Otoliths of sub-adult Lake Sturgeon *Acipenser fulvescens* contain aragonite and vaterite calcium carbonate polymorphs

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Abstract

In this study we quantified the percent $CaCO_3$ polymorph composition in otoliths of larval and juvenile Lake Sturgeon *Acipenser fulvescens* via X-ray microdiffraction. Sagittal otoliths of sub-adults were primarily composed of aragonite (>90%) while the lapilli otoliths were 100% vaterite. This is the first time the presence of aragonite in otoliths has been reported in an acipenseriform and is surprising given that the ability to form aragonite otoliths was not thought to have evolved until the separation of teleost and holostean species from other Actinopterygian fishes (e.g., sturgeon, paddlefish, gar).

Key Words

Juvenile, Larvae, X-Ray Diffraction

Report

Otoliths are calcified structures that form by accumulation of biominerals deposited in layers on a proteinaceous matrix in the inner ear of fishes (Secor *et al.*, 1995; Campana, 1999). These paired structures function as hearing and balance systems and have become powerful tools used in fisheries management due to their morphological properties and characteristics of biomineralization (Campana, 2005; Pracheil *et al.*, 2014; Cook *et al.*, 2018). Otoliths form concentric rings (annuli) in proportion to the somatic growth of the individual fish, forming species-specific shapes that are used for identification and ageing purposes (Campana, 1999; Cook *et al.*, 2018). In addition, elemental signatures in otoliths form when divalent cations (e.g., Sr^{2+}, Ba^{2+}, Mg^{2+}, Zn^{2+}) from the environment or diet are substituted for calcium (Ca^{2+}) in the calcium carbonate ($CaCO_3$) matrix due to their similar chemical properties (i.e. atomic size, valence charge; Campana, 1999; Palace *et al.*, 2007; Loewen *et al.*, 2016). These elemental signatures have proven useful as natural tags enabling reconstruction of environmental life histories such as identification of natal origin of adult and larval fishes (e.g., Thorrold *et al.*, 2001; Elsdon and Gillanders, 2003; Loeppky and Davoren, 2018).

Calcite, aragonite, and vaterite are the crystalline polymorphs of $CaCO_3$ and make up the primary composition of otoliths (Carlström, 1963; Gauldie, 1993; Campana, 1999; Pracheil *et al.*, 2017). Importantly, crystal densities and lattice structures vary with each polymorph and, thus, the concentration per unit volume of Ca also varies, with aragonite being the densest and vaterite being the least dense (Chakoumakos *et al.*, 2016). At present, studies investigating otolith chemistry assume $CaCO_3$ polymorph composition is homogeneous across the entire otolith, which can potentially confound inferences that are made on life-history events when interpreting these chemical signatures (Melancon *et al.* 2005; Tzeng *et al.* 2007; Pracheil *et al.*, 2017). Despite this, $CaCO_3$ composition is not commonly determined through validated techniques and only a few studies have determined the bulk $CaCO_3$ polymorph composition of otoliths relative to the large number of studies on otolith microchemistry and microstructure (Pracheil *et al.*, (2017). Recently, Pracheil *et al.*, (2017) used neutron diffraction on intact whole otoliths. The non-destructive nature of neutron diffraction allows the possibility of quantitatively measuring the percent polymorph composition of fish otoliths without altering or destroying the otolith, thus, preserving it for further microchemical analyses. The ability to quantify the $CaCO_3$ polymorph composition of otoliths, and potentially identify the mechanisms involved in crystallization, will provide new insights into the life history of fishes and improve our confidence with inferences made on range-wide habitat use based on elemental signatures in otoliths.

Lake sturgeon *Acipenser fulvescens* Rafinesque 1817 is a primitive fish species found in North America occupying the Laurentian Great Lakes, Hudson Bay, and Mississippi River drainages. The otoliths of chondrostean fishes (i.e. Acipenseriformes and Polypteriformes), including *A. fulvescens*, were previously described as being composed entirely of vaterite (Carlström, 1963; Gauldie, 1993), however, Pracheil *et al.* (2017) recently demonstrated that adult *A. fulvescens* otoliths actually contained a substantial percentage of calcite (~18-35%). These findings were surprising given the ability to form calcite otoliths had not previously been reported in any sturgeon species (Pracheil *et al.*, 2017). It is unclear, however, if this shift in $CaCO_3$ polymorph phase was due to ontogenetic development, a physiological response to changes in environmental conditions, or some other unknown factor.

