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How to remove the brain of Amazonian manatee (*Trichechus inunguis*) calves preserving the skull for morphological analysis

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ABSTRACT

Although there are several studies on the skull of Amazonian manatees (*Trichechus inunguis*) to better understand the anatomy, physiology, and behavior of these animals, the analysis of the brain has been often neglected. Typically, in osteological studies, the brain is discarded to preserve the integrity of the skull. One of the main reasons for this neglect of the brain is the lack of adequate dissection protocols to allow the extraction of the intact brain while preserving the integrity of the skull. In this study, we present a simple step-by-step protocol for a comprehensive procedure of brain extraction and fixation in manatee calves while ensuring the preservation of the skull structure to the best possible extent for further studies. The protocol is based on an incision at the posterior part of the skull, extending laterally toward the parietal bone until reaching the frontal bone, followed by removing the upper portion of the skullcap to extract the brain. After the procedure, the removed skull portion can be reconstituted to preserve the entire skull structure. The protocol also offers adaptations to simplify the methodology according to the reality of places with little laboratory structure, allowing the preservation of rare tissues with limited resources and/or in areas of difficult access. Our proposed methodology enables maximum utilization of the collected animal, which not only aligns with ethical and practical considerations, but also makes material available for a detailed description of the manatee brain, and a better understanding of the neuroanatomy of aquatic mammals in general.

KEYWORDS: Sirenia; neuroanatomy; dissection protocol; osteology, aquatic mammals

Como remover o cérebro de filhores de peixe-boi amazônico (*Trichechus inunguis*) preservando o crânio para análises morfológicas

RESUMO

Embora existam diversos estudos em crânios de peixes-boi amazônicos (*Trichechus inunguis*) a fim de melhor compreender a anatomia, fisiologia e comportamento desses animais, análises do cérebro tem sido negligenciadas. Tipicamente, em estudos osteológicos, o cérebro é descartado para preservar a integridade do crânio. Um dos principais motivos para essa negligência com o cérebro é a falta de protocolos adequados de dissecação que permitam a extração do cérebro intacto enquanto preserva-se a integridade do crânio. Neste estudo, apresentamos um protocolo simples, passo a passo, para um procedimento de extração e fixação do cérebro em filhotes de peixe-boi, garantindo a preservação da estrutura do crânio ao máximo possível para estudos futuros.. O protocolo é baseado em uma incisão na parte posterior do crânio, estendendo-se lateralmente em direção ao osso parietal até alcançar o osso frontal, seguido pela remoção da porção superior da calota craniana para extrair o cérebro. Ao final do procedimento, a porção removida pode ser reconstituída de forma a manter a estrutura completa do crânio. O protocolo também oferece adaptações para simplificar a metodologia de acordo com a realidade de locais com pouca estrutura laboratorial, permitindo a preservação de tecidos raros com recursos limitados e/ou em áreas de difícil acesso. A metodologia proposta permite a máxima utilização do animal coletado, o que não apenas está em conformidade com considerações éticas e práticas, mas também torna o material disponível para uma descrição detalhada dos cérebros dos peixes-boi e, consequentemente, a melhor compreensão da neuroanatomia de mamíferos aquáticos em geral.

PALAVRAS-CHAVE: sirênios; neuroanatomia; protocolo de dissecção, osteologia, mamíferos aquáticos

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INTRODUCTION

The Amazonian manatee (*Trichechus inunguis* Natterer, 1883) belongs to the Sirenia order and is endemic to the Amazon Basin in South America (Rosas 1994; Amaral et al. 2023). It is an herbivorous, fully aquatic mammal and, uniquely, the only sirenian that inhabits exclusively freshwater environments. Despite being the smallest member of its group, calves are born weighing 10-15 kg and measuring 85-105 cm and can grow up to 2.75 meters long and weigh as much as 450 kilograms (Best 1983; Best 1984; Amaral et al. 2010).

