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THE EFFECTS OF COMMON METHODS OF SOFT TISSUE REMOVAL ON SKELETAL REMAINS: A COMPARATIVE ANALYSIS

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The Effects of Common Methods of Soft Tissue Removal on Skeletal Remains: A Comparative Analysis

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Abstract

The removal of soft tissue from skeletal remains is a process familiar to a wide array of scientific fields and the methods used to perform it are likewise numerous yet inconsistent. In forensic investigations and crime labs across the country, there lacks a standardization for this process. This lack of standardization pairs with a distinct lack of literature on the potential benefits and risks associated with each method as well as basic information on the proper amount of additives, temperatures, or time estimations. In a forensic context, human remains may be the only evidence available, which makes any damage or loss of material particularly detrimental, and this lack of knowledge on the effects of common methods not only negligent, but dangerous.

In this research, domesticated pig (*Sus scrofa domesticus*) limbs were obtained as a human proxy to study the effects of five distinct but commonly used flesh removal methods: dermestid beetles (*Dermestes lardarius*), distilled-water boil, bleach boil, ammonia simmer, and enzyme-based detergent simmer. Each pig limb was weighed and measured before being randomly selected for one of the five methods with each method being done in three separate trials. Each method was evaluated based on a set of specific criteria, focusing primarily on time efficiency, cost, damage, and the effects on DNA extraction from the remaining bone sample.

While the dermestid beetles had the longest time-expectancy, they caused the least amount of damage to the bone surface and DNA quality. The bleach, while severely hindering the ability to amplify DNA, was the quickest of the methods and cleaned them the most efficiently. While the ammonia was the most potent of the methods, it was efficient, low-cost, and left amplifiable DNA. No method performed the worst in every criterion evaluated, nor did any method perform the best. Each method proved to have different advantages and disadvantages, whether the disadvantages were higher cost, long time expectancy, or destruction of DNA. The results of this research highlight how differently each method performs and how easily bone material can be affected. Method selection is a decision that can severely impact later research and analysis, and demands to be done with more consideration and awareness of the potential risks and desired results.

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Table of Contents

List of Figures

List of Tables

1.0 Introduction

The removal of soft tissue is a process familiar to members of a wide array of scientific fields and contexts, for skeletal remains that are both human and non-human. Due to the highly variable goals and intentions behind flesh removal, the methods used are likewise numerous and inconsistent. In forensic investigations and crime labs across the country there lacks a standardization for this process, sometimes even within a single crime lab; multiple different methods can be used within one facility depending on resources available, or the specific researcher's preference. This lack of standardization pairs with a distinct lack of literature on the potential benefits and risks associated with each method, as well as even basic agreed upon information and instruction on the proper amount of additives, temperatures, or time estimations. Unlike in taxidermy or a museum, in a forensic context human remains may be the only evidence available, which makes any damage or loss of material particularly detrimental, and this lack of knowledge on the effects of common methods not only negligent, but dangerous.

The following thesis project focuses on several commonly used soft tissue removal methods, documenting in detail the amount of additive used, the amount of time each took, and the effects the method had on the resulting skeletonized bones. Because the goals and intentions behind soft tissue removal tend to vary widely between fields and contexts, the results are designed with a specific emphasis on the viability and practicality of the method within a forensic context, but the general information can be relevant for any field familiar with the task.

1.1 Common Methods of Soft Tissue Removal

The removal of soft tissue from skeletal remains is not something reserved for forensic fields of research, nor only performed on human remains. The need for soft tissue removal on skeletal specimens spreads across a multitude of different fields and serves a variety of diverse functions. Flesh removal is used in museums to prepare bones for display and for comparative research, and hunters clean their kill. Even strictly narrowing it to an anthropological context, it still serves a variety of purposes. Body farms and physical anthropologists use recently skeletonized remains of modern populations to perfect and update the markers used in biological profile construction and analysis. Due to the wide array of fields and intentions behind the removal of soft tissue from skeletal remains, there is an equally wide array of potential methods to perform the job that are regularly in use.

A single literature search in a search engine can show you the wide variation of methods employed, diversified by materials available, time constraints, and preference of the individual performing the task (Mann and Berryman 2011). What is clearly lacking in the literature, however, are studies specifically looking at the task of soft tissue removal itself. Many use the remains, but few explain the possible effects the method may have on the bone, some even neglect to address how they performed the flesh removal, paying no attention to the possible effects this choice could have on the results.

Oftentimes, multiple methods must be employed to properly clean remains, particularly in methods that are thought to be less destructive but far more time consuming. There are methods more physically demanding such as manually removing flesh with a scalpel and dissection tools, a method commonly seen as inappropriate for human remains and also requiring

a high amount of handling and additional methods to deem properly cleaned. More popular methods include those that require little physical labor and preparation on the part of the researcher, such as traditional standstill maceration and heated maceration treatments. These types of methods, being the ones that are more commonly used within a forensic application, will be largely what is focused on in the following pages, including traditional maceration, heated chemical treatments, heated enzymatic treatments, and dermestid beetles.

1.1a Maceration

Maceration may be the most well-known and oldest of the soft tissue removal methods and although it will not be attempted in its traditional form in the following study, its notoriety warrants a discussion to explain the arguments against it. Maceration is a term used in a variety of different contexts, from food to biology, but at the core, the definitions are all basically the same thing: the process by which a substance is soaked in a water solution relying on bacterial growth to break down the material (Mairs et al 2004). In its simplest form within skeletal preparation, this is simply employed by soaking a fleshed bone in a closed container of water kept at a constant lukewarm temperature (Couse and Connor 2015). The application of maceration may also involve the addition of additives such as ammonia or dish detergent creating a solution to quicken the process and assist in the removal of grease that otherwise remains on the bone in just a water soak (Couse and Connor 2015; Frank et al 2015; Mairs et al 2004). The soaking of remains can take weeks to months depending on the prior condition of the sample. Due to the long-anticipated time frame required for maceration, human remains are not

generally exposed to this treatment in a forensic context, it is however commonly used in an educational context due to the non-invasive nature and low cost.

This method, evaluated solely in its traditional state, is not able to completely clean bones or remove all the fat and grease left behind; additives or additional post-labor is necessary to produce the desired result. The long-term soaking in water can furthermore cause a staining on the bones that may cause taphonomic or traumatic damage to be overlooked or mislabeled. The time expectancy is only one of many concerns with the method, however. The bacterial break down process produces foul odor gases and must be changed regularly to prevent mold growth that may damage the bones (Searfoss 1995). This procedure involves physically removing the separating tissues from the bone and with no heat to disinfect the remains, the bacterial growth can risk the health and safety of both the researcher and the remains themselves (Mairs et al 2004).

Despite the concerns with standstill traditional maceration, a multitude of maceration variations exist, with varying levels of success. In a recent study, Crouse and Conner (2015) separate maceration techniques into two categories: “physical maceration” and “warm water maceration.” They entirely ignore the older, time-consuming room temperature water only method. In their study they look at feline skulls and bear paws, examining the effects of two physical maceration techniques and three warm water techniques. The physical maceration techniques employed included physically removing the soft tissue by hand with a scalpel and forceps before employing a scouring pad and water to remove any residual tissue left over. While this may be a minimally expensive technique, the amount of handling required and potential for damage to the bone surface that could later obstruct trauma analysis causes the method to be impractical. The method additionally requires other methods to be used in conjunction, with a

softening phase where remains were still exposed to chemical solutions to assist in loosening the flesh from the bone needing to occur beforehand and additional water application after. The second physical maceration technique used by Crouse and Connor was power washing, using as the name suggests a power washer to spray the bones clean. It almost goes without saying to note that while this method may be time efficient, it is potentially destructive to the skeletal remains, not to mention costly and shambolic. The amount of required handling and potential damage makes both these physical maceration methods completely inappropriate for use in a forensic context, a fact acknowledged by the researchers (Couse and Connor 2015), and therefore it has not been considered for comparison in the following study.

Heated maceration methods have long been the more favored and investigated method due to the shorter time frame and non-invasive nature, with simmering and boiling techniques utilizing a variety of additives becoming common practice (Couse and Connor 2015). The additives range from using household cleaning products such as dish and laundry detergent to chemicals such as sodium perborate and carbonate, while still others remain utilizing a heated water solution on its own (Lee et al 2010; Uhre et al 2015).

While maceration techniques vary widely in terms of physical labor and additives used, the simple conclusion across the literature is clear: the traditional method of soaking remains for several months is not applicable in a forensic context, nor are physical methods that require exposing the bones to physical handling and destructive results. The simmering techniques are thought to be more ideal in respect to both time and safety, although the wide variety of additives have their own effects that must be kept in consideration when selecting a soft tissue removal method.

1.1b Chemical Additives

The addition of cleaning elements to simmering or boiling water, as previously addressed in the discussion of more traditional maceration techniques, is not an uncommon concept in forensic anthropology when it is necessary to remove the soft tissue from remains. Unfortunately, oftentimes these additives are grouped together with no differences assigned to them and little mention of the different effects they could have on the remains themselves. Couse and Connor (2015) are no exception to this with the two additives attempted in warm maceration techniques being grouped together in the same section and the only difference between the two being mentioned was the slightly more efficient performance of one over the other.

The sole consistency that the chemical additives seem to possess is the tendency to rely on base solutions due to their reaction with the acidic molecules in the skin and ability to dissolve fatty acids and oils, causing increased breakdown of soft tissue and decreased greasiness in the final result. The common additives range from enzyme-based laundry detergents, to ammonia, to sodium perborate and carbonate (Ecklund 2007). Documents regarding these methods vary from simmering to soaking overnight, to alternating between different solutions. The interest in what method is most efficient has certainly increased in recent years (Ecklund 2007; Frank et al 2015; Mann and Berryman 2012), but there still lacks any universally agreed protocol or even cohesive agreement on which additives may be more damaging than others. While many of these methods and additives need to be explored in more detail, there are various factors that have a higher priority over others and the literature reflects this; macroscopic damage affecting the ability to do DNA extraction and identification analysis (Ecklund 2007; Frank et al 2015; Lynn and Fairgrieve 2009).

Bleach

Bleach, with an active ingredient of sodium hypochlorite, is perhaps the most commonly known of additives, and perhaps the most controversial of them. There are a variety of different ways to involve bleach in the flesh removal process, from soaking directly in bleach to creating a water solution and simmering it under careful monitoring (Mann and Berryman 2012). While some of these methods have been used since the 1980s by the University of Tennessee body farm and Dr. Bill Bass, generally hailed with great success, the ability to extract DNA from human remains has caused the method to face new reasons for criticism (Ecklund 2007). While the simmering of remains is already a method that must be dealt with under great care and supervision, the bleach can severely hinder the ability to extract amplifiable DNA from the samples (Eklund 2007; Rennick et al 2005). In a method explanation done by Mann and Berryman (2012), they demonstrated a method differing from the others mentioned, involving neither boiling temperatures nor diluting the bleach in a water solution, instead the fleshed remains were placed straight into household bleach. They deemed this method quick and efficient but acknowledged that it is only practical in a circumstance where DNA extraction is not a factor. While bleach methods are commonly criticized and just as commonly defended, the search for a substitute has led to a variety of different chemical materials being employed in the soft tissue removal process with far less data on the potential effects of them.

Ammonia

Household ammonia is a diluted solution of water and ammonia hydroxide that is considered a weak base and while not currently found in forensic context of flesh removal, is a

process that can be commonly found outside of it (Hoffmeister and Lee 1963; National Park Service 2006). It is typically used for the last stage to remove any remaining grease after remains have had their soft tissue removed by dermestid beetles or bacterial maceration (Lee et al 2010). In several select publications, however, there has been a documentation of the use of dilute ammonia in a simmering water solution throughout the entire soft tissue removal process of mammalian specimens (Hoffmeister and Lee 1963; National Park Service 2006). The method was shown to be a quick and effective method in mammalian skeletal preparation but has not had any documented use on human remains. This method, like other heated maceration methods, does require careful and diligent monitoring so as not to let the solution boil or overcook. The heating of ammonia additionally causes an increased amount of pungent odor that can produce toxic and suffocating fumes that may be a health and safety risk to researchers while monitoring and disposing of the solution (CDC 2011).

