New Approaches to the study of Comparative Neuroanatomy in Marine Vertebrates using MRI: The Whale shark, Rhincodon typus, as a Case Study

K. E. Yopak[1], and L. R. Frank[1]
[1]Center for Scientific Computation in Imaging, UCSD, La Jolla, CA, United States

Introduction MRI technology has recently emerging as an effective investigative tool for non-invasive visualization and quantification of the internal anatomy of fishes [e.g. 1], as well as studies on comparative brain anatomy of vertebrates [2-5]. Cartilaginous fishes emerged at the onset of the vertebrate radiation and offer a unique opportunity to explore the evolution and elaboration of the vertebrate brain, including our own. Throughout the past 200 years, various technologies and methods have emerged to assist in the collection, quantification, and dissemination of neuroanatomical data. Traditional invasive techniques typically distort the precise relative positions of tissue structures by tearing and shearing, altering information on the internal architecture. Moreover, when examining rare and/or valuable specimens, invasive methodologies become problematic, as often specimens must be left intact for preservation or future study. Magnetic Resonance Imaging (MRI) is unique in its ability to non-invasively acquire high-resolution 3D data from soft tissue structures. While these cutting-edge technologies and methods are extensively developed for their applications in human brain research, their utility in comparative marine biology remains largely unexplored.

This study examines the use of MRI to obtain high-resolution image data in an important but rare and damaged brain specimen, wherein digital reconstruction allowed for non-invasive quantification of brain organization in the whale shark, *Rhincodon typus* [4]. *R. typus* is a cartilaginous, epipelagic, filter feeding member of the Orectolobiformes order in the class Chondrichthyes. This species is one of only three sharks to have evolved its highly specialized lifestyle, feeding solely on planktivorous material in the open ocean, which highlights it as a key species in the study of evolutionary trends within cartilaginous fishes.

Specimen Prep and Imaging Anatomical MR scans for brains from 2 individual specimen of *R. typus* were performed on a Bruker 7Tesla small animal scanner with gradient strengths of 46Gauss/cm. Brains were excised from the chondrocranium and fixed in 10% formalin, after which, brains were transferred to 1 X PBS + 0.01% sodium azide for at least 14 days to remove excess fixative before transferring to fresh 1 X PBS + 0.01% sodium azide with the addition of 5 mM of the contrast agent Prohance (Bracco Diagnostics Inc.) for a further 7 days at 4° C. Equilibrating the tissue in this contrast agent achieves a significant reduction in the longitudinal relaxation time (T1) of the sample and a corresponding increase in the SNR efficiency of the data acquisition. MR image data was acquired from contrast-enhanced, fixed brains.

Image Segmentation The data were digitally segmented using ITK-SNAP [6]. The five major brain structures (telencephalon, diencephalon, mesencephalon, cerebellum, and medulla oblongata) were identified and individually segmented (fig 1). As both brains were hemisected prior to scanning, total brain volume was obtained by mirroring the brain along its true bilateral division digitally. These data were then compared to existing data on over 60 species existing in the literature [7, 8]. All relative size structure data was normalized with a weighted factor analysis (θ) [9, 10] and connectivity between species was determined using a hierarchical cluster analysis and multidimentional scaling (MDS) ordinations.

Results The brain of *R. typus* was characterized by a small brain in comparison to other cartilaginous fishes, with a relatively reduced forebrain and mesencephalon and a relatively enlarged diencephalon, occupying 40%, 7%, and 8% of the brain, respectively [4]. Its most notable characteristic was a large and highly foliated cerebellum, a trait previously documented only in agile, predatory shark species [7] (fig 1). Hierarchical cluster analysis and MDS showed evidence of convergent evolution in the brains of *R. typus* and another planktivorous yet taxonomically divergent species, the basking shark (*Cetorhinus maximus*) [4].

Conclusions Strong correlations between brain patterns and various ecological factors have been found in other vertebrate groups (ie bird, bony fishes, and mammals), including diet, habitat, and social behavior. Our previous research has found similar relationships between ecology and brain organization in cartilaginous fishes [4, 7, 8, 11]. As sharks are basal vertebrates, it is critical for the study of neuro-evolution to understand how both phylogeny and adaptive processes have shaped the shark brain. The study of species with unique behavioral and morphological specializations is critical when teasing apart evolutionary trends, yet becomes difficult, as often these species are extremely rare and/or difficult to obtain. MRI facilitated the acquisition an analysis of non-invasive, high-resolution images in this rare shark species, which had previously been impractical. How these approaches can potentially transform the way other researchers view, quantify, and disseminate neuroanatomical data in basal vertebrates will be discussed.

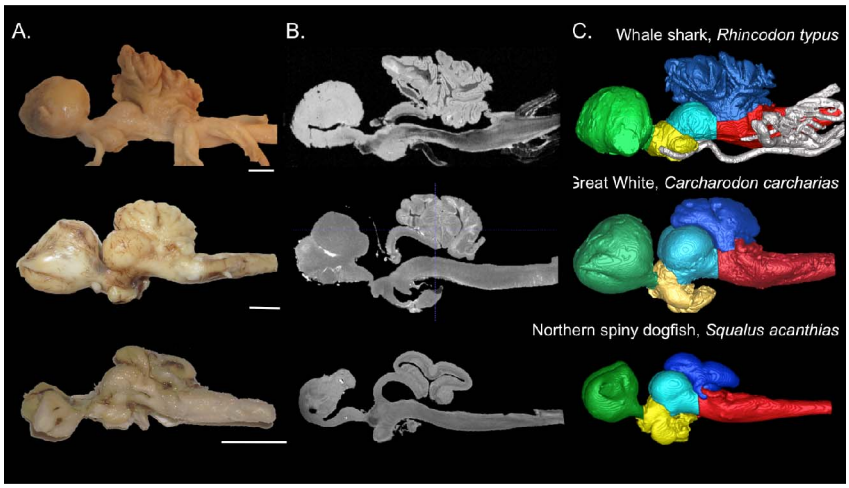

Fig 1. The brains of three shark species in lateral view (**A**) Gross morphology, (**B**) Sagittal slice of MR data, (**C**) Digital segmentation. KEY: Green = telencephalon, yellow = diencephalon, cyan = mesencephalon, blue = cerebellum, red = medulla, white = cranial nerves (if applicable)

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