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Impact of voxel size on microCT morphometric analysis of the toadlet *Brachycephalus ephippium*

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ABSTRACT

Brazilian Rainforest is the habitat of many species of the genus *Brachycephalus*, among them is the pumpkin toadlet, *Brachycephalus ephippium*. X-ray microcomputed tomography is a nondestructive imaging technique which allows the visualization and analysis of internal microstructures of various samples, and has been applied on the study of these animals, enabling thorough description and characterization of new species. *Brachycephalus ephippium* is of particular interest for it was the first *Brachycephalus* species to be discovered, which makes it a common interspecies comparative basis. Cranial morphological landmarks used in biological research are very small (0.1-1mm), hence the effects of voxel size on microCT images can be relevant, and this is what was investigated in this work. Effects of reconstruction voxel size were evaluated in both visual and quantitative aspects, which showed that an intermediary voxel size could be chosen on similar microCT applications without significant loss of information, but with great processing and storage gain, optimizing the application of the technique in such works.

Keywords: **microCT, segmentation, Brachycephalus, voxel size, reconstruction.**

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1. INTRODUCTION

X-ray microcomputed tomography (microCT) is a nondestructive imaging technique, which allows visualization and analysis of internal microstructures of a vast assortment of samples. The technique has been extensively used in small animal applications in a wide variety of biological research fields [1-5].

Brachycephalus (Anura: Brachycephalidae) is a genus of miniaturized frogs endemic to the Brazilian Rainforest of the northeast, southeast and south of Brazil [6]. These are diurnal animals, that can be found amidst leaf litter and most of them can be only found in montane forests. Furthermore, many of these species can only be found in one or few adjacent mountain tops, something that, along with the destruction of their habitat and relatively low reproductive rate, makes them particularly vulnerable to extinction [7].

MicroCT enables the visualization of internal bone microstructures, and its applications on *Brachycephalus* species have been vast, varying from investigations on auditory properties, morphological evolutionary patterns such as hyperossification and new species characterization [6-10].

In taxonomy works on *Brachycephalus*, it is not only necessary to compare specimens of the allegedly new species with one another, but with well-known species specimens as well. Thus, the description of a new species often requires the inspection of several specimens [6, 10]. The samples of this work are from the first described *Brachycephalus* species, *Brachycephalus ephippium*, also known as the Pumpkin Toadlet [11], which makes this species a comparison basis in many taxonomy studies. So far, 35 species of these small frogs have been discovered, 22 in the last decade [6, 9], and the efforts in this field have not subsided.

As far as taxonomy and evolutionary studies are concerned, the cranium is a region of great importance. Morphological landmarks are often used to characterize specimens, since one is often concerned with skull shape variations [6-11], and this motivated the focus of this work on the skulls of these animals.

Reconstruction and scanning (or acquisition) voxel sizes are important parameters on the application of MicroCT. Investigations have been conducted on how the variation of these parameters affect microCT results on bone samples [12-14], although none have been done on species of the genus *Brachycephalus*. Acquisition voxel size relates to the quality and level of detail that can be resolved in the raw data, whereas reconstruction voxel size is the effective voxel size of the 3D image reconstruction, and can be greater than scanning voxel size, mainly to avoid computational costs [14].

This work aimed to investigate the impact of the variation of the reconstruction voxel size on the morphological analysis of *Brachycephalus ephippium* cranial regions, using microCT qualitative and quantitative results. For that, raw data projections were reconstructed with three different effective voxel sizes, via reconstruction software. Herein, 9.5 μm represented the gold standard effective reconstruction voxel size, in accordance with the revised literature [6-10].

Cranial morphology and morphometry are of great importance in evolutionary research [11]. Morphometric analysis was used to quantify the influence of increased reconstruction voxel size on cranial morphometry, in order to investigate if the use of bigger than usual reconstruction voxel sizes could produce significant variation in image results regarding both visualization and analyses where the voxel, being the smallest object in a 3D image, is accounted for.

To increase reliability, morphometric analyses were performed using two different segmentation methods (Otsu and Global). The results provided useful information towards the optimization of applications regarding this kind of samples in the future.

