Journal of FISH BIOLOGY
Published for the Fisheries Society of the British Isles

Journal of Fish Biology (2012) **80**, 1292–1299
doi:10.1111/j.1095-8649.2011.03188.x, available online at wileyonlinelibrary.com

Micro-computed tomography: an alternative method for shark ageing

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Micro-computed tomography (microCT) produced 3D reconstructions of shark *Carcharhinus brevipinna* vertebrae that could be virtually sectioned along any desired plane, and upon which growth bands were readily visible. When compared to manual sectioning, it proved to be a valid and repeatable means of ageing and offers several distinct advantages over other ageing methods.

Key words: 3D reconstructions; sagittal sectioning; spinner shark; tomographic imaging; vertebral centra.

Increases in commercial fishing effort targeting sharks have prompted worldwide concern regarding the status of shark stocks, and have highlighted the need for sustainable exploitation through appropriate fishery management (Barker & Schluessel, 2005; Baum & Blanchard, 2010; Ferretti *et al*., 2010). Growth rates, natural mortality rates and longevity, and hence the resilience of shark stocks to various levels of fishing mortality, can all be estimated using age and size data. Accurate methods of shark ageing are, therefore, essential for comprehensive assessment and management of exploited shark populations.

Age determination in sharks is most commonly achieved *via* analysis of growth bands in vertebral centra using a range of specific methods (Cailliet, 1990; Cailliet *et al*., 1983). Techniques such as X-ray imaging (Liu *et al*., 1999), centrum surface micro-topography (Carlson & Parsons, 1997) and staining (Wintner & Cliff, 1995; Officer *et al*., 1996) have been applied to derive age estimates using whole vertebrae. While the suitability of whole vertebrae has been demonstrated for the ageing of young sharks (MacNeil & Campana, 2002), accuracy in the cases of older individuals is limited by: (1) difficulties in resolving tightly grouped banding on the outer margins of vertebrae, (2) obscuring of growth bands on opposing halves due to vertebral geometry and (3) variability in birthmark clarity (MacNeil & Campana, 2002; Cailliet & Goldman, 2004; Goldman, 2004). Consequently, the use of whole

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vertebrae for verification of growth band periodicity *via* marginal increment and centrum edge analyses is prone to inaccuracy, particularly given the need for precise characterization and measurement of the critical areas of the centrum outer margin (Cailliet *et al*., 2006).

In light of these limitations, the analysis of sagittally cut vertebral sections (generally $< 0{\cdot}6$ mm thick) has underpinned the majority of shark ageing studies to date. Unenhanced sections have typically produced the best readability across a range of shark species (Wintner *et al*., 2002; Carlson & Baremore, 2005; Carlson *et al*., 2006; McAuley *et al*., 2006). Techniques such as calcium-binding stains (Piercy *et al*., 2007), X-ray micro-radiography (Simpfendorfer *et al*., 2002; Joung *et al*., 2008), submersion in ethanol (Bishop *et al*., 2006), histology (Natanson *et al*., 1995) and metal substitution (Gelsleichter *et al*., 1998) have been used, however, in attempts to enhance growth band clarity. Despite its widespread use and acceptance as the preferred method of shark ageing (Goldman, 2004), manually obtaining sagittal sections from vertebral centra is a destructive sampling process and is vulnerable to the inherent variability in section quality associated with manual processing.

This study aimed to assess the use of micro-computed tomography (microCT) as a valid and repeatable alternative technique for age determination in a species of carcharhinid. MicroCT utilizes X-ray technology to produce image stack reconstructions of 3D objects from which virtual sections can be visualized and extracted at any orientation. The suitability of the microCT method was thus assessed *via* direct comparisons between manually cut sagittal sections and three-dimensional virtual sections imaged from whole vertebrae, across a range of sizes and ages of shark.

The spinner shark *Carcharhinus brevipinna* (Müller & Henle 1839), a species distributed widely throughout warm temperate and tropical shelf waters of the world (Last & Stevens, 2009) and caught commercially in coastal waters of eastern Australia, was the fish studied. A total pool of 166 *C. brevipinna* was separated according to three vertebral diameter size classes: 12–16, 20–24 and 28–32 mm (measured using digital callipers). These vertebral size classes were chosen to correspond with two vertebrae diameter frequency histogram modes from observed commercial catch data from 2008 to 2009 (W. G. Macbeth, unpubl. data). For each size class, vertebrae samples from eight individuals were randomly selected, providing a total of 24 individuals for assessment. The selected individuals ranged in size from 132 to 257 cm total length (L_T) and had a male:female ratio of 1·4:1.