The main objective of this study was to investigate the initial crystallographic formation of otoliths during early life-history stages and to describe a technique that could be used to quantify the otolith $CaCO_3$ polymorph composition of larval (28 days post fertilization, dpf) and juvenile (78 dpf) *A. fulvescens*. Owing to their small size (20-40 µm), X-ray microdiffraction (µXRD) was chosen because of its ability to measure powder diffraction patterns on a small amount of material in a relatively short amount of time leaving the otolith sample intact.

All procedures conducted on fish were approved by the Animal Care Committee at the University of Manitoba permit# F15-007 in accordance with guidelines established by the Canadian Council for Animal Care.

Fish in this study were the laboratory-reared progeny of wild spawning adult *A. fulvescens* captured by gill net downstream of Pointe du Bois on the Winnipeg River (50°18'06.6"N, 95°32'29.5"W) in Manitoba, Canada. Spawning was induced in both males and females by the administration of two doses of gonadotropin releasing hormone (GnRH; 10 $mg.kg^{-1}$ and 5 $mg.kg^{-1}$) 24 hr prior to manually stripping eggs and milt. Once collected, the gametes were transported separately to the Animal Holding Facility at the University of Manitoba where ~15 ml of eggs were fertilized with 200 ml of mixed sperm from at least two males. Fertilized eggs were then transferred to MacDonald tumbling jars for incubation with a constant supply of City of Winnipeg dechlorinated tap water maintained at ~16°C. Once hatched (7-10 days), live larvae were transferred to flow-through holding tanks for on-growing under normal hatchery conditions (i.e. ~16°C, 12 hr light:dark photoperiod).

Larval and juvenile *A. fulvescens* were sampled by randomly selecting individuals using a dip net from multiple tanks at 28 dpf and 78 dpf. Fish were immediately euthanized in an overdose of MS-222 buffered with equal volumes of sodium bicarbonate (250 ppm) then preserved in 95% ethanol. A single left or right sagittal otolith was randomly removed from each individual using an *Olympus SZX7* dissecting microscope mounted with *Olympus SZX-PO* polarized lenses to aid with detection of otoliths. Owing to the delicate nature of removing otoliths from larval fish and the fragility of the otoliths in this species at this age, only the sagittal otoliths (28 dpf: n = 8; 78 dpf: n = 15) could be removed from the majority of fish, although lapilli otoliths were removed where possible (n = 2; Table 1). Once removed, otoliths were transferred to a microscope slide with a centimeter square grid divided into 25 equal squares and covered with a piece of double-sided tape then transported to Oak Ridge National Laboratory (ORNL) in Tennessee for µXRD analysis (CITES permit #18CA00085FONHQ).

Otoliths were measured for polymorph composition using X-ray powder diffraction. This technique uses the scattering of X-rays off the atoms of a polycrystalline structure (i.e. otolith). The scattered X-rays register on a detector producing a Debye-Scherrer diffraction pattern that can be used to gain information on the structure of the crystals or the identity of a crystalline substance (i.e. polymorph composition; Fig. 2A-C). The methods for using this technique are as follows: individual otoliths were removed from the tape and suspended in a 300 μm diameter Molecular Dimensions LithoLoop with a drop of Paratone oil. Powder diffraction data was collected using a Rigaku

XtaLAB PRO diffractometer equipped with graphite monochromated Mo Kα radiation (0.7107 Å) operated at 50KV and 40 mA, a Dectris Pilatus 200K detector, and Rigaku Oxford Diffraction CrysAlisPro software. This X-ray diffractometer has a kappa goniometer and is typically used for single-crystal diffraction studies, but it is equally suited for powder microdiffraction. The X-ray beam collimation was chosen to ensure each otolith was fully within the beam. The otoliths were spun around the phi axis to improve powder averaging and the exposure time was 180 s. The goniometer 2-theta and omega positions were set to collect data out to 0.7 Å. The resulting Debye-Scherrer patterns were radially integrated to generate 2-theta-intensity powder diffraction data suitable for Rietveld refinement. Quantitative phase analysis by the Rietveld method (Rietveld, 1969) was done using the Fullprof Suite software package (Fig. 1D; Rodríguez-Carvajal, 2001), using reference structural data for aragonite from the Inorganic Crystal Structure Database and the structural data of vaterite proposed by Wang and Becker (2009) and empirically validated for adult Lake Sturgeon otoliths by Chakoumakos *et al.* (2016).

