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Bone microstructure and bone mineral density are not systemically different in Antarctic icefishes and related Antarctic notothenioids

Amir M. Ashique[1] | Oghenevwogaga J. Atake[1] | Katie Ovens[2] | Ruiyi Guo[1] |
Isaac V. Pratt[1] | H. William Detrich III[3] | David M. L. Cooper[1] |
Thomas Desvignes[4] | John H. Postlethwait[4] | B. Frank Eames[1]

[1]Anatomy, Physiology, and Pharmacology, University of Saskatchewan, Saskatoon, Saskatchewan, Canada

[2]Augmented Intelligence & Precision Health Laboratory (AIPHL), McGill University, Montreal, Quebec, Canada

[3]Marine and Environmental Sciences, Northeastern University Marine Science Center, Nahant, Massachusetts, USA

[4]Institute of Neuroscience, University of Oregon, Eugene, Oregon, USA

Correspondence
B. Frank Eames, Anatomy, Physiology, and Pharmacology, University of Saskatchewan, 2D01-107 Wiggins Rd, Saskatoon, SK, S7N 5E5, Canada.
Email: b.frank@usask.ca

Abstract

Ancestors of the Antarctic icefishes (family Channichthyidae) were benthic and had no swim bladder, making it energetically expensive to rise from the ocean floor. To exploit the water column, benthopelagic icefishes were hypothesized to have evolved a skeleton with "reduced bone," which gross anatomical data supported. Here, we tested the hypothesis that changes to icefish bones also occurred below the level of gross anatomy. Histology and micro-CT imaging of representative craniofacial bones (i.e., ceratohyal, frontal, dentary, and articular) of extant Antarctic fish species specifically evaluated two features that might cause the appearance of "reduced bone": bone microstructure (e.g., bone volume fraction and structure linear density) and bone mineral density (BMD, or mass of mineral per volume of bone). Measures of bone microstructure were not consistently different in bones from the icefishes *Chaenocephalus aceratus* and *Champsocephalus gunnari*, compared to the related benthic notothenioids *Notothenia coriiceps* and *Gobionotothen gibberifrons*. Some quantitative measures, such as bone volume fraction and structure linear density, were significantly increased in some icefish bones compared to homologous bones of non-icefish. However, such differences were rare, and no microstructural measures were consistently different in icefishes across all bones and species analyzed. Furthermore, BMD was similar among homologous bones of icefish and non-icefish Antarctic notothenioids. In summary, "reduced bone" in icefishes was not due to systemic changes in bone microstructure or BMD, raising the prospect that "reduced bone" in icefish occurs only at the gross anatomic level (i.e., smaller or fewer bones). Given that icefishes exhibit delayed skeletal development compared to non-icefish Antarctic fishes, combining these phenotypic data with genomic data might clarify genetic changes driving skeletal heterochrony.

Amir M. Ashique and Oghenevwogaga J. Atake, these authors contributed equally to this work.

1 | INTRODUCTION

Environmental changes can drive species evolution as natural selection acts on trait variants that promote survival in novel niches. As the Drake Passage and Tasmanian Gateway opened roughly 35 million years ago, the Antarctic Circumpolar Current formed, physically isolating the Southern Ocean from temperate oceans and leading to the progressive cooling of Antarctic waters (Eastman, 1993a). Ultimately, temperate fish species vacated the Southern Ocean, thereby opening novel ecological niches that were subsequently filled by notothenioid fishes, which had evolved an anti-freeze glycoprotein that protected their tissues in this frigid environment (Chen et al., 1997; DeVries, 1971). Among notothenioids, Antarctic icefishes (family Channichthyidae, suborder Notothenioidei) possess additional phenotypes that might be related to these cold, oxygen-saturated waters, including "white" blood resulting from pseudogenization of hemoglobin genes (Braasch et al., 2015; Cocca et al., 1995; Daane et al., 2020; Devries & Eastman, 1978; Eastman, 1993b).

Notothenioids were ancestrally benthic, living on the ocean floor, and descended from ancestors that had lost their swim bladder, a gas-filled organ that provides fish with neutral buoyancy (Eastman, 1993b). Some notothenioid lineages evolved strategies that secondarily reduce body density, thus improving their ability to exploit pelagic habitats. For example, the icefishes *Chaenocephalus aceratus* and *Champsocephalus gunnari* have a percent buoyancy (a measure comparing weight in water vs. weight in air) that was closer to neutral density than related benthic notothenioids, such as *Notothenia coriiceps* and *Gobionotothen gibberifrons* (Eastman & Sidell, 2002). Density-reducing features can include increases in substances less dense than water, such as lipids, but also the reduction of dense body parts, such as bones, teeth, and scales. Changes to icefish skeletons often were hypothesized as a response to an evolutionary pressure to occupy different trophic environments in the water column, but the exact nature of the supposed skeletal changes in adults needs clarification.

In Antarctic icefishes, "reduced bone" and skeletal heterochrony, in which a descendent species shows a shift in the timing of developmental events compared to an ancestor (de Beer, 1958; Gould, 1977), have been widely reported, raising the possibility that the latter might drive the former. The conclusion that icefishes have "reduced bone" mostly relies upon gross anatomic studies, analyses of percent ash weight of whole bodies, and non-quantitative Alizarin red staining (Devries & Eastman, 1978; Eastman & DeVries, 1981; Iwami, 1985; Voskoboinikova, 2001; Żabrowski, 2000). Clearly, some icefish bones are greatly reduced or even missing (Iwami, 1985; Żabrowski, 2000). Similarly, ash-weight experiments can reflect changes at the gross anatomic level (i.e., overall bone mass), collectively indicating the size and density of the skeleton in relation to the rest of the body and/or the relative proportion of cartilage to bone in the adult skeleton. Interestingly, histological and molecular studies of skeletogenesis in icefishes demonstrated a delay in developmental timing of endochondral ossification relative to non-icefish Antarctic notothenioids (Albertson et al., 2010; Voskoboinikova, 2001). Even though heterochrony is often correlated to morphological changes (de Beer, 1958; Gould, 1977), a full understanding of how adult icefish bones might have been altered as a result of this developmental shift in timing remains unclear.

While icefishes indeed have smaller and fewer bones at the gross anatomic level (Iwami, 1985; Żabrowski, 2000), "reduced bone" could in principle also result from each bone appearing less dense in at least two specific ways. The first case, termed "bone tissue density," refers to how much mineralized bone fills the total volume of a given bony skeletal element, reflecting bone microstructure (Dempster et al., 2013). For example, two mammalian long bones of the exact same gross anatomic size can contain very different amounts of bone tissue distributed near the surface (compact bone) or internally as trabeculae (cancellous bone), thus having different bone tissue density. Features of bone tissue density can be measured in three dimensions (3D) using micro-CT parameters, most directly by bone volume fraction, but also by bone surface area-to-volume ratio, structure linear density, and structure thickness (Bouxsein et al., 2010). Collectively, these measurements reflect bone microstructure. The second case, termed "bone mineral density" (BMD), reflects how much mineral is in each given volume of bone tissue. This tissue-level BMD can also be measured directly in 3D using micro-CT by calibrating imaging data with standard hydroxyapatite controls (Bouxsein et al., 2010). To be clear, tissue-level BMD is different from clinical measures of BMD, which are often two-dimensional projections across a volume, reflecting bone tissue density (Dempster et al., 2013).

Current literature involving non-quantitative data suggested that icefishes have altered bone microstructure and BMD. Two recent histological studies of bones visually suggested a decrease in bone microstructure in Antarctic icefishes, because icefish bones appeared to have fewer thin bony rods (analogous to trabeculae in mammalian long bones) compared to non-icefish Antarctic species (Eastman et al., 2014; Meunier et al., 2018). Icefishes even have been suggested as a natural evolutionary model of osteoporosis, a systemic disease of progressive bone loss in aging humans (Albertson et al., 2009; Kawalilak et al., 2014). Regarding BMD, recent micro-CT and CT imaging suggested that icefish bones had decreased BMD (Daane et al., 2019; Eastman et al., 2014), but these studies were also not quantitative.

Here, we used histology and quantitative assessments of micro-CT data to test the hypothesis that a change in bone microstructure and/or BMD occurred during the evolution of Antarctic

icefishes. Representative craniofacial bones (i.e., ceratohyal, frontal, dentary, and articular) were analyzed in two Antarctic icefish species (the blackfin icefish *Chaenocephalus aceratus* and the mackerel icefish *Champsocephalus gunnari*) and two non-icefish Antarctic notothenioid species (the bullhead notothen *Notothenia coriiceps* and the humped notothen *Gobionotothen gibberifrons*; see Figure 1 for evolutionary relationships among these fishes). Results failed to support the hypothesis, suggesting that icefish bones did not evolve via systemic changes to bone microstructure or BMD, such as occurs during osteoporosis when humans have decreased levels of estrogen, a circulating osteogenic substance (Kawalilak et al., 2014). We

discuss how these findings relate to heterochrony as a putative developmental mechanism underlying Antarctic icefish bone evolution.

