bol.cient.mus.hist.nat. 28(2), julio-diciembre, 2024. 87-101. ISSN: 0123-3068 (Impreso) ISSN: 2462-8190 (En línea)

BOLETÍN CIENTÍFICO
CENTRO DE MUSEOS
MUSEO DE HISTORIA NATURAL

The use of multi-enzymatic detergent for cleaning flesh from bones of small mammals preserved in fluid and frozen

Alexandra Maria Ramos Bezerra (Dr.) [1], Halícia Celeste (MSc.) [2,3] y Andreza Soeiro (MSc.) [4]*

Abstract

The scientific collections of Mammals mainly consist of preserved specimens in spirit or taxidermized skins and skeletons. Cleaning bones is one of the most important steps in preserving the dried osteological material. Necrophagous insects are predominantly used to clean delicate bones such as skulls of small mammals but, maintaining the colonies of these insects can be challenging. In order to find an alternative and simple approach to safely clean small mammal bones, multi-enzyme detergent was tested. Although this method is known for its application in large mammals and forensic studies, it has never been attempted on small mammals in scientific collections. Different concentrations of a multi-enzymatic detergent to deflesh small mammal skulls were tested. After removing excess flesh, the skulls were placed separately in containers filled with the solution up to three times the volume of the skull. They remained immersed for 2.5 to 24 hours at 24°C–37°C. The final cleaning was done using a soft toothbrush and tweezers under a stereoscope. The results were satisfactory, especially with the highest concentration, although all other concentrations were effective in degradation of soft tissue, even in formalin-preserved specimens, apparently without damaging bone structures. To prevent damage to the skulls, it is recommended to check them hourly until the tissue has been completely removed.

Keywords: fluid preserved specimens, scientific collections, skull, vertebrates.

*FR: 19-X-2023. FA: 22-X-2024.

[1] Mastozoologia, Coordenação de Zoologia, Museu Paraense Emílio Goeldi, Campus de Pesquisa, Belém, PA, Brazil, amrbezerra@hotmail.com
orcid.org/0000-0002-7972-5535 **Google Scholar**
[2] PPG Zoologia, UFPA/ Museu Paraense Emilio Goeldi. Av. Perimetral 1901. Terra Firme. Belém, PA, Brazil, haliciaceleste@gmail.com
orcid.org/0000-0002-1429-2222
[3] Laboratório de Ecologia e Zoologia de Vertebrados, Instituto de Ciências Biológicas, Universidade Federal do Pará, Belém, PA, Brazil.
[4] Mastozoologia/COZOO, PIBIC/CNPq, Museu Paraense Emilio Goeldi. Av. Perimetral 1901. Terra Firme. Belém, PA, Brazil, bio.acsn@gmail.com
orcid.org/0000-0002-3281-790X
* Corresponding author

CÓMO CITAR:
Bezerra, A.M.R., Celeste H., Soeiro A. (2024). The use of multi-enzymatic detergent for cleaning flesh from bones of small mammals preserved in fluid and frozen. *Bol. Cient. Mus. Hist. Nat. Univ. Caldas, 28*(2), 87-101. https://doi.org/10.17151/bccm.2024.28.2.5

El uso de detergente multi-enzimático para limpiar la carne de los huesos de pequeños mamíferos preservados en fluido y congelados

Resumen

Las coleciones científicas de mamíferos principalmente consisten en animales preservados con cuerpos o partes en médios líquidos, así como peles taxidermizadas y huesos limpios. La limpieza de los huesos es uno de los pasos más importantes antes de colocarlos junto a otros espécimenes em la colección seca. Preferentemente, se utilizan insectos carnívoros para limpiar huesos delicados, como los cráneos de pequeños mamíferos, pero mantaner sus colonias puede ser un desafío. Con el objetivo de encontrar un método alternativo y sencillo para limpiar de manera segura los huesos de pequeños mamíferos, probamos el detergente multi-enzimático. Aunque este método es conocido para grandes mamíferos y estudios forenses, nunca se había intentado en pequeños mamíferos preservados en colecciones científicas. Probamos diferentes concetraciones de un detergente multi-enzimático para limpiar la carne de los cráneos de pequeños mamíferos. Despúes de retirar el exceso de carne, colocamos los cráneos por separado en recipientes llenos de la solución, hasta tres veces el volumen del cráneo. Permanecieron sumergidos durante 2.5 hasta 24 horas a uma temperatura de 24°C–37°C. La limpieza final se realizo con un cepillo de dientes suave y pinzas bajo estereoscopio. Los resultados fueron satisfactorios, especialmente con la concentración más alta, aunque todas las demás fueran eficaces en la degradación de los tecidos blandos, incluso en especímenes fijados en formol, aparentemente sin dañar las estructuras óseas. Para evitar daños en los cráneos, recomendamos verificarlos cada hora hasta que se haya eliminado por completo el tejido.

Palabras clave: especímenes preservados en fluido, colecciones científicas, cráneos, vertebrados.

Introduction

In natural history collections, terrestrial vertebrate specimens are primarily preserved as whole wet specimens (also known as fluid or spirit collections) or taxidermied as stuffed or opened skins and osteological preparations. The latter type of preparation generally requires cleaning the flesh (mostly muscular tissue) from the skull and postcranial skeleton bones before placing the specimen in the dry collection cabinets and drawers (Barquez et al. 2021). Despite the undisputed importance of preserving flesh tissue attached to bones and skin for DNA sampling of historical and ancient specimens (Mandrioli 2016; Abreu-Junior et al. 2020), appropriate cleaning of flesh from bone pieces intended for dry collections is required. Incompletely removed flesh can promote fungal growth and attract carnivorous invertebrates, which can feed on the flesh and damage the skins and delicate bones of specimens housed in the collection (Muñoz-Saba et al. 2020). Furthermore, properly cleaned skulls and skeletons allow for more accurate morphological analyses (Timm et al. 2020), such as the observation of sutures, scars, and foramina, mainly in small-bodied species.

