The effect of vaterite deposition on sound reception, otolith morphology, and inner ear sensory epithelia in hatchery-reared Chinook salmon (*Oncorhynchus tshawytscha*)

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Abstract: The inner ear of teleost fishes contains three calcareous structures (otoliths) that are part of the organs for hearing and balance. The largest of these structures, the sagitta, is usually composed of calcium carbonate crystals in the form of aragonite, but the calcium carbonate also occurs less frequently in a clear crystallized form called vaterite. We investigated the functional consequences of otolith crystal structure on hearing in juvenile Chinook salmon (*Oncorhynchus tshawytscha*) using the auditory brainstem response technique. A significant loss of sensitivity (2.5–6.5 dB) occurred within the primary hearing range (100–300 Hz) among salmon that had at least one vateritic sagitta. Auditory thresholds were not significantly different in fish with one vs. two vaterite sagittae. Crystallized sagittae were significantly larger and less dense than their aragonite counterparts. Saccular epithelium shape and hair bundle orientation patterns did not differ between saccules with different crystal types. There was, however, a propensity for the saccular epithelia from vateritic sagittae to have fewer sensory hair bundles. We conclude that significant hearing loss was associated with the occurrence of vateritic sagittae and suggest that hearing loss is caused by the lower density of the vaterite otoliths.

Résumé : L'oreille interne des poissons téléostéens contient trois structures calcaires (otolithes) qui font partie des organes de l'ouïe et de l'équilibre. La plus grande de ces structures, la sagitta, est généralement composée de cristaux de carbonate de calcium sous forme d'aragonite, mais le carbonate de calcium peut aussi se présenter plus rarement sous une forme cristalline claire nommée vatérite. Nous avons étudié les conséquences fonctionnelles de la structure cristalline des otolithes sur l'ouïe chez de jeunes saumons chinook (*Oncorhynchus tshawytscha*) à l'aide de la technique de la réaction du tronc cérébral auditif. Une perte significative de sensibilité (2,5–6,5 dB) se produit dans la zone principale d'audition (100–300 Hz) chez les saumons qui possèdent au moins une sagitta en vatérite. Il n'y a pas de différence significative de seuil auditif entre les poissons qui ont une ou deux sagittas en vatérite. Les sagittas cristallisées sont significativement plus grandes et moins denses que les sagittas en aragonite. La forme de l'épithélium sacculaire et les patrons d'orientation des touffes de poils ne diffèrent pas dans les saccules qui contiennent différents types de cristaux. Il y a, cependant, une tendance chez les épithéliums sacculaires associés aux sagittas en vatérite à posséder moins de touffes de poils sensoriels. Nous concluons qu'il y a une importante perte auditive associée à la présence de sagittas en vatérite et nous croyons que la perte auditive est causée par la densité moindre des otolithes en vatérite.

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Introduction

Otoliths are calcium carbonate structures found in the ears of teleost fishes. The inner ear contains three connected semicircular canals and three otolithic end organs: the saccule, lagena, and utricle, each of which contains, respectively, an otolith called the sagitta, asteriscus, and lapillus. The precise function of each end organ is uncertain, but the saccule, often the largest of the three, is the primary auditory receptor in many teleost species (reviewed in Popper and Fay 1999; Popper and Lu 2000). The utricle is generally associated with vestibular function, although it serves an auditory purpose in some species (Denton et al. 1979; Blaxter et al. 1981; Popper et al. 2003). The function of the lagena is largely unknown, although it is likely to have an auditory role in some, if not all, fishes (Lu et al. 2003; Meyer et al. 2004). Each otolithic end organ contains a sensory epithelium populated with mechanoreceptive hair cells that is overlain by the otolith. Relative motion between the sensory epithelium and the otolith stimulates the hair cells, providing the fish with information about body position and the surrounding auditory environment (see Popper and Lu 2000; Popper et al. 2003; Ladich and Popper 2004).

All fish otoliths are composed of calcium carbonate crystals suspended in a protein matrix. Different crystal polymorphs are linked with each type of otolith; calcium carbonate is usually deposited as aragonite in the sagitta and lapillus, but it occurs in a clear, crystallized form called vaterite in the asteriscus (Campana 1999; Falini et al. 2005). Vaterite is also the principle polymorph observed in many abnormal, or "crystallized", sagittal otoliths (Gauldie et al. 1997).

Very little is known about the mechanisms that cause the switch from aragonite to vaterite in sagittal otoliths, nor is there much information regarding the functional impact of this switch. As environmental disturbance is known to affect developmental stability in fishes (Valentine et al. 1973; Bestgen and Bundy 1998; Cardinale et al. 2004), some investigators have hypothesized that exposure to acute or chronic stress deriving from mechanical trauma (Strong et al. 1986), starvation (Payan et al. 2004), density effects (Casselman 1990), or temperature stress (Johansson 1966) can promote the development of this aberrant otolith phenotype. Indeed, in hatcheries, where one or more of these factors tend to be magnified, as many as 80% of the fish produced have crystallized sagittae (Bowen et al. 1999; Tomas and Geffen 2003). Thermal stress has been associated with the occurrence of crystallized otoliths in captive fish and may play a central role in their formation (Gauldie 1986, 1996; Oxman et al. 2005). It has been suggested that vaterite expression may also have a genetic basis (Gauldie 1986; Sweeting et al. 2004), but preliminary data indicate that heritable control of the switch is unlikely (Oxman et al. 2005). Given that these stressful conditions also occur naturally, it is not surprising that vateritic sagittae are frequently observed in free-ranging populations, though in much lower proportions (Sweeting et al. 2004).