2 | MATERIALS AND METHODS

2.1 | Specimen collection and initial preparation

Live Antarctic icefishes (*Chaenocephalus aceratus* and *Champsocephalus gunnari*), and non-icefish notothenioid fishes (*Notothenia coriiceps* and *Gobionotothen gibberifrons*) were collected in March 2012 via bottom trawling of the continental shelf (150–180 m deep) from the *ARSV Laurence M Gould* near Low and Brabant Islands in the Palmer Archipelago. These species were the abundant species in these waters that were captured by trawling at that time. Specimens were maintained in running seawater (−1 to +1°C) in transfer tanks and brought to Palmer Station, Antarctica, where they were euthanized via overdose with Tricaine (MS-222), decapitated, and immediately frozen for intercontinental transport. Standard length and weight of each fish indicated that all were adults (Table S1). After thawing, skull bones of interest (i.e., ceratohyal, frontal, and mandibular; Figure 1) were dissected and fixed overnight in 4% paraformaldehyde in PBS, dehydrated in a graded ethanol series, and maintained in 70% ethanol at 4°C until analyzed. The initial focus of this study was on mandibular bones, so unfortunately frontals and ceratohyals of G. gibberifrons and C. gunnari were not prepared for analyses. See Table S1 for sample sizes of each bone.

2.2 | Micro-CT analyses

Bones were imaged at room temperature using a Skyscan 1172 high-resolution micro-CT scanner (Bruker-Skyscan). The bone specimens were washed with 70% ethanol to remove residual tissue debris, and then polystyrene foam chips were used to position firmly the bone segments in a polypropylene sample holder containing 70% ethanol. Images were acquired using the following settings: magnification 10 (pixel size = 26.6 μm), voltage 49 kV, current 205 μA, 120 ms exposure time, 180° angular range, 0.1° rotation step, 5 average frames/view, and 0.5 mm aluminum filter. Image data sets were processed using NRecon Software (Skyscan, version: 1.6.9.18) to generate serial two-dimensional reconstructions. Binarization (thresholding of greyscale images) of micro-CT images was done by local thresholding (Dufresne, 1998), resulting in threshold pixel greyscale values (minima) of: 84 for frontal bone images of all species; 100 for ceratohyal bone images of all species; and 80 for all images of the mandibular bones (articular, dentary) of all species. Binarized images were then shrink-wrapped using CT-Analyzer (Skyscan, version 1.14.4.1+).

Before 3D morphometric analyses were performed, images of mandibular bones underwent segmentation using CT-Analyzer. To separate the articular and the dorsal (tooth-bearing) and ventral portions of the dentary, these regions were specified as regions of interest (ROIs) by drawing outlines around them on representative
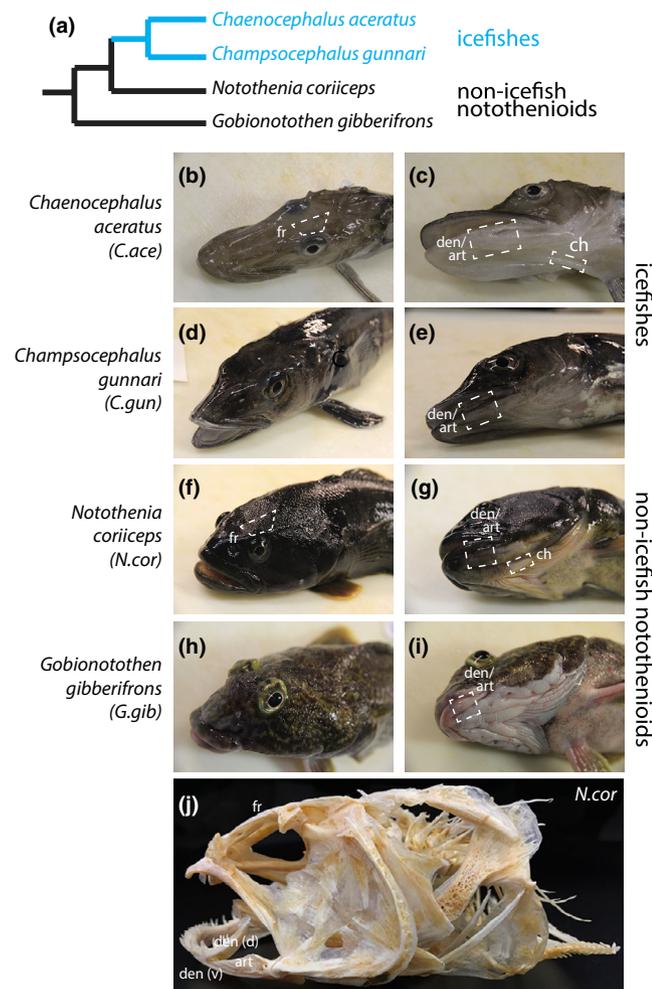
FIGURE 1 Sampled Antarctic fish species and bones. (a) Phylogenetic relationships among the Antarctic fish species examined (Near et al., 2018). Dorsolateral (b, d, f, h) and ventrolateral (c, e, g, i) views illustrate positions of sampled bones in the icefishes *Chaenocephalus aceratus* (b, c) and *Champsocephalus gunnari* (d, e), and the non-icefish notothenioids *Notothenia coriiceps* (f, g) and *Gobionotothen gibberifrons* (h, i). J. Skull of *N. coriiceps* illustrates positions of bones (ceratohyal not visible). Abbreviations: art, articular; ch, ceratohyal; den(d), dorsal portion of dentary; den(v), ventral portion of dentary; fr, frontal. Total lengths of specimens in panels (b–i) are as follows: *C. aceratus* =43.2 cm; *C. gunnari* =43.4 cm; *N. coriiceps* =33.0 cm; and *G. gibberifrons* =37.4 cm

micro-CT 2D virtual slices. ROIs were then interpolated to all other slices to generate volumes of interest (VOIs) using the interpolation function of CT-Analyzer. Adjustments to this automatic function were made to some slices by re-drawing outlines when ROIs did not separate properly. Similar segmentation was done to remove tooth tissues from the dorsal dentary (See Figure S2 for examples of VOI renderings of the tooth-bearing portion of the dentary pre- and post-segmentation).

For quantitative measurements, anatomical landmarks, such as the location of the articular relative to the dorsal and ventral portions of the dentary, were used to compare homologous regions of each bone across species. Because it is hard to distinguish compact and cancellous bone in fishes, regardless of whether the bone forms by intramembranous or endochondral ossification (Cubbage & Mabee, 1996; Eames et al., 2013; Huysseune, 2000), quantitative measurements were obtained without respect to these distinctions. To measure bone microstructure, the following common outputs of CT-Analyzer were used: bone volume fraction (BV/TV = bone volume over total volume), bone surface area-to-bone volume ratio (BS/BV), structure linear density (St. Li. Dn), and structure thickness (St. Th; Figure S1; Bouxsein et al., 2010). The latter parameters (St. Li. Dn and St. Th) are analogous to trabecular number and trabecular thickness, but only bony rods in cancellous bone are defined as trabeculae (Dempster et al., 2013). Since our measurements also included compact bone (Gray & Williams, 1989), we avoided using the term "trabecular." Tissue-level BMD was analyzed by the guidelines presented by the American Society for Bone and Mineral Research (Bouxsein et al., 2010). Briefly, a water phantom (Hounsfield unit (HU) of zero) and two custom phantoms of defined density (0.25 g/cm^3 and 0.75 g/cm^3 of calcium hydroxyapatite (HA)) were scanned at the above settings to establish coefficients for calibrated density-specific analyses. All bones were scanned from both left and right sides of the head of the same individual. No significant left-right differences were quantitated for any parameter of the sampled bones (data not shown), so values for each parameter were calculated by averaging respective left and right measurements for that individual.

2.3 | Histology

Bone samples (after micro-CT scanning) underwent whole-mount or section histology as described herein. Briefly, overnight acid-free 0.01% Alizarin red staining marked calcified bone in whole head samples (Eames et al., 2011); skeletal elements were dissected subsequently for brightfield imaging. Alternatively, samples for sectioning underwent demineralization in 19% EDTA, paraffin embedding, and sectioning to 10 μm thickness. Bone and cartilage were highlighted with Milligan's Trichrome staining protocol, as follows. Milligan's mordant was prepared in a fume hood, combining 150 ml Milligan's Solution A (3% potassium dichromate in dH$_2$0) with 50ml Milligan's Solution B (hydrochloric acid in 95% EtOH (1:10)) and allowing it to sit at RT for at least 30 min (but no more than 4 h). After dewaxing and rehydration, slides were stained in mordant for 5 min

(in fume hood), dH$_2$0 for 1 min (in fume hood), 1% acid fuchsin for 30 s, dH$_2$0 for 30 s, 1% phosphomolybdic acid for 2 min, 2% orange G for 30 s, dH$_2$0 for 1 min, 1% acetic acid for 2 min, 1% Aniline blue for 3 min, and 1% acetic acid for 3 min. Aniline blue stains tightly wound collagen fibers (typically Col1 or Col2), so bone/dentine will stain dark blue, while cartilage stains light blue; acid fuchsin stains in magenta muscle and also tightly-wound collagen fibers of previously mineralized tissues (after demineralization) (Atake et al., 2019; Eames et al., 2007; Nielsen et al., 1998).

2.4 | Statistical analyses

Principal component analysis (PCA) was performed using the stats, factoextra v1.0.7, and ggplot2 v3.3.2 packages in R v3.6.1. Univariate statistical analysis was performed using SPSS V.22 (SPSS). The distribution of each microstructural parameter was tested for normality using the Shapiro–Wilk test. Since data for mandibular bones were obtained from multiple species, one-way analysis of variance (ANOVA) was used to test for differences in a given microstructural or BMD measurement among species, and Tukey HSD and Games-Howell were used for subsequent post hoc analyses between each species, depending upon whether homogeneity of variance assumption was met or not, respectively. Since data for ceratohyal and frontal bones were measured from only two species, independent sample t-tests were used to test for differences in a given microstructural or BMD measurement between each species.