2. MATERIALS AND METHODS

2.1. Specimens

The specimens used in this work are deposited in the C. F. B. Haddad collection, Universidade Estadual Paulista, Rio Claro, São Paulo, Brazil, and were collected by field researchers in São Francisco Xavier, São Paulo, Brazil. Animals of this collection are anesthetized before being euthanized and the use of specimens in this study was made possible via loan from their curators. Three samples of *Brachycephalus ephippium* were analyzed. The snout-vent length variation within samples is 12.81-12.99 mm, cranial length and width between 4.42-5.01 mm and 5.23-5.88 mm, respectively. A photograph of a *Brachycephalus ephippium* is presented in Fig. 1, where the small amphibian is seen on a leaf in Serra dos Órgãos, Teresópolis, Rio de Janeiro, Brazil.

Figure 1: *Brachycephalus ephippium (Serra dos Órgãos, Teresópolis, Rio de Janeiro, Brazil, December 2008).*
Source: Courtesy of Célio F. B. Haddad, São Paulo State University, Brazil

2.2. MicroCT scans and analysis

To prevent the samples from moving during scans – which could lead to faulty reconstructed images – a thin, polystyrene mold was used to secure the sample. Due to its low density compared to the specimens bone structure, it can be easily separated from bone during analysis.

Scans were performed using a phoenix v|tome|x 300 system (General Electric). Its microfocus X-ray directional tube (300 kV/500 W maximum voltage/power) has a tungsten target and filament, and a cooling system provides high beam stability at high power and small beam sizes [15]. Detection is accomplished by a digital GE DXR250RT detector, with a 1024 x 1024 pixel matrix and 200 µm pixel size. System setup for voltage and current was 50 kV and 180 µA, respectively. The effective acquisition pixel size was 9.5 µm. Scans were made with 1000 projections, with full rotation (360º), and corresponding 0.36º step. For each projection, a total of 5 frames were obtained and averaged, with 250 ms exposure time, and skip of 2. This setup led to a scanning time of 42 minutes per specimen.

Subsequent to the acquisition, raw image data were reconstructed using the manufacturer reconstruction software, datos|x reconstruction, with three isotropic voxel sizes: 9.5 µm, 19 µm and 38 µm. The reconstructed volumes were then converted into image stacks of 2D slices. After that, the images were rotated so the cranium would be centered and aligned, and exported as seen in the transaxial plane (Dataviewer v. 1.5.4).

Analyses were conducted using CTAn (Bruker). Primarily, the definition of a region of interest (ROI) was chosen so the analysis would consider the cranial region only. ROI selection is often done slice by slice (using interpolation) in a 2D interface, which can be highly time consuming, although this could be optimized by a suitable pre-scan sample positioning and software alignment. The latter allowed the use of a ROI in the shape of the smallest square containing the sample. By applying it to the whole image stack, a volume of interest (VOI) in the form of a rectangular prism was generated, and the same VOI was used for each specimen throughout all different voxel sizes analyses.

Next, the VOI image stack was segmented using two different segmentation methods: Global and Otsu. Segmentation is one of the most vital steps in microCT image analysis, in which the binarization threshold is defined – the point in the gray scale histogram that separates object and non-object material into black and white pixels, and vice-versa –, and its choice is of great importance for obtaining the best possible analysis results. There is a wide variety of different segmentation methods. The Global method is widely used in imaging applications on bones. It is a manual method, therefore it depends on operator skill in order to achieve satisfactory results, as it consists on the threshold choice by histogram manipulation and the comparison between the original gray scale image and the segmented one [2, 13, 14]. Otsu method is an automatic segmentation method, based on an algorithm which assumes that images have two classes of pixels and, from the gray level histogram (considered bimodal), it calculates the threshold that optimizes the separation between the two classes, so that the dispersion between them is minimum [16, 17].

