphidromy (Ohta cited in Secor 2002). Similarly, migrations in vertical directions may be undertaken by both prey and predators, respectively to avoid predators or seek prey (Bayly 1986), or to seek out a chemocline (Hamner et al. 1982). Hence, small-scale movements within groundwater estuaries associated with the presence of a chemocline may be important ecologically, especially as these clines are associated with rich microbiological communities that may fix energy chemoautotrophically (Pohlman et al. 1997, Humphreys 1999) and attract biofilm grazers. The ambient Sr concentration is higher in marine than in fresh water (Odum 1957, Tzeng and Tsai 1994, Tzeng 1996) and this is reflected in the concentration of elements deposited in the accreting otoliths of fish (Fenton & Short 1992) with the Sr:Ca ratio being an especially useful indicator anadromy (Campana 1999). This ratio in ostracod

valves is also useful to determine the palaeosalinity of lakes (Chivas et al. 1985). There is considerable evidence that the Sr:Ca ratio in the otoliths of fishes are higher in marine than in freshwater environments (Casselman 1982, Kalish, 1990, Secor 1992, Tzeng & Tsai 1994, Tzeng 1996, Tzeng et al. 1997) and so the ratio can be used as an indicator of the movement of individuals between marine and freshwater (Campana 1999, Cheng and Tzeng 1996, Shen et al. 1998). Hence, it is a pertinent parameter to examine the small scale movement between fresh and seawater that may be made by *Milyeringa* in the anchialine waters they inhabit. Recent experiments have shown that a marine fish, *Leiostomus xanthurus*, had otolith Sr/Ca and Ba/Ca ratios deposited in proportion to their ratios in ambient waters and, as temperature significantly affected Sr incorporation, that more subtle environmental variations might be recoverable from otolith preparations (Bath et al. 2000).

In the absence of detailed biological or population work on *M. veritas*, the current work was conducted to establish whether useful biological information can be obtained from the cavefish otoliths. Particularly whether individuals inhabit a single water type, either seawater or fresh water, throughout life, alternatively, that individuals may move between water bodies of different types, either at different stages through the life cycle or irregularly.

Materials and methods

The sagittal otoliths were dissected from 15 specimens of *Milyeringa* already in the collection of the Western Australian Museum and preserved in 75% alcohol and which were collected from water of known salinity at the time of capture, some sites providing access to a range of salinities at the same location (Table 1). *Milyeringa* were sampled from nine locations within the coastal karst of the Cape Range peninsula, Western Australia (approx 22° S; 114° E) and they include individuals from separate genetic stocks (Adams & Humphreys 1993). Tests on the otoliths of other species have shown that the effect of alcohol preservation on the elemental concentration of sagitta is least on Calcium and Strontium (Procter & Thresher 1998).

Owing to the often complex physico-chemical profile in the groundwater and the various sources of the specimens, TDS (total dissolved solids, mg l^{-1}) is used here as a surrogate for salinity, as is common practice amongst limnologists (Boulton and Brock 1999). This was measured from water sampled at the time of capture from about the same depth as the fish, or it was estimated from conductivity profiles of the water column or, in the data intensive Exmouth borefield, TDS was interpolated between those in closely adjacent bores.

To establish the relationship between TDS and Sr:Ca ratio the Ca and Sr concentrations in water samples of local seawater and from seven groundwater sites were measured using Method 3210B (American Public Health Association 1998) by an analytical laboratory (Chemistry Centre (WA) method designation iMET1WCICP).

Otolith preparation and quantitative measurements of Ca and Sr largely follow Tzeng et al. (1997). Briefly, otoliths removed from the gudgeons were washed in distilled water and dried in air. The otoliths were embedded in thermo-epoxy (Epofix resin) and cured for 40 min at 70 °C. Embedded otoliths were ground from the proximal side of the sagittal plane of the fish until the primordium of the otolith was revealed. For microprobe analysis, the polished otoliths were coated under vacuum with a layer of carbon for electron conductance. The embedding, grinding and coating procedures were similar to those used elsewhere (Tzeng & Tsai 1994). Quantitative measurements of the concentration of Ca and Sr in otolith of the gudgeon were conducted using an electron microprobe microanalyzer (EPMA) equipped with wavelength dispersive spectrometers (JXA- 8900R, JEOL, Co. Ltd., Japan). Measurements were made at 10 μm intervals along a transect from the primordium to the edge of the otolith as this has been found to best discriminate between marine and fresh waters in other species (Tzeng et al. 1997). The beam conditions were 15 keV for the acceleration voltage, 3 nA for the current, and a 5×4 μm rectangular beam. Standards used for Ca and Sr calibration were synthesised aragonite ($CaCO_3$) and natural strontianite ($SrCO_3$:NMNH-R-10065), respectively. After microchemistry analysis, the otoliths were polished to remove the carbon layer and etched

with 5% EDTA for 1 min to reveal the potential daily or annual increments.

In the absence of growth data for the fish (Humphreys 2001), or experimentally marked otoliths with clear growth increments, the ages of the fish are unknown. However, as each otolith was scanned from the core to its periphery the data comprise a time series of Sr:Ca ratio against the relative age of the fish. The quality control method of Cumulative sum analysis (Woodward & Goldsmith 1964) is used to examine the trends of the radial profile of Sr:Ca ratio in the otoliths of *M. veritas*, as used for other time series data (Ibanez et al. 1993, Le Fevre-Lehoerff et al. 1995, Beaugrand et al. 2000).