Several methods for cleaning flesh from bones have been described in the literature (Muñoz-Saba et al. 2020), although the selected method to remove flesh from bones could affect the preservation of amplifiable DNA (Silverman 2018). The best-known, long-term, and widely used techniques in vertebrate collections include the use of scavenging beetles, specifically dermestids (*Dermestes* spp.) (Tiemeier 1939; Searfoss 1995; Bezerra 2012; Timm et al. 2020), and basic maceration, which consists of soaking the bones in cold water and changing the water daily (Muñoz-Saba et al. 2020). The first method is very efficient. Beetles clean flesh quickly and perfectly (Gomes y Mendes-Oliveira 2015; Timm et al. 2020; Muñoz-Saba et al. 2020), but the insect colony is very sensitive to changes in temperature and humidity, inbreeding depression, seasonal population fluctuations, diseases, and predation by other invertebrates (Muñoz-Saba et al. 2020). It is possible that in less than 24 hours, a colony that was once alive and efficient can have almost all of its individuals dead (pers. obs.). Also, this method is not as effective for cleaning specimens preserved in formalin or alcohol, requiring additional steps to achieve efficient bone cleaning without damaging the colony (Timm et al. 2020; Muñoz-Saba et al. 2020). The second most commonly used method is maceration which is also efficient, but time-consuming, often taking weeks and producing an unpleasant odor, and frequently requiring an isolated area designed for the process. It can also be particularly difficult when used on small skulls, especially in young or juvenile specimens, as it can easily decouple bones and teeth from the alveoli. Other common methods are hydrogen peroxide (H2O2) and boiling water (as suggested in Aggarwal et al. 2016). However, the first method tends to overbleach the bones, inhibiting morphological observation of scars and sutures, while the second method can cause severe damage to the delicate skulls of most small mammal species, and hot dissolved fat can adhere to skull surfaces (Muñoz-Saba et al. 2020).

Most morphological studies in mammalogy, particularly those focused on taxonomy, traditionally analyze skins, including color pattern and structure of fur, as well as skull and teeth (Avila-Pires 2011; Barquez et al. 2021). Additionally, the increase in fieldwork over the last decades, mainly driven by environmental impact assessment studies (most of them have few researchers available for proper preparation of the specimen), has led to the collection of a large number of specimens, mainly small non-flying mammals, prepared for wet storage in scientific collections or as skins with the respective non-defleshed carcasses (Moraes Neto et al. 2015; pers. obs.).

In the search for a bone cleaning method that is easy to use, not depending on dermestids, and avoiding damage of the cranial structures of small mammal skulls, it is important to highlight the blog 'Jakes's bones' by the young naturalist and self-proclaimed 'bone collector' Jake McGowan-Lowe (www.jakes-bones.com, ©2009-2016 Jake McGowan-Lowe, active up to date – accessed October 2023). Among the various methods he describes and applies, the use of biological washing powder caught our attention. This

method involves the use of a detergent (available in liquid or powder form) which contains enzymes to improve and accelerate the removal of organic particles. These enzymes can include proteases, amylases, cellulases, and lipases, which are commonly used for proper cleaning of medical equipment and tools, and other tasks such as laundry, dishwashing, and industrial equipment cleaning in the food industry (Valls et al. 2011; Hede 2020). Therefore, the aim of this study is to provide a step-by-step report of the controlled use of a multi-enzyme detergent for cleaning flesh from small mammal skulls of the orders Chiroptera, Didelphimorphia, and Rodentia.

Materials and Methods

Samples

Skulls from small mammal specimens of the orders Chiroptera, Didelphimorphia, and Rodentia housed at Museu Paraense Emílio Goeldi (Belém, Pará, Brazil), were extracted for experimentation to determine the required concentrations of multi-enzymatic detergent solution to dissolve flesh from bones, and the time taken for each step of the procedure. Skulls were extracted from whole preserved specimens or carcasses. The specimens were preserved in 70% ethanol, and some were previously frozen or injected with 10% formalin, as detailed in Table 1. The specimens used in the experiment were predominantly adults (individuals with all teeth erupted and functional). They were collected over a range of dates, from three to nearly 40 years ago, and various preservation methods had been used, including frozen (n = 10) and cryopreserved (n = 15) specimens, as described below and listed in Table 1. The skull length of the analyzed species ranged from 21.20 mm to 65.89 mm (Supplementary Data SD1). Collection data, preservation methods, and analyzed specimens are described as follows:

Didelphimorphia (n = 15): skulls used were from species *Marmosops marina* (n = 10), *Marmosa demerarae* (n = 1), and *Monodelphis americana* (n = 4) collected between 2018 and 2020 in Paragominas, Pará state, Brazil. The carcasses of five specimens (Table 1) were initially placed into zip lock bags and kept immersed in liquid nitrogen (inside a cryo-Dewar) at -120°C for up to 20 days, and then transferred to a -80°C freezer where they remained for 10.5 months. Their skulls were separated from the postcranial carcasses and placed in 70% ethanol, where they remained until the present experiment. The remaining 10 specimens were preserved as whole bodies in a -20 °C freezer from the time of collection until preparation of the skin for taxidermy and the subsequent detachment of the skulls from postcranial carcasses. These skulls were also placed in 70% ethanol, where they remained until the start of the present experiment.

Chiroptera (n = 19): skulls used were from from bat specimens of the genus *Artibeus* (Phyllostomyidae, Stenodermatinae) were used in this experiment. Ten specimens of *A. planirostris* collected during 1984 in Tucuruí, Pará state, five specimens of *A.*

obscurus and four specimens of *A. lituratus* collected in 2004 at the Amanã and Mamirauá Sustainable Development Reserves, Tefé, Amazonas state, Brazil. All specimens were fixed in 10% formalin and preserved in 70% ethanol.

