Forensic Taphonomy and Stable Isotopes: An Examination of δ^{13}C and δ^{15}N in Decomposing Skin, Muscle, and Grave Soil as an Indicator of Post Mortem Interval.

by

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Abstract

Forensic taphonomy studies the circumstances surrounding death and subsequent processes of decomposition. In medicolegal death investigations, two pieces of information are essential- the identity of the deceased and the post-mortem interval (PMI). Current methodology uses stable isotopes in hard, decomposition-resistant tissues to provenance the remains for identification. Stable isotopes have not yet, however, been examined as a forensic tool for estimating PMI in soft tissues from recovered remains.

This dissertation reports unique data on stable isotope ratios of $\delta^{13}C$ and $\delta^{15}N$ in three studies. The first study examined human skin and muscle stable isotope ratios from nine human donor bodies over the first 20 days of outdoor decomposition in Texas. I found $\delta^{15}N_{muscle}$, with mean starting values of 9.5‰, increased by 2.5‰ by day 10 and plateaued beyond the 20-day study. Second, I measured $\delta^{13}C$ and $\delta^{15}N$ in two studies using underlying grave soils in an area known as the cadaver decomposition island (CDI) in the first 30 days of decomposition (PMI_{30}) and over longer periods up to 900 days PMI (PMI_{900}). In the PMI_{30} study, surface gravesoil $\delta^{15}N$ increased from a mean value of 3.3‰ to 8.2 ‰ by days 10-15, peaking at 9.9‰ at day 20, and remained elevated beyond 30 days. In the PMI_{900} study, gravesoils starting with 2-4‰ $\delta^{15}N$ increased by 15-20‰ after the first month, eventually declining to 9-11‰ by 600-900 days. The third study in New Brunswick, Canada generated preliminary regionally specific data for porcine analogues. As with the first Texas human decomposition study, the porcine analogue tissues confirmed $\delta^{15}N_{muscle}$ showed the greatest promise as a forensic tool with mean starting values of 4.1‰ increasing by 0.5‰ by day-6 and remained elevated beyond the end of the 18-day experiment.

My novel work provides baseline data confirming the potential of using stable isotopes in soft tissues, specifically $\delta^{15}N_{muscle}$, to provide quantifiable information applicable to medicolegal death investigations. Future research can use my data as a proof-of-concept framework to refine methodologies that provide more accurate PMI estimates that are a key piece on real-world forensic casework.

Dedication

This dissertation is dedicated to Dr. William Roy Miles, *my Rummy*.

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List of Symbols, Nomenclature, or Abbreviations

ADD: Accumulated degree days

AIC: Akaike information criteria

BLS: Bovine liver standard

CFIRMS: Continuous flow isotope-ratio mass spectroscopy

DOC: Dissolved organic carbon

DON: Dissolved organic nitrogen

FACTS: Forensic anthropology centre at Texas State

FARF: Forensic anthropology research facility

IAEA: International Atomic Energy Agency

MLS: Muskellunge muscle standard

NH_4-N: Ammonium nitrate

NO_3-N: Nitrate nitrogen

NRN: Ninhydrin reactive nitrogen

PMI: Post mortem interval

TBS: Total body score

TSD: Time since death

V-PDB: Venne pee-dee belemnite

CHAPTER 1 INTRODUCTION TO FORENSIC TAPHONOMY

Forensic taphonomy, the branch of forensic anthropology dealing with the processes of human decomposition to determine circumstances and time-of-death, is a relatively new discipline that has become a focus of both experimental research and retrospective analysis of forensic cases for the past forty years. However, despite advances in our understanding of human decomposition, there are limitations and unknown factors within the discipline, specifically, estimating time since death in recovered human remains has not been clearly addressed in research or in experimental design. In this introduction chapter, I first present a literature review on decomposition addressing the major factors that impact taphonomic processes and issues of standardized decomposition data. The second part of the introduction provides a framework for investigating new avenues of stable isotope ratio analyses beyond their current use in provenancing recovered human remains and into the realm of PMI (post-mortem interval) data for real-world applications.

1.1. Issues using standardized forensic decomposition data

a. <u>Post-mortem changes</u>

One of the most critical questions addressed in forensic taphonomic research is estimating the PMI of a deceased individual. The time between death and discovery of a body is important, particularly in forensic cases where determining the time of death may have legal implications. Whether PMI experiments are conducted on human donor bodies or porcine analogues (using *Sus scrofa domesticus* pigs as research substitutes), the basis for collecting this type of information involves subjective, qualitative visual observations of decomposition and translating them into a standardized quantifiable set of data that can be statistically analyzed. In the following section, I will discuss decomposition progression and the development of standardized decomposition variables which have their own biases and limitations.

The process of human decomposition is well documented in outdoor studies (Goff 2009, Mann *et al.* 1990, Parks 2011, Prieto *et al.* 2004, Zhou & Byard 2011). Immediately following death, three post-mortem changes begin: livor mortis, the settling of bodily fluids to gravitationally dependent areas of the body; algor mortis, the cooling of the body to equal that of its environment; and rigor mortis, a temporary stiffening of the muscles caused by biochemical changes to muscle contractile proteins after death (Gunn 2009). These early post-mortem changes usually occur in a timeframe of hours or days following death. After these early changes have occurred, then decomposition follows, normally visible within 48 hours after death.

The stages of normal decomposition follow a predictable pattern (Gunn 2009). The first stage is fresh, when the body does not exhibit significant visual evidence of decomposition, such as odour or discoloration. The fresh stage is followed by the bloat phase, where autolytic and bacterial enzymes, particularly in the gut, result in large volumes of putrefactive gases accumulating in the intestines and abdomen to the point where the abdominal organs may rupture and be released from the peritoneal cavity. Bloat will then progress into active decay, where the greatest mass loss occurs as tissues are subjected to intense insect activity and ultimately liquefied and liberated from the body. Once peak tissue mass loss occurs, there is less substrate available for bacteria and insects to feed on and decomposition slows; the body then enters the advanced decay stage. Eventually, decomposition will reduce the soft tissues of the body until only bone and other decomposition-resistant tissues like hair, fingernails, and tendons remain. This is the skeletonized stage, and these bony elements can remain for years if undisturbed. These stages of decomposition have been universally observed in human remains in various locations, but standardization of decomposition stages and timing is required for meaningful comparison of collected data.

b. <u>Total Body Score (TBS) and Accumulated Degree-Days (ADD)</u>

The first major study to assign a numerical value to a series of visual descriptive stages in the decomposition progress was by Galloway *et al.* (1989) describing the cadaver as a single unit with an alphanumeric value assigned to the whole body within five sequential stages and subcategories of decomposition. A later study by Megyesi *et al.* (2005) took this initial descriptive decomposition work looking at the whole cadaver as a unit and modified it to create the total body score (TBS) system. This differs from the Galloway *et al.* (1989) method by instead of assigning an alphanumeric value to the entire body, TBS separately scores the decomposition of the head, trunk, and limbs and then sums those scores to calculate the TBS. Separate regions of the body decompose at different rates based on the availability of skin, muscle, fat, and natural orifices. A totally fresh cadaver is denoted by a TBS score of 3, while a fully skeletonized body has a TBS score of 35. The combined efforts of Galloway *et al.* (1989) and Megyesi *et al.* (2005) in standardizing decomposition observations were ground-breaking in forensic taphonomy. It is not an exaggeration that they significantly shaped the discipline; according to the abstract and citation database Scopus, these two works have been cited 219 and 419 times, respectively, at the time of writing this chapter.

Megyesi *et al.* (2005) took the concept of decomposition standardization one step further by correlating the visual TBS data with accumulated degree-days, ADD. ADD are a combined product of thermal energy and time, where the elapsed time in days is multiplied by the mean daily temperature. In theory, this allows elapsed time since death and temperature to factor into PMI, as temperature is the single most important factor affecting decomposition (Buchan & Anderson 2001, Goff 2009). ADD is used in other disciplines, such as entomology and fish husbandry, where organism growth and metabolism are a direct function of temperature (Schoenly & Hall 2002, Schoenly *et al.* 2007, Meier-Augenstein 2010). For example, if an insect grows to a specific point in 100 ADD, then that amount of growth should be able to be reached via 10 days at 10ºC, or 4 days at 25ºC, or any combination thereof where time in days is multiplied by the mean temperature yielding a result of 100 ADD. However, what works for insects and fish during tissue growth does not necessarily work for human decomposition, which is by its nature, a destructive process in a larger more complex organism.

c. <u>Climate and regionally-specific taphonomic standards</u>

Initial results from Megyesi *et al.* (2005) showed great promise when TBS and ADD were initially correlated to a statistical degree of significance. They created a formula where TBS scores could produce an ADD PMI estimate with a reported r^2=0.845 ($p<0.001$). Unfortunately, subsequent research has found this formula does not hold true in other regions of the world, and is further hampered for practical use as it used retrospective forensic case files with photos (a single point in time at discovery of the remains) from Michigan and Indiana instead of a series of in-person observations on real cadavers. Despite the methodology involved in the TBS system where several data collectors could introduce a bias with respect to subjectivity in assigning score values, the variability in multiple observers was examined and found that in people with at least some training or experience with the TBS system, this variation was minimal and ultimately insignificant (Dabbs *et al.* 2016). If observer data are not a source of error in the TBS and ADD equation, what else should be considered when applying it to forensic cases?

One consideration with using ADD measures of decomposition data and equating it to PMI is the mathematical nature of the equation. First, the equation is reliant on temperature. Below a species-specific thermal threshold, metabolism in microbes and insects cannot occur, so decomposition halts once cadaver tissues fall below that temperature. However, if human tissues become frozen, which routinely occurs in temperate and northern climates, decomposition is significantly impacted. Instead of decomposition simply resuming once the tissues warm up, the biochemical aspects of the freeze-thaw cycle create ice crystals in the cells which ultimately impacts PMI estimates in two ways (Roberts & Dabbs 2015). First, thermal energy applied to a frozen body has the initial heat input directed toward the thawing process instead of immediately re-starting decomposition. This results in frozen bodies taking longer to reach the same stage of decomposition as non-frozen bodies. As a result, the ADD PMI estimates based on TBS are underestimated. Second, the patterns of decomposition are altered. Frozen bodies may only minimally or not bloat at all, display increased dehydration and desiccation of tissues leading to increased insect activity, and discoloration of the abdominal skin is reduced or absent. In short, bodies subjected to the freeze-thaw cycle are affected by external agents of decay, decomposing from the outside-in, and thus experience rapid decomposition processes. Bodies not experiencing freezing conditions decompose from the inside-out, impacted to a greater degree by internal agents of tissue loss (Micozzi 1986).

While the Megyesi *et al.* (2005) equation calculating PMI seemed promising, many studies have reported results that significantly differ when comparing known ADD values with those calculated from TBS scores. Regional differences have been reported from Spain (Prieto *et al*. 2004), South Africa (Myburgh *et al.* 2013, Forbes *et al.* 2019), Australia (Marhoff *et al.* 2016), and Texas (Suckling *et al.* 2016). Vass (2011), attempting to improve on the Megyesi *et al.* (2005) equation to estimate PMI, devised two calculations using temperatures and a constant based on skeletonization time: one for surface remains, and another for buried remains that incorporates local air/soil humidity, visual decomposition, and adipocere (grave wax) formation. This paper was presented as a universal post-mortem interval formula, but it too has been discredited for other regions, specifically Canada (Cockle & Bell 2015). Research continued on the creation and validation of formulae to reliably predict PMI from not only outdoor surface remains, but also indoor, buried, and submerged remains (Buekenhout *et al.* 2018, Ceciliason *et al.* 2018, van Daalen *et al.* 2017). A new method of human PMI calculations uses a more holistic approach combining external and internal organ features to create an Entire Body Score (EBS). EBS is then factored into calculation of TSD (time since death) values measured in days, with promising results (Hayman & Oxenham 2017).

A final point of consideration regarding PMI equations that use TBS and ADD is the divide between those studies that have the advantage of using human experimental donor bodies versus those that are limited to porcine analogues. The next section of my introduction chapter discusses the specifics between human and pig anatomy, tissue composition, and decomposition rates and patterns between the species. Those differences must also be addressed when applying human-derived PMI equations to animal subjects. Work from Tennessee, Texas, South Africa, and Australia have provided extensive data that ADD estimates of PMI derived from humans do not apply to porcine analogues (Dautartas *et al.* 2018, Knobel *et al.* 2018, Myburg *et al.* 2013, Suckling *et al.* 2016). To increase validity of PMI estimates based on visual data, separate porcine-centric TBS scoring methods have recently been proposed to increase scientific rigor. Fitzgerald & Oxenham (2009) developed a degree of decomposition index (DDI) system for porcine decomposition that permits scoring of eight body regions instead of three. The DDI method also permits assigning scores in incremental units (1.0, 1.1, 1.2, 1.3, etc.) instead of the simpler, integer assignments (1, 2, 3, etc.) used by Galloway *et al.* (1989) that must be treated as ordinal rankings in statistical analyses (Cockle & Bell 2015). Spies *et al.* (2020) implemented a body segment approach and found significant impacts on decomposition rates whether the porcine analogues were clothed or not. This is an important consideration given most taphonomic research is conducted on unclothed porcine analogues while most forensic cases involve clothed human remains. While porcine studies are useful predictors of human taphonomy, validation studies on human cadavers are needed to ensure forensic relevance and scientific rigor.

While the hope of finding a fast and easy equation to accurately provide estimates of PMI in ADD from TBS were initially encouraging, forensic taphonomists now recognize that decomposition is a complex process with numerous factors that can alter its progression and rates that are not addressed in the current literature (Dawson *et al.*

2023). Recognition that we must concurrently acquire more data on the factors impacting decomposition while establishing separate regional taphonomic standards and equations has strong support and is the subject of current investigation.

1.2. Forensic taphonomy use of porcine proxies

a. <u>Anatomical differences between pigs & humans</u>

The ethics of using animal models as human surrogates in forensic research was reviewed by Mole & Heyns (2018). They summarize the ethical questions of using human research subjects if there are suitable animal model species available, and the use of animal models at all if there are other alternatives. Taking a closer literature survey at peer-reviewed forensic research from 2012-2016, they found 35.3% used rat models, and 29.3% used pigs. However, a breakdown of forensic research reveals that taphonomic and PMI studies made up 24.2% of all forensic research, which were almost entirely reported using porcine analogues.

Taphonomic studies using porcine analogues instead of human subjects avoids ethical concerns and bypasses local legislation where the use of humans in non-medical research is prohibited. It also evades objections from the adjacent landowners who may be unwilling to have decomposing human remains close to their homes. Taphonomic literature makes extensive use of porcine analogues, but the question needs to be addressed as to whether pigs and humans are sufficiently similar to provide meaningful decomposition data that can be applied across species.

Pig and human digestive systems differ in some respects. In contrast to a human's average intestinal length of 7.5 m, pigs have 23 m of intestine. Humans and pigs also have very different digestive vasculature, lymphoid tissues, coiling patterns within the abdominal cavity, and bacterial loads (Connor *et al.* 2017). Since decomposition is highly reflective of autolysis of tissues as well as endogenous bacterial activity from the gut microflora, the potential exists for patterns and rates of decomposition between human and pig models to differ.

Adipose tissue is an important factor during decomposition. There are similarities between human and porcine deposition of fat tissues in the body for subcutaneous fat around the abdomen, and omental fat around the heart and kidneys (Baumgartner *et al.* 1995, Dunshea & D'Souza 2015). Both humans and pigs have an average body fat of around 20% with variation between individuals likely (Dunshea & D'Souza 2015, Meeuwsen *et al.* 2010). Complex triglycerides stored as body fat will eventually break down into glycerol and fatty acid components. However, closer examination of the composition of porcine and human adipose tissues reveals specific differences at the molecular level. Dietary fats have some influence on human fatty tissues and are mostly triglycerides predominated by unsaturated fatty acids that include oleic, linoleic, and laurel acids. Pig fat triglycerides, by comparison, are mostly saturated fatty acids such as stearic acid in husbandry rearing facilities with a more standardized diet of commercial feed (Notter *et al.* 2009). While the same groups of fat molecules were present in both species, their relative proportions within their respective adipose tissues are significantly different. Given both similarities and differences between humans and pigs, researchers using porcine analogues need to have those data replicated with human donor bodies when permitted to either support or refute the porcine results to be valid in forensic cases.

b. Taphonomic validation studies between porcine analogues and human donor bodies

Despite the existence of only ten forensic research facilities that use human donor bodies for decomposition research, the data gleaned from these experiments is significant, particularly studies validating porcine results with human subjects. Interestingly, these results have been inconsistent in terms of whether porcine data parallels human data. Notter *et al.* (2009) examined the differences in decomposing adipose tissues between the two species. Porcine adipose tissue not only decomposes faster than human adipose tissue, but after 30 days human adipose tissue has a mineral content of mostly potassium and sodium, while decomposed porcine adipose tissue contains more potassium and magnesium. Because a human donor body represents an intense but short-lived influx of nutrients into a localized environment, the impact of this difference in composition of human and porcine adipose tissues residual compounds remains unknown.

