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

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Abstract

Sus scrofa domesticus limbs were obtained as a human proxy to study the effects of five distinct materials used in published methods of flesh removal: *Dermestes lardarius* beetles (also referred to as dermestids), distilled-water boil, bleach boil, enzyme-based detergent simmer, and ammonia simmer. Each method was evaluated based on a set of specific criteria, focusing on time efficiency, macroscopic damage, and the effects on DNA preservation and potential for future analysis.

While the dermestid beetles had the longest time-expectancy and were the most labor-intensive method, they caused minimal damage to the bone surface and did not appear to affect the DNA preservation. Heated maceration methods sped up the process considerably, but that often led to decreased DNA quantity and minimal to severe amounts of macroscopic damage. The ammonia simmer method was the only method tested that was found in zoological literature but did not appear to have any published use within the forensic field, operating occasionally instead as a degreasing agent. While the ammonia method required the most safety precautions, the method was efficient both in time and tissue removal, and left amplifiable DNA, perhaps indicating a potential future in more forensic contexts. In contrast, the enzyme-based detergent method, often praised in published literature, performed poorly in multiple categories of evaluation.

Each method proved to have different advantages and disadvantages, with no method performing the best or worst in every evaluated criterion. The results of this research highlight how differently each method performs and how easily skeletal material, and the DNA within, can be affected by maceration techniques. Method selection can severely impact later analysis and the choice should be made with consideration and awareness of the potential risks and desired results.

Keywords: *Forensic Anthropology; Forensic Science; Maceration; Defleshing; mtDNA*

Introduction

Maceration, or the removal of soft tissue from skeletal remains, is a process familiar to members of a wide array of scientific fields and contexts. Flesh removal is used in museums to prepare bones for display and for comparative research, hunters may prepare remains for showcasing, and body donation research facilities use recently skeletonized remains of modern individuals to refine and update the markers used in biological profile construction and analysis. Additionally, forensic anthropologists al- so use these techniques during casework to reduce fleshed or decomposing individuals to skeletal remains for analysis. Due to the wide array of fields and goals behind the removal of soft tissue from skeletal remains, there are an equally wide array of developed methods to perform the task.

However, no consistent standard exists for forensic applications of tissue removal, which leads to a wide range of protocols and varied guidance in the am-

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ount of additives, temperatures, and timing. While the interest in method efficacy has increased in recent years (Ajayi et al., 2016; Ecklund, 2007; Frank et al., 2015; Mann & Berryman, 2012), there remains a lack of cohesive agreement on which additives may perform best in similar scenarios or which methods have the most negative impact on skeletal remains and DNA viability. While many of these methods and additives are used outside of the forensic sciences, safety and consistency in forensic analysis is of the utmost importance. The bulk of the literature reflects primarily on macroscopic damage that affects trauma analysis, as well as methods that may complicate DNA extraction (Ecklund, 2007; Fenton et al., 2003; Frank et al., 2015; King & Birch, 2015; Lynn & Fairgrieve, 2009).

Heated maceration methods have long been the preferred and more investigated method due to their shorter duration and less manually laborious nature than unheated methods. Simmering and boiling techniques utilizing a variety of additives have become common practice (Couse & Connor, 2015). The additives range from household cleaning products such as dish and laundry detergent to directly adding chemicals such as sodium perborate or carbonate, while still other methods utilize heated water with no additional additives (Lee et al., 2010; Uhre et al., 2015).

The following research focuses on several commonly used soft tissue removal methods, involving four heated maceration methods and one method utilizing *Dermestes lardarius* beetles, hereafter referred to as dermestids. The amount of additives used, the duration to completion each maceration method took, and the effects the method had on the resulting skeletonized material were scored and documented in detail. The results in this study were then used to create a method selection flowchart, empha sizing the different advantages and disadvantages of the tested methods. 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 to any field familiar with the task.

Materials and Methods

Research Sample

The research sample consisted of 17 domesticated pig (*Sus scrofa domesticus*) limbs from the North Dakota State University agricultural program. The pig limbs were primarily the hock portion of the leg and contained fragments of two long bones, either the tibia and fibula or radius and ulna depending on whether it was a hind or front limb. The pigs were all from the same environment and were butchered in the same manner at approximately the same age, all being just under a year old.

The 17 pig hocks were assigned a maceration method at random, with three hocks being assigned to each category and the remaining two being reserved for manual soft tissue removal to allow for a DNA comparison with samples that did not undergo one of the tested methods. Control DNA samples were not taken prior to the maceration of the pig hocks to avoid additional variables and considerations in regard to specific element and region of bone (Antinick & Foran, 2018). In lieu of a true control sample, consistency was attempted by ensuring all pigs were the same age from the same environment and butchered at the same time

with the same technique to limit background variation as much as possible (Arismendi et al., 2004). While this is not a true control sample, whereby samples from each pig hock would have been collected prior to treatment, the two untreated pig hocks can be used for comparison by providing an estimation and expectation of the DNA preservation and degradation resulting from the tested methods.

