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Utility of quantitative micro-computed tomographic analysis in zebrafish to define gene function during skeletogenesis

Julia F. Charles^{1,*}, Meera Sury^{2,3}, Kelly Tsang³, Katia Urso¹, Katrin Henke², Yue Huang², Ruby Russell³, Jeffrey Duryea³, and Matthew P. Harris^{2,*}

¹Department of Medicine, Brigham and Women's Hospital

²Department of Genetics, Harvard Medical School; Department of Orthopaedics, Boston Children's Hospital

³Department of Radiology, Brigham and Women's Hospital

Abstract

The zebrafish is a powerful experimental model to investigate the genetic and morphologic basis of vertebrate development. Analysis of skeletogenesis in this fish is challenging as a result of the small size of the developing and adult zebrafish. Many of the bones of small fishes such as the zebrafish and medaka are quite thin, precluding many standard assays of bone quality and morphometrics commonly used on bones of larger animals. Microcomputed tomography (microCT) is a common imaging technique used for detailed analysis of the skeleton of the zebrafish and determination of mutant phenotypes. However, the utility of this modality for analysis of the zebrafish skeleton, and the effect of inherent variation among individual zebrafish, including variables such as sex, age and strain, is not well understood. Given the increased use and accessibility of microCT, we set out to define the sensitivity of microCT methods in developing and adult zebrafish. We assessed skeletal shape and density measures in the developing vertebrae and parasphenoid of the skull base. We found most skeletal variables are tightly correlated to standard length, but that at later growth stages (>3 months) there are age dependent effects on some skeletal measures. Further we find modest strain but not sex differences in skeletal measures. These data suggest that the appropriate control for assessing mutant phenotypes should be age and strain matched, ideally a wild-type sibling. By analyzing two mutants exhibiting skeletal dysplasia, we show that microCT imaging can be a sensitive method to quantify distinct skeletal parameters of adults. Finally, as developing zebrafish skeletons remain difficult to resolve by radiographic means, we define a contrast agent specific for bone that enhances resolution at early stages, permitting detailed morphometric analysis of the forming skeleton. This increased capability for detection extends the use of this imaging modality to leverage the zebrafish model to understand the development causes of skeletal dysplasias.

*co-corresponding authors.

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Keywords

Zebrafish; microcomputed tomography; bone; development; phenotypic variation; bone matrix specific contrast

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1. Introduction

All vertebrates share the capacity to make bone. Teleost species, including the zebrafish, *Danio rerio*, have fundamental cellular mechanisms of bone formation and remodeling as seen in other vertebrates. The fish skeleton is metabolically active, undergoing osteoclast mediated resorption and remodeling throughout life ¹. At the cellular level, zebrafish bone contains the three major bone cell types: osteoclasts, osteoblasts and osteocytes. Although fish can use other sources besides the skeleton for calcium regulation, remodeling of the ossified skeleton is similarly required for growth and fracture healing ¹. Additionally remodeling of the skeleton is key for the correct shaping of fish bones during development. Zebrafish, medaka and other small laboratory fish models are complementary systems to rodent models for studies of skeletogenesis, with advantages including ease of genetic manipulation, visualization of bone cells in real time during development, the large number of progeny and relatively low cost of husbandry ^{2,3}.

1.1 Zebrafish as a model organism for the study of skeletal biology

The zebrafish, *Danio rerio*, is a common research model to investigate genetic regulation of vertebrate development and the mechanistic causes of human disease. Much of the informative power of the zebrafish stems from the identification and characterization of mutants with specific phenotypes and the subsequent identification of the specific genetic changes underlying the mutant phenotype. The majority of these studies have primarily centered on mechanisms of embryogenesis and formation of the early body plan. Studies in developing zebrafish have provided great insight into key genetic and cellular aspects of skeletogenesis ^{3,4}. Large genetic screens have been carried out in zebrafish to identify genes affecting formation and patterning of the larval skeleton ⁵⁻⁷. Through these screens, it has been found that many genes regulating skeletal formation are conserved with other vertebrates, and common skeletal pathologies can be observed both in fish and human ³. Recently, work has begun to explore the utility of this model to understand processes of late development. Building on initial forays into analysis of mutations affecting the adult skeleton ⁸, more systematic screens are in progress to expand analysis to the genetic regulation of later aspects of skeletal development and remodeling. Through such screens, zebrafish mutants resembling human bone disorders such as osteogenesis imperfecta ^{9,10}, Bruck syndrome ¹¹, and Raine syndrome ¹² have been identified. With the advent of efficient genome editing techniques, reverse genetic approaches, in which mutations in specific genes of interest are created, are becoming more frequent ¹³ including in zebrafish (*e.g.* ¹⁴). The ease of engineering specific mutations in the zebrafish is likely to bolster the systematic use this model in evaluating the relevance of genetic variants identified in patients and of candidate genes identified through genome wide association studies.¹⁵⁻¹⁷