1.1c Enzyme-Based Additives

A newer but increasing in popularity additive within heated maceration is enzyme-based detergents such as Biz® or Persil®. The enzyme-based methods utilize the presence of proteolytic enzymes to induce protein decomposition of the proteins present in the composition of muscle and soft tissue. While some studies try to implement the addition of pure enzymes to be in control of the type and amount being added to the solution, and some maintain not worth the significant additional cost (Uhre et al 2015). A majority of these techniques rely on laundry detergents that contain enzymes as a stain-remover, but because of the nature of detergent patents, the specific ingredients and amounts of them are not generally available to the public,

causing guess work in the amount of detergent to use and the correct temperature of the heated solution to activate the enzymes without denaturing them.

The most common brand of detergent used for soft tissue removal processes is Biz®, perhaps due to its wide availability and low cost, as well as published successful results (Leeper 2015). This method, like many of the heated maceration methods, comes with a great deal of controversy on its results. In the 70s and 80s, the method was widely documented to be efficient and not cause any damage to the resulting bones throughout hundreds of uses (Mooney et al 1982, Ossian 1970); later studies disagreed with these assessments and demonstrated resulting damage to smaller, more delicate bone material (Shelton and Buckley 1990). Despite the controversy, however, the method continues to be used in a variety of contexts, still claiming the method to be much gentler on the skeletal remains and DNA quality than other harsher chemical additives (Lee et al 2010; Mairs et al 2004; Simonsen et al 2011; Uhre et al 2015).

1.1d Dermestid Beetles

Dermestes lardarius, as well as occasionally used *Dermestes maculatus*, are a small species of beetle found worldwide that is commonly used in skeletal preparation due to its dietary habit of eating decomposing remains. Universities and museums commonly have large colonies contained in a tank to assist in the preparation of specimens, and the zoological museum on the University of Montana campus is no exception. This method, while occasionally employed in the cleaning of human remains, is not the quick process sometimes illustrated on crime show television and is instead a lengthy process that, similar to maceration, cannot fully clean the bones without additional labor.

An article from 2014 addresses the lack of data on this specific method of soft tissue removal, specifically employing the beetles to clean human mandibles and maxillae (Charabidze et al). They acknowledge the common use of this method in taxidermy and zoology, but comment on a distinct neglect in a forensic context. They estimated the amount of time required to completely skeletonize samples to be about two to three total weeks, conceding that while longer than many other methods in use, it tends to require less handling and does not cause any damage to the DNA extraction process. While this study does make a good argument for the usage of dermestids and shows that, even in a forensic context, the timeline may not be completely debilitating, it does not include other aspects of the dermestid use process that may involve a longer time frame. Dermestid beetles prefer dried flesh to fresh and giving them fresh material and organs can actually be very harmful to the colony health as a whole (Schroeder et al 2002). To ensure beetle health while using them in the removal of flesh, the remains must be stripped of a majority of the flesh material and then dried before exposing the colony to the new specimen. If the material was found outside and already has some insect activity, the remains should further be frozen for at least 24 hours so as not to expose the colony to any other insect species. This not only involves physical handling that Charabidze et al (2014) praises this method for not needing, but also requires additional time for preparation before being able to let the beetles begin their process. Additionally, even after several weeks of exposure, the remains may still need separate cleaning after being removed. In the case where the beetles do not need to survive, and time is no constraint, perhaps this can be approached differently, but if the idea is to keep the cost low, the proper care for the colony is essential and therefore adds both time and handling to the method.

All of this considered, however, the method is still a far more hands-off approach to the removal of flesh from skeletal remains and involves less exposure to foreign chemical material than other commonly employed methods. While the time frame may be an unfortunately long one, the benefits may outweigh the detriments under the proper circumstances. While not expected to measure up in either time efficiency or effectiveness, the method is still a common one and therefore will be attempted as a part of this study to determine if more physically laborious methods may still be more beneficial to the remains than a time efficient one.

1.2 Use of Domesticated Pigs as a Human Proxy

Due to the controversial and sensitive nature of human-based research, human samples are more often not a possibility than in the occasions where they are. This has caused researchers in a number of different fields to search for a suitable substitute. For many years, particularly in biomedical and surgical fields, this job fell on monkeys and dogs, but in more recent history domesticated pigs have almost completely replaced any other animal substitute (Swindle et al 2012). Pigs have been shown in a multitude of examples to be a trusted model for human organs and systems to provide surgical training for organ transplants, lab techniques, and other various surgeries to avoid initial training on living human specimens (Smith and Swindle 2006). In the last thirty years, pigs have been used in studies as varying in field as dermatology, cardiology, and biomedical research looking into musculature and transplantation. Collectively through these they have been able to show that a pig's organ system is 80 to 90 percent similar to humans in anatomy and function, leading to, despite the obvious anatomical differences, pigs and humans being comparable in more ways than one (Swindle 2007).

This similarity and comparability of pig anatomical and physiological systems has additionally not gone unnoticed by the forensic realms of research. For years, a variety of different animals were used in decomposition studies ranging from lizards to elephants (Catts and Goff 1992). However, the similarity in organ structure, size, and function, in addition to the skin similarity, in pigs led them to become the preferred animal model for human decomposition and entomology studies (McIntosh et al 2017). Catts and Goff (1992) list four main requirements for an animal sample: "The animal model must closely approximate the pattern of human corpse decomposition, be relatively easy to obtain, be inexpensive, and not tend to arouse public objections." Since studies in the 1960s and 1970s by Jerry Payne showed the similar internal anatomy, fat distribution, omnivorous diet, and lack of fur the pig has been the clear favorite and most promising surrogate for mimicking human decomposition (Scholeny et al 2007). While humans would be a far preferred resource, the sensitive nature of body farms is only recently becoming a more accepted factor and even presently, there are several "body farms" who solely use pigs as their research specimen of choice.

In more recent research there has been some confliction and criticism over the use of pigs as a human substitute in decomposition and post-mortem interval studies due to comparison studies that demonstrate a clear difference in the decomposition rates (Connor et al 2017, Knobel et al 2018). These studies are in their early stage and data from a study being conducted at the University of Tennessee Knoxville has yet to be peer-reviewed and published (Knobel et al 2018). In these recent studies, they have found that due to environmental factors like scavenging and bug activity there is a significant dissimilarity in the decomposition processes between domesticated pigs and human remains in the later stages of decomposition (Connor et al 2017). Both published studies acknowledged that in cooler temperatures where bug activity was less

active in general and in the early stages of decomposition, the comparison between the decomposition processes still appeared to be homogeneous enough to track trends (Connor et al 2017; Knobel et al 2017). The concern at the heart of this new research is the construction of an accurate post-mortem interval for a human based on pig decomposition may not be reliable enough to be used in court proceedings. These results have not in any way questioned the similarity in skeletal composition that pigs have to humans, which is the basis for examination within this study. Additionally, because the domesticated pigs utilized in the following study were fresh and not exposed to any environmental factors where the issues with the comparison seem to heavily rely, and with the limited availability of repetition of these results, the recent controversy over pig decomposition comparison was not considered to play a heavy result-impacting role.

1.3 Research Goals and Significance

After the point a body can be visually identified and before the point a body is completely decomposed is a large window of time, and it is often throughout that time period that a forensic anthropologist may be called in to consult on a forensic case. A number of factors can be determined from skeletal remains ranging from age and sex estimation to trauma analysis and taphonomic evaluation. These factors can be the difference between being able to identify the individual and having one more cold case to be stored in a closet or drawer gathering dust leaving families lacking closure and murderers wandering free. The preservation of as much evidence as possible is crucial and for a forensic anthropologist to perform their job efficiently, the soft tissue needs to be removed quickly and safely (Fenton et al 2003).

While the ability to construct a biological profile from the remains is important, what may additionally be crucial in the identification process is the ability to extract DNA from the remains. While it is always recommended to take a DNA sample before any additional soft tissue removal processes may be employed, the condition of the soft tissues can be contaminated by bacteria or decomposed to the point where endogenous DNA is compromised and additional samples must be taken at a later stage. Therefore, it is always important to consider the possibility of needing to extract additional samples further down the road of analysis and therefore DNA preservation should be considered as soft tissue removal methods are selected. Rennick et al (2005) examined the effects of various preparation methods on the extraction of DNA yields to determine the safety of common methods of distilled water boil, bleach, and powdered detergent, and supported the criticism of bleach but oddly found the powdered detergent to allow the largest segments to be amplified between the three methods. This study will be readdressed with the addition of other methods and considerations to compare the DNA extraction ability among other methods.

This project is not attempting to be a bold declaration of what method is the most efficient in every circumstance, but rather provide a useable set of data detailing time expectations, effectiveness, and viability in a forensic context. With these results, I hope to be able to show that seemingly minimal decisions can have severe consequences that need to be examined and considered more closely. From DNA extraction to forensic investigation to nonhuman remains going to a museum, the methods employed have a direct impact on later researchers. When the sensitivity and critical importance of skeletal remains within a forensic context is taken into consideration, the lack of any specific protocols or even a clear

understanding of the expectations and repercussions of the methods being utilized is a dangerous and negligent oversight that demands rectification.

2.0 Materials and Methods

2.1 The Research Sample

Domesticated pigs (*Sus scrofa domesticus*) were selected as the specimens for study due to their long history of usage as a human sample substitute in other realms of forensic research. Due to the difficulty in obtaining human samples for this type of research as well as the widely documented similarities in pig decomposition, pigs were determined to be a suitable substitution in the defleshing of skeletal remains comparison (Catts and Goff 1992; McIntosh et al 2017; Scholeny et al 2007).

A total of seventeen pig hocks were obtained from the North Dakota State University Meat Lab located in Fargo, North Dakota. The hocks were received with the trotter already removed, but with the skin remaining intact to leave as much similarity to a complete limb as possible. The pigs provided were all from the North Dakota State University agricultural program and therefore all had access to the same living environment and were all butchered at approximately at the same age. Although the exact age was unknown, due to the state of fusion between epiphyseal plates, the pigs were all determined to be of juvenile age between 4 months and one year. The sex of the pigs was not determinable from the remains present but due to the juvenile age and similar size of all remains, this was not deemed to be a relevant factor for consideration in the comparison.

Due to limitations from the sample source, both hind and front hocks were received and used as a part of the total sample. As it was not possible to determine hind from front limbs before the flesh was removed, the samples were distributed completely at random. The bones

being observed throughout the defleshing methods were then identified as either the tibia and fibula or radius and ulna depending on the portion of the pig the hock in question originated. Figure 2a shows the bone morphology of the sample in question.

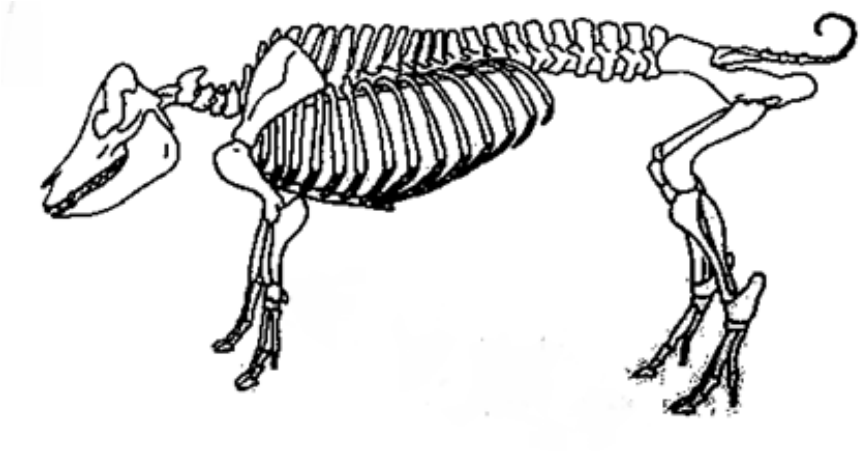

Figure 2a: Image of the anatomy of a pig (*Sus scrofa*) to show the bone structure and morphology of the research specimens (Searfoss 1995).

2.2 Flesh Removal Lab Area and General Materials

The four heated maceration methods being analyzed were performed in a similar manner in a make-shift lab setup within a ventilated garage (Figure 2b). An electric camping burner was used with a 12qt stainless-steel stockpot containing two gallons of liquid for each individual method. Temperature was closely monitored in both the lab area as well as the liquid solution itself using two separate digital cooking thermometers to ensure a consistent temperature throughout each test performed. Additionally, a strainer basket insert was placed inside the pot to prevent the samples from laying on the base where direct contact with the heat source may have influenced the final damage to the resulting bones.