Tested Maceration Methods

Five soft tissue removal methods, consisting of four heated maceration methods and one method utilizing a colony of dermestids, were selected for study based on methods found in previously published literature and researcher experience (**Table 1**). All the heated maceration method materials, including start-up equipment, are widely accessible and relatively low cost. Cost could be additionally lowered if tap water, as opposed to the distilled water used in these trials, and off-brand additives were used, and materials were purchased in bulk quantities.

All heated maceration methods were performed in a small, ventilated room with as many variables kept constant across methods as possible. Pig hocks subject to heated maceration methods did not undergo any manual processing (Couse & Connor, 2015). The heated methods were performed using an 18qt stockpot containing a two-gallon solution made from distilled water and the specific additive. The amount varies based on the documented amounts in previously published literature (**Table 1**). The household ammonia method was adapted and modified by the researcher from previous published use in a zoological context (Hoffmeister and Lee, 1963) as well as personal experience working in skeletal preparation within a zoological museum. In forensic uses, ammonia tends to be used as a degreaser after the application of a primary method (Lee et al., 2010), but in zoological preparation solutions of up to 50% ammonia can be used in a simmer for the entire process (Hoffmeister and Lee, 1963). The researcher's own experience following zoological museum protocols involved soaking delicate remains in a room temperature ammonia and water mixture for several days to remove residual tissue and grease that the dermestid colony leaves behind. Personal experience with use of ammonia and published uses in zoological contexts were combined to create the method performed in this study.

Two methods, distilled water and 6.25% bleach solution, were kept at a low boil, while the 12.25% household ammonia and the 10% enzyme-based detergent methods were kept at a lower simmering temperature for the duration of the test. A laundry detergent with an accessible ingredient list was utilized to confirm that both lipase and protease enzymes were present in the selected detergent (Uhre et al., 2015). Method temperatures were based on those used in selected references (**Table 1**). The tests were monitored at half-hour checkpoints for temperature checks and photos to ensure consistency of temperature throughout the duration of the method and to document the bone surface. The defleshed bone was then dried in a fume hood for 48-72 hours before being placed within the dermestid tank, in accordance with the museum protocol where the colony was housed (Schroeder et al., 2002). The pig hocks were exposed to the dermestid colony one at a time, but because the primary purpose of the colony was for museum specimen preparation, there

Table 1: Table showing the different additives, amounts, and references of the four heated maceration methods attempted, as well as literature references for the dermestid beetle method and manual soft tissue removal.

	Percentage of Additive Within Solution	Amount of Additive	Approximate Starting Temp	Reference
Manual Soft Tissue Removal	N/A	N/A	Room Temperature	Couse & Connor, 2015
Dermestes lardarius Colony Exposure	N/A	N/A	Room Temperature	Ajayi et al., 2016; Charabidze et al., 2014; Schroeder et al., 2002
Distilled Water	0%	None	99.0° C	Lee et al., 2010; Rennick et al., 2005; Uhre et al., 2015
Enzyme-based Laundry Detergent	10.0%	1.6 cups per gallon	75.0° C	Eklund, 2007; Lee et al., 2010; Mooney et al., 1982; Nawrocki, 1997
Bleach	6.25%	1 cup per gallon	99.0° C	Eklund, 2007; Nawrocki, 1997; Rennick et al., 2005
Household Ammonia	12.25%	2 cups per gallon	90.0° C	Modified based on Hoffmeister & Lee 1963; National Park Service 2006

were other materials present throughout the duration. The dermestid beetle method had a much longer timeframe expectancy and therefore the exposed pig hocks were checked only once a day.

The methods were evaluated on criteria including time efficiency, DNA concentration quantity, ease of application, macroscopic damage, and effectiveness, with scoring tables based on those used in previous studies for the qualitative criteria (Couse & Connor, 2015; King & Birch, 2015; Lee et al., 2010; Steadman et al., 2006; **Table 2**). Criteria were selected and scored based on previous studies as well as the necessary requirements of bone preparation techniques within forensic usages (Couse & Connor, 2015; King & Birch, 2015; Lee et al., 2010; Steadman et al., 2006).

Time efficiency was considered to be the time each specimen was directly exposed to the specified method. Any pre- or post-treatments required by the

method were not included in the time efficiency calculation. Effectiveness and macroscopic damage were qualitatively assessed after each method was completed.

