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Otolith mineralogy affects otolith shape asymmetry: a comparison of hatchery and natural origin Coho salmon (*Oncorhynchus kisutch*)

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Abstract

Many aspects of natural and hatchery origin salmonid genetics, physiology, behaviour, anatomy and life histories have been compared due to the concerns about what effects domestication and hatchery rearing conditions have on fitness. Genetic and environmental stressors associated with hatchery rearing could cause greater developmental instability (DI), and therefore a higher degree of fluctuating asymmetry (FA) in various bilaterally paired characters, such as otoliths. Nonetheless, to appropriately infer the effects of DI on otolith asymmetry, otolith mineralogy must be accounted for. Vateritic otoliths differ substantially from aragonitic otoliths in terms of mass and shape and can artificially inflate any measurement of FA if not properly accounted for. In this study, measurements of otolith asymmetry between hatchery and natural origin Coho salmon *Oncorhynchus kisutch* from three different river systems were compared to assess the overall differences in asymmetry when the calcium carbonate polymorph accounted for 59.3% of otoliths from hatchery origin *O. kisutch* was vateritic compared to 11.7% of otoliths from natural origin *O. kisutch*. Otolith mineralogy, rather than origin, was the most significant factor influencing the differences in asymmetry for each shape metric. When only aragonitic otoliths were compared, there was no difference in absolute asymmetry between hatchery and natural origin *O. kisutch*. The authors recommend other researchers to assess otolith mineralogy when conducting studies regarding otolith morphometrics and otolith FA.

KEYWORDS
asymmetry, Coho salmon (*Oncorhynchus kisutch*), hatcheries, mineralogy, morphometrics, otoliths, salmonid enhancement, vaterite

1 | INTRODUCTION

Fluctuating asymmetry (FA) is one of the three forms of asymmetry found in bilaterally paired characteristics (Palmer & Strobeck, 1986; Van Valen, 1962), and refers to small, random deviations from perfect left–right symmetry (Palmer & Strobeck, 1986; Swaddle, 2003). FA is commonly used as a proxy measurement for developmental instability (DI), which is defined as the inability of an individual to prevent developmental "accidents" from being expressed in the phenotype (Mather, 1953; Van Valen, 1962; Van Dongen, 2006).

When an individual is prevented from buffering against deviations from symmetry, FA increases (Mather, 1953; Palmer, 1996; Van Dongen, 2006). When FA increases, this may be correlated with an increase in DI as a result of genetic and environmental stressors experienced by an individual (Lens *et al.*, 2002; Palmer, 1994).

FA measurements are also commonly used as a proxy (inverse) measurement of fitness (Brown *et al.*, 2008; Palmer & Strobeck, 1986) and are generally trait-specific (Clarke, 1998; Soulé, 1967; Soulé & Cuzin-Roudy, 1982). Some deviations from symmetry are more detrimental to the fitness of the organism than others, and therefore these deviations are more buffered against during the development of the individual (Clarke, 1995; Palmer & Strobeck, 1986). Regardless, the selective landscape and the functional significance of traits must be properly characterized to accurately interpret the significance of FA seen within any trait in terms of DI or fitness (Bergstrom & Reimchen, 2000).

FA measurements have been conducted on many structures within the Teleostei, including bony plates (Bergstrom & Reimchen, 2000), the lateral line (Planidin & Reimchen, 2021) and otoliths (Koeberle *et al.*, 2020; Lychakov & Rebane, 2005; Mille *et al.*, 2015). Otoliths are bilaterally paired sensory structures within the inner ear of many fish species and serve a role in hearing, proprioception and gravity sensation (Popper & Lu, 2000). Generally, there are three paired otoliths on the left and right sides of the head: (a) the lapillus, (b) asteriscus and (c) sagitta (Carlström, 1963). The sagittal otoliths (hereafter, represented by the term “otolith”) are typically the largest otoliths in non-ostariophysan teleosts and can vary substantially in shape among evolutionary lineages and functional groups (Carlström, 1963; Nolf, 1993; Tuset *et al.*, 2003).

Most commonly, otoliths are composed of calcium carbonate, in the form of aragonite, and an organic matrix (Degens *et al.*, 1969), which gives the structures a density of 2.94–2.95 g cm^{-3} (Tomás & Geffen, 2003). Nonetheless, the calcium carbonate found within the otoliths can also take the form of vaterite, which has a density of 2.54–2.65 g cm^{-3} (Campana & Thorrold, 2001; Tomás & Geffen, 2003). Once vaterite formation occurs in the otolith matrix, the process appears to be irreversible (Reimer *et al.*, 2017). When compared to aragonitic otoliths, vateritic otoliths are larger in terms of length, width, perimeter, area and volume, but they have a lower mass, making them less dense (Oxman *et al.*, 2007; Tomás & Geffen, 2003). Due to this difference in density, vateritic otoliths can impair fish hearing and alter behaviour in many teleost species (Oxman *et al.*, 2007; Reimer *et al.*, 2016; Vignon & Aymes, 2020).

Otolith asymmetry occurs when the mass and shape of the paired otoliths of an individual are not equal (Koeberle *et al.*, 2020; Lychakov & Rebane, 2005; Mille *et al.*, 2015), and this usually results in FA (Lychakov *et al.*, 2006; Lychakov & Rebane, 2005; Tomás & Geffen, 2003). Otolith shape asymmetry is complex with a wide assortment of variables used in the broader literature: simpler morphometrics like Feret length (OL) and width, shape indices such as rectangularity and descriptors from the outline of the otolith (Gagliano *et al.*, 2008; Geladakis *et al.*, 2021; Koeberle *et al.*, 2020; Tuset *et al.*, 2003). Differences in FA can be measured by examining the mean of unsigned/absolute asymmetry of the paired features, or by comparing the variance of signed asymmetry of the paired features (Geladakis *et al.*, 2021; Palmer & Strobeck, 1986).

One well-documented difference between hatchery and natural origin salmonids is the higher prevalence of vaterite in the otoliths of hatchery salmonids (Gauldie, 1986; Reimer *et al.*, 2016; Sweeting *et al.*, 2004). The cause of vaterite formation in salmonids is not well understood, but leading hypotheses include increased growth rates and rearing densities (Austad *et al.*, 2021; Reimer *et al.*, 2017). Concerningly, strong deviations from mass and shape symmetry may negatively impact the survival rate of salmonids due to a decrease in hearing capabilities (Lychakov & Rebane, 2005; Oxman *et al.*, 2007; Popper & Lu, 2000; Reimer *et al.*, 2016; Vignon & Aymes, 2020). Thus, the environmental and genetic stressors that increase DI in salmonids and increase asymmetry in paired otolith morphometrics may lead to decreased fitness (Oxman *et al.*, 2007; Valentine *et al.*, 1973).