Histological and transgenic tools to examine skeletal tissues during embryogenesis in zebrafish are well developed. These can also be applied to analyses of post-embryonic developmental processes³. However, the use of zebrafish and other small laboratory fishes to investigate skeletogenesis and skeletal dysplasias has been limited by the lack of systematic analytical techniques to assess bone quality and shape, especially in late developmental stages. Thus the majority of genetic analyses in the zebrafish report effects on the adult skeleton based on gross morphological phenotypes, without substantial quantitation of bone shape, volume or mineral density. It remains unclear what normal variation is present within different genetic backgrounds and how the presented mutant phenotypes compare with normal variation. These reports point to an overall deficiency in our knowledge of the control of skeletogenesis in zebrafish and utility of modeling disease conditions.

1.2 Micro-computed tomography for the assessment of zebrafish skeletal phenotypes

MicroCT acquires a series of 2 dimensional x-ray projections over more than 180 degrees of rotation of an object. Individual projections are reconstructed to produce a 3D tomographic image. Through this imaging, accurate quantitative analysis of mineralized tissue can be performed to assess size, thickness, structure, mineralization and overall quantity of bone. This imaging modality is used extensively in skeletal research to characterize the rodent skeleton and standardized parameters and published parameters are available to the bone community¹⁸. Because of its small size, many of the bones of the developing and mature adult zebrafish skeleton are quite thin, and previous microCT analysis tools were not sufficient to accurately characterize these bones. However, the advent of a new generation of software, increases in beam strength, and detector sensitivity have now made detection feasible in most laboratory facilities.

Adult zebrafish have an average length of 2.5–3cm in length. This small size makes microCT scanning of whole adult fish possible on standard table top instruments, though at relatively low resolution. High-resolution images of specific regions of interest (ROI) can be obtained with a fraction of the time and memory requirements compared to whole fish scans, allowing for local high precision measurements in the zebrafish of bone size, shape and density. Although microCT methods are gaining popularity in the analysis of adult zebrafish¹⁹, there is often little quantitative analysis of these data. Rather, microCT is commonly used as an imaging substitute for whole mount Alizarin red staining (e.g.^{2,20}). In cases where quantification is presented, the intrinsic variation of these quantitative measurements in the zebrafish is unclear (e.g.^{10, 21}). However, it is unclear what variation exists within and between strains, different sexes, and different aged adults that may affect conclusions based on these measures. These values are essential to adequately define the experimental use of microCT to study the zebrafish skeleton, especially in cases of altered gene function and pathology.

We set out to develop a standardized microCT analysis method to reproducibly quantify specific vertebral elements in order to support an analysis of skeletogenesis in the adult zebrafish. Here, we examine the utility and reproducibility of microCT analysis in the mature zebrafish and examine intrinsic biological variation of vertebral measures caused by

differences in sex, age, standard length, and laboratory strain. Further, we demonstrate that quantification of skeletal elements by microCT is a sensitive method to detect changes in shape and remodeling in two existing genetic models of skeletal dysplasia, showing the versatility of this methodology to assess phenotypic variation due to mutation. Additionally, to facilitate microCT analysis of earlier developmental stages in zebrafish, we define a novel contrast methodology that permits sensitized analysis of mineralization in zebrafish during development. This method overcomes imaging limitation of younger specimens and expands the utility of zebrafish as a genetic model to understand development and remodeling of the skeleton.

2. Methods

2.1 Fish Husbandry

Zebrafish (*Danio rerio*) were raised under standard conditions²². The *bmp1a/wel*^{31169 23} and *csfr1a^{mh5}* (Caetano-Lopes J, *in review*), loss of function mutants in *bone morphogenetic protein 1a* and *colony stimulating factor receptor 1a*, were identified in ENU mutagenesis screens based on abnormal adult morphology. For microCT analysis, fish of different strains and mutant genotypes were fixed in 3.7% formaldehyde in PBS for 24 hours at the indicated ages, and sex and standard length (length from tip of nose to beginning of the caudal fin) were determined. Fish were subsequently stored in 70% ethanol until scanning.

2.2 Micro-computed Tomography

Samples were immobilized with foam wrapping in a 11mm diameter sample tube and scanned in 70% ethanol using a μ CT 35 (Scanco Medical). For detailed measurement analyses, fish were imaged at an isotropic voxel size of 6 μ m using an X-ray tube potential of 55 kVp, an 0.5mm aluminum filter, a X-ray intensity of 0.145 mA and an integration time of 600 ms per slice for vertebrae and 800ms for scans of parasphenoid bones.