DNA Extraction and Quantification

After each method trial was completed, the macerated pig hock remains were labeled, dried for a 24-hour period, and frozen (-20ºC) until the DNA extraction process could be completed. Each treated pig hock contained fragments from two long bones, either the tibia and fibula or the radius and ulna. Each of these bone fragments were lightly sandpapered to remove any potential surface contamination and then drilled with a 3/8" brad-point drill-bit to collect 0.50g of bone powder. The drill bits were thoroughly rinsed and soaked in bleach for ~30 minutes before being allowed to dry between uses. Bone powder was collected into new, sterile, labeled 1.5ml microcentrifuge tubes and sealed and stored at room temperature until DNA extraction was performed approximately one week later. All surfaces were thoroughly cleaned with bleach between each sample (Silverman, 2018).

DNA extraction was done following a protocol provided with the purchased QIAamp DNA Micro Kit (QIAGEN, 2010) acting solely as a proxy in place of more traditional forensic methods in an attempt to estimate preservation and degradation of DNA within treated samples. The extracted DNA included two samples per pig hock, one for each long bone. The extracted DNA samples were amplified with the PCR protocol: 8.58 μl of H2O, 2.4 μl dNTP, 0.18 μl forward primer, 0.18 μl reverse primer, 1.5 μl of 10X PCR MgCl2 Buffer, 0.45 μl MgCl2, and 0.08 μl of platinum Taq. A total of 13.37 μl of the prepared mix was aliquoted into the 0.2mL tubes 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'GCCTAAATCTCCCCTCA ATGGTA -3') and CO2susR2 (5'AGAAA-GAGGCA-AATAGATTTTCG-3') (Lahiff et al., 2001; Pangallo et al., 2010). PCR was completed for sixty cycles with a 58ºC touchdown annealing temperature. Two 2% agarose electrophoresis gels were run to confirm that amplified DNA was present in all post-PCR reaction product samples before any sequencing or quantification was done. If a clear band of the expected size (in comparison with a 100bp ladder) was shown in the agarose gel for each of the 38 extractions (two DNA extractions from each of the 15 method-tested pig hocks and four from each of the two physically macerated pig hocks), the samples from the PCR product were prepared for sequencing to ensure the amplified DNA present in the samples was the targeted *Sus scrofa* mitochondrial DNA (Silverman, 2018).

Extra care was taken with the DNA analysis, following standards commonly found in ancient DNA laboratories, to enable the accessibility of even minute amounts of DNA. This included extra cycles in PCR, gel analysis, and targeting mitochondrial DNA due to its high copy number. Because of the potential for some of the samples to yield very low quantities of DNA, it was necessary to ensure that even if the amounts were very small, it would be possible to detect any and all potential DNA that could be useful in forensic or other analyses.

Of the 38 DNA extractions that were sent for sequencing at UM's Murdoch Sequencing Core, 36 came back with DNA sequences that were then up-

Table 2: Description of the qualitative criteria and score values used to analyze each method. Scoring tables were modified from those used in previously published literature (Couse & Connor 2015; Lee et al., 2010; Silverman, 2015; Steadman et al., 2006).

Effectiveness of Method		Macroscopic Damage		Ease of Application	
Score	Description	Score	Description	Score	Description
1	Bones were cleaned completely only by the method tested with no presence of grease.	1	Bones show no sign of macroscopic damage or alteration.	1	Application of method is easy to follow, requires no past experience, and needs no supervision.
2	Bones were mostly cleaned, may have involved some additional effort by the researcher. Little to no grease present.	2	Slight alterations such as a single crack or spot of visible water staining on the cortical bone are present.	2	Application of method is simple to follow and requires no past experience. It may require limited prior knowledge of the method or minimal amounts of supervision.
3	Some cartilage and a minimal amount of grease may still be present, but still mostly cleaned.	3	Mild alterations on bone are present, such as multiple cracks and visible water staining on multiple areas of the bone. Exterior of the bone may feel dried out.	3	Application of method requires some knowledge of or experience with method or specific guidelines to follow. May require intermittent supervision or minimal labor.
4	Cartilage remains on bone, grease may still be present, interior of bone may still have some bone marrow present.	4	Significant alterations present such as large cracks, dried out and rough exterior, slight porosity, and visible water staining.	4	Method application requires knowledge or experience with the method. Requires some amount of consistent supervision or moderate labor.
5	Cartilage and tissue still present on bones. Material is not completely cleaned.	5	Severe water damage or tissue staining present, chipping or severe cracking visible. Bones feel dry and rough. Increased porosity. Bone structure is compromised, and water staining spots are saturated and soft.	5	Method application requires prior knowledge and experience with the method and time-consuming labor and supervision before, during, or after the method.

loaded into Sequencher 5.4.6 for editing and analysis. Two extractions failed to produce sequence data, but other DNA extractions from the same samples did sequence and the failed sequences were considered to be caused by error. All 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. DNA concentration quantification was done using a Qubit® dsDNA BR Assay Kit (ThermoFisher). Extracted DNA from all 38 of the DNA samples 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 extracted DNA and the standards each had 10μL of the standard and 190μL of the buffer. If a sample was too low to provide a readable value, 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 value (Silverman, 2018).