Aragonitic and vateritic otoliths have been known to differ in terms of macrostructure for decades (Mugiya, 1972; Strong *et al.*, 1986). In fish populations where aragonite–aragonite (AA), aragonite–vaterite (AV) and vaterite–vaterite (VV) pairs occur, the otolith mineralogy needs to be accounted for to avoid confounding results involving macrostructure. This is especially important for researchers to consider due to the growing number of publications in the fields of otolith morphometrics and otolith asymmetry (Nazir & Khan, 2021). In the past 5 years alone (2017–2021), 388 journal articles were published on otolith morphometrics and/or otolith asymmetry [Web of Science search: 18 May 2022 using the search terms otolith* AND (morphometrics OR shape OR asymmetry)]. Although there are studies that do account for otolith mineralogy (*e.g.*, Neves *et al.*, 2017; Passerotti *et al.*, 2020), 97% of the 388 studies do not indicate how, if at all, this was taken into account. Although it is possible that this issue was addressed before publication, such as removing visually aberrant otoliths (A. Bose, pers. comm., 24 May 2022), it is impossible for the reader to know without follow-up correspondence.

In one example of a study in which otolith mineralogy was not accounted for, Koeberle *et al.* (2020) found increased otolith FA in hatchery origin Chinook salmon *Oncorhynchus tshawytscha* (Walbaum 1792) over natural origin *O. tshawytscha*. These differences were attributed to differences in DI between the hatchery and natural rearing environments. Nonetheless, both aragonitic and vateritic otoliths were being used in the study without being differentiated (I. Arismendi, pers. comm., 21 May 2020). Differences in otolith mineralogy between hatchery and natural origin *O. tshawytscha* were a more likely explanation for the differences in otolith FA, especially without a described link between DI and vaterite formation. There is an overall inadequate accounting of otolith mineralogy in the broader literature, and this has implications for studies of otolith morphometrics and otolith shape asymmetry (Strong *et al.*, 1986; Vignon, 2020).

This study aims to explore the relationship between FA and otolith mineralogy across hatchery and natural origin salmonids. The objectives of this study were to (a) compare the proportion of AA, AV and VV otolith pairs between hatchery origin and natural origin Coho salmon *O. kisutch* (Walbaum 1792) from three hatchery facilities and (b) determine how the absolute asymmetry of otolith morphometrics

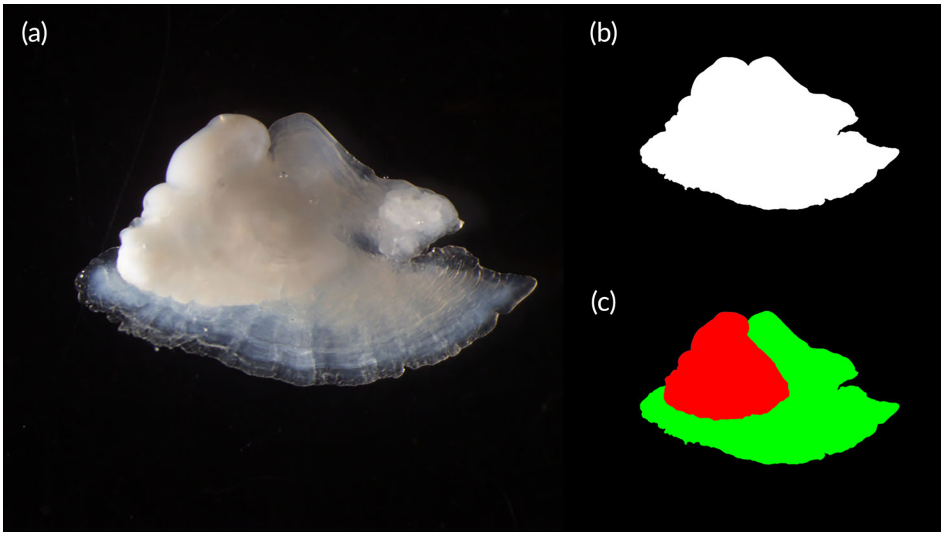

FIGURE 1 (a) An unedited photograph of a left otolith from a hatchery origin Coho salmon from the Chilliwack (CH) River hatchery. (b) The otolith has been highlighted in pure white against a pure black background to increase the effectiveness of the ShapeR package. (c) Regions of the otolith that are visually aragonitic are coloured in red, and regions of the otolith that are visually vateritic are coloured in green. The background is coloured in black

changes with the asymmetry in vaterite coverage in otolith pairs and various other factors, such as origin, hatchery, sex and fork length (FL). To address these objectives, otolith pairs from hatchery and natural origin adult *O. kisutch* returning to Big Qualicum (BQ), Chilliwack (CH) and Quinsam (Q) hatcheries (British Columbia, Canada) in the year 2018 were collected and compared. The authors also investigated continuous metrics of vaterite content to see how FA varies with increasing differences in vaterite content between otolith pairs, which has not been done previously. The authors aim to show the importance of otolith mineralogy in conducting studies of otolith macrostructure and recommend that researchers account for this variable in their own research to avoid misinterpreting their data and drawing faulty conclusions.

2 | METHODS

2.1 | Sample collections

Otoliths were collected from adult *O. kisutch* at the BQ, CH and Q hatcheries. *O. kisutch* were selected from fish euthanized by hatchery personnel for multiple broodstock egg takes from 30 October until 19 December 2018; the sampling was incidental to their euthanasia. *O. kisutch* were considered to be natural origin spawners if they had an intact adipose fin and hatchery origin spawners if they lacked an adipose fin. Individual *O. kisutch* were measured (FL, ±1 mm), and otolith pairs were removed, washed with deionized water and cleaned of any excess organic material and moisture. Otoliths were stored dry after collection. The calcium carbonate polymorph of all otoliths extracted at the hatcheries was recorded *via* visual assessment. Aragonite was visually characterized as an opaque, white mineral, whereas vaterite was visually characterized by a clear, colourless mineral. These assessments were done in the field and later validated under a microscope. VV pairings were originally not to be kept as the authors were interested in collecting fish with at least one aragonitic otolith; nonetheless, some of these pairs were inadvertently kept due to miscommunication of methods among samplers. Therefore, the authors have more VV pairings in their frequency of occurrence ($n = 362$) data than in their shape or vaterite coverage data ($n = 247$).