The skull is one of the most studied structures in the Amazonian manatee. Analyses of its components, such as the tympanic bulla and the occipital condyles, have helped to shed light on ecological, behavioral, and morphological issues (Domning 1978; Domning and Hayek 1986; Castelblanco-Martínez et al. 2014; Barros et al. 2017; Valdevino et al. 2021). Additionally, cranial endocasts have been widely used as a tool in neuroanatomy to provide information about the brain anatomy of diverse fossil taxa, including sirenians (O'Shea and Reep 1990; Macrini 2023). Also, the nervous system in manatees has several peculiarities that are of great interest to researchers, particularly from evolutionary-developmental biology and comparative neuroanatomical perspectives (O'Shea and Reep 1990; Popov and Supin 1990; Kelava et al. 2013; Charvet et al. 2016). Manatees are known for their distinctive smooth cerebral cortex and thick gray matter, which may be of relevance to better understand the mechanism that generates cortical gyrification (Mota and Herculano-Houzel 2015). They are also noted for their small brain-to-body size ratio and low encephalization quotients, which have been the subject of discussions regarding their cognitive abilities (O'Shea and Reep 1990; Reep and Bonde 2006). In this sense, studying the brain morphology of manatees is not only of interest to address the structure and function of their unique neuroanatomical features, but also provide valuable data to broaden comparative studies of mammalian brains in general.

However, despite their considerable potential, studies focused on the nervous system of manatees are notably sparse. Published neuroanatomical descriptions of the West Indian manatee (*Trichecus manatus*, Linnaeus, 1758) (Reep and O'Shea 1990; Marshall and Reep 1995) are decades old and thus did not incorporate modern techniques not available at that time. Furthermore, detailed information regarding the dissection and fixation procedures used in these studies is no longer accessible, hindering further analysis. For the Amazonian manatee specifically, to the best of our knowledge, there are no published detailed descriptions of its brain anatomy. This likely results from the logistic challenges associated with collecting the brains of aquatic mammals, especially in the challenging climatic and logistic conditions of the Amazon, combined with a lack of scientific funding for studying the neuroanatomy of these animals in South America.

Another factor that contributes to the scarcity of neuroanatomical studies of manatees is the invasive nature of the usual approaches for brain extraction, which typically damage the skull, making it unsuitable for future morphological studies. There is thus a trade-off between extracting the intact brain and preserving the integrity of the brain case. As a result, and given the usual lack of the necessary materials and protocols, in most cases, the brain is discarded to preserve the bone structure for osteological investigations. A methodology for obtaining material that simultaneously enables both osteological and neuroanatomical research would therefore be highly valuable.

To address this challenge, we have developed and proposed in here a simple and cost-effective protocol to remove the brain from manatee calves while preserving the main components of the skull. It includes a detailed description of a removal and fixation procedure compatible with non-invasive approaches, such as magnetic resonance imaging (MRI), as well as histologically invasive approaches, such as immunohistochemistry (Herculano-Houzel and Lent 2005). Our main objective was to pave the way for expanding the diversity of materials deposited in anatomical collections for future research on manatee brains and to maximize the utilization of collected specimens, enabling investigations through diverse modern neuroanatomical methodologies.

MATERIAL AND METHODS

We dissected five Amazonian manatee calves from the Aquatic Mammals Laboratory at the National Institute of Amazonian Research (Instituto Nacional de Pesquisas da Amazônia - INPA), Manaus, Brazil. This research facility serves as a hub for receiving, rehabilitating, and facilitating scientific studies of endangered orphaned animals. The procedures were authorized by the Scientific Breeding Facility of Instituto Brasileiro do Meio Ambiente e dos Recursos Naturais Renováveis (IBAMA) under process # 21105.004653/1984-60. The skulls were obtained from animals that did not survive the rehabilitation process and were collected during necropsy procedures. The study was conducted under the aegis of the recently established 'Rede Brasileira de Neurobiodiversidade' (neurobiodiversidade.org), created specifically to enable the collection and analysis of aquatic mammal brains in Brazil.

The age of the calves ranged from 0.5 to 1 year. Their sex, body size, and weight are informed in Table 1. Two whole individuals were frozen immediately after death and kept in a freezer for no more than three months before the procedure, while the other three had only the skull cleaned (with no skin and muscles) and placed in formalin immediately after death for at least 24 hours before dissection (Table 1). For brain extraction, we developed a protocol based on prior examinations of the cranial anatomy and position of the brain within the skull of the Amazonian manatee, using

the aquatic mammal collection at INPA. Our goal was to establish a cutting plan that would enable the extraction of the whole brain while preserving the integrity of the skull to the greatest extent possible. To carry it out, the following tools are required: scalpel, microsurgery scissors, dental forceps pliers, hacksaw blade, spatula, metal spoon, tweezers, and epidural needles (Figure 1). The following reagents are required for the fixation and storage steps: 10% formalin or 4% formaldehyde (PFA), phosphate buffer, sodium azide, and deionized water (see composition in the Supplementary Material, Tables S1-S4). All the reagents and equipment must be made available beforehand. In institutions that receive stranded manatees and manatee deaths are deemed likely, it is recommended that a staff veterinarian or resident researcher be trained in the procedure.