Three kinds of diffraction patterns were distinguished in our studies of *A. fulvescens* otoliths (Figure 1A-C); those that had Debye-Scherrer rings with uniform intensity correspond to otoliths with fine-grain size (~ 1 micron) and randomly oriented grains (Fig. 1A). Those that had uneven intensity on one or more Debye-Scherrer rings indicate that the grains making up the otolith have a preferred crystallographic texture (Fig. 1B); i.e., the crystal grains are aligned in a single direction. For the vaterite lapilli otoliths this crystallographic texture is extreme. The third type of pattern has both Debye-Scherrer rings plus spots, arising from single-crystal diffraction of much larger crystal grains of calcite (> ~100 microns). This third type was not present in the larval and juvenile otoliths reported here but is found from otoliths of older fish (Loeppky et al., 2018).

The average percent $CaCO_3$ polymorph composition by mass of each otolith was calculated and the proportion of $CaCO_3$ polymorphs at 28 and 78 dpf was determined. Statistical differences (i.e., percent composition differences between 28 dpf and 78 dpf) were calculated using a one-way ANOVA. Results indicated the average polymorph composition for larval *A. fulvescens* sagittal otoliths at 28 dpf was 72.8% aragonite and 27.2% vaterite (±6.6% SE; Figure 1). At 78 dpf the proportion of aragonite to vaterite in juvenile *A. fulvescens* otoliths increased significantly ($F_{1,21}$=8.85, p=0.007) to 91.4% aragonite and 8.6% vaterite (±2.9% SE). Interestingly, both lapilli otoliths that were analyzed from the juvenile fish were pure vaterite (Figure 1E).

Results from this preliminary study indicate that the sagittal otoliths from larval and juvenile *A. fulvescens* contain a substantial proportion of the $CaCO_3$ polymorph aragonite (~43-98%). Indeed, the majority of the sagittal otoliths contained >90% aragonite. To our knowledge, this is the first report of confirmed aragonite crystallization in the otoliths of a chondrostean species (i.e. Acipenseriformes and Polypteriformes) and is surprising given that the ability to form aragonite otoliths was not previously known to occur in primitive fishes. Hitherto, the crystallization of aragonite otoliths was not thought to have evolved until the separation of teleosts from other Actinopterygian fishes (e.g., sturgeon, paddlefish, gar; Carlström, 1963; Gauldie, 1990, Fermin et al., 1998; Pracheil *et al.*, in press). Although small calcite and aragonite inclusions have indeed been observed in the otoliths of other acepenserids, they were believed to be an artifact of preservation or a transformation to more stable crystalline forms post-dissection rather than true observations of non-vaterite otoliths (Carlström, 1963, Lychakov, 1995, Schulz-Mirback, 2018). Mechanical and thermodynamic testing of vaterite transformation confirmed, however, that although the vaterite polymorph found in Lake Sturgeon otoliths is metastable, it is indeed resistant to transformation to more stable polymorphs under routine methods (Pracheil et al., 2017).

The results presented here, along with the findings from Pracheil *et al.*, (2017), indicate that the capacity to precipitate all three forms of $CaCO_3$ polymorphs is present in the earliest phylogenetic branches of Actinopterygii and that the molecular control of these mechanisms is likely retained throughout the evolutionary tree of fishes rather than having evolved anew in teleost and holostean species. Our finding counters the classification that Carlström had suggested in 1963 stating, “the sturgeons and teleosts each formed completely homogenous groups” and could be classified based on the $CaCO_3$ composition of their otoliths. These results, however, support evidence that the molecular infrastructure needed to synthesize any polymorph is present throughout the evolutionary lineage of fishes (Pracheil *et al.,* in press).

The change in proportional composition of the sagittal otoliths from the larval to juvenile stage suggests an ontogenetic shift may be occurring where original otolith nucleation initiates as one polymorph then changes as the fish ages. Further studies are required to understand the mechanisms involved in regulating polymorph composition during early life history and throughout ontogeny. Note that the otoliths from wild-caught adult *A. fulvescens* analyzed in Pracheil *et al.* (2017) did not contain any proportion of aragonite polymorphs, although these were all from adult fish. This could be representative of potential population effects or perhaps due to the individuals analyzed in this report having been reared in a hatchery setting. A similar occurrence was reported in the otoliths of hatchery raised Atlantic salmon *Salmo salar* L. 1758, where significant vaterite inclusions were identified in comparison with the typically pure aragonite sagittal otoliths of wild conspecifics (Reimer *et al.*, 2016). The underlying cause for this vaterite inclusion in hatchery-reared fish, however, is currently unknown, although it has been linked to rapid growth and diet (Reimer *et al.*, 2016). Also, it could be that there was a small, but undetectable amount of aragonite in the *A. fulvescens* otoliths measured by Pracheil *et al.* (2017), but that amount is effectively below the detection limits of Reitveld refinement on neutron diffraction data, given neutron beam exposure time and size of the otolith being analyzed. Lower neutron diffraction detection limits and higher polymorph composition precision estimates can be achieved through longer neutron beam exposures whereby a larger amount of diffraction data can be collected even on small amounts of polymorphs. Other potential reasoning for detecting aragonite in the otoliths analyzed in our study may include an influence of environmental gradients, or a genetic influence of heritability. Further investigation is required to answer these questions and will be the focus of future studies.