2.2 | Photographs

Otoliths were briefly submerged (maximum 3 min) in a plastic Petri dish filled with Super-Q deionized water (MilliporeSigma, Burlington, MA, USA; www.emdmillipore.com). The distal sides of the otoliths were photographed on a black background using an Olympus SZX16 stereoscope, DP26 camera and CellSens Standard software (Olympus, Shinjuku, Tokyo; www.olympus-global.com) at 16× magnification (Figure 1a). In every photo, each pixel represents an area of 3.6 by 3.6 μm. Broken otoliths were not photographed and, therefore, not used in morphometric analyses. Out of the 267 otolith pairs collected and retained, seven AA (6%), eight AV (7%) and five VV (15%) otolith pairs were not used in subsequent morphometric analyses. Two hundred and forty-seven remained intact.

2.3 | Morphometrics and shape metrics

Two hundred and forty-seven intact otolith pairs were utilized for morphometric analysis. Automatic measurements of the OL, Feret width (OW), otolith perimeter (OP) and otolith area (OA) of the

TABLE 1 Measurement formulas for otolith shape indices used in this study

Otolith measurement type	Measurement formula
Rectangularity	$OA/(OL \times OW)$
Circularity	$OP^2/4\pi \times OA$
Roundness	$(4 \times OA)/(\pi \times OL^2)$
Ellipticity	$(OL - OW)/(OL + OW)$
Aspect ratio	OL/OW
Areal density	Mass/OA

Abbreviations: OA, otolith area; OL, Feret length; OP, otolith perimeter; OW, Feret width.

otoliths were collected using the R package *ShapeR* (Libungan & Pálsson, 2015). Vateritic otoliths were difficult to resolve with *ShapeR* due to their transparency. Thus, Adobe Photoshop 2020 (Adobe Incorporated, San Jose, CA, USA) was used to modify the otolith photos to make the otolith appear pure white (hexidecimal value #FFFFFF) and the background pure black (hexidecimal value #000000; Figure 1b). Otoliths were weighed with a Mettler Toledo ME104 analytical balance (Mettler Toledo, Columbus, OH, USA) to the nearest 0.1 mg.

Rectangularity, circularity, roundness, ellipticity, aspect ratio and areal density were calculated from directly measured variables using their relevant equations (Saygin *et al.*, 2017; Tuset *et al.*, 2003; Table 1). Rectangularity is defined as the area of an object divided by its minimum enclosing rectangle; thus a perfect rectangle would have a value of 1. Circularity is defined as the perimeter squared divided by the area of the object; a perfect circle would have a value of 4π as the equation is squared: $4\pi^2r^2/\pi r^2$. In this study, the authors slightly modified circularity by adding 4π to the denominator, and thus perfect circles yielded a value of 1 rather than 4π. Roundness is similar to circularity in that it records departures from a circle, but compares a ratio of OA to OL^2 instead of a ratio of OP^2 to OA. Ellipticity compares changes in the two axes of the ellipse (OL and width specifically). Aspect ratio is the ratio of OL to OW. Finally, areal density was calculated as the otolith mass divided by the OA.

Elliptic Fourier descriptors were produced using the *ShapeR* package in R (Libungan & Pálsson, 2015). The same outline which produced the simpler morphometrics (OL, OW, OP and OA) also contains a matrix of *x* and *y* coordinates of the outline, which can be used to produce 48 coefficients of 12 harmonics. Each harmonic was broken down into an *x* and *y* component, and each *x* and *y* component was broken down into a sine and cosine function, respectively; thus, there were four coefficients per harmonic labelled through a d (Tort, 2003). The first three coefficients of the first harmonic were omitted from the analysis as these were used to standardize outlines by size, rotation and starting point (Libungan & Pálsson, 2015). Six low-order harmonics (2–7) were selected for this analysis as these accounted for over 98.5% of the total variation of the otolith outline. The amplitudes of these low-order harmonics were calculated for both the left and the right otoliths using the following equation found in Tort (2003):

$$\mathrm{Amp}_n = 0.5\sqrt{a_n^2 + b_n^2 + c_n^2 + d_n^2}$$

Overall, a wide assortment of variables were considered in our assessment of otolith asymmetry to mirror what has been investigated in other publications that address the subject (Gagliano *et al.*, 2008; Geladakis *et al.*, 2021; Koeberle *et al.*, 2020; Palmer *et al.*, 2010; Tuset *et al.*, 2003).

2.4 | Vaterite measurements

When otoliths were extracted at the hatcheries, a qualitative assessment of whether the otoliths were aragonitic (no vaterite apparent) or vateritic (vaterite apparent) was made. This assessment was based on the presence or absence of vaterite on the otolith to the naked eye. Across all hatcheries, qualitative assessments of 362 otolith pairs were made. These observations made up the frequency of occurrence data among these hatcheries.

In the lab, Adobe Photoshop 2020 (Adobe Incorporated, www.adobe.com) was used to differentially colour pixels that represented aragonite and vaterite as determined by an experienced technician. Aragonite was coloured in pure red (hexidecimal value #FF0000), and vaterite was coloured in pure green (hexidecimal value #00FF00; Figure 1c). The green and red colours used were from the RGB colour swatch on Adobe Photoshop 2020 (Adobe Incorporated, www.adobe.com). A script was run in Python (Python Software Foundation, Wilmington, DE, USA, www.python.org) to measure the ratio of green pixels to red pixels to measure the percentage coverage of vaterite on the otolith. Vaterite coverage measurements were conducted on all whole otoliths that were retained from hatchery *O. kisutch*. This amounted to 247 otolith pairs. Inter-observer bias was under ±1%. There was 100% agreement between field and lab assessments of vaterite presence.

2.5 | Statistical

A Cochran–Mantel–Haenszel χ^2 test for count data was conducted to test the hypothesis of whether AA, AV and VV otolith pairs were equally represented in both natural and hatchery origin *O. kisutch* across different hatcheries (Cochran, 1954; Mantel, 1963). A Woolf test was conducted to check that the assumption of equal association across the strata was met. These tests were conducted using the otolith mineralogy determined qualitatively at the hatchery, 362 *O. kisutch* in total. For the remaining analyses, the data set with quantitatively measured vaterite was used, 247 *O. kisutch* in total.