While the occurrence of vaterite sagittae is well documented, virtually nothing is known regarding the structural and functional implications of vaterite deposition. Daily growth increment formation is not impaired in vaterite sagittae, suggesting that some aspects of otolith growth are maintained in vaterite otoliths (Campana 1983). However, alterations in otolith morphology resulting from vaterite deposition, especially with regards to density and shape, could potentially affect hearing, orientation, and behavior. Vaterite is less dense than aragonite (Northwood and Lewis 1968), and it is often deposited in an irregular pattern on the otolith (Zhang et al. 1995; Bowen et al. 1999). This combination of altered density and morphology may impair the sagitta's ability to interface with the sensory hair cells on the epithelium, and (or) it may influence epithelial morphology, both of which could compromise the sensory capabilities of the animal. Any such limitation to an individual's ability to perceive, orient, and interact within its environment could ultimately affect survival. Vaterite formation may be a compensatory response to stress, but the physiological, behavioral, and ecological consequences remain unknown.

This study compared the hearing ability, sagittal morphology, and structure of the saccular epithelium from juvenile Chinook salmon (*Oncorhynchus tshawytscha*) with normal and crystallized sagittae to determine if vaterite deposition affected the structure and function of the inner ear.

Materials and methods

Experimental animals

One hundred fall-run juvenile Chinook salmon from brood year 2004 were collected from the Coleman National Fish Hatchery (CNFH) in Anderson, California, for use in hearing tests designed to evaluate the affect of vateritic sagittal otoliths on sound reception. The CNFH was chosen because it had a history of producing large numbers of fish with crystallized otoliths (R. Barnett-Johnson, unpublished data). Fish were placed in insolated Styrofoam fish boxes containing 10 °C super-oxygenated water, stress coat, and ammonia blocker and transported unfed to the Bioacoustics Laboratory at the University of Maryland. Upon arrival, the fish were placed in an 800 L circular tank with a water temperature of 16 °C and maintained on a diet of Oregon-moist pellets, Biomoist grower, and EWOS microfeed. The salmon were given 10 days to acclimate to their new environment prior to the start of the hearing trials.

Auditory brainstem response (ABR)

The auditory thresholds of 40 randomly sampled juvenile salmon (average weight = 7.07 g) were measured in a class A soundproof room using ABR. This technique is a noninvasive electrophysiological method of measuring neural responses to auditory stimuli and is commonly used for measuring hearing in fishes and other vertebrates (Kenyon et al. 1998; Higgs et al. 2001; Smith et al. 2004). All ABR tests were conducted blind regarding otolith composition of the test fish.

Each fish was immobilized with Flaxedil (gallamine triethiodide, Sigma, St. Louis, Missouri) at a concentration of approximately 2 $\mu g \cdot g^{-1}$ body mass, restrained in a mesh sling, and suspended underwater in a 19 L plastic vessel. The fish was suspended so that the top of the head was approximately 6 cm below the surface of the water and 22 cm above an underwater speaker (University Sound UW-30, Lubell Labs, Columbus, Ohio). A reference electrode was

placed cranially between the nares, while a recording electrode was placed on the dorsal midline surface of the fish approximately at the posterior edge of the opercula. A ground electrode was placed in the water near the body of the fish to reduce background noise.

Sound stimuli were presented and ABR waveforms were collected using a TDT physiology apparatus with SigGen and BioSig software (Tucker-Davis Technologies Inc., Alachua, Florida). Sounds were computer-generated via TDT software and passed through a power amplifier connected to the underwater speaker. The calibration of each frequency used was conducted using a calibrated underwater hydrophone (sensitivity of –211 ± 3 dB re 1 $V \cdot \mu Pa^{-1}$; 0.003–100 kHz, omnidirectional; Type 10CT, G.R.A.S. Sound & Vibration, Vedbæk, Denmark). Sound stimuli were projected at 100, 200, 250, 300, 400, 600, 800, and 1000 Hz frequencies. Each pure tone burst had a 2 ms rise and fall time, was 15 ms in duration, and was gated through a Blackman window. Responses to each tone burst at each sound pressure level (SPL) were collected using the BioSig software, with 1000 responses averaged for each presentation.

An auditory threshold level was established for each frequency by determining the minimum SPL at which an ABR waveform could be detected (Higgs et al. 2001; Smith et al. 2004). During the first step of each hearing trial, a fish was presented with a tone of a given frequency at an initial SPL of 130 dB re 1 μPa. If an ABR waveform was observed during this initial exposure, the SPL was attenuated in 10 dB intervals until the ABR amplitude started to decrease. Once this effect was noted, the SPL was attenuated in 5 dB steps until the ABR amplitude was close to that of the background noise level and no discernible response could be detected. The last detectable ABR waveform was considered representative of the minimum auditory threshold response for the frequency being presented. These steps were conducted at each frequency for each test subject to generate individual audiograms.

Inner ear dissection, otolith morphology, and otolith composition

Following ABR recording, each fish was measured (standard and total length), weighed, and euthanized with an overdose of buffered MS-222 (tricaine methanesulfonate; Sigma). The dorsal cranium was removed to expose the ears, and the entire head was fixed in 4% paraformaldehyde (Sigma) for 1 h at 4 °C to firm tissue and facilitate the removal of the inner ear. This brief exposure to fixative had no affect on otolith structure (D.S. Oxman, unpublished data). After fixation, both ears were dissected from the head and the sagittae removed from the saccular pouch. The saccular epithelia were carefully trimmed and stored in 0.1 $mol \cdot L^{-1}$ phosphate buffer (PB) at 4 °C for further processing. In some cases, the asteriscus was removed from the lagena so that its shape and structure could be examined qualitatively.

Sagittal morphology was analyzed using OPTIMAS image analysis software v. 6.5 (Media Cybernetics 1996) to quantify length (mm), breadth (mm), area (mm^2), perimeter (mm), and circularity. The circularity index was defined as the ratio of the perimeter squared divided by the area (Media Cybernetics 1996). An index of 16 indicates a square boundary, whereas a true circle will have a minimum value of 4π. Sagittal mass (mg) was measured with a Mettler-Toledo® AL54 balance accurate to 0.0001g. Volume was estimated from otolith mass and density (e.g., $V = M/D$) using a density measurement of 2.93 $g \cdot cm^{-3}$ for aragonitic sagittae and 2.65 $g \cdot cm^{-3}$ for vateritic sagittae (Campana and Thorrold 2001). These density estimates were verified by T. Kircher and K. Severin at the Advanced Instrumentation Laboratory, Department of Geology and Geophysics, University of Alaska, Fairbanks.