The anatomical nomenclature of bone structures follows Domning and Hayek (1986), and Hoson et al. (2009) for suture terminology. The protocol described in here can be applied to Amazonian manatees of any developmental stage but is particularly recommended for the extraction of the brains of calves, which are more susceptible to damage than those of adults.

Table 1. Information about the specimens of Amazonian manatee (*Trichechus inunguis*) used in this study. NA = Not available information.

Animal identification	Sex	Standard length* (cm)	Body weight (kg)
Pb #291	Male	84	10
Pb #293	Male	83	11
Pb #294	Male	92	14
Frozen head 1	NA	NA	NA
Frozen head 2	NA	NA	NA

*Standard length = length in straight line from the tip of its snout to the base of its tail.

Figure 1. Dissection tools used in the process of brain removal of Amazonian manatee (*Trichechus inunguis*) calves.

RESULTS

The brains of the two individuals that were frozen postmortem were apparently well preserved externally but not internally, and disintegrated during the removal process. Therefore the protocol described below is based on the dissection of the three individuals that had their skulls cleaned and immediately immersed in formalin after death.

Dissection protocol

1 - Separate the head from the rest of the body using a scalpel or sharp dissecting knife through the junction between the occipital condyle and the first cervical vertebra (Figure 2a,b).

2 - Remove the skin and muscles from the head for a clear visualization of the frontal, parietal, and occipital bones using the tweezers, scalpel and surgical spatula (Figure 2c, 3). As the cranial sutures are not totally closed in calves (Figure 3c), the brain can be seen through the bones. The removal of the brain immediately after death is not indicated in the case of calves, as their brains are exceedingly fragile and may not withstand the forces applied during removal.

3 - After the removal of the skin and muscles, inject fixative into the most internal regions of the brain through the foramen magnum and nasal bone openings using an epidural needle. This will aid in the fixation of the inner parts of the brain (Figure 3c) as the fixative does not reach all regions of the tissue at the same rate (Gage et al. 2012; Latini et al. 2015; Loomis 2016). Then immerse the whole skull in 4% paraformaldehyde or 4% formalin for at least 24 hours before initiating the brain dissection process, thus avoiding the potential leakage of sensitive structures, such as the cerebellum, through the foramen magnum during the subsequent skull-cutting process. Use a volume of fixative 10 to 20 times greater than the volume of the skull being preserved, covering the whole structure, as a low liquid volume often results in poor fixation (Everitt and Gross 2006; Loomis 2016).

The status of the fixation process can be assessed by observing the color of the brain tissue. Fresh tissue is soft, rich in blood, and exhibits a reddish appearance. Upon fixation, the tissue becomes harder and takes on a slightly pale brownish hue (Figure 4). Additionally, the consistency of the brain tissue can be inspected by gently palpating it with the fingers. Unsuccessful fixation will result in a pinkish color due to the presence of blood. If the brain does not achieve the consistency and color typical of fixed material after 24 hours, the fixative needs to be replaced.

4 – To separate the top of the skull, use a hacksaw blade and start the cut in the posterior part of the skull, at the level of the supraoccipital-exoccipital synchondrosis, which is not yet fused in calves (Figure 3c). Then continue on both sides towards the parietal bone until the frontal bone, after the coronal suture. Laterally, the cut plane goes along the suture between the parietal and squamosal bones (Figure 3a), where

the bone is less dense, reducing the risk of accidental damage from applying too much pressure to harder skull regions. The supraoccipital-exoccipital synchondrosis, is the last region on the skull to begin the ossification process (Valdevino 2016). This cutting line also protects delicate structures like the tympanic bulla and other important bone regions.