To assess whether the different measurements displayed FA, directional asymmetry or anti-symmetry, the mean and modality of the data were tested among each otolith pair type (AA, AV and VV). A one-sample permutation *t*-test with 9999 Monte–Carlo permutations was run on the signed asymmetry of each measurement type to assess significant deviations from a mean of zero, which would imply directional asymmetry. Hartigan's dip test of unimodality was run on the signed asymmetry of each measurement type to assess significant deviations from unimodality, which would minimally imply bimodality and thus antisymmetry (Hartigan & Hartigan, 1985). For each dip test, *P*-values were calculated *via* 9999 Monte–Carlo permutations. Measurements that had means not significantly different from zero and modalities not significantly different from unimodality were considered to display FA.

The authors produced three matrices of absolute asymmetry values for simpler morphometrics (OL, OW, OP and OA), shape metrics (rectangularity, circularity, roundness, ellipticity, aspect ratio and areal density) and the amplitudes of harmonics 2–7. Non-metric multidimensional scaling (nMDS) plots were produced using Bray–Curtis dissimilarity matrices of the three matrices outlined for all the measured otoliths ($N = 247$) and of only the AA paired otoliths ($N = 100$). Each of the six matrices was also transformed into a Euclidean dissimilarity index. One-way permutational MANOVA (PERMANOVA; Anderson, 2001) was used to test the null hypotheses that none of the associated factors (absolute difference in vaterite, origin, hatchery, sex and FL) were significantly associated with the overall differences in otolith shape of all measured otolith pairs. PERMANOVAs are a multivariate technique that is used to assess if the centroids and dispersion of groups of variables differ from one another. Differences among the centroids, *i.e.*, a location effect, are meaningful in the sense that this indicates differences in multivariate space among groups. Differences in dispersion, *i.e.*, a dispersion effect, indicate a greater variation in data points across multivariate space between groups. To ensure a significant location effect was identified, PERMANOVA models were checked for homogeneity of dispersion by using beta dispersion tests on each grouping variable (Anderson, 2006). A PERMANOVA was conducted on each of the six dissimilarity matrices, three for all otolith pairs and three for only AA pairs. For all PERMANOVAs, 9999 Monte–Carlo permutations were conducted. Interaction effects between the various factors were included in the first iterations of the models, and if they were not significant, they were removed and not reported.

Another way to test the differences in FA is to assess the homogeneity of variances of the signed asymmetry values [*i.e.*, Var (Left–Right)]. Permutation tests were used to compare the variances across groups of interest. There were three sets of permutations done on three sets of comparisons, all otoliths between the two origins, AA paired otoliths between the two origins, and AA *vs*. AV otolith pairs of all fish. These permutation tests had 9999 Monte–Carlo permutations used to compare against the real result. Permutation tests were used as there were departures from normality within the groups for some of the measurements.

A standard α value of 0.05 was used for statistical tests unless otherwise specified. All statistical analyses were conducted in R (R Core Team, 2022). Cochran–Mantel–Haenszel χ^2 and Woolf tests were done in the R package *vcd* (Meyer et al., 2020). One-sample permutation *t*-tests were conducted using the *perm.t.test* function in the R package *MKinfer* (Kohl, 2020). Hartigan's dip tests were conducted using the function *dip. test* in the R package *diptest* (Maechler, 2021). Matrices were created using the *as.matrix* function in base R. The nMDS plots were calculated using the R package *vegan* (Oksanen *et al*., 2019). PERMANOVAs were calculated using the function *adonis* within the *vegan* package (Oksanen *et al*., 2019). Euclidean dissimilarity indices were produced using the function *veg.dist* in the *vegan* package (Oksanen *et al*., 2019). Levene's tests were conducted with the *levene.test* function in base R (R Core Team, 2022). χ^2 tests were conducted using the *chisq. test* function in base R (R Core Team, 2022). All plots were created using the R package *ggplot2* (Wickham, 2016).

2.6 | Ethics statement

O. kisutch are routinely humanely euthanized each year for broodstock collections by hatchery personnel at each hatchery location. The authors collected otoliths from the euthanized *O. kisutch* used for broodstock collections, and thus, no additional fish were killed for this study.

3 | RESULTS

3.1 | Otolith mineralogy within rearing environments

The otolith mineralogy was significantly associated with the origin of the *O. kisutch* across the three river systems ($\chi^2 = 65.74$, $df = 2$, $P < 0.001$). The assumption of equal association across the strata was met ($\chi^2 = 0.62$, $df = 2$, $P = 0.73$). The main differences between the expected and observed values were that AA pairs were overrepresented in natural origin *O. kisutch*, and AV and VV pairs were overrepresented in hatchery origin *O. kisutch* (Supporting Information Appendix S1). Across all river systems, 40.7% of hatchery origin *O. kisutch* otoliths were aragonitic and 59.3% were vateritic, whereas 88.3% of natural origin *O. kisutch* otoliths were aragonitic and 11.7% were vateritic. There were also differences in the frequency of AA, AV and VV pairs across hatchery and natural origin *O. kisutch* ($P < 0.001$). The main hatchery driving this trend was the CH hatchery, as 54.3% of their hatchery origin otoliths were aragonitic in comparison to 29.7% of BQ hatchery origin otoliths and 34.8% of Q hatchery origin otoliths.

3.2 | Determining the type of asymmetry displayed

When examining all the *O. kisutch* otoliths, the mean of signed asymmetry of OL, OW, rectangularity and roundness was significantly different from zero ($P < 0.001$ for all), as the left otolith was larger in terms of OL and OW on average. In contrast, rectangularity and roundness were right biased. None of the measurements had modalities significantly different from unimodality. When the authors examined AA otolith pairs only, the mean of signed asymmetry of OL, OW, OP, rectangularity, circularity, roundness and Harmonic 6 was

TABLE 2 Differences in the mean values of measurements between aragonitic and vateritic otoliths

Measurement	Aragonitic otoliths	Vateritic otoliths
OL (mm)	5.594 ± 0.356	5.841 ± 0.388
OW (mm)	3.251 ± 0.201	3.624 ± 0.265
OP (mm)	14.919 ± 0.969	18.705 ± 2.258
OA (mm^2)	11.966 ± 1.167	13.657 ± 1.587
Otolith mass (g)	1.354E−2 ± 0.956E−2	1.208E−2 ± 0.171E−2
Areal density ($g\ mm^{-2}$)	1.129E−3 ± 0.088E−3	8.874E−4 ± 1.019E−4
Rectangularity	6.580E−1 ± 0.935E−1	6.447E−1 ± 0.296E−1
Circularity	6.775E−1 ± 0.548E−1	5.052E−1 ± 1.033E−1
Roundness	4.877E−1 ± 0.328E−1	5.101E−1 ± 0.410E−1
Ellipticity	2.646E−1 ± 0.255E−1	2.342E−1 ± 0.334E−1
Aspect ratio	1.723 ± 0.094	1.617 ± 0.116