To determine the crystalline composition (aragonite or vaterite) of each otolith, left and right sagittae were examined under a Leica MZ6 stereomicroscope equipped with reflected and transmitted light so that the percentage of vaterite present could be estimated visually (Fig. 1). Otoliths were assigned a score from 1 to 4 based on the percentage of vaterite present as described by Gauldie (1986) and modified as follows: 1, a completely aragonitic otolith; 2, vaterite deposition composed ≤33% of the otolith; 3, vaterite deposition exceeded 33% but was less than 66% of the otolith; and 4, otolith almost entirely vaterite (≥66%). Scoring was conducted blindly without a priori knowledge of ABR performance. Otoliths with scores of 1 and 2 were considered aragonitic (A), whereas those scored as a 3 or 4 were categorized as vateritic (V). To validate our visual scores, an image analysis system was used to quantify percent vaterite, defined as (1 – area of aragonite) / total otolith area.

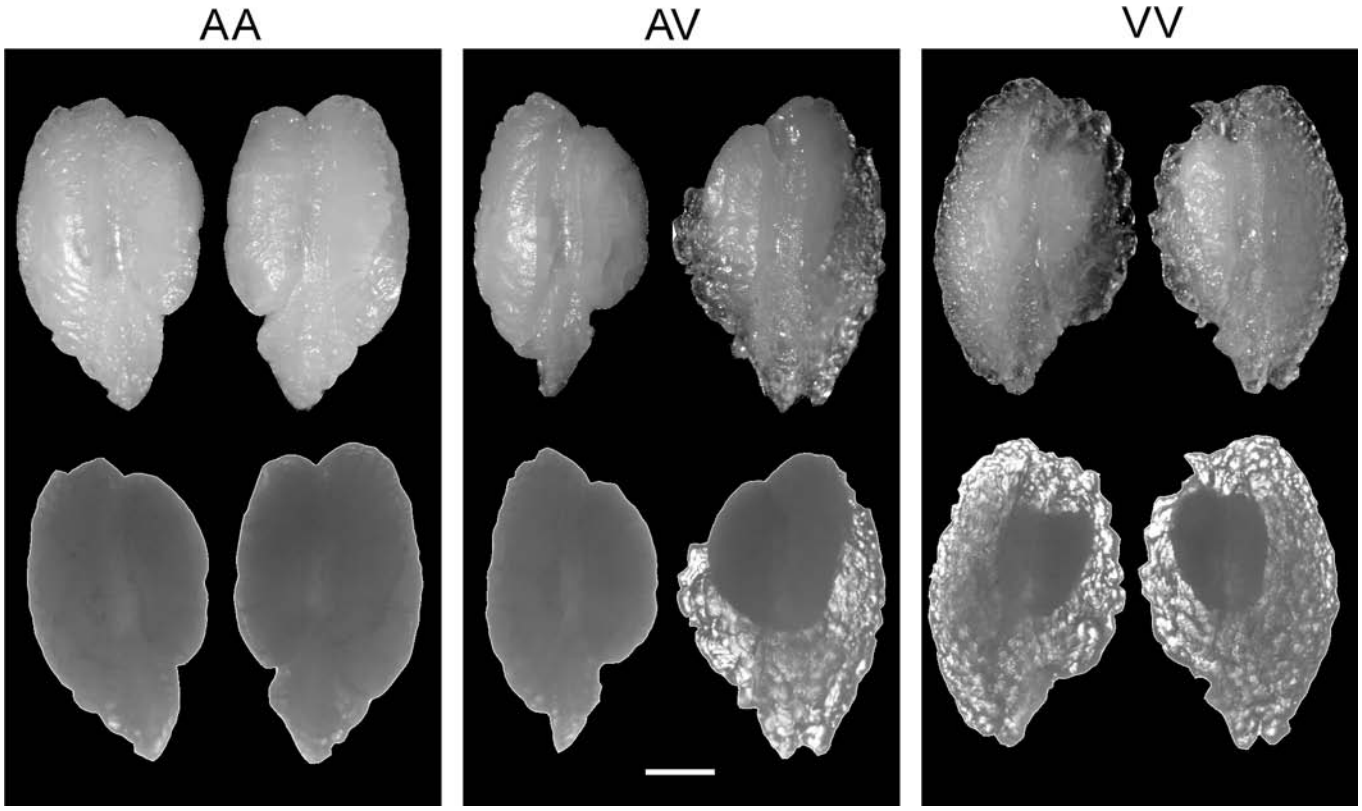
Otolith scores representing each tested individual were placed into one of three categories, defined as AA (both sagittae aragonitic), VA (one of each), and VV (both sagittae vateritic; Fig. 1). Hearing profiles associated with each fish were subsequently compared based upon these categories. Audiograms from individuals with two normal otoliths (AA) were averaged to provide baseline information on auditory bandwidth and sensitivity for normal juvenile Chinook salmon.

Soft tissue examination

To assess the morphological impact associated with vaterite deposition, saccular epithelia associated with both calcium carbonate forms were fluorescently labeled and viewed with either a compound epifluorescence or confocal microscope so that the density of sensory hair bundles, bundle orientation, and saccular shape (length:width ratio, *L*:*W*) could be quantified and compared between otolith types. Sensory epithelia were double-labeled for β-tubulin and F-actin, two major protein components of sensory hair bundles, using the method described in Lu and Popper (1998). All solutions were diluted in PB, and all steps were performed at room temperature unless specifically noted. Epithelia were treated with 0.5% Triton-X (Sigma) and incubated at 4 °C overnight in β-tubulin antibody (mouse monoclonal IgG clone D66, Sigma) at a concentration of 1:350 in PB. Tissue was then rinsed in PB and placed in secondary antibody (Alexa 633 goat anti-mouse, Invitrogen, Carlsbad, California) for 90 min. Tissue was again rinsed and counter-stained with Alexa 488-conjugated phalloidin (Invitrogen), which binds F-actin. Tissue was rinsed, mounted, and coverslipped with ProLong Antifade Gold (Invitrogen).