Statistical Analysis

The starting weights of each hock portion were used in a one-way single factor ANOVA statistical test sorted by their assigned methods to ensure there was no significant difference in weight between sample groups. Additionally, the start weights were compared against their time efficiency in a Pearson's R correlation test to determine if weight of the specimen had an impact on the time efficiency of each method. DNA concentration values were subjected to a Kruskal-Wallis test to determine if the concentration values from the tested methods were significantly different from the samples that only underwent manual soft tissue removal.

Results

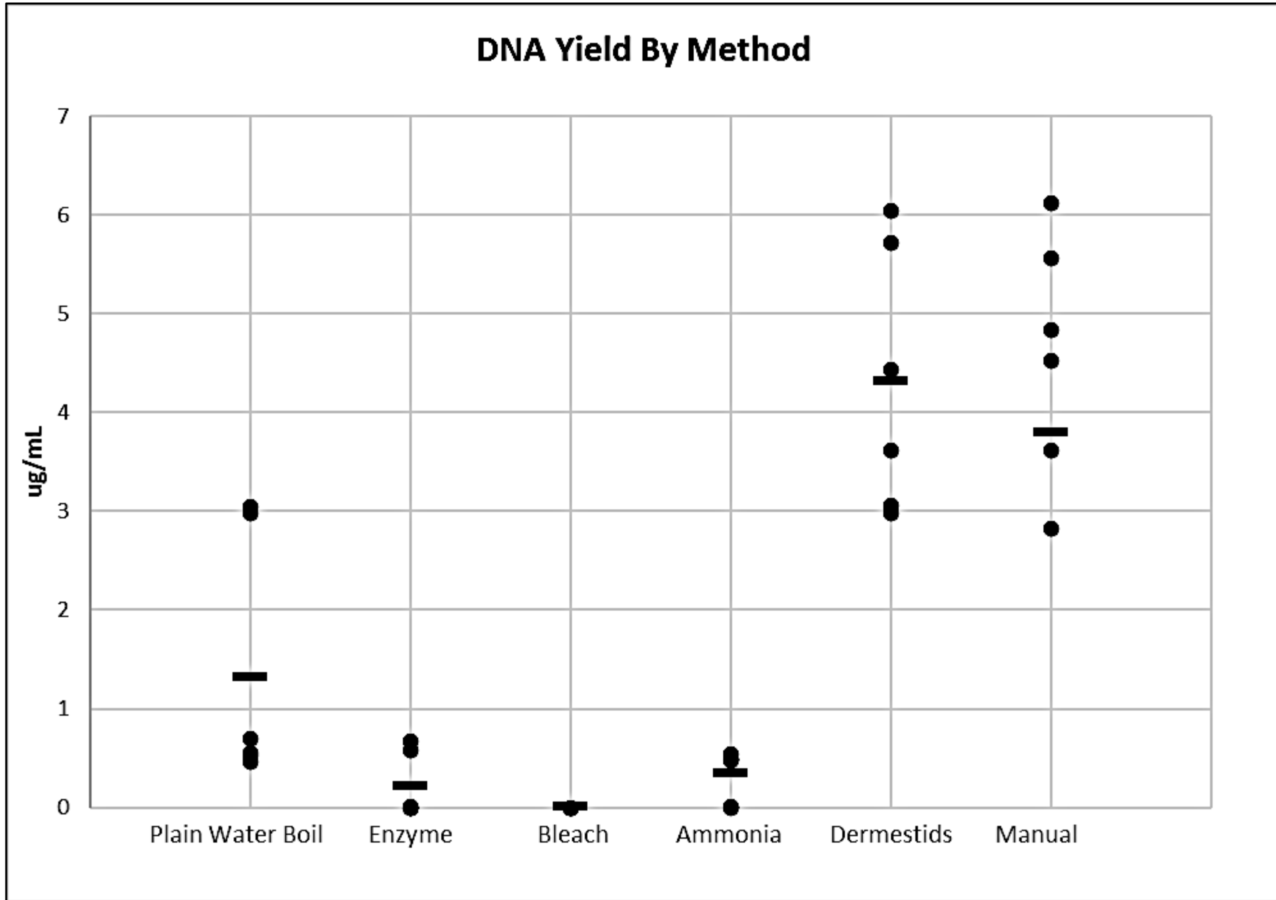
Initial Weight Distribution

The weight of the 17 pig hocks ranged from 340.00g – 586.00g ($\bar{x}$ = 432.88g, s = 16.84). A one-way single factor ANOVA showed no statistically significant difference in the start weights of the pig hocks across the different soft tissue removal methods ($F(5,11) = 0.7768$, $p = 0.5836$). When the pig hocks were calculated against their completion times within their assignment method group in a Pearson's R correlation test, all methods individually showed minimal to no correlation between initial weight and completion time.