Rodentia ($n = 80$): skulls used were from the species *Necromys lasiurus* ($n = 9$), *Oxymycterus delator* ($n = 1$) (Sigmodontinae), and *Proechimys* cf. *roberti* ($n = 1$) (Echimyidae) collected during 2016 in Ribeirão Cascalheira, state of Mato Grosso, Brazil. Skulls of the species *Oxymycterus* sp. ($n = 3$), and *N. lasiurus* ($n = 1$) collected during 2017 in Brasília, Federal District, Brazil. All specimens were preserved as whole carcasses in 92% commercial ethanol. A set of rodent specimens ($n = 65$) was collected and preserved as specimens of the order Didelphimorphia (as described above), including 10 specimens that were cryopreserved in liquid nitrogen (Table 1). The set was composed of skulls of sigmodontines *Calomys expulsus* ($n = 10$), *Hylaeamys megacephalus* ($n = 2$), *Hylaeamys yunganus* ($n = 5$), *N. lasiurus* ($n = 16$), *Oecomys* gr. *catharinae* ($n = 1$), *Oecomys* gr. *paricola* ($n = 7$), *Oecomys roberti* ($n = 2$), *Oecomys* sp. ($n = 1$), *Oligoryzomys microtis* ($n = 7$), *Pseudoryzomys simplex* ($n = 6$), and *Rhipidomys emiliae* ($n = 3$), and of equimid rodents of the species *Echimys chrysurus* ($n = 1$), *Makalata didelphoides* ($n = 1$), *Mesomys stimulax* ($n = 1$), and *Proechimys roberti* ($n = 2$).

General standard for cleaning flesh

In this experiment, the multi-enzymatic detergent Poderoso® from ©Kelldrin Group was used. This detergent contains five enzymes: protease, amylase, peptidase, cellulase, and lipase, with a minimum proteolytic activity of 0.07 mL-1.min.-1 and an amylolytic activity of 0.02 mL-1.min.-1 (Kelldrin 2022). The selection of this product brand was not based on any specific reason, but rather on the fact that it is readily available in Brazilian retailers as it is a Brazilian product. The manufacturer recommends a 0.2% dilution of the detergent in deionized water with pH range of 6.0–9.0 pH- at temperatures between 30°C and 40°C for cleaning medical tools, equipment, and floors (Kelldrin 2022).

Before immersing the skulls in the multi-enzymatic detergent solutions, the superficial muscles (lateral masseter, temporalis, frontalis, and occipitalis) and the eyes were carefully removed manually under a stereomicroscope in order to optimize the multi-enzymatic action. The complete workflow of the experiment is illustrated in Figure 1. Three different dilutions of multi-enzymatic detergent solution were prepared: 5%, 10%, and 15% (for example, 5 ml detergent in 100 ml water, 10 ml in 100 ml, and 15 ml in 100 ml, respectively). To standardize the test for size, shape, and anatomical structure, as well as to assess the effectiveness of flesh cleaning, at least one species or genus with adult specimens from each order of small mammals was selected to test the three solutions: genus *Marsomosops* (didelphid opossums), genus *Artibeus* (bats), and *N. lasiurus* (cricetid rodents) (Table 1).

Each skull was placed inside a labelled glass container filled with the designated solution, ensuring that the volume reached up to three times that of the skull. Then, the containers were placed in an oven set at 37°C or in a room maintained at a temperature range of 24°C to 26°C. The specimens were exposed to these conditions for a maximum of 24 hours (Table 1), with a duration of seven hours per day, corresponding to the laboratory working hours. Hourly inspections were carried out to monitor the cleanliness of the samples (verifying that there was no gelatinized flesh) and to assess any potential damage to the skulls. In addition, during these inspections, the solution was stirred for one minute to ensure a uniform mixing of the dilution and to accelerate the flesh degradation process (Simonsen et al. 2011). It is important to note that the duration of seven hours per day was chosen based on the available laboratory worktime. Therefore, caution was advised to avoid any potential negative effects of an over-enzymatic action on the bone structures, as the experiment was aimed to establish the optimal cleaning time needed to remove flesh from the skulls without compromising their integrity.

The skulls were removed from the solution and placed in a cooler environment at 18°C (room temperature controlled by a split air conditioning system), stopping the enzymatic action until the next day, when the skulls were returned to their respective containers with the multi-enzymatic detergent solution. Before being placed back in the container, an initial cleaning step was carried out under the stereomicroscope using thin-tipped medium-sized tweezers. This step was performed to remove muscle fibres from some skulls with less gelatinous flesh (Figure 1). Subsequently, the skulls were removed from the solution and rinsed under running water in a tray. A soft-headed toothbrush was used to gently remove the gelatinous flesh during rinsing. After this step, the skulls were immersed in 92% ethanol for 30 min to stagnate any ongoing proteolytic process before final cleaning.

The cleaning process was finished under the stereomicroscope, with the help of thin-tipped medium-sized tweezers and a small scalpel (blade number 12). Subsequently, skulls were immersed again in 92% ethanol for 24 hours to ensure the complete cessation of the proteolytic action of the multi-enzymatic detergent. After this period, the skulls were analyzed again under the stereomicroscope. If the skulls were completely clean of flesh, they were dried at a temperature of 18°C. The analysis under stereomicroscope also aimed to identify any signs of bones or teeth detachment or visible cracking resulting from the enzymatic detergent maceration. The clean skulls and mandibles received their respective catalogue numbers with a 0.01 ink pen. The temperature (in °C) during the experiments was measured using an analog thermometer.