In the present study, both of the lapilli otoliths analyzed were composed of pure vaterite. Morphologically they appeared to have precipitated in a completely spherical shape and the crystallites that make up the spherulites have extreme preferred crystallographic orientation. This can be quantitatively determined by further analysis of the diffraction patterns (Chakoumakos *et al.*, 2018). The biogenic formation of vaterite *in vivo* can be compared and contrasted to laboratory synthesis as vaterite spherulites are used in laser trapping and optical tweezer applications. Inferences made on the biological relevance of the lapilli otoliths having different polymorph composition than the sagittal otoliths require further study.

Although we have now identified that *A. fulvescens* have the capacity to precipitate all three forms of $CaCO_3$ polymorphs, an ability that was not previously known to occur in a chondrostean species, the underlying mechanisms involved in polymorph precipitation and precipitation in *A. fulvescens* and other fishes remain unknown. The ability to quantify polymorph composition in the otoliths of larval and juvenile *A. fulvescens* using µXRD provides a unique opportunity to investigate these mechanisms in acipenserids and could potentially be applied to any fish species. The capacity for this technique to be used on small sized otolith samples (<500vµm) in a non-destructive manner allows for an understanding of the role development and the environment may play on otolith composition throughout early life history. Furthermore, although the molecular mechanisms involved in protein expression linked to polymorph precipitation have been identified in a teleost species (Söllner *et al.*, 2003), identifying these mechanisms in a primitive fish species such as *A. fulvescens* would provide insight into the evolution of otoliths and their role in hearing and balance among modern fish species and higher vertebrates.

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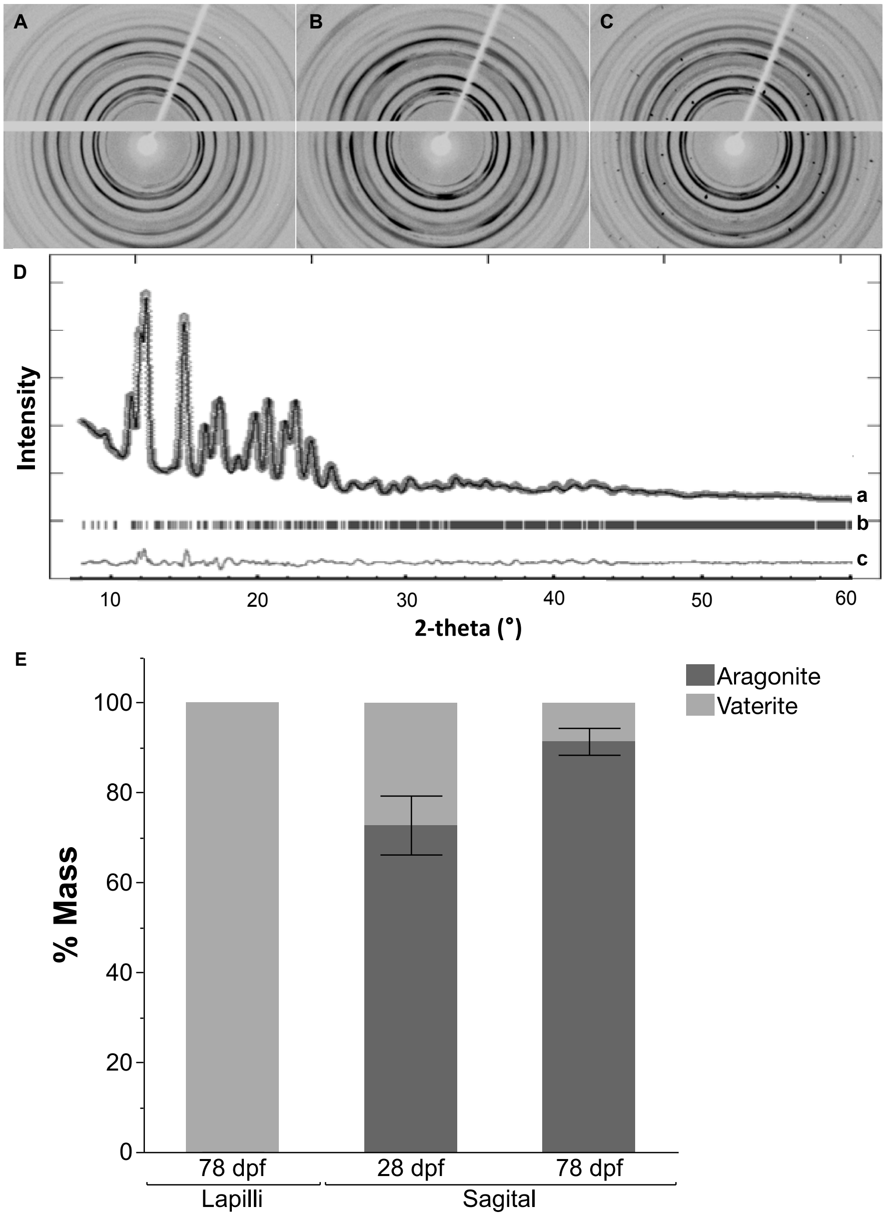