DNA Yields and Sequencing

The extracted DNA concentrations calculated by the Qubit are shown in **Figure 1**. Averages were calculated using both the broad range and high sensitivity assay concentration values. All samples produced a significantly different DNA concentration when compared with the samples that had the soft tissue manually removed, except for the dermestid samples ($\chi 2 = 0.15$, $p = 0.69854$). This demonstrated the DNA quality was not significantly impacted by the dermestid method in comparison to the samples that were not exposed to a tested treatment method, as opposed to the heated

Figure 1: Scatter graph showing the average extracted DNA concentration in µg/µL of each method tested. Averages are shown by solid black line; black circles represent individual specimen results.



maceration methods that all showed significantly lower yields ($p < .05$; **Figure 1**).

The bleach samples failed to produce any readable results using the broad range assay and the enzyme-based detergent method only produced readable results for two of the six samples. The distilled water boil and the ammonia samples both produced readable results for most of the samples tested, although the concentration yields were significantly lower than that of the physically macerated samples. In total, twelve samples failed to produce readable results using the broad range assay, but all produced results using the high sensitivity assay. These results are summarized in the below table (**Table 3**). The samples with readable DNA concentration yields using a broad range assay were considered to be less significantly impacted by the method. No high sensitivity assay result exceeded 0.0003 µg/µL.

Although the Qubit calculated concentration values for the samples varied, with some readings as low as 0.0001 µg/µL, all but two of the samples were able to be amplified and sequenced by the University of Montana Genomics Core. All sequences queried 99% for

Susscrofa mitochondrial DNA, demonstrating that even the methods that had a severe impact on the DNA yields, such as the bleach boil methods, were still able to produce DNA extractions that could be amplified and sequenced for at least mtDNA.

Scored Criteria Analysis

The methods were all scored based on the criteria descriptions listed in **Table 2** and averaged for comparison (**Table 4**). Time efficiency is specifically documented in **Figure 2**. Even excluding preparation time, the dermestid method took the longest to complete, ranging from three to eight days. The heated maceration methods were all completed in less than a single day, with the bleach and ammonia averaging under four hours per sample (**Figure 2**). On average, the distilled water boil method took an hour longer than the ammonia and bleach, while the enzyme-based laundry detergent simmer method took over twice as long as the bleach boil method.

Overall, all methods performed effectively, with a majority of the tissue left behind being cartilage between epiphyseal plates and joints. The enzyme-based detergent method in particular failed to remove cartilage, and all long bones treated with this method failed to separate from adjacent bones. The distilled water boil method also had cartilage remaining on all three pig hocks tested with the method, but the amounts were less than that of the enzyme method, and the bones present separated from one another. The dermestid specimens were left with minimal amounts of remaining tissue that was mostly located on the interior of the bone. The other heated maceration methods (bleach and ammonia) were completed with little to no visible tissue and with minimal amounts of cartilage remaining on several of the treated bones. Specifically, the ammonia simmer and the bleach boil method each had one pig hock of the three retain some remaining remains from a majority of the tested methods did not have a greasy texture or a lingering odor. The bleach and ammonia methods both resulted in no detectable grease, either visually or to the touch, cartilage on the epiphyses. The resulting remains from a majority of the tested methods did not have a greasy texture or a lingering odor. The bleach and ammonia methods both resulted in no detectable grease, either visually or to the touch, nor any noticeable odor. The pig hocks

Table 3: Table showing how many samples from each method required the high sensitivity assay to produce readable DNA concentration yields and how many samples were able to be sequenced.

	Manual Soft Tissue Removal	*Dermestes lardarius* Colony Exposure	Distilled Water Boil	Enzyme-Based Detergent Simmer	Bleach Boil	Ammonia Simmer
Readable yields with broad range assay	8/8	6/6	6/6	2/6	0/6	4/6
Readable yields with high sensitivity assay	N/A	N/A	N/A	4/4	6/6	2/2
Sequenced	7/8	5/6	6/6	6/6	6/6	6/6

exposed to the dermestid colony did have a slight greasy texture upon removal, but after following museum protocol and being rinsed with tap water and lightly brushed with a toothbrush, the greasiness dissipated. The enzyme-based detergent method similarly had to be brushed and rinsed after method completion to remove the slime and grease left over from the soapy solution it was heated in. A slight odor of detergent lingered on the bones. Excluding the enzyme-based method, no other samples had any evidence of long-term odor or grease.