Table 1. Solution dilution (%), Time (h/min) and Temperature (Room [24°C–26°C], Oven [37°C]) for flesh cleaning* the of the small mammal skulls. Including the mammalian order and species (some at genus level), voucher number (young or juveniles in bold), collection year (Year), preservation type (1 = cryopreserved, 2 = 10% formalin solution, 3 = 70% ethanol, 4 = 92% ethanol). Abbreviation: N = number of processed skulls. Voucher acronyms: ARB = filed number of Alexandra M. R. Bezerra (to be deposited in the MPEG mammal collection); MPEG = Museu Paraense Emilio Goeldi, Belém, Pará, Brazil.

Order	Species	Dilution	Time	Temperature	Voucher	Year	N	Preservation type
Didelphimorphia	*Marmosa demerarae*	5%	24h	Room	MPEG 46205	2020	1	1, 3
Didelphimorphia	*Marmosops marina*	5%	4h	Oven	MPEG 46180, 46193	2019	2	3
Didelphimorphia	*Marmosops marina*	5%	24h	Room	MPEG 46203, 46208, 46209, 46220	2020	4	1, 3
Didelphimorphia	*Marmosops marina*	10%	4h15min	Oven	MPEG 46160, 46162, 46163, 46165	2019	4	3
Didelphimorphia	*Monodelphis americana*	5%	4h	Oven	MPEG 46168, 46170, 46182	2019	3	3
Didelphimorphia	*Monodelphis americana*	10%	2h30min	Oven	MPEG 46124	2018	1	3
Chiroptera	*Artibeus planirostris*	5%	16h	Oven	MPEG 32074, 32075, 32078	1984	3	2, 3
Chiroptera	*Artibeus obscurus*	5%	16h	Oven	MPEG 37754	2004	1	2, 3
Chiroptera	*Artibeus lituratus*	5%	16h	Oven	MPEG 37757, 37764	2004	2	2, 3
Chiroptera	*Artibeus planirostris*	10%	16h	Oven	MPEG 32080, 32081, 32083	1984	3	2, 3
Chiroptera	*Artibeus planirostris*	10%	7h	Oven	MPEG 32079	1984	1	2, 3
Chiroptera	*Artibeus obscurus*	10%	16h	Oven	MPEG 37736, 37756, 37758	2004	3	2, 3
Chiroptera	*Artibeus planirostris*	15%	16h	Oven	MPEG 32084, 32085, 32086	1984	3	2, 3
Chiroptera	*Artibeus obscurus*	15%	16h	Oven	MPEG 37750	2004	1	2, 3
Chiroptera	*Artibeus lituratus*	15%	16h	Oven	MPEG 37759, 37766	2004	2	2, 3

Order	Species	Dilution	Time	Temperature	Voucher	Year	N	Preservation type
Rodentia	*Calomys tener*	5%	2h25min + 3h40min = 6h05min	Room + Oven	MPEG **46172**	2019	1	3
Rodentia	*Calomys tener*	5%	2h25min + 4h40min = 7h05min	Room + Oven	MPEG **46171,** 46173, 46191, 46198, 46200, 46202	2019	6	3
Rodentia	*Calomys tener*	5%	24h	Room	MPEG 46206	2020	1	1, 3
Rodentia	*Calomys tener*	10%	2h30min	Oven	MPEG **46123, 46127**	2018	2	3
Rodentia	*Echimys chrysurus*	5%	24h	Room	MPEG **46213**	2020	1	1, 3
Rodentia	*Hylaeamys megacephalus*	15%	3h	Oven	MPEG 46154, 46155	2018	2	3
Rodentia	*Hylaeamys yunganus*	5%	4h30min	Oven	MPEG 46159, 46175	2019	2	3
Rodentia	*Hylaeamys yunganus*	5%	24h	Room	MPEG 46211	2020	1	1, 3
Rodentia	*Hylaeamys yunganus*	10%	4h30min	Oven	MPEG 46177, **46179**	2019	2	3
Rodentia	*Makalata didelphoides*	5%	24h	Room	MPEG **46204**	2020	1	1, 3
Rodentia	*Mesomys stimulax*	10%	4h47min	Oven	MPEG 46174	2019	1	3
Rodentia	*Necromys lasiurus*	5%	2h25min + 3h40min = 6h05min	Room+Oven	MPEG **46187, 46188, 46189,** 46195	2019	4	3
Rodentia	*Necromys lasiurus*	5%	4h47min	Oven	MPEG 46161, 46166, 46167, 46185, 46201	2019	5	3
Rodentia	*Necromys lasiurus*	5%	17h	Oven	ARB 839, 840, 841	2016	3	4
Rodentia	*Necromys lasiurus*	5%	24h	Room	MPEG 46214	2020	1	1, 3
Rodentia	*Necromys lasiurus*	8%	24h	Room	ARB 873	2017	1	4
Rodentia	*Necromys lasiurus*	10%	4h47min	Oven	MPEG 46186, 46197	2019	2	3
Rodentia	*Necromys lasiurus*	10%	17h	Oven	ARB 842, 843, 849	2016	3	4
Rodentia	*Necromys lasiurus*	15%	4h	Oven	MPEG 46137, 46139, 46144, 46152	2018	4	3