Figure 1. (A-C) Debye-Scherrer diffraction patterns and (D) Rietveld fitting of X-ray microdiffraction (µXRD) of juvenile *Acipenser fulvescens* otoliths. Diffraction patterns show three types of crystallographic structure: (A) uniform intensity rings indicating randomly orientated grains, (B) uneven intensity rings indicating preferred orientation, and (C) spots plus rings indicating large single-crystal grains. (D) Rietveld refinement from 28 dpf sagittal otolith (* in Table 1) showing: (a) µXRD pattern-curve (dashed grey line) fitted to known pattern for vaterite and aragonite (solid dark line); (b) positions for predicted Bragg's peaks (i.e. pronounced peaks on curve); (c) difference between modelled and observed diffraction patterns. (E) Mean (±SE) otolith $CaCO_3$ polymorph composition by mass of larval (28 dpf) and juvenile (78 dpf) *A. fulvescens*. Sagittal otoliths (28 dpf n=8; 78 dpf n=15) were primarily composed of aragonite (>60%) at both time points while the lapilli otoliths (n=2) were 100% vaterite.

Table 1. Refined phase fractions by mass (M%, SD in parentheses) of whole sagittal and lapilli otoliths in larval (28 dpf) and juvenile (78 dpf) *Acipenser fulvescens*.

Otolith	**Age (dpf)**	**Aragonite (M%)**	**Aragonite R_{Bragg}**	**Vaterite (M%)**	**Vaterite R_{Bragg}**
Sagittal	28	92.6 (3)	0.04	7.4 (1)	0.06
Sagittal	28	42.5 (3)	0.05	57.5 (2)	0.04
Sagittal	28	61.1 (3)	0.04	38.9 (1)	0.04
Sagittal	28	74.3 (2)	0.03	25.7 (1)	0.04
Sagittal*	28	57.7 (3)	0.04	42.3 (3)	0.04
Sagittal	28	82.8 (1)	0.03	17.2 (1)	0.04
Sagittal	28	73.3 (1)	0.03	26.7 (1)	0.04
Sagittal	28	97.9 (4)	0.04	2.1 (1)	0.05
Sagittal	78	90.4 (2)	0.04	9.6 (1)	0.06
Sagittal	78	98.6 (2)	0.03	1.4 (1)	0.03
Sagittal	78	98.9 (3)	0.03	1.1 (1)	0.03
Sagittal	78	98.7 (3)	0.05	1.3 (1)	0.03
Sagittal	78	97.8 (2)	0.04	2.2 (1)	0.05
Sagittal	78	95.4 (2)	0.05	4.6 (1)	0.06
Sagittal	78	90.1 (2)	0.04	9.9 (1)	0.08
Sagittal	78	98.8 (2)	0.04	1.2 (1)	0.03
Sagittal	78	94.3 (3)	0.03	5.7 (1)	0.06
Sagittal	78	96.6 (3)	0.04	3.4 (1)	0.07
Sagittal	78	98.1 (4)	0.05	1.9 (1)	0.05
Sagittal	78	90.5 (4)	0.05	9.5 (1)	0.06
Sagittal	78	69.4 (3)	0.04	30.6 (2)	0.04
Sagittal	78	94.4 (2)	0.04	5.6 (1)	0.07
Sagittal	78	59.4 (3)	0.04	40.6 (1)	0.04
Lapilli	78	0.0	-	100.0	0.27
Lapilli	78	0.0	-	100.0	0.16

** Otolith used in Fig. 1D*

R_{Bragg}, Measure of the agreement between the calculated and observed pattern for aragonite and vaterite polymorphs (lower values = better fit).

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