Samples were viewed with a Zeiss Axioplan microscope and photographed with a Zeiss AxioCam MRc5 camera. Images were processed with Zeiss AxioVision AC software.

Fig. 1. Representative left and right sagittal otolith pairs collected from juvenile Chinook salmon (*Oncorhynchus tshawytscha*) used in hearing experiments. Pairs consisted of two aragonitic sagittae (AA), one aragonitic and one vateritic sagitta (AV), or two vateritic sagittae (VV). Images were illuminated with reflected (top row) and transmitted light (bottom row). The scale bar is 500 μm and applies to all panels.



For measurements of whole epithelia, samples were photographed with a 2.5× objective, and length and width measurements were performed off-line. Length was measured from the rostral to the caudal tip of the saccular epithelium. Width was measured at the widest point of the rostral portion of the epithelium. The *L:W* ratio was then calculated for each epithelium to describe shape.

Hair bundle density was measured in three 10 000 μm^2 regions of each saccule, one each in the rostral, central, and caudal regions. Epithelia were photographed with a 40× dry objective, and AxioVision software was used to draw 10 000 μm^2 boxes around each area. All hair bundles within each box were counted. Counts in the three areas were added together to obtain one density estimate for each epithelium. To assess hair bundle orientation, epithelia were imaged with a Zeiss 510 confocal microscope. Bundle polarization was mapped for the entire epithelium on overlapping fluorescent micrographs (taken with a 40× objective) of actin–tubulin-labeled epithelia. The position of the kinocilium (labeled with anti-tubulin) was noted relative to the location of the stereocilia (labeled with phalloidin). The direction of depolarization was marked as the side of the hair bundle containing the kinocilium. Mapping was performed blind with respect to otolith type.

Statistical analyses

To determine if juvenile Chinook salmon used here were developmentally and morphologically similar among treatments groups (AA, VA, VV) and thus comparable, total length (TL), standard length (SL), and mass of fish were compared using a one-way analysis of variance (ANOVA). It was assumed that gender had no affect on hearing ability. The significance level for this and all other statistical tests was set at the 0.05 level. SAS ® 9.1 (SAS Institute Inc., Cary, North Carolina) was used for all statistical analyses.

A stepwise approach was used to analyze the relationship between otolith type and auditory threshold. Initially, the effect of otolith type (e.g., treatment) and frequency on auditory threshold level was examined using a factorial ANOVA (Proc GLM SAS) to establish if there were any significant effects or interactions of otolith type on auditory sensitivity over all frequencies. This factorial ANOVA model was defined as

$$(1) \qquad Y_{ijk} = \mu + O_i + H_j + O_i H_j + \varepsilon_{ijk}$$

where Y_{ijk} was the threshold level of sound detected, O_i was the treatment, H_j was the frequency of sound used as a stimulus, O_iH_j was their interaction, and ε_{ijk} was the error. The interaction term compared the slopes associated with each otolith complement over all frequencies. An insignificant interaction meant the slope of each line (e.g., treatment) was identical. Bonferroni-corrected post hoc pairwise comparisons were conducted in conjunction with this and all subsequent ANOVA analyses.

The second step involved using Chinook salmon with a normal complement of otoliths (AA) as controls to define

their primary hearing range (i.e., those frequencies at which the threshold of sound detection was the lowest and most sensitive). The auditory profile indicated that hearing thresholds among fish with aragonite sagittae were most sensitive over the 100, 200, 250, and 300 Hz signals, but declined dramatically between 300 and 400 Hz. To confirm this observation, an ANOVA was conducted to test hearing sensitivity of fish with a normal complement of otoliths over the 100–400 Hz range. The following model was used:

$$(2) \qquad Y_{ij} = \mu + H_i + \varepsilon_{ij}$$

Once the primary hearing range was identified, the model (1) ANOVA was used to compare the auditory thresholds of each treatment group throughout this frequency range and to determine how the presence of vaterite affected hearing in the most sensitive region of the Chinook audiogram.

A linear regression was used to model auditory acuity of control fish against stimulus for frequencies ranging between 400 and 1000 Hz to assess threshold levels among normal fish at the extreme range of their auditory capabilities. A factorial ANOVA involving all otolith pair configurations was subsequently conducted using model (1) to determine how otolith complement affected sensitivity at these high frequencies.

An analysis of covariance (ANCOVA) employing SL as a covariate was used to assess the effect of otolith composition on otolith morphology by comparing length, breadth, area, perimeter, mass, volume, and circularity between aragonitic and vateritic sagittae. Because all morphological descriptors were bilaterally symmetrical within AA and VV treatment groups (ANCOVA; $p \geq 0.36$), left and right sagittae were pooled according to their structural classification. The mass of each otolith was standardized for size by dividing it by the area measurement, and the resulting ratios were compared between treatments using the ANCOVA.

The shape of the saccular epithelium associated with each type of otolith (as defined by the *L:W* ratio) and hair bundle densities were compared between treatments using a two-tailed *t* test. Left and right saccules were pooled within treatments for these comparisons. A linear regression was conducted to determine if hair bundle counts associated with vateritic and aragonitic sagitta were correlated with saccular shape.

Results

Fish mass (ANOVA, $F_{[2,37]} = 1.54$, $p = 0.23$), TL ($F_{[2,37]} = 1.38$, $p = 0.26$), and SL ($F_{[2,37]} = 1.29$, $p = 0.29$) were similar among treatment groups. Therefore, any differences observed in hearing ability or ear structure among groups cannot be attributed to size differences. Of the 40 juveniles tested, 20 (50%) had two aragonitic sagitta (AA), 12 (30%) had two vateritic sagitta (VV), and 8 (20%) possessed one of each otolith type. All visual scores of otolith composition were validated by image analysis.