5 - After completing the cut all around the skull, use dental forceps pliers to carefully remove the skullcap and expose the dorsal part of the brain (Figure 3d). The brain is enveloped by thin membranes called meninges, which must be removed carefully to avoid cutting into the brain during the extraction. In these animals, the meninges were strongly adhered to both

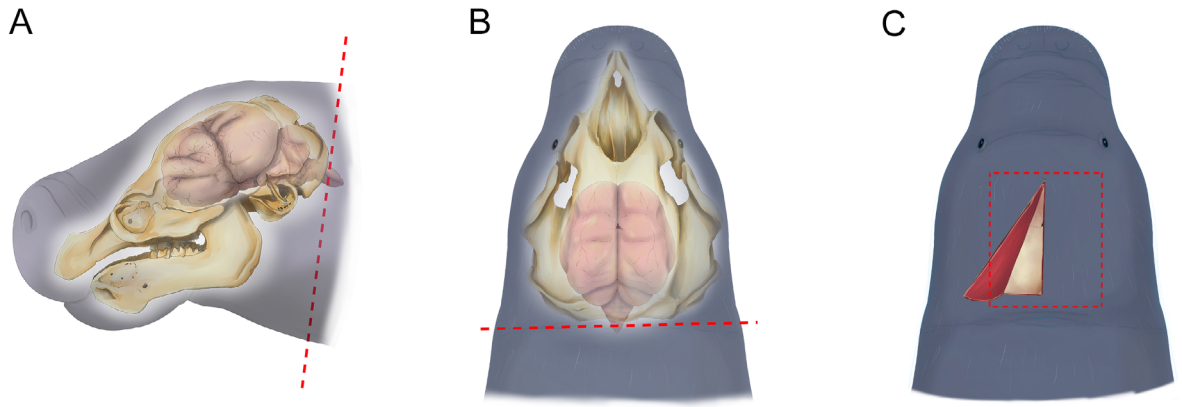

Figure 2. Line of incision (dashed line) behind the occipital condyle to separate the head from the body of Amazonian manatees, showing the position of the brain inside the skull in lateral (**A**) and dorsal (**B**) view; **C** – Sketch showing the removal of skin and muscles from the head to allow the visualization of the skull bones. Illustrations by Gabriel Melo-Santos.