Order	Species	Dilution	Time	Temperature	Voucher	Year	N	Preservation type
Rodentia	*Necromys lasiurus*	15%	17h	Oven	ARB 858, 864, 868	2016	3	4
Rodentia	*Oecomys* gr. *catharinae*	15%	4h	Oven	MPEG 46151	2018	1	3
Rodentia	*Oecomys roberti*	10%	2h30min	Oven	MPEG 46126	2018	1	3
Rodentia	*Oecomys roberti*	10%	4h47min	Oven	MPEG 46164	2019	1	3
Rodentia	*Oecomys* gr. *paricola*	5%	24h	Room	MPEG 46215	2020	1	1, 3
Rodentia	*Oecomys* gr. *paricola*	5%	2h25min + 4h40min = 7h05min	Room + Oven	MPEG 46176, **46183**, **46184**	2019	3	3
Rodentia	*Oecomys gr. paricola*	10%	2h30min	Oven	MPEG 46133, 46141, **46148**	2018	3	3
Rodentia	*Oecomys* sp.	15%	4h	Oven	MPEG 46178	2019	1	3
Rodentia	*Oligoryzomys microtis*	5%	2h25min + 4h40min = 7h05min	Room + Oven	MPEG 46169, **46196**	2019	2	3
Rodentia	*Oligoryzomys microtis*	5%	24h	Room	MPEG 46207, 46212, 46216	2020	3	1, 3
Rodentia	*Oligoryzomys microtis*	10%	2h30min	Oven	MPEG **46140, 46153**	2018	2	3
Rodentia	*Proechimys roberti*	5%	24h	Room	MPEG **46210**	2020	1	1, 3
Rodentia	*Proechimys roberti*	10%	4h30min	Oven	MPEG **46181**	2019	1	3
Rodentia	*Proechimys* cf. *roberti*	8%	17h	Oven	ARB 834	2016	1	4
Rodentia	*Oxymycterus delator*	8%	17h	Oven	ARB 860	2016	1	4
Rodentia	*Oxymycterus* sp.	8%	24h	Room	ARB 872, 874, 876	2017	3	4
Rodentia	*Pseudoryzomys simplex*	5%	2h25min + 4h40min = 7h05min	Room + Oven	MPEG **46190, 46194**	2019	2	3
Rodentia	*Pseudoryzomys simplex*	10%	4h47min	Oven	MPEG **46192**, 46199	2019	2	3
Rodentia	*Pseudoryzomys simplex*	15%	2h30min	Oven	MPEG 46125, **46150**	2018	2	3
Rodentia	*Rhipidomys emiliae*	10%	2h30min	Oven	MPEG 46149	2018	1	3
Rodentia	*Rhipidomys emiliae*	15%	3h	Oven	MPEG 46156, 46158	2018	2	3

* Time before the final brushing and the cleaning under the stereomicroscope.

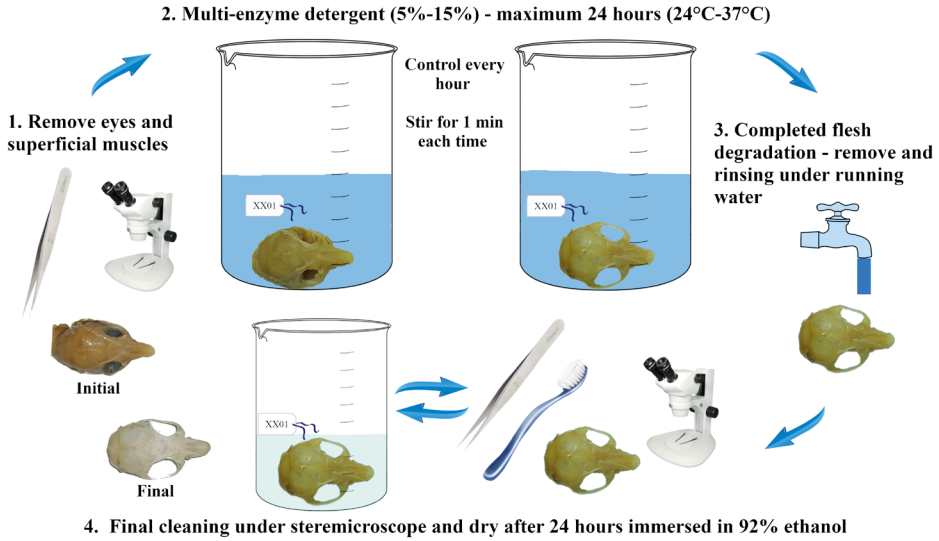

Figure 1. Workflow for clearing flesh from skulls applying multi-enzymatic detergent, using the skull of a specimen of *Oecomys* gr. *paricola* (MPEG 46215) as an example. PNG pictures from www.cleanpeng.com.

Other tests performed

After testing three different solution dilutions, a further intermediate dilution between the lower concentration solutions previously used of 5% and 10% was tested. This intermediate dilution consisted of 8% of the multi-enzymatic detergent (that is, 8 ml detergent in 100 ml water). All skulls were subjected to the same process of evaluating the effectiveness for flesh cleaning and identifying any visible macroscopic damage, following the steps described above (Table 1).

Results

A total of 54 skulls were analyzed using a 5% solution concentration, 6 skulls at 8% solution concentration, 33 skulls at 10% solution concentration, and 21 skulls at 15% solution concentration. All concentration solutions of the multi-enzymatic detergent effectively cleaned the flesh from the skulls (Figure 2, Supplementary Data SD2), without any limitation due to the time elapsed since specimen collection or the type of specimen preparation (Table 1). The degree of proteolytic action on tissues appears to be inversely proportional to the concentration of the solution, with higher concentration resulting in more gelatinous conversion of flesh tissues in the skulls, such that higher dilutions require less time for the dissolution of tissues (Figure 3).