The resulting segmented slices were then analyzed for commonly bone morphometric parameters: Bone Volume (BV), Bone Surface (BS), Bone Surface to Bone Volume ratio (BS/ BV), Trabecular Pattern factor (Tb.Pf), Trabecular Number (Tb.N), Trabecular Separation (Tb.Sp) and Number of Objects (Obj.N). This procedure was applied for all nine data sets. This same analysis procedure was conducted for all reconstructed image stacks.

The output from an analysis presents the average values of morphometric parameters for each data set. To increase reliability, these values were then averaged to obtain the final value for each examined parameter, taking the three corresponding results for that given parameter, voxel size and segmentation method.

3. RESULTS

Original and segmented 2D slice images, for both Global and Otsu segmentation methods, were noticeably impacted with the increase of voxel size (Fig. 2).

In Fig. 2 it is possible to compare 2D slices in original gray scale (a-c) with binarized images, segmented using Global (d-f) and Otsu method (g-i), of original reconstruction voxel size of 9.5 μm (a, d, g) and degraded larger voxels 19 μm (b, e, h) and 38 μm (c, f, i). Images binarized using Otsu algorithm (Fig. 2-g, Fig. 2-h, Fig. 2-i) presented overall more overestimated results (translated as more white pixels) than Global (Fig. 2-d, Fig. 2-e, Fig. 2-f) in all image sets. For the biggest voxel size studied (38 μm), Global method better represents the original image. From the smallest (9.5 μm) to the intermediary (19 μm) voxel size, Global method images differed considerably, whereas for Otsu segmentation this did not occur. When compared, the intermediate (Fig. 2-e, Fig. 2-h) and the biggest voxel size segmented images (Fig. 2-f, Fig. 2-i), the Global method presents less difference between images than Otsu.