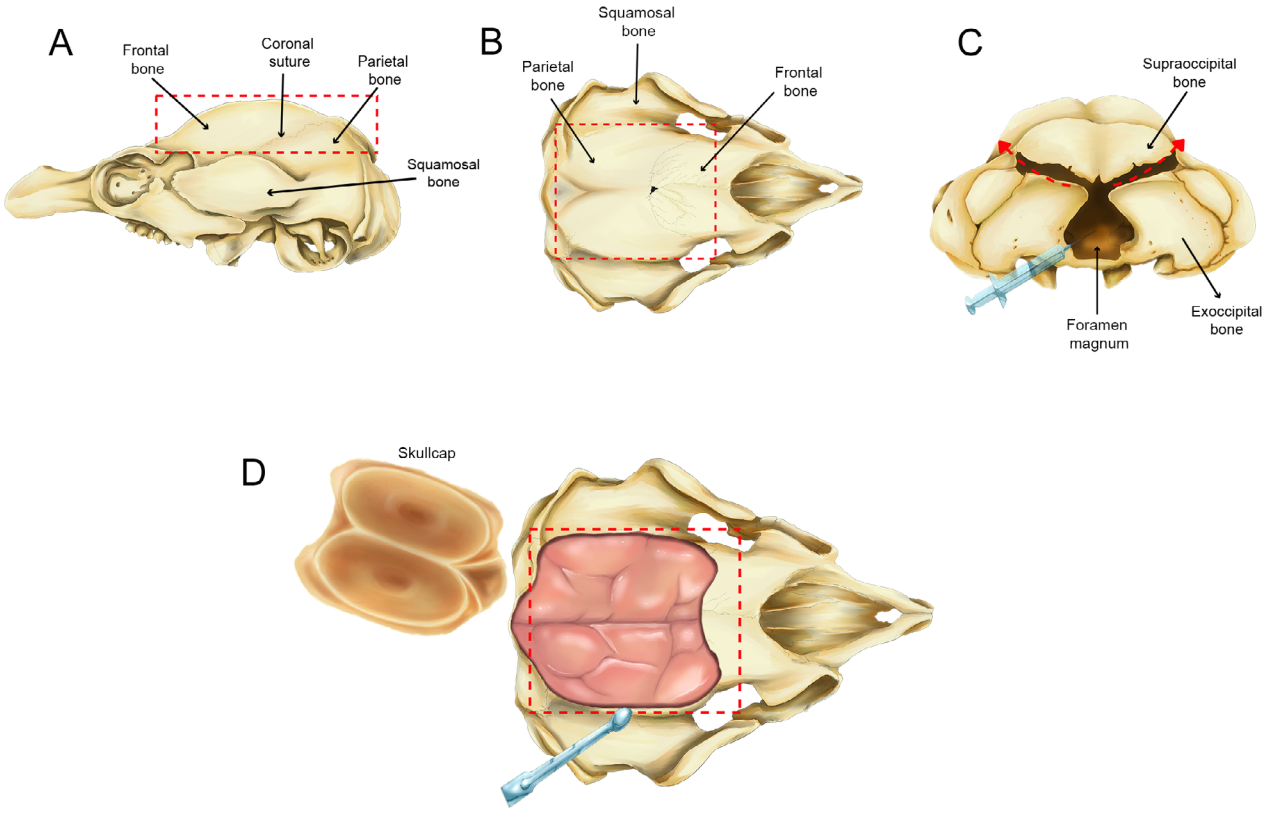

Figure 3. Delimitation of the cutting area (dashed line) of the head of Amazonian manatees in lateral (**A**), dorsal (**B**, **D**), and posterior (**C**) view. The supraoccipital-exoccipital synchondrosis is the ossification area between the supraoccipital bone and exoccipital bones shown in the red dotted line. The syringe in (**C**) indicates the injection point for fixative into the brain through the foramen magnum. The spatula in (**D**) indicates the insertion movement to release the brain from the skull after fixation. Once brain extraction is complete, the skullcap can be returned to its original position, maintaining the complete structure of the skull. Illustrations by Gabriel Melo-Santos.

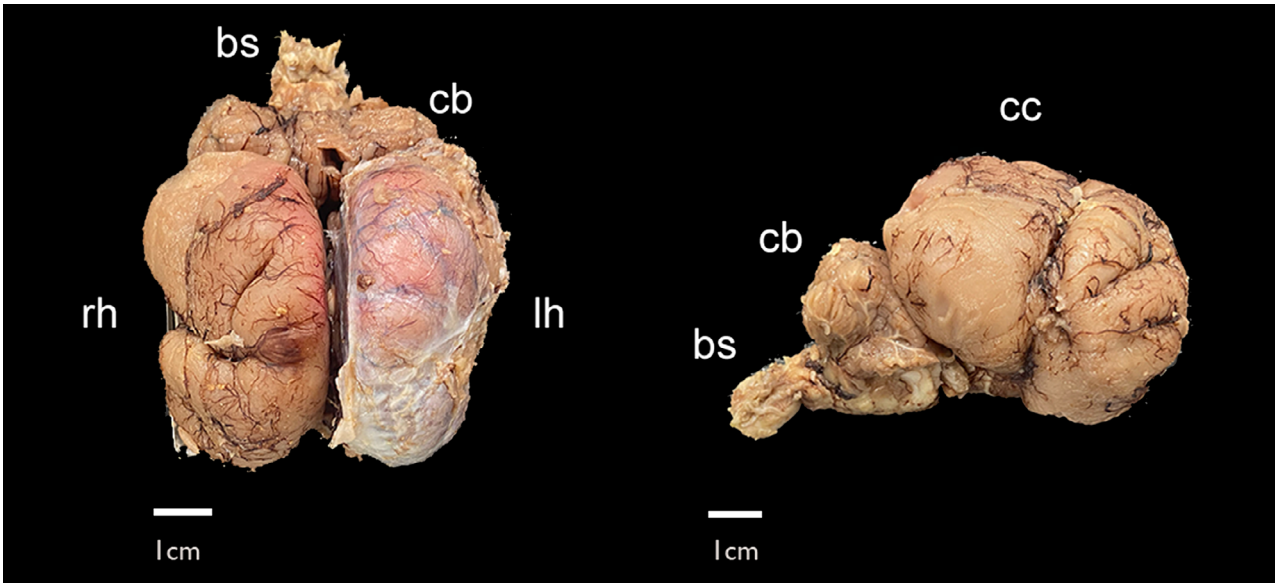

Figure 4. Brain of an Amazonian manatee calf in dorsal (left) and lateral (right) views after its removal from the skull. The right hemisphere in the dorsal view still conserves the meninges. Abbreviations: bs: brainstem; cb: cerebellum; cc: cerebral cortex; rh: right hemisphere; lh: left hemisphere. Scale bar = 1cm.