For parasphenoid bone analysis, a semi-automated contouring approach was used to outline a portion of the parasphenoid bone delineated by easily identifiable anatomic features. This region of interest (ROI) was thresholded using a manually determined global threshold that set the bone/non-bone cut-off at 452.7 mgHA/cm³. BMD and BV/TV (bone volume/tissue volume within the contoured region of interest) were calculated using software supplied by the manufacturer. BMD measurement on the μ CT 35 was calibrated to phantoms of known hydroxyapatite density by the manufacturer and quality control against external phantoms was performed weekly. For vertebral analysis, custom software was used to determine the 3-D microstructural features of C1 and C2 vertebrae in a semi-automated fashion. Three points were manually identified on both the anterior and posterior edges of the vertebrae, centrum, as shown for the anterior edge in Figure 1C. For each set of three points, a unique circle was defined that passed through each of the three points. The radius of the vertebral opening was defined as the average radius of the two circles and the length as the distance between the centers of the circles. The width of the neural arch was defined as the average distance between opposite sides of the structure as shown by the red lines in Figure C; the height of the arch was defined as the distance between the center of the vertebral opening and the

joining point of the neural arches. Arch area was calculated as the product of width and height. Bone density of the centra was calculated as the average gray scale values in a volume of interest normalized to units of mgHA/mm³ using a calibration phantom. Volume rendered images of vertebral bodies were generated using custom software (Figure 1) or Amira software package, version 6.0 (FEI) (Figures 5 and 6).

Images of silver nitrate contrast stained samples were obtained using a Skyscan 1173 microCT machine (Bruker). Fish were imaged at an isotropic voxel size of 7.14µm with an X-ray source voltage of 70 kV, 80 uA current and exposure time of 1500 ms. Fish were imaged over 240 degrees, using a 0.2 degree rotational step and images reconstructed with software supplied by the manufacturer. Volume rendered images pre-and post-contrast stained samples were generated using Amira software package, version 6.0 (FEI). For each image, the range of linear attenuation coefficient values displayed was adjusted using Amira's "colormap" function to optimize signal to noise from soft tissue structures, thus maximizing detection of mineralizing structures.

2.3 Statistical Analyses

Intraclass correlation co-efficients (ICC) were calculated with the 'two way random mode' using the R package *psych*. Further statistical analyses were performed with GraphPad Prism version 6. Pearson correlation, Kruskal-Wallis test with Dunn's post-test for multiple comparisons, or Mann-Whitney U test were used as indicated in the figure legends.

2.4 Whole mount silver nitrate contrast staining for mineralized matrix in zebrafish

Zebrafish at different time points during juvenile development were fixed in formaldehyde and stored in 70% ethanol. Samples at known standard length were rehydrated into dH₂O and pigmentation was bleached with 6% H₂O₂ for 15 minutes. Samples were washed 3 times in dH₂O and placed into Silver nitrate solution (AgNO₃) at the indicated concentration. For the majority of work, 1% AgNO₃ was used as the working concentration. Staining was performed in multi-well cell culture dishes on top of a standard gel lightbox for 45 minutes to an hour; absolute time was judged by the extent of staining in fin rays and the level of background that arose. To stop staining, samples were rinsed in dH₂O at least 3 times and stored at 4°C until scanned. Prior to scanning, the eyes were removed as these showed retention of AgNO₃ and increased the background signal in scans.

3. Results

Primarily using a microCT-35 (Scanco Medical), we scanned over 200 individual adult fish of different strains, genotypes, sexes, and ages to understand the intrinsic variation within skeletal elements and the sensitivity of microCT to detect and quantify changes in these parameters. We focused our analysis on two separate anatomical regions: 1) the first and second caudal vertebrae (C1 and C2, Figure 1A), as these are often centers of deformation in cases of spinal curvature in fishes and 2) the parasphenoid bone of the cranium, as the formation and extension of this bone during development is correlated with skull proportion (PS, Figure 1B). These bones represent different skeletogenic tissues, having both endochondral (parasphenoid) and intramembraneous bone ossification (vertebrae). While

vertebra centra do not contain trabecular bone, the interior of the centra does undergo remodeling. Both element types additionally grow by accretionary growth.

Individual fish were fixed and maintained in 3.7% formaldehyde to minimize shrinkage due to fixation and artifacts of shape change that can occur with alcohol based fixatives²⁴. ROI including the first and second caudal vertebrae (C1 and C2, respectively, Figure 1A) or the parasphenoid bone (Figure 1B) were scanned at an isotropic voxel size of 6 μm . We measured length, radius, volume and bone mineral density (BMD) of the vertebral centra, as well as angle and area of the neural arch as diagrammed in Figure 1C using custom software. The rostral half of the parasphenoid bone (dotted line in Figure 1B) was analyzed for bone volume relative to tissue volume (BV/TV) and bone mineral density using software supplied by Scanco.

3.1 Reproducibility of measures

One source of error influencing the precision of microCT methods can arise from error induced in processing and selection of ROI by individual observers. To address the precision and reproducibility of our quantification methods, scans were analyzed by two independent observers to determine intra- and inter-observer variance of measurements. Additionally, to assess repositioning reproducibility, 12 individual fish were scanned and analyzed twice with the same microCT instrument, with the second evaluation of the samples being performed 2 months after the initial analysis.