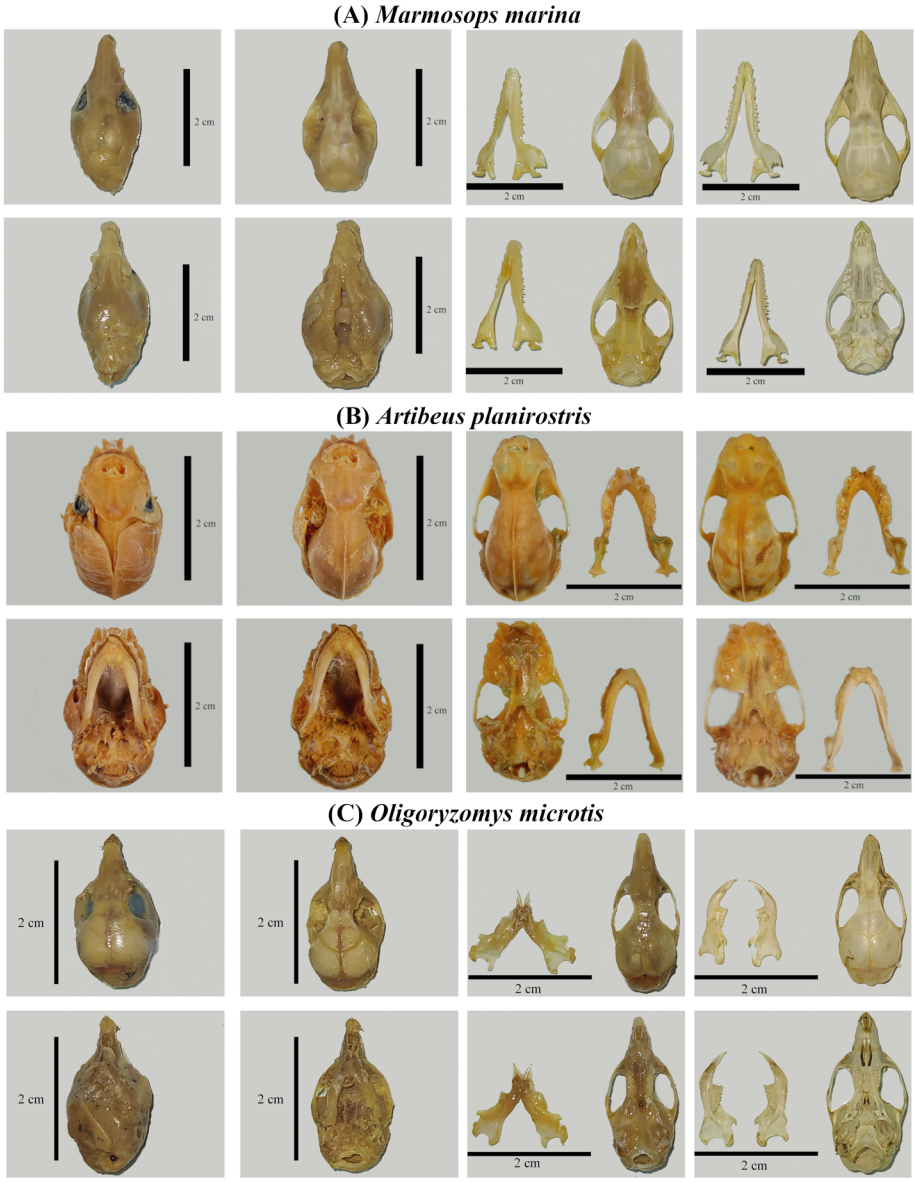

Figure 2. Skulls of some specimens during the complete multi-enzymatic detergent cleaning process. From left to right: 1st. skull with flesh and eyes before starting the cleaning process; 2nd. after the first manual cleaning to remove excess flesh and eyes; 3rd. after some hours immersed in a multi-enzymatic detergent solution, with the mandible detached from the skull; 4th. skull and mandible dried after complete cleaning. (A) opossum *Marmosops marina* (MPEG 46203), (B) bat *Artibeus planirostris* (MPEG 32079), and (C) rodent *Oligoryzomys microtis* (MPEG 46212) (details in Table 1). More examples can be found in Supplementary Data D2.

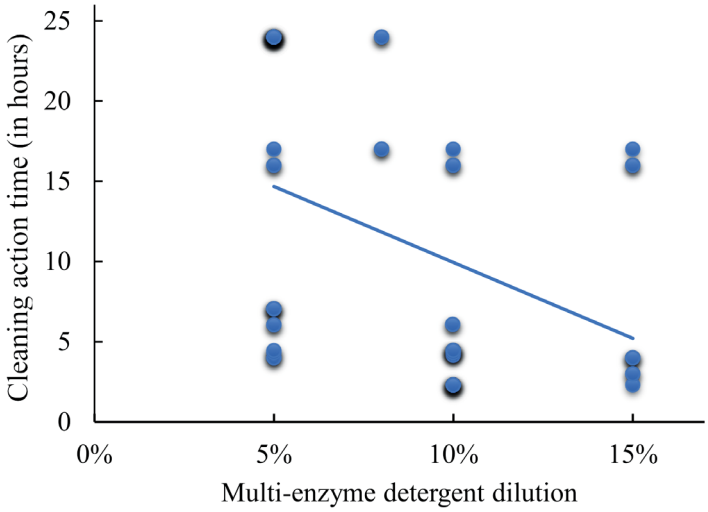

Figure 3. Graph showing the time (in hours) required for each skull cleaning (blue dots) with the multi-enzymatic detergent solution, where the trend curve indicates the shortest time required for higher solution concentrations. The shaded areas around the blue dots indicate the accumulative number of skulls cleaned.

No damage or weakening was observed in the teeth and skull structures of the adult specimens fixed in 10% formalin solution and 70% ethanol fluid preserved, or preserved only in 92% ethanol. However, skulls that were cleaned using the highest concentration solutions (such as 10% and 15%) showed a color alteration: the gelatinized flesh on these skulls turned translucent green (the multi-enzymatic detergent itself is light blue), including along cranial sutures. However, this color disappeared after rising under running water and removing the gelatinized flesh with a delicate brush. On the other hand, all cryopreserved specimens had to be removed from the solution in approximately 7 hours (7h05min) because of the noticeable weakness of the bones due to freezing with liquid nitrogen already before the test (the postcranial skeletons and the skins of these specimens were also placed in the same cryo-Deweler and became dehydrated and brittle). As previously mentioned, the final cleaning process was carried out under stereomicroscope after immersing the skulls in 92% ethanol for 24 hours. Once completely cleaned, the skulls were dried at a temperature of 18°C.