Departures of the Sr:Ca ratio from the mean trend along the radius of the otolith were measured by excursions beyond the 99% confidence intervals of the true mean Y of the residual values of the linear regression of the Sr:Ca ratio on radial distance from the core of the otolith.

Results

Storage effects

There is no evidence of decalcification in the material used, nor was there evidence of surface erosion of the otoliths. Examination of the raw data shows that the mean calcium concentration in different otoliths ranged between 34.7 and 39.1 wt % (Table 2, mean 37.2, SD 1.87) similar to material examined elsewhere.

Water chemistry

Local seawater had $Sr:Ca = 19.42 \times 10^{-3}$. Nonetheless, in different areas of the Cape Range coastal plain there were marked changes in Sr:Ca ratio of the groundwater (range $4.80 - 19.72 \times 10^{-3}$: local seawater 19.42×10^{-3}) which overall was positively associated with the TDS (log) ($F_{s1,12} = 26.42$, $p = 0.0002$, $r = 0.83$).

Otoliths were examined from cavefish of a range of size (standard length $9 - 48$ mm) and water chemistry (TDS 1000-25600 mg l^{-1}), and which were sampled from various habitats (caves, boreholes, sinkholes) (Table 1). The mean Sr: Ca ratio within a given sagitta otolith reflects the TDS of the location where it was collected (Figure 1: log TDS, $Fs_{1,11} = 28.92$, $p = 0.0002$), despite the sometimes complex physico-chemical profiles.

There is no significant relationship between cavefish size and TDS ($Fs_{1,10} = 1.05$, $p = 0.33$), thus there is no tendency for the cavefish to segregate by TDS according to their size. There is no

Table 2. Statistics of the Ca and Sr concentrations (weight %) and the Sr:Ca ratio ($\times 10^{-3}$) derived from the radial scans from the core to the periphery of the otoliths and measured every 10 μm from the core along the radius of the otolith.

Sample	Mean Sr/ a ($\times 10^{-3}$)	SE	n	Min	Max	Calcium Mean	SD	Range	Strontium Mean	SD	Range
1151B	8.52	0.17	49	5.5	11.1	34.9	0.61	$33.3 - 35.9$	0.3	0.04	$0.19 - 0.39$
1151C	11.01	0.39	55	6.6	18.6	35	0.65	$33.3 - 35.9$	0.38	0.09	$0.24 - 0.65$
1182	7.06	0.13	103	3.47	10.2	38.6	0.61	$37.2 - 40.0$	0.27	0.05	$0.14 - 0.39$
1182A	7.97	0.2	62	4.4	13.6	38.7	0.69	$36.7 - 39.9$	0.31	0.06	$0.17 - 0.50$
3194	3.45	0.14	64	0.48	5.8	38.7	0.95	$32.5 - 40.1$	0.13	0.05	$0.02 - 0.22$
3943	11.43	0.38	53	7.4	19.7	34.7	0.62	$33.1 - 35.9$	0.4	0.09	$0.25 - 0.68$
3949	13.9	0.32	82	6.83	21.3	38.4	0.08	$34.2 - 39.7$	0.53	0.11	$0.26 - 0.82$
4281	10.51	0.32	40	5.4	15.2	39.1	0.36	$38.2 - 39.9$	0.41	0.08	$0.21 - 0.60$
4622	4.48	0.16	36	2.84	6.7	38.6	1.21	$35.3 - 40.0$	0.17	0.03	$0.11 - 0.26$
4664	4.28	0.12	36	2.91	5.8	38.6	0.87	$36.5 - 40.1$	0.12	0.03	$0.12 - 0.22$
4677	13	0.41	36	3.92	16.3	38.7	0.62	$37.2 - 39.5$	0.5	0.06	$0.37 - 0.63$
5231	4.42	0.1	67	1.9	6.2	36.5	0.75	$33.8 - 37.7$	0.16	0.03	$0.07 - 0.23$
5232	7.72	0.23	54	4.8	12.4						
9915a*	5.95	0.18	59	3.51	10.1	38.1	0.69	$35.6 - 39.0$	0.23	0.05	$0.13 - 0.36$
9915b*	6.03	0.21	44	3.48	8.51	38.5	0.42	$37.6 - 39.4$	0.23	0.05	$0.13 - 0.33$
9916a*	2.64	0.25	17	0.76	4.63	36.7	0.14	$34.7 - 38.2$	0.1	0.04	$0.03 - 0.17$
9916b*	2.75	0.3	15	1.27	5.53	37.9	0.42	$37.0 - 38.4$	0.11	0.04	$0.05 - 0.21$

*Suffixes a and b denote duplicate analyses of an otolith along a separate radius; only the first of each pair is considered in regressions.
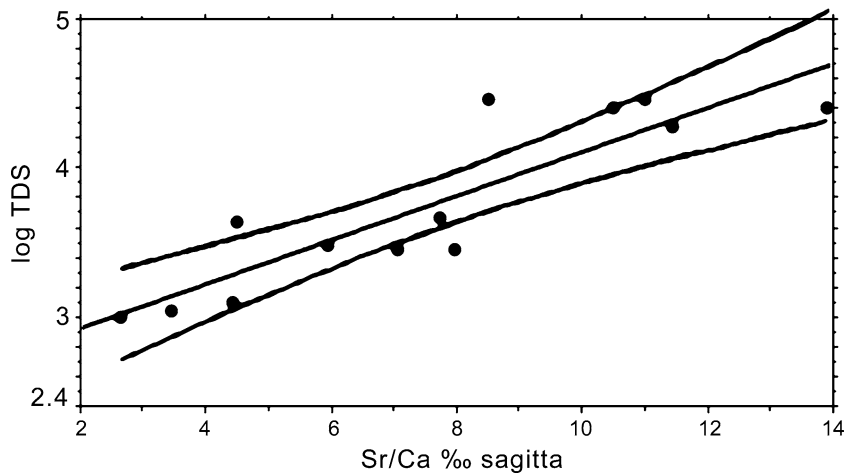
Figure 1. Mean $Sr/Ca \times 10^{-3}$ of sagitta of *Milyeringa veritas* regressed on TDS (log). Error bars denote the 95% CI of the true mean Y. Four sets of data from Table 2 are excluded: 9915B and 9916B as they were replicate scans of the same sagitta, and 4464 and 4677 because the TDS was measured only at the time the bores were drilled to below the karst and when the groundwater profile would have been mixed.