Figure 2b: Lab setup in ventilated garage. All equipment pictured.

The dermestid samples were processed in the Philip L. Wright Zoological Museum (University of Montana) prep room, where a ten-gallon tank of *Dermestes lardarius* are kept in a well maintained and supervised environment for museum samples. Due to the use of the tank for museum sample prep, the tank was occupied by other samples throughout the course of the testing that may influence the total time each test took both in general and in comparison with each other. However, all three samples were tested in the same storage container in the same location within the tank to ensure as much consistency throughout the tests as possible.

Physically macerated control samples were used only for a control sample for DNA yields and therefore were done at a separate time than the rest of the samples. Both controls had their soft tissue removed with a standard dissection kit within the researcher's residence. They were left to dry for 24 hours before being frozen until use for the DNA extraction.

Nitrile gloves were worn during all handling of the samples and equipment, fleshed or defleshed. During heated maceration methods, safety goggles and a P95 chemical odor respirator were always utilized to protect against possibly potent fumes. After each sample was completed,

all materials were thoroughly cleaned with tap water and a bleach based dish detergent to prevent any chemical transfer between treatments. The samples were dried out overnight and then weighed, photographed, and analyzed before being frozen until needed for DNA extraction.

2.3 Before Treatment

Prior to exposure to one of the five soft tissue removal treatments being examined, all samples were given a random number designating their method selection. Each sample was then photographed, measured, and weighed using a digital scale. The starting weights of each sample were used in a one-way single factor ANOVA statistical test sorted by their potential methods to ensure that there was no significant difference in weight between the sample groups. The starting weight was designated to be the immediate weight after receiving them from the butcher, with no other physical alterations performed on them. Weights were also taken after flesh removal was done physically for both the dermestid and control samples, although they were not factored in due to that weight loss being determined as a part of the method itself.

2.4 Soft Tissue Removal Methods

The methods chosen for study were based on cost, time efficiency, and commonality of usage in published literature. A total of five methods were tested and analyzed, excluding the two samples that underwent physical maceration to be used as a DNA control sample. The methods chosen were 1) *Dermestes lardarius* beetles, 2) distilled water boil, 3) enzyme based

simmer, 4) ammonia simmer, and 5) bleach boil. Each method was done in three separate repetitions to evaluate the consistency and accuracy of the results.

For all methods aside from the dermestid beetles, distilled water was used as a majority of the heated liquid solution. Additive amounts were determined based on the approximated average of what is common in available published research detailed in Table 2a. All heated maceration methods were maintained at the desired heat with a camping burner with heat settings that were on a numbered scale from 1 – 9, followed by a MAX option. The temperature was monitored at half hour checkpoints throughout the test to ensure the temperature remained consistent.

Table 2a: Defleshing method additive amounts and temperature ranges.

	Distilled Water Boil	**Enzyme-Based Simmer**	**Bleach Boil**	**Ammonia Simmer**
Solution Composition	100% Distilled Water	10% Tide Laundry Detergent	6.25% Clorox Bleach	12.5% Household Ammonia
Amount of Additive	None	1.6 Cups per Gallon	1 Cup per Gallon	2 Cups per Gallon
Average Starting Temp	99.8° C	78.3° C	99.8° C	89.3° C
Average Starting pH	7.0	7.6	8.6	9.6

2.4a Method 1: *Dermestes lardarius* Beetles

The first method performed is the most distinct of the five methods analyzed as it is the only non-maceration method tested, as well as the most researcher-intensive (Charabidze et al 2014). The *Dermestes lardarius* beetle, also called simply dermestids, tank available was the one

that is currently in use in the Philip L. Wright Zoological Museum on the University of Montana campus. The museum uses the dermestids to finish cleaning specimens that have been skinned and skeletonized for museum and research use. Because of the current and constant use of the tank for specimen preparation, the pig samples being tested within the tank were not the only food source for the beetles and could directly impact the final results through genetic cross-contamination from the other skeletonizing samples in the tank or by causing a longer time frame for the samples to be cleaned.

Following museum protocol, the pig hocks were skinned and had a majority of soft tissue removed by hand. This was done carefully with a basic home dissection kit by the researcher and soft tissue was only removed to the point where it could be ensured no macroscopic damage was done to the bone itself (See Figure 2c for before and after example). After soft tissue removal was done on a sample, they were placed in a fume hood to dry out for a minimum of 24 hours before being placed in an open storage container within the tank containing the dermestid colony.

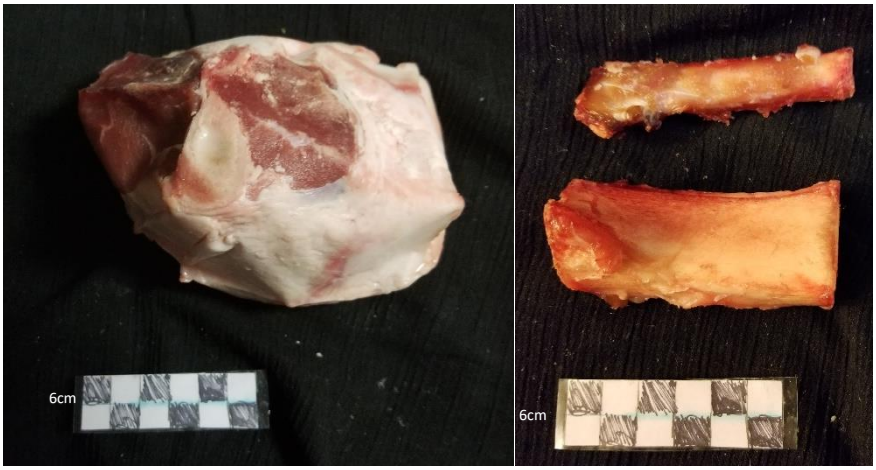

Figure 2c: Sample before physical removal was done (left) and sample after flesh removal but before beetle exposure (right).

Only one sample was placed in the tank at a time to try to retain as much consistency as possible between the three, and the samples were placed in the same container in the same location of the tank. However, the amount of other available food sources within the tank could not be controlled for and may have influenced the time it took for cleaning as it got further into the semester and additional samples were placed in the tank by museum volunteers.

Due to the longer time expectancy with a physical removal method like dermestids, the samples were not checked as frequently as known to be quicker methods. Materials were checked approximately every 24 hours and photos taken, although at times the sample was hardly visible due to beetle coverage. The samples were not touched throughout the process to avoid disturbing the beetles and their process. Because the dermestid tank is within a campus building, the samples were not able to be checked on the weekends and were therefore removed from bug exposure at the end of the day on Friday and replaced in the tank first thing in the morning on Mondays. This time removed from exposure was excluded from a final time calculation.

After the remains had shown no change between two checkpoints and bug activity had decreased significantly, the bones were removed and had all live beetles physically removed from them. To assist in disrupting the still present beetles and remove the minimal amount of grease, the bones were lightly brushed with a toothbrush and tap water. The remains were left to dry in a high-walled dish so no beetles could escape the controlled area, but as some dermestids are very small and like to hide in dark corners such as the nutrient foramen or inside the hollowed portion of the bone, after drying the remains were placed in a labeled paper lunch bag and frozen until needed for DNA extraction.

The dermestid beetle soft tissue removal method requires a great deal more handling both in the preparation before treatment and the removal of living bugs from the remains during post-treatment. These additional preparations were done following the protocol of the caretakers of this specific dermestid tank and may vary considerably for other tanks, therefore the total time calculated during final analysis only included the time period the sample was directly exposed to the beetles within the tank.

2.4b Method 2: Distilled Water Boil

Heated maceration methods were all performed in a similar way with the same equipment. The first of the heated maceration methods was done with just distilled water and no additional chemical additives. Two gallons of distilled water were put into a 12qt stainless-steel pot with a fitting lid and an adjustable strainer insert. The strainer insert was completely submerged in the liquid solution, only hovering about 2 inches from the bottom of the pot, but increased ease of material removal and prevented any material from laying on the bottom of the pot where it would be in direct contact with the heat source. The temperature and pH level of the solutions were taken every half hour during strict checkpoints with digital cooking thermometers and a digital pH tester designed for aquariums and swimming pools. The pH tester was calibrated in between samples and the pots, strainers, and toothbrushes were washed with water and a bleach based household dish soap then left to soak in tap water overnight.

The water was brought to a boil before the pig material was added and the temperature was maintained throughout the process. Due to the speed at which the material consistency can

change, the pot was checked every half hour to monitor temperature, pH levels, and weight. At the aforementioned every half hour checkpoints, the material was briefly removed from the boiling solution and photographed before being immediately returned to the pot. After the material had not visibly changed between two checkpoints, the material was immediately extracted from the water, rinsed in tap water, lightly brushed with a toothbrush, and then laid out on a sheet pan lined with paper towels to dry out overnight. Once completely dried, the materials were weighed, photographed, and frozen in a labeled paper bag until needed for DNA extraction.

2.4c Method 3: Enzyme Based Simmer

The remaining three heated maceration methods were all performed in a similar fashion to that of the distilled water boil method. While previous documentations of the enzyme-based method used Biz® detergent (Ecklund 2007; Nawrocki 1997; Ossian 1970), because of the inability to obtain the type of enzymes and other ingredients present within Biz®, Tide® Original Liquid Detergent was used as a replacement (Procter & Gamble, n.d.). A similar percentage solution to published Biz-based methods was used with the Tide® replacement, using 1.6 cups per gallon for a 10% concentration solution. Rather than bringing the solution to a boil, to maintain protease enzyme activity the solution was brought to 75 – 80 degrees Celsius before adding the pig sample. Maintaining a non-boiling temperature proved difficult and so the temperature was checked every fifteen minutes to maintain consistency. The sample was still only physically removed from the pot during each of the half hour checkpoints. In the case of the enzyme-based samples, they were all removed after two checkpoints of no visible change. This

involved leaving a great deal of cartilage still remaining between the long bones and the epiphyses, but they showed no visible advancement in breakdown between checkpoints. After deemed complete, the bones were removed using the strainer insert and thoroughly rinsed in tap water to remove any remaining detergent. Similar to the distilled water boil, the bones were left to dry overnight and then photographed and frozen until DNA extraction.

2.4d Method 4: Ammonia Simmer

For the third heated maceration method, household ammonia was used based on the National Park Service's Museum Protocol (National Park Service 2006). While ammonia hydroxide tends to be more commonly found in the degreasing portion of skeletonizing mammalian specimens during museum prep, there have been several published pieces over the years demonstrating its use in heated maceration; particularly within taxidermy and hunting circuits (Hoffmeister and Lee 1963). Although its use has not been documented within a forensic context, the descriptions of time efficiency and ease found in mammalian publications, and its common use as a degreaser, created a viable alternative method that could be compared against other more common options.

Due to the lack of a clear methodology for this method in a forensic context, the amounts varied considerably across other published methods. A solution concentration of 12.25% was ultimately decided on, using 2 cups of household ammonia per gallon of distilled water. Due to the fumes heating ammonia can cause and warnings in documents using this method, this was done keeping the temperature at a simmer and not letting it boil at any point, adding the sample once the solution reached 85-90 degrees Celsius. Similar to the other methods, the sample was

monitored at half hour interval checkpoints for weight, temperature, pH, and photographs. After the sample was completed, the bones were left to dry before being frozen until DNA extraction. Of all the methods, ammonia hydroxide did involve the greatest number of safety precautions, both in handling of the solution as well as disposing of it after, so while not adding additional cost or time, the method did require additional consideration and caution.

2.4e Method 5: Bleach Boil

The final heated maceration method used in the analysis was one of the most commonly found in a variety of forms within forensic literature: the bleach boil method (Mann and Berryman 2011; Nawrocki 1997; Rennick, Fenton, and Foran 2005). Bleach is found as an additive within many different approaches to maceration and soft tissue removal, whether it be heated or stagnant. Clorox® brand bleach was selected for use in this study due to its commonality and accessibility. While many documented amounts of the additive are available in published research, the moderate average seemed to fall around 1 cup per gallon for a 6.25% concentration.