Intra- and inter-observer measurements made on the parasphenoid (Figure 1D) and vertebrae centra (Figure 1E) are highly reproducible. In contrast, vertebral arch measures exhibited substantial variance between observers (Figure 1F), which likely reflects the subjectivity in assigning landmarks used by the automated imaging processing program to calculate arch angle and area. Similarly, repositioning between scans introduces variability in arch measures (Figure 1F). This likely reflects differences in rendering of the terminal components of the arch in different scans, affecting the placement of landmarks on the reconstruction. The confidence interval for variability in BMD after repositioning was wider than for other measures (Figure 1D, 1E). This may reflect the fact that morphometric and gray scale measurements have different inherent variation. Additionally, small variations in the material used immobilize fish for scanning could contribute to between-scan variability by increasing the x-ray filtration creating subtle beam hardening artifacts. The Pearson correlation coefficients for all comparisons are listed in Supplemental Table 1. A comparison of C1 and C2 vertebrae showed that skeletal measurements of these vertebrae are highly correlated (Supplemental Table 2). Thus, for subsequent figures we report data from C2 for simplicity. Taken together, our reproducibility analysis suggests that measures of the vertebrae centrum and parasphenoid are robust across observer and scan position. While vertebral arch measures are more variable, it remained unclear if this precluded sensitivity of this metric when performed by a single observer (see below).

3.2 Correlation of skeletal element measurements with standard length

Zebrafish attain reproductive maturity at ages between 2.5 and 3 months. Their rate of development can vary depending on many environmental and genetic variables. Even within

a tank, variation in developmental rate among sibling fish is obvious. Previous staging of post-embryonic development of the zebrafish has established that analysis of the standard length (STL) of a fish is one of the best measures of its developmental stage²⁵. It remains unclear if this standard extends to characteristics manifesting beyond reproductive maturity.

Analysis of over 90 wild type zebrafish, ages 3 to 10 months of mixed sex and strain backgrounds, shows strong positive correlation of skeletal element measurements with STL (Figure 2). The angle of the neural arch of the vertebrae is an exception to this general correlation with angle showing limited change in differently sized fish. Thus similar to previous standard tables of post-embryonic development in the zebrafish²⁵, we normalized primary measurement data for STL for all measures except neural arch angle.

3.3 Influences of sex on skeletal metrics in adult zebrafish

Sex has substantial effects on skeletal development in mammals. Mice show significant sex variability in bone mass, for example¹⁸, thus, measures to address the effect of altered gene function in mouse skeletal development must be controlled for sex of the experimental populations. Previous analyses of skeletal parameters in other model fishes such as the medaka have also shown sex-specific differences in skeletogenesis²⁶. Zebrafish have adult sex-specific phenotypes recognizable in size and coloration. We thus asked if zebrafish also exhibit sex-specific variation in skeletogenesis that would necessitate control of this variable in analyses of skeletal traits. Comparison of measurements derived from wild type male and female fish at 3, 5, 9, and 10 months of age did not reveal significant systematic sex differences. A representative analysis of parasphenoid and vertebral skeletal parameters of 5 month-old male and females without significant sex difference is shown (Figure 3). As no systematic differences in skeletal metrics due to sex of the individual were seen in adult zebrafish, in further analyses we pooled measurements derived from male and female fish.

3.4 Age-related alteration in bone formation during maturation of adult zebrafish

Zebrafish continue to grow in size during adulthood, although the rate of growth tapers as they reach reproductive maturity. Our analysis shows that longitudinal and radial growth in vertebrae can be attributed to isometric increase in size and can be normalized by STL (Figure 4A, 4B). However, STL normalized vertebral volume, BMD, and parasphenoid BMD increase significantly as zebrafish mature beyond 3 months of age (Figure 4C–E). We repeated these analyses on age-related variation in a second wildtype strain (WIK) and observed similar trends (Figure 4F–H). This suggests that, at least for vertebral volume and BMD, the use of age-matched controls is critical for detecting gene or treatment effects on skeletal parameters, even after normalizing for STL. Although we did not extensively compare skeletal measurements between different laboratory strains, 5 month-old AB and WIK strain fish differ significantly in BMD. This suggests that differences likely exist between strains and the most conservative approach would be to compare sibling experimental groups to best control for strain background.