relationship between the mean and the coefficient of variation of the Sr:Ca ratio ($Fs_{1,13} = 2.007$, $p = 0.18$).

The Sr:Ca ratio at 10 μm intervals from the core of the otolith to its periphery is shown for 15 individual *Milyeringa* in Figures 2 and 3. Two replicated scans show the general consistency of the changes in the Sr:Ca ratio along separate radii of the same otolith (Table 2 and Figure 3).

The scans exhibit several distinct attributes. The plots in Figures 2 and 3 show that the mean level of the Sr:Ca ratio differs fourfold between individual *M. veritas*, ranging from 2.64 to 13.9×10^{-3} (Table 2), and that the form of the scans differs between individuals. While some plots show a relatively constant Sr:Ca ratio level throughout the growth of the otolith (Figure 3: #3194, #4664), others fluctuate widely but stay at about the same level overall (Figure 2: #5231), while others show a distinct trend, with the Sr:Ca ratio either declining (Figure 2: #5232) or increasing (Figure 3: #1182) during growth. In some individuals there are distinct, presumably prolonged, changes in the Sr:Ca ratio as indicated by the variance (Table 2 and Figure 2, #3943 and #1151C; Figure 3, #3949) which are inferred to indicate a habitat with higher or lower TDS respectively.

The trend analyses (Figures 2, 3, right columns) suggest that some individuals have undergone life cycle shifts in the TDS environment. For example,

Milyeringa #3943 from Bundera Sinkhole (Figure 2), the deep anchiline cave that is known to have a TDS range between 20 000 and 36 000 mg l^{-1} TDS. This individual is inferred to have started life in the deep marine water ('a' in Figure 2), moved to the brackish surface (b), returned to marine water below the halocline (c) and then inhabited the broad halocline (d) for half the growth of the otolith. The Sr:Ca ratio never approaches the low level found in cavefish #5231 from the freshwater cave C-215.

The two cavefish from Kubura Well seemingly had different life experiences. Gudgeon #1151C apparently started life in seawater (e) and then seems to have moved into and remained within a halocline in later life (f). By contrast, #1151B seems to have remained largely within the halocline of Kubura Well throughout life. Finally, #5231 in cave C-215 continually has a Sr:Ca ratio $< 6.5 \times 10^{-3}$.