Audiograms

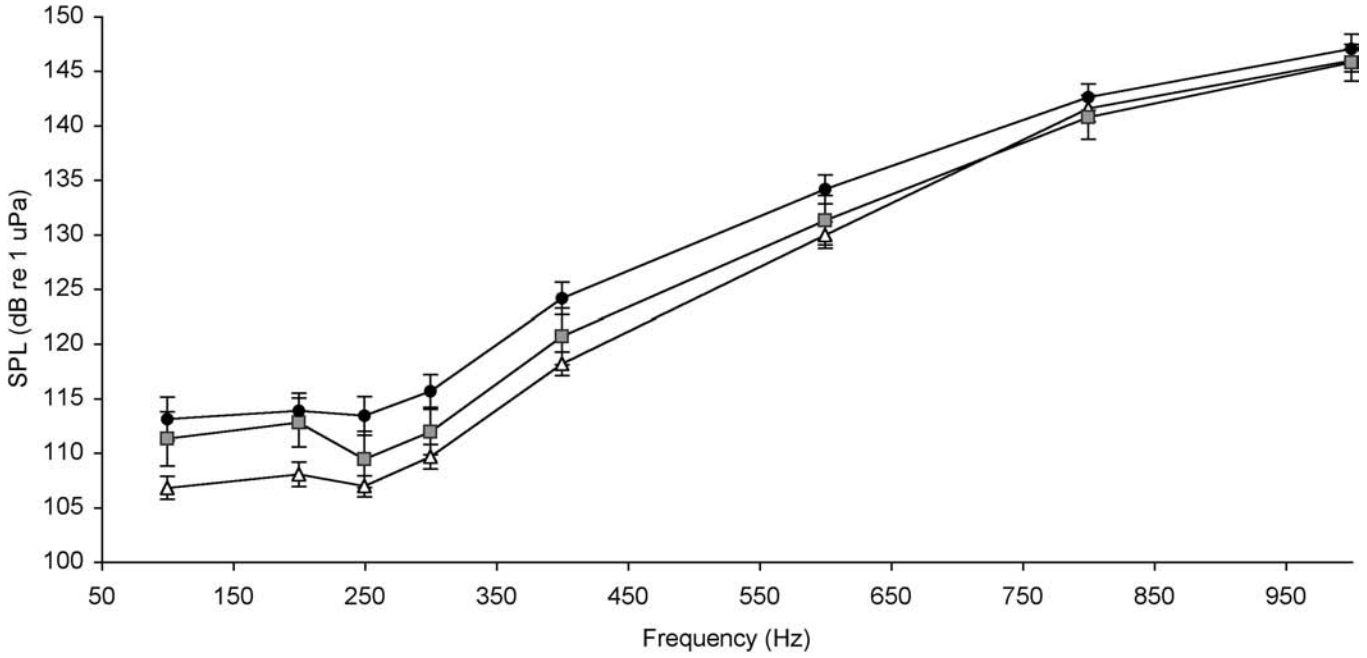
Overall trends in the ABR audiograms indicated the most sensitive portion of the hearing range, those frequencies at which the threshold of detection of sound was the lowest, occurred between 100 and 300 Hz in all treatment groups and that auditory thresholds increased linearly between 400 and 1000 Hz (Fig. 2). There was also a correlation between otolith type and hearing sensitivity; fish with normal otoliths possessed the most sensitive hearing ability over the entire range of tested frequencies, but sensitivity decreased as the number of vateritic sagittae present increased (Fig. 2). Hearing sensitivity differed significantly among groups over all frequencies based on otolith configuration ($F_{[2,283]} = 21.78$, $p < 0.00001$) and frequency of stimulus ($F_{[7,283]} = 254.05$, $p < 0.00001$; model 1). The slope associated with each otolith configuration was similar among treatments ($F_{[14,283]} = 0.85$, $p = 0.61$), indicating that frequency affected hearing ability the same way in all fish.

Among juvenile Chinook salmon with two normal otoliths (AA), the threshold level of detection increased with increasing frequency over the 100–400 Hz range ($F_{[4,93]} = 20.18$, $p < 0.00001$; model 2). The post hoc pairwise comparison, however, indicated that threshold levels needed to detect the 100, 200, 250, and 300 Hz stimuli were similar ($p \geq 0.60$), and a significantly greater SPL was required to detect the 400 Hz stimulus ($p < 0.00001$). This confirmed the empirical observation that sound detection among normal juveniles declined sharply between 300 and 400 Hz and that the most sensitive portion of the auditory range occurred below 300 Hz.

Elevated hearing thresholds were significantly correlated with the presence of vateritic sagittae. Individuals possessing vateritic otoliths had significantly greater hearing thresholds over the most sensitive portion of the hearing range (e.g., 100–300 Hz) relative to fish with normal otoliths ($F_{[2,143]} = 18.87$, $p < 0.00001$; model 1). Hearing thresholds tended to increase as the number of vateritic otoliths present increased (Fig. 2). Using AA individuals as a reference, the greatest loss in acuity was observed among VV individuals, which suffered from a 5.5–6.5 dB loss in sensitivity throughout the primary hearing range (Fig. 2). Hearing among VA-bearing Chinook tended to be intermediate between the other two groups, with a 2.5–4.5 dB loss of sensitivity relative to the controls. Post hoc pairwise comparisons demonstrated that the auditory acuity of AA-bearing Chinook was greater than that of individuals with one ($p < 0.01$) or two vateritic otoliths ($p < 0.00001$), whereas the presence of one or two vateritic otoliths resulted in the same level of hearing impairment when compared with each other ($p = 0.11$). In other words, the presence of one or two vateritic otoliths significantly impaired hearing over those frequencies in the most sensitive portion of the hearing range, but the loss of sensitivity associated with vateritic sagittae was the same, regardless of the number of vateritic sagittae involved.