Similar to the distilled water boil method, the solution was brought to a temperature of 99 degrees Celsius before adding the pig sample. Checkpoints were every half hour and involved checking the temperature, pH, weight, and photographing the sample. The bleach method was found to require additional supervision as the process was so quick that the bones could go from nearly fully fleshed to complete within a single half hour checkpoint. After completion, the bones were thoroughly rinsed in tap water and brushed lightly with a toothbrush to remove any

residual bleach from the surface of the bones. They were then similarly left to dry before being frozen until needed for the DNA extraction process.

2.4f Method 6: Physical Maceration Used on Control Samples

Of the 17 pig samples used in this study, 2 of them were reserved for DNA control samples and therefore could not undergo a soft tissue removal method that involved any foreign substances. Because bone powder was the substance being tested for its DNA yields, the soft tissue still needed to be removed from the control samples until the bone surface was accessible. Similar to the first step of the dermestid method, the two samples underwent a physical maceration performed by the researcher with a standard dissection kit (Couse and Connor 2015). Physical maceration was performed until a large enough section was accessible for the drill to come into direct contact with the bone surface. Care was not specifically taken to avoid nicking the bone or causing any macroscopic damage, as these samples were not being analyzed by the criteria the other methods were subjected to.

2.5 DNA Extraction and Quantification

As samples completed their defleshing process, they were placed in labeled paper bags detailing their number, starting weight, final weight, and total method exposure time. These paper bags were sorted by method and stored in a freezer until used for the DNA extraction process. As soon as all samples had been completed, the two long bone fragments from each sample were brought into the Snow Modern DNA Lab at the University of Montana for the remainder of the study.

2.5a: Bone Powder Drilling

Each individual pig sample was separated into two DNA extraction attempts; one from each long bone. Due to the sample available, the long bones did vary between pairs of tibiae and fibulae versus pairs of radii and ulnae depending on the original pig hock used (Table 2b further expands on which bones contributed to each sample). Each long bone was drilled with an 18v cordless drill and 3/8" bradpoint drill-bit to collect 0.50g worth of bone powder. The bones were lightly sandpapered down in the area of the drill location and a series of bradpoint drill-bits were used in rotation to allow for the drill-bit to be bleached, rinsed, and dried in between each sample to prevent any potential cross-contamination.

Photographs were taken before and after each sample underwent the drilling process. The drill location was done on the distal end of the bone when it was available and the drilling was kept to the ends of the bone as much as possible. While consistency in drill location was attempted between the samples, at times a second or third hole would need to be drilled due to the thickness of the cortical bone available. Location of multiple drill location was determined by the thickest areas of each specific bone to ensure the lowest amount of holes possible were used in the process. A total of 0.50 gram of powdered bone was collected from each sample.

Although approximately 0.50g was consistently collected from each bone, the density varied considerably. Of the thirty-four samples, seven of them required the drilled powder to be separated into two 1.5ml microcentrifuge tubes rather than one. Bone powder was collected into the labeled 1.5ml microcentrifuge tubes and stored at room temperature until DNA extraction could be performed approximately one week later.

2.5b DNA Extraction: QIAamp Protocol

DNA extraction was done following a protocol provided with the purchased QIAamp DNA Micro Kit (QIAGEN 2010). Although the extractions were done in two separate batches due to the capacity of equipment, with 20 samples being done in both Batch 1 and Batch 2, the same protocol was used in both instances and therefore will be described in the following paragraphs as if all were performed simultaneously.

New 1.5ml microcentrifuge tubes were labeled in a similar naming mechanism to the original bone powder tubes further detailed in Appendix A. The original tubes containing 0.50g of drilled bone powder were taken one at a time and had 0.10g placed in the new labeled tubes. The remaining 0.40g of bone powder was saved in the event that any extraction would need to be duplicated.

After bone powder separation, 360µl buffer ATL, 20µl Proteinase K, and 1µl rehydrated carrier RNA was added to each individual tube and pulse vortexed for 10 seconds to ensure the materials were mixed. All samples were then placed in a 56-degree Celsius heat block for an 18-hour incubation period. After incubation, the microcentrifuge tubes were removed from the heat block and spun in a centrifuge to make sure all liquid was in the bottom of the tube. An additional 300µl of buffer AL was added and vortexed until it created a homogeneous solution. After another brief incubation period of 10 minutes at 70 degrees Celsius, the tubes were centrifuged and the supernatant pipetted into the included QIAamp MinElute® columns labeled with the same identifiers described in Table 2b. Additional wash buffers were added to the columns, centrifuged and placed in new tubes between each addition. A dry run was done in the centrifuge at top speed to ensure no ethanol remained within the column. The column was then

moved to a final tube labeled with the same identifier and had 50µl buffer AE added. After a final run in the centrifuge, the extracted DNA was then lidded tightly and refrigerated for later use.

2.5c PCR and Sequencing

All the extracted DNA samples were subjected to the same PCR protocol: 8.58 µl of H_2O, 2.4 µl dNTP, 0.18 µl Forward Primer, 0.18 µl Reverse Primer, 1.5 µl of 10X PCR $MgCl_2$ Buffer, 0.45 µl $MgCl_2$, and 0.08 µl of Taq. A total of 13.37 µl of the prepared mix was aliquoted into the PCR 0.2mL tube and had 1.5 µl of the associated sample's extracted DNA added. The primers used in the PCR phase targeted a 212 base pair fragment of the *Sus scrofa* mitochondrial Cytochrome c Oxidase subunit II gene; CO2susF2 (5' GCCTAAATCTCCCCTCAATGGTA - 3') and CO2susR2 (5'AGAAAGAGGCAAATAGATTTTCG-3') (Lahiff et al 2001; Pangallo et al. 2010).

Two 2% agarose electrophoresis gels were run to confirm that amplified DNA was present in all post-PCR reaction product samples before any sequencing was done. If the agarose gel did not display a clear band for a sample, the sample was exposed to a second PCR attempt. If a clear band of the expected size (in comparison with a 100bp ladder) was shown in the agarose gel for each of the thirty-eight extractions (two DNA extractions for each of the fifteen samples and four for each control sample), the samples from the PCR product was prepared for sequencing to ensure the extracted DNA present in the samples was actually the targeted *Sus scrofa* mitochondrial DNA.

Preparing the PCR samples for sequencing was done using USB® ExoSAP-IT® PCR Product Cleanup protocol. 5µl of the post-PCR reaction product was combined with 2µl of the ExoSAP-IT reagent and then incubated at 37˚C for 15 minutes to break down the remaining primers and nucleotides. The incubation temperature was increased to 80˚C for an additional 15 minutes to inactivate the ExoSAP-IT. All 38 samples were then taken to the University of Montana Genomics Core for DNA sequencing using the forward pig primer.

Of the 38 samples that were sent for sequencing, 36 of them came back with DNA sequences that were then uploaded into Sequencher 5.4.6 for editing by eye and analysis. All of the finalized sequences were run through Basic Local Alignment Search Tool (BLAST) registered to the National Center for Biotechnology Information (NCBI) GenBank to search for a corresponding sample based on nucleotide matches.

2.5d Qubit and DNA Yield Quantification

DNA concentration quantification was done using a Qubit® dsDNA BR Assay Kit. Extracted DNA from all thirty-eight of the working DNA extracts as well as the single sample were quantified for initial DNA concentration values. Thin walled 0.5mL PCR tubes were used for each of the samples as well as the two standards provided with the kit. A Qubit® dsDNA BR Buffer was added to each of the tubes so that each tube contained a total of 200µL. DNA samples contained 195µL of the buffer and 5µL of the associated DNA and the standards each had 10µL of the standard and 190µL of the buffer.

To begin, the Qubit® 4.0 Fluorometer setup screen was used to select double stranded DNA and broad range coordinating with the assay kit used. The standards were both read to

calibrate the machine and each sample was read. If a sample provided a result that was out of range and too low to provide a readable sample, they were tested a second time with a new buffer mix to ensure it was not user error. If the sample still failed to provide a readable result, they were tested on a later day using a high sensitivity assay (Qubit® dsDNA 1X HS Assay Kit) to read the lower concentration result.

3.0 Results

The core purpose of this study was to analyze the negative impact various soft tissue removal methods can have both on the bone surface as well as on DNA extraction, while also keeping in consideration the effectiveness, time efficiency, and cost of each method. Five methods of soft tissue removal in total were analyzed and scored using a set of specific criteria similar to the organization of a study done by Couse and Connor (2015). The criteria and description of score values are listed below (Table 3a).

Table 3a: Description of criteria and score values used to analyze each method.

Cost of Method	
Score	**Description**
1	No Cost
2	$0.01 - $1.00
3	$1.01 - $5.00
4	$5.01 - $10.00
5	Over $10.00

Time Efficiency of Method	
Score	**Description**
1	Under 1 Hour
2	1 Hour – 4 Hours
3	4 Hours – 10 Hours
4	10 Hours – 24 Hours
5	Over 24 Hours

Effectiveness of Method	
Score	**Description**
1	Bones were cleaned completely only by the method tested with no presence of grease.
2	Bones were mostly cleaned, may have involved some additional effort by the researcher. Little to no grease present.
3	Some cartilage and a minimal amount of grease may still be present, but still mostly cleaned.
4	Cartilage still remains on bone, grease may still be present, interior of bone may still have some tissue present.
5	Cartilage and tissue still present on bones. Material is

Macroscopic Damage	
Score	**Description**
1	Bones show no sign of macroscopic damage or alteration.
2	Slight water damage may be present, some slight cracking.
3	Some mild water damage and slight cracking visible. Exterior of bone may feel dried out.
4	Severe water damage present, increased porosity. Slight cracking.
5	Severe water damage or tissue staining present, chipping or

	not completely cleaned.

	severe cracking visible. Bones feel dry and rough. Increased porosity.

Quantifiable DNA	
Quantifiable DNA	**Description**
Score	**Description**
1	3.00 µg/µl +
2	1.001 µg/µl – 2.999 µg/µl
3	0.251 µg/µl – 1.000 µg/µl
4	0.001 µg/µl - 0.250 µg/µl
5	> 0.000 µg/µl

In addition to the listed criteria, the availability of the equipment, practicality, and versatility of use within different lab environments were also considered which will be expanded on in the discussion and conclusions. The criteria of effectiveness and damage were scored through qualitative observation, whereas the cost, time efficiency, and DNA yield were scored through quantitative calculation. The results are gathered first by looking at each method individually, followed by an overall comparison sorted by each specific criteria listed in Table 3a.

3.1 Initial Sample Weight Distribution

Upon purchase, each of the seventeen pig limbs were measured and weighed using a digital kitchen scale. The weight of the samples varied from 340.00g – 586.00g with a mean of 432.88g and a standard error of 16.84. The starting weights of each sample were used in a one-way single factor ANOVA statistical test sorted by their potential methods to ensure that there

was no significant difference in weight between the sample groups. The ANOVA showed no statistically significant difference in the start weights of the pig samples across the six soft tissue removal methods including the control samples; $F(5, 11) = 0.7768$, $p = 0.5836$.

3.2 Results by Method

Each method will be addressed individually by each of the criteria analyzed within the study, before being addressed in comparison between the methods.

3.2a *Dermestes lardarius* Results

Cost

An already thriving colony of *Dermestes lardarius* beetles in a tank cared for by the Philip L. Wright Zoological Museum on the University of Montana campus was available for research use and therefore there was no assigned cost for this method. Access to a dermestid colony is not always available and therefore the cost for this method could vary based on the ability to start a new colony or access to an already existing one.

Time Efficiency

The dermestid samples varied widely between the three tested specimens, ranging from 3 days to nearly 8 days. Due to the tank being on campus, the building was locked over the weekends. When the samples were not finished by Friday afternoon, they were removed from direct exposure to the beetles and were replaced first thing on the following Monday morning. The time where the samples were not directly exposed to the beetles were not factored into the

final time calculation. This also excluded the additional time it took for the researcher to prep the samples to be placed in the tank as well as the process of removing the live beetles after the method was complete. The preparation tasks could vary based on the assigned protocols and care for the specific tank being used, but could add a significant amount of time and labor required for the method and should be kept in consideration if one is planning on using it.

Effectiveness

The bones were cleaned of a vast majority of all soft tissue by the dermestid beetles (Figure 3a). Some samples had some minimal amounts of tissue remaining on the bones as well as some cartilage that remained on the distal ends of the long bones. Additionally, the completed bones remained greasy to the touch and had to be lightly brushed with a dampened toothbrush using tap water after removal from the tank.