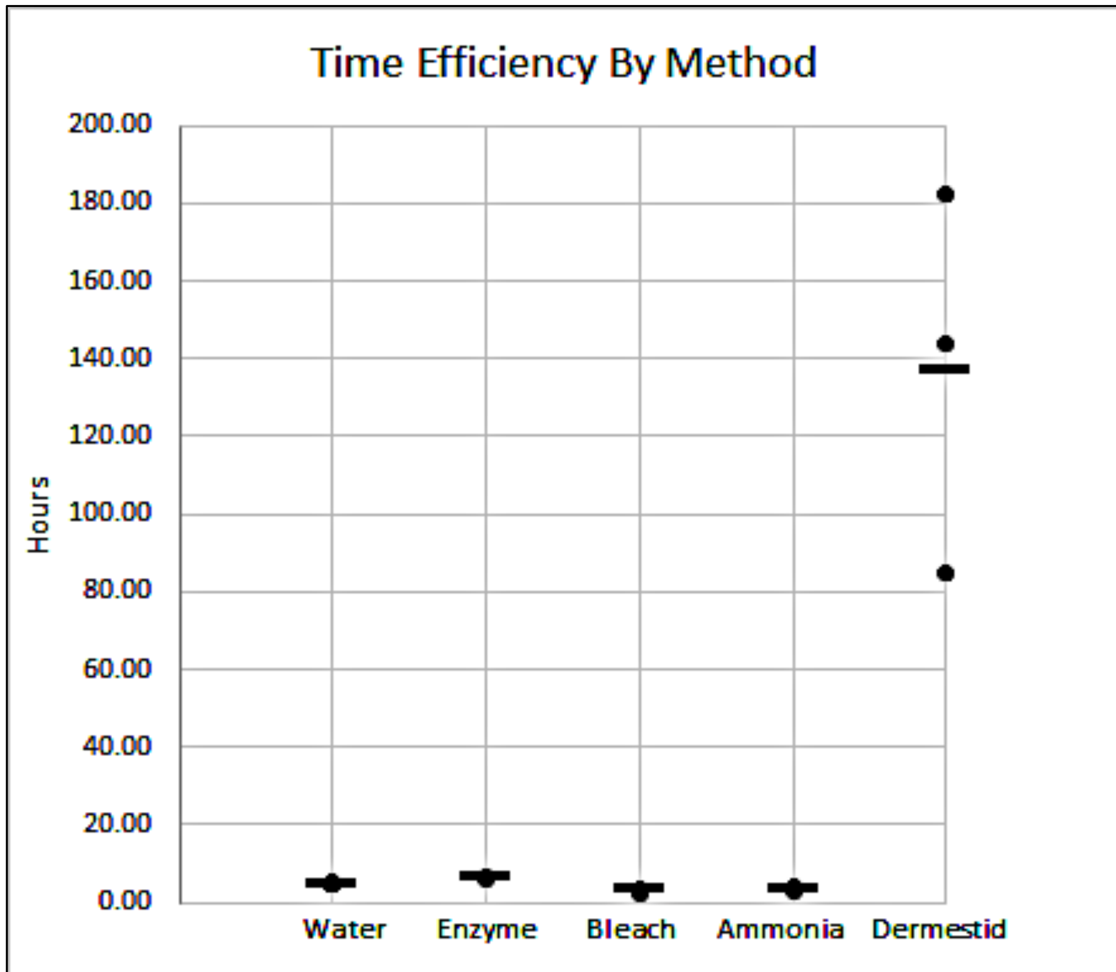
In regard to macroscopic damage, the ammonia and bleach methods produced the best results, with little to no visible damage on the resulting bones. Both methods caused slight color alteration on the resulting remains, with the bleach causing slight whitening on several bones and the ammonia method resulted in an obvious altered whitening to all tested samples. The dermestid method showed no macroscopic damage or alterations but did result in slight amounts of tissue staining on all the samples. The two remaining heated maceration methods, distilled water and enzyme-based, both showed visual macroscopic damage as a result of the treatment. The distilled water method left the bones with minimal to moderate water damage

Table 4: Starting and final weight ranges and averages, the time to completion ranges and averages, the DNA concentration value ranges and averages, and the average score values of all the samples within a method for each of the criteria in Table 2.