3.5 Use of microCT to characterize the genetic regulation of zebrafish skeletogenesis

We chose two genetic models, loss-of-function mutants of *bmp1a* and *csf1ra*, to test the sensitivity of our microCT method for detecting changes in bone size, shape and

mineralization in adult zebrafish, Loss-of-function mutations in *bone morphogenetic protein 1 (bmp1)* cause autosomal recessive osteogenesis imperfecta²⁷ whereas *Csfr1* is essential for osteoclastogenesis in mammals, and defects in its signaling can cause osteopetrosis²⁸. Adult fish of *bmp1a* and *csfr1a* mutants were compared to wildtype siblings from each clutch at 6 months of age (Figure 5 and 6; Supplementary Figures 1 and 2, respectively).

bmp1a mutants show obvious defects in skeletogenesis and can be shorter than wildtype siblings²³, and their vertebral morphology is grossly abnormal (Figure 5A–D). Taking into account this phenotype, we considered the possibility that STL normalization was not appropriate for the analysis of changes in proportion of skeletal elements in the adult. We calculated the ratio of the vertebral length to radius (Figure 5D) as an indicator of vertebral deformity. Our analysis showed a significant difference in this ratio between *bmp1a* mutants and wild type siblings. As an alternative strategy for normalization, we use the diameter of the eye as a standardized measure as the relative proportion of the orbit remained unaffected. Confirming previous analysis¹⁰, altered Bmp1a function led to an increase in BMD of both skeletal structures (Figure 5F). Vertebrae from *bmp1a* mutants were found to have increased volume and radius but shorter length of C2 (Figure 5E–H). Corresponding measures of neural arch metrics showed a shallower angle with a measurable increase in area of the arch (Figure 5I, 5J). An increase in bone volume and BMD were also observed for the parasphenoid (Supplemental Figure 1). Although most metrics, apart from neural arch angle, were normalized for orbit size to take into consideration changes in proportion in the mutant, similar differences were detected normalizing with STL (Supplemental Figure 2).

Csfr1a mutants represented the other extreme that the obvious deficiencies in *bmp1a* as no obvious phenotype could be detected by visual inspection (Figure 6A–D). As *csfr1a* mutant fish did not show significant changes in proportion of the vertebrae (Figure 6D), all data for this mutant were normalized by STL. We found that *csfr1a* mutants exhibited higher vertebral volume (Figure 5E) without alterations in length, radius or BMD (Figure 5F–H). Distinctive alterations in the angle and area of the neural arch were also observed (Figure 6I and J). Although not immediately observable, these changes can be seen in volume renderings that match quantitative measures (Figure 6A and B). No differences were seen in the parasphenoid bone (Supplemental Figure 3). Thus, microCT analyses are sensitive to identify changes in bone density and shape in adult zebrafish models of known skeletal dysplasias with varied strength of effect.

3.6 Enhancing sensitivity for detection of hypomineralized bone during zebrafish development

Despite a fully patterned skeleton, skeletal traits in younger samples are difficult to image by microCT as the degree of mineralization is insufficient for detection. We investigated methods to enhance the contrast between mineralizing matrix and soft tissue in the developing and adult zebrafish while retaining methodological ease of analysis.

Silver nitrate specifically binds to the organic phosphate matrix of bone. As silver has a high atomic number (Z=47) it is expected to attenuate the x-ray beam and increase detection sensitivity of matrix in samples. Using a simple whole mount protocol on whole fixed zebrafish, young juvenile samples (~10mm STL) treated with silver nitrate solutions (0.1–

1%) showed specific labeling of forming bones throughout the developing and adult zebrafish (Figure 7A). Silver nitrate affects deep tissues as well as those peripheral, penetrating to stain vertebrae. Analysis of silver nitrate stained specimens did not show significant labeling of non-skeletal structures, except for sparse staining of the retina. Importantly, a comparison of young (10mm STL, approx 30dpf) juveniles stained with silver nitrate or alizarin red showed comparable patterns of staining between methods suggesting similar sensitivity of silver nitrate in whole mount preparations (Figure 7B–C). Unlike the amorphous staining of calcium phosphate by alizarin red, silver nitrate showed stellate staining pattern across bone-forming fronts in the dermatocranium (Figure 7B'–C').

We tested the utility of silver nitrate contrast for detection of skeletal structures by microCT during juvenile development of the zebrafish. From staggered developmental stages starting at 13mm STL, a single wild type individual was scanned at a nominal resolution of 7µm prior to, and again just after staining with silver nitrate. As the skull encompasses a large number of bones, some quite thin, skull imaging provides a good test of utility of this staining method. Skeletal elements of young juvenile zebrafish skull at 13mm STL were limited and difficult to resolve by microCT. However, when stained with silver nitrate, the extent of bone growth can easily be visualized in these same individuals (Figure 7D). Contrast staining with silver nitrate allowed for detection of early ossification centers and increased resolution of bone boundaries within the skull, enabling tracking of particular skeletal elements over development. Silver nitrate staining of older fish also enhanced detection and quality of the resolution of skeletal edges. Scans of silver nitrate treated skulls are available as a broader resource dataset on Facebase.org.