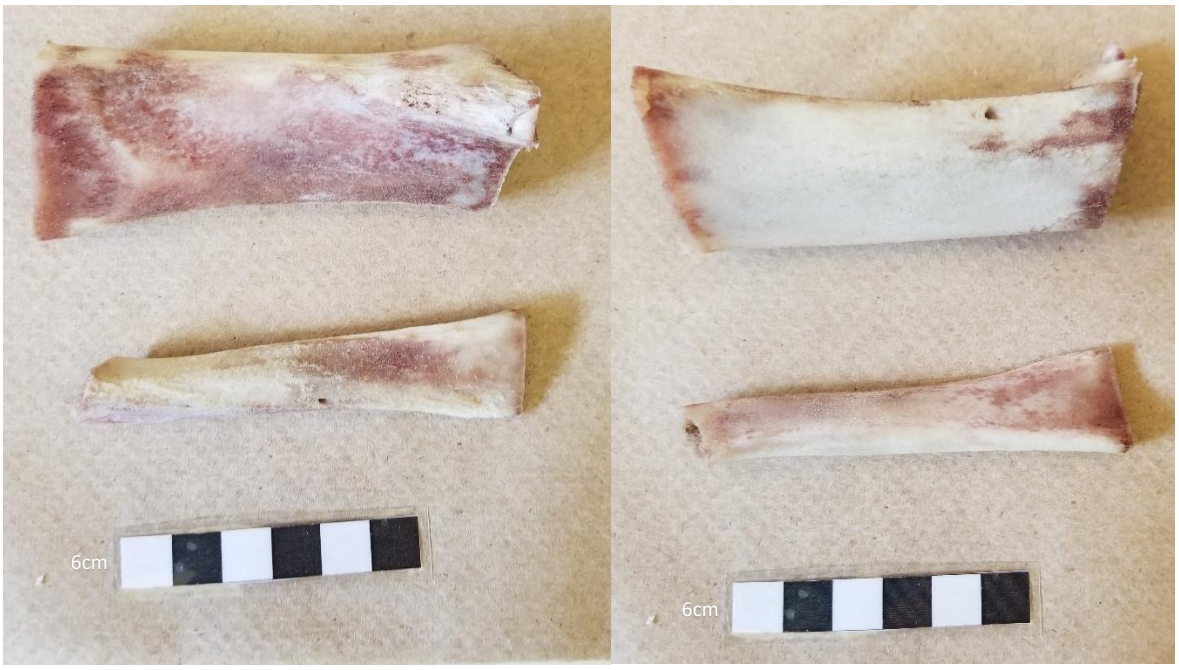

Figure 3a: Sample 013, tibia (top) and fibula (bottom) after completion of dermestid method. Some tissue remains on the bone in addition to some tissue staining.

Macroscopic Damage

There was no evidence of any surface damage to the samples exposed to the dermestid beetles, although some slight tissue staining remained on areas of the bone (Figure 3a).

DNA Yield

The bone powder was drilled when possible from a location where there was no residual tissue or tissue staining remaining on the bone. The extracted DNA was quantified for two bones per sample using the Qubit® dsDNA BR Assay Kit creating a total of six quantified extractions. The concentration was calculated in µg/µl and all yielded high DNA concentrations, ranging from 2.98 µg/µl to 6.04 µg/µl ($\overline{x}$ = 4.31, s = 0.5422).

3.2b Distilled Water Boil Results

Cost

The heated maceration methods all used the same set of purchased lab equipment and therefore was not tallied in any of the cost analysis for each of the methods. Distilled water was purchased for use in this method and was the only additional cost. Two gallons of water were used for each of the three tests run and distilled water is typically around $0.40 a gallon making for the cost per sample under $1.00.

Time Efficiency

The distilled water boil samples all took roughly the same amount of time to complete, ranging from 4.72 hours to 5.27 hours ($\overline{x}$ = 5.00, s = 0.1590).

Effectiveness

The samples tested in the distilled water boil method were for the most part completely rid of all soft tissue, both exterior and interior (Figure 3b). One of the three samples was removed from the boiling solution still containing some amount of cartilage on the distal end of both long bones but was otherwise clean. The bones did not seem to have any greasiness to them nor a strong odor. The texture of the bones was coarse and dried out and minimal tissue staining remained on several of the bones.

Figure 3b: Sample 002 after being removed from the distilled water solution and thoroughly dried. Clear water damage is visible and there is a clear chip present at the top of the fibula.

Macroscopic Damage

The distilled water boil samples all showed a mild to severe amount of water damage that is indicated by the arrow in Figure 3b and one sample was left with a minimal amount of dark

tissue staining. Additionally the cortical bone had a coarse and dried out texture with increased porosity and cracking on the distal ends of the long bones.

DNA Yield

In the first batch of extractions, the extraction for sample 101A failed to amplify during PCR and therefore that sample was extracted a second time. Both samples were still used during the quantification process. All samples from the distilled water boil method, including the one that did not PCR amplify, produced a readable result using the Qubit® ranging from 0.464 µg/µl to 3.04 µg/µl ($\overline{x}$ = 1.31 s = 0.4425).

3.2c Enzyme-Based Simmer Results

Cost

Tide® Liquid Original laundry detergent was purchased for the enzyme-based simmer method due to the ability to find an easily accessible document containing a list of ingredients present in the detergent, as well as the documented presence of protease and amylase enzymes. The price for Tide® products tends to vary widely based on location and size of the bottle, so the total cost was calculated using the personal cost to the researcher at $0.20 a fluid ounce. The 10% concentration in each two gallon sample required 3.2 cups of detergent in addition to the purchased distilled water. The $5.12 cost of 3.2 cups of detergent at $0.20 a fluid ounce plus the $0.80 for the distilled water made for a total cost for the enzyme-based simmer method $5.92 per sample.

Time Efficiency

The length of time it took for the samples exposed to the enzyme-based simmer method to be complete were all very similar, ranging from 5.83 hours to 6.75 hours ($\bar{x}$ = 6.38, s = 0.2815).

Effectiveness

The enzyme-based simmer method samples were not completely cleaned when removed. Many of them had remaining cartilage on the exterior and minimal soft tissue still present in the interior of the bone. The solution of detergent and water was very slippery and opaque causing issues during the checkpoints for the sample. Additionally, the samples exposed to this method were the only heated maceration method samples that came back with a greasy texture and odor.

Figure 3c: Samples 004 (A) and 005 (B) after being completed with the enzyme-based detergent simmer method and dried. Cartilage is not broken down on sample 004 and severe water damage can be seen in sample 005.

Macroscopic Damage

The samples tested with the enzyme-based simmer method all showed severe water damage and cracking (Figure 3c). The water damage appeared to completely saturate the bone composition and reduced the cortical bone to a mush-level consistency. The method left the bones with a very dry and coarse texture causing some visible striations upon the bone and seemed to increase the porosity throughout the metacarpal present

DNA Yield

When the bone powder was drilled from the enzyme-based simmer method samples, there was an increased complication due to the severe water damage and saturation of the bones. The water damage to the bones proved to be saturation compromising the cortical bone composition. In places where the bone surface was clearly affected by the liquid solution, the drill would go straight through the bone with little to no resistance and the bone composition was reduced to an almost mush-like consistency. When quantifying the DNA extraction from each of the samples, only two of the six samples produced a readable result, 114A and 115B, with a concentration calculation of 0.664 µg/µl and 0.572 µg/µl respectively. When the other four samples were tested using the high sensitivity assay, they were able to be read, with the overall results ranging from 0.002 µg/µl to 0.664 µg/µl ($\bar{x}$ = 0.2062 s = 0.1308).

3.3d Bleach Boil Results

Cost

A wide variety of brands of bleach exists, but due to the wide availability and notability Clorox® regular bleach was used. Similar to the other chemical additives, the price can vary widely by location and amount being purchased, and therefore an average was used, calculating it at $0.10 a fluid ounce. The bleach boil method used 2 cups of bleach for a cost $1.60 and was added to the $0.80 distilled water cost making for a total cost of $2.40 per sample.

Time Efficiency

The bleach boil method samples ranged from 2.52 hours to 3.57 hours ($\bar{x}$ = 3.12, s = 0.3131).

Effectiveness

The bleach boil method left the samples clean with little to no water damage or remaining cartilage. The bones appeared to experience some slight coloration changes along the edges and were lighter in appearance than the enzyme-based and distilled water samples, but the majority of the bone remained a natural color and appearance (Figure 3e). The interior showed slight tissue staining but no remaining tissue was present on the interior or exterior of the bone. There was no greasiness and the texture of the bone was smooth and natural.

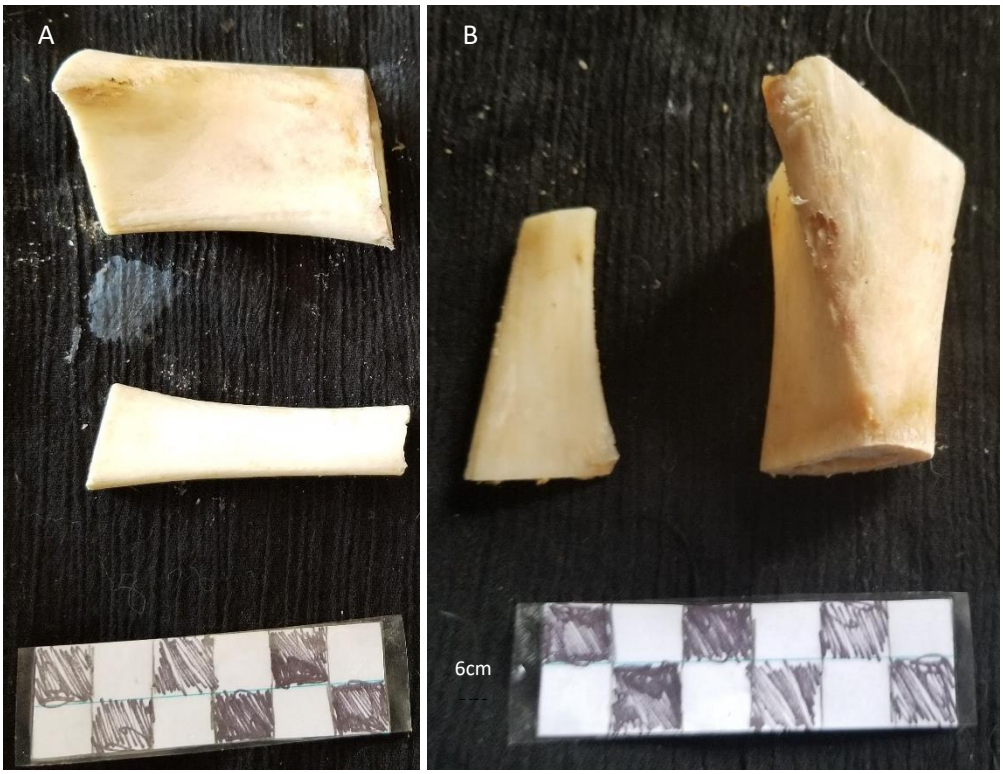

Figure 3d: Samples 007 (A) and 008 (B) after being completed with the bleach boil method and thoroughly dried. Bones are clean with some slight whitening present.

Macroscopic Damage

The samples tested with the bleach boil method showed little to no macroscopic alterations (Figure 3d). Several of the bones showed slight signs of whitening but not to a severe degree. There was no visible cracking or cortical bone alterations on the exterior or interior of the bone, aside from some slight tissue discoloration on the interior of one of the bones.

DNA Yield

Although the DNA extractions amplified during the PCR process for all samples, none of the samples produced a readable result when put through the Qubit® process with the broad range assay. Using the high sensitivity assay, all samples produced minimal readable results ranging from 0.001 µg/µl to 0.002 µg/µl ($\bar{x}$ = 0.0015 s = 0.0000).

3.2e Ammonia Simmer Results

Cost

Top Job® Basic Clear Ammonia was used for the ammonia simmer method. Similar to the enzyme-based method, household ammonia can be found at a wide variety of prices so only the one used was considered costing $0.02 a fluid ounce. The ammonia simmer method used a 12.25% concentration requiring 32 fluid ounces of the household ammonia in addition to the distilled water. This amount of ammonia cost $0.64 and was added to the $0.80 distilled water cost making for a total cost of $1.44 per sample.