Variation from the trends in the Sr:Ca ratio are illustrated by plotting the residual values of the regression of Sr:Ca ratio on the distance from the core (right columns of Figures 2, 3). Excursions of the Sr:Ca ratio beyond the 99% confidence intervals are deemed to show biologically significant changes in Sr:Ca ratio. Such changes are seen (Figures 2, 3) in the otoliths of most of the cavefish irrespective of the mean Sr:Ca ratio.
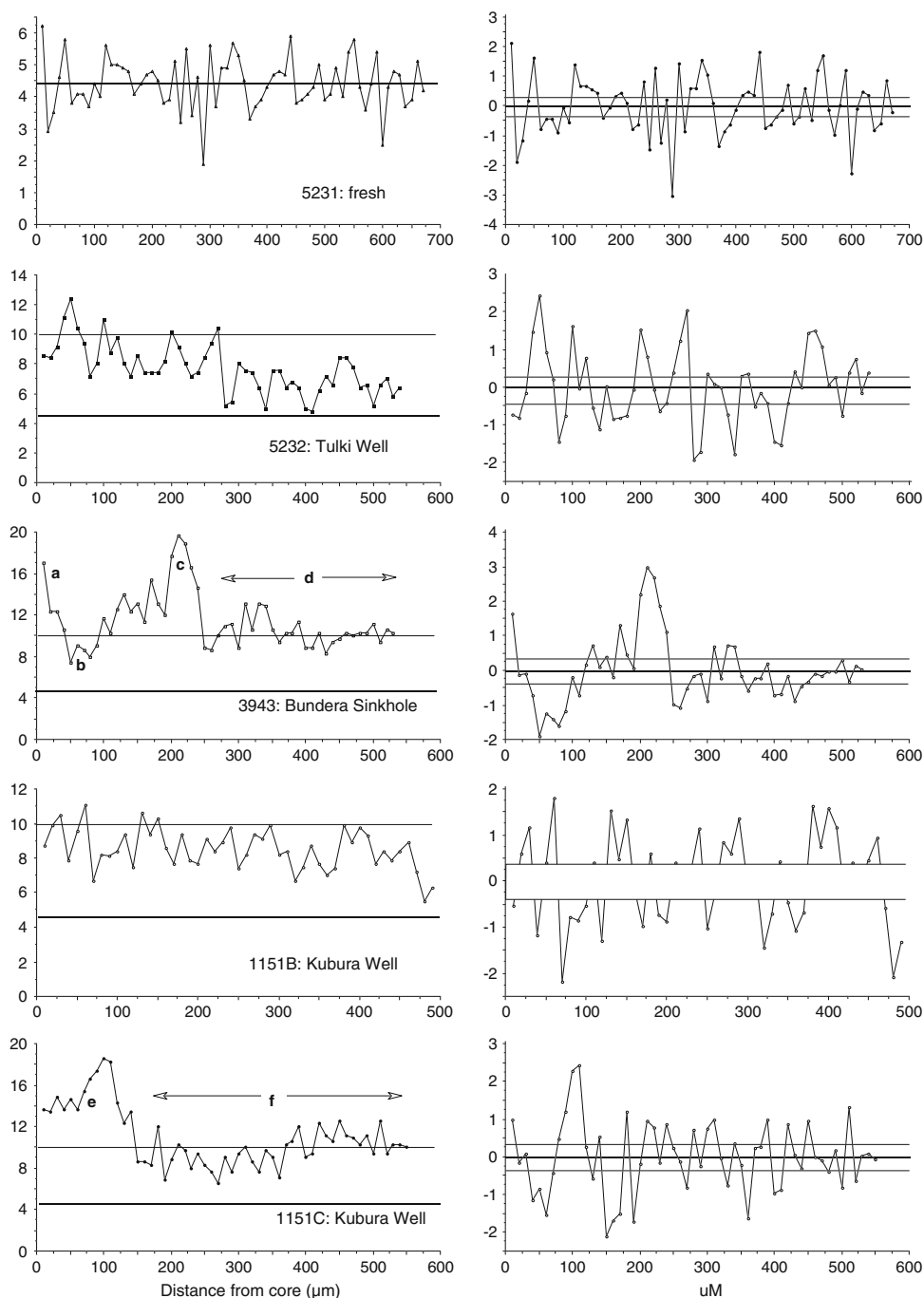
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Figure 2. Left column: Sr:Ca ($\times 10^{-3}$) radial profiles in the sagittal otolith of five *Milyeringa veritas* from waters of differing physico-chemical characteristics. A value of Sr:Ca 5×10^{-3} is the upper boundary seen in gobies from freshwater (see text). The dotted line denotes the higher of the mean Sr:Ca ratios seen in *Milyeringa* from a freshwater cave C-215 (upper). The horizontal line at a Sr:Ca $= 10 \times 10^{-3}$ is used in discussion to distinguish estuarine inhabitants (Sr:Ca $= 5 - 10 \times 10^{-3}$) from marine waters Sr:Ca $> 10 \times 10^{-3}$). Right column: Cumulative sum analysis (Woodward & Goldsmith 1964) of the trends of the radial profile of Sr:Ca ratio in the otoliths of *M. veritas*. Note that an upward inflexion in the trend line denotes a change to generally greater Sr:Ca ration (inferring inhabiting water of greater salinity), and *vice versa* with a downward inflexion. Fish identification numbers (Table 1) are from top to bottom: 5231; 5232; 3943; 1151B; 1151C.
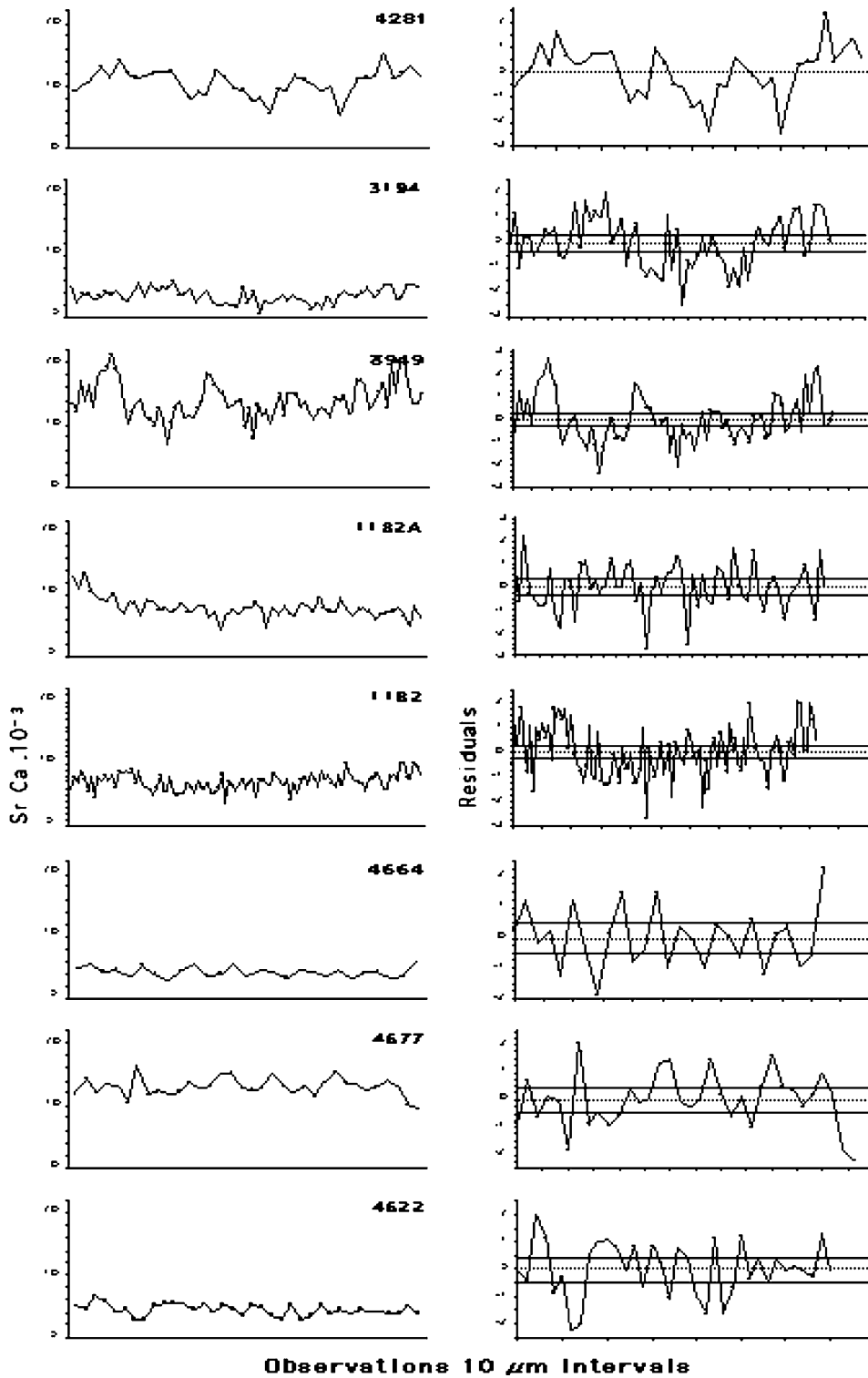
Sr Ca.10⁻³