Note: Error represented by S.D.
Abbreviations: OA, otolith area; OL, Feret length; OP, otolith perimeter; OW, Feret width.

significantly different from zero ($P < 0.001$ for all), as the left otolith was larger in terms of OL, OW and OP on average. Rectangularity, roundness and circularity were right biased. None of the measurements had modalities significantly different from unimodality. For AV pairs, the mean of signed asymmetry of OL ($P = 0.042$), rectangularity ($P < 0.001$) and harmonic 3 ($P = 0.038$) was significantly different from zero, with the left otolith being marginally larger than the right one on average. OP ($P = 0.017$), OA ($P = 0.031$), mass ($P = 0.003$), areal density ($P < 0.001$) and circularity ($P = 0.042$) had modalities that significantly differed from unimodality. When compared AV otolith pairs where the vateritic otoliths were over 50% vateritic, only the signed asymmetry of rectangularity significantly differed from zero ($P < 0.001$). OW ($P = 0.015$), OP ($P < 0.001$), OA ($P < 0.001$), mass ($P < 0.001$), areal density ($P < 0.001$) and circularity ($P = 0.001$) had modalities that significantly differed from unimodality. AV pairs therefore display DA for 1–3 of the 17 measurements, antisymmetry for 5–6 of the 17 measurements and FA for 8–11 of the 17 measurements. VV otolith pairs had mean signed asymmetry values that significantly differed from zero for OL ($P = 0.007$), OW ($P = 0.031$) and rectangularity ($P = 0.025$) with no significant deviations from unimodality. Left otoliths were again larger on average. These data follow what the authors expected to see from these different calcium carbonate polymorphs in terms of whether they displayed FA, directional asymmetry or antisymmetry (Supporting Information Appendices S2–S5).

3.3 | Models of otolith asymmetry

On average, vateritic otoliths were larger than aragonitic otoliths. Specifically, OL was 4.4% bigger, OW was 11.5% bigger, OP was 25.4% bigger and OA was 14.1% bigger. Nonetheless, aragonitic otoliths had higher masses by 12.1% and larger areal densities by 27.2%. When compared to aragonitic otoliths, vateritic otoliths had lower values for rectangularity, circularity, ellipticity and aspect ratio, whereas a higher value for roundness (Table 2).

For the PERMANOVA model of simple morphometrics (OL, OW, OP and OA) of all otoliths, the origin and percentage difference in vaterite were the only significant factors within the model ($\text{Pseudo-F}_{\text{origin}} = 35.672$, $P_{\text{origin}} < 0.001$; $\text{Pseudo-F}_{\text{\%vaterite}} = 406.678$, $P_{\text{\%vaterite}} < 0.001$). The percentage difference in vaterite alone accounted for 58.9% of the variation in the distances between points in the dissimilarity index. The quantitative measurement of vaterite explained more of the variation in the data than the qualitative assignment of vaterite or aragonite, though both were significant (Pseudo-F = 146.47, $P < 0.001$, 51.0%). The homogeneity of dispersion did not hold true for origin or otolith pair type ($P < 0.001$ for both). When examining the ordinated data, the authors found both a location effect and a dispersion effect for the otolith pair type (Figure 2). It was unclear if there was anything more than a dispersion effect for the origin of the *O. kisutch*. When only the AA otolith pairs were considered, origin, FL, hatchery and sex were not significantly related to the multivariate spacing of the data points in the dissimilarity index. There was a significant dispersion effect between the sexes ($P = 0.049$), but upon review of an associated ordination plot, there was no observable-associated location effect.

For the PERMANOVA model of the shape metrics (rectangularity, circularity, roundness, ellipticity and aspect ratio), the origin, percentage difference in vaterite and hatchery were the significant factors within the model ($\text{Pseudo-F}_{\text{origin}} = 11.982$, $P_{\text{origin}} < 0.001$; $\text{Pseudo-F}_{\text{\%vaterite}} = 150.138$, $P_{\text{\%vaterite}} < 0.001$, $\text{Pseudo-F}_{\text{hatchery}} = 2.854$, $P_{\text{hatchery}} = 0.025$). There was a significant difference in dispersion between the hatcheries ($P = 0.016$), so it was unclear if there was any location component associated with the differences between the hatcheries. There was also a significant difference in dispersion between the paired otolith types and origin ($P < 0.001$ for both). Upon review of the ordination plot, there was a clear location effect alongside a significant dispersion effect for the otolith pair type, but it was unclear if this was anything other than a dispersion effect for the origin (Figure 3). The percentage difference in vaterite accounted for 36.7% of the total variation in the distances between points in the dissimilarity index. The qualitative otolith-type assessment accounted for less of the overall variation between the data points in the dissimilarity index (Pseudo-F = 63.829, $P < 0.001$, $R^2 = 33.2\%$). When only the AA otolith pairs were considered, origin, FL, hatchery and sex were not significantly related to the multivariate spacing of the data points in the dissimilarity index. There were no significant differences in dispersion between the various grouping variables.

For the PERMANOVA model of the amplitudes of the lower-order Fourier harmonics, the origin, percentage difference in vaterite and FL were the significant factors in the model ($\text{Pseudo-F}_{\text{origin}} = 12.439$, $P_{\text{origin}} < 0.001$; $\text{Pseudo-F}_{\text{\%vat}} = 52.145$, $P_{\text{\%vat}} < 0.001$, $\text{Pseudo-F}_{\text{FL}} = 3.189$, $P_{\text{FL}} = 0.044$). Both origin and otolith pair type failed to meet the assumption of homogeneity of dispersion; nonetheless, when looking at the associated nMDS plots, otolith pair type clearly has a location and dispersion effect, whereas origin appears to only have a

FIGURE 2 Non-metric multidimensional scaling (nMDS) plot of the unsigned asymmetry values for Feret length (OL), Feret width (OW), otolith perimeter (OP) and otolith area (OA). Bray–Curtis dissimilarity calculations were used. Dimensions were reduced to two dimensions, with a maximum number of iterations set at 999, and the maximum number of random starts set to 500. A solution was reached within 35 iterations. Aragonite–aragonite (AA) pairs are represented by blue (#648FFF), aragonite–vaterite (AV) pairs are represented by pink (#DC267F) and vaterite–vaterite (VV) pairs are represented by yellow (#FFB000). These colours were chosen from the IBM colour blind safe palette. Hatchery-reared individuals are indicated by circles, and naturally reared individuals are indicated by triangles. $N = 247$. Otolith pairing: ●, AA; ●, AV; ●, VV. Clip: ●, Hatchery; ▲, natural