Although none of the fish representing each treatment group were sensitive to frequencies between 400 and 1000 Hz, stimuli presented with sufficient intensity could be detected (Fig. 2). This decline in sensitivity was approximately linear, and a regression of hearing threshold on stimulus for fish with normal sagittae modeled a line for which both intercept and slope were significant ($p < 0.00001$) with an adjusted squared multiple (R^2) of 0.80. The intercept (100.6 ± 2.0 dB) was near the level observed in the normal auditory range, and the slope was 0.048 ± 0.003 dB·Hz^{-1}, which meant that in the range including 400–1000 Hz, audi-

Fig. 2. Audiograms of juvenile Chinook salmon (*Oncorhynchus tshawytscha*) with two normal sagittae (AA; triangles), one crystallized sagitta (VA; squares), and two crystallized sagittae (VV; circles). The sound pressure level (SPL) was measured in decibels (dB). Significantly elevated auditory thresholds were observed in salmon possessing at least one vaterite otolith (ANOVA, $p < 0.01$). Elevated thresholds associated with vateritic sagittae were most pronounced in the low-frequency portion of the Chinook salmon's hearing range (up to 400 Hz), the most sensitive segment of the salmon audiogram. Error bars denote ±1 standard error.



tory acuity diminished at a rate of 0.046 dB per 1 Hz increase in stimulus frequency.

Auditory detection thresholds at higher frequencies (400–1000 Hz) where hearing sensitivity was low were also significantly affected by otolith configuration ($F_{[2,146]} = 3.96$, $p = 0.021$) and frequency ($F_{[1,146]} = 425.01$, $p < 0.00001$; model 1). The amount of hearing loss tended to increase as the number of vateritic sagittae increased, and within each treatment, acoustic sensitivity decreased as frequency increased (Fig. 2). A post hoc pairwise comparison of effects of different otolith types showed that individuals with two vateritic otoliths had a poorer ability ($p = 0.006$) to detect sound throughout the high frequency range than did normal fish, whereas the detection ability of fish possessing one vateritic and one normal otolith did not differ from either normal fish ($p = 1.0$) or fish possessing two vateritic otoliths ($p = 0.17$). We therefore concluded that VA was intermediate between the two symmetrical types in its effect on auditory sensitivity at the high-frequency end of the Chinook salmon audiogram.

Otolith morphology

Vateritic sagittae ($n = 29$) were longer (+0.12mm; ANCOVA, $F_{[1,76]} = 10.42$, $p = 0.002$) and wider (+0.22 mm; ANCOVA, $F_{[1,76]} = 90.80$, $p < 0.0001$) on average than the normal aragonitic form ($n = 49$; Table 1). Consequently, they had a greater area (+0.45 mm^2, ANCOVA, $F_{[1,76]} = 53.38$, $p < 0.0001$), perimeter (+1.02 mm, ANCOVA, $F_{[1,76]} = 73.64$, $p < 0.0001$), and volume (+0.06 mm^3, ANCOVA, $F_{[1,76]} = 14.46$, $p < 0.0001$). Because the greater size of vateritic sagittae was driven primarily by an increase in breadth, they tended to be squarer than normal otoliths (ANCOVA for circularity, $F_{[1,76]} = 50.01$, $p < 0.0001$). There was no significant difference between the average mass of aragonitic and vateritic otoliths (ANCOVA, $F_{[1,76]} = 0.194$, $p = 0.661$). The mass:area ratio, however, was significantly lower for vateritic otoliths (0.60) than for aragonitic otoliths (0.72: ANCOVA, $F_{[1,76]} = 98.27$, $p < 0.0001$), indicating vateritic sagittae were significantly lighter per square millimetre than the aragonitic form.

Although only a few asterisci were examined, there was a tendency for those associated with vateritic sagittae to be either reduced (Fig. 3) or completely absent. In contrast, ears with aragonite sagittae always contained an asteriscus that appeared qualitatively normal. The prevalence of diminished or missing asterisci was not quantified.

Sensory epithelia

Vaterite deposition may have had a minimal affect on the development of the sensory epithelia. On average, fewer saccular hair bundles were associated with vateritic sagitta (mean = 407.15 ± 45.48 per μm^2, $n = 20$) than with the aragonitic form (mean = 431.45 ± 34.86 per μm^2, $n = 22$). These differences, however, were not significant (t test with unequal variances; $p = 0.062$). The shape of the saccular epithelium associated with vateritic sagitta, as defined by the L:W ratio (mean = 6.39 ± 0.57, $n = 9$), was no different than that observed among aragonitic sagitta (mean = 6.12 ± 0.68, $n = 15$; t test with unequal variances, $p = 0.306$). Linear regression indicated no correlation existed between hair bundle density and saccular shape in Chinook salmon with normal ($R^2 = 0.08$) or crystallized ($R^2 = 0.22$) otoliths.

Table 1. Mean length, breadth, area, perimeter, circularity, mass, mass:area ratio (*M*:A), and volume for aragonitic and vateritic sagittae from juvenile Chinook salmon (*Oncorhynchus tshawytscha*).

Sagitta	Sample size	Length[a] (mm)	Breadth[b] (mm)	Area[b] (mm^2)	Perimeter[b] (mm)	Circularity[b]	Mass[c] (mg)	*M*:*A*[b] ($mg \cdot mm^{-2}$)	Volume[b] (mm^3)
Aragonitic	49	2.26 (0.16)	1.30 (0.08)	2.01 (0.24)	6.17 (0.44)	19.02 (0.79)	1.44 (0.17)	0.72 (0.04)	0.49 (0.003)
Vateritic	29	2.38 (0.14)	1.52 (0.11)	2.46 (0.27)	7.19 (0.55)	21.04 (1.66)	1.46 (0.22)	0.60 (0.06)	0.55 (0.007)

Note: Standard deviations are provided in parentheses.
[a]ANCOVA: $p = 0.002$.
[b]ANCOVA: $p < 0.0001$.
[c]ANCOVA: $p = 0.661$ (not significant).