Residuals

4281

3194

3949

1182A

1182

4664

4677

4622

Observations 10 μm Intervals

Figure 3. Left column: Sr:Ca ($\times 10^{-3}$) radial profiles in the sagittal otolith of 10 *Milyeringa veritas* from waters of differing physico-chemical characteristics − all plotted to the same vertical scale range of $0 - 22$ Sr:Ca ($\times 10^{-3}$). Right column: Cumulative sum analysis (Woodward & Goldsmith 1964) of the trends of the radial profile of Sr:Ca ratio in the otoliths of *M. veritas*. Note that a positive change in slope indicates a change to a higher Sr:Ca ratio, which is indicative of a move to more saline water, and *vice versa*. The horizontal dotted line denotes zero cumulative sum. Data are plotted by observation number from the core of the sagitta which were sampled at $10 \,\mu m$ intervals. Note that the last four rows represent paired samples from two fish scanned along separate radii of the same otolith. Fish identification numbers (Table 1) are from top to bottom: 4281; 3194; 3949; 1182A; 1182; 4664; 4677; 4622; 9916A; 9916B (duplicate); 9915A; 9915B (duplicate).

The number of cycles extending beyond the 99% confidence intervals on the residual plots (Figures 2, 3) are related to the length of the segment scanned and they occur on average $55.1 \,\mu m$ apart with rather narrow limits (SE 3.62, range $36 - 84$, $n = 15$); these data are shown as frequency and cumulative frequency plots in Figure 4.

Otolith growth

The sectioned sagitta of *Milyeringa* of the few specimens from sites partly exposed to diffuse natural light showing marked banding (Figure 5: bottom right: Kubura Well) that may be growth increments that can often be read from otoliths of surface fish and provide detailed individual life history information. In contrast, sagitta of cavefish from most of the sites, which were necessarily in total darkness, showed a complete absence of concentric banding (Figure 6).

Discussion

Otoliths stored under some conditions can undergo post mortem changes (Procter and Thresher 1998). No evidence was found to suggest major effects of storage on the levels of Ca and Sr in the otoliths, a finding in agreement with Procter and Thresher (1998) who found that the measured concentrations of Ca and Sr to be relatively insensitive to the range of procedures they tested. For example, they recorded Ca values within the range of $38 - 42$ wt %, while Campana (1999) recorded Ca values of 38.0 (SD 2.05 wt %) in marine species and 40.7 (SD 2.58 wt %) in freshwater species. Similarly, Procter and Thresher (1998) recorded Sr values within the range of $0.1 - 0.7$ wt %, and Campana (1999) recorded Sr values of 0.21 (SE 0.013 wt %) in marine species

and 0.07 (SE 0.011 wt %) in freshwater species and 0.19 (SE 0.007 wt %) in estuarine species. The Sr values recorded here lie within the bounds recorded in intensive studies and do not indicate excessive post mortem effects (Figure 7).

In the absence of growth data for the cavefish (Humphreys 2001), or experimentally marked otoliths with clear growth increments, the ages of the cavefish are unknown. However, as each otolith was scanned from the core to its periphery, the plots in Figures 2 and 3 are of Sr:Ca ratio against the relative age of the cavefish.

Studies of eels show that the Sr:Ca ratio depends both on the life history stage and as a response to different environmental salinities. For American eels, *Anguilla rostrata*, Jessop et al. (2002) found that Sr:Ca ratio $> 5 \times 10^{-3}$ indicated estuarine residence while Sr:Ca ratio < 4 indicated freshwater residence. In the Japanese eel, *Anguilla japonica*, the marine phase Sr:Ca ratio varied between 7 and 18×10^{-3} and that three life cycle phases could be categorised following metamorphosis: 1, freshwater phase with Sr:Ca ratio $< 4 \times 10^{-3}$; 2, seawater phase with Sr:Ca ratio variously $> 5 \times 10^{-3}$; 3, estuarine phase with Sr:Ca ratio varying from 0 to 10×10^{-3} (Tzeng et al. 2002, Shiao et al. 2003), or Sr:Ca $4 - 10 \times 10^{-3}$ in seawater and Sr:Ca $< 4 \times 10^{-3}$ in freshwater, while in estuarine habitats the Sr:Ca ratio fluctuated between the two (Tzeng et al. 2003). Shen et al. (1998) considered the Sr:Ca ratio in goby otoliths to be indicative of the water inhabited by the fish, being Sr:Ca 5×10^{-3} (10×10^{-3} in original which is here corrected for molarity) in fish living in estuarine conditions, with values of Sr:Ca greater or less than 5×10^{-3} to be indicative of marine and freshwater conditions respectively.