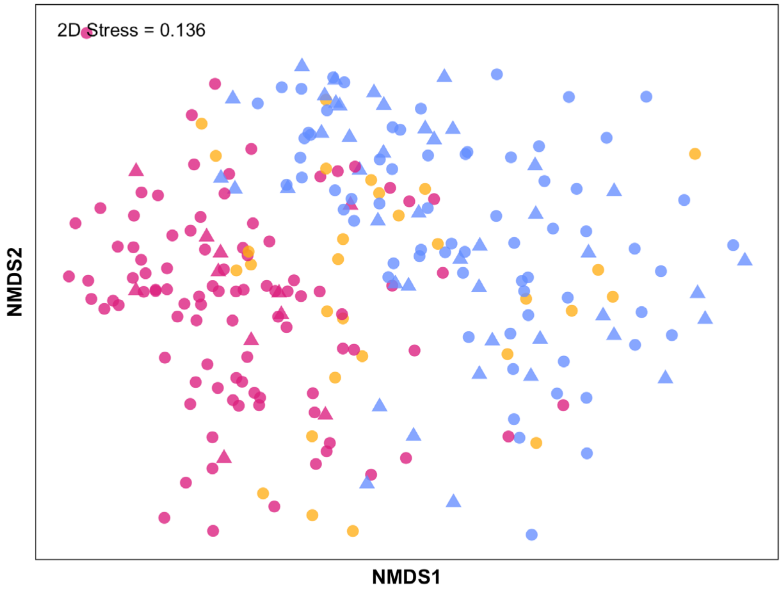

FIGURE 3 Non-metric multidimensional scaling (nMDS) plot of the unsigned asymmetry values for rectangularity, circularity, roundness, ellipticity and aspect ratio. Bray–Curtis dissimilarity calculations were used. Dimensions were reduced to two dimensions, with a maximum number of iterations set at 999, and the maximum number of random starts set to 500. A solution was reached within 20 iterations. Aragonite–aragonite (AA) pairs are represented by blue (#648FFF), aragonite–vaterite (AV) pairs are represented by pink (#DC267F) and vaterite–vaterite (VV) pairs are represented by yellow (#FFB000). These colours were chosen from the IBM colour blind safe palette. Hatchery-reared individuals are indicated by circles, and naturally reared individuals are indicated by triangles. $N = 247$. Otolith pairing: ●, AA; ●, AV; ●, VV. Clip: ●, Hatchery; ▲, natural

dispersion effect (Figure 4). The percentage difference in vaterite accounted for 16.8% of the distance between points in the dissimilarity index. The qualitative otolith-type assessment accounted for more of the overall variation between the data points in the dissimilarity index (Pseudo-F = 27.569, $P < 0.001$, $R^2 = 17.7\%$). When only the AA otolith pairs were considered, origin, FL, hatchery and sex were not

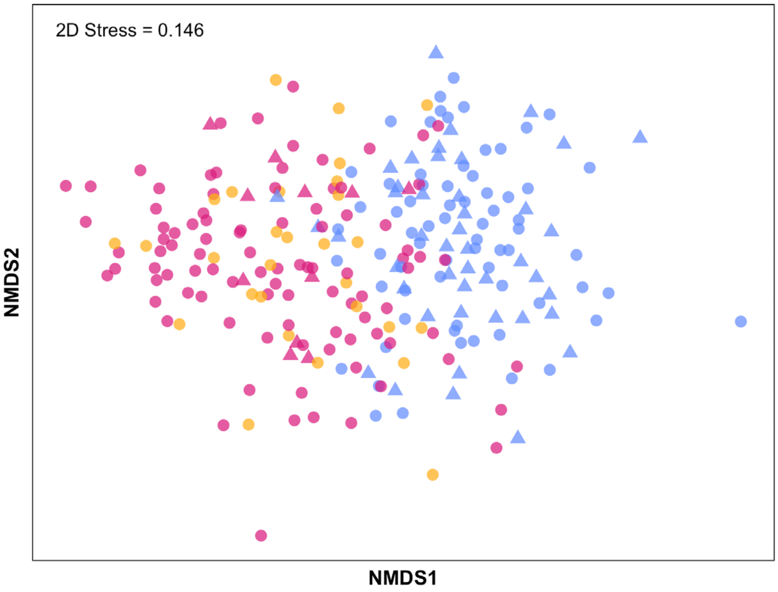

FIGURE 4 Non-metric multidimensional scaling (nMDS) plot of the unsigned asymmetry values for the amplitudes of harmonics 2–7. Bray–Curtis dissimilarity calculations were used. Dimensions were reduced to two dimensions, with a maximum number of iterations set at 999, and the maximum number of random starts set to 500. A solution was reached within 20 iterations. Aragonite–aragonite (AA) pairs are represented by blue (#648FFF), aragonite–vaterite (AV) pairs are represented by pink (#DC267F) and vaterite–vaterite (VV) pairs are represented by yellow (#FFB000). These colours were chosen from the IBM colour blind safe palette. Hatchery-reared individuals are indicated by circles and naturally reared individuals are indicated by triangles. $N = 247$. Otolith pairing: ●, AA; ●, AV; ●, VV. Clip: ●, Hatchery; ▲, natural

TABLE 3 Variance of the signed otolith asymmetry for each measurement type for hatchery and natural origin *Oncorhynchus kisutch*

Measurement	Natural origin	Hatchery origin	% Difference	Significance
OL (mm)	3.247E−2	2.859E−2	−11.95	ns
OW (mm)	1.611E−2	1.220E−2	−24.27	ns
OP (mm)	2.468E−1	1.443E−1	−41.53	ns
OA (mm^2)	1.041E−1	1.764E−1	69.45	ns
Otolith mass (g)	1.576E−6	0.521E−6	−66.97	ns
Areal density (g mm^{-2})	8.378E−9	4.564E−9	−45.52	ns
Rectangularity	7.645E−4	4.260E−4	−44.28	*
Circularity	1.611E−3	0.740E−3	−54.09	**
Roundness	7.831E−4	4.225E−4	−46.05	ns
Ellipticity	4.971E−4	3.463E−4	−30.34	ns
Aspect ratio	6.928E−3	4.768E−3	−31.18	ns
Harmonic 2	6.402E−5	3.757E−5	−41.32	ns
Harmonic 3	1.924E−5	2.593E−5	34.77	ns
Harmonic 4	2.782E−5	2.339E−5	−15.92	ns
Harmonic 5	1.383E−5	1.202E−5	−13.09	ns
Harmonic 6	7.779E−6	9.942E−6	27.81	Ns
Harmonic 7	5.483E−6	4.438E−6	−19.06	Ns