From each *C. brevipinna*, a section of three to five vertebrae was sampled from the cervical region of the vertebral column (*i.e.* anterior to the first dorsal fin), stored on ice and then frozen upon return to the laboratory. In preparation for ageing, vertebrae samples were thawed, manually cleaned of excess soft tissue, separated into individual centra and soaked in a 5% sodium hypochlorite solution until all remaining soft tissue had been removed. Soak time varied from 15 to 45 min depending on the size of the centra. Cleaned vertebrae were rinsed thoroughly in tap water and then stored in 70% ethanol.

One vertebra from each *C. brevipinna* was chosen at random, removed from the alcohol and air-dried in preparation for scanning. Specimens were scanned using an Xradia (www.xradia.com) MicroXCT-400 X-ray micro-tomography system. The scanning system was set to a source energy of 120 keV, with a flux of 83 μA for all scans. To provide some phase enhancement to the resulting tomographic projections,

the source and ×0·5 scintillator or objective were set at 150 and 200 mm from the specimen, respectively. This scanning geometry resulted in a pixel size of 24·41 μm with the cooled CCD camera being used in its binning 2 mode, and tomographic projection images of 1024 × 1024 with a field of view of 25 mm × 25 mm. The camera exposure was set at 1·0 s and a total of 360 projection images were obtained during each scan of *c*. 42 min. All projection images were at 16 bit grey-scale depth and the resulting raw X-ray projection file was 4·1 GB in size.

Projection image data sets were reconstructed into axial-slice image stacks using a filtered back-projection algorithm implemented in graphical processing unit (GPU) hardware and supplied with the scanner. Corrections were made for rotational misalignments (*i.e.* centre shift), beam hardening and ring artefacts. The resulting reconstructed image stacks were of variable thickness depending on the size of the vertebrae: 400 slices for small, 512 slices for medium and 670 slices for the large. Average reconstruction times were < 5 min for all the specimens.

The data visualization software VG Studio Max 1.2 (Volume Graphics; www.volumegraphics.com) was used to visualize the axial slice stacks in full 3D context. This software permitted complete 3D visualization and facilitated extraction of virtual sections at any orientation through the specimen using digital clipping planes. For quantitative ageing assessment, virtual sections clipped along the sagittal plane to include the vertebral focus were extracted from all the vertebral specimens.

Following microCT scanning, the same vertebral centra were sagittally sectioned to 0·5–0·6 mm thickness using an Isomet low-speed diamond blade saw (www.buehler.com). Sections were fixed to a glass slide with waxed resin and examined under reflected light using an Olympus SZ dissecting microscope fitted with a digital camera (http://microscope.olympus-global.com/).

Growth bands were counted on microCT virtual sections and manually cut sections on two independent occasions (readings 1 and 2) by one reader without prior knowledge of the size of each individual *C. brevipinna*. A growth band was defined as a band-pair, comprising one opaque and one translucent band (Cailliet *et al*., 2006). For the purposes of this study, the term age count is used to denote estimates of age based on the assumption of annual band-pair deposition in the absence of age validation for this species. Age counts were derived by counting fully formed translucent bands occurring after the birthmark; the latter being denoted by an angle change on the centrum face (Goldman, 2004). The readability of each microCT virtual and manually cut section was scored according to the following definitions: 1, all growth bands well defined and visible; 2, almost all bands visible, clear interpretation possible; 3, most bands visible, interpretation reliable to within ± 1; 4, bands visible, majority difficult to interpret and 5, unreadable.

A combination of methods was used to evaluate bias and precision in age counts between-reading and between-method (Cailliet & Goldman, 2004). Bias was investigated using age-bias plots and Bowker's test of symmetry to determine whether observed count differences were systematic or due to random error (Hoenig *et al*., 1995; Campana, 2001). Precision estimates were calculated using the coefficient of variation (c.v.) (Chang, 1982). Age counts obtained from reading 1 were used for between-method analyses.