On the Cape Range peninsula the Sr:Ca ratio of the groundwater was positively related to its TDS. Characterisation of the TDS to which a given
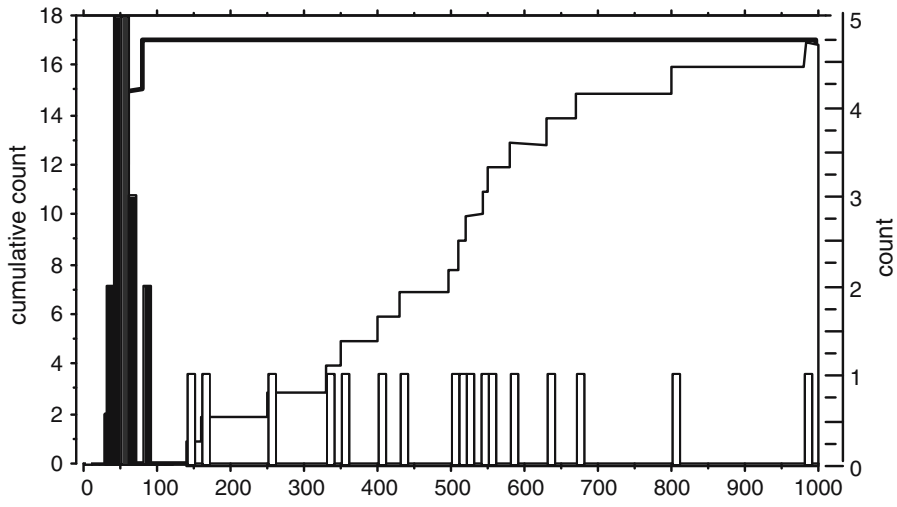
Figure 4. Frequency distribution and cumulative frequency distribution of the length of the otolith radial scans (open bars and fine line) and of the mean separation distance of excursions beyond the 99% confidence intervals in the residual plots in Figures 2 and 3 (solid bars and bold line).
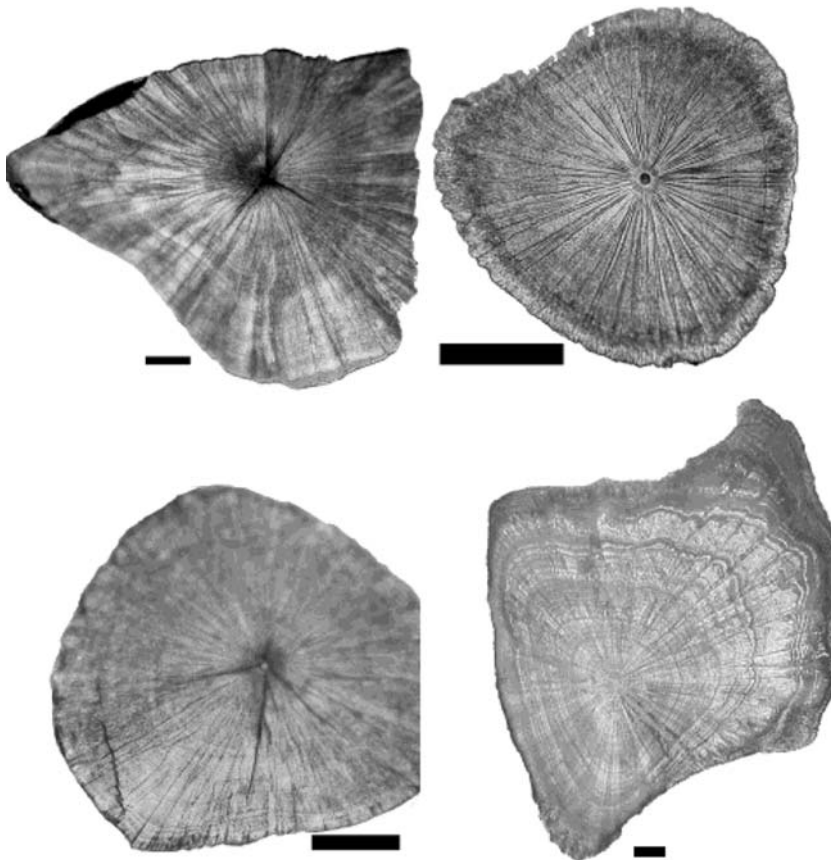