3 | RESULTS

3.1 | Histological analyses of homologous bones of icefish versus non-icefish

To evaluate the underlying causes of "reduced bone" in Antarctic icefish, we compared craniofacial bones of four species of relatively high abundance in waters of the West Antarctic Peninsula: two icefishes (*Chaenocephalus aceratus* and *Champsocephalus gunnari*) and two non-icefish notothenioids (*Notothenia coriiceps* and *Gobionotothen gibberifrons*; Figure 1). Four bones were analyzed, two forming by endochondral ossification (ceratohyal and articular), and two forming by intramembranous ossification (frontal and dentary; Figure 1; Eames et al., 2013).

In contrast to previous reports (Albertson et al., 2009; Devries & Eastman, 1978; Eastman, 1993b; Iwami, 1985), Alizarin red staining intensity was similar in many of the craniofacial bones from the four species. Despite some apparent differences in specific bones, generally a similar intensity of staining was observed among the ceratohyal, frontal, dentary, and articular bones and among Antarctic fishes (Figure 2a–h), suggesting that overall BMD of icefish bones was not lower than BMD in related notothenioid bones. Qualitatively, increased Alizarin red staining was apparent in the dentary and articular of the *G. gibberifrons* mandible compared to homologous bones of the other species (Figure 2e–h).

icefish   non-icefish
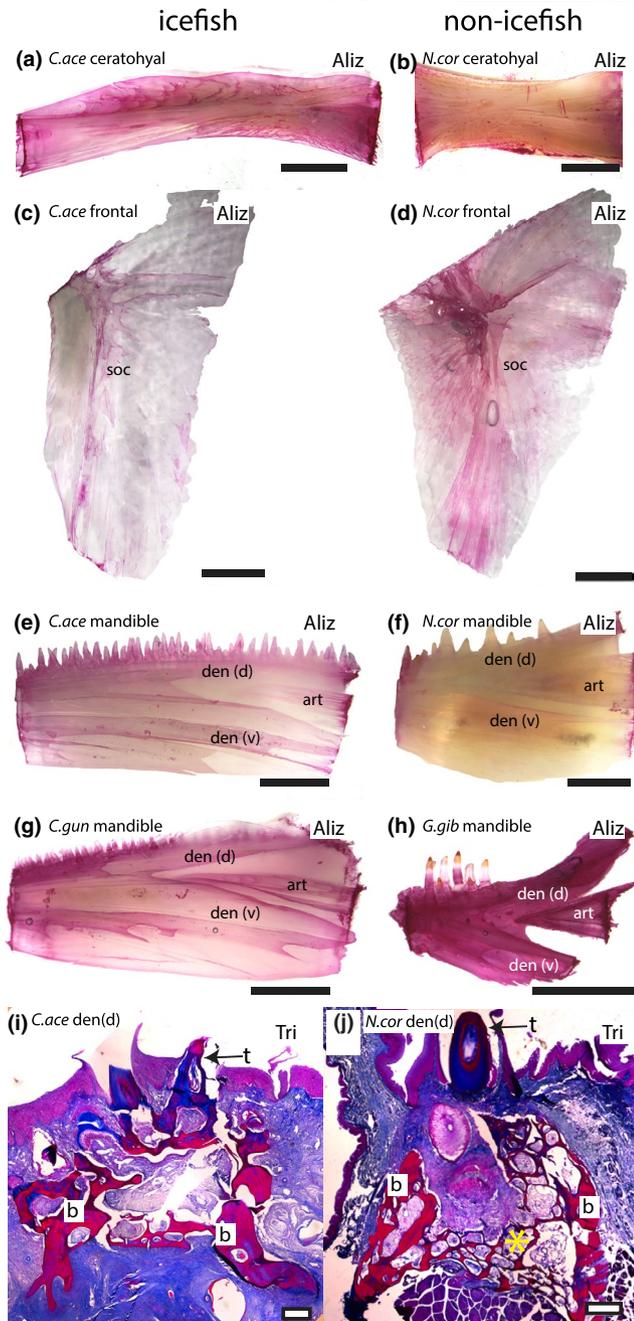
FIGURE 2 Histological analyses revealed similar staining patterns among Antarctic icefishes and non-icefish notothenioids. Alizarin red staining intensity was similar among bones of icefishes *C. aceratus* (a, c) and *C. gunnari* (e, g) and related notothenioids *N. coriiceps* (b, d) and *G. gibberifrons* (f, h), although some increase in Alizarin red staining appeared near the supraorbital canal (soc) of the *N. coriiceps* frontal (d) and the *G. gibberifrons* mandible (h). The apparent relative decrease in Alizarin red staining of *N. coriiceps* ceratohyal (b) and mandible (f) likely reflects increased opacity of remaining surrounding tissues. Milligan's Trichrome staining of the dorsal portion of the dentary in *C. aceratus* (i) and *N. coriiceps* (J) mandibles suggested increased bony rods (yellow *s) in *N. coriiceps* bone. In Milligan's Trichrome, Aniline blue stains tightly wound collagen fibers (typically Col1 or Col2), so bone/dentine will stain dark blue, while cartilage stains light blue; acid fuchsin stains tightly-wound collagen fibers of previously-mineralized tissues (after demineralization) and also muscle magenta (Atake et al., 2019; Eames et al., 2007; Nielsen et al., 1998). Both species had similar staining patterns and intensities among tissues of bone (b) and tooth (t), including similar amount of acid fuchsin (magenta) staining in mineralized bone, suggesting similar BMD. Abbreviations: Aliz, Alizarin red; art, articular; b, bone; d, dorsal; den, dentary; soc, supraorbital canal; t, teeth; Tri, Trichrome; v, ventral. Scale bars: a–h, 6.25 mm; I,J = 500 μm

portion of the dentary in the icefish *C. aceratus* appeared to have fewer thin rods of bone than those of the non-icefish notothenioid *N. coriiceps* (Figure 2i, j; yellow * in J indicates bony rods). Regarding qualitative measures of BMD, however, no differences were observed. Both icefish and non-icefish dentaries had similar intensities and relative areas of acid fuchsin staining, which stains collagen fibers of previously-mineralized regions of bone magenta under Milligan's Trichrome, even after sample demineralization (Figure 2i, j; Atake et al., 2019; Eames et al., 2007; Egerbacher et al., 2006).

3.2 | Micro-CT images of homologous bones of icefish versus non-icefish

Micro-CT images of a variety of Antarctic fish head bones suggested some differences in bone microstructure between icefish and non-icefish notothenioid species, but these differences were not consistent across bones and species (see Table S1 for sample sizes of each bone analyzed). The number and thickness of bony rods in the ceratohyal of the icefish *C. aceratus* did not appear to be different from the ceratohyal of the non-icefish *N. coriiceps* (Figure 3a, b). However, some other bones appeared to have fewer thin bony rods in some icefishes, compared to non-icefish notothenioids, especially when comparing reconstructed slices of the micro-CT data (Figure 3, Movies S1–S3). For example, the *C. aceratus* frontal seemed to have fewer thin rods of bone, compared to the *N. coriiceps* frontal (Figure 3c, d). Similarly, the dentary of the icefishes *C. aceratus* and *C. gunnari* appeared to have fewer thin bony rods than the non-icefish notothenioid *N. coriiceps*, but about the same number as the non-icefish *G gibberifrons* dentary (Figure 3e–h), so these results

Some differences in overall anatomy of bones, however, were apparent among species. For example, the *N. coriiceps* frontal had large ventral struts from the supraorbital canals, but these were absent or substantially reduced in *C. aceratus* frontals ("soc" in Figure 2c, d; see also Figure 3c, d). Such differences might explain the apparent denser Alizarin staining in *N. coriiceps* frontal, compared to *C. aceratus*. As previously reported (Eastman et al., 2014; Iwami, 1985; Żabrowski, 2000), the ethmoid of *C. aceratus* was not ossified, whereas the *N. coriiceps* ethmoid was well-ossified (data not shown). Cartilage persisted in ceratohyals of both *C. aceratus* and *N. coriiceps* (data not shown).

Section histology suggested potential differences in bone microstructure, but not BMD, between icefish and non-icefish bones. Qualitatively, many histological sections of the dorsal

icefish

non-icefish

FIGURE 3 Micro-CT images of sampled bones from icefishes and non-icefish notothenioids. 3D renderings of micro-CT data demonstrate surfaces of homologous portions of the *C. aceratus* ceratohyal (a), frontal (c), and dentary and articular (e); *N. coriiceps* ceratohyal (b), frontal (d), and dentary and articular (f), *C. gunnari* dentary and articular (g); and *G. gibberifrons* dentary and articular (h). Virtual sections through these bones (a'-h'; obtained from plane of red dashed lines in a–h) suggested less bony rods in some bones of icefishes, compared to bones of non-icefish relatives, but not consistently fewer in icefishes. For ceratohyal images, a' and b' virtual slices are rotated clock-wise 90° from the corresponding orientation of the (a) and (b) renderings. Abbreviations: art, articular; d, dorsal; den, dentary; soc, supraorbital canal; v, ventral. Scale bars, 3 mm

did not consistently support the hypothesis that icefish bones have altered bone microstructure.