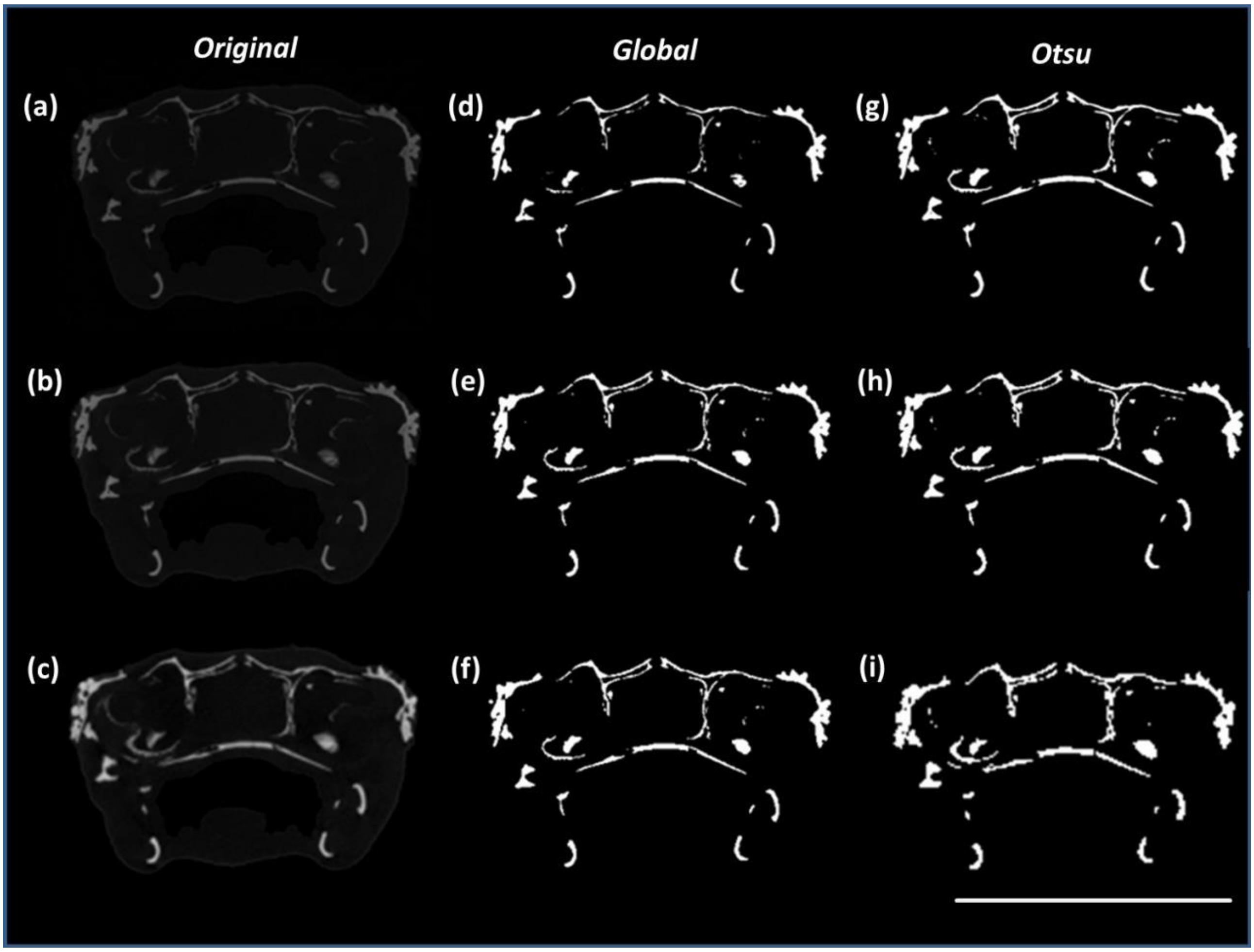

Figure 2: *2D slices of the same position can be compared. Scale bar: 5 mm.*

A qualitative inspection of voxel sizes 9.5 μm and 38 μm reconstructions evidenced the difference in quality between them, perceived as image blur and inferior detail differentiability on the 38 μm image. The 19 μm voxel size reconstructions presented considerably less loss of bone surface information compared to the gold standard.

The impact of voxel size and the chosen segmentation method varied according to the studied morphometric parameter (Fig. 3). Tb.Sp, BS, BV and BS/ BV showed no significant differences between results for all voxel sizes concerning the segmentation methods. Both voxel size and segmentation method appeared to have no effect on Tb.N values. However, Tb.Pf and Obj.N outputs had strong differences regarding both segmentation method and voxel sizes.