The saccular orientation of sensory hair bundles was mapped from nine ears (five aragonite, four vaterite) of five fish. Both aragonite and vaterite saccular epithelia displayed the standard four-quadrant orientation pattern characteristic of most hearing generalist fishes (Fig. 4; Popper 1977; Buran et al. 2005). Qualitatively, this orientation pattern was not substantially disturbed in ears with crystallized sagittae.

Discussion

The deposition of calcium carbonate in its crystalline vateritic polymorph was significantly correlated with altered sagittal structure and auditory function. Vateritic sagittae from juvenile Chinook salmon were bigger and less dense than the normal aragonitic form, and their occurrence was associated with moderately altered saccular epithelia and reduced asteriscus development. Most dramatically, the presence of vateritic sagittae was correlated with a statistically significant decrease in auditory sensitivity. This hearing loss was most pronounced in the low-frequency end of the juvenile Chinook's hearing range (100–300 Hz), which is the most sensitive portion of the salmon audiogram. Although such correlations do not prove causation, they provide a solid foundation from which we can formulate hypotheses regarding the functional consequences of vaterite formation.

The presence of vaterite was correlated with a significant decrease in auditory sensitivity, but the functional cause of the degradation remains speculative. Fewer saccular hair bundles were associated with vateritic sagittae, but it is unlikely this affected hearing because these counts were not significantly different than those from normal otoliths. Although a variety of other physiological factors may be involved, differences in density between calcium carbonate polymorphs may be key to understanding the mechanical origins of this loss. Mass may have been similar between types, but vateritic otoliths were significantly lighter per unit area and less dense ($2.65\ g \cdot cm^{-3}$) than the aragonitic form ($2.93\ g \cdot cm^{-3}$) because their larger size resulted in a greater volume without the significant addition of weight. The larger size of the crystallized sagitta, whether it was the result of compensatory physiological mechanisms or an artifact of its crystalline structure, did not appear to make up for its lesser density. This lack of compensation is not surprising, since any such mechanism would be limited by space and the physical properties of the vaterite. It is this loss of density resulting from vaterite formation that likely caused the hearing impairment observed among vateritic individuals. This assumes, of course, that there is a relationship between otolith density and hearing.

Otoliths are typically the densest part of a fish (approximately three times greater than that of the entire fish), and that density is believed to play a role in their function as sound and motion detectors (Popper et al. 2003). As sound vibrations impinge upon a fish, its body moves along with the motion of the water. The inertia of the very dense otolith in its chamber results in differential movement between it and the less dense sensory epithelium, resulting in shearing of sensory hair bundles and stimulation of the auditory nerve (Popper and Fay 1993, 1999). A sagitta of lower density would potentially lag behind the movement of the epithe-

Fig. 3. Asterisci recovered from a juvenile Chinook salmon (*Oncorhynchus tshawytscha*) whose left ear contained a vateritic sagitta (left image), while the right ear had a normal aragonitic sagitta (right image). Scale bar is 100 μm.

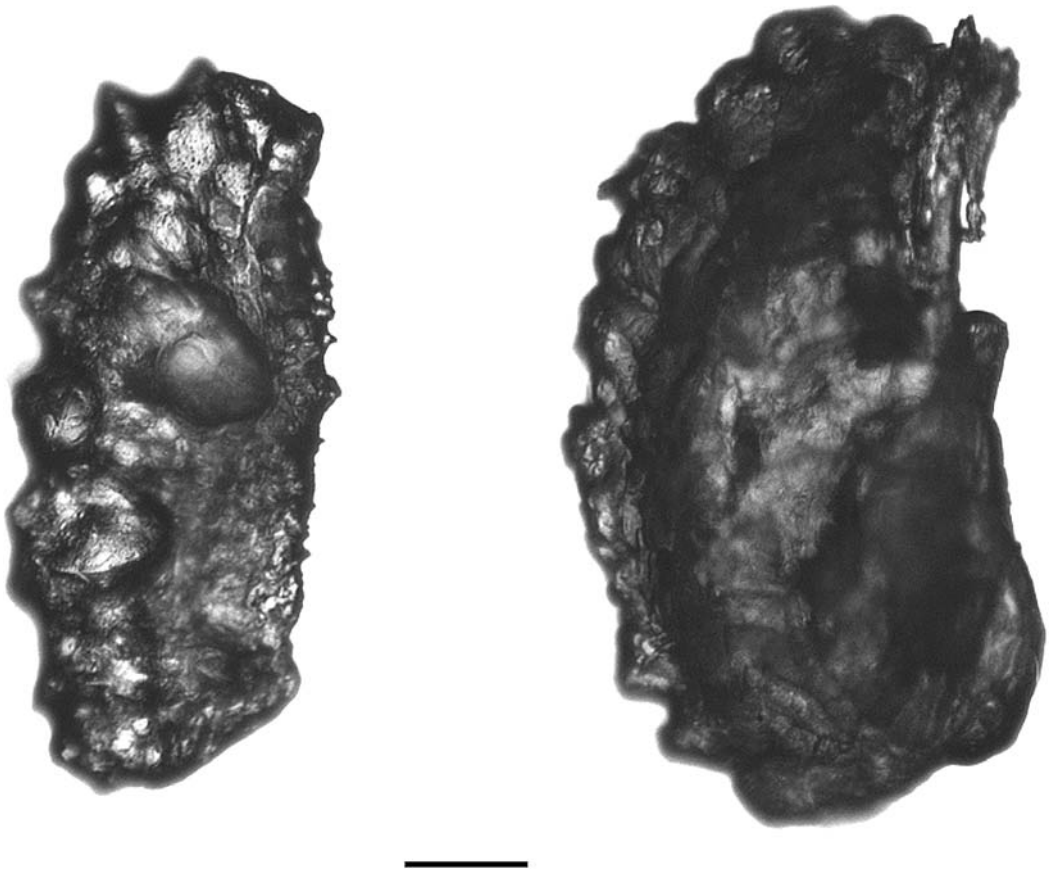
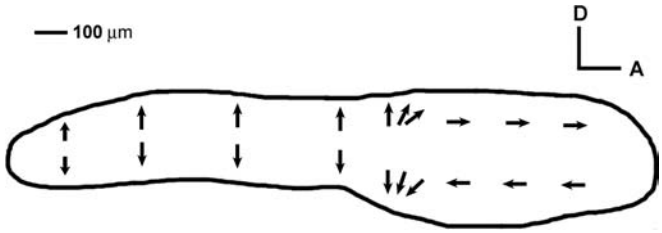