General anatomic features of most bones were very similar (Figure 3), but as noted previously (Figure 2c, d), ventral projections from the supraorbital canals distinguished frontals of the non-icefish *N. coriiceps* from the icefish *C. aceratus* ("soc" in Figure 3c, d). No qualitative differences in pixel greyscale values, an indicator of BMD, were apparent in the same bones across species in these identically-scanned micro-CT images (Figure 3).

3.3 | Principle component analysis of micro-CT data suggested microstructural features differed by individual bones, not by species

To determine whether "reduced bone" in icefishes included changes to bone microstructure and/or BMD, the collective 3D datasets for mineralized portions of the micro-CT-imaged bones were analyzed quantitatively using CT-Analyzer (Bouxsein et al., 2010). Specifically, bone volume fraction (BV/TV), bone surface
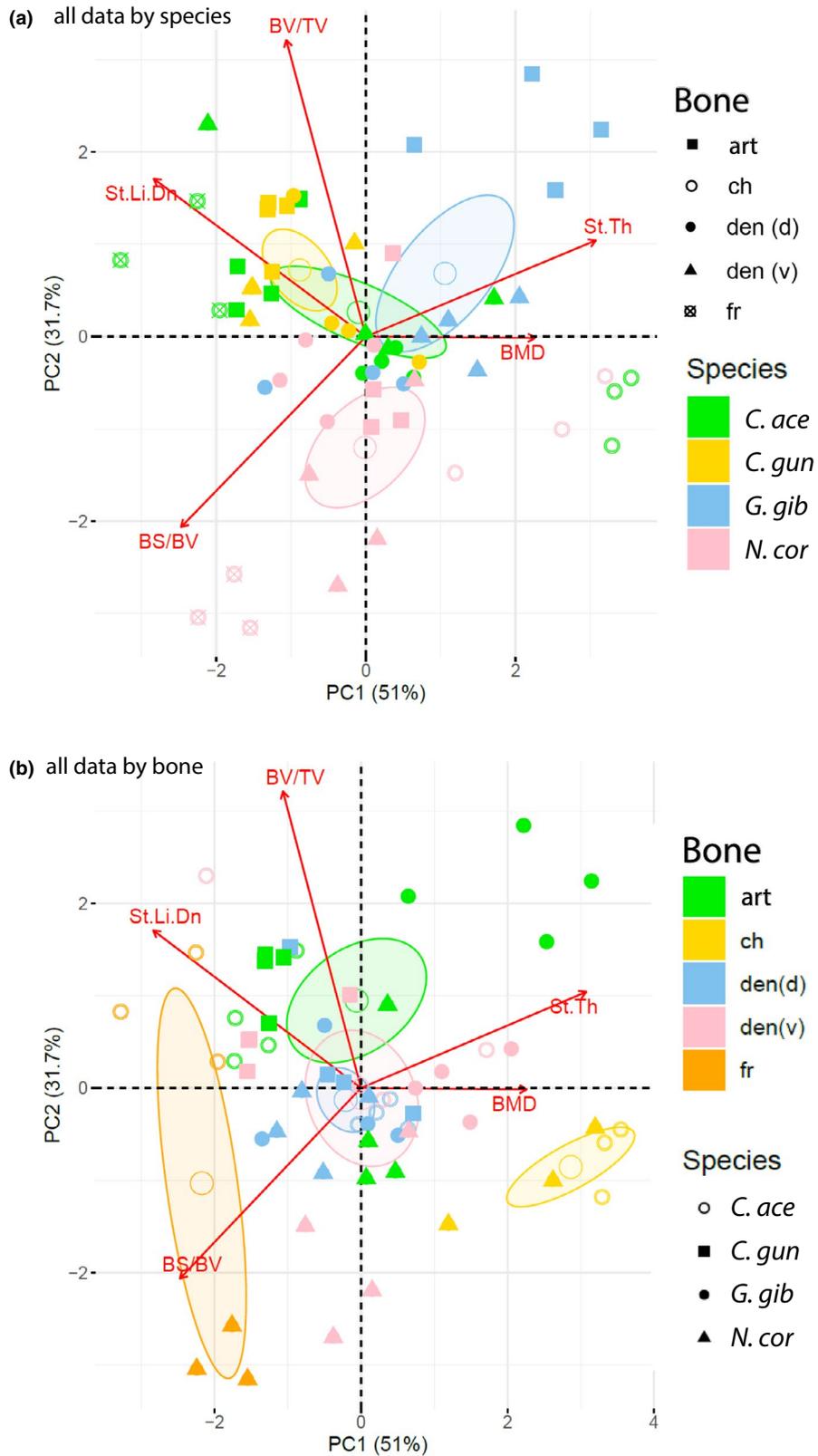
FIGURE 4 Principle component analysis (PCA) of all micro-CT data suggested that variations among samples were best captured by grouping data by individual bones, not by species. PCA biplots show the first two PCs of the data variance. PC scores of samples (dots) are colored based on the grouping of data by: species of fish (a) or the distinct bone (b). 95% confidence ellipses are shown for each grouping in the same color as the samples. These ellipses separated samples better by bone (b) than by species (a). Loadings of the data variables are shown as red arrows. The further away these loadings are from a PC origin, the more influence they have on that PC

area-to-bone volume ratio (BS/BV), structure linear density (St. Li. Dn), and structure thickness (St. Th) were used to quantitate microstructure of Antarctic fish bones (see Methods; also see Table S2 for all quantitative micro-CT data). Because the tooth-bearing dorsal portion of the dentary might be under different selective pressures than the ventral portion of the dentary, these regions were considered separately by micro-CT VOI segmentation (see Methods).

When all bone microstructure parameters and BMD were considered, a PCA of all data grouped by species did not separate icefish from non-icefish notothenioid species, even though the first two PCs covered 83% of the data variance (Figure 4a). This analysis is perhaps the most direct refutation of the hypothesis that changes to bone microstructure and BMD underlie "reduced bone" in icefishes. In contrast, the same PCA grouped by individual bones resolved the data better than grouped by species; the ceratohyal and frontal had collective bone microstructure and BMD features that were likely statistically different from other bones (Figure 4b). Both of these results did not change when BMD data were omitted (data not shown), specifically suggesting that bone microstructure did not underlie "reduced bone" of icefishes. Because PCAs demonstrated that individual bones had unique microstructural features, regardless of the species analyzed, further analyses focused on individual bones.

The PCAs of all data from individual bones suggested some differences among species, but these differences did not typically separate bones of icefishes from non-icefish notothenioids (Figure 5). Bone microstructure and BMD data did not distinguish ceratohyals of the icefish *C. aceratus* from the non-icefish *N. coriiceps* (Figure 5a). In addition to the gross anatomic differences between frontal bones of *C. aceratus* and *N. coriiceps* (Figures 2, 3), a PCA of bone microstructure and BMD data completely separated frontals of these two species (Figure 5b). However, individual PCAs of both the dorsal and ventral portions of the dentary failed to distinctively segregate icefish from non-icefish bones (Figure 5c, e). All species had distinct articulars in the PCA considering bone microstructure and BMD data (Figure 5d). The mandibular bones sampled interact physically and might operate together as a functional unit. PCAs that included all mandibular bone data together, however, also failed to clearly separate icefishes from non-icefish notothenioids (data not shown). Generally, PCAs failed to distinguish bones of both icefishes from both non-icefish notothenioids, regardless of whether BMD data were included with the bone microstructure data (Figure 5; data not shown for PCAs without BMD).

3.4 | Quantitation of micro-CT images demonstrated that bone microstructure of icefish bones was not consistently different compared to homologous non-icefish bones

Bone volume fraction, the most direct measure of bone tissue density, was not generally different between icefish and non-icefish bones. No difference in bone volume fraction was observed for the ceratohyals of *C. aceratus* and *N. coriiceps*, whereas the *C. aceratus* frontal had significantly higher bone volume fraction than that of *N. coriiceps* ($p < 0.01$, $F = 4.0$, df = 4; Figure 6a, b). Some statistical differences in bone volume fraction occurred among bones of the mandible, but these differences were not consistent (i.e., not always higher or lower), and the differences were not always between icefish and non-icefish bones (Figure 6c).

Bone surface area-to-volume ratio, another parameter of bone microstructure, was not consistently different in icefish versus non-icefish bones. Surface area-to-volume ratio was similar in the ceratohyal, but was significantly decreased in the frontal, of *C. aceratus* compared to *N. coriiceps* ($p < 0.01$, $F = 3.7$, df = 4; Figure 7a, b). Also, the surface area-to-volume ratio of the dorsal portion of the dentary was significantly lower in the icefish *C. aceratus* than in the non-icefish notothenioid *N. coriiceps* ($p < 0.05$, $F = 2.0$, df = 3; Figure 7c). While the same trend was observed when comparing the dorsal dentary of the icefish *C. gunnari* to homologous regions of non-icefish dentaries, the differences were not statistically significant. No consistent trends were observed for the articular and the ventral portion of the dentary. For example, the articulars of the icefishes *C. aceratus* and *C. gunnari* had a significantly higher surface area-to-volume ratio than the non-icefish *G. gibberifrons* articular ($p < 0.01$ for each comparison, $F = 0.73$, df = 3), but so did the *N. coriiceps* articular ($p < 0.01$, $F = 0.73$, df = 3; Figure 7c).