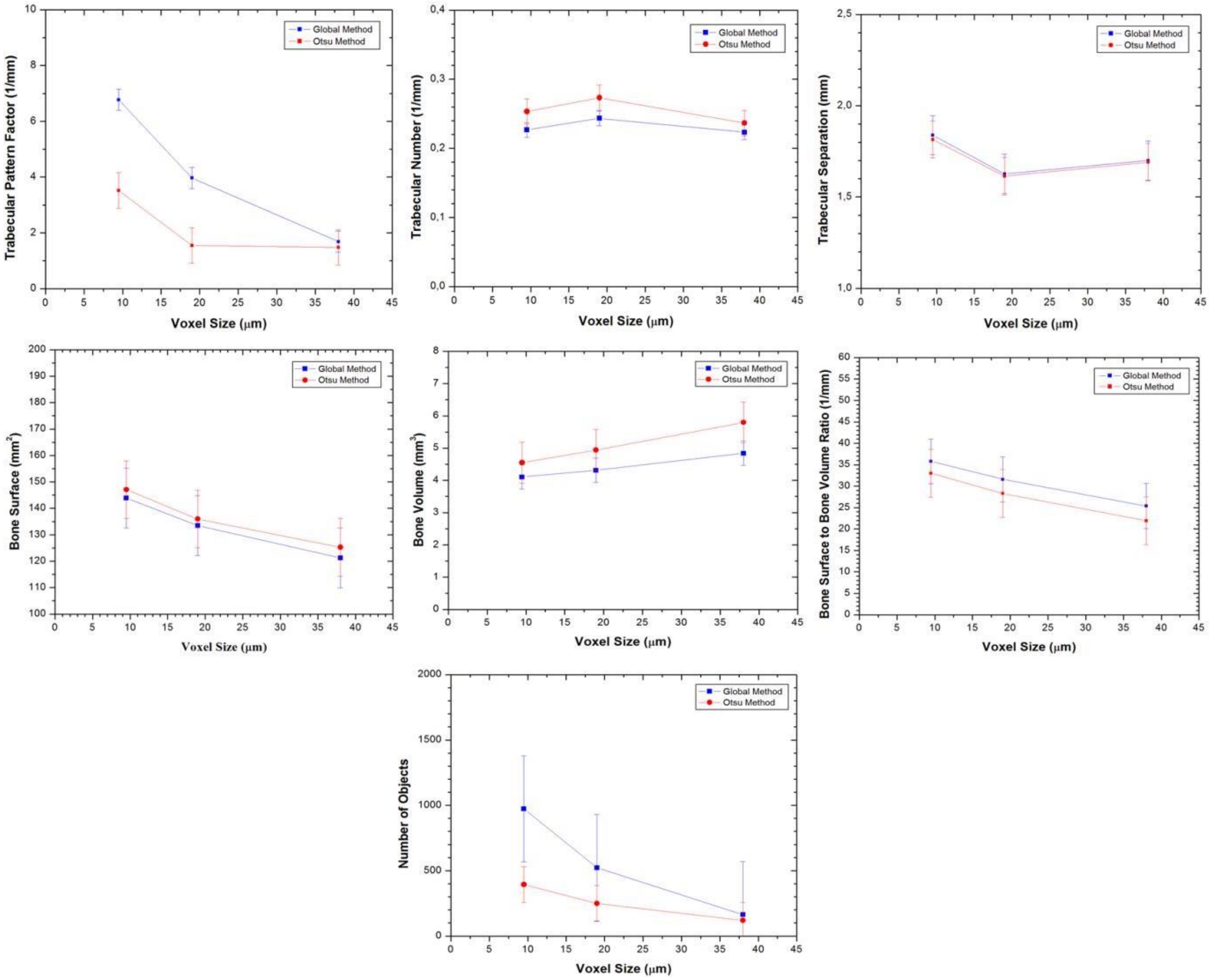

Figure 3: *Graphs showing the variation of morphometric parameters for the different voxel size and segmentation methods evaluated.*

Using the Otsu method, the results for Obj.N acutely differed in voxel sizes 9.5-19 μm (-37%), between 19-38 μm the discrepancy was even higher (-52%), and the information loss from this parameter to the 9.5-38 μm was of 70%. For the Global method, the same three comparisons resulted in the decrease of 46%, 69% and 83%, respectively. Tb.Pf also varied remarkably in the Global method, which showed the decrease of 41%, 57%, and 75%, in the intervals previously mentioned. The Otsu reduction was of 56%, 4% and 58%.

4. DISCUSSION

This work evaluated the effects derived from the variation of reconstruction voxel size on bone morphometric parameters on the cranium of *Brachycephalus ephippium* frogs. Voxel size importance is well established in literature, and studies have also been conducted to investigate the impact and difference of acquisition and reconstruction voxel size [1-5, 13, 14].

Most parameters analyzed showed significant difference in the 9.5-38 µm voxel size variation with both methods, but not as much for the 9.5-19 µm. For instance, as BS decreased, BV increased, in both segmentation methods, with the increase in voxel size. Although the difference between 9.5-19 µm (5-8 % for BV and 7-8 % for BS) was not significant, the 9.5-38 µm (17-27 % and 41-47 %) presented notable variation. Obj.N and Tb.Pf had their values strongly affected, compared to 9.5-38 µm (70-83 % and 58-75 %, respectively). Tb.Pf is a connectivity parameter and as it approaches zero more connected trabeculae are observed [18], evaluating the differences in the absolute values between Global and Otsu method, 3.25 mm^{-1} at 9.5 µm and 0.20 mm^{-1} at 38 µm, and how intense was the decrease (for 9.5 µm is 4.02 and 2.38 times greater than at 38 µm, for Global and Otsu methods, respectively), it is arguable that most information regarding trabecular connectedness is lost when using this voxel size.