Figure 5. Sectioned sagitta of the cave fish, *M. veritas*, showing marked banding in fish from site partly exposed to natural light (bottom right: Kubura Well) in contrast to the absence of concentric banding in fish from sites in total darkness. Left column: 9915 dark no tide; 4664 dark, tide affected. Right column: 9916 dark no tide; 1182: light tidal. Scale bar 100 μm.
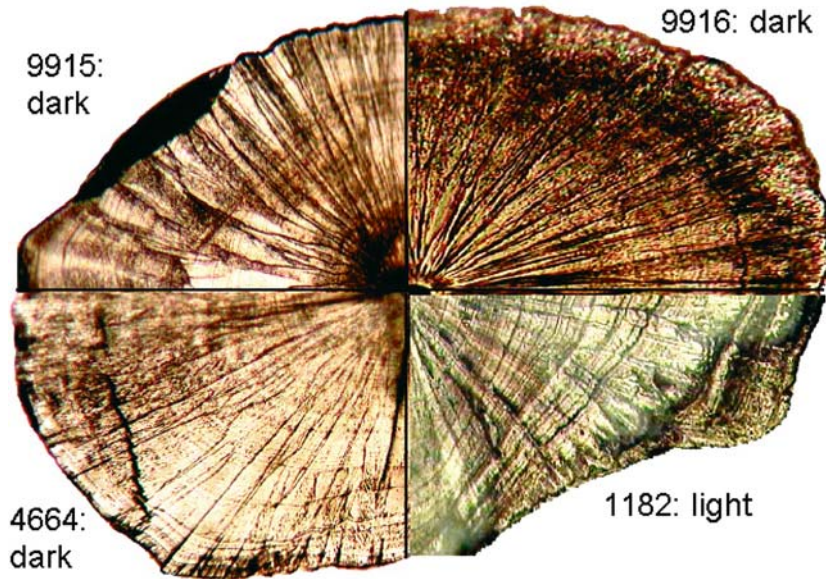
Figure 6. Segment details from Figure 5 showing marked banding in fish from site partly exposed to diffuse natural light (bottom right: Kubura Well) compared with fish from sites in total darkness (boreholes: 4464, 9915, 9916) some with tidal influence (4664). These segments are distorted in shape for display purpose.
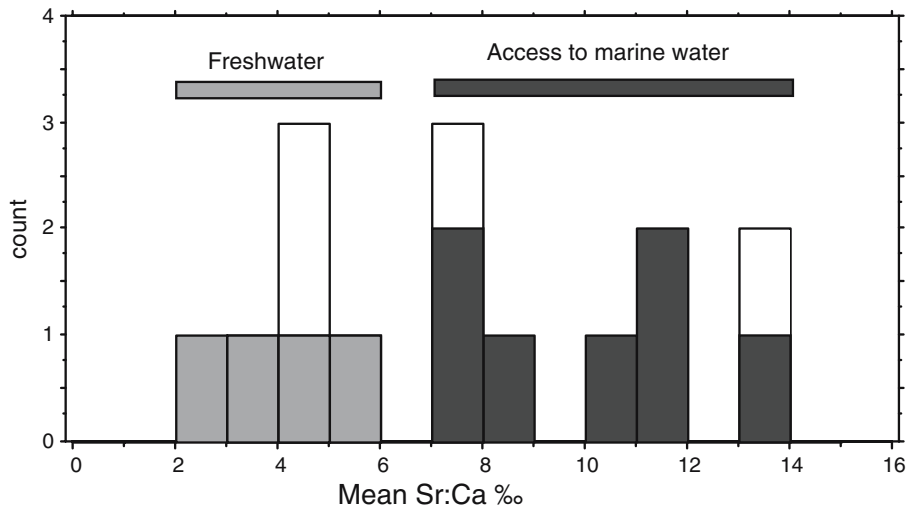

Figure 7. Frequency distribution of the mean Sr:Ca ($\times 10^{-3}$) ratio in the sagittal otolith of 15 *Milyeringa veritas*. Fish from freshwater has a mean Sr:Ca ratio of $2.6 - 5.9 \times 10^{-3}$ (left solid bar and light shading), while those fish from sites in which the vertical profile provided known access to 50% seawater had mean Sr:Ca ratio of from 7.1 to 13.9×10^{-3} (right solid bar and dark shading). Fish denoted by unshaded parts of histogram are from sites for which insufficient habitat data are available pertaining to marine water access.

cavefish is exposed at the time of collection is compounded by the marked TDS gradients in some of the profiles (Humphreys 1999). Despite this, *Milyeringa* from different water types exhibit mean Sr:Ca ratios closely correlated with the environmental TDS. Although the 'freshwater' in this arid area tends to brackish (Table 1), cavefish from these sites had a mean Sr:Ca ratio of

$2.6 - 4.4 \times 10^{-3}$ (Table 1 and Figures 2, 3), while those cavefish from water with a TDS close to that of seawater, or from sites in which the vertical salinity profile provided such access, had mean Sr:Ca ratios of from 8.5 to 13.9×10^{-3} (Figure 5). Tzeng et al. (2002) similarly established such values for fish known to enter freshwater. In the following discussion, for brevity, we infer that at the time of deposition of that section of the otolith, a Sr:Ca ratio exceeding 10×10^{-3} implies that when that part of the otolith was being deposited the cavefish inhabited the marine part of the groundwater estuary. Similarly, a Sr:Ca ratio less than 6×10^{-3} implies the cavefish inhabited a freshwater environment, and between these values a brackish environment. We recognise that this has not been tested experimentally in *Milyeringa*.

Cavefish 5231 in cave C-215 has an Sr:Ca ratio consistent with the cave containing amongst the freshest water known from Cape Range peninsula (Humphreys 1994). This individual is interpreted as having remained continually in freshwater but to have undergone significant (Figure 2) but minor changes in its environment. These relatively minor changes are likely to be associated with groundwater recharge resulting from the irregular episodic torrential rainfall characteristic of this arid region (Humphreys et al. 1989, Humphreys 1990).

The apparent movement of gudgeon #5232 between different water masses is interpreted as a habitat shift rather than a change in water quality at the site because three TDS readings taken at that location show little variation (three readings between 1983 and 1994, ranged between 4600 and 5000 mg l^{-1} (Humphreys & Adams 1991, Humphreys 1994).