The bone microstructure parameter of structure linear density was similar in the ceratohyal, but was significantly increased in the frontal, of *C. aceratus* compared to *N. coriiceps* ($p < 0.01$, $F = 1.9$, df = 4; Figure 8a, b). Structure linear density in the dorsal portion of the dentary was not statistically different among *C. aceratus*, *C. gunnari*, *N. coriiceps*, and *G. gibberifrons* (Figure 8c). On the other hand, the articulars of both icefishes *C. aceratus* and *C. gunnari* had significantly increased structure linear density compared to the non-icefish *N. coriiceps* ($p < 0.01$ for each comparison, $F = 7.6$, df = 3), but only the *C. gunnari* was significantly increased compared to the non-icefish *G. gibberifrons* ($p < 0.05$, $F = 7.6$, df = 3; Figure 8c). A similar trend for increased structure linear density was revealed in

FIGURE 5 Principle component analysis (PCA) of micro-CT data suggested that bone microstructure and bone mineral density measures could separate some individual bones by species. PCA biplots show the first two PCs of the data variance. PC scores of samples (dots) are colored based on the species of data for the ceratohyal (a), frontal (b), dorsal portion of dentary (c), articular (d), or ventral portion of dentary (e). 95% confidence ellipses for each grouping are shown in the same color as the samples. Loadings of the data variables are shown as red arrows

(a) ceratohyal data by species

(b) frontal data by species

(c) dentary (d) data by species

(d) articular data by species

(e) dentary (v) data by species
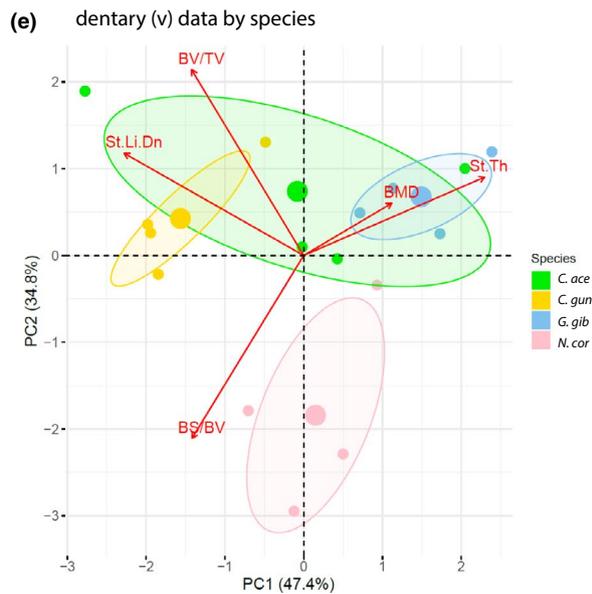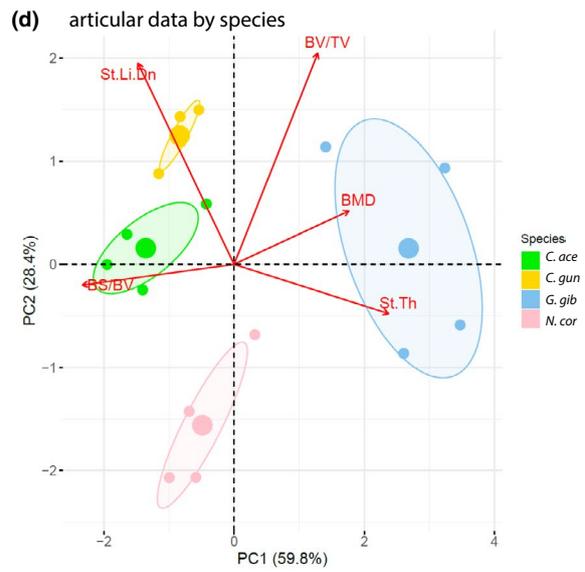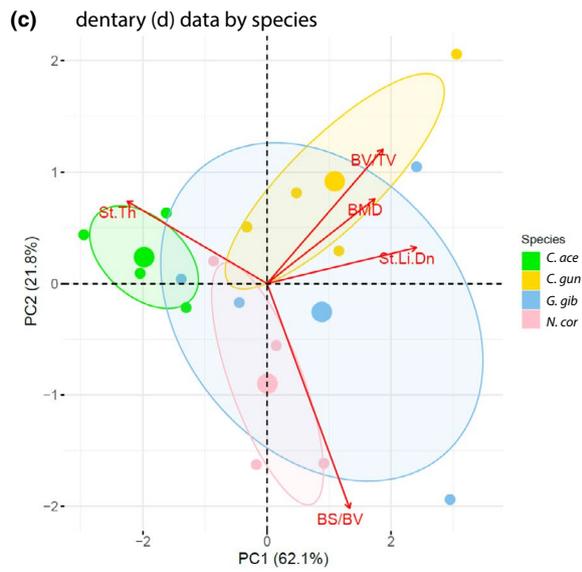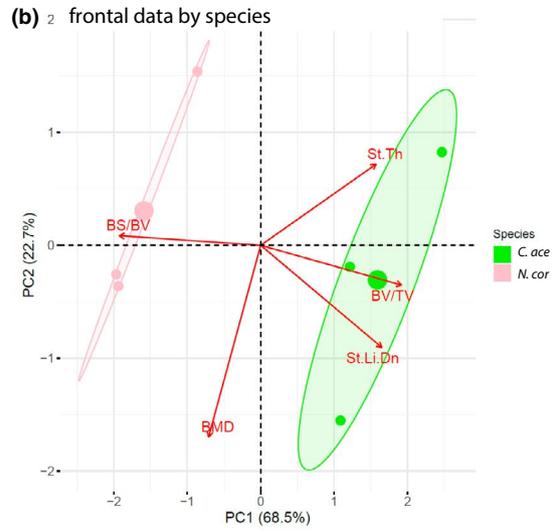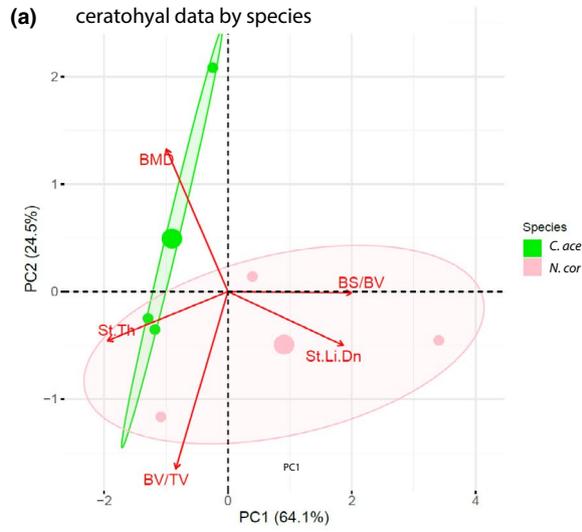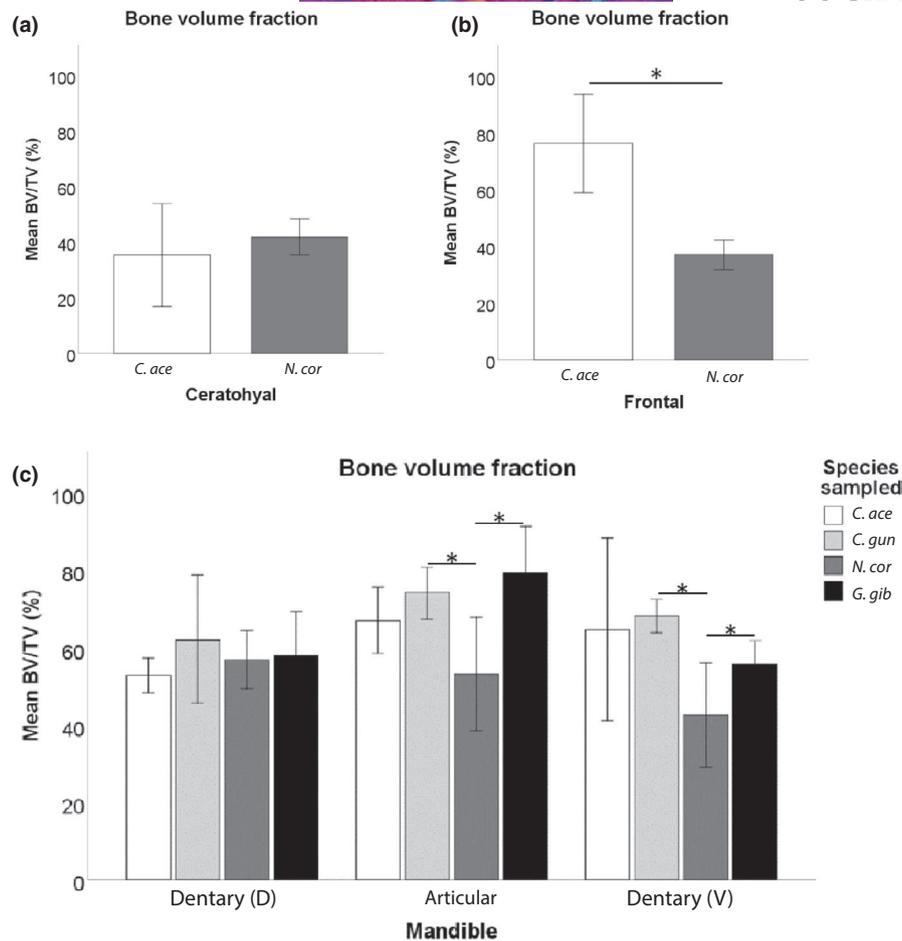
FIGURE 6 Icefish bones generally do not have altered bone microstructure (i.e., bone volume fraction) compared to homologous non-icefish bones. Bone volume fraction was calculated in ceratohyals of *C. aceratus* and *N. coriiceps* (a); frontals of *C. aceratus* and *N. coriiceps* (b); and mandibular bones (dorsal (D) and ventral (V) portions of the dentary and the articular) of *C. aceratus*, *C. gunnari*, *N. coriiceps*, and *G. gibberifrons* (C). *indicates statistically significant comparison ($p < 0.05$). Error bars are 95% confidence intervals

the ventral dentaries of both icefishes compared to non-icefishes, but only the *C. gunnari* data were statistically significant ($p < 0.01$ and $p < 0.05$ vs. *N. coriiceps* and *G. gibberifrons*, respectively, $F = 3.3$, df = 3), likely due to the large variation among *C. aceratus* ventral dentaries (Figure 8c).