MicroCT scanning produced high-resolution, 3D images representative of the four vertebral ageing templates employed in the literature: whole vertebrae, radiograph, half vertebrae and sagittal section (Fig. 1). The quality and resolution of microCT

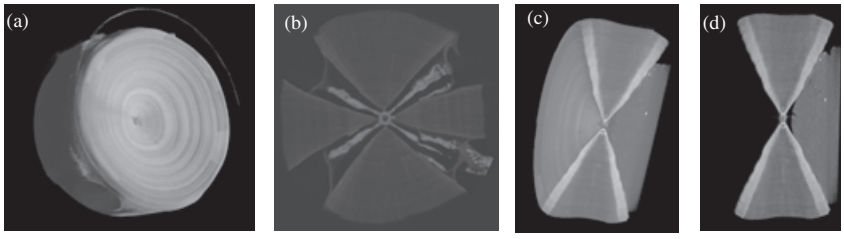

FIG. 1. Reconstructed 3D virtual images of: (a) a whole vertebra (b), a radiograph, (c) a half vertebra and (d) a sagittal section of *Carcharhinus brevipinna* following microCT scanning of one vertebra.

output were sufficiently high such that the growth bands were visible for each of the four image types.

All microCT virtual sections had discernible growth bands extending along the corpus calcareum from the birthmark to the centrum edge that were directly comparable to those on manually cut sections (Fig. 2).

Growth band clarity was similar between methods, with mean ± S.E. readability for microCT and manual sections scored as $2{\cdot}6 \pm 0{\cdot}1$ and $2{\cdot}8 \pm 0{\cdot}1$, respectively.

Age-bias plots and Bowker's test of symmetry identified no systematic between-reading bias in age counts for manually cut sections ($\chi^2 = 9{\cdot}33$, d.f. $= 9$, $P > 0{\cdot}05$) and microCT virtual sections ($\chi^2 = 9$, d.f. $= 10$, $P > 0{\cdot}05$) [Fig. 3(a), (b)] or between-method bias ($\chi^2 = 13$, d.f. $= 12$, $P > 0{\cdot}05$) [Fig. 3(c)] across the age range 2–19 years. Precision estimates were considered acceptable (C.V. values < 11) for all three comparisons (Fig. 3) (Campana, 2001).

While microCT is already an established technology for imaging mineralized animal tissues (Neues & Epple, 2008), this study marks its first application to the ageing of elasmobranchs. MicroCT-generated sections provided comparable and repeatable

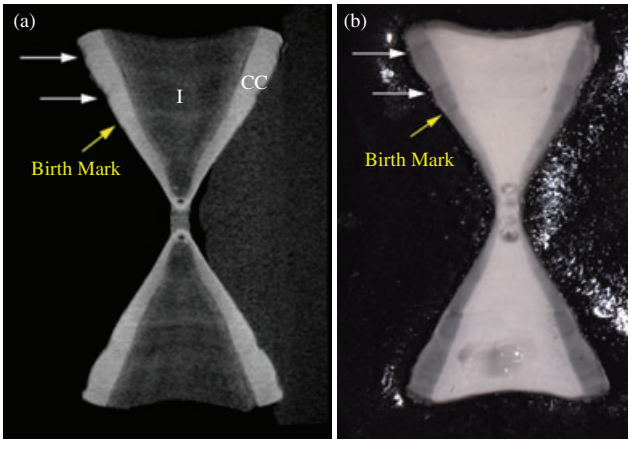

FIG. 2. Visual comparison of a (a) microCT virtual section and (b) manually cut section from the same vertebra of *Carcharhinus brevipinna*. ⟶, fully formed translucent growth bands. I, intermedialia; CC, corpus calcareum.