Time Efficiency

The ammonia simmer method had a slight amount more variability between the samples than the other heated maceration methods ranging from 2.78 hours to 4.05 hours ($\overline{x}$ = 3.46, s = 0.3694). A Pearson's R statistical test was performed to determine if the weight of the bones played a factor in the variable time expectancy. This resulted in a R value of 0.538 and a COD of 0.289, suggesting a slight to moderate positive correlation, showing almost 30% of the variation in time expectancy could be explained by the sample's heavier weight.

Effectiveness

The samples subjected to the ammonia simmer method were spotless with no visible water damage or remaining cartilage (Figure 3d). The texture of the cortical bone remained smooth and had a natural feel to them with no greasiness or porosity.

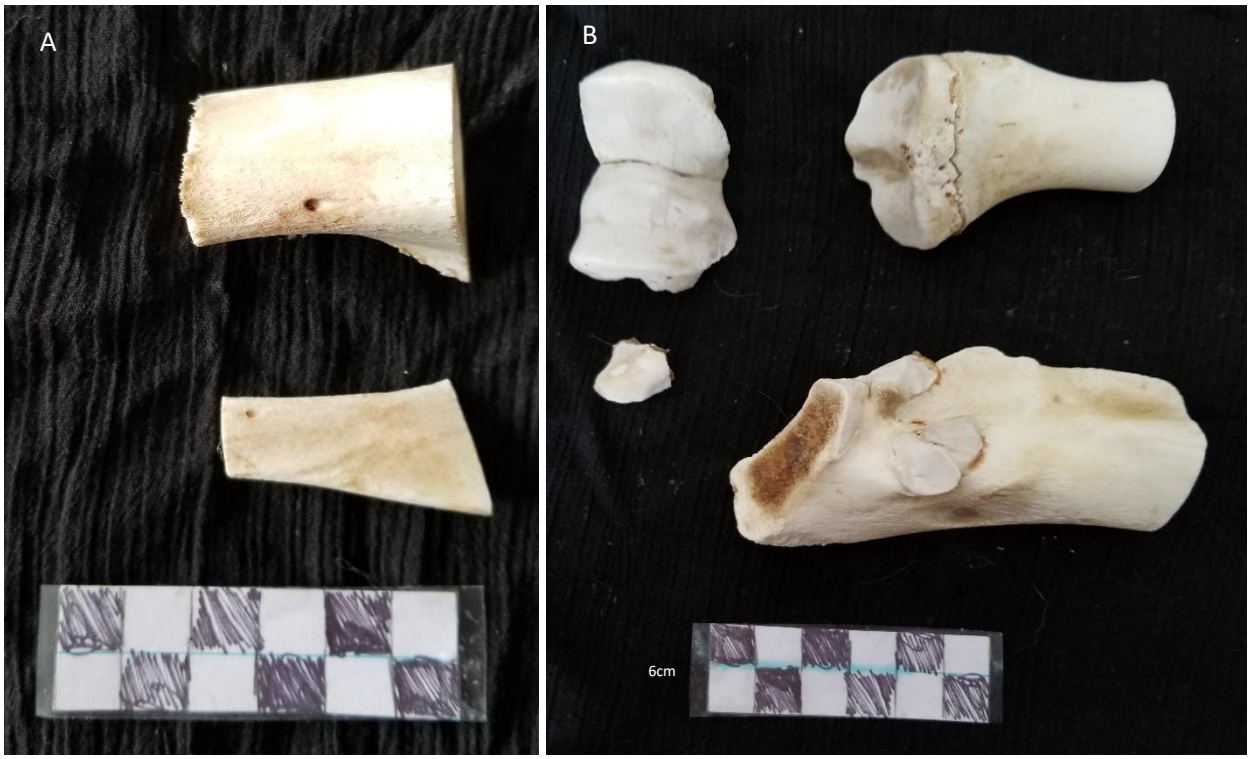

Figure 3e: Samples 010 (A) and 012 (B) after being completed with the ammonia simmer method and thoroughly dried. Bones are clean with very apparent whitening.

Macroscopic Damage

The bones completed using the ammonia simmer method showed little to no macroscopic alterations aside from the severe whitening evident on all the bones (Figure 3e). The small flake of bone in Sample 012 showed no sign of damage despite being far thinner than any other bone being tested. There was some slight tissue staining remaining on the bone but nothing that appeared excessive or obstructed the view of the bone.

DNA Yield

Of the six ammonia simmer samples, only two failed to produce a readable result during the quantification process. Due to the anomaly, these two samples were run through the process twice to certify the results of the sample were not due to a researcher error. The concentration

calculation for the samples that had a readable result ranged from 0.472 µg/µl to 0.544 µg/µl ($\bar{x}$ = 0.497, s = 0.0160). Once the high sensitivity assay was performed, the other two samples were able to be given numerical results with the new results ranging from 0.002 µg/µl to 3.04 µg/µl ($\bar{x}$ = 0.3317, s = 0.1050).

3.3 Method Comparison

3.3a Scoring System Criteria

The methods were all scored based on the criteria descriptions listed in Table 3a and totaled for comparison (Table 3b). Additional details on each of the method's specific results can be found in the above Results by Method section.

Table 3b: Methods scored by trait using the scoring system listed in Table 3a.

	Cost Per Sample	Time Efficiency	Effectiveness	Macroscopic Damage	Quantifiable DNA	Total Score
Dermestid	1	5	3	1	1	11
Distilled Water Boil	2	3	2	4	2	13
Enzyme-based Simmer	4	3	4	5	4	20
Bleach Boil	3	2	1	1	5	12
Ammonia Simmer	3	2	1	2	3	11

3.3b Final Weight

Similar to how the samples were compared against each other in the initial sample analysis to determine if there was a significant difference between sample weight, the final weights were subjected to a single-factor ANOVA test. The tested weights were separated by bone type and sample method to determine if there was a significant difference among the end results. The ANOVA test showed no statistical significance in the final weights between the samples of the different methods; $F(4, 10) = 0.1853$, $p = 0.9407$; $F(4,10) = 0.6883$, $p = 0.6164$.

Additionally, Pearson's R was calculated for all samples to determine if the starting weight had any impact on the time efficiency calculation. This was with number pairs of the original weight average and time efficiency average of each method and resulted in an R value of – 0.5339 and a coefficient of determination (COD) of 0.285. This indicates a slight negative correlation meaning that based on the limited amount of data, there is a pattern suggesting the greater the initial weight, the less time each sample took to complete. The COD calculation states that 28.5% of the variation in completion time can be explained by the weight difference with heavier samples showing to take less time. When the samples were calculated within their own methods, this same negative correlation was not found and all methods individually, aside from the ammonia samples that showed a moderate positive correlation, showed minimal to no correlation between the starting weight and the time efficiency. This original negative correlation result is likely due to the limited amount of number pairs calculated.

3.3c Time Efficiency Comparison

The time efficiency was calculated and averaged for each of the five tested methods and compared against one another. Using a Kruskal-Wallis H test, the time efficiency for all five methods showed statistically significant difference when compared all together; $H(4) = 26.6323$, $p = 0.00002$. The tests were then taken in pairs to compare them to demonstrate a significant difference between each. The only methods that did not show a statistically significant difference were the bleach and the ammonia samples; $\chi 2(2) = 0.2402$, $p = 0.8103$. All other method pairs were statistically significantly different from one another.

The dermestid method took on average 136.92 hours with the range being from 3 to 7 days, while all other methods took under 7 hours. For this reason, the dermestid average time efficiency was not included in the below chart showing the time efficiency of the four heated maceration methods (Figure 3f)**.**

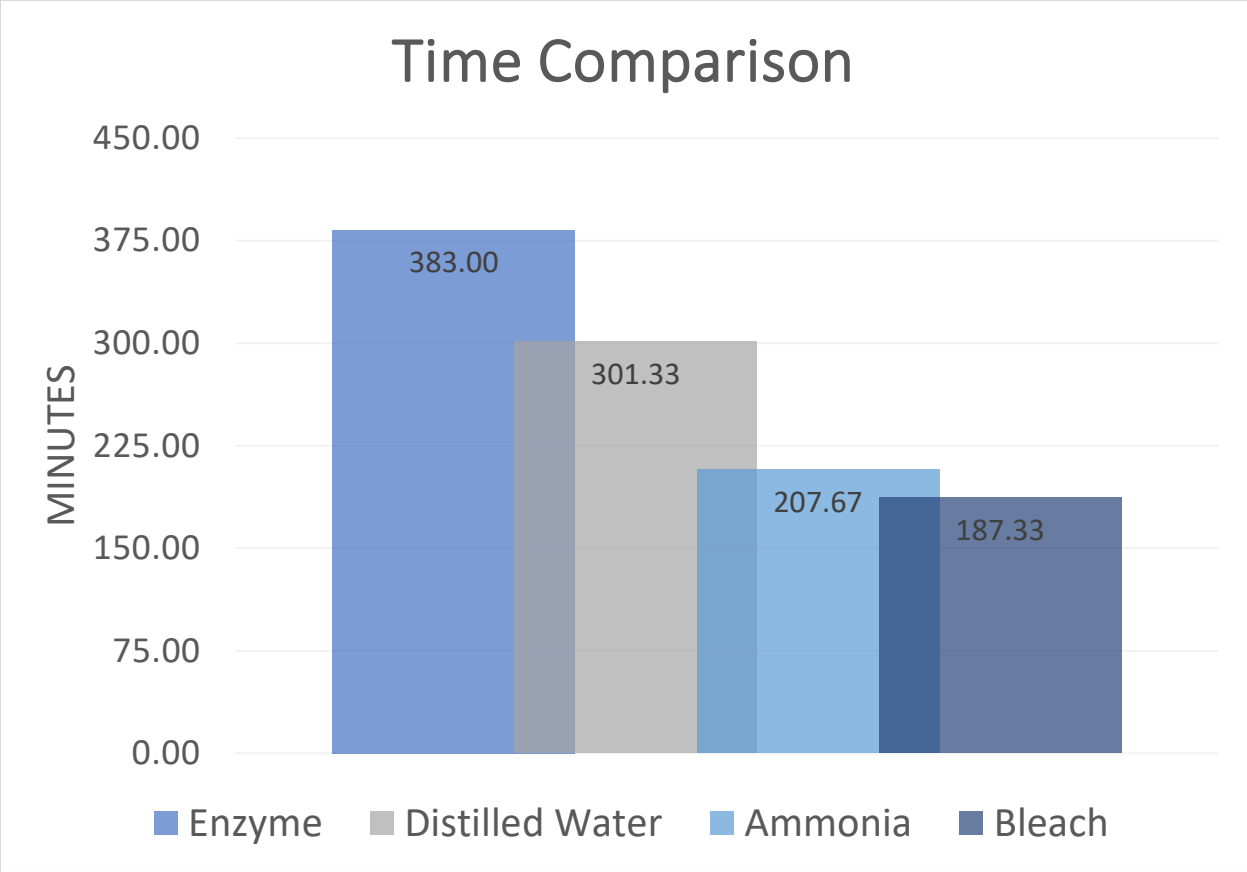

Figure 3f: Bar chart showing average time efficiency for each method tested.

3.3d DNA Yields and Sequencing

The extracted DNA concentration calculated by the Qubit were averaged by method and are shown in Figure 3g. Averages were calculated using both the broad range assay concentration values as well as the results read using the high sensitivity assay. In the case of the ammonia and enzyme-based samples, there was a mix of zeros and readable calculation values effecting the resulting average of the readable values that were available. A Kruskal-Wallis H test was conducted between each group of samples to determine if there was a significant difference in the DNA concentration values. The only samples to not produce a statistically significant result were the control samples from the dermestid samples; $\chi 2(2) = 0.15$, p =

0.69854. This demonstrated the DNA quality to not have been significantly impacted by the dermestid method, opposed to the heated maceration methods that all showed significantly lower yields (Figure 3f).