Finally, structure thickness was not consistently different in icefish bones compared to non-icefish bones. The ceratohyal and frontal bones of the icefish *C. aceratus* had similar structure thickness to those of the non-icefish *N. coriiceps* (Figure 9a, b). Structure thickness of the dorsal and ventral portions of the dentary was not statistically different among *C. aceratus*, *C. gunnari*, *N. coriiceps*, and *G. gibberifrons* (Figure 8c). The articulars of the icefishes *C. aceratus* and *C. gunnari* had significantly lower structure thickness than the non-icefish *G. gibberifrons* ($p < 0.01$ for each comparison, $F = 2.0$, df = 3), but structure thickness of the non-icefish *N. coriiceps* articular was statistically similar to both icefishes (Figure 9c). In summary, no consistent bone microstructure differences were apparent in icefish bones, compared to non-icefish notothenioids.

3.5 | Quantitation of micro-CT images revealed similar BMD between homologous bones of icefish versus non-icefish

Bone mineral density has been hypothesized specifically to be lower in Antarctic icefishes compared to their non-icefish relatives (Albertson et al., 2009; Devries & Eastman, 1978; Eastman, 1993b; Iwami, 1985). In contrast, the icefish bones studied here did not consistently have lower BMD than non-icefish bones. The ceratohyal and frontal of the icefish *C. aceratus* did not have lower BMD compared to the BMD of homologous bones in the non-icefish *N. coriiceps* (Figure 10a, b). BMD of the dorsal portion of the dentary was statistically lower in the icefish *C. aceratus* compared to the non-icefish *G. gibberifrons* ($p < 0.05$, $F = 2.0$, df = 3), but not compared to the non-icefish *N. coriiceps* (Figure 10c). Also, BMD of the dorsal dentary of the icefish *C. gunnari* was significantly higher than that of the other icefish tested, *C. aceratus* ($p < 0.05$, $F = 2.0$, df = 3), but was not lower than the dorsal dentary BMD in the non-icefish tested. BMD of the articular was statistically lower in the icefish *C. aceratus* compared to either *N. coriiceps* or *G. gibberifrons* ($p < 0.05$
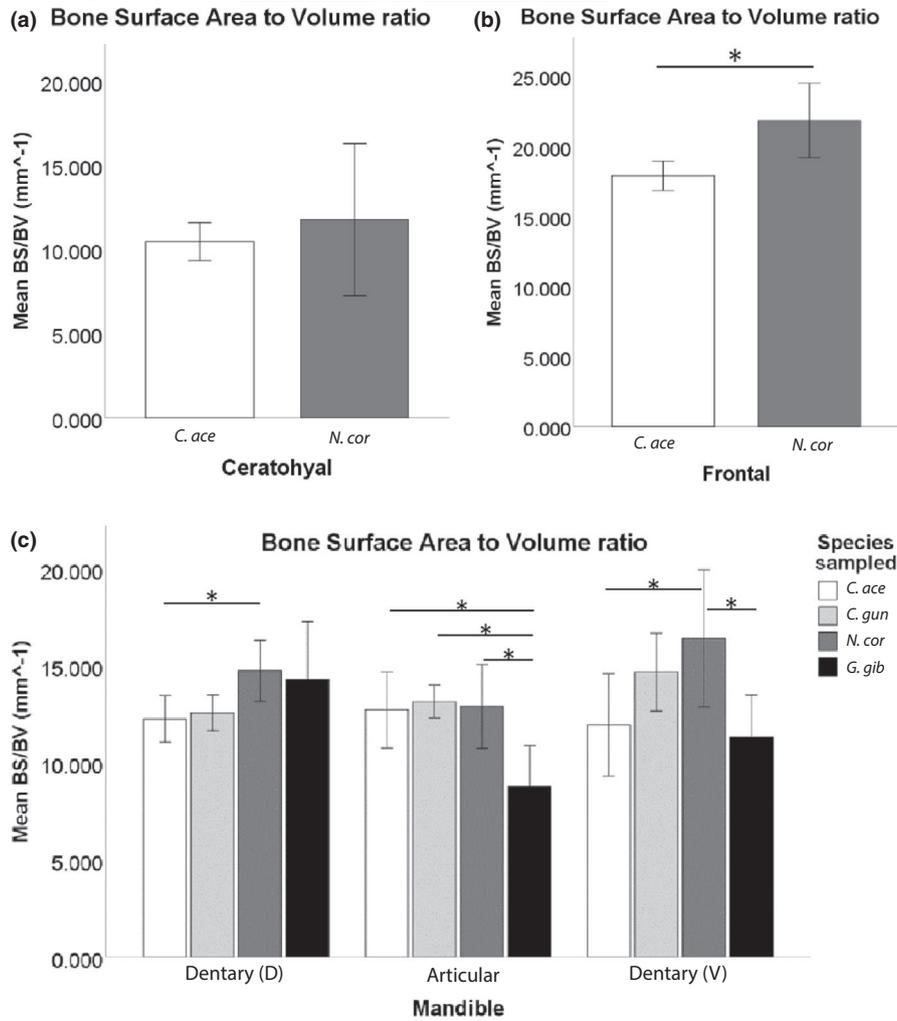
FIGURE 7 Icefish bones generally do not have altered bone microstructure (i.e., surface area-to-volume ratios) compared to homologous non-icefish bones. Bone surface area-to-volume ratio was calculated in ceratohyals of *C. aceratus* and *N. coriiceps* (a); frontals of *C. aceratus* and *N. coriiceps* (b); and mandibular bones (dorsal (D) and ventral (V) portions of the dentary and the articular) of *C. aceratus*, *C. gunnari*, *N. coriiceps*, and *G. gibberifrons* (c). *indicates statistically significant comparison ($p < 0.05$). Error bars are 95% confidence intervals

and $p < 0.01$, respectively, $F = 1.1$, df = 3), but BMD in the articular of the icefish *C. gunnari* was not statistically lower than the articular BMD of *N. coriiceps* or *G. gibberifrons* (Figure 10c). While the BMD of the ventral portion of the icefish *C. aceratus* dentary was significantly lower than the non-icefish *G. gibberifrons* ($p < 0.01$, $F = 1.4$, df = 3), it was not significantly lower than the non-icefish *N. coriiceps*, and the BMD of the homologous bone in the icefish *C. gunnari* was not statistically different from the non-icefish *N. coriiceps* and *G. gibberifrons* (Figure 10c).

4 | DISCUSSION

For decades, the skeletons of Antarctic icefishes (family Channichthyidae) were reported to have "reduced bone" compared to related non-icefish species (Devries & Eastman, 1978; Eastman & DeVries, 1981; Iwami, 1985; Voskoboinikova, 2001; Żabrowski, 2000). While this conclusion was supported by gross skeletal anatomy,