Discussion

The effectiveness of using a multi-enzymatic detergent to remove flesh from small mammal skulls, obtained from fluid and frozen specimens, was researched in this

study. The results were very satisfactory, especially when the highest concentration (15%) was used, although all tested concentrations (5%, 8%, and 10%) were effective for tissue degradation. Similar to the findings in this study, Simonsen et al. (2011) also found that stirring the solution increased the time needed for the cleaning process. Although no macroscopic damage was observed on the bones and teeth of both the oldest specimens (collected in 1984) and the newest specimens (collected in 2020), it is recommended to monitor the samples every hour during the cleaning process. This is crucial because it is not always possible to know how the specimen was originally preserved, as is the case with the cryopreserved specimens tested here. The proteolytic action of the enzymes could potentially damage the bones and detaching sutures and teeth.

It is also recommended to consider the use of lowest concentration solutions on some specimens, mainly for specimens that are juvenile or have slender skulls, such as the rodents of the genus *Calomys* and smaller didelphids of the genera *Marmosops* and *Monodelphis*. Silverman (2018) found severe water damage and coarse texture on the long bones and metacarpals of *Sus scrofa* after using a flesh removal method by simmering a 10% concentration solution of protease -amylase based detergent. The method used in this study differs in some aspects from the one used by Silverman: first of all, the detergent solution was not simmered, the skulls were just immersed in it; secondly, after rinsing the skulls with water, they were immersed in 92% ethanol for 24 hours, effectively stopping any ongoing proteolytic process.

The use of protease and lipase enzymes has been useful for accelerate maceration methods in forensic science (Mairs et al. 2004; Simonsen et al. 2011; Uhre et al. 2015). Several studies have also reported the use of common household detergent to remove soft tissues from human bones (Uhre et al. 2015) and other mammalian species, such as *Sus scrofa* (Husch et al. 2021)*, Mus musculus* and *Rattus norvegicus* (Simonsen et al. 2011). However, the joint use of detergent and enzymes, in the case of the multi-enzymatic detergent for cleaning flesh from bones in mammal preparations in natural history collections, has been minimally investigated (Fernández-Jalvo y Monfort 2008, Leeper 2015), and its standardized use for small mammals has not been described so far. In fact, this study used a dilution solution similar to that of Leeper (2015), who also tested 5%, 10%, and 15% concentrations of the commercial enzyme-active laundry detergent (Biz®) to thoroughly clean white-tailed deer (*Odocoileus virginianus*) skulls, but at a temperature between 75°C and 80°C during the process. Since small mammals have more delicate bones and teeth, it is recommended to use an optimal temperature for enzymes, around 25°C to 37°C. *Final Considerations.* Multi-enzymatic detergent is expensive compared to dermestid and other methods (Silverman 2018), it requires more careful storage, and has a shorter shelf-life time (3M™ 2018; Kelldrin® 2022). However, a five-liter bottle of multi-enzymatic detergent (like the one used in this experiment) can produce

approximately 33 to 100 liters of solution when diluted (from highest to lowest dilution used here, respectively). This quantity allows for the easy cleaning of hundreds of small mammal skulls (depending on the dilution factor), regardless of the preservation type used, including formalin and other methods where dermestids are not as effective. Furthermore, although the samples for DNA extraction and quality have not been tested in this study, previous studies have shown that the use of detergent and enzymes preserve the DNA (Uhre et al. 2015), with sequenced samples queried between 99% and 100% for mitochondrial DNA of the target species (Silverman 2018). It is expected that this study serves as an important tool for curatorial management of scientific collections.

Acknowledgments

We are thanks to José de S. Silva Jr "Cazuza" for providing us complete access to the mammal collection in MPEG; Celso Moraes and Airton da Luz for supporting in the MPEG laboratory collection; Alberto Akama and Guillherme Garbino made valuable comments on the previous version of the manuscript. ACSN received fellowship from PIBIC/CNPq/MPEG (processes 145331/2017-2, 128589/2018-3, 128572/2019-1, 152753/2020-6); ARB received research fellowship from PCI/CNPq/MPEG 2019-2023; HC received PhD fellowship from CNPq (process 142891/2021-5).

Supplementary Data

Supplementary Data SD 1. Skull length range data for (CBL, CIL = condylobasal length, GLS = greatest length of skull, ONL = occipitonasal length, or SL = Skull length) of the species used in the study. The measurements (in millimeters) are from the literature, unless otherwise specified.

Supplementary Data SD 2. Some other specimens that were cleaned during the experiment.

References

3M (2018). *Boletim técnico 3M Detergente Multienzimático 4E*. Saint Paul, Minnesota, USA. https://multimedia.3m.com/mws/media/1589158O/technical-bulletin-multienzyme-detergent-4e.PDF. Accessed 21 June 2023.