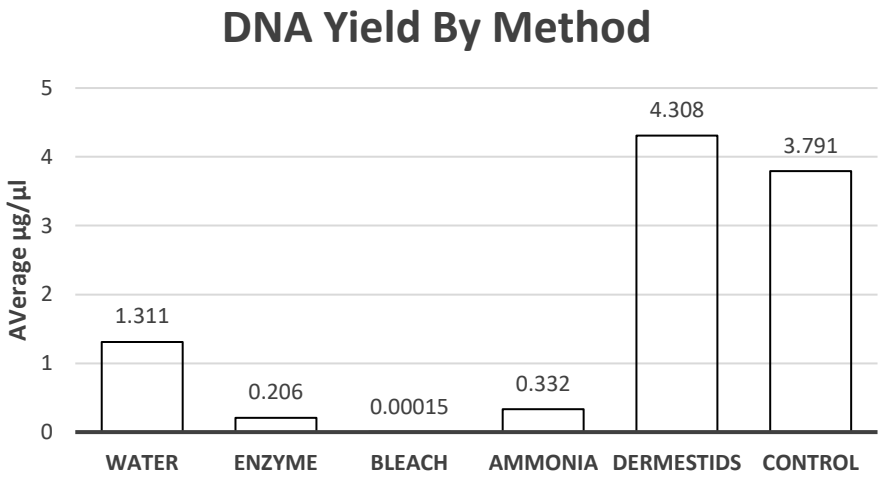

Figure 3g: Bar chart showing the average extracted DNA concentration in µg/µl of each method tested.

The 38 total DNA extractions underwent PCR and DNA sequencing to ensure that the DNA being quantified and amplified were all the desired *Sus scrofa* DNA. Two DNA extractions failed to produce analyzable sequence data, but the other DNA extractions from those samples produced results and the failed sequences were considered to be caused by researcher error. The sequences produced by the University of Montana Genomics Core were all uploaded into Sequencher 5.4.6 for editing and analysis. These uploaded sequences were run through BLAST, registered with the NCBI GenBank. This database was used to search for the sample nucleotide query and detect the nearest nucleotide match. All sequenced samples queried 99% - 100% for *Sus scrofa* mitochondrial DNA and an example of the BLAST results are shown below (Figure 3h).

Job title: PIB-109-A03

RID DF2M10M6015 (Expires on 04-20 02:12 am)

Query ID lcl|Query_243039
Description None
Molecule type nucleic acid
Query Length 190

Database Name nr
Description Nucleotide collection (nt)
Program BLASTN 2.8.0+

Descriptions

Sequences producing significant alignments:

Description	Max score	Total score	Query cover	E value	Ident	Accession
Sus scrofa breed Kele mitochondrion, complete genome	233	233	71%	1e-57	99%	gi|1374427572|MF143597.1

Alignments

Sus scrofa breed Kele mitochondrion, complete genome
Sequence ID: **gi|1374427572|MF143597.1** Length: 16828 Number of Matches: 1
Range 1: 7826 to 7960

Score	Expect	Identities	Gaps	Strand	Frame
233 bits(258)	1e-57()	134/136(99%)	1/136(0%)	Plus/Plus	

Features:

```
Query  39    CAATAATTNNTAACATTATTTATTTTATTCCAACTAAAAATCTCAAACTACTCATACCCA  98
Sbjct  7826  CAATAATTA-TAACATTATTTATTTTATTCCAACTAAAAATCTCAAACTACTCATACCCA  7884
Query  99    GCAAGCCCAGAATCAACCGAACTCAAAACTCAAAAACATAGCACCCCTTGAGAAATAAAA  158
Sbjct  7885  GCAAGCCCAGAATCAACCGAACTCAAAACTCAAAAACATAGCACCCCTTGAGAAATAAAA  7944
Query  159   TGAACGAAAATCTATT  174
Sbjct  7945  TGAACGAAAATCTATT  7960
```

Figure 3h: Image of Sample 109A results to demonstrate the results of DNA sequencing data run through NCBI GenBank BLAST.

4.0 Discussion

This study examined five commonly used soft tissue removal methods, specifically looking at the cost, time efficiency, effectiveness, and risk of damage macroscopically and to DNA quality. Domesticated pig (*Sus scrofa*) limbs were used to conduct all methods and each method was done in three repetitions for a total of fifteen methods with two additional limbs being used for a DNA control sample. The results of each specific criteria examined across all five methods are summarized in the following section followed by an evaluation of each method on its own highlighting each's advantages and disadvantages.

4.1 Summary of Results

Cost

The cost of each soft tissue removal method was calculated based on the cost for each individual sample and did not include the cost for start-up equipment such as the electric camping burners, stockpots, and thermometers. If a method did not require any additional costs after basic equipment was purchased, there was a zero dollar cost assigned to the sample. The dermestid method utilized an existing dermestid colony currently on the University of Montana campus and was therefore free to access. It should be noted that the cost to purchase and maintain a dermestid colony can vary. While feeding and caring for the beetles once the colony is thriving is relatively cheap and low maintenance, the startup costs can range from under fifty dollars to several hundred depending on the size colony and type of tank purchased.

The only calculated cost for each heated maceration method was the distilled water used in all heated maceration methods and the chemical or enzymatic additives. The cost of these materials can also vary based on the brand, amount being used per sample, and the amount being purchased at once. The cheapest of the heated maceration methods was the distilled water boil and this method could, in fact, be of no cost if tap water were to be used rather than distilled water. The ammonia method was only slightly more expensive than the distilled water method and the bleach was about three times the cost. The most expensive method was, by far, the enzyme-based detergent simmer method, costing over twice as much as the bleach method and six times the amount of the distilled water boil method.

Time Efficiency

Time efficiency was calculated from the time each sample was directly exposed to its specified method. Any pre- or post-treatments required by the method were not included in the time efficiency calculation. This means that the time it took to skin and strip the dermestid samples of excessive soft tissue, dry them in the fume hood before exposure, and remove live bugs from the bones after they were completed were not calculated into the final dermestid time efficiency calculation. It should be kept in mind that the dermestid method is more labor intensive than the other methods analyzed in this study. The dermestid method also took the longest to process, ranging from 3 to 8 days. During times where the method was not completed by Friday afternoon, the sample was removed from bug exposure until the following Monday morning so continual observation would still be possible. The colony also remained in consistent use throughout the process and the additional sources of food within the tank may have affected the speed at which the beetles processed the samples.

The heated maceration methods were all completed in less than one day and were able to be constantly monitored throughout the entire process. The bleach method was the quickest of the methods but the ammonia method was only about a quarter of an hour slower on average and they were the only two methods that did not have a significant time difference between them. The distilled water method took over an hour longer than both the bleach and ammonia methods and the longest method, the enzyme-based simmer, took over an hour longer than the distilled water and was over twice as long as the bleach boil method. The time efficiency calculations showed all the methods to be statistically significant different from each other, excluding the bleach and ammonia methods that did not have a significant difference.

Effectiveness

The effectiveness was qualitatively calculated by the amount of soft tissue removed only by the method tested and the degree of greasiness remaining on the resulting bone. In general, all of the methods removed the soft tissue from the bone, with the only remaining tissue left behind being cartilage between epiphyseal plates and joints. The enzyme-based method seemed to particularly struggle with the breakdown of cartilage and all of the long bones tested with this method failed to separate from the other bones present. The dermestid samples, as well, had some remaining tissue, mostly on the interior of the bone. Had the samples been left in the dermestid colony for a longer period, this remaining tissue may have eventually been digested by the beetles, but once the process had slowed down enough to show no visible change between two checkpoints, the remains were removed. The other heated maceration methods were removed with little to no visible tissue, although some still had small amounts of cartilage remaining.

Despite expectations, most of the resulting samples did not have a greasy texture; even the distilled water boil samples resulted in little to no grease remaining on the bones. The bleach and ammonia methods both resulted in no detectable grease or odor, with all the soft tissue being completely removed. The ammonia did significantly whiten all the samples exposed to it, while the bleach method seemed to only slightly alter several of the bones tested. The dermestid samples did have a slight greasy texture to them upon removal, but after being rinsed and lightly brushed with a toothbrush the greasiness mostly dissipated. The enzyme-based method similarly had to be brushed and rinsed after removal from the detergent solution to remove the slime and grease leftover from the soapy solution it was heated in and the slight odor of detergent lingered on the bones. Excluding the enzyme-based method, no other samples seemed to suffer from any long-term odor or grease and all largely performed well.

Macroscopic Damage

Macroscopic damage was qualitatively assessed after completing treatment to look for any alterations visible on the bone. This primarily focused on water damage, visible cracking, and bone texture. The ammonia and bleach samples had the best results with little to no visible damage on the resulting bones. Both methods, however, caused color alteration on the samples, with the bleach causing slight whitening on several bones and the ammonia resulting in noticeable whitening on all the samples with the bones having an obvious unnatural color to them. The dermestid samples showed no macroscopic damage or alterations but some minimal amounts of tissue and tissue staining remained on all samples.

The two remaining heated maceration methods, distilled water and enzyme-based, caused water damage, cracking on the distal ends, and coarse, dried out textures. The distilled water method left the bones with some minimal to medium level water damage as well as some light

amounts of cracking. The enzyme-based method performed the worst in this category, leaving severe water damage and saturation that affected later analysis of the bone as well as increased porosity and noticeable cracking. The surface was left with a dried out, sandpaper like texture that at times left visible striations along the bone. Aside from the enzyme-based method, the minimal resulting damage on all other samples did not appear to obstruct any view or analysis of the bones.

DNA Yields

The DNA yields were calculated by measuring the concentration of DNA with a Qubit® dsDNA BR Assay Kit. The results of the concentration values were recorded in µg/µl and compared against eight DNA extractions done on four separate bones from two physically macerated control samples. The bleach samples failed to produce any readable results using the broad range assay and the enzyme-based method only produced two readable results of the six samples. The distilled water boil and the ammonia samples both produced readable results for most to all of the samples tested, although the concentration yields were statistically significantly lower than that of the control samples.

The samples that did not produce readable results using the broad range assay were tested a second time using a Qubit® dsDNA 1X HS Assay Kit and were read using the high sensitivity option on the Qubit. All samples produced readable results using the high sensitivity assay, but of the twelve samples that were tested, no reading exceeded .0003 µg/µL.

The DNA concentrations were then subjected to a Kruskal-Wallis Test to determine if the method results were significantly different from that of the controls. The only method that did not produce statistically significant results from that of the control samples were the samples

exposed to the dermestid method. All the heated maceration methods were significantly lower than that of the control samples, but the distilled water boil samples were the least affected, showing significantly higher readings than that of any of the heated additive methods.

DNA Amplification and Analysis

The DNA sequencing was done by the University of Montana Genomics Core using the post-PCR reaction product and forward pig primers. Although the Qubit calculated concentration values for the samples did vary considerably, with some readings as low as 0.0001 μg/μL, all but two of the samples were able to be amplified and sequenced. The two samples that failed to sequence were Sample 113B and Sample 116Aa, neither of which were samples that had to be read using a high sensitivity assay and the duplication of the two failed samples both amplified and produced sequence data that was able to be analyzed. The initial failed amplification was likely due to researcher error at some stage of the process.

All sequences were uploaded into Sequencher 5.4.6 and run through BLAST, registered with NCBI GenBank, and all samples queried 99% for *Sus scrofa* mitochondrial DNA, demonstrating that even the methods that had a severe impact on the DNA yields such as the bleach boil methods, still were able to produce DNA extractions that could be amplified and sequenced.

Availability and Practicality

All equipment used was easily available and not expensive, however the methods varied in amount of preparation required and start up equipment. A dermestid colony requires some level of care and consistent maintenance, not to mention has a more expensive start-up cost than

the other methods tested, and combining that with the long time needed to complete the defleshing, it may not be practical in a forensic context. The heated maceration additives were all relatively cheap and easy to purchase, but required short-term constant supervision to ensure the solution did not overheat or overcook. The fumes and odors ranged throughout the methods, with the enzyme-based method and bleach method just smelling potently of laundry detergent or chlorine respectively. The ammonia method had the most potent and toxic fumes that could be a potential health and safety risk if not handled properly. The method was the only one that required safety goggles and a face mask to comfortably operate within the same room as the sample, even with the ventilation within the lab area. Although a mask was worn throughout all methods, they were not nearly as potent and did not have the same level of toxicity within the fumes. If the ammonia method were to be carried out in a laboratory setting, it would be best done in a fume hood.

The ability to dispose of the solution safely is also something to consider. The distilled water and enzyme based methods could both be disposed of down a standard household drain, but the bleach and ammonia methods had to have extra care taken during disposal. Depending on the plumbing and water supply of a building, it is not always advisable to pour toxic chemicals down a standard drain, particularly in the large amount needed to perform such methods. All solution waste in this study was able to be disposed of at a local chemical waste facility, but without the proper safety measures, both the bleach and ammonia methods may be a health and safety risk to that of the facility and the researchers. It is recommended to look at CDC's website or contact them directly for protocols and warnings for proper disposal of any chemical product.