the brain and the braincase, so use a blunt object without sharp tips, such as a spatula or metal spoon, to very gently and carefully separate the membrane from the brain tissue. Microsurgery scissors can also be used to cut the meninges and facilitate their separation from the skull. This precaution is necessary to avoid potential damage to the tissue while separating the brain from the skull.

6 - Keep the skull in dorsal orientation (Figure 3b) and, with the aid of the surgical spatula and metal spoon, release the rest of the brain from the skull to allow its removal without any damage to the tissue (Figure 3d). Carefully slide your fingers between the bone-brain boundary in the laterals, and also underneath the brainstem and cerebellum, and gently lift the posterior part of the brain. Be careful not to apply excessive pressure to the brain tissue.

7 - Immediately after removing the brain, place it in a container filled with PBS in sodium azide (PbAz), and sealed with a hermetic cover, for storage until analysis. Alternatively, a 30% sucrose solution at 4 °C can be used to dehydrate the tissue for cryoprotection. After dehydration, the brain sinks to the bottom and can then be stored in an antifreeze solution at -20 °C. This step is beneficial for immunohistochemical analyses, as it gives greater flexibility for conducting experiments (Corthell 2014).

8 – After the brain is removed, the skull can undergo further cleaning and preparation for inclusion in an osteological collection. If the upper part of the skull was removed with a clean cut (Figure 3d), it can be easily fitted back into place, preserving the original skull morphology for anatomical, imaging and/or histological studies.

DISCUSSION

Our dissection protocol for brain extraction in Amazonian manatees represents a novel methodology that allows for the removal of the brain in good condition while preserving the skull for a variety of modern morphological studies. It enables analyses of brain tissue using both invasive and non-invasive techniques, such as immunohistochemistry and magnetic resonance imaging, respectively.

The brain tissue is soft, delicate, and prone to degradation. It is surrounded by the meninges, which, among several other functions, provide a protective covering for the underlying neural tissue of the brain and the spinal cord (Decimo et al. 2012; Dasgupta and Jeong 2019). Based on previous necropsies in the context of the Rede Brasileira de Neurobiodiversidade, we have observed that in other aquatic mammals, such as dolphins, the meninges surrounding the brain are not as tightly attached to the brain and skull as in the Amazonian manatee calves. This observation reinforces the recommendation of immersing the head for brain removal in a bucket filled with 4% paraformaldehyde (PFA) or 10% formalin (preferentially PFA, if planning to perform immunohistochemistry analyses (Corthell 2014; Loomis 2016) to allow the brain to gain consistency and better withstand handling during the procedure. Further research is necessary to elucidate if this is a general species-specific feature or just related to the calf stage**.**

One of the main concerns when fixating neural tissue is selecting an appropriate fixative that preserves the brain tissue as closely as possible to its living state while maintaining its suitability for the intended analysis. Also, reducing the time interval between death and fixation is crucial for maintaining

tissue integrity (Insausti et al. 2023). While freezing is often more practical than other preservation methods and is commonly chosen when available, our experiments revealed that, in the case of brain tissue, it caused significant degradation of the sample even before the extraction procedure was completed. The tissue loses its consistency and fails to fixate internally, resulting in the complete loss of the sample. Besides that, freezing the sample even after fixation in formalin is not a good choice since it leads to the formation of ice crystals in the brain sample and consequent damage to the tissue, rendering the organ unsuitable for further procedures (Rosene and Rhodes 1990).

The use of 4% PFA is generally considered the gold standard approach to preserve cellular morphology and antigenicity (Li et al. 2017; Konno et al. 2023). However, due to the logistical challenges involved in preparing this solution — since 4% PFA cannot be ordered as a working solution, requiring prior preparation, and must be kept cold—previous tests showed that, alternatively, the use of formalin at low concentrations (4%) as fixative also has provided satisfactory results in immunohistochemistry and neuroimaging studies.