ash weight measurements of whole bodies, and limited Alizarin red staining, other potential changes to icefish bones need clarification. One study of the spiny icefish *Chaenodraco wilsoni* demonstrated gross skeletal changes, such as expansion or retention of cartilage (instead of undergoing endochondral ossification) and reduction in size (or even absence) of whole bones (Żabrowski, 2000). Indeed, here we report similar gross anatomical data in *C. aceratus* (e.g., reduced complexity of frontal bone and unossified ethmoid). Regarding features of bone that might change at a smaller scale, three recent qualitative studies suggested a decrease in bone microstructure and BMD in Antarctic icefishes, compared to non-icefish Antarctic species (Daane et al., 2019; Eastman et al., 2014; Meunier et al., 2018). Because "reduced bone" in icefish is so widely reported, and some genes potentially underlying these changes have been implicated recently (Daane et al., 2019), we sought to quantitate for the first time the sub-anatomic features of icefish bones. Specifically, we tested the hypothesis that, compared to related Antarctic notothenioids, bones of Antarctic icefish had altered bone microstructure and BMD.
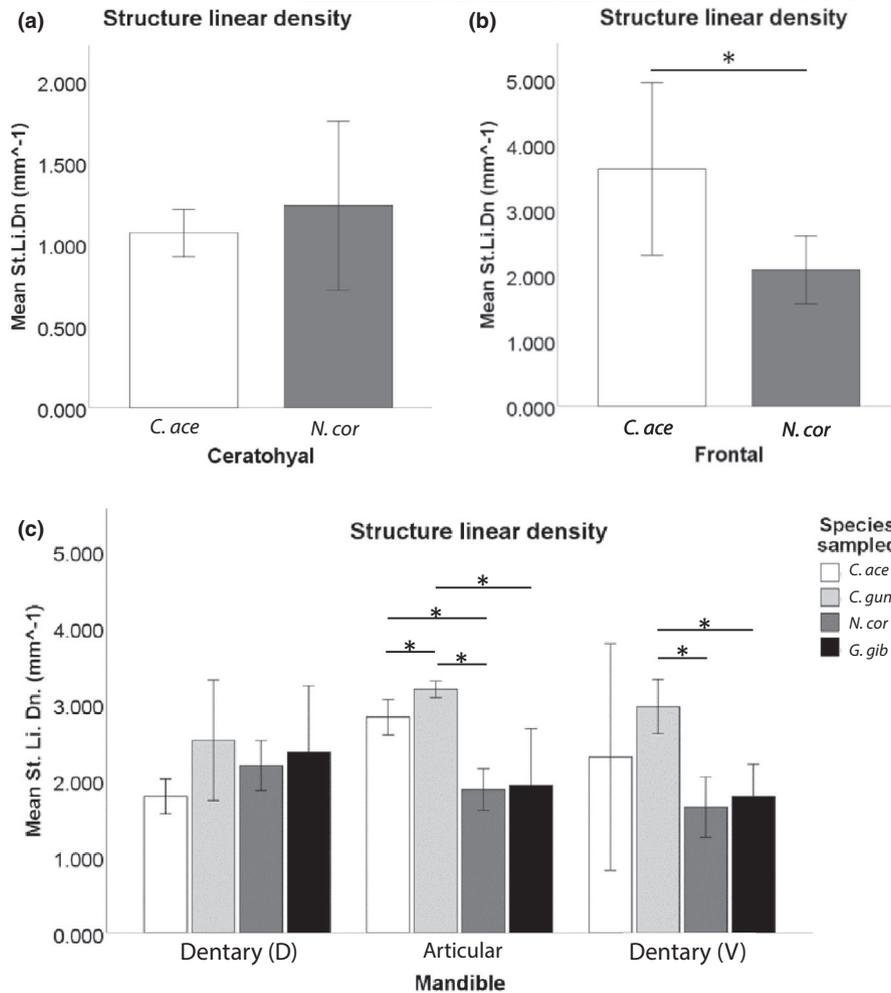
FIGURE 8 Icefish bones generally do not have altered bone microstructure (i.e., structure linear density) compared to homologous non-icefish bones. Structure linear density was calculated in ceratohyals of *C. aceratus* and *N. coriiceps* (a); frontals of *C. aceratus* and *N. coriiceps* (b); and mandibular bones (dorsal (D) and ventral (V) portions of the dentary and the articular) of *C. aceratus*, *C. gunnari*, *N. coriiceps*, and *G. gibberifrons* (C). *indicates statistically significant comparison ($p < 0.05$). Error bars are 95% confidence intervals

Despite a few pieces of supporting evidence, our data demonstrated that bone microstructure was not systemically altered in icefishes, compared to non-icefish Antarctic notothenioids. Histological sections of the icefish *C. aceratus* dentary seemed to have fewer thin bony rods than the dentary of the non-icefish *N. coriiceps*. Also, micro-CT images of some icefish bones visually suggested a decrease in bony rods. For the first time, however, micro-CT data allowed quantitative, three-dimensional analyses of bone microstructure across several craniofacial bones from two icefish and two non-icefish species. Some quantitative measures of individual bones from certain icefish species demonstrated altered bone microstructure, but most measures of bone microstructure were not consistently different in icefishes across all bones and species analyzed, suggesting that the histological and micro-CT slice data were not necessarily representative of the larger sample analyzed. For example, all measurements of the frontal trended towards altered bone microstructure in *C. aceratus* compared to *N. coriiceps*, although not all measures were significantly different. Interestingly, the *C. aceratus* frontal also

had major changes to its gross anatomy, so perhaps altered bone microstructure is more common in icefish bones with major anatomical changes. Structure linear density was significantly higher in the articular of both icefishes (*C. aceratus* and *C. gunnari*) compared to non-icefish notothenioids (*N. coriiceps* and *G. gibberifrons*), but other measures of bone microstructure in the articular did not show a consistent pattern of changes between icefishes and non-icefishes. Generally, most analyses did not support the hypothesis that icefish bones have altered bone microstructure compared to closely related notothenioids. As such, these data do not support the hypothesis that icefishes might serve as an evolutionary model for osteoporosis (Albertson et al., 2009; Beck et al., 2021; Kawalilak et al., 2014).

Technical limitations might explain the absence of consistently altered bone microstructure in icefish bones compared to non-icefish bones from our quantitative, 3D analyses. Perhaps additional biological replicates would have given more significant results, especially considering the possibility that the multivariate statistics employed might produce false negatives. In most cases, however, quantitative
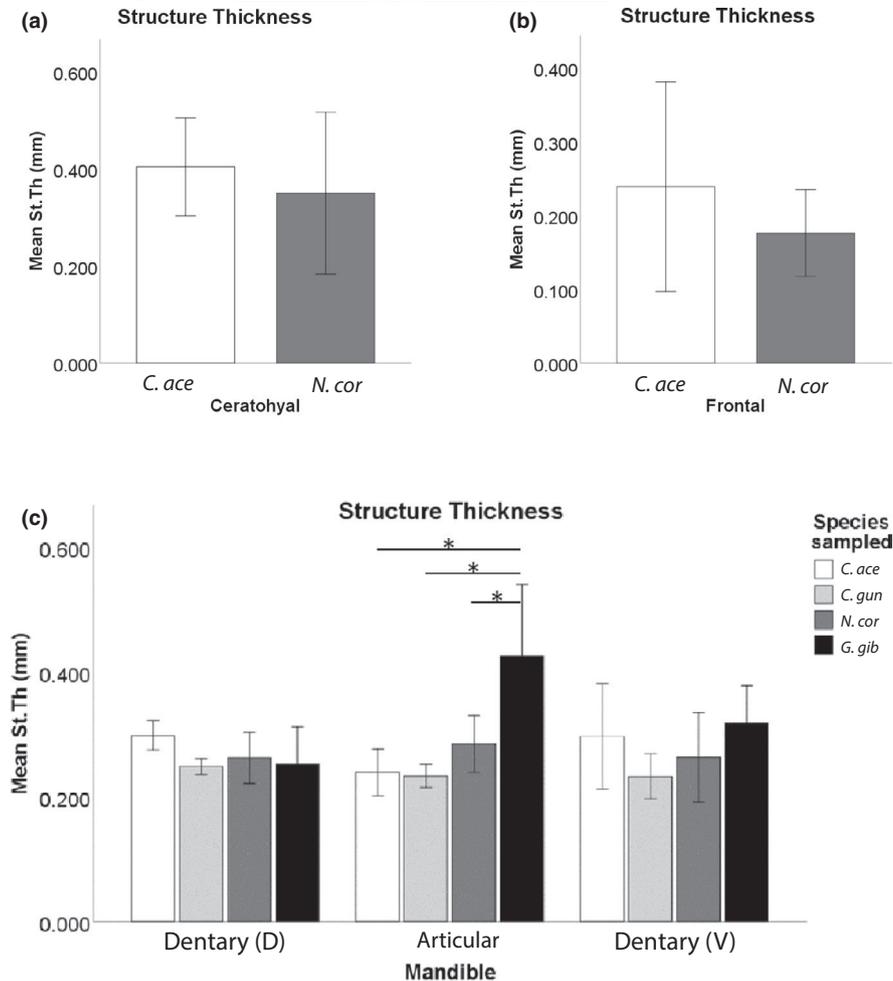
FIGURE 9 Icefish bones generally do not have altered bone microstructure (i.e., structure thickness) compared to homologous non-icefish bones. Structure thickness was calculated in ceratohyals of *C. aceratus* and *N. coriiceps* (a); frontals of *C. aceratus* and *N. coriiceps* (b); and mandibular bones (dorsal (D) and ventral (V) portions of the dentary and the articular) of *C. aceratus*, *C. gunnari*, *N. coriiceps*, and *G. gibberifrons* (C). *indicates statistically significant comparison ($p < 0.05$). Error bars are 95% confidence intervals

comparisons did not trend consistently in a direction supporting the hypothesis of altered bone microstructure in icefishes. Although the micro-CT scan settings were at the highest resolution for this desktop model, in principle scanning at higher resolution might reveal more subtle differences in bone microstructure. Also, given inhomogeneous microstructure along a given bone, comparisons might have been skewed if regions of each bone analyzed were not exactly homologous, despite efforts to use anatomical landmarks in our sample dissections and micro-CT VOI definitions. Finally, the microstructural parameters analyzed here (e.g., BV/TV or St. Li. Dn) simply might not capture what the human eye appears to detect qualitatively, although the human act of visual perception has been shown to find patterns that do not exist, defined as apophenia (Wickham et al., 2010).

In addition to these potential technical limitations, many biological factors that influence bone microstructure might not segregate cleanly between the Antarctic icefishes and non-icefish notothenioids sampled here. For example, pelagic and benthic lifestyles might impart different selective pressures on the bony skeleton.