A qualitative comparison is shown in Fig. 4. The 19 µm reconstruction voxel size presents a subtle difference compared to the gold standard, whereas the 38 µm one shows considerable information loss. Cranial structures of interest in *Brachycephalus* studies are commonly around 0.1-1 mm [6-11], hence differences of this magnitude could lead to considerable loss of information, and a minimum discretization of three to four elements across the thickness of individual trabeculae is recommended to minimize numerical errors [13]. In accordance with the quantitative analysis, the 38 µm voxel size image is visually deformed, whereas the 19 µm represents no visual or quantitative relevant loss compared to the gold standard.

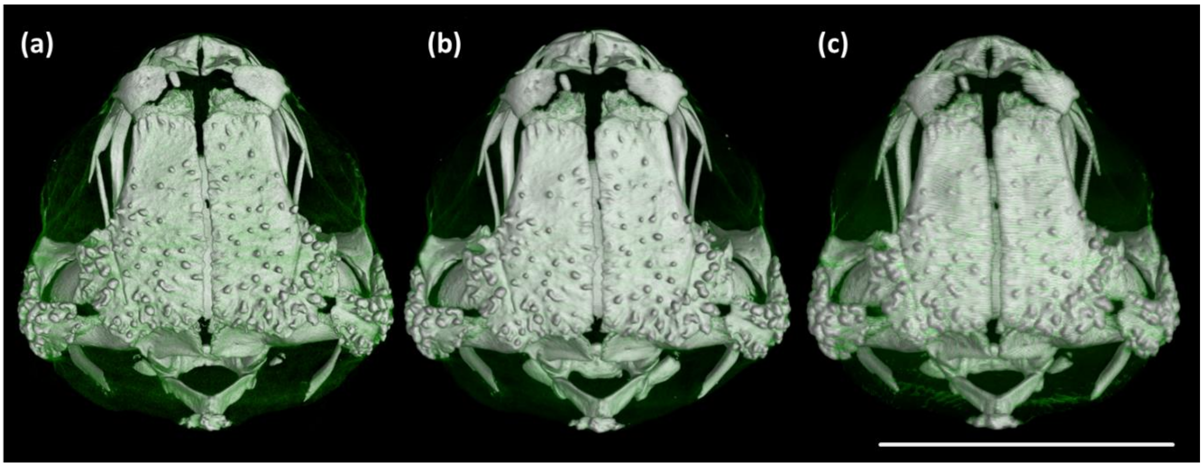

Figure 4: *Reconstructed volumes of voxel sizes: (a) 9.5 μm, (b) 19 μm and (c) 38 μm Scale bar: 4 mm.*

Imaging facilities dwell on concerns regarding reconstruction, data processing and storage, which could amount to high cost and be more time consuming. A 9.5 μm voxel size reconstructed volume occupies more than 5 GB of hard disk, comparatively with an intermediary 19 μm reconstruction, comprised of less than 300 MB per sample. As for the analysis, approximately an hour was needed for the 9.5 μm voxel size sample data, using a high-end computer, while 19 μm reconstructions lasted less than 10 minutes per sample data. This could be irrelevant if working with a small number of samples, though the use of several samples on the same study is more common [6].

The results provided compelling evidence that the application of the technique could be optimized for these samples, by utilizing an intermediary 19 μm reconstruction voxel size, saving considerable amount of processing time and storage space, whilst keeping most relevant quantitative and visual information. However, the use of minimum acquisition voxel size is recommended to best accommodate this inference, considering no variations of such kind were performed, accordingly no remarks can be made about how the variation of acquisition voxel size can affect the specimens microCT morphological analysis. A possible continuation of this work could focus on the latter, by performing scans with the different voxel sizes studied here and comparing the results, or even changing other acquisition parameters in order to improve the use of the technique for future related research.

5. CONCLUSION

Microcomputed tomography is a powerful nondestructive imaging technique, which enables visual and quantitative analysis of internal microstructures of objects, providing valuable data in small animal applications. The segmentation method choice is not to be taken lightly as it represents a great challenge in analysis performed by microCT. Although capable of providing high resolution images, the application in small animals could benefit from the use of higher, intermediary, reconstruction voxel sizes in large scale analysis, optimizing both storage and processing of the reconstructed data.

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