Studies of skeletal muscle tissue show similar variability to that observed in adipose tissue. Lab studies have been carried out with small amounts (1.5 g) of frozen human, pig, cow (*Bos taurus*), and lamb (*Ovis aries*) skeletal muscle tissue buried in soil to look at decomposition (Stokes *et al.* 2013). While no single animal analogue's muscle was a reliable surrogate for human muscle, it is interesting to note that lamb muscle, not pig muscle, had decomposition results closest to human muscle for tissue mass loss, soil chemistry, and soil microbial activity. In contrast, forensic entomologists found no difference in the growth and developmental rates of blowflies when reared in the lab whether fed human muscle tissue or minced pork (Bernhardt *et al.* 2017). It is notable that both experiments were conducted in controlled lab environments, which may or may not be reflective of data from whole cadavers in the field.

Whole animal carcasses now dominate the forensic taphonomic literature, with mixed results in terms of whether porcine analogues decompose in a similar manner to humans. The field of forensic entomology has had the greatest success in using porcine analogues when sampling and validating the action of forensically important insects on both species of remains. Schoenly & Hall (2002) undertook a series of studies in the US to validate the use of porcine analogues for the purposes of increasing scientific rigor in forensic entomology in legal cases. They found forensically important species of insects colonize human and porcine remains similarly with no significant differences found until after skeletonization occurred. There were differences between size classes of porcine analogues, with greater parallels between humans and porcines of over 50 lbs (~ 23 kg). These results were later confirmed by Schoenly *et al.* (2007) when testing four entomological sampling methods with human and porcine remains. They found slight, but not statistically significant, preference of the insects to colonize human bodies over porcine analogues.

A more recent entomological study from Australia used both humans and porcine analogues to compare decomposition rates and olfactory compound profiles for training cadaver-detection canines (Knobel *et al.* 2018). They found porcine analogues decomposed differently and more quickly than humans, with porcine insect activity occurring over the entire carcass resulting in a more consistent mass loss down to bony remains. Insect activity on the human donor bodies, on the other hand, had initial colonization in the orifices of the face and groin. Because of this, as porcine analogues progressed to more uniformly skeletonized remains, humans displayed mummified front torsos while the back of the torso continued into active decay with more tissue loss. Entomological experimentation not only has the advantage of similarity between porcines and humans regarding insects present at each cadaver, but also indirectly provided information on differences in actual decomposition patterns and rates between the two cadaver species.

In other studies, porcine analogues were found to be close approximations to human decomposition, but with important differences. Connor *et al.* (2017) noted several disparities between the decomposition of both test subjects. Porcines were found to have a more homogenous decomposition as compared to the high variability in the human donors. One reason put forth for this difference was the heterogeneity in human diets as compared to the more uniform diets provided to porcines at rearing facilities. A second significant difference between porcines and humans was observed during the bloat stage of decomposition. During bloat, abdominal gases accumulate during progression of autolytic cellular enzyme activity and gut microbe metabolism. During bloat, 60% of the porcine carcasses experienced abdominal rupturing due to the increase in abdominal pressure, while none of the human cadavers' abdomens ruptured. The authors hypothesized that the most likely cause of this rupture seen in porcines is due to differences between digestive anatomy. Porcines have over triple the intestinal length of humans. When factoring in length with the highly coiled pattern of the intestines within the abdomen, porcines have a much greater surface area for gut microflora to produce post-mortem putrefactive gases to levels of pressure sufficient to split muscle and skin. These differences between species, however, do not negate the usefulness of using porcine analogues in taphonomic research when human donor bodies are not available. Data from porcine analogues should therefore be interpreted with caution before application to human remains, as human validation studies of porcine data are required for rigorous medicolegal interpretation and use.

1.3. Forensic uses of stable isotopes

a. Provenancing human remains with δ^2H and δ^{18}O

Forensic applications of analyzing stable isotope for explosives, pharmaceuticals, and food items are well documented (Benson *et al.* 2006, Fry 2006, Meier-Augustein 2010). However, forensic application of stable isotope analysis to human tissues has been a recent development (Ehleringer *et al*. 2008). From a forensic standpoint, identification of recovered human remains is a priority. While stable isotope analysis of human tissues does not have the power to discriminate and identify individual people as with DNA profiling, it can offer important clues as to the victim's life history and travel patterns, and can be used on both living and deceased individuals. In many forensic cases the decedent may have been murdered, mutilated, dismembered, or subjected to a myriad of physical or chemical methods to hinder both discovery and identification of the decedent. Fortunately, stable isotope assays can be used when there is no soft tissue remaining on a body and investigators must work with bony remains and decomposition-resistant tissues like hair and fingernails.

i. Teeth and bones

Teeth are very resilient and persist in skeletonized remains long after soft tissue decomposition ends. This is due to the presence of bioapatite (hydroxyapatite made by a living organism) in tooth enamel (Meier-Augenstein 2010). Formed on the external tooth surface in such a way to resist the forces of chewing, enamel is a heavily mineralized substance forming the hardest substance in the body. During tooth formation, enamel-producing cells degenerate with eruption of the initial set of deciduous teeth, so changes to the teeth do not occur over time once formed (Marieb & Hoehn 2018). Established enamel is a static substance with a composition that reflects a finite period of time in which the teeth developed.

Given that tooth enamel is not subjected to physiological modifications by the body over time, teeth can be used for stable isotope analysis to get information about an individual's geographical location during early life. Part of enamel formation includes heavy mineralization of hydroxyapatite crystals that contain both hydrogen and oxygen, and we know that isotope ratios of δ^{2}H and δ^{18}O in animal tissues are reflective of local sources of drinking water and precipitation (Fry 2006, Hobson *et al.* 2012). Values of enamel δ^{13}C should reflect the carbon sources of the diet during tooth formation of carbonates, especially those for C-$_3$ and C-$_4$ plants, so analysis of human tooth enamel can lead investigators to narrow down the location of an unidentified deceased individual's early life (Meier-Augenstein 2010).

One example of the use of stable isotopes in tooth enamel is reported by researchers in Japan with unidentified skeletons of World War II soldiers in an effort to separate Japanese and American fighters in mixed assemblages (Someda *et al.* 2016). By using δ^{18}O and δ^{13}C enamel of the war dead and comparing them to extracted teeth from Japanese and American individuals (of a similar age during the war), investigators were able to reliably discriminate Japanese remains with a mean enamel δ^{13}C of -13.25‰ and δ^{18}O of -5.56‰ from American remains with mean values for δ^{13}C of -9.87‰ and δ^{18}O -

6.87‰. Authors attributed the source of these varied values to different sources of dietary water, C_4 corn, and corn-fed beef between the Japanese and American soldiers.

More recently, smaller-scale isoscapes for tap water have been created, resulting in greater precision in correlating tooth enamel and drinking water. Warner *et al.* (2018) created isoscapes for both δ^2H and $\delta^{18}O$ of tap water throughout the state of Mississippi. They found both the municipal water sources and tooth enamel revealed a gradient of enrichment of δ^2H and $\delta^{18}O$ in southeastern county residents, whereas northwestern county residents were more depleted in both water isotopes. As more local isoscapes of drinking water are created and refined, forensic investigators can become more precise with assigning areas of origin and residence based on tooth enamel data.

As with enamel, bone is also a strong component of human remains that has been used in forensic isotope analysis. Bones are a macroscopically decomposition-resistant tissue and can remain for centuries despite the loss of other soft tissues when recovered. Bone matrix is approximately half organic and cellular material, mostly collagen proteins, and half inorganic mineralized salts, including carbonates and phosphates (Marieb & Hoehn 2018). In life, the combination of these components gives bones their strength and durability. Bone is also a dynamic tissue where old bone is broken down and new bone matrix formed, known as bone-remodeling, although not all bones in the adult are capable of this dynamic. In this respect, bone differs from tooth enamel as bone has the potential to change over time compared to the static composition of enamel. This creates interesting avenues of stable isotope analysis with forensic implications.

Bone is used for stable isotopic analysis, but there are inherent limitations to its interpretation. While bone can undergo remodeling, the breakdown and rebuilding of bone matrix is slow, and therefore slow to reflect changes in dietary intake and migration. Skeletal elements of bone in an adult femur, including collagen protein fibers, have a 3% annual turnover rate, taking as long as 25-30 years to turnover completely (Hedges *et al.* 2007). These slow rates of change detectable in bone make them useful for long-term studies in anthropology and archaeology but have also become useful in forensic investigations.

A forensic case from Dublin, Ireland in 2005 involved a decapitated and dismembered set of human remains (Meier-Augenstein & Fraser 2008). A forensic pathologist was able to estimate the remains were from an African male. Both the inner region of the femur (with newer bone matrix) and the outer region (with older bone) were analyzed for stable isotopes of δ^{2}H and δ^{18}O. Due to the slow remodeling rates found in the femur, investigators were able to take the difference between the isotopic values of the inner and outer bone and back-calculate two important pieces of information about the deceased: his likely region of origin, and how long he had been in Dublin. Data showed that the femur was likely from the Horn of Africa (Ethiopia, Somalia, Kenya, Eritrea, Uganda and eastern Sudan), and had been in Dublin for approximately 6.3 years ($\pm$ 2.9 years). This narrowed down the potential pool of decedents, providing justification for authorities to conduct familial DNA testing for identification. Eventually the victim was identified as being originally from Kenya, immigrating to Dublin seven years prior. In this case, the stable isotope analysis of the femur bone was a key piece of data in the ultimate resolution of the case with successful conviction of the murderers.

While tooth enamel and bone matrix are good resources for assessing the permanent and long-term deposition of stable isotopes, forensic investigators are also interested in gaining information on stable isotopes in tissues that are more quickly affected by changes in water and food intake. Hair and fingernails have become more useful for this avenue of forensic stable isotope research.

ii. Hair and fingernails

A decedent's diet plays a large role in the composition of hair and fingernails for several reasons. First, these keratinized structures exist in an anatomical system separate from the bloodstream and are not subject to fluctuations dictated by rapid, short-term changes in blood chemistry. Second, they grow continuously throughout life, with fingernails growing an average of 0.2 cm per month, while human scalp hair grows at a much faster rate of 1.1 cm per month, making it the preferred tissue for analysis (Lehn *et al.* 2015). Hair also correlates more strongly than fingernails to water isotopes (Fraser & Meier-Augenstein 2007). Third, the creation of hair requires protein synthesis which must incorporate the body's own amino acids, most of which are derived from dietary proteins. Formation and deposition of proteins in continually growing keratinized structures, together with the available amino acids in the tissues, can provide a record of the proteins throughout the concurrent growth of the hair, and thus the stable isotopes of ^{13}C and ^{15}N along the length of the hair shaft over time. In much the same way as ^{13}C and ^{15}N are useful, hair growth also records the amounts and ratios of ^{2}H and ^{18}O throughout the creation of new hair matrix, because the quantity and ratios of water molecule stable isotopes ingested from local drinking water dictate those same isotopic values present in the hair of the remains (Table 1.1).

Table 1.1 Compiled data for stable isotopes found in human hair divided by region (mean ±SD).

Continent	δ^{2}H ‰	δ^{13}C ‰	δ^{15}N ‰
Europe	-74.7 ± 9.8	-20.74 ± 0.66	8.59 ± 0.64
North America	-78.2 ± 9.0	-17.98 ± 0.57	8.31 ± 0.38
South America	-69.3 ± 13.6	-17.78 ±1.57	8.97 ± 0.66
Asia	-72.3 ± 10.1	-19.98 ± 0.91	8.54 ± 1.31
Africa	-39.8 ± 12.6	-15.94 ± 2.79	8.26 ± 1.15
Australia & New Zealand	-55.7 ± 10.3	-19.71 ± 1.18	9.02 ± 0.94

(Data adapted from Hulsemann *et al.* 2015, Kusaka *et al*. 2016, Lehn *et al.* 2015)

Unlike bone that reflects changes in protein composition over a time frame of decades, hair can display isotopic changes over shorter intervals of days and weeks. If sampling hair plucked from the root, changes in the matrix can be analyzed immediately. However, it is important to recognize that if samples are obtained from hair clipped at the scalp level, this hair takes on average six to twelve days to grow sufficiently from the root bulb and out of its follicle to a length that can be cut at the scalp surface. As a result, cut hair should be processed keeping in mind the general accepted standard of a two-week delay between the time of change (i.e., different food, different water source) and when detectable stable isotope changes are seen (Mekota *et al.* 2006). This ability of hair to record isotopic variations over time, coupled with its relative ease of sampling using non-invasive methods, makes human hair an excellent source of forensic information.

Data are now published on the stable isotope ranges for human hair in locations throughout the world. Summary review papers by Hulsemann *et al*. (2015) and Lehn *et al.* (2015) compiled data from numerous papers (totaling several hundred samples) to give regional differences of δ^{13}C and δ^{15}N by country and continent. They report that, despite having comparatively fewer samples from African nations, there is significant correlation between $\delta^{13}C$ and latitude as well as the quantity of C_4 plants in the diet, which is confirmed in other studies (Kusaka *et al.* 2016). These continental results for hair and fingernails are being further refined to enable more precise identification of an individual's movements over time on the scale of weeks and months. A cold case from Newfoundland, Canada, discussed below, exemplifies this work.

In 2001, a skull and some associated vertebrae were recovered from Minerals Road, Conception Bay South, with subsequent excavation and evidence collection conducted by the Royal Newfoundland Constabulary (RNC) and the Chief Medical Examiner. Preliminary examination concluded that the time-since-death could be from one to ten years, and the deceased was likely that of a Caucasian male in his mid-to-late 20s. An RCMP forensic artist completed a facial reconstruction, and DNA samples were analyzed. Despite this information, the victim remained unidentified, and the case went cold until 2007 when samples were submitted for stable isotope analysis. The tooth enamel revealed $\delta^{18}O$ values that were significantly enriched when compared to Newfoundland water sources, indicating that he likely did not spend his childhood locally. Data from the hair revealed several additional clues: sequential analysis of his hair revealed fluctuating $\delta^{2}H$ values that taken together with Canadian waterscapes, showed a clear pattern of his movements over the last 19 months of his life where he moved regularly between Newfoundland, eastern Canada, south Ontario, and back again in an almost annual cycle (Meier-Augenstein 2010). Genetic genealogy was most recently used in early 2023 and revealed the victim to be of Cuban ancestry (Syed 2023). Law enforcement continues to investigate, but to date the individual remains unidentified despite this body of specific additional information.

b. Potential for $\delta^{13}C$ and $\delta^{15}N$ in soft tissue decomposition and PMI research

Decomposition does not significantly impact the harder tissues discussed above, like hair and fingernails. Although their resistant structure gives information on regions of origin and migration beyond the completion of decomposition, they are of little to no use when it comes to estimating PMI. Aside from identifying the deceased, knowing the elapsed time between recovery and death gives forensic investigators important information that can include or exclude perpetrators. To date, PMI has been researched using qualitative methods such as the TBS grading system translated into quantitative data correlated with time and temperature in ADD. The first part of this chapter discussed these methods and their limitations in depth, but I wish to reiterate that there is no current universally confirmed methodology to accurately estimate PMI across regional, climatic, and seasonal situations. This is why I have chosen stable isotopes as a tool to examine PMI in forensic taphonomy.

Hair and nail $\delta^{2}H$ and $\delta^{18}O$ are reflective of municipal water sources in a given area, with sequential changes in these stable isotopes recorded in the keratin and collagen of hair and nails, and forensic research has made good use of this principle. But what about decomposing soft tissue? I chose to focus on skin and muscle tissues for two reasons: first, in cases of advanced decomposition, internal organs liquefy very quickly and are not possible to be sampled, whereas skin and muscle persist much longer than the viscera. Second, skin and underlying muscle is easy to access for sampling given their superficial locations, and if no forensic investigator is available to sample at the scene, recovery personnel could be trained to sample these tissues. Viscera sampling would require much more invasive technical sampling methods deep into the body cavities that would interfere with autopsy protocols collecting post-mortem evidence and require specialized expertise. For these reasons, skin and muscle were the obvious choices for preliminary stable isotope sampling for PMI data.

During decomposition, the human body experiences a significant decrease in the amount of total carbon and nitrogen in the remaining cadaver as autolytic enzymes, bacterial degradation, and possibly insect activity consume the tissues. As the body's nitrogen liquefies, it can be traced and analyzed from underlying grave soils to give indications of PMI (Fancher *et al.* 2017). Conversely, much of the body's carbon released from the tissues during decomposition is not easily captured and measured since most of it is liberated as CO_2 gas (Benninger *et al.* 2008). Applying the principles of fractionation of ^{13}C and ^{15}N during assimilation of nutrients into soft tissues, and that heavier isotopes require more energy in a closed system to react to enzymes, it is a realistic possibility that sampling of soft tissues during human decomposition will result in a gradual enrichment in soft tissues and underlying soils for both $\delta^{13}C$ and $\delta^{15}N$ as decomposition progresses. There is an important avenue of research to be undertaken in correlating carbon and nitrogen stable isotope enrichment over time that may ultimately form the basis of using biochemical markers of the tissue themselves to predict or even back-calculate a more reliable PMI estimate as opposed to current TBS methods. There are existing biochemical markers of tissues that have been used to some degree of precision to estimate PMI based on cerebrospinal fluid, synovial fluid, and vitreous humour of the eye (Madea 2005, Salam *et al.* 2012), but these methods are limited to a short post-mortem interval, measured in hours. Forensic researchers need more reliable objective tools to estimate PMI with longer time frames after death.