Although all four species studied here occupy demersal habitats, the icefish *C. gunnari* has a low percent buoyancy (2.90 ± 0.23%) and is considered benthopelagic, while the icefish *C. aceratus* and non-icefish notothenioids *N. coriiceps* and *G. gibberifrons* have relatively higher percent buoyancies (3.19 ± 0.52%, 4.34 ± 0.26%, and 4.54 ± 0.36%, respectively) and are considered benthic (Eastman, 2020). Perhaps sampling of bones from different icefish and non-icefish notothenioid species with more contrasting percent buoyancies and habitats would produce different results. Also, bone microstructure changes might have been specific to the external (cortical) surface of bones or to the internal (more trabecular-like) surfaces. Alternatively, any changes might have simultaneously increased in one domain and decreased in the other domain. In these cases, our measurements might not accurately detect changes, because we did not separate these domains in our analyses. However, no specific differences were apparent in external versus internal features of the bones sampled. Finally, different bones are under different functional constraints, and some bones may be able to evolve more than others if one of these functional constraints is
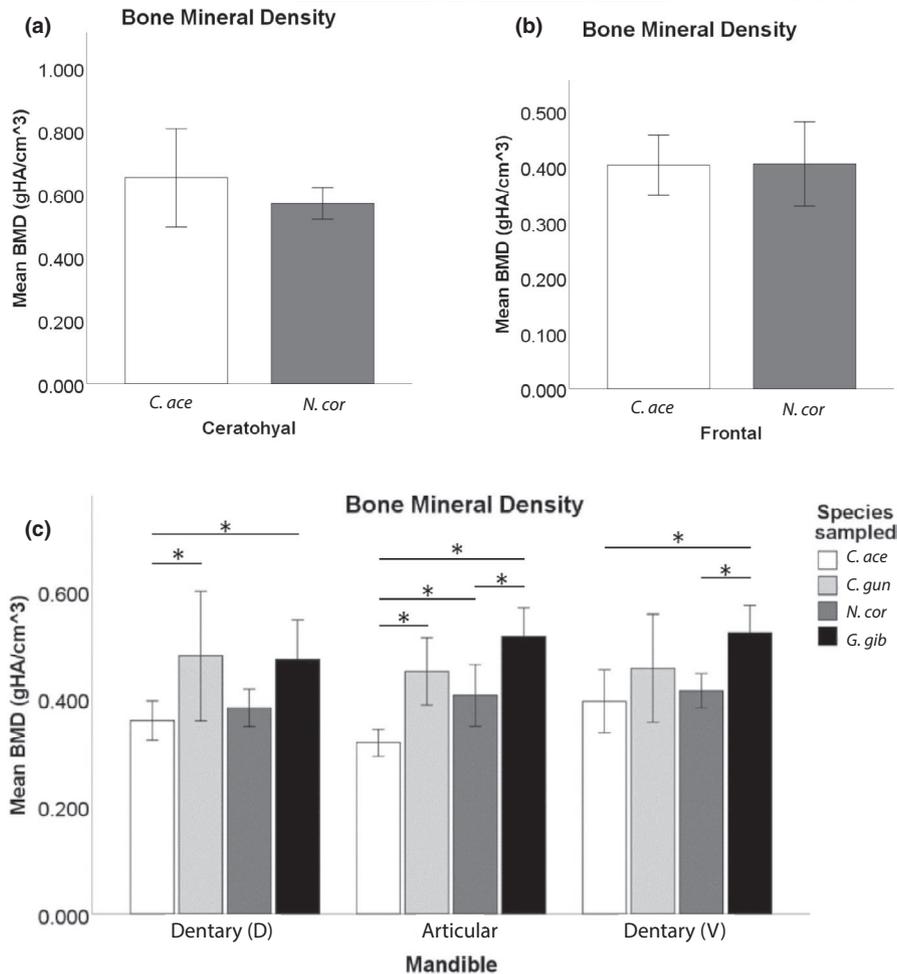
FIGURE 10 Icefish bones have similar bone mineral density as homologous non-icefish bones. Bone mineral density was calculated in ceratohyals of *C. aceratus* and *N. coriiceps* (a); frontals of *C. aceratus* and *N. coriiceps* (b); and mandibular bones (dorsal (D) and ventral (V) portions of the dentary and the articular) of *C. aceratus*, *C. gunnari*, *N. coriiceps*, and *G. gibberifrons* (C). *indicates statistically significant comparison ($p < 0.05$). Error bars are 95% confidence intervals

reduced. Our analyses included bones that presumably function differently, though, and our data were best distinguished by individual bones, not by species. For example, the dentary functions in prey capture and mastication, so varying diets among fishes (e.g., soft-bodied fish and krill, or hard-shelled benthic invertebrates) might confound the data comparing dentaries. In all four studied species here, however, prey capture mostly involves catching and holding the prey with the jaws and swallowing it whole. In another example, the frontal protects the braincase. Perhaps bone microstructure differences in the *C. aceratus* frontal reflects relaxed functional constraints in the sluggish lifestyle of icefishes compared to the more active behavior of the non-icefish *N. coriiceps*. Further quantitative research on additional bones, such as vertebrae or fin bones, of additional Antarctic icefishes and related clades is needed to clarify how modifications to bone microstructure might have evolved selectively during this natural history experiment.

Perhaps surprisingly, given previous qualitative reports, our histological and micro-CT data demonstrated no consistent decrease in BMD in icefishes, compared to BMD in non-icefish Antarctic nototheniods. Percent ash weights of whole bodies generally are lower in icefishes compared to their relatives (Devries & Eastman, 1978; Eastman & DeVries, 1981), but these results might reflect changes to gross skeletal anatomy, not necessarily BMD. Indeed, the absence of ventral projections from the supraorbital canals of frontal bones in *C. aceratus*, described here, adds to the long list of anatomical changes in the icefish neurocranium (Iwami, 1985). Previous imaging studies that visually suggested lower BMD in icefishes were carried out on whole bodies or whole heads, which can confound BMD quantitation, and the imaging data were not calibrated with mineral density standards (Daane et al., 2019; Eastman et al., 2014). Our histological analyses did not suggest that BMD differed between icefish and non-icefish bones. Alizarin red staining was not typically more intense in non-icefish bones, compared to icefish bones. Also, no clear differences in the area or intensity of acid fuchsin, which reflects mineralization of skeletal tissues in Milligan's Trichrome protocol (Atake et al., 2019; Egerbacher et al., 2006), were seen in sections of *C. aceratus* dentary, compared to *N. coriiceps*. Despite many suggestions that icefish bones have lower BMD, our

quantitative micro-CT data demonstrated clearly that icefish bones have similar BMD as homologous bones of related Antarctic clades.

As a point of reference, the BMD of the Antarctic icefishes and related notothenioids studied here collectively were much lower than the BMD of many other vertebrates. Despite different selective pressures on the skeleton in land and sea, BMD in both mammals and bony fishes range from 0.8 to 1.3 g of hydroxyapatite per cm^3 (gHA/cm^3), with some bony fishes near the lower end of that range (Atkins et al., 2014; Boivin & Meunier, 2002; Cohen et al., 2012; Nuzzo et al., 2002). The fact that mineral density of mineralized (non-dental) tissues in cartilaginous fishes, such as sharks and skates, also occurs in this range (Atake et al., 2019; Peignoux-Deville et al., 1982) suggests that a tissue mineral density of 0.8–1.3 gHA/cm^3 is a conserved trait among vertebrates. While the BMD of *C. aceratus* and *N. coriiceps* frontals were 0.6–0.7 gHA/cm^3, the BMDs of the other bones sampled were 0.3–0.5 gHA/cm^3 in both icefishes and non-icefish notothenioids. These data argue strongly that the common ancestor to these four species already had a reduction in BMD. To elucidate exactly when evolutionary modifications of the highly conserved vertebrate trait of BMD occurred, quantitative comparisons among other notothenioids and related groups are needed. Indeed, recent micro-CT or CT imaging suggested that a substantial reduction in BMD had already occurred in the ancestor of Eleginopsioidea before adaptations to constant cold waters (Daane et al., 2019; Eastman et al., 2014).

Changes to developmental timing, or heterochronies, have long been implicated in major evolutionary transitions (de Beer, 1958; Hall, 2003), but specifically linking heterochronic traits in vertebrates with their genetic underpinnings remains elusive. Reduced bone in Antarctic icefish skeletons might be a good model system, since it has been thought to occur through a developmental delay, termed paedomorphosis, when juvenile traits of an ancestor are retained in the adult of a descendant (de Beer, 1958; Voskoboinikova, 2001). Indeed, delayed skeletal development in icefish embryos was demonstrated clearly, compared to embryos of a related notothenioid (Albertson et al., 2010). Perhaps analyzing bones from additional life stages, such as juveniles, would reveal stronger evidence for bone microstructural changes than the adult analyses here. However, collectively our data argue that heterochronic shifts in skeletal development of icefishes are associated with gross anatomical changes, such as smaller bones or retention of cartilage, not smaller-scale changes to bone microstructure or BMD. Interestingly, genes related to bone formation, including *col1a1*, *col1a2*, and *trip11*, recently were identified to have undergone episodic diversifying selection on the branch leading to the icefish clade (i.e., in the last common ancestor of icefishes; Daane et al., 2019). Here, we have summarized quantitative features of bones in icefishes and related Antarctic clades, so combining these data with genomic resources might reveal the molecular mechanisms of heterochronic changes during skeletal evolution.

ORCID

Isaac V. Pratt https://orcid.org/0000-0001-7839-3922
David M. L. Cooper https://orcid.org/0000-0002-3175-9170
Thomas Desvignes https://orcid.org/0000-0001-5126-8785
John H. Postlethwait https://orcid.org/0000-0002-5476-2137
B. Frank Eames https://orcid.org/0000-0002-8200-3760

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.