Note: Percentage difference indicates the relative percentage difference between hatchery and natural origin *O. kisutch*, with natural origin *O. kisutch* as a baseline. Significance was determined by permutation analysis; ns, not significant. Only aragonite–aragonite (AA) pairs were considered.
Abbreviations: OA, otolith area; OL, Feret length; OP, otolith perimeter; OW, Feret width.
$^{*}P < 0.05$.$^{**}P < 0.01$.$^{***}P < 0.001$.

significantly related to the multivariate spacing of the data points in the dissimilarity index. There were no significant differences in dispersion between the various grouping variables.

The absolute difference between the left and right structures is one of two ways to compare the difference in FA, the other being comparing the signed asymmetry values. When comparing the

TABLE 4 Variance of the signed otolith asymmetry for each measurement type for aragonite–aragonite (AA) and aragonite–vaterite (AV) otolith pairs

Measurement	AA otoliths	AV otoliths	% Difference	Significance
OL (mm)	0.301E−1	1.509E−1	401.16	***
OW (mm)	0.141E−1	1.652E−1	1074.96	***
OP (mm)	0.184	15.459	8324.52	***
OA (mm^2)	0.147	3.656	2380.33	***
Otolith mass (g)	0.979E−6	3.833E−6	291.56	***
Areal density ($g\ mm^{-2}$)	0.633 E-8	7.000 E-8	1005.85	***
Rectangularity	0.554E−3	1.222E−3	120.42	***
Circularity	0.107E−2	3.632E−2	238.81	***
Roundness	0.574E−3	1.949E−3	239.67	***
Ellipticity	0.421E−3	1.708E−3	305.51	***
Aspect ratio	0.584E−2	2.052E−2	251.25	***
Harmonic 2	0.048E−3	1.476E−3	2992.39	***
Harmonic 3	0.232E−4	2.241E−4	867.62	***
Harmonic 4	0.249E−4	2.661E−4	968.25	***
Harmonic 5	0.127E−4	2.010E−4	1480.19	***
Harmonic 6	0.090E−4	1.431E−4	1487.35	***
Harmonic 7	0.049E−4	1.032E−4	2003.12	***

Note: Percentage difference indicates the relative difference between AA and AV otolith pairs, with AA otolith pairs as a baseline. Significance was determined by permutation analysis; ns, not significant. Both hatchery and natural origin fish were included in the analysis.

Abbreviations: OA, otolith area; OL, Feret length; OP, otolith perimeter; OW, Feret width.

*$P < 0.05$.**$P < 0.01$.***$P < 0.001$.

variances of the signed differences between all the 17 shape factors between natural origin and hatchery origin *O. kisutch*, there were significant differences in variance between OW ($P = 0.007$), OP ($P = 0.004$), OA ($P = 0.043$), areal density ($P = 0.027$), circularity ($P = 0.003$) and all the harmonics ($P < 0.05$). In all but one of these instances, hatchery origin *O. kisutch* had a higher variance compared to the natural origin *O. kisutch* (Supporting Information Appendix S6). Specifically, 11 of the 17 variables considered had significantly different variances, with many percentage relative differences above 100%. Nonetheless, when the authors compared only aragonitic otolith pairs, only the variance in circularity was significantly different between hatchery and natural origin *O. kisutch* ($P = 0.007$; Table 3). In this one instance, the variance of the natural origin *O. kisutch* was higher than the hatchery origin *O. kisutch* (1.611E−3 *vs*. 7.396E−4).

When comparing AA and AV pairings to each other irrespective of origin, the variances of the signed differences in the otolith measurements were significantly different with the AA pairs having lower overall variance ($P < 0.001$ for all metrics; Table 4). Instead of looking at the left–right asymmetry of the AA *vs*. AV pairs, the variance in the difference between the aragonitic and vateritic otolith values was compared to the variance in asymmetry of the AA otolith pairs. This removed the added variance caused by vateritic otolith appearing on both the left and right sides of the head. Even still, the variance between the AV pairs was significantly higher than the AA pairs ($P < 0.001$).

4 | DISCUSSION

4.1 | Comparisons between hatchery and natural origin salmonids

In this study, significant differences in otolith shape and otolith shape asymmetry were observed between hatchery and natural origin *O. kisutch*. This confirms similar findings in previous studies that demonstrated that some aspects of the hatchery rearing environment are impacting the growth of otoliths in *O. tshawytscha* (Koeberle *et al*., 2020). There were differences among the three hatcheries, which could be related to differences in hatchery rearing practices. Based on our unpublished observations, vaterite prevalence between brood years in *O. kisutch* from the same hatchery can vary by over 30% without appreciable differences in hatchery rearing practices (Quindazzi *et al*., unpubl. data, Nov 2020). The links between these practices and vaterite incidence require further study. The authors also found that vaterite formation occurred over five times as often in hatchery origin compared to natural origin *O. kisutch*, which is consistent with other findings (Reimer *et al*., 2016; Sweeting *et al*., 2004). When they incorporated the absolute difference in vaterite coverage between the left and right otoliths, otolith mineralogy was the main factor explaining the variation between otolith asymmetry across all measurements, not origin. Although origin was still significant in models that considered all otolith pair types, this was likely due to the heterogeneity in dispersion between hatchery and natural origin data points rather than an actual location-based effect between the centroids of the groups in this factor. All other factors (sex, hatchery and FL) were not significantly related to the absolute asymmetries of the otolith shape data outside of some confounding dispersion effects.

When only the aragonitic otoliths were considered, origin, sex, hatchery and FL were all not significantly related to the dissimilarity of the absolute asymmetries of the shape metrics. This result supports the finding that origin does not influence otolith FA. In instances where there was a difference in variance between hatchery and natural origin AA pairs, contradictory to what might be expected, natural origin *O. kisutch* had more variability. This indicates that differences in otolith mineralogy, rather than origin, account for the differences in otolith asymmetry. The results of this study suggest that the proximal cause of shape and mass differences is vaterite formation in hatchery origin fish. This is concerning because vaterite formation in hatchery origin salmonids is associated with hearing loss (Oxman *et al*., 2007; Reimer *et al*., 2016; Vignon & Aymes, 2020), which is an important sensory modality (Popper, 1996; Popper & Lu, 2000).