While there are no data yet published for human soft tissue stable isotopes during decomposition, there is evidence for the utility of stable isotopes and soft tissues, namely, experiments on stable isotope sample preservation methods for marine animals. Stable isotope samples are commonly frozen until analyzed, so verification that isotopic values will not change over prolonged periods of storage was needed. Burrows *et al.* (2014) took samples of killer whale skin and blubber from an animal that had washed up on shore and subjected them to storage at room temperature, 4ºC, -20ºC, and -80ºC. Most interestingly, the killer whale skin left to decompose at room temperature showed significant enrichments in both δ^{13}C and δ^{15}N over time. These elevated levels were detectable at day three after sampling and continued to increase until the end of the experiment at day 14; δ^{15}N increased 6.4‰ over this time while δ^{13}C increased 1‰. They concluded that stranded killer whales could be sampled up until three days after death before the stable isotopes for carbon and nitrogen were significantly affected and should not be considered reliable.

Yurkowski *et al.* (2017) performed a similar experiment to validate sample storage methods for Greenland shark, lake trout, and ringed seal muscle tissue. Samples left to decompose at room temperature with open lids produced significant enrichments in both δ^{13}C and δ^{15}N detectable by day eight of the study for all three species. The authors conclude by urging caution when sampling muscle from animals that have been left to decompose for over a week. Parallel findings of muscle enrichment of δ^{13}C and δ^{15}N during decomposition were found in grouper, rabbitfish, mantis shrimp, and crab within 120 hours after death (Perkins *et al.* 2018), as well as in dolphins and turtles (Payo Payo *et al.* 2013) and beavers (Keenan & DeBruyn 2019). These isotope-focused studies provide preliminary evidence that both fish and mammalian tissues are enriched in carbon and nitrogen stable isotopes over the course of decomposition.

There is obvious value to investigate deceased human skin and muscle tissues that may illuminate the relationship between $\delta^{13}C$ and $\delta^{15}N$ at a given point in the decompositional process. While these changes may occur at different rates for $\delta^{13}C$ and $\delta^{15}N$, and may even occur at different rates when comparing muscle to skin tissues, quantifiable and accurate stable isotope predictors of decomposition, and thus PMI, would open a new avenue of forensic taphonomic research. However, thoughtful experimental design and application of results is necessary. Depending on the variability in the data, if these enrichment rates can be evaluated in one region, they can also be replicated in other forensic research jurisdictions to provide regionally-specific enrichment rates. The legal implications for forensic work of this type may provide powerful tools and advancement in PMI research in application to cases of medicolegal significance.

c. Forensic methodologies and the *Daubert* standard

The current dissertation work was designed to investigate the potential for stable isotopes to be refined into a forensic PMI methodology for medicolegal death investigations. Should the resulting data support the utility of stable isotopes in this respect, its eventual use as a legally accepted PMI estimation tool will be subject to the US Supreme Court's *Daubert* standard (*Daubert v. Merrrell Pharmaceuticals, Inc.* 1993). As more forensic evidence is introduced at trials, a concurrent effort to eliminate "junk science" emerged to ensure data introduced as evidence is unbiased and scientifically recognized as being reliable and rigorous. The *Daubert* case established a set of guidelines that judges should use when considering the evidentiary value of scientific evidence. The ruling laid out a framework where forensic evidence comes from an expert witness based on sufficient data or facts, the expert's knowledge and experience will help understand those data or facts, the expert testimony is based on reliable principles and methods, and the expert has applied those principles to the facts of the case to render an expert opinion.

Decomposition data is now more routinely considered as forensic evidence in criminal cases. Henssge and Madea (2007) refined the forensic science principles that decomposition and PMI estimation methods should meet when introduced as expert testimony: study variables are quantifiable, the method can be assessed mathematically, factors that quantifiably contribute to the data are considered, and data should include validation of precision with associated error estimates. Therefore, establishing the utility of soft tissue stable isotope changes over decomposition as an estimate of PMI with sufficient data, validation of the methods, and inclusion of meaningful error ranges are essential for its inclusion in legal contexts. This dissertation is the first step toward the justification, refinement, and legal use in the future in medicolegal death investigations.

1.4 Summary

Stable isotope fractionation has many applications in biological research and has provided invaluable information to forensic sciences and medicolegal death investigations. In addition to the current use of stable isotope analysis of δ^{2}H and δ^{18}O in hair and fingernails to determine regional information on a deceased individual, there is potential for δ^{13}C and δ^{15}N to be another forensic tool to estimate PMI. There is evidence that decomposition's fractionation processes in skin and muscle cause enrichments in both $\delta^{13}C$ and $\delta^{15}N$ over time in other organisms. If significant correlations can be achieved between these two variables in skin and muscle from human remains, stable isotopes may hold the key to unlocking an avenue of new forensic research that can quantify the time since death in an accurate way with increased precision as compared to the variable results that current PMI estimation methods provide.

My dissertation presents data that quantifies the changes in $\delta^{13}C$ and $\delta^{15}N$ in three chapters. The first chapter study investigates human donor body skin and muscle tissues decomposing outdoors in Texas over the early PMI of 20 days. The second chapter work examines Texas human gravesoils in two studies over a short PMI of 30 days and an extended PMI of 900 days of decomposition. The third chapter presents data from a study using skin and muscle tissues from porcine analogues in Fredericton, Canada. In these three chapters, I predict that $\delta^{13}C$ and $\delta^{15}N$ will both result in elevated (enriched) levels over time as decomposition progresses in samples of skin, muscle, and gravesoils. Two published studies I authored that led to my current research focus are presented in Appendices B and C which contribute to the discipline of forensic taphonomy. Appendix B reports pilot data using porcine analogue neonate biometrics as measures of decomposition in soil and manure interments, and Appendix C is a review article outlining a framework for future experimental design that more accurately reflects forensic casework realism.

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CHAPTER 2 FORENSIC TAPHONOMY AND STABLE ISOTOPES: δ^{13}C AND δ^{15}N ENRICHMENT PATTERNS IN HUMAN SKIN AND MUSCLE DURING OUTDOOR DECOMPOSITION.

2.1 Abstract

Stable isotopes in hard, resistant tissues are routinely used to provenance human remains for identification, but stable isotopes have not been investigated for their application to soft tissue decomposition in a forensic taphonomy context. This study examined stable isotope trends in nine human donor bodies from San Marcos, Texas over the first three weeks of decomposition in the two soft tissues most commonly recovered from decomposed remains- skin and muscle. Isolated enrichment points for $\delta^{13}C_{muscle}$ and $\delta^{15}N_{skin}$ were found, but a clear trend emerged with significant increases in $\delta^{15}N_{muscle}$ where a mean start value of 9.5‰ increased after day-10 by 2.5‰ and plateaued thereafter beyond day-20. The $\delta^{13}C_{skin}$ did not show any discernable pattern of enrichment or depletion throughout the study. This work provides evidence for future uses of stable isotope analysis of soft tissues in forensic case work with possible post-mortem interval (PMI) estimation applications.

2.2 Introduction

Forensic taphonomy examines post-mortem changes in a body to determine the circumstances surrounding death as well as the time elapsed between death and discovery of the remains; this period is called the post-mortem interval (PMI). Human decomposition has been well-documented in the past 40 years at forensic anthropology research facilities using human remains for taphonomic research on the progression of fresh, bloat, active decay, advanced decay, and dry remains stages (Knobel *et al.* 2018, Shirley *et al.* 2011, Wescott 2018). Despite these strides, forensic taphonomy still lacks a universal method of examining quantitative, tissue-specific changes that can be applied across many climatic and interment conditions to give forensically accurate and reliable PMI estimates for the early decomposition phase. Biochemical tissue markers have been used with some success to estimate PMI from cerebrospinal fluid, synovial fluid, and vitreous humour of the eye, but these methods are limited to very short PMI windows measured in hours (Madea 2005, Salam *et al.* 2012). Forensic researchers would benefit from more reliable, objective tools to estimate PMI to reconstruct a timeline of events, help identify the victim, and assist in the potential purport ratios of crime (Baliso *et al.* 2023).

Law enforcement would benefit greatly from having a universal taphonomic PMI formula, but the decedent must also be identified at the outset of any forensic case. Forensic cases now employ stable isotope analysis to provenance human remains (Lehn *et al.* 2015, Meier-Augenstein & Fraser 2008). Current provenancing methods analyze decomposition-resistant tissues of hair, fingernails, bones, and teeth to obtain relative measures of δ^2H and δ^{18}O to give sequential information about an individual's water intake through ingested food and drink that are then incorporated into continually growing or changing tissues. Results can be compared to existing isoscapes (stable isotope data on known water sources) to narrow an unknown decedent's region of origin and estimate where and when they have lived and travelled, thus giving investigators valuable information to help identify the remains by narrowing down the potential pool of unidentified individuals (Ehrlinger *et al.* 2008, Warner *et al.* 2018). However, current forensic applications of hard tissue stable isotopes do not address the issue of PMI estimates. In the early stages of decomposition there are several tissue types remaining in the body that may hold evidence for analysis. There is potential for targeting these available soft tissues to improve PMI estimate methodologies.

Stable isotopes are extensively used in ecological studies of animal migration patterns from δ^2H and δ^{18}O in bird feathers and fish scales similar to their use in human provenancing techniques (Hobson 1999, Hobson *et al.* 2012, Landsman *et al.* 2018). Additionally, skin and muscle values for δ^{13}C and δ^{15}N provide insight on animal trophic status which can trace an organism's spatial and temporal position in an ecological food web (Darimont & Reimchen 2002, Reimchen *et al.* 2002). Briefly, both carbon and nitrogen are assimilated up the food web through increasing trophic levels from autotroph to herbivore to carnivore by enrichments of 1-2‰ for δ^{13}C and 3-4‰ of δ^{15}N for each increasing trophic level (Kelly 2000, Meier-Augenstein 2010). Such shifts in enrichment or depletion have not yet been investigated in forensic taphonomy to assess stable isotope ratio changes in human soft tissue decomposition over time. Given the current forensic focus of stable isotopes on hard tissues, it is worth examining the potential of other remaining tissues to not only provenance the remains but also to explore PMI implications of soft tissues that are degraded over the course of decomposition.

During decomposition, the human body experiences a significant decrease in the amount of total carbon and nitrogen in the cadaver as autolytic enzymes, bacterial degradation, and insect activity degrade and consume the tissues. As the body's sequestered nitrogen is released via liquefaction of tissues, it can be traced and analyzed from underlying grave soils to give indications of PMI (Fancher *et al.* 2017). Conversely, much of the body's carbon released from the tissues during decomposition is not easily captured and measured because much of it is liberated as CO_2 gas (Benninger *et al.* 2008). Applying what is known about fractionation of $\delta^{13}C$ and $\delta^{15}N$ during assimilation of consumers versus that of prey, given that heavier isotopes require more energy in a closed system to react with enzymes compared to lighter isotopes of the same element, it is likely that sampling of soft tissues during human decomposition will result in a gradual enrichment of both $\delta^{13}C$ and $\delta^{15}N$ as decomposition advances.

While there are no data published yet for humans during decomposition, there is evidence for the utility of stable isotopes and soft tissues from experiments on stable isotope sample preservation methods for marine animals. Stable isotope samples are commonly held in frozen storage until analyzed, so it was necessary to verify that isotopic values do not change over prolonged periods of storage. Burrows *et al.* (2014) sampled killer whale skin and blubber from an animal that had washed up on shore and found significant enrichments in both $\delta^{13}C$ and $\delta^{15}N$ over time as decomposition progressed. These elevated levels were detectable three days after sampling and continued to increase until the end of the experiment at day 14; $\delta^{15}N$ increased 6.4‰ over this time while $\delta^{13}C$ increased 1‰. They concluded that stranded killer whales could be sampled up to three days after death before the stable isotopes for carbon and nitrogen were significantly affected and should be considered unreliable.

Yurkowski *et al.* (2017) performed a similar experiment to validate sample storage methods for Greenland shark, lake trout, and ringed seal muscle tissue. Significant enrichments in both $\delta^{13}C$ and $\delta^{15}N$ were detectable by day eight of the study for all three species. The authors conclude by urging caution when sampling muscle from animals left to decompose longer than a week. Parallel findings of muscle enrichment of

$\delta^{13}C$ and $\delta^{15}N$ during decomposition were found in grouper, rabbitfish, mantis shrimp, and crab within five days after death (Perkins *et al.* 2018). Related findings in beaver *(Castor canadensis)* carcasses report significant enrichment of $\delta^{15}N$ in several soft tissues with progression through the fresh, bloat, and active decay stages (Keenan & DeBruyn 2019). These four published works provide preliminary evidence that both fish and mammalian tissues are enriched in carbon and/or nitrogen stable isotopes over the course of decomposition.

The purpose of this study is to utilize current knowledge of marine animal and mammal decomposition and apply it to human forensic taphonomy to specifically address our hypothesis that stable isotope ratios of $\delta^{13}C$ and $\delta^{15}N$ increase in human skin and muscle as decomposition progresses. If so, I wish to address two specific questions: (1) At what point is tissue enrichment first detectable during decomposition, and (2) how long does the tissue enrichment last in the early PMI? These results will yield important data that furthers our knowledge of the rates and processes for human tissue decomposition.

2.3 Materials & Methods

a. <u>Outdoor facility</u>

Research was conducted at the Forensic Anthropology Research Facility (FARF), in San Marcos, Texas. FARF occupies 26 acres of secured and fenced outdoor area within Freeman Ranch, an agricultural research facility and working farm managed by Texas State University-San Marcos. Body donations are obtained through a voluntary donation program with the expressed documented permission of the deceased individual and/or the consent of the next of kin for the purposes of forensic research, managed by the Forensic Anthropology Centre for Taphonomic Research (FACTS) at Texas State University.

b. <u>Weather</u>

The study occurred over the summer of 2016 and fall of 2017. In the 2016 study period, there was a single day of rainfall of 18 mm, with a daily mean temperature of 30°C (range 26°C to 37°C). The 2017 study period experienced two days of rainfall (6 mm and 17 mm), with a daily mean temperature of 26°C (range 19°C to 34°C).

c. <u>Donors</u>

Nine human donor bodies were studied for a 20-day period of observation. All nine donors were of European ancestry, died from various causes, and varied in their age, sex, height, and weight (Table 2.1). Donors were placed under exclusion cages to prevent large scavenging activity by birds or mammals but still permit free access to sunlight, airflow, and insects as normal taphonomic processes (Suckling *et al.* 2016).

Table 2.1. Demographic information of nine human donor bodies studied at FARF, San Marcos, Texas.

Age	Sex	Cause of Death	Height (cm)	Weight (kg)	BMI
82	Male	Cerebrovascular accident	172	73	24.7 normal
78	Male	Parkinson's disease	157	54	21.9 normal
66	Male	Multiple myeloma	178	102	32.2 obese
85	Male	Cardiovascular disease	178	75	23.7 normal
56	Female	Metastatic ovarian cancer	152	65	28.1 overweight
71	Male	Esophageal cancer	161	58	22.4 normal
30	Female	Dehydration & cancer	157	41	16.6 underweight
72	Male	Cerebrovascular accident	177	63	20.1 normal
62	Male	Blunt force trauma	180	114	35.2 obese

BMI = weight (kg)/height(m)^2; BMI < 18.5 underweight, 18.5-24.9 normal, 25.0-29.9 overweight, >30.0 obese.

d. Decomposition data

The day the decedent was placed in the FARF outdoor facility was considered day zero for the experiment as some donors were brought to the facility from a distance and required overnight refrigeration at 4°C before placement in the field the following day. In addition to time in days, ADD (Accumulated Degree Days) were also calculated during decomposition by summing the mean daily temperatures for each body donor for their respective 20-day study period. Total Body Score (TBS) was calculated by visual criteria outlined by Megyesi *et al.* (2005) where the head, trunk, and limbs are assigned separate point values based on defined physical characteristics (see Appendix A). A completely fresh body has a minimum TBS of 3, and a completely skeletonized body has a maximum TBS of 35.

e. Stable isotopes- $\delta^{13}C$ and $\delta^{15}N$

Soft tissue sampling for stable isotope analysis was performed every five days by excising a small amount of superficial skin and underlying muscle (<1 cm^3) and securing them in individual 1.5 ml Eppendorf tubes with snap-caps, then immediately stored in a -20°C freezer. Skin and muscle were collected from the upper arm and thigh regions on both left and right sides of the body for a total of four replicates per donor for both tissue types (left arm, right arm, left thigh, right thigh). At the conclusion of the experiment, frozen sample tubes were thawed at room temperature with open caps for three hours, and then housed in a drying oven at 60°C for a minimum of 48 hours to completely desiccate. These dried samples were pulverized to a powder of uniform consistency.