Fig. 4. Mapping of hair bundle orientation in the Chinook salmon (*Oncorhynchus tshawytscha*) saccule. The hair bundle orientation pattern shown is for both vaterite and aragonite saccular epithelia, which were not substantially different from one another. Arrows indicate the depolarizing direction for hair bundles in each region.



lium to a lesser degree, decreasing the differential movement between the saccular epithelium and its otolith. It would therefore require a greater force (e.g., higher intensity stimulus) to stimulate the sensory epithelium and trigger a neural response. We hypothesize that because vateritic sagittae are less dense than the normal aragonitic form, they are less able to appropriately stimulate the sensory hair cells, thereby decreasing auditory sensitivity.

Although the density hypothesis is the most likely explanation, other physiological factors associated with vaterite formation could have contributed to the hearing loss. Vaterite deposition significantly increased sagittal size, which could have restricted otolith movement in the saccular chamber and inhibited sound reception. Vaterite chemistry may also have played a role in elevating auditory thresholds. Calcium concentrations in vaterite and aragonite sagittae are the same, but vaterite suffers from potassium and sodium depletion (Tomas and Geffen 2003). If this phenomenon results in surplus concentrations of K^+ and Na^+ in the blood and endolymph of the inner ear, it could impair hair cell and nerve function to the detriment of auditory sensitivity. One cannot rule out the possibility that a combination of these factors caused the observed hearing loss.

In a few instances, we observed that the lagenar otolith (asteriscus) was significantly reduced in size or missing entirely from ears that had crystallized sagittae. It is interesting to note that the teleost asteriscus is usually composed of vaterite and not aragonite (reviewed in Campana 1999). This observation suggests that the processes that induced vaterite deposition among sagittal otoliths also affected those that regulate the formation of the asteriscus. It is unclear, however, if this phenomenon was permanent or temporary. It is possible that asteriscus development was simply delayed. The function of the lagena is largely unknown, so the consequences of a reduced or absent asteriscus are not well understood. Preliminary evidence in goldfish, however, suggests that the lagena plays a role in directional hearing at low frequencies (Lu et al. 2003; M. Meyer, Bioacoustics Laboratory, University of Maryland, College Park, MD 20742, USA, personal communication). Therefore, fish with

vateritic sagittae and diminished or missing asterisci may have deficiencies in both hearing sensitivity and sound source localization. Regardless, it is likely that the abnormal development of two out of three otoliths is detrimental to inner ear function and, consequently, survival.

The functional impact of abnormal otolith development on salmon life history may be considerable. By affecting auditory sensitivity, vateritic sagittae could impair a fish's ability to successfully perceive and interact with its environment. Aquatic organisms live in a dense medium where sound waves travel efficiently, but visibility is often limited. Fish consequently rely on motion and vibration detection to establish orientation, maintain equilibrium, and interpret their surroundings. Decreased auditory sensitivity could alter behavior in ways that reduce a fish's ability to locate food, shelter, and conspecifics while avoiding predators, thus decreasing their chances of survival.

If possession of crystallized sagittae affects hearing to such an extent that it increases mortality rate, then the production of vaterite-bearing individuals in captive environments may affect the ability of such programs to conserve wild populations. Large numbers of vateritic individuals are frequently produced by hatcheries, and their occurrence has been correlated with the stressful conditions often present within such habitats, including high population densities, temperature fluctuation, noise, vibration, disease, poor water quality, and inadequate nutrition (Sweeting et al. 2004). Exposure to such acute and chronic stressors is known to influence many aspects of fish biology, including development (Beacham 1988; Danzmann and Ferguson 1988) and morphology (Valentine et al. 1973; Hard et al. 1999; Campbell 2003). If hatchery conditions increase the frequency of vateritic sagittae and if the hearing impairment associated with these otoliths is detrimental to survival, the resulting increase in mortality could compromise the ability of hatchery programs to augment fisheries and protect wild stocks. The proportion of vateritic individuals within a population that survive to adulthood, however, remains unknown.

The influence of vateritic sagittae cannot always be detrimental because they are occasionally recovered from adult fishes (Johansson 1966; Bowen et al. 1999; Tomas and Geffen 2003). This raises an intriguing possibility; fish have some way to compensate for the hearing loss associated with vaterite deposition, or the hearing loss has little or no biological consequence to the fish. It is possible that physiological and behavioral mechanisms exist that correct for such impairment, but no such compensation was observed during this study. It is possible that fish with vateritic sagittae develop sensory or behavioral modifications to compensate for the hearing loss, perhaps relying instead on alternative senses. Similar mitigation is not unheard of among wild animals and has been reported in a variety of marine mammals (Schusterman et al. 2000). Schooling fishes may be particularly well suited for dealing with abnormal otolith development in some members of the group because they can rely on the cumulative, enhanced senses and actions of numerous individuals to make up for the hearing loss.

Though many of the relationships described in this study were correlative and not causative, there is strong evidence that vaterite deposition has a negative impact on auditory sensitivity in juvenile Chinook salmon, particularly over the most sensitive portion of their hearing range. The insights and hypotheses presented here provide a foundation for further examinations regarding the causes and effects of vaterite formation. Future work will seek to determine the functional mechanism(s) responsible for the hearing loss associated with vateritic sagitta, examine the causes and mechanics of vaterite formation, and assess its potential affect on behavior and survival. These studies may have serious implications for hatchery practices that induce high levels of stress in cultured salmonids.

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