	Starting Weight (Average)	Final Weight (Average)	Time in Hours (Average)	DNA Concentration (Average)*	Effectiveness	Macroscopic Damage	Ease of Application
Dermestes lardarius	424-540g (473g)	38-51g (43.5g)	84.73-182.25h (136.92h)	2.98-6.04 µg/µL (4.31 µg/µL) $p = 0.699$	3	1	5
Distilled Water Boil	340-439g (396g)	45-54g (50g)	4.72-5.27h (5.00h)	0.46-3.04 µg/µL (1.311 µg/µL) $p = 0.020$	2	4	2
Enzyme-Based Simmer	378-424g (401g)	58-87g (68g)	5.83-6.75h (6.38h)	0.00-0.66 µg/µL (0.21 µg/µL) $p = 0.002$	4	5	3
Bleach Boil	358-494g (406g)	33-80g (49g)	2.52-3.57h (3.12h)	0.00-0.00 µg/µL (0.00 µg/µL) $p = 0.002$	1	1	3
Ammonia Simmer	342-586g (472g)	31-99g (73g)	2.78-4.05h (3.46h)	0.00-0.54 µg/µL (0.33 µg/µL) $p = 0.002$	1	2	3

*Samples with soft tissue manually removed had DNA concentrations ranging from 0.96-6.12 µg/µL. P-values reported with DNA concentration values are Kruskal-Wallis significance test results in comparison with the samples that were not exposed to a tested treatment.

Figure 2: Scatter graph showing time efficiency of each heated maceration method. Averages are shown by a solid black line; black circles represent individual specimen results.



shown in small visible water stains on the cortical bone that in two instances showed saturation and compromised bone integrity, as well as several small cracks on two of the three tested pig hocks. The enzyme-based detergent as well as increased porosity and noticeable cracking on at least one element from each pig hock. The saturation caused a decrease in solidity of the bone composition, which caused difficulty when drilling the bones for DNA. The bone surface was left with a dried out, sandpaper-like texture that in some instances left visible striations along the bone. Aside from the samples treated with the enzyme-based method, the minimal damage produced from all other treatments did not appear to obstruct any analysis of the bone or affect long-term storage potential.

Discussion

Results found within this study in a majority of cases appeared to largely parallel that of prior research. The literature in time efficiency, while variable due to the difference in sample specimen and temperatures utilized, all largely praised heated maceration methods for their speed and specifically found higher temperatures and boiling, when done carefully with adequate supervision, to be

more efficient than the low or no heat alternatives (King & Birch, 2015; Nawrocki, 1997).

While many of these results parallel that found in prior studies, there were also several differences found within this study that either differ or directly contradict that found in prior research. Most significantly, the enzyme-based detergent method tested within this study appeared to cause serious degradation to the DNA, a point directly in opposition with prior attempts at this method (Lee et al., 2010; Steadman et al., 2006). Additionally, the pig hocks exposed to the enzyme-detergent method were among the most macroscopically damaged and the bone quality was significantly compromised, a factor not noted in any prior published usages. While the enzyme-based method is one that has been praised in past documented uses (Lee et al., 2010; Simonsen et al., 2011), this research did not see the same level of successful results, with the method taking longer, proving less effective, and causing more damage both macroscopically and to the DNA quality than previously reported. In previous publications utilizing this method, it has been mentioned that a protease and lipase in the correct concentration and ratio are the most effective, however, laundry detergents do not always specify the amount or specific enzymes included (Uhre et al., 2015). The inability to clearly compare the detergents makes it difficult to conclude if the failure of the method is due to a difference in specific enzyme ratios present within the detergent selections or the temperature reaching too high of a degree for proper enzyme activation. The poor results of the method within this study call for a reevaluation of the detergent-based methods within the field to identify what brands of detergent work most effectively, without causing damage, and why.

The current study did demonstrate the expected decrease in DNA preservation in the bleach tested samples, corroborating with other similar studies (Steadman et al., 2006), but while several studies saw decrease in bone quality or surface alteration in samples with bleach, the bleach-tested hocks within this study showed no bone alteration or surface texture changes and macroscopically appeared to be in excellent condition. This could be due in part to other studies testing the bleach solution at lower temperatures, consequently causing the elements to have a much longer exposure time, versus the boiling temperature used within the present study where samples were exposed to the bleach solution for a maximum of under four hours (King & Birch, 2015; Steadman et al., 2006).

The ammonia simmer method, while occasionally found in zoological research or as a degreaser after a primary method (Fenton et al., 2003; Lee et al., 2010; Steadman et al., 2006), is not one that could be located in published forensic research and therefore the results could not be compared. The promising performance by the ammonia within this study opens opportunity for a new heated maceration method and could benefit from additional research and data collection through repetitions of the method described in this study, as well as other variations with temperature and amount of additive.