The time required for fixation is another concern and depends on the size of the brain and the fixative used (Carter and Shieh 2015; Loomis 2016). It is crucial to ensure that the brain tissue becomes fixed enough to allow for manipulation without disintegration. However, avoiding over-fixing is also important to preserve the tissue for histological analysis, such as immunohistochemical staining (Corthell 2014). This timing must be adjusted to adapt to specific needs and circumstances. Previous studies on humans have shown that extending fixation time to 72h still allows techniques like Nissl-staining (Insausti et al. 2023). However, smaller brains, such as those of manatees, tend to overfix more quickly. Further tests are necessary to determine the appropriate fixation time for different morphological techniques.

As a post-fixation step, freezing the tissue for slicing techniques or sample storage can be an alternative option after the PFA/formalin fixation process rather than PbAz. To cryoprotect the tissue in this case, sucrose can be used to push the water molecules out of the tissue, preventing ice crystal formation and preserving the tissue available for further morphological analysis (Corthell 2014). However, for neuroimaging studies, preliminary results have demonstrated that sucrose-infiltrated brain tissue preparation may alter the water diffusion properties of the tissue (Mullins et al. 2013). This may prevent imaging methodologies such as diffusion tensor MRI, which relies on the anisotropy of the diffusion of water molecules to investigate connectivity in the brain. In this context, preserving the brain in PBS or PbAz after the fixation process is preferable. PbAz not only acts as an antimicrobial preservative (de Prisco et al. 2022) but also enhances the contrast between tissues of their nuclear spin relaxation times, resulting in more clearly defined images (Wielenga et al. 2023). Although the post-fixation time and reagents were adapted to immunohistochemistry studies mentioned here, post-fixation time for other techniques can be up to five years stored simply in paraformaldehyde/formalin (Insausti et al. 2023; Nardi et al. 2023). However, it should be noted that such prolonged exposure may lead to deformation and shrinkage of the tissue which may, for example, confound volumetric measurement studies (Su et al. 2014; de Guzman et al. 2016; Insausti et al. 2023; McKenzie et al. 2024). These effects are still under debate but must be considered when determining the appropriate post-fixation duration to ensure the integrity of morphological data in long-term storage.

In rare instances, neuroimaging studies can be performed *in situ*, right after the animal's death. This allows the immediate analysis of the brain and has the advantage of investigating the organ in its correct position within the skull, with no distortions related to the brain removal and fixation (Montie et al. 2007). However, given the challenges of carcass transportation, preparing the brain for *ex-situ* analysis is more frequently the best choice. An alternative approach is to perform *in situ* brain imaging in materials that are preserved in formalin for a long time, as in the case of museums or research collections. However, for these materials, the immunohistological analysis may be unsuitable. In light of this, our approach provides researchers with the necessary time, equipment, and resources to perform comprehensive imaging, histological, and osteological analyses, yielding a wide range of valuable data for further research.

The acquisition of high-quality brain tissue, along with the preservation of the skull, is a significant advancement in the field. Both elements are crucial for comprehending the evolution of mammalian brains and the potential cognitive capacities of the species (Domning and Hayek 1986; Bauer and Reep 2022; Henaut et al. 2022), but in many cases, one must be chosen at the expense of the other. Our protocol introduces a standardized method for whole brain extraction, increasing the availability of this organ in zoological collections, and potentially minimizing methodological discrepancies among comparative anatomical descriptions. This standardization is crucial as varying protocols can affect the accuracy, reproducibility, and comparison across different neuromorphological studies (G.Vonsattel et al. 1995; Ioannidis 2011; Klapwijk et al. 2021).

We expect this work will open the door to various neuroanatomical studies on Amazonian manatees including the potential analysis of adult specimens. The extracted manatee brains can be used to produce detailed neuroanatomical descriptions, high-fidelity cortical surface reconstructions from structural MRI, white matter connectivity mapping through imaging techniques such as diffusion tensor imaging (DTI), and precise cellular counting for the various brain regions using techniques such as the isotropic fractionator (Herculano-Houzel and Lent 2005). In terms of comparative neuroanatomy, studying Amazonian manatee brains allows for comparative

analyses within the Sirenia group (Chapman 1875; Marshall and Reep 1995; Reep and O'Shea 1990; Reep et al. 1989; Sarko and Reep 2007; Bauer and Reep 2022; Macrini 2023) as well as other mammals (Manger et al. 2012), providing valuable insights into the relationship between evolutionary and neuromorphological aspects of this group.