Abreu-Júnior, E.F., Pavan, S.E., Tsuchiya, M.T.N., Wilson, D.E., Percequillo, A.R. y Maldonado, J.E. (2020). Museomics of tree squirrels: a dense taxon sampling of mitogenomes reveals hidden diversity, phenotypic convergence, and the need of a taxonomic overhaul. *BMC Evolutionary Biology*, *20*, 77. https://doi.org/10.1186/s12862-020-01639-y

Aggarwal, N., Gupta, M., Goyal, P.K. y Kaur, J. (2016). An alternative approach to bone cleaning methods for anatomical purposes. *International Journal of Anatomy and Research*, *4*(2) 2216–2221. https://doi.org/10.16965/ijar.2016.181

Avila-Pires, F.D. (2011). Mudanças nas práticas de coleta e estudos de mamíferos a partir do século XIII. *Filosofia e História da Biologia, 6*, 211–226. https://www.abfhib.org/FHB/FHB-06-2/FHB-6-2-03-Fernando-Dias-de-Avila-Pires.pdf

Barquez R.M., Díaz, M.M., Berrizbeitia, M.F.L. y Mollerach, M.I. (2021). *Colección de Mamíferos Lillo: un manual de procedimientos para la preparación y conservación de mamíferos y anexos*. Publicaciones Especiales PDBA N° 6, Tucumán, Argentina. http://hdl.handle.net/11336/156758

Bezerra, A.M.R. (2012) [2013]. Coleções Científicas de Mamíferos. I - Brasil. *Boletim da Sociedade Brasileira de Mastozoologia*, 65, 19–25.

Fernándes-Jalvo, Y. y Monfort, M.D.M. (2008). Experimental taphonomy in museums: preparation protocols for skeletons and fossil

vertebrates under the scanning electron microscopy. *Geobios*, *41*,157–181. https://doi.org/10.1016/j.geobios.2006.06.006

Gomes, L.B. y Mendes-Oliveira, A.C. (2015). Montagem e manutenção de colônias de besouros do gênero *Dermestes* (Coleoptera, Dermestidae) Linnaeus, 1758 para preparação de esqueletos usados em coleções biológicas. *Boletim da Sociedade Brasileira de Mastozoologia*, *73*, 37-41.

Hede, D.P. (2020). *A beginner's guide to enzymes in detergents*. Household Care, Novozymes. *Bagsværd*, Denmark. https://biosolutions.novozymes.com/en/dish/insights/article/beginners-guide-enzymes-detergents. Accessed 21 June 2023.

Husch, C., Berner, M., Goldammer, H. y Lichtscheidl-Schultz, I. (2021). Technical note: a novel method for gentle and non-destructive removal of flesh from bones. *Forensic Science International*, *323*, 110778. https://doi.org/10.1016/j.forsciint.2021.110778

Kelldrin (21 June 2020). *Poderoso Detergente Multienzimático*. https://kelldrin.com.br/produto/poderoso-detergente-multienzimatico/

Leeper, B.J. (2015). *Evaluation of current methods of soft tissue removal from bone*. Dissertation, University of Pittsburgh, Pittsburgh, Pennsylvania, USA. http://d-scholarship.pitt.edu/26725/1/Leeper_ETD_2015_2.pdf. Accessed 21 June 2023.

Mairs, S., Swift, B. y Rutty, G.N. (2004). Detergent: an alternative approach to traditional bone cleaning methods for forensic practice. *The American Journal of Forensic Medicine and Pathology*, *25*, 276–284. https://doi.org/10.1097/01.paf.0000147320.70639.41.

Mandrioli, M. (2016). MUSEomica: quando la genomica entra in museo. *Quaderni del Museo Civico di Storia Naturale di Ferrara*, 4, 53–70. https://storianaturale.comune.fe.it/modules/core/lib/d.php?c=pqEP3

Moraes Neto, C.R, Lazar, A. y Pessôa, L.M. (2015). Taxidermia de pequenos mamíferos não voadores previamente preservados em meio líquido. *Boletim da Sociedade Brasileira de Mastozoologia*, *73*, 92–94.

Muñoz-Saba, Y., Sánchez-Nivicela, J.C., Sierra-Durán, C.M., Vieda-Ortega, J.C., Amat-García, G., Munoz, R., Casallas-Pabón, D. y Calvo-Roa, N. (2020). Cleaning osteological specimens with beetles of the genus *Dermestes* Linnaeus, 1758 (Coleoptera, Dermestidae). *Journal of Natural Science Collections*, 7, 72–82. http://www.natsca.org/article/2584

Searfoss, G. (1995). *Skulls and Bones: a guide to skeletal structures and behavior of North American mammals*. Stackpole Books, Mechanicsburg, Pennsylvania, USA.

Silverman, E.S. (2018). *The effects of common methods of soft tissue removal on skeletal remains: a comparative analysis*. Master's Thesis, University of Montana, Missoula, USA. https://scholarworks.umt.edu/cgi/viewcontent.cgi?article=12240&context=etd Accessed on 12 May 2023.

Simonsen, K.P., Rasmussen, A.R., Mathisen, P., Petersen H. y Borup, F. (2011). A fast preparation of skeletal materials using enzyme maceration. *Journal of Forensic Science*, *56*(2), 480–484. https://doi.org/10.1111/j.1556-4029.2010.01668.x

Tiemeier, O.W. (1939). The dermestid methods of cleaning skeletons. *The University of Kansas, Science Bulletin*, *26*, 377–383. https://www.biodiversitylibrary.org/partpdf/38656

Timm, R.M., McLaren, S.B. y Genoways, H.H. (2020). Innovation that changed Mammalogy: dermestid beetles-the better way to clean skulls. *Journal of Mammalogy*, *101*, 923–925. https://doi.org/10.1093/jmammal/gyaa067

Uhre, M.L., Eriksen, A.M., Simonsen, K.P., Rasmussen, A.R. Hjort, B.B. y Lynnerup, N. (2015). Enzymatic maceration of bone: a gentler technique than boiling. *Medicine, Science, and the Law, 55*, 90–96. https://doi.org/10.1177/0025802414532246

Valls, C., Pujadas, G., Garcia-Vallve, S. y Mulero, M. (2011). Characterization of the protease activity of detergents: laboratory practicals for studying the protease profile and activity of various commercial detergents. *Biochemistry and Molecular Biology Education*, *39*, 280–290. https://doi.org/10.1002/bmb.20488