4. Discussion

The zebrafish is gaining popularity as an experimental genetic model to understand gene function in skeletal development. The use of zebrafish and other laboratory fish models to study aspects of skeletogenesis and mineralization has been both advanced, but also hampered by their small size and numerous thin bones. Zebrafish is useful for analysis of transgenes and gene expression *in vivo*, but their skeletal structures remain at the lower end of detection by common radiographic techniques used in analyses of mammalian bone. This is especially evident in analyses of developmental stages prior to formation of the reproductively mature adult (approximately 3 months or less than 17mm STL). In larger species, microCT has proven to be a powerful methodology for analysis of complex structures, including the skeleton or vascular system²⁹. These measures are important to understand gene function in the etiology of skeletal dysplasias.

High-resolution, and detailed scans of zebrafish and medaka have been accomplished through use of synchrotron radiation micro-computed tomography (SR microCT^{30,31}, <http://zfAtlas.psu.edu/>). However, limited access and cost for such beam sources makes this method impractical, especially when considering the large number of samples needed for phenotypic analysis and analysis of variation. Use of conventional table-top microCT machines overcomes these limitations, with loss of resolution compared to SR microCT methods. With increasing access, the use of microCT has become more commonplace in zebrafish studies. However, it remains unclear how the precision of the method compares to

intrinsic variance between different stages and sex of zebrafish as well as between strains. Efforts to define these variables are crucial as they will influence ability to accurately define phenotypes being compared. To extend the utility and validate the use of microCT in zebrafish, we performed a systematic analysis of the robustness of the experimental methodology both in variation arising as a component of the analysis as well as intrinsic variation with the fish.

The majority of skeletal features showed excellent correlation for both intra- and inter-observer repeated measures. Correlation was weaker for neural arch metrics, presumably due to the manual and subjective nature of placing landmarks used by the algorithm to calculate arch angle and area. Sample repositioning was assessed by analysis of independent scans obtained on the same fish 2 months apart. For the majority of features, measurements are highly consistent between scans.

We then addressed variation in vertebral and parasphenoid measures arising from variables inherent to the zebrafish, including age, sex and genetic background. Similar to previous staging of post-embryonic development, we find that all metrics used to define skeletal elements, apart from neural arch angle, showed strong scaling with STL. Thus, intra-clutch variability caused by tank effects and population density can be normalized simply for length of the fish. SL normalization also allows between-clutch comparisons as long as fish are of similar background strain and age.

Mutations in *bmp1a* in zebrafish cause skeletal phenotypes that model the pathologies observed in human patients, with disorganized bone albeit higher bone density¹⁰. We previously identified ENU generated loss-of-function mutations within *bmp1a* (R227X) in zebrafish²³. Although this mutation causes an obvious skeletal phenotype in fish, with reduced body length and distinctive cranial morphology, the quantitative effect of this mutation on bone mass, BMD and vertebral morphology remains undefined. Loss-of-function mutations in *csfr1a* in zebrafish lead to a distinct pigmentation phenotype³², but only a mild skeletal phenotype involving shape changes of the neural arches in 11 month-old fish³³. However, the observed changes were not quantified nor compared with normal variance, thus it is unclear if this observation depicts a significant change or if it is within the normal variance for zebrafish. We identified a new ENU induced allele of *csfr1a* leading to a loss-of-function³⁴. In contrast to *bmp1a*, this new mutant had little to no grossly obvious skeletal deficiencies. Thus, this mutant allowed us to assess sensitivity of our methods to identify subtle changes in skeletal phenotype through use of microCT imaging and analysis.

Analysis of mutants with skeletal deformities raised an interesting caveat to normalization by length. In mutants affecting skeletal growth, body proportion is frequently altered leading to a 'stumpy' phenotype. In these cases, standard length is no longer an independent measure of staging between fish. Alternative measures, such as orbital diameter, can potentially be used for normalization. Using these methods, we demonstrate that we can effectively detect changes in bone quality in a model of osteogenesis imperfecta and *csfr1a*-mediated osteoclast deficiency (Figures 5 and 6).

Unlike mice that demonstrate distinct sex differences in skeletal metrics¹⁸, skeletal measures in male and female zebrafish were indistinguishable. Interestingly, we observed changes in bone density as a function of age during what is generally thought of as the isometric phase of adult growth. Although skeletal size metrics are relative to overall size, density of bone increases during mature growth of the fish. In general, STL normalized skeletal measures were similar between strains examined (AB, WIK, mixed backgrounds). BMD was significantly different between strains at 5 months of age, however resolved thereafter.

Comparison of age-matched individuals remain important for the majority of measures. Given these findings, we would recommend the use of wildtype siblings as the control group when assessing magnitude of effect on the skeleton, rather than a comparison with data from unrelated wild-type individuals. However, normalization to STL of the fish can allow approximate comparisons of age-matched zebrafish across sex and between strains. This is useful, as comparisons can be made even between individuals that differ in size due to their raising conditions/density.