4.2 Method Summary

Dermestids

In a museum specimen preparation context, the dermestid method is very popular and very effective. The method requires little additional labor if specimens already have skins removed and there is a consistent food source for the samples; the maintenance of the colony winds up being a significantly lower cost than if the vast amount of samples had to be skeletonized by some other method. The additional appeal is that when carefully supervised, remains can be removed at any point of the flesh removal process if the specimen is desired to be left with ligaments intact.

The factors that cause it to appeal to that of museums, taxidermists, and hunters are some of the same factors that make it impractical for the use in a forensic context. A sample needing to have the soft tissue removed is likely to be that of one that has already begun the decomposition process and has prior insect activity. To maintain the health of the colony, excess flesh must be removed and all prior insect activity must be killed off before being exposed to the dermestids. This causes additional time, labor, and excessive handling of the remains that could otherwise be avoided.

The dermestid method does, however, have some very significant strengths that cannot be ignored. It is the only method tried within this study to produce DNA yield results insignificant from that of the control samples and the lack of macroscopic damage to the remains is an important thing to consider. Under certain circumstances, the dermestid beetle method can be very useful and should not be a method completely ignored just based on the time it can take.

Distilled Water Boil

Boiling in distilled water proved to be a relatively quick and easy method, with the remains finishing in under 6 hours opposed to the nearly 6 day average of the dermestids. The method was extremely low cost and if tap water was used in the place of distilled water, the method would be of next to no cost. The bones that resulted from the water boil method tended to have moderate water damage and some minimal cracking along the ends of the bone, as well as a dried out and coarse texture along the bone surface. The damage did not seem to obstruct analysis of the bone, but it should be kept in consideration that the water damage can indicate saturation that severely affects the bone composition as was seen in the enzyme-based method.

Although the method did seem to result in some amount of damage to the bones, there was no greasy residue left on the bones and they did not seem to be left with any foul odor. The method itself, similarly, had a slight odor of cooked meat but the fumes were not potent or harmful to the eyes and the odor did not linger after the method was complete. While still significantly different from that of the control samples, all samples from the distilled water boil had readable DNA concentration results and performed well above the other heated maceration methods.

The distilled water boil method, similar to all heated maceration methods, required constant supervision to ensure timely removal, but overall was not labor intensive. While the level of damage to the surface of the bone is cause for concern, the low cost and ability for DNA extraction still leaves it up for viable consideration when selecting a soft tissue removal method.

Enzyme-based Simmer

While there are many publications praising the performance of enzyme-based detergent simmering methods, the present study did not see any evidence of them. The difference in

detergent selection may have played a significant role as it is not possible to see what common ingredients are found in the detergents in other frequently cited publications describing the method (Ecklund 2007; Nawrocki 1997; Ossian 1970). The detergent method was by far the most expensive, costing well over twice the cost of the bleach method, and aside from the dermestids, took the longest.

In addition to the cost and length of time, the bones were not completely rid of soft tissue, with cartilage remaining on all tested samples. Additionally, the samples saw the most macroscopic damage, with severe water saturation compromising the bone composition which negatively impacted the DNA extraction process when drilling for bone powder. This difficulty in drilling through the water saturation of the bone may explain the lack of readable results during the DNA quantification process. Only two of the six samples produced a readable result and both were significantly lower than that of the controls or even the distilled water boil method.

The solution itself was also difficult to work with, being slimy and slippery in texture and opaque in appearance making for a great deal of difficulty when attempting to check the progress of the sample at checkpoints and leaving a slimy texture to the final results. The fumes from the solution, while not painful or toxic, did carry a strong odor resembling that of a laundromat that lingered in the lab area and on the bones themselves.

The enzyme-based method was the only of the methods to perform poorly in all criteria looked at and there is nothing positive to report about the method in its tested form. In future repetitions of this study it is advised to experiment with different types of enzyme based detergents at varying temperatures to determine if the effects caused by this method are due to a

unique ingredient within the Tide® detergent or if these effects can be found by multiple different enzyme-based methods.

Bleach Boil

The bleach boil samples were significantly faster than all except the ammonia method taking just over 3 hours on average to complete. The method was right in the middle as far as cost, but if buying off-brand bleach or in bulk amounts, the price per sample would be expected to significantly drop. Of the heated maceration methods, the bleach samples had the least macroscopic damage visible with little to no water damage and no tissue remaining on them. Several of the bones did appear to have a whitened appearance, but it was not present on all of the bones nor was it severe.

The solution odor smelled strongly of chlorine, but it was no stronger than that of a hotel room containing a swimming pool and it did not linger on the bones nor in the lab area. However, by the end of the method the solution was greasy and difficult to clean off of the equipment. Additional care needed to be taken during disposal of the solution after method completion and without the presence of a fume hood or suitable ventilation, the method may not be safe to perform indoors. The primary deterrent to the bleach boil method, however, comes from the damage to the DNA quality. Unexpectedly, none of the six bleach samples produced a readable DNA concentration result demonstrating that the DNA concentration was heavily affected by the tested method.

Aside from the damage to the DNA yields and safety, the bleach boil method performed the best of all of the examined criteria. With its quick and efficient results, when DNA preservation is not a concern, these results support previous literature defending the value of

bleach during soft tissue removal, even potentially within a forensic context depending on the circumstances.

Ammonia Simmer

The samples exposed to the ammonia simmer method took only slightly longer on average than the bleach boil samples, with bleach and ammonia being the only methods to not have statistically significant differences in their time efficiency calculations. Additionally the cost was only slightly above that of the distilled water and over a dollar cheaper than that of the bleach. Although significantly lower than that of the control samples, unlike the bleach, four of the six samples also produced a readable DNA concentration result, so while affected by the method, the DNA quality appeared to still be mostly in a usable state.

While the bones from the ammonia method showed no signs of macroscopic damage, including tissue staining or water damage, as well as no remaining tissue or cartilage, they all were severely whitened by the process, which could be an undesirable result depending on the circumstances. The prominent downside to the ammonia simmer method is the toxicity and potency of the fumes. Of all methods employed, the ammonia was the most potent and toxic of the methods, requiring a face mask as well as safety goggles to even be in the same room and even then caused discomfort during checkpoints and disposal. The odor did not linger on the bones or in the lab area and although the solution became greasy by the end, it did not leave any detectable residue on the bones.

While there is no publication documenting this method's usage on human remains, in the proper lab area with the proper safety precautions, this method could be of great value. It was not far off from the quickest method, taking less than 3.5 hours to complete the described pig limb

on average, was cheap, and aside from whitening the bones, did not cause any visible alterations to the bone. The DNA results, while still significantly lower than that of the control and distilled water samples, were still able to be read and quantified. The results here demonstrate that this method should be further examined for its use within a forensic context and in future replications should be tried in different concentration amounts to better examine its effects.

5.0 Conclusion

5.1 Method Selection

The process of soft tissue removal is one that it seems everyone has created their own specific technique for, depending on their access to resources and their own experience. More often than not the selection of method is one that is made on a whim without much thought. Every method has advantages and disadvantages and there is no simple answer to which method is necessarily best, but there is always a method that is going to be best suited to one's desired results. Rather than basing the selection on experience or what one is used to, there is a need to better consider the long term effects on the bone and whether the potential risks are worth the potential benefits.

5.2 Research Significance

This study highlights the time expectations, effectiveness, and DNA extraction quality of several of the more commonly methods utilized within current research. The comparisons and detailed descriptions available here are an early attempt at rectifying the gap in published literature on the benefits and risks. This study was not designed to be one that could provide a conclusive determination of the best performing soft tissue removal method, but rather the beginning of a roadmap for choosing a method that best suits a researcher's needs, expectations, and desires of the final product. This highlighting of the need for greater consideration and awareness of the long term effects a soft tissue removal selection may have on the skeletal

material is one that is not limited to that of a forensic context, but is relevant to that of any field employing this process.

5.3 Limitations

This study was conducted using 17 domesticated pig hocks as a substitute to human remains. While pigs have commonly been used in place of human remains in decomposition studies, until a similar method is tried on human remains, it is impossible to know if the species difference impacted any of the results presented in this study. Additionally the sample size of 17 allowed for only 3 repetitions for each method and therefore could cause trends or patterns in the data to be obstructed or overlooked.

There are many methods of soft tissue removal available and this examined only five of them with only one concentration of each additive being used. Different results for additives could be produced by trying them at different concentration levels, temperatures, or different skeletal elements. The selection of Tide® detergent as the enzyme-based additive could have caused a significant difference in the results than previously documented enzyme-based detergents such as Biz® due to differing ingredients between the two. This study does not necessarily demonstrate the best or worst performing soft tissue removal methods available and can only account for the results produced by the tests performed.

As previously stated, the dermestid tank was in constant use by the museum volunteers and staff, causing for a varying amount of other food sources within the tank at any given time. Each sample took longer than the previous and this could be the increase in samples being prepped and placed in the colony by museum volunteers as the semester progressed. In an ideal

situation, the sample being tested would be the only food source available to the colony to better control outside variables and increase the accuracy of the time expectancy.

5.4 Recommendations for Future Research

The samples tested here were a very small portion of a nonhuman mammal and comparative studies will need to be done with other and larger areas of the specimen and ideally with human remains to determine the accuracy of the results. This study also only addressed five specific methods and one concentration level. In future research, addition methods and analyzed criteria should be added and compared with these results as well as repetitions of the methods tested here to ensure consistency and replicability. In particular, other methods of enzyme-based detergents should be tested at different concentrations and temperatures to further examine the differences in detergent performance. The positive performance of household ammonia in this study could lead to a significant impact in the way soft tissue removal is currently conducted and should be further investigated at varying levels of concentration and temperature to document additional effects of the method.

Appendix A

Table A.1: The identification number and bone of each bone powder sample.

Test ID	Method	Bone	Bone Powder ID	DNA Extraction ID	Test ID	Method	Bone	Bone Powder ID	DNA Extraction ID
001	PWB	Ulna	001A	101A/101Aa*	**010**	Ammonia	Tibia	010B-1	010B
001	PWB	Radius	001B-1	101B	**010**	Ammonia	Tibia	010B-2	
001	PWB	Radius	001B-2		**011**	Ammonia	Ulna	011A	011A
002	PWB	Fibula	002A	102A	**011**	Ammonia	Radius	011B-1	011B
002	PWB	Tibia	002B	102B	**011**	Ammonia	Radius	011B-2	
003	PWB	Ulna	003A	103A	**012**	Ammonia	Ulna	012A	112A
003	PWB	Radius	003B	103B	**012**	Ammonia	Radius	012B	112B
004	Enzyme	Ulna	004A	104A	**013**	Dermestid	Fibula	013A	113A
004	Enzyme	Radius	004B	104B	**013**	Dermestid	Tibia	013B-1	113B
005	Enzyme	Ulna	005A	105A	**013**	Dermestid	Tibia	013B-2	
005	Enzyme	Radius	005B	105B	**014**	Dermestid	Fibula	014A	114A
006	Enzyme	Ulna	006A	106A	**014**	Dermestid	Tibia	014B-1	114B
006	Enzyme	Radius	006B	106B	**014**	Dermestid	Tibia	014B-2	
007	Bleach	Fibula	007A	107A	**015**	Dermestid	Fibula	015A	115A
007	Bleach	Tibia	007B-1	107B	**015**	Dermestid	Tibia	015B-1	115B
007	Bleach	Tibia	007B-2		**015**	Dermestid	Tibia	015B-2	
008	Bleach	Fibula	008A	108A	**016**	Control	Fibula	016A	116A/116Aa*
008	Bleach	Tibia	008B	108B	**016**	Control	Tibia	016B	116B/116Bb*
009	Bleach	Fibula	009A	109A	**017**	Control	Fibula	017A	117A/117Aa*
009	Bleach	Tibia	009B	109B	**017**	Control	Tibia	017B	117B/117Bb*
010	Ammonia	Fibula	010A	110A					

*Aa and Bb designate the samples that had two extractions performed. Control samples were done twice to ensure a comparable control sample and 001A failed in the first extraction attempt.

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