Lipid was extracted from both skin and muscle samples prior to analysis to remove potential bias due to variation in lipid content between samples. Lipid extraction methodology involved the submersion of the powdered samples with a 2:1 chloroform to methanol solution for a minimum of 30 minutes. The supernatant was removed with a disposable pipette and discarded, while the sample at the bottom of the tube was retained. This lipid extraction procedure with the 2:1 chloroform: methanol solution was repeated at least three times or until the supernatant was clear and colorless (Fischer-Rush *et al.* 2021). Stable isotope ratios were analyzed using continuous flow isotope-ratio mass spectrometry (CFIRMS) to obtain values of ^{13}C and ^{15}N.

Isotopic data are reported in per mil (‰) and calculated using the following delta (δ) notation calculation:

$$\delta\ (‰) = ((R_{sample} - R_{standard}) / R_{standard}) \times 1000\ ‰$$

where R_{sample} is the ratio of the heavy to light isotope contained in the sample, and $R_{standard}$ is the ratio of the heavy to light isotope in the reference material established by the International Atomic Energy Agency (IAEA, Vienna, Austria). The universal standard of $\delta^{13}C$ used Vienna Pee-Dee Belemnite (V-PDB (Craig 1957)) and $\delta^{15}N$ used atmospheric air (AIR (Mariotti 1983)), respectively.

In-house secondary standards for BLS (Bovine Liver Standard) and MLS (Muskellunge Muscle Standard) were calibrated against IAEA (International Atomic Energy Agency) standards to normalize stable isotope values. To assess analytical accuracy, check standards of nicotinamide, N_2 and CH_7 were also analyzed. Furthermore, 5-7% of samples were run with replicates to monitor instrument drift over time. Analytical precision was better than 0.2‰ for $\delta^{13}C$ and 0.3‰ $\delta^{15}N$ following repeated analysis.

f. Statistics

Statistical analyses were performed using R (R Core Team 2013) to assess significant changes in $\delta^{13}C$ and $\delta^{15}N$ for several comparisons. To address my first question if human skin and muscle become more enriched as they decompose, I used the general linear model to detect effects of decomposition progression factors on stable isotope data for both tissue types. To address my second question to determine at what point in the decomposition process these effects become significant, Tukey's Student t-tests (paired samples) compared sampling days for each tissue and isotope. Significance was established at $\alpha = 0.05$.

2.4 Results

a. Accumulated Degree Day (ADD) data

ADD data were calculated by summing the daily mean temperatures (°C) for each individual donor body over the 20-day sampling period. Because the cadavers had different start dates, daily temperature fluctuations resulted in varied ADD values for each five-day sampling date. ADD values are not identical, although they show similar mean increases over time. There is some overlap between sampling at 15 and 20 days because the ADD values are a representation of accumulated thermal energy over time and daily temperatures vary (Table 2.2).

Table 2.2. Accumulated degree day (ADD) and ranges for each five-day sampling interval for nine human donor bodies at FARF, San Marcos, Texas.

Sampling day	Mean ADD (±SE)	ADD range
0	0	0
5	144.8 (± 4.5)	119-165
10	286.2 (± 11.2)	235-335
15	430.6 (± 16.5)	371-502
20	569.8 (± 18.6)	466-647

b. Total body score (TBS) data

The mean TBS over time of decomposition increased sharply from a mean TBS of 3 at day-0 to a TBS of 15 by day-5 during the bloat phase. As the bodies moved through post-bloat and through active decay, a mean TBS of 20 was found for days 10-15. This sharp increase gradually slowed by day 20 to a mean TBS of 22 (Figure 2.1).

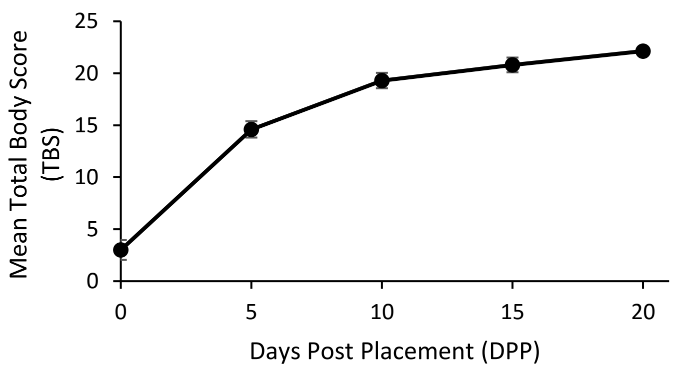

Figure 2.1. Mean body scores (TBS± SE) as per Megyesi *et al.* (2005) for nine human donor bodies sampled at five-day intervals during 20 days of outdoor decomposition at FARF, San Marcos, Texas.

c. Statistical analysis- GLM and AIC scoring of model factors

Data were first assessed using the General Linear Model (GLM) to examine the effect of each factor on the data. I then used model selection based on the Akaike information criteria (AIC), which helped identify the simplest model explaining the data. Multiple models were tested for each combination of stable isotope and tissue sampled.

AIC scores showed factors of year, sex, side, and location did not have significant impacts on stable isotope values for any of the four model parameters studied: $\delta^{13}C_{skin}$, $\delta^{13}C_{muscle}$, $\delta^{15}N_{skin}$, $\delta^{15}N_{muscle}$ (Table 2.3). Consequently, because no differences were detected between comparisons of males and female donor bodies, left and right sides, or arm and leg samples for either isotope in either skin or muscle, data remained pooled for subsequent analysis.

For each donor body at a given sampling day, four replicate samples were averaged to obtain that donor's mean isotopic value (right arm, right leg, left arm, left leg). Progression of decomposition, as measured by time in days, was a significant factor in the models for enrichment of $\delta^{13}C_{muscle}$, $\delta^{15}N_{skin}$, and $\delta^{15}N_{muscle}$ (*$p < 0.0001$*), but was not significant for $\delta^{13}C_{skin}$, indicating it is neither enriched nor depleted over time.

Table 2.3. General linear model (GLM) factors tested in human skin and muscle for nine human donor bodies over 20 days of outdoor decomposition in San Marcos, Texas.

Parameters tested	Day	Year	Sex	Side	Location
$\delta^{13}C_{skin}$	-	-	-	-	-
$\delta^{13}C_{muscle}$	0.06 (0.01)*	-	-	-	-
$\delta^{15}N_{skin}$	0.02 (0.006)*	-	-	-	-
$\delta^{15}N_{muscle}$	0.14 (0.01)*	-	-	-	-

*Numbers report GLM coefficient (standard error) for factors having significant effects on the model; * denotes p<0.0001 level of significance; - denotes factor removed from the model.*

d. Stable isotope data analysis

The nine human donor bodies showed overall stable isotope increasing enrichments over the course of the 20-day study (Table 2.4). Exceptions were observed in three donors' $\delta^{13}C_{skin}$, and one donor's $\delta^{15}N_{muscle}$. Mean values for nine human donor bodies were calculated and used for further analysis (Figure 2.2).

Table 2.4. Mean $\delta^{13}C$ and $\delta^{15}N$ stable isotope ratios for nine human donor bodies' skin and muscle at the start and end of twenty days of outdoor decomposition at FARF, San Marcos, Texas.

Donor	$\delta^{13}C$ (‰) skin		$\delta^{13}C$ (‰) muscle		$\delta^{15}N$ (‰) skin		$\delta^{15}N$ (‰) muscle	
	Start	End	Start	End	Start	End	Start	End
1	-16.90	*-17.09*	-18.51	-17.75	11.94	12.41	8.52	11.75
2	-17.15	-17.11	-19.34	-17.20	12.38	12.91	8.98	12.42
3	-18.30	-16.13	-17.69	-15.46	11.72	12.50	12.62	*12.18*
4	-16.41	-16.21	-17.96	-16.54	12.52	12.61	10.06	12.38
5	-18.49	-16.44	-17.02	-15.49	12.36	12.75	9.82	11.73
6	-16.28	*-16.37*	-18.21	-16.95	12.84	13.07	9.70	12.79
7	-17.20	*-17.80*	-18.10	-17.97	12.59	12.82	8.44	11.66
8	-16.56	-16.30	-17.74	-16.23	12.71	12.88	10.23	12.10
9	-15.89	-15.52	-17.42	-16.67	11.58	12.29	9.26	12.24

Numbers in italics denote depletion of stable isotope in a tissue at the end of the 20-day study.

Skin was higher (enriched) in both total $\delta^{13}C$ and $\delta^{15}N$ relative to muscle throughout the 20 days of the experiment, with the greatest differences between the two tissues at day-0 for both isotopes (Figure 2.2). The difference between skin and muscle isotope values became smaller as decomposition progressed. A relatively consistent measure of mean $\delta^{13}C_{skin}$ with decomposition was noted with no significant enrichment detected over 20 days (-16.4 to -16.8±0.2‰), while a significant enrichment of mean $\delta^{13}C_{muscle}$ was detected at day-10 when compared to day-0 (-18.0 vs -17.0±0.2‰).

Significant enrichment of $\delta^{15}N_{skin}$ was observed over the course of decomposition, first detected at 15 days with an increase from 12.3±0.1‰ to 12.9±0.2‰. Greater increases were detected in the mean enrichment of $\delta^{15}N_{muscle}$ at sampling day-10 (12.0±0.3‰) compared to day-0 (9.5±0.3‰). This increase leveled off between days 10-

20 when enrichment levels of $\delta^{15}N_{muscle}$ became more comparable to $\delta^{15}N_{skin}$ levels. The same general trends in enrichment continued after 30 days (Miles, unpublished data).

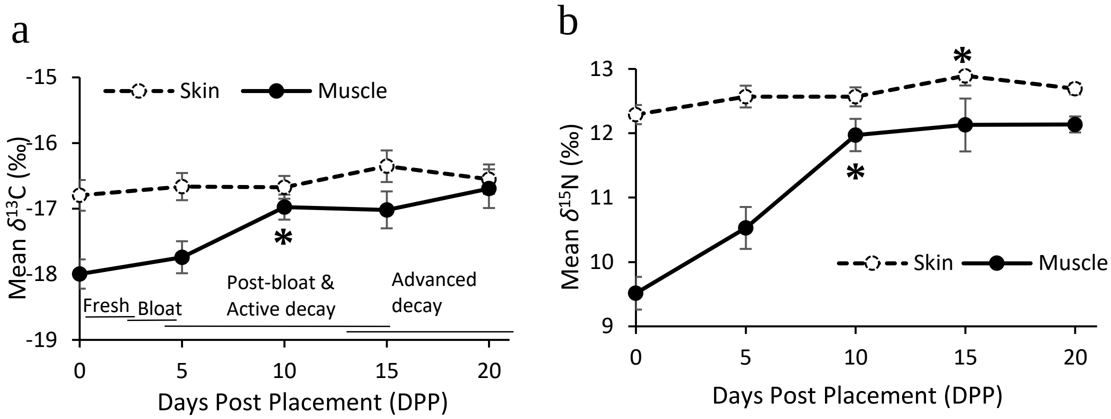

Figure 2.2. Mean (a) $\delta^{13}C$ and (b) $\delta^{15}N$ (‰ ± SE) for nine human donor bodies'skin and muscle samples at five-day intervals during 20 days of outdoor decomposition at FARF, San Marcos, Texas. * Asterisk denotes the first point at which a significant difference was found when compared with day 0; $p < 0.05$).

In addition to the relative abundances of $\delta^{13}C$ and $\delta^{15}N$, the mean *changes* in those relative abundances in tissues over time were calculated (Figure 2.3). This enables the comparison of changes in enrichment rates while accounting for the variations in mean isotope content of each donor's tissues at day-0. A series of T-tests determined at what points significant enrichments were detectable in tissue-specific stable isotope data between subsequent sampling days (i.e., when compared with each stable isotope data value collected five days prior). Results showed no comparative change in $\delta^{13}C_{skin}$ over time, with a single comparative enrichment point for $\delta^{13}C_{muscle}$ between day-5 (0.3±0.1‰) and day-10 (1.0±0.2‰) for a total mean increase of roughly 0.7‰. The $\delta^{15}N_{skin}$ data showed a single point of comparative enrichment between day-0 and day-5

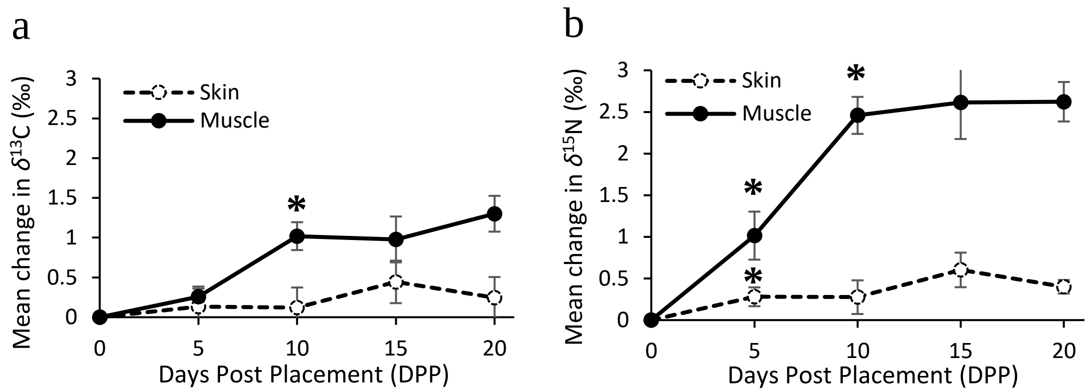

Figure 2.3. Mean difference in (a) $\delta^{13}C$ and (b) $\delta^{15}N$ (‰ ± SE) for nine human cadavers' skin and muscle samples at five-day intervals during 20 days of outdoor decomposition at FARF, San Marcos, Texas. * Asterisk denotes a significant enrichment in a given sampling day's mean change when compared with the previous sampling five days prior (paired T-tests, $p < 0.05$).

of 0.3‰, while $\delta^{15}N_{muscle}$ stable isotopes showed the much greater increases of 2.5‰ as well as two separate points at which comparative enrichments occurred over the 20-day study between 0-5 days (~1‰), and again at 5-10 days (~1.5‰). These comparative levels of enrichment generally levelled off and stopped increasing to a significant level after 10 days and levelled off by day 20 at a cumulative ~2.6‰ increase since day-0, and remained plateaued at that level beyond day 30 (Miles, unpublished data).

2.5 Discussion

Stable isotopes of $\delta^{13}C$ and $\delta^{15}N$ in decomposing human skin and muscle were studied to determine if increased enrichment occurred, and at what point during the decomposition process these increases were significant. The most intensive decomposition processes occur during the late bloat and active decay stages (Gunn 2009), and elevated TBS scores coincident with the greatest slope increases were confirmation of anticipated decomposition advancement. In conjunction with TBS score increases,

$\delta^{13}C_{muscle}$ and $\delta^{15}N_{muscle}$ were elevated, although the point at which these elevated enrichments were detected varied. An unmistakable enrichment trend was found in muscle tissues, detectable for $\delta^{15}N_{muscle}$ by day 5, and $\delta^{13}C_{muscle}$ and at day-10. The $\delta^{15}N_{skin}$ also showed an initial increase by 10 days, but no such increase was seen in $\delta^{13}C_{skin}$. This result suggests that muscle tissue decomposition has more predictable and quantifiable stable isotope ratio changes in the early post-mortem period when compared to skin, while skin stable isotope ratios had small increases, or not at all, regardless of decomposition stage.

Soon after death, the body experiences a large flux in the integrity of the soft tissues due to three major factors. First, endogenous biochemical factors initiate intracellular degradation. This process includes a loss in stability of intracellular lysosome membranes that would normally isolate autolytic enzymes from the rest of the intracellular contents. Post-mortem, once these membranes degrade and fail, lysosomal enzymes are released leading to autolysis or self-digestion of the soft tissues (Gunn 2009). Second, microbial agents contained within the body and introduced from the decomposition environment begin to metabolize the tissues. Most important are the body's own endogenous putrefactive microbes, particularly those normally restricted to the distal gut, that translocate post-mortem and cause rapid anaerobic destruction of soft tissues (Carter *et al.* 2007). Third, the onset of insect activity on the remains as larval stages, especially maggots, feed voraciously on soft tissues in the early stages of decomposition. This is a key factor in tissue degradation that starts in the bloat phase and intensifies during the active decay stage where the greatest tissue mass loss is evident (Matuszewski *et al.* 2014). These three factors contribute to the greatest changes of soft tissue decomposition, including obvious changes in the remains of odours, discolouration, gas accumulation, liquefaction, and mass loss. It follows that if enrichments for both $\delta^{13}C$ and $\delta^{15}N$ occur, they should occur during these bloat and active decay periods of intense decompositional degradation in skin and muscle. This is precisely what my data reflected.