Previous microCT approaches using zebrafish were confined to well ossified adult stages. One of the strengths of zebrafish as an experimental model is the ability to detail changes through development. Contrast agents such as phosphotungstic acid and iodine have shown great utility in making even soft tissues detected and quantifiable to tomography³⁵. These methods however require substantial post-scanning processing *in silico* to segment tissues of interest as all tissues are labeled. Here, we define a new contrast method for whole-mount analysis that is specific to forming bone through its differential staining of phosphate-related bone matrix. Importantly, as the stain is specific to areas undergoing mineralization, segmentation of rendered data sets to isolate skeletal structures is not necessary as is the case with other commonly used contrast agents.

Contrast agents such as iodine or phosphotungstic acid are used to differentially increase beam attenuation of tissues within the sample making soft tissues more electron dense^{35–37}. The majority of contrast-stained techniques, however, require substantial *in silico* segmentation of the rendered data by differential density to identify and analyze tissues of interest^{35–37}. The whole mount staining technique using silver nitrate allows for specific differential staining of bone matrix during development and permits rapid and specific analysis of osteogenesis. One potential drawback to this contrast technique is that measures of hydroxyapatite are no longer valid to quantitate bone density directly as incorporation of the silver enhances the density of skeletal elements. However, comparative analysis of relative density can be made, such as density measures presented as a percentage of staged sibling or wildtype controls. Like all visualization methods, care should. Use of alternate whole mount staining techniques such as Alizarin Red can control for this variation. Use of silver nitrate in whole mount preparations thus supports the use of microCT radiographic imaging to track bone formation through development in the zebrafish, not just in adult forms.

Together, these findings support the utility of radiographic techniques such as microCT to quantitatively detail the developing skeleton of the zebrafish. Further, we demonstrate that

these methods are sensitive to characterize specific changes in the formation and patterning of the skeleton induced by altered gene function. Combining mutant analysis and experimental tools available in the zebrafish model with the ability to detail and quantify skeletal growth and form by tomography permits detailed understanding of the mechanisms of skeletal development and pathologies associated with altered gene function and in disease. As these data can be easily archived and data mined beyond initial experiments, the use of microCT for broad scale phenotyping provides a lasting resource dataset for interrogation of skeletogenesis with zebrafish and in comparison to other taxa (*e.g.* mouse, zebrafish skull development at www.facebase.org, chondrichthians sharksrays.org/).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- Zebrafish, *Danio rerio*, is an emerging model organism for the study of skeletogenesis
- Use of zebrafish to study skeletogenesis are limited by lack of quantitative analysis to define normal and altered bone formation in mutants
- We utilize microCT to develop a quantitative analysis method for morphometric properties and bone mineral density
- We assess skeletal variation inherent in the zebrafish providing foundation for mutant analysis
- We detail a novel use of contrast agent to facilitate the detection and analysis of forming zebrafish skeleton during development.

Figure 1. Skeletal parameters of the zebrafish measured and their reproducibility

A. Representative 3D image of an adult wildtype zebrafish scanned at 18 μ m resolution on a Bruker Skyscan 1173 with the analyzed vertebrae C1 and C2 marked. **B.** *In silico* hemisection of an adult zebrafish skull reconstruction scanned as in (A) demonstrating the parasphenoid bone used in this analysis. Analysis was limited to the posterior portion of the bone with the rostral limit determined by an anatomic landmark (dotted line demarcates the medial landmark used to delineate anterior end of measurement). **C.** Representative schematic and images of caudal vertebrae (C1) with the semi-automatically generated markings used for analysis. **D–F.** Robustness of measure as measured by variance in results between scans and correlation of measurements made by observers on the same scan. Two independently obtained scans were analyzed by the same observer to assess repositioning reproducibility of measurements, n=12. A selection of scans was analyzed by either two

independent observers (interobserver reproducibility, n= 61 vertebrae; n=20 parasphenoid) or by the same observer on two independent occasions (intraobserver reproducibility, n=21 vertebrae; n=17 parasphenoid bone). Intraclass correlation coefficients (ICC) with upper and lower bounds are shown for intraobserver comparisons (gray circles), interobserver comparisons (blue circles) and repositioning reproducibility (red circles) for the parasphenoid (**D**), vertebrae (**E**), or vertebral arch (**F**).

C

Correlation of skeletal measures with STL				
Structure	(n)	slope	<i>r</i>	p value
Vertebrae				
radius	94	0.007 ± 0.0006	0.77	<0.0001
length	94	0.026 ± 0.0013	0.89	<0.0001
volume	94	0.004 ± 0.0003	0.76	<0.0001
BMD	94	6.663 ± 1.243	0.49	<0.0001
neural arch area	94	0.005 ± 0.0007	0.57	<0.0001
neural arch angle	94	-0.419 ± 0.118	-0.35	0.0006
Parasphenoid				
BV/TV	122	0.008 ± 0.0015	0.43	<0.0001
BMD	122	6.064 ± 1.369	0.38	<0.0001

Figure 2. Zebrafish bone parameters correlate with standard length

A–C. Correlation of C1 vertebral and parasphenoid bone parameters with standard length (STL) of adult fish in 94 (vertebrae) and 122 individuals (parasphenoid) from wildtype zebrafish of various strains, ages 3 to 10 months. Correlation was examined by calculation of the Pearson correlation coefficient, *r*. Most parameters of both parasphenoid and vertebral arches showed tight association with standard length. **A.** C1 length versus STL. **B.** Neural arch angle was one of the few measures where correlation was less than 0.4. **C.** Summary of metrics used in this study and their correlation with standard length of the fish.