Overall, each method tested within this study was able to perform the task of skeletonizing the *Sus scrofa* limbs efficiently in a relatively short amount of time, but each method has advantages and disadvantages associated with it. The bleach boil method performed

quickly and efficiently but did significantly affect the DNA quality, and in a situation where that is a concern, this method should be removed from consideration. The dermestids and ammonia simmer method both performed well with little damage or added concern, but with the dermestids taking days opposed to the hours of a heated maceration method. While the distilled water boil method did cause more morphoscopic damage than several of the other methods, the integrity of the bone largely remained intact. The time efficiency and cleanliness of each method did vary moderately, but all methods could be a viable selection in most general situations. While all the methods, even the bleach boil, were able to be sequenced for DNA, it should be kept in mind that the DNA analysis methods used were those common in ancient DNA laboratories in anticipation of the reduced DNA quantity. The low DNA yields of the bleach boil still indicate the method to not be appropriate in the event DNA will be required from the remains after the method is completed. Additionally, while the enzyme-based detergent method samples similarly were all able to be sequenced, the surface damage and saturation to the bone that the method caused made cutting the bone for DNA very difficult and caused even further damage to the element. This, along with the low average DNA yield of the tested samples, suggest that the enzyme-based detergent method as it was performed here is also not a preferable method choice when DNA viability is a factor needing to be considered. **Figure 3** outlines a basic method selection flowchart based on the results within this study.

The process of soft tissue removal is one that varies significantly, with even the same additive having documented usages of very different concentrations (King & Birch, 2015; Steadman et al., 2006). There is a need to consider the long-term effects on the bone and whether the potential risks are worth the potential benefits. While one should always seek to limit any damage to skeletal material, all methods will have some lasting impact on the end result and should be considered as a method is selected.

Conclusion

This study highlights the time expectations, effectiveness, and DNA extraction quality of several of the more common methods utilized within current research as well as additionally testing a less common method of a sole ammonia simmer. This study aims to be the beginning of a roadmap for choosing a method that best suits a researcher's needs, expectations, and desires of the final product, while introducing the ammonia simmer method to a forensic context and highlighting previously unnoted issues with enzyme-based detergent maceration. Beyond forensics, the awareness and consideration of the long-term effects soft tissue removal methods may have on skeletal material is relevant across any field performing skeletonization or storing the resulting remains. A majority of methods in some way have been shown to violate the skeletal preparation standards set forth within the field, whether they deteriorate the surface, alter the material, or render the bone unfit for DNA analysis or long-term storage. Similar to former studies, these results demonstrate that no single method can successfully satisfy all the requirements of an ideal skeletonization method (King

Figure 3. Method selection flowchart based on the results presented in this study.

Sample in need of maceration
Will DNA ever be required from the sample?
Yes
No
Is there a rush to complete the maceration?
Yes
Is there fume hood access, or high ventilation?
Is there concern of creating macroscopic damage on the sample?
Yes
Bleach
No
Yes
No
No
***Dermestes lardarius* Colony Exposure**
Household Ammonia
Distilled Water
Enzyme-based Laundry Detergent

& Birch, 2015; Scientific Working Group for Forensic Anthropology, 2011). While the method that is likely to do the least amount of harm to both the skeletal material and DNA preservation should always be the chosen method, the priorities of what constitutes the least amount of harm may vary based on the desired results and intended future of the remains.

Results and comparisons detailed here are greatly limited by the small sample size and the usage of pig hocks as a human proxy. While there has been recent research showing dissimilarities in decomposition processes between pig and human remains, a large bulk of this research has focused on the later stages of decomposition and environmental processes, such as scavenging and bug activity. While the concerns raised in this recent research have significant implications for post-mortem interval studies, early stages of decomposition and skeletal composition have still been said to be homogenous enough for research purposes (Connor et al., 2017; Knobel et al., 2018).

With only three repetitions of each method as done in the present study, trends or patterns within the data may be obstructed or overlooked. Furthermore, there are many methods of soft tissue removal available and this study examined only five methods with only one concentration of each additive. Different results

for additives could be produced by using different concentration levels, temperatures, or different skeletal elements. The enzyme-based detergent method tested in this study did not perform to the same quality as other previously published usages and may warrant a reevaluation in the field. While better results may be found in other variations of detergent (Ajayi et al., 2016; Lee et al., 2010; Simonsen et al., 2011) or by directly adding enzymes to the water solution (King & Birch, 2015; Uhre et al., 2015), further examination of different detergents, concentrations, and temperatures are needed to identify the limitations of this method.

Future research is recommended with additional methods, additives, concentrations, and criteria for further data collection, as well as repetitions of the methods tested here to ensure consistency and replicability. In particular, the poor performance of enzyme-based detergent within this study warrants further evaluation of detergents at different concentrations and temperatures to further examine the differences in detergent performance. The use of household ammonia, a new method in the forensic context, is recommended to be further investigated at varying levels of concentration and temperature to document additional effects of the method. Additionally, the DNA extraction method utilized in this study was used as a proxy to estimate preservation and degradation of DNA within treated samples, more traditional forensic methods of DNA extraction should be tested in order to better determine the viability of the methods in a forensic context.

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