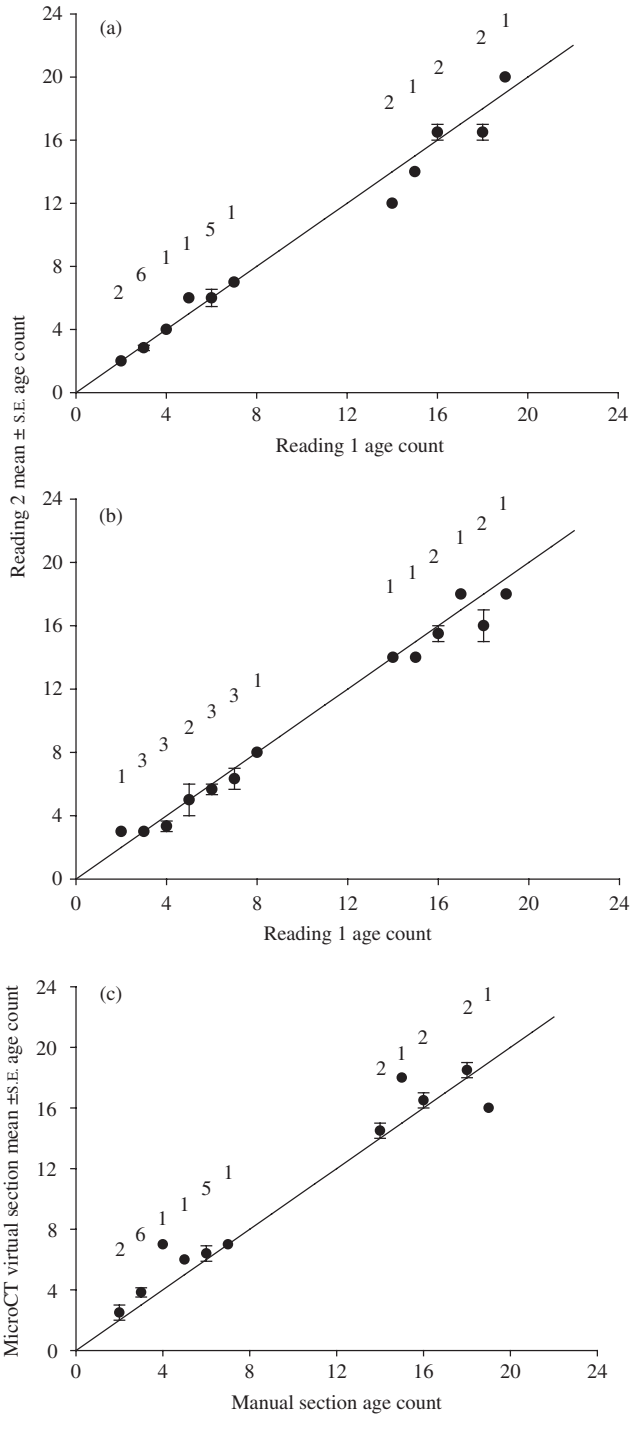

FIG. 3. Age-bias plots of *Carcharhinus brevipinna* vertebral age counts from: (a) two independent readings of manually cut sections (C.V. = 5·58), (b) two independent readings of microCT virtual sections (C.V. = 7·00) and (c) two independent methods (C.V. = 10·7). Sample sizes and one-to-one equivalence lines are shown.

age counts relative to manually produced sections across a wide age range of *C. brevipinna.* The microCT method, like manual sectioning, is capable of resolving tight banding on the centrum outer edge, a critical factor in the accurate ageing of older individuals and calculation of marginal increment ratios. In the case of larger vertebrae, microCT was observed to improve growth band resolution in the intermedialia, particularly near to the centrum edge, when compared to manual sections, although this coincided with comparably reduced readability along the corpus calcareum (see Fig. 2 for vertebral zone locations).

This research identified several distinct advantages of microCT over manual sectioning. First, it is a non-destructive technique that can provide a reliable age count without affecting the structural integrity of the vertebral sample. While it therefore permits unlimited multiple virtual sectioning from unlimited angles and perspectives, researchers would need to maintain consistency with respect to the angle and perspective used among vertebrae when ageing individuals of a particular species. This method also permits an archive of the intact vertebrae should novel vertebral analysis techniques involving whole vertebrae be developed in the future. Second, variables inherent to manual processing such as section width and location are eliminated, as the digital sectioning of the virtual vertebra can be precisely specified at the desired width or location. Third, microCT eliminates the need to adjust light source and light angle during reading, a potential source of between-reader variability. Finally, although cleaned prior to scanning in this study, the low intrinsic X-ray contrast of non-mineralized tissues (Metscher, 2009) means that vertebral samples can be scanned in an uncleaned state without affecting the quality or resolution of the microCT output, hence substantially reducing sample processing time.

Owing to limited financial resources, this research was performed on only 24 vertebral samples from only one carcharhinid species. Optimal ageing methods can, however, be species-specific (Cailliet & Goldman, 2004), and so a more robust methodology for evaluating any given shark ageing technique would encompass larger sample sizes and, ideally, more than one species. The nature of the microCT method is such that a longer scan time (or greater number of projection images) translates to higher quality output, but at correspondingly higher cost. During this study, a compromise was reached between scan time (and therefore cost) and data quality resulting in growth band clarity being comparable to manually prepared sections. Employment of a longer scan time per vertebral sample would, however, probably have improved virtual section readability.

We would like to thank the NSW commercial shark fishers and NSW Department of Primary Industries scientific observers for the provision of vertebrae samples, and M. Yerman for assistance in the laboratory. This work was jointly funded by NSW Department of Primary Industries, Northern Rivers Catchment Management Authority and The University of Sydney.

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