Figure 3. Standard length adjusted vertebral and parasphenoid bone measurements do not vary with sex

5 month old male and female WIK strain fish, n=6–7, were analyzed and compared.

Standard length (STL) adjusted BMD at either **A.** vertebrae centra or **B.** parasphenoid shows no difference between sex. **C.** Summary of metrics used in this study with sex-specific means and p values determined by the Mann-Whitney U test for between sex comparison.

Figure 4. Age and strain dependence of standard length adjusted vertebral and parasphenoid bone measurements

A–C. Standard length (STL) adjusted measurements in AB fish are graphed versus age in months, $n=9-10$ per group. C2 vertebral length (A) and radius (B) are insensitive to age. However, vertebral volume (C) and BMD of both vertebrae (D) and parasphenoid (E) vary significantly with age. * $p < 0.04$, ** $p < 0.002$, *** $p < 0.0009$, **** $p < 0.0001$ by Kruskal-Wallis with Dunn's post-test for multiple comparisons. F–H. Strain differences between AB and WIK are noted at some ages examined. STL adjusted C2 volume measures diverge by 9–10 months whereas STL adjusted BMD values differ significantly at 5 months but not 9–10 months. ** $p=0.005$, *** $p < 0.0003$ (Mann-Whitney U test).

Figure 5. Quantitation of *bmp1a* skeletal deficiencies

Vertebrae of wildtype (*wt*) and *bmp1a*^{-/-} siblings were examined at 6 months of age, n=10–11. **A–B.** Representative sagittal section and lateral view of rendered C2 and adjacent vertebra. **A.** *wt* and **B.** *bmp1a*^{-/-} vertebrae. **C.** Representative frontal view of rendered vertebrae from 6 month-old *wt* and *bmp1a*^{-/-} fish. **D.** Comparison of the C2 length to radius ratio from *wt* and *bmp1a*^{-/-} siblings suggesting vertebral deformity in *bmp1a*^{-/-} fish. As this will influence STL normalization, eye diameter obtained from scans was used as an independent measure of size to adjust bone parameters for development. **E–J.** Comparison of C2 vertebral bone measurements from *wt* and *bmp1a*^{-/-} fish: volume (**E**), BMD (**F**), length (**G**), radius (**H**), neural arch angle (**I**), and area (**J**). With the exception of neural arch angle, all the parameters are presented normalized to the orbit diameter (orbit dia).

**p<0.01, ***p<0.001, ****p<0.0001 (Mann-Whitney U test).

Figure 6. Detection of a *csflra* skeletal phenotype

Vertebrae of wildtype (*wt*) and *csflra*^{-/-} siblings were examined at 6 months of age, n=18–19. **A–B.** Representative sagittal section and lateral view of rendered C2 and adjacent vertebra. **A.** *wt* and **B.** *csflra*^{-/-} vertebrae. **C.** Representative frontal view of rendered vertebrae from 6 month-old *wt* and *csflra*^{-/-} fish. **D.** Ratio of length to radius is similar for *wt* and *csflra*^{-/-} vertebrae. **E–J.** Comparison of vertebral parameters in *wt* and *csflra*^{-/-} fish: volume (**E**), BMD (**F**), length (**G**), radius (**H**), neural arch angle (**I**), and area (**J**). With the exception of the angle, all the parameters are presented normalized to STL. *p<0.05, ***p<0.001, ****p<0.0001 (Mann-Whitney U test).

Figure 7. Increasing detection sensitivity of microCT in zebrafish through use of selective contrast agent for skeletal tissues

A. Whole mount preparations of similar sized wildtype juveniles with different concentrations of silver nitrate. Bottom, size matched unstained control zebrafish. **B.** Dorsal view of 1% silver nitrate stained wildtype zebrafish skull (10mm STL); **B'** inset of staining showing stellate pattern at edge of forming bones. **C.** Size matched, alizarin red stained juvenile; **C'** inset of staining at comparable area as **B'** showing smooth boundaries of stained bones. **D.** Developmental series of zebrafish prior (top) and after (bottom) contrast staining with silver nitrate. No specific staining was detected for specimens <13mm in the absence of contrast staining (data not shown). *AR*, Alizarin Red; *PBS*, phosphate buffered saline.