4.2 | Vaterite formation and development instability

Vaterite replacement of aragonite in otoliths is a well-documented phenomenon, especially in hatchery origin fishes (Campana, 1983; David *et al*., 1994; Palmork *et al*., 1963; Reimer *et al*., 2016; Sweeting *et al*., 2004). There are many hypotheses regarding vaterite substitution in the otoliths of hatchery origin fishes. The leading hypothesis is that increased growth rates seen in hatchery origin fish in comparison to natural origin fish promote the secretion of vaterite in the otolith matrix (Reimer *et al*., 2017). The exact relationship between growth and vaterite formation may be related to other factors, such as overexpression of certain genes which may mitigate vaterite formation (Chalan *et al*., 2022). Once vaterite formation begins, it appears to be irreversible (Reimer *et al*., 2016; Tomás & Geffen, 2003). The presence of vaterite crystals seems to either promote the formation of more vaterite directly or indirectly through interaction with otolith proteins (Kalka *et al*., 2019; Poznar *et al*., 2020; Tohse *et al*., 2009). This interaction can result in patterns such as half of the otolith being composed of vaterite and the other half being composed of aragonite (*e.g*., Austad *et al*., 2021; Campana, 1983). The exact mechanism promoting an inconsistent switch from the precipitation of aragonite to vaterite is likely due to environmental factors within the hatchery rearing environment (Austad *et al*., 2021; Reimer *et al*., 2017).

Vaterite coverage drives differences in otolith asymmetry between hatchery and natural origin salmonids, but it is not clear if there is a connection between vaterite formation and DI. Both the genotype and the environment have an impact on the aggregate effects of DI, which then yields differences in FA in phenotypes of different populations (Klingenberg, 2019; Lens *et al*., 2002; Palmer, 1994). The formation of otoliths themselves and the calcium carbonate polymorphs they are made of are known to be under both genetic and environmental control (Loeppky *et al*., 2021). If vaterite formation and DI are linked, we will be unable to measure this through differences in otolith FA. This is because vaterite formation continues outside the presence of the initial environmental stressor which started it. Thus, vaterite coverage is not linearly associated with DI and by proxy FA. Furthermore, AV otolith pairs are more asymmetric than VV otolith pairs, and thus if we use otolith FA as a proxy of DI, we would find that vaterite coverage and DI do not covary. AA and VV otolith pairs seem to display FA, with VV pairs having higher FA than AA pairs, likely due to the inconsistent coverage of vaterite between otoliths. Nonetheless, the authors did have relatively few VV pairs due to their collection process and the brittle nature of vaterite, which led to proportionally more VV pairs being lost in comparison to AA or AV pairs. The authors would likely need to access more VV pairs to be more certain about the extent of their FA in comparison to AA pairs.

When only aragonitic otoliths were considered, there were almost no significant differences between hatchery and natural origin *O. kisutch*, with natural origin fish having higher FA in the instances where differences did occur. This result would seem to indicate that outside of vaterite formation, there do not seem to be other hatchery stressors that ultimately produce an increased amount of FA between the left and right otoliths of hatchery origin *O. kisutch* compared to natural origin *O. kisutch*. Although it is possible that DI is somehow related to vaterite formation, there does not appear to be unaccounted for hatchery rearing effects that increase the FA in the otolith shape metrics.

4.3 | Otolith FA

Otolith mineralogy must be accounted for when conducting studies of otolith FA. When FA is considered, it is assumed that the underlying structure of the data set has a mean of zero and non-directional variations around that mean. Vateritic and aragonitic otoliths differ substantially from each other in terms of macrostructure and have an equal chance of being on the left or right side of the head. This means, although the signed mean will still be zero, there will be a bimodal distribution of the left–right differences; thus, AV pairs demonstrate antisymmetry rather than FA. Differences in otolith shape are maximized when one otolith is fully vateritic, but the other otolith is fully aragonitic. As a result, the authors suggest that it is imperative to consider otolith mineralogy when investigating otolith shape asymmetry, and otolith morphometrics more broadly. Otolith mineralogy can be accounted for using a variety of methods. Visual inspection provides a surface level indication of whether otoliths are aragonitic or vateritic, but this is the least one ought to do. More specialized techniques can provide better quantitative data, such as micro-Raman spectroscopy and neutron diffraction (Wood *et al*., 2022). Regardless, failure to partition, these otoliths into calcium carbonate polymorphs will result in researchers misinterpreting data and drawing flawed conclusions. In future, the authors recommend the researchers to state how otolith mineralogy was considered.

Otolith FA may not be useful for measuring DI. The link between FA and stress is perhaps more complicated than comparing simple univariate measurements (Van Dongen, 2000). This has led the authors, as well as many others, to adopt a more rigorous multivariate account of FA in otoliths (Palmer *et al.*, 2010). There may be a publication bias in terms of finding a correlation between increases in FA in relation to some increased factor of stress, even in cases of multivariate comparisons (Díaz-Gil *et al.*, 2015; Palmer, 2000). As an example, in this study, the authors found almost no differences between hatchery and natural origin *O. kisutch* which would have likely remained unpublished. Stress is certainly an area of concern more broadly in the world of aquaculture due to routine handling of fish, stocking densities, poor water quality and increased intraspecific aggression (Braithwaite & Salvanes, 2010; Huntingford *et al.*, 2006). These factors also impact salmonids, and standard rearing conditions are generally stressful for hatchery salmonids (Cogliati *et al.*, 2019; Fagerlund *et al.*, 1981; Sharpe *et al.*, 1998). Inbreeding at the hatchery could also increase FA through increased homozygosity (Palmer & Strobeck, 1986; Vøllestad *et al.*, 1999). Despite the increased stress of the hatchery environment, the authors found no subsequent increase in otolith FA. This seems to support the notion that otolith FA metrics are not useful for studying DI (Díaz-Gil *et al.*, 2015).

4.4 | Conclusions

In this study, the authors found that the absolute difference in vaterite coverage was the greatest predictor of otolith asymmetry. When they compared only AA otolith pairs, there were no differences between hatchery and natural origin *O. kisutch*. Vateritic otoliths are five times more common in hatchery origin *O. kisutch* than their natural origin counterparts. The results highlight that researchers must account for otolith mineralogy when conducting studies of otolith morphometrics and otolith asymmetry on hatchery origin and natural origin fish.

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