These examples of apparent individual movements between water of different quality suggest the younger cavefish inhabit water of greater TDS. In those cases where such an outcome is possible, the cavefish were apparently in more saline water in early life, as shown by the mostly marked negative change in slope of the trend analyses (Figures 2, 3). Such an outcome could result from a preference for more marine waters in early life, or else from an unrelated preference for deeper, more stable habitat, where more marine water happens to occur. However, overall there was no significant relationship between cavefish size and TDS, an outcome not necessarily inconsistent with the

above, as many individuals were in habitats lacking access to high TDS water. Where no major change in Sr:Ca ratio occurred and irrespective of the mean TDS of water inhabited, the periphery of the otolith has lower Sr:Ca ratio suggesting that the fish may select water of marginally lower TDS later in life (regression of Sr:Ca on distance from otolith core; 15 of 17 cases had negative slopes of which 10 were significant ($p < 0.05$), while only two cases exhibited a positive slope, one significant $p = 0.014$). It is suggested that these more minor changes may be a life stage effect. This contrasts with the commonly observed increase in otolith Sr:Ca with fish age (Campana 1999).

Daily growth increments can be read from otoliths of most species of fish and provide detailed individual life history information, the value of which would be greatly enhanced if it could be associated with comparable stable isotope profiles (Radtke et al. 1996). Unlike the case with surface living fish (Tzeng et al. 2002, 2003), fine growth increments in the otoliths of *Milyeringa* were not apparent in fish from permanently dark sites (deep caves and bores; Figures 5, 6) which is in accord with the lack of periodicity common in subterranean animals inhabiting total darkness (Langecker 2000) owing to the absence of diurnal cues (photoperiod). Cave fish may provide good material for validating the effect of photoperiod on the daily growth increment in fish, especially because some banding is seen in cavefish from sites that were in part exposed to daylight (shallow caves) (Figure 5), suggesting that the cavefish may still be responsive to such cues.

There is little basis for even an order of magnitude calibration of growth rate in *Milyeringa* (Humphreys 2001) or the otoliths. However, most individuals express a cycling in the otolith Sr:Ca ratio over a period represented by ~55 μm of otolith growth (Figure 4). While minor changes are also seen in surface dwelling fish, the cause of which is unclear (Jessop et al. 2002), physiological periodicity is unusual in a cave animal (Lamprecht and Weber 1985, 1992) and several possibilities warrant consideration.

(1) The surface TDS of the groundwater and the depth of the freshwater lens are likely to be affected by recharge from rainfall, an effect that is exacerbated in karst areas by the lack of soil to buffer to the flow of recharge water. However, the

scans through the otolith shows fairly regular periodicity in the Sr:Ca ratio which is inconsistent with the irregular episodic major rainfall events, sometimes separated by many years (Humphreys et al. 1989, Humphreys 1990), that lead to recharge of groundwater in this arid area.

(2) Water temperature effects on metabolism affecting Sr:Ca ratio are unlikely as cave and groundwater temperatures usually remain close to the mean annual surface temperature. For example, the temperature range in C-215 was 0.65 °C over 33 days (W.F. Humphreys and R.D. Brooks, unpublished). However, at one site only, the broadly exposed Bundera Sinkhole, vertical movement through the thermocline within this anchialine cave could result in a change of body temperature of 4.5 °C within 6 m. However, as meta-analysis did not support a correlation between temperature and Sr:Ca in either salt or freshwater fishes (Campana 1999), even at this unique site the effects of temperature on Sr:Ca are likely to be trivial. Note, however, that temperature did significantly affect Sr incorporation in the otoliths of the marine fish, *Leiostomus xanthurus*, under experimental conditions, despite otolith Sr:Ca and Ba:Ca ratios having been deposited in proportion to their ratios in ambient waters (Bath et al. 2000).

(3) Food availability effects on metabolism affecting Sr:Ca ratio are possible in open sites due to changes in the influx of epigean food. For example, the region has extreme temperatures in summer (often >40 °C screen temperature) that would limit movement of epigean invertebrates (Humphreys and Feinberg 1995). However, C-215 and most bore sites are distant from surface inputs and would not be so influenced.

(4) Being an anchialine system the habitat of *Milyeringa* is affected, to a varying extent, by marine tides and this is expressed in semi-diurnal, semi-monthly and semi-annual cycles that may alter TDS at a given location in a periodic manner. The semi-annual cycle would seem a likely candidate to be associated with the changes seen in the Sr:Ca ratio of *Milyeringa* otoliths.

This work posed the question as to whether individual cave gudgeon, *M. veritas*, moved through the range of salinities found within the groundwater estuary they inhabit, either facultatively, or in obligate and predictable life stage movements, as is the case in many fishes found in surface estuaries. Although the data are sparse, individual *Milyeringa* clearly occupy waters of vary different TDS, ranging from sea water to freshwater. Individuals seem able to live consistently for long periods in waters of the same type without having to move between waters of different TDS. Hence, they do not exhibit obligatory diadromous behaviour by moving between the marine water and fresher parts of the groundwater estuary. It is not known whether these categories of behaviour represent different subpopulations that consistently occupy a given water type. Conversely, some individuals seem to move between waters having quite different TDS, apparently moving one or more times through large changes in TDS with no apparent preference to moving into water of greater or less TDS. The Sr:Ca signature in the sagitta is sufficiently information rich to suggest that an extended study, combined with stable isotope profiles, would yield considerable information on the biology of the Australian cavefish. This information is currently intractable owing to the subterranean habitat and conservation status of the fishes.

Acknowledgements

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