CONCLUSIONS

This study offers the first detailed method for extracting and fixing manatee brains while preserving the skull for future morphometrical investigations. Our research addresses the scarcity of data on aquatic mammal brains, particularly in Brazil, and provides the means to increase the material available for multivariate studies aimed at advancing our understanding of manatee neuroanatomy, contributing to the broader field of mammalian neuroanatomy.

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SUPPLEMENTARY MATERIAL

Avelino-de-Souza *et al.* How to remove the brain of Amazonian manatee (*Trichechus inunguis*) calves preserving the skull for morphological analysis

Table S1. Composition of the phosphate buffer (PB) 0.4M used in the protocol for skull-preserving removal of the whole brain of Amazonian manatees (*Trichechus inunguis*).

Ingredient	Total volume (L)		
	1	5	10
Distilled water (L)	0.5	2.5	5
Anhydrous di-potassium hydrogen phosphate [K_2HPO_4] (g)	56	280	560
Sodium di-hydrogen phosphate [$NaH_2PO_4\ H_2O$] (g)	10.6	53	106
Distilled water	adjust to 1L	adjust to 5L	adjust to 10L

Check pH to be between 7.2-7.4.
The solution can be stored at 4°C for up to 6 months.
To prepare the working solution of 0.1 M, dilute 1:4 with distilled water, i.e., 250 ml of PB 0.4 M plus 750 ml of distilled water generates 1 liter of PB 0.1 M.
To prepare the solution, the following equipment is necessary: magnetic stirrer to dissolve the components, pH-meter, precision balance, and a glass Pyrex bottle.

Table S2. Composition of the 0.1 M sodium azide (NaAz) solution used in the protocol for skull-preserving removal of the whole brain of Amazonian manatees (*Trichechus inunguis*).

Ingredient	Total volume (L)		
	1	5	10
0.1 M PB (L)	1	5	10
Sodium azide (g)	1	5	10

SAFETY NOTE: Sodium azide must be handled and prepared carefully due to its toxic and carcinogenic nature. It is crucial to avoid inhaling its dust and avoid using metal utensils during its manipulation. Safety guidelines for this substance are available at <https://www.ehs.harvard.edu/sites/default/files/lab_safety_guideline_sodium_azide.pdf>.

The solution can be stored in the refrigerator or at room temperature for up to one year.
To prepare the solution, the following equipment is necessary: magnetic stirrer to dissolve the components, precision balance, and a glass Pyrex bottle.

Table S3. Composition of the 4% paraformaldehyde (PFA) in 0.1M PB solution used in the protocol for skull-preserving removal of the whole brain of Amazonian manatees (*Trichechus inunguis*).

Ingredient	Total volume (L)		
	1	5	10
Distilled water	0.75	3.75	7.5
PFA powder stir (g)	40	200	400
NaOH 10M	drops	drops	drops
0.4M PB (L)	0.25	1.25	2.5

PREPARATION: Heat distilled water to 60 °C, add PFA and stir. Neutralize pH with the NaOH 10M drop by drop until the solution becomes clear, then add 0.4 M PB. The output solution should equal pH = 7.2. Filter the solution using filter paper and store at -20 °C up to 1 month. If kept in the refrigerator (at 4 °C) it must be used in up to one week.
To prepare the solution, the following equipment and material is necessary: fume hood, precision balance, heated stir plate, Erlenmeyer flask (500 ml), magnetic stir bar, pH meter, filter paper, glass Pyrex bottle. The following EPIs are necessary: gloves, lab coat, mask, eye protection.

Table S4. Composition of the 30% sucrose solution that can be used in the protocol for skull-preserving removal of the whole brain of Amazonian manatees (*Trichechus inunguis*).

Ingredient	Total volume (L)		
	1	5	10
0.1 M PB (L)	1	5	10
Sucrose (kg)	0.3	1.5	3

Complete with 0.1PB to the final volume.
The solution can be stored at 4°C for up to 1 month.
The following equipment will be needed: stirring plate, magnetic stirrer, glass Pyrex bottle.