Enrichment of stable isotopes occurs due to fractionation of the elements, which means the lighter isotopes of ^{12}C and ^{14}N are being preferentially (more easily) metabolized than the heavier isotopes of ^{13}C and ^{15}N because lighter isotopes require less energy to shift between phases of use. As such, proportionately more of the heavier isotopes are expected to persist in substrate during metabolism, whether those biochemical alterations are due to lysosomal autophagy, microbial putrefaction, or maggot feeding activity. This result was confirmed in my work; muscle was significantly enriched in both heavier stable isotope ratios of $\delta^{13}C$ and $\delta^{15}N$ over time as decomposition progressed. Elevated levels of heavy isotopes are limited to the early PMI (0-15 days), after which the enrichment rates slow and plateau once most of the soft tissues are putrefied and the body enters the advanced and drier decay phases of decomposition. This correlation between the advancement of soft tissue decomposition with the increases in $\delta^{13}C$ and $\delta^{15}N$, especially in muscle, show that there is potential for stable isotopes to be employed beyond their current uses for provenancing human remains in hard tissues. By taking a wider forensic view of stable isotopes from hard tissue provenancing to soft tissue taphonomy, a much larger scope of research possibilities emerges with a new focus on post-mortem interval studies.

Extrapolating these results raises the question of whether a skin or muscle biopsy sample from recovered remains may yield information regarding the PMI of the body. If verified, use of stable isotopes would be a significant step forward in advancing the quantitative methods available to obtain accurate and precise PMI estimates with medicolegal implications. Two significant works in forensic taphonomy research developed PMI equations with the advantage of easy applications. The first work by Megyesi *et al.* (2005) centers on using TBS values to calculate an ADD-based PMI interval established from retrospective forensic case analysis data, but its findings are proving to be limited when applied to human remains in various geoclimatic regions (Forbes *et al.* 2019, Marhoff *et al.* 2016, Myburgh *et al.* 2013, Suckling *et al.* 2016). Further reviews of this method of PMI estimation have critiqued it on a statistical basis as it only allows for integer scoring and no partial values, causing it to be more of an ordinal ranking system than a sequential scoring system impacting its usability in real forensic cases (Cockle & Bell 2015). Issues have also been raised as to the interchangeability of the independent and dependent variables that induce changes to the regression slope, invalidating the model (Moffatt *et al.* 2016).

A second important work in PMI estimate equations from Vass (2011) developed two equations: one for aerobic decomposition based on variables of degree of decomposition, temperature, humidity, and a second equation calculating anaerobic decomposition incorporating factors of adipocere coverage, temperature, and soil moisture. However, like the Megyesi *et al.* (2005) method, the Vass (2011) equation can significantly under- or over-estimate the PMI when applied outside the geoclimatic region in which it was originally developed (Cockle & Bell 2015). While work is advancing on other scoring systems to turn qualitative observations into quantitative decomposition data, there is still a distinct need to develop a reliable way of obtaining a narrow and forensically relevant PMI range. Given the variability of different human bodies particularly in height and mass (Matusewski *et al*. 2014, Roberts *et al.* 2017, Spicka *et al.* 2011), and how their decompositional processes vary in response to environmental variables including temperature, moisture, and oxygen availability (Anderson & Bell 2014, Carter *et al.* 2010, Roberts & Dabbs 2015), I feel it may be more prudent to quantify the biochemical responses of soft tissue decomposition advancement (in this study, stable isotope ratios) from recovered remains rather than quantifying a range of environmental factors in an attempt to create a one-size-fits-all prediction to how each unique body may respond to these variables.

My study has shown that human $\delta^{13}C_{muscle}$ and $\delta^{15}N_{muscle}$ increase as decomposition progresses, which is a valuable indication of the potential for expanding stable isotope research into soft tissues. This elevated $\delta^{15}N_{muscle}$ enrichment is detectable by day-5, and by day-15 enriches to such a degree that it becomes more similar to $\delta^{15}N_{skin}$. It appears possible to develop methodologies with stable isotope ratios to tell if a forensic case has been decomposing in an outdoor environment for less than or greater than fifteen days. However, we are not yet at the point where a single muscle sample can be analyzed to yield an exact PMI for forensic application. Whereas this study had the advantage of knowing the stable isotope values of skin and muscle when the experiment began at day-0, it would be improper to take a single stable isotope ratio and attempt to assign an accurate PMI to that sample without knowing the at-death values, as well as knowing the variability in a population for those same isotopes for meaningful comparison. This potential will be addressed in future work to determine if correlations exist between soft tissues and hard tissues that may provide a reference point on which to base additional computations of PMI based on stable isotopes when decomposing human remains are recovered in forensic cases.

Suggestions for furthering this area of investigation include increasing the sample size of human donors to improve statistical power of the study, increasing the sampling frequency from every five days to every two to three days to get a more precise profile of stable isotope ratio changes, and performing the experiment under a variety of geoclimatic regions and interment conditions. This is particularly true for areas with freeze-thaw cycles as temperature and moisture are arguably the two most important driving factors controlling decomposition rates, and we know previously frozen bodies decompose differently than unfrozen bodies (Roberts & Dabbs 2015). Other useful data include testing between groups of donors with known eating habits (i.e., vegans, vs vegetarians, vs omnivores) that may reveal differences by dietary groups at the time of death and how rates of enrichment may vary, and concurrent testing with adult pigs of appropriate size to see if there are parallels between humans and their standard proxies that would validate their use in future forensic stable isotope work.

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CHAPTER 3 HUMAN CADAVER DECOMPOSITION ISLANDS AND FORENSIC TAPHONOMY: GRAVESOIL δ^{13}C and δ^{15}N ENRICHMENT PATTERNS IN SHORT (30 DAY) AND EXTENDED (900 DAY) POST-MORTEM INTERVALS.

3.1 Abstract

Soil forensics examines edaphic properties of evidentiary value in the immediate vicinity of human remains in a region called the cadaver decomposition island (CDI). As a body decomposes, soil in the CDI is permeated with liquified human nutrients and becomes gravesoil. My study is the first to specifically investigate how stable isotopes of carbon (δ^{13}C) and nitrogen (δ^{15}N) change in human gravesoils during decomposition over the post-mortem interval (PMI). Gravesoil samples were analyzed for % C, δ^{13}C, % N, and δ^{15}N in CDIs over the initial 30-day PMI (PMI_{30}) and over longer PMIs up to 900 days (PMI_{900}). Gravesoil % C and δ^{13}C did not show any significant changes with decomposition progression in the PMI_{30} study. By days 10-15, gravesoil mean % N more than doubled from 0.4% to 1.05%, while mean gravesoil δ^{15}N increased from 3.3‰ to 8.2‰ by day 10, up to a high mean of 9.9‰ on day 20. Both % N and δ^{15}N remained significantly elevated for the entire PMI_{30} period. In the PMI_{900} study, gravesoils increased from 0.2-0.8% N to a high of 1.9% N after two to three months, then down to 0.5-0.6% in the 600-900 day time frame. Gravesoil δ^{15}N originating at 2-4‰ was enriched by 15-20‰ after the first month, eventually declining to 9-11‰ after 600-900 days. I conclude that δ^{15}N in human gravesoils has promise as a forensic tool to detect a CDI and correlate it with decomposition stage that adds to existing methods in estimating PMI, even if the body is absent.

3.2 Introduction

Forensic taphonomists study mechanisms of human decomposition, in part, to provide law enforcement with accurate and reliable PMI (post-mortem interval) estimations using both qualitative and quantitative assessment of decomposing tissues (Buchan & Anderson 2001, Chandrakanth *et al.* 2013, Megyesi *et al.* 2005, Tumram *et al.* 2011). Multi-disciplinary collaborations have expanded their focus from modelling the environment's impact on cadaver decomposition (Vass 2011) to instead examine how tissue decomposition impacts and changes the surrounding environment with particular attention paid to gravesoils under a body. Whereas gravesoils have previously had value as trace evidence in criminal investigations (Wiltshire 2009), the biochemical impacts of a decomposing body are being recognized as having meaningful and quantifiable impacts on gravesoils with potential forensic significance.

Outdoor decomposition provides a unique opportunity to examine the impacts of decomposing tissues on gravesoil. From an ecological perspective, a decomposing body acts as a brief but intense influx of high-quality nutrients containing 50-75% water, 20% proteins, 10% lipids, 1% carbohydrates, and 5% minerals (Carter *et al.* 2008, Ioan *et al.* 2017). Tissue breakdown due to cellular autolytic enzymes, endogenous microbes, and insect activity reduces large macromolecules of proteins, carbohydrates, and lipids into carbon, nitrogen, and other chemicals. The extent to which these nutrients enter the surroundings depends on the progression of decomposition through the fresh, bloat, active decay, advanced decay, and dry remains stages, with the greatest mass loss occurring in the active decay stage (Carter & Tibbett 2008). This mass loss is a direct result of soft tissue liquefaction when decomposition fluids rich in human-derived nutrients enter the underlying soil.

In recent decades, gravesoil science examined the potential for soils under a decomposing body to reveal information about PMI. In the broader context of forensic taphonomy, research indicates gravesoils can be indicators of not only the presence of human remains in an outdoor location, but potentially how long the body was present even if the remains have been moved due to criminal activity or animal scavenging (Aitkenhead-Peterson *et al.* 2012, Emmons *et al.* 2017, Perrault & Forbes 2016). The area where decompositional body fluids have infused into soils beneath a body, termed a cadaver decomposition island (CDI), experiences a significant input of body-derived nutrients in the early PMI, particularly in carbon and nitrogen. Over time, the amounts of varying forms of carbon and nitrogen in the CDI gravesoil will eventually decrease back to levels seen before the decomposing body was present (Fancher *et al.* 2017, Van Belle *et al.* 2009).

Many studies assessing the amounts of carbon and nitrogen levels associated with CDI gravesoils use porcine analogues, owing to regional permitting regulations. In these studies, CDIs showed elevated amounts of nitrogen over time. Benninger *et al.* (2008) monitored CDI gravesoils under decomposing pigs for 100 days and detected total nitrogen increased from the starting ~0.25% N to 0.45% N 14 days after deposition, then peaking at more than double the starting nitrogen with 0.6% N between days 21-42. Gravesoil returned to ambient nitrogen levels by day 100. No discernable trend of elevated carbon was found, as those same gravesoil samples had large variations in total carbon at the control sites prior to the study starting.

In addition to measures of total nitrogen in gravesoils, specific forms of nitrogen have been reported in the literature, including ninhydrin-reactive nitrogen (NRN), nitrate nitrogen (NO_3-N), and ammonium nitrate (NH_4-N). Carcass fluids from decomposing porcines were found to significantly elevate gravesoil NRN levels after four days of decomposition (Spicka *et al.* 2011). Van Belle *et al.* (2009) report porcine gravesoils starting with less than 20 μg N/g soil were elevated in NRN by day 3 (~37 μg N/g soil), peaking at days 10-14 (~90 μg N/g soil) and remained elevated beyond the end of the 100-day study. With decomposing rats (*Rattus rattus*), NRN was similarly elevated in gravesoils at day 7 and remained elevated at the end of the 28-day experiment (Carter *et al.* 2008).

Complementing these previous short-term studies, Fancher *et al.* (2017) published long-term data for human CDIs compared to control soils of ~6 μg N/g soil where NH_4-N levels in CDIs were significantly higher 14 days after decomposition (111 μg N/g soil), while NO_3-N increased from 36 μg N/g soil to 245 N/g soil by 171 days. These measures of nitrogen returned to control levels after 732 and 851 days, respectively. These temporal data studies were conducted in specific research locations, so the broader forensic application of these findings may be dependent on regional variation in climates, temperatures, precipitation, and soil types.

Knowing the time that it takes surface gravesoils to become significantly elevated in nitrogen is useful in a gravesoil ecology context, but there are also forensic applications in knowing the spatial extent of elevated nutrient levels in human CDIs. Determining the extent and rates that cadaver nutrients from liquefied decomposing tissues permeate nearby gravesoils, both vertically and horizontally in CDIs, has only been recently investigated. CDIs were examined at 20 cm and 50 cm radius intervals around decomposing porcines in Canada (Perrault & Forbes 2016). Carcass nutrients were elevated compared to control soils at 20 cm radius for phosphorus and sodium by 195 ADD (accumulated degree days); carbon and nitrogen were not assessed. Adult human bodies placed on surface soil plots of 1 m x 2.5 m in Texas showed significantly elevated levels of dissolved organic carbon and nitrogen after 248-288 days of decomposition along a distance gradient that extended out to the periphery of the plot boundaries (Aitkenhead-Peterson *et al.* 2012). This was the case whether the bodies were autopsied or not, or whether they were placed under exclusion cages or not, and therefore subject to large scavengers.

Another study in Texas (Fancher *et al.* 2017) sampled CDI gravesoils from 0-15 cm depths under donor bodies in two separate locations with different soil types. Surface gravesoils showed significantly more dissolved organic carbon (DOC) in gravesoils by day 6, peaking at days 14 and 132 for the two soil types, and stayed elevated over the course of the study. They found dissolved organic nitrogen (DON) peaked at days 132 and 263 for the two soil types and remained elevated at end of the study. When examining deeper layers of the same gravesoils, depths to 15 cm under the body also showed increased DON, NH_4-N and NRN, albeit delayed in timing and to a lesser degree compared to surface CDI gravesoils. These results show elevated carbon and nitrogen in gravesoils of different types and depths, but sampling was performed at a single point in time for cadavers placed at different times in the facility. To date, no longitudinal studies have been done that monitor the rate of nutrient input into gravesoils continuously over time. There are still many unanswered questions about cadaver nutrient influx in CDIs in other avenues of forensic taphonomy of gravesoils not yet studied, namely soil stable isotope analysis.

Stable isotope ecology deals with minute fractions of heavy isotopes of common elements to show how much of a particular compound is present and how both heavy and light isotopes are used and shift in an environment. Regular isotopes, such as ^{12}C and ^{14}N react at different rates when compared to their heavier isotopes of ^{13}C and ^{15}N that require more kinetic energy to react in the same way. Because of this, light and heavy isotopes are used at different rates in a process called fractionation (Fry 2006). This fractionation of stable isotopes can result in either the heavy isotope accumulating in a reaction, known as enrichment, or used in greater proportions than the lighter isotope, causing the heavy isotope to become depleted in a system. As a body decomposes, significant biochemical changes occur in the tissues, fractionation being one of them. The previous chapter of this dissertation showed human muscle becomes enriched in $\delta^{15}N$ with decomposition. The fate of these cadaver-derived nutrients remains unknown, but as tissues liquefy and seep into the cadaver decomposition island beneath the remains, it is very likely that the increased $\delta^{15}N$ from muscle leads to an increase in soils' $\delta^{15}N$ ratio beneath human remains, but to date this has not been investigated.

My previous work in chapter 2 of this dissertation showed that human skeletal muscle becomes significantly enriched in $\delta^{15}N$ over the course of decomposition as early as five days post-mortem. The influence of stable isotopes of carbon and nitrogen from decomposing human tissues on CDI gravesoils is currently unknown, but fish and mammal data provide insight into this question. Evidence from fish ecology studies report enrichments of $\delta^{15}N$ in riverbank soils and nearby vegetation from decomposing salmon carcasses distributed by bear foraging (Reimchen *et al.* 2003). In addition to fish, mammal carcasses have a similar enrichment effect on CDIs. Keenan *et al.* (2019) report gravesoils under decomposing beaver (*Castor canadensis*) carcasses ("carrion hotspots") had elevated levels of total nitrogen and $\delta^{15}N$ (by 7.5±1.0‰) a year after deposition. A longitudinal study of temporal and spatial gravesoil enrichments associated with human forensic taphonomy has not yet been published but would provide forensic data conspicuously lacking in the literature. If such an enrichment pattern can be detected and monitored over the decomposition progress (and beyond skeletonization), it would be a huge step forward in validating the use of stable isotopes in a new aspect of CDI forensic case work.

The aim of this study is to investigate the liberation of both $\delta^{13}C$ and $\delta^{15}N$ from liquefied human tissues through natural outdoor decomposition into CDI gravesoils. I wish to answer three main questions based on current gaps in forensic taphonomy: (1) Is there a quantifiable increased enrichment of these heavy isotopes in CDI gravesoils over time and if so, at what point in the early PMI is this enrichment first detectable, (2) Do detectable levels of these increasingly enriched stable isotopes differ with greater lateral and horizontal distances from the body and (3) how long do these enrichments persist over extended PMI periods? If significant, these temporal and spatial data may provide meaningful information that adds to our understanding of CDI gravesoil dynamics as well as supporting the use of stable isotopes as another tool that can be used for PMI estimates in forensic casework.

3.3 Materials & Methods

Research was conducted at the Forensic Anthropology Research Facility (FARF), in San Marcos, Texas. FARF occupies 26 acres of secured and fenced outdoor area within Freeman Ranch, an agricultural research facility and working farm managed by Texas State University-San Marcos. Body donations are obtained through a voluntary donation program with the express documented permission of the deceased individual and/or the consent of the next of kin for the purposes of forensic research, managed by the Forensic Anthropology Centre for Taphonomic Research (FACTS) at Texas State University. Donor bodies are placed under exclusion cages to prevent large scavenging activity by avian or canid animals but still permit free access to sunlight, airflow, and insects as normal taphonomic processes (Suckling *et al.* 2016). The day the donor body was placed in the FARF outdoor facility was considered day 0 for the experiment, as some donors were brought to the facility from a distance and required overnight refrigeration at 4°C before placement in the field the following day.

a. <u>Soil sampling</u>

i. Gravesoils sampled during the early PMI period from day 0-30 (PMI_{30})

Six donor bodies were placed at FARF and permitted to decompose naturally under exclusion cages to obtain data for the early PMI from 0-30 days. At five-day intervals, samples were taken from gravesoils in three locations in each CDI- Spot A on the surface soil directly under the donor's hip (0 cm depth, 0 cm out), Spot B was 2.5 cm under the surface soil and directly by the donor hip (2.5 cm depth, 0 cm out,), and Spot C on the surface soil at a distance 5 cm out from the body's hip (0 cm depth, 5 cm out; Figure 3.1a). Samples for these locations were repeated on the left and right sides of the body as duplicates for each sampling day. The groin/hip region of the body was selected due to its ease of access for sampling underlying gravesoil as well as the likelihood it would contain a significant amount of liquefied nutrients from the body. This region is often the first and most obviously impacted during the decomposition process due to overlying abdominal putrefaction. Samples were not collected at additional depths past 2.5 cm due to substantial underlying rock.

For sample spots A and C, the surface substrate was gently cleared away to remove obvious vegetation and leaf litter to ensure only soil was collected. Spot B soil was disturbed as little as possible to obtain accurate samples at 2.5 cm depth, and sampling points were not re-used; each sampling day created a new alteration to the soil surface.

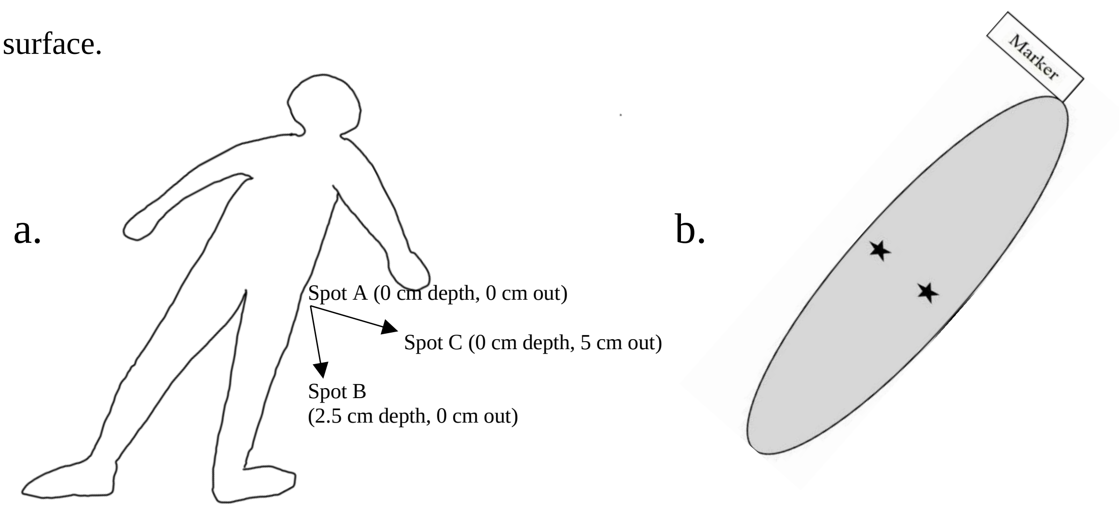

Figure 3.1. Diagrams of (a) prone donor body in the early PMI (PMI_{30}) gravesoil study, and (b) wooden marker at the head of a CDI for the extended PMI (PMI_{900}) gravesoil study in San Marcos, Texas. Uppercase letters A, B, C represent sampling spots within the short period PMI_{30} CDIs near a donor, while black stars indicate extended period PMI_{900} sampling CDI locations without donor remains present.

ii. Gravesoils sampled over an extended PMI of up to 900 days (PMI_{900})

In most cases, the visual evidence of the CDI persists long after the body has been removed due to soil staining. This allowed for sampling of shorter PMI gravesoils where bodies were still present, as well as extended PMI gravesoils where the donor body had been removed. Sampling of 43 extended period PMI_{900} gravesoils involved clearing away the surface vegetation and leaf litter from an area ~10 cm to the left and right of the center of the CDI to approximate where the groin of the donor would be located, and sampled for surface and 2.5 cm depth soils (Figure 3.1b). This was done with replicates for as many different donor CDIs with varying PMIs as possible. Control samples were obtained from soils where bodies had never been placed and used for comparisons with the PMI_{900} soil samples.

For both the early PMI_{30} and extended PMI_{900} studies, each sample removed ~1 cm^3 of soil from its respective location by use of a small gardening spade and a lab-grade curved spatula-like scoop. Soil was secured in individual 1.5 ml Eppendorf tubes with snap-caps then immediately stored in a -20°C freezer. The spade and spatula scoop were thoroughly rinsed with distilled water and paper lab wipes between samples to minimize cross-contamination of samples.

b. Stable isotopes- $\delta^{13}C$ and $\delta^{15}N$

Frozen sample tubes were allowed to thaw at room temperature with open caps for three hours, and then housed in a drying oven at 60°C for a minimum of 48 hours to completely desiccate the samples rendering them biologically inert for isotopic analysis. Dried samples were pulverized to a uniform powdered consistency and lipid were extracted to avoid potential influence on carbon stable isotope ratios. Lipid extraction methodology involved the submersion of the powdered samples with a 2:1 chloroform to methanol solution for a minimum of 30 minutes. The supernatant was removed with a disposable pipette and discarded, while the sample at the bottom of the tube was retained. This lipid extraction procedure with the 2:1 chloroform: methanol solution was repeated at least three times or until the supernatant was clear and colorless (Fischer-Rush *et al.* 2021). Stable isotope ratios were analyzed using continuous flow isotope-ratio mass spectrometry (CFIRMS) to obtain values of ^{13}C and ^{15}N.

Isotopic data are reported in per mil (‰) and calculated using the following delta (δ) notation calculation:

$$\delta\ (‰) = ((R_{sample} - R_{standard}) / R_{standard}) \times 1000\ ‰$$

where R_{sample} is the ratio of the heavy to light isotope contained in the sample, and $R_{standard}$ is the ratio of the heavy to light isotope in the reference material established by the International Atomic Energy Agency (IAEA, Vienna, Austria). The universal standard of $\delta^{13}C$ used Vienna Pee-Dee Belemnite (V-PDB (Craig 1957)) and $\delta^{15}N$ used atmospheric air (AIR (Mariotti 1983)), respectively.

Four in-house secondary standards - Corn Meal Standard ($\delta^{13}C$: -13.3‰, $\delta^{15}N$: 14‰), Ephedra Plant Standard ($\delta^{13}C$: -30.9‰, $\delta^{15}N$: 0.4‰), Spirulina standard ($\delta^{13}C$: -24.9‰, $\delta^{15}N$: 12.9‰), and Seaweed Plant standard ($\delta^{13}C$: -28.4, $\delta^{15}N$: 21.1‰), calibrated against IAEA (International Atomic Energy Agency) standards, were used to normalize stable isotope values. To assess analytical accuracy, check standards of acetanilide, N_2 and CH_7 were also analyzed. Furthermore, 5-7% of samples were run with replicates to monitor instrument drift over time. Analytical precision was below 0.3‰ for both $\delta^{13}C$ and $\delta^{15}N$ following repeated analysis.

Statistical analysis was performed using R (R Core Team 2013) to determine significant changes in % C, $\delta^{13}C$, % N, and $\delta^{15}N$ for several comparisons: the general linear model for an effect of decomposition progression (days), and Tukey's Student's t-tests (paired samples) to compare between sampling days for each isotope at each sampling location during the early post-mortem interval study. Significance was established at α=0.05.

3.4 Results

Elemental and isotopic composition of soil collected at CDIs revealed a considerable impact of liquefied human tissue nutrients into adjacent gravesoils. While carbon showed highly variable results for both PMI_{30} and PMI_{900} gravesoils, rapidly detectable elevated measures of mean % N and $\delta^{15}N$ in the initial PMI_{30} phase persisted well into the extended PMI_{900} study.

a. Gravesoils sampled during the early PMI period from day 0-30 (PMI_{30})

CDI soils exposed to decompositional fluids in the first 30 days showed different results for both elemental and isotopic measurements of carbon and nitrogen. GLM analysis did not identify a statistically significant relationship between decomposition progression and either mean soil % C or mean soil $\delta^{13}C$ (Table 3.1). Mean measures for nitrogen, however, showed distinct increases over time. Decomposition affected the mean % N at spots A (0 cm down, 0 cm out) and B (2.5 cm down, 0 cm out), but no such effect was detected for spot C (0 cm down, 5 cm out). Mean $\delta^{15}N$, however, was significantly impacted by the decomposition process over time for all three soil spots sampled.

Table 3.1. Summary of general linear model *p* values for five mean gravesoil measurements for three sampling spots in six human cadavers' grave soils over the first 30 days of outdoor decomposition (PMI_{30}) at FARF, San Marcos, Texas.

Mean soil measure	Spot A (0 cm out-0 cm down)	Spot B (2.5 cm down-0 cm out)	Spot C (0 cm down-5 cm out)
$\delta^{15}N$	<0.0001	<0.0001	0.03
% N	0.006	0.003	-
% C	-	-	-
$\delta^{13}C$	-	-	-
C:N	0.01	-	-

- in table denotes no significant effect of time (and therefore decomposition progression) was detected ($p>0.05$).

Each sampling day's gravesoil mean measurement was compared to that of the starting day soils at day 0. I found no discernable enrichment pattern of gravesoils with either % C or $\delta^{13}C$ over time except for a single point of enrichment for % C at spot A on day 25 (Figure 3.2).

Unlike gravesoil carbon data, clear patterns emerged with % N and $\delta^{15}N$. The first point in time when mean % N was significantly higher compared to day 0 gravesoil was detected on day 15 for spot A samples, corresponding to the end of active decay and beginning of advanced decay phase of cadaver decomposition. Mean % N increased from 0.43% on day 0 to 1.05% by day-15, more than doubling the initial total nitrogen gravesoil content. Spot B samples were significantly higher in % N as well but detectable much later, first at day-30 well into the advanced stage of cadaver decomposition. This increase in nitrogen at spot B was less pronounced than that at spot A, with a day-30 mean percent nitrogen of 0.49% N compared to 0.31% at day-0. Samples collected from spot C were not found to be higher in % N at any point in the 30-day study period.

Mean values for spot A $\delta^{15}N$ gravesoils showed significant enrichments at day-10 in the active decay stage of decomposition, five days earlier than the first elevated levels of mean % N were detected. The original day-0 mean $\delta^{15}N$ of 3.3‰ increased to 8.2‰ at day-10, an overall increase of 4.9‰ that remained significantly elevated throughout the 30-day study. In addition to spot A, gravesoils at spots B and Site C also had significantly elevated mean $\delta^{15}N$ levels detectable at day-15. Spot B's mean $\delta^{15}N$ at day-0 of 4.7‰ increased to 8.0‰ by day-15 (an increase of 3.3‰), while spot C's day-0 mean $\delta^{15}N$ of 3.3‰ increased to 7.4‰ in the same 15-day period (an increase of 4.1‰).

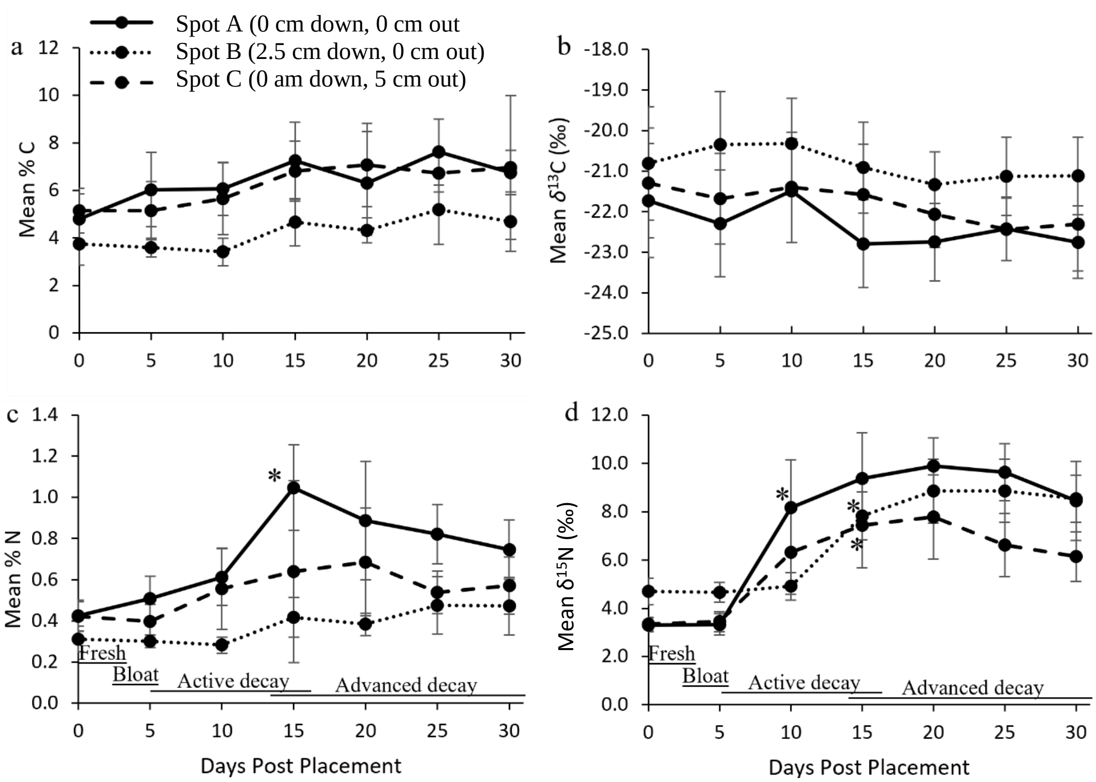

Figure 3.2. Mean values of (a) percent carbon (%±SE), (b) $\delta^{13}C$ (‰±SE), (c) percent nitrogen (%±SE), and (d) $\delta^{15}N$ (‰±SE) for three sampling spots adjacent to six human cadavers' grave soils over the first 30 days of outdoor decomposition (PMI_{30}) at FARF, San Marcos, Texas. * Asterisk denotes the first point at which a significant mean increase was detected compared to day 0; $p < 0.05$).

When examining the overall C:N ratio of gravesoils at the three body-adjacent sampling spots, we found the mean C:N over the course of decomposition decreased, given the relatively stable levels of carbon over the PMI_{30} period while the mean nitrogen content increased over the same time frame. This reduction in mean C:N from 10.9 to 9.3 was detected only for spot A surface soils closest to the body on day-25 and day-30 of the study. During this time, the body was entirely in the advanced decay decomposition stage (Figure 3.3).

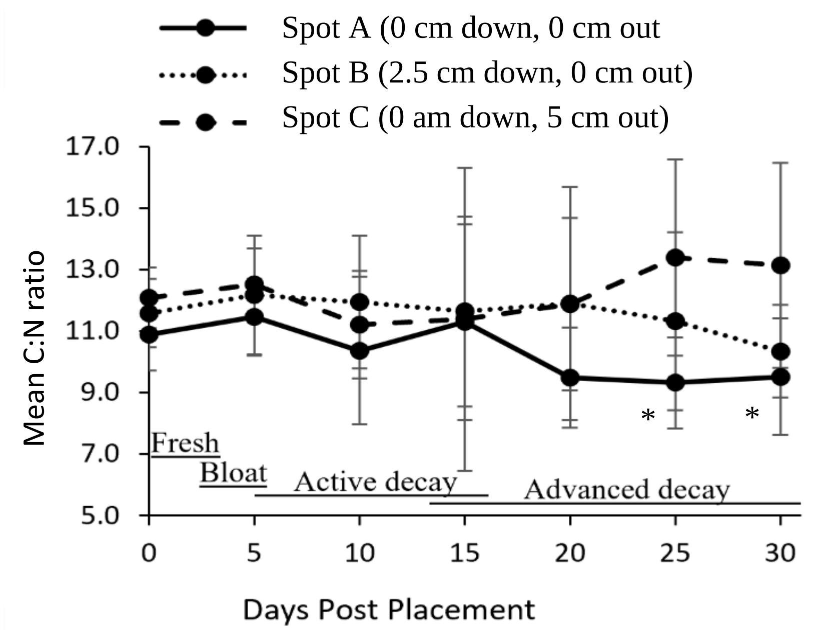

Figure 3.3. Mean values of C:N carbon to nitrogen ratio (±SE) for three sampling spots adjacent to six human cadavers' grave soils over the first 30 days of outdoor decomposition (PMI_{30}) at FARF, San Marcos, Texas. * Asterisk denotes a significant mean C:N change when compared to day 0; $p < 0.05$).

b. Gravesoils sampled over an extended PMI of up to 900 days (PMI_{900})

CDIs in the longer PMI_{900} study revealed differences between the two forms of carbon and nitrogen. Control soil % C had a narrow range of values (2.2-9.8%) compared to the samples collected from gravesoils approaching 100 days of decomposition with values in excess 30% C (Figure 3.4). These elevated surface gravesoils did not decline to the same range as control soils until 600-900 days of decomposition. Samples from those same CDI gravesoils taken at 2.5 cm depth showed no such spikes in % C with values between 2.1% and 9.3% throughout the extended PMI_{900} period, which aligned with the control soil range at the outset of the study.

In contrast, control soil values for $\delta^{13}C$ varied more significantly between -18.0‰ and -26.3‰. This large variation in initial $\delta^{13}C$ for the surface gravesoils mirrored the overall range of samples throughout the extended PMI period, although interestingly, the surface CDI gravesoils were depleted when compared to the corresponding CDI soils sampled at 2.5 cm depth.

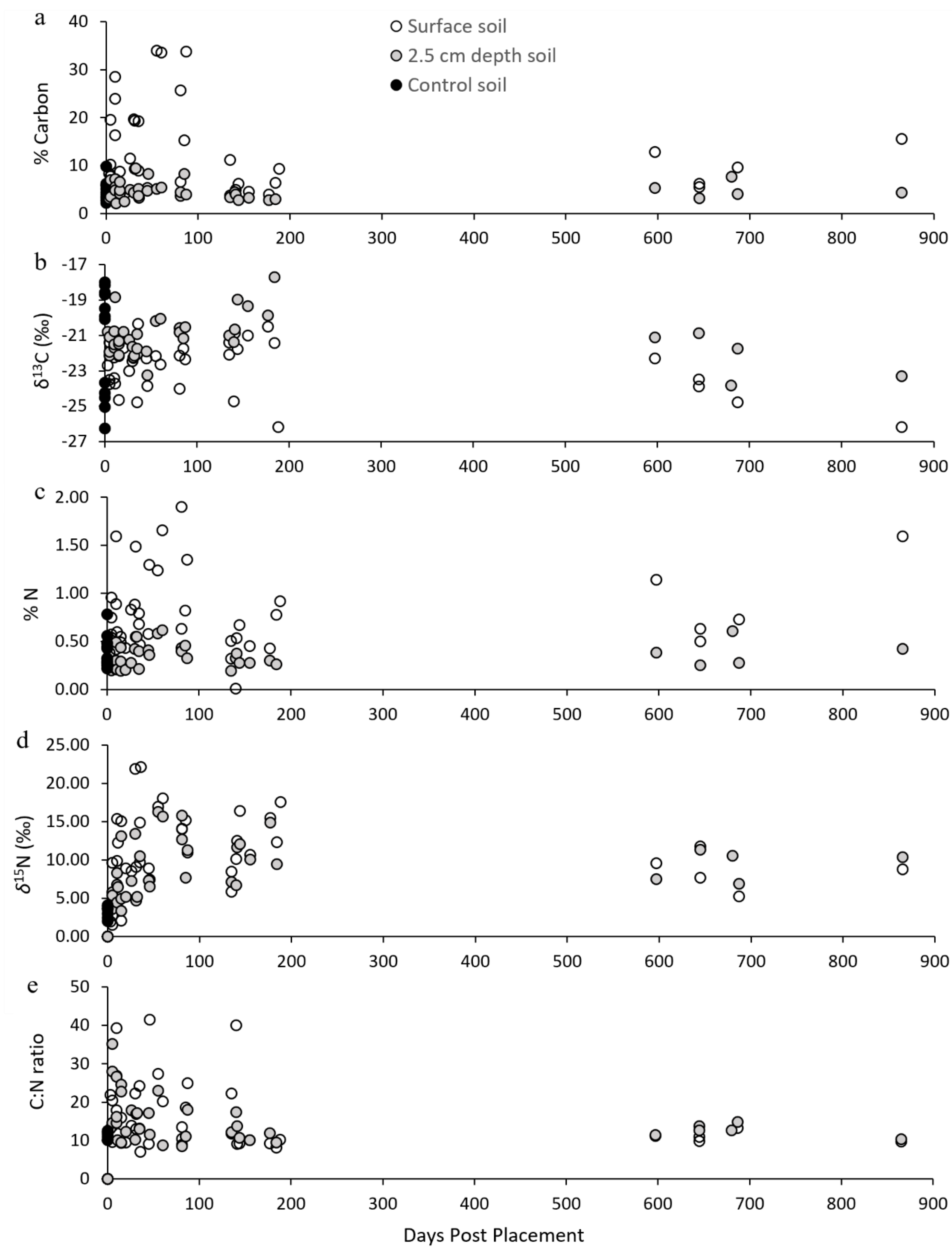

Figure 3.4. Measured values of soil carbon (a) percent carbon and (b) δ^{13}C (c) percent nitrogen (%±SE), (d) δ^{15}N (‰±SE), and (e) C:N for individual cadaver gravesoils with various known post-mortem intervals (PMI_{900}) of outdoor decomposition at FARF, San Marcos, Texas (n=43).

Values for both forms of nitrogen showed much narrower ranges of values when compared with their corresponding carbon values. Control soils had 0.2-0.6 % N, while surface CDIs soils peaked at 1.9% at day 81, a four-to-fivefold increase, eventually declining back to control values between 600-700 days post-mortem. Surface gravesoils had consistently greater % N than the 2.5 cm depth soils at the same PMIs. Like the % C values, % N sampled at 2.5 cm depth did not fall outside the control soil value range so no significant elevation in % N was observed over the PMI_{900} study.

Distinguishing itself from the trends found with $\delta^{13}C$, the control $\delta^{15}N$ soils were more consistent with values from 2.0-4.1‰. This gives a range of ~2.0‰ for control soil $\delta^{15}N$, whereas the mean $\delta^{13}C$ in control soil varied by ~8.3‰. With this tighter range of comparative control soil values, a clear trend of gravesoil enrichment for $\delta^{15}N$ appeared almost immediately after cadavers were allowed to decompose, reaching more than 20‰ between 20 and 30 days of decomposition for surface gravesoils, and ~16‰ for the 2.5 cm depth soils around day 60. In general, the surface gravesoils were enriched when compared to the corresponding 2.5 cm depth soils over the PMI_{900} period. Both surface and 2.5 cm depth gravesoils did not lessen in $\delta^{15}N$ to match that of the control soils and remained elevated throughout the PMI_{900} study.

Examination of C:N in CDI gravesoils during 900-day PMI periods shows very narrow ranges for control soils (10.0-12.5) while surface soils C:N peaked from days 41-46 (>40), and 2.5 cm depth soils peaked at day-5 (>35). However, since these scatter plots show individual cadaver data points, this may be an outlier. If so, the next peak in 2.5 cm depth soils occurs at day-15 with a value of 24. Both surface and 2.5 cm depth gravesoils appear to return to control soil C:N values by 200 days.

3.5 Discussion

This study addresses three major questions: (1) Is there a quantifiable enrichment of $\delta^{13}C$ and $\delta^{15}N$ in CDI gravesoils over time, and if so, at what point in the early PMI is this enrichment first detectable, (2) Do detectable levels of enriched $\delta^{13}C$ and $\delta^{15}N$ differ with increasing lateral and horizontal distances from the body, and (3) How long do these $\delta^{13}C$ and $\delta^{15}N$ enrichments persist over the longer PMI_{900}? The data have provided clear evidence of how $\delta^{13}C$ and $\delta^{15}N$ are affected both temporally and spatially in human gravesoil CDIs.

Question 1 was to determine whether $\delta^{13}C$ and $\delta^{15}N$ are enriched in human gravesoils as decomposition advances, and when it is detectable. My results indicate that of the two stable isotopes studied, $\delta^{13}C$ and $\delta^{15}N$, significant enrichments were found with $\delta^{15}N$ gravesoils in both PMI_{30} and PMI_{900} studies, providing evidence for its use in forensic taphonomy. While a cadaver contains both carbon and nitrogen, these substances are liberated into the surrounding CDI environment differently during decomposition. Benninger *et al.* (2008) found gravesoils under decomposing porcines were significantly elevated in nitrogen first at day 14 of the experiment and remained elevated compared to control soils beyond 72 days. Carbon did not show significant increase in gravesoils despite the large quantity of carbon contained in a body. This was ostensibly due to large fluctuations in carbon for both tissues and soils, with most carbon liberated from decomposition in the form of volatile gases, particularly CO_2. The current study aligns with those findings whereby a significant amount of nitrogen, whether it be in total % N or $\delta^{15}N$, enters the CDI in appreciable quantities detectable in the PMI_{30} by days 10-15 during the active decay phase of decomposition. Active decay is when the greatest loss of mass from a cadaver occurs due to autolysis, bacterial degradation, and maggot larvae feeding so elevated levels of nitrogen in various forms were anticipated to increase during this stage. Soil δ^{15}N is significantly elevated in the active decay stage of decomposition at days 10-15. Soil δ^{13}C did not show any such enrichment over time.

The second component of this study addressed how far and how fast any stable isotope enrichment would be detectable not only at surface gravesoils directly adjacent to a cadaver, but also at a depth 2.5 cm under the body or 5 cm out from the body. No clear pattern emerged with the three sampling spots for δ^{13}C. Soil δ^{15}N not only showed increased levels at spot A collected at the surface and adjacent location, but also at spot B (2.5 cm down) and spot C (5 cm out from the body). However, the timing of these increases differed. Spot A showed significantly elevated δ^{15}N by day-10, while spots B and C experienced a delay in elevated δ^{15}N until day-15. This stands to reason given that as tissues decompose and liquefy, they gradually purge from the body into the CDI and slowly permeate into the soil. The data are corroborated by studies using both porcines and humans reporting cadaver-derived nutrient data in CDI gravesoils (Aitkenhead-Peterson *et al.* 2012, Benninger *et al.* 2008, Carter *et al*. 2008, Emmons *et al.* 2017, Fancher *et al.* 2017, Perrault & Forbes 2016, , Spicka *et al.* 2011, Van Belle *et al.* 2009). Cadaver and carcass nutrients permeate gravesoils horizontally and laterally over time, first detectable in surface soils with subsequent elevated nutrient levels detected at both lateral and horizontal distances from the body, dependent on soil type present. Gravesoil δ^{15}N was significantly elevated in surface gravesoils compared to control soils by day-10. Elevated δ^{15}N was found after a slight delay by day-15 for gravesoils at a depth of 2.5 cm under the body (spot B), and 5 cm out from the body (spot C).

Finally, extended period PMI_{900} gravesoils were examined to see how long these elevated levels of $\delta^{13}C$ and $\delta^{15}N$ persist, especially those CDIs where the body was removed. As with the early PMI_{30} study, $\delta^{13}C$ in extended PMI_{900} period did not show any trends in enrichment because $\delta^{13}C$ in the control soils had a wide range of values that prevented meaningful comparisons to the sampled gravesoils. Control samples for $\delta^{15}N$, however, had a very narrow range of values, making higher levels obvious in the data and more meaningful. Gravesoils sampled at both the CDI surface and 2.5 cm depth showed substantial increases very early in the PMI_{900} study that persisted in soils until 600-700 days of decomposition. These data support other studies looking at longer PMI cadaver-derived nutrients in gravesoils. Data reported for forms of human tissue nitrogen in Tennessee show a return to control gravesoil levels by 700-800 days (Fancher *et al.* 2017). Stable isotopes were analyzed under decomposing beaver carcasses where elevated $\delta^{15}N$ was detected one year after deposition at the site (Keenan *et al.* 2019). The discrepancy between elevated $\delta^{15}N$ in beaver CDI gravesoil for one year and my study's human gravesoil elevated $\delta^{15}N$ detectable after two years is likely explained not only by the difference in soil types under the decomposing tissues but also the sheer size discrepancy between beavers and humans. With humans having on average two to four times the mass of an adult beaver, there is more soft tissue mass to decompose, so a greater volume of liquified nutrients is available to enter the adjacent soils and prolong nitrogen enrichment.

The data presented provide insights into the process through which nitrogen from decomposing organic material enters, is cycled through, and is liberated from the soil via the nitrogen cycle. In gravesoil, nitrogen enters the soil from liquefied decomposing tissues, shifting the soils from aerobic to anaerobic conditions (Carter & Tibbett 2008). Once in the soil, nitrogen can be converted through atmospheric nitrogen, ammonia, ammonium, nitrites, and nitrates by soil microbe metabolism. However, the soil's oxygen content will determine how this cycle functions depending on whether the conditions are aerobic or anaerobic. In anaerobic conditions, ammonium cannot be converted to nitrites and nitrates by nitrifying microbes as this is an aerobic process; it is only when gravesoils revert to aerobic conditions that nitrification can resume (Fancher *et al.* 2017). This causes a lag in CDIs resuming the aerobic stages of the nitrogen cycle until further soil microbes gradually reintroduce more oxygen to the gravesoil. This delay in resuming aerobic metabolism explains the persistence of nitrogen, and in my study specifically δ^{15}N, by approximately two years. An extended period of high δ^{15}N indicates that samples with such drastic levels of enrichment, ~20‰ for surface soil and 15‰ for 2.5 cm depth soil, indicate δ^{15}N's potential to establish the presence of a cadaver in a specific location for two years, even if the body has been moved to conceal it or if it has been scavenged and dispersed. This evidence has practical utility for forensic investigators even in the absence of human remains at the scene. However, it should be acknowledged that there is a conspicuous gap in the available CDIs sampled with no data available in the 200 to 600-day PMI range. Further studies of this nature should include additional data to provide a more comprehensive dataset. The δ^{15}N becomes rapidly enriched in gravesoils and persists to 600-700 days before returning to levels seen in control soils. Due to the wide range of both control and gravesoil δ^{13}C values, it is difficult to draw any strong carbon isotope ratio conclusions from this aspect of the study.

In summary, $\delta^{15}N$ has tangible promise as a forensic tool supplementing current trends in gravesoil science in determining not only the presence of decomposing remains, but also how long remains decomposed in that location. The temporal and spatial extension of the rates of $\delta^{15}N$ liberation into CDI gravesoils is readily detected in the active decay phase of decomposition by days 10-15, depending on the spot sampled. This isotopic ratio enhancement endures up to two years. Additional studies with greater sample sizes and with varied soil types could establish the utility of soil $\delta^{15}N$ as another method in which forensic scientists and investigators can use with evidentiary value, a method that is easy to collect and relatively inexpensive compared to other chemical analyses currently used.

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CHAPTER 4 FORENSIC TAPHONOMY, PORCINE ANALOGUES, AND STABLE ISOTOPES: CHANGES IN PIG SKIN AND MUSCLE $\delta^{13}C$ & $\delta^{15}N$ DURING THE EARLY POST-MORTEM INTERVAL IN ATLANTIC CANADA.

4.1 Abstract

This study aimed to provide preliminary regionally-specific taphonomic data in Atlantic Canada using porcine analogues. Six adult porcines were left outdoors to decompose naturally in late summer, sampled every three days to measure $\delta^{13}C$ and $\delta^{15}N$ in both skin and muscle over a total of twelve days. Over the course of the study, as porcines transitioned through the fresh, bloat, and active decay stages of decomposition, $\delta^{15}N_{muscle}$ became enriched from a mean starting value of 4.1‰, first detectable at day-6 in the bloat phase by a mean change of ~0.5‰. No such enrichments were found in $\delta^{15}N_{skin}$, $\delta^{13}C_{skin}$, or $\delta^{13}C_{muscle}$. This snapshot-in-time study provides important preliminary evidence that porcine muscle tissues, like human tissues, become enriched in $\delta^{15}N$ as decomposition progresses and is detectable in the very early post-mortem interval. Future research can expand these findings to further elucidate the potential of stable isotopes as a quantifier of post-mortem interval in the under-studied region of Atlantic Canada.

4.2 Introduction

Forensic taphonomy, the study of peri-mortem circumstances and post-mortem decomposition of human remains, is a critical component to death investigations as the location and condition of the body at discovery provide essential evidence supplementing traditional law enforcement techniques (Basilo *et al.* 2019). One way forensic taphonomy can provide meaningful information is with the post-mortem interval (PMI). PMI is key to determining time elapsed between time of death and recovery, playing a role in confirming last known sightings, witness statements, and confirming or disproving suspect alibis.

Estimating PMI over the past two decades has become increasingly complex, given the seemingly infinite intrinsic and extrinsic factors that can affect decomposition rates and processes. Quantitative biochemical markers of post-mortem tissues have been identified but are limited in use to very short PMIs of a few hours (Madea 2005, Salam *et al.* 2012). Over longer PMIs, temperature and moisture are recognized as the two most important factors governing decomposition through the fresh, bloat, active decay, advanced decay, and skeletonization process (Carter *et al.* 2007). Forensic taphonomists must also be keenly aware of factors including peri-mortem trauma, sunlight, freeze-thaw cycles, insect activity, and scavenging that may accelerate or retard decomposition progress and impact PMI estimates (Carter *et al.* 2007, Cockle & Bell 2015, Meyer *et al.* 2013, Roberts & Dabbs 2015, Wescott 2018). Attempts to universally quantify PMI were developed and modified over time (Megyesi *et al*. 2005, Vass 2011) but no single method precisely predicts PMI in all situations and locations (Bygarski & LeBlanc 2013, Forbes *et al.* 2019, Knobel *et al.* 2018, Korgesaar *et al.* 2022, Myberg *et al*. 2013). PMI research has shifted to establishing regionally-specific taphonomy data, given the wide range of global bio-geoclimatic conditions that impact human decomposition.

Most forensic taphonomy data come from relatively warm climates of Tennessee and Texas, USA as well as Australia and South Africa; Canada, a larger and colder country, unfortunately lacks a robust body of taphonomic data. While a few published articles exist on Canadian taphonomy, they come from wide-ranging areas of different geoclimatic conditions with small sample sizes, often with a focus on forensic entomology rather than taphonomy (Anderson 2011, Bygarski & LeBlanc 2013, Gill 2005, Meyer *et al.* 2013, Michaud & Moreau 2011). A single Canadian single facility using human donors as taphonomic research subjects opened in 2020 but requires time to generate decomposition data sets that are applicable to its location in Trois-Rivieres, Quebec. While generally informative, those data are of limited utility throughout Canada, a large nation of almost 10 million km^2 encompassing five major biomes: tundra, boreal forests, mountain forests, grasslands, and deciduous forests. These biomes create highly variable climates ranging from record cold winters (-63.0°C) to hot summers (49.6°C; Environment Canada 2023). Using the Köppen-Geiger climate classification, Canada lacks a tropical (group A) zone, but contains arid (B), temperate (C), continental (D), and polar regions (E; Peel *et al.* 2007). By reviewing published taphonomy data from Tennessee, Texas, Sydney, Australia (zone Cfa- humid subtropical), and Cape Town, South Africa (zone Csb- temperate, dry warm summer), data from those warmer locations may not be applicable to the entire varied Canadian landscape to accurately estimate PMI in forensic cases.

As of 2023, Canada has a population of over 40 million people, with 1.8 million people living in Atlantic Canada (made up of the four most easterly provinces of New Brunswick, Nova Scotia, Prince Edward Island, and Newfoundland) with most land mass in the Dfb climate zone (Statistics Canada 2023). Atlantic Canada does not yet have a compilation of decomposition studies in the literature, with one published taphonomy project in Nova Scotia (Brown & Peckmann 2013), and an entomologist that collaborates on forensic projects in New Brunswick (LeBlanc *et al.* 2021, Michaud & Moreau 2011, Moreau *et al.* 2021). Atlantic Canada has a need for current and specific taphonomic standards relevant to that region- there are currently 1030 missing persons, and 13 sets of unidentified human remains in addition to both solved and unsolved homicides in the region (Canada's Missing 2022, RCMP 2023).

The aim of my third study examines outdoor decomposition in porcine analogues in Atlantic Canada to obtain very preliminary taphonomic data with specific attention to changes in $\delta^{13}C$ and $\delta^{15}N$ in porcine skin and muscle over the early post-mortem period. Stable isotope ratios in decomposing human skin and muscle in Texas provided promising results with distinct $\delta^{15}N$ enrichment in muscle tissues over the early PMI of 20 days (Chapter 2). Based on this potential for stable isotope use in taphonomy work in soft human tissues in Texas, we now examine their forensic application in Atlantic Canada as a pilot study. Based on the Texas human cadaver study, a similar trend of enrichment for $\delta^{15}N$ is anticipated in porcine analogue muscle as decomposition advances, with no discernable enrichment trend in skin $\delta^{15}N$, skin $\delta^{13}C$, or muscle $\delta^{13}C$.

4.3 Materials & Methods

This study was conducted in September 2019 on private land inside the city of Fredericton, New Brunswick, Canada (Figures 4.1, 4.2). The area was secured by a lockable nine-foot chain link fence restricting access. New Brunswick falls within the temperate broad-leaved forest category but is sufficiently northern to contain boreal forest regions as well. The study site is an open natural grassland penetrated by full sunlight adjacent to a large, forested area with mature trees on one side. Small barn-like sheds for equipment storage are adjacent to the site.

Six healthy domestic pigs (*Sus scrofa*) weighing 25-35 kg were purchased after being dispatched by a captive-bolt pistol gun ("bolt gun") shot to the head and transported to the study site within two hours for field deployment. All carcasses were positioned on their right sides, heads pointing southwest, 10 m apart as permitted by the study area and uncaged (Figure 4.3). For identification, carcasses were randomly numbered 1-6 with metal toe tags on the front left hoof.

a. <u>Weather data</u>

Weather data recorded from the nearest Environment Canada weather station was accessed used to determine the daily maximum and minimum temperatures, from which the daily mean was calculated. The daily mean temperature for each day of the experiment was used for summation of the accumulated degree-day (ADD) thermal energy over time. Data for rainfall, humidity, and cloud cover were also collected.

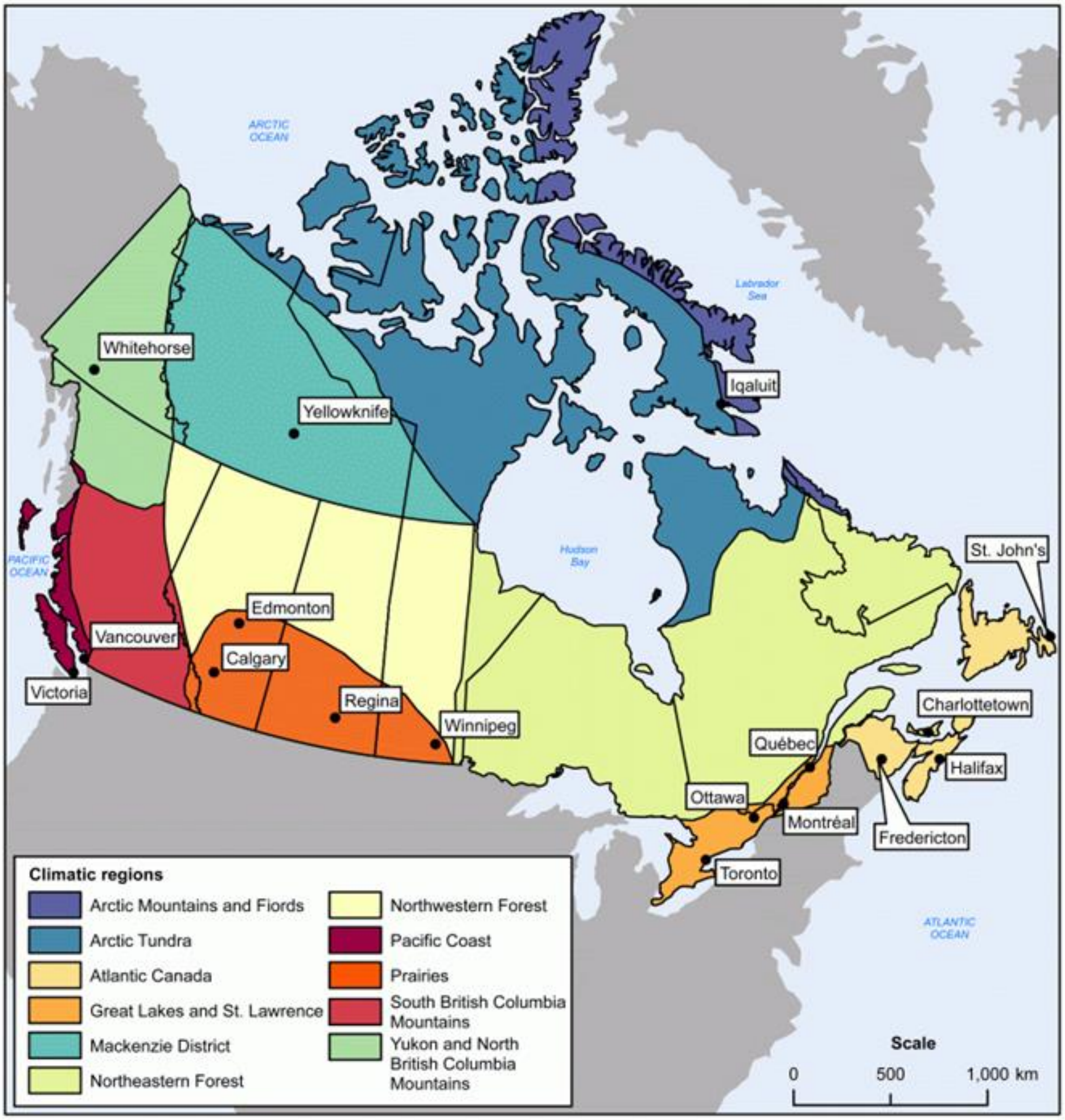

Figure 4.1. General climatic regions of Canada map (Environment Canada 1998).

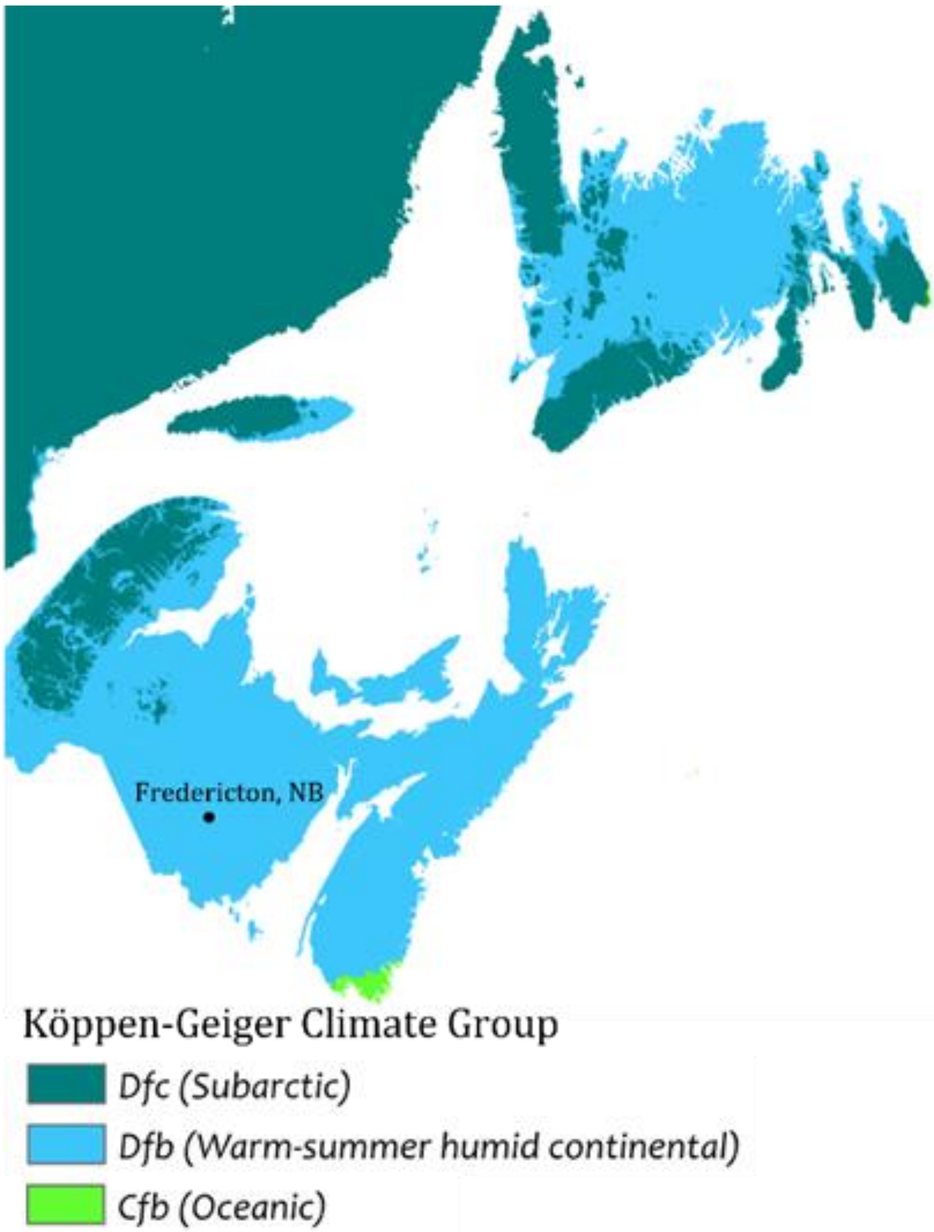

Figure 4.2. Köppen-Geiger Climate map of Atlantic Canada showing study location in Fredericton, New Brunswick (adapted from Petersen 2016).

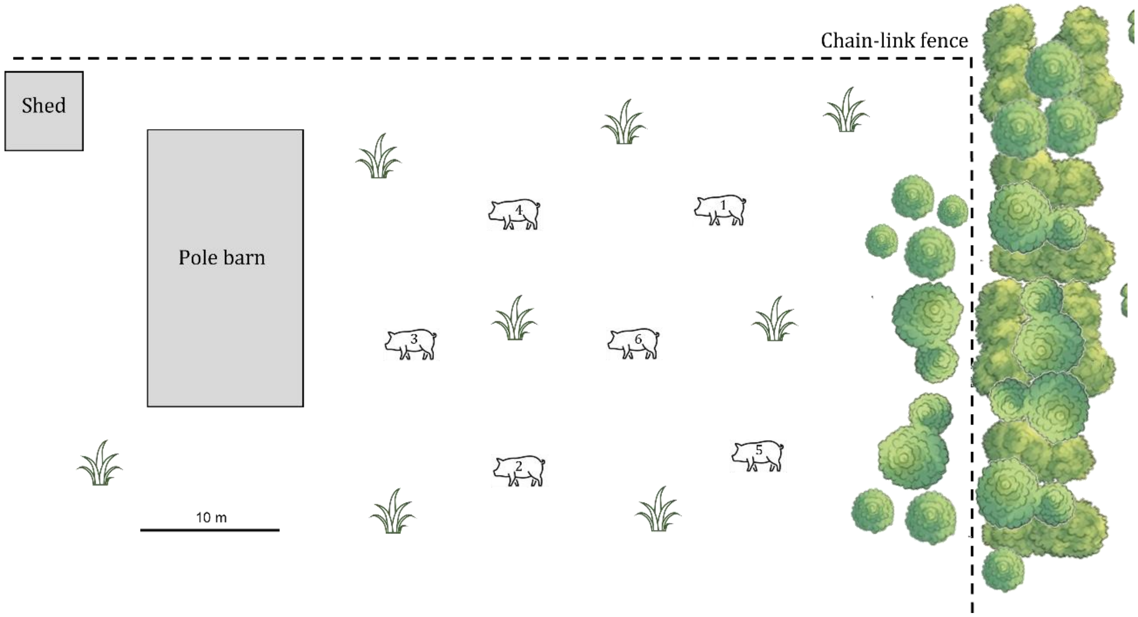

Figure 4.3. Aerial schematic diagram of field study site showing placement of six pigs in a grassy area surrounded by chain link fencing, surrounding woodland, and adjacent storage structures.

b. <u>Total Body Score (TBS) data</u>

Each porcine analogue was photographed on each sampling day (three-day intervals) to record physical appearance and visual decomposition changes as per Megyesi *et al.* (2005) method. The TBS scoring approach assigns a numerical score based on standardized criteria. A fresh carcass has a TBS score of 3, and a completely skeletonized remains has a TBS score of 35. Photos were taken of each carcass on sampling days in multiple views including the entire body from above, back, hind, and head, as well as close-up photos showing isolated areas of tissues when needed. Photos were captured using a Samsung Galaxy A53 mobile phone camera in the highest resolution setting (16MP, 3468 x 4624). Images were downloaded to a Dell laptop for visual analysis.

c. Stable isotopes- $\delta^{13}C$ and $\delta^{15}N$

Soft tissue sampling for stable isotope analysis was performed every three days by excising a small amount of superficial skin and underlying muscle (<1 cm^3). These were secured in individual 1.5 ml Eppendorf tubes with snap-caps and, then immediately frozen at -20°C. Skin and muscle were collected from the left rump/leg of the carcass with four samples taken per carcass on each sampling day- two replicates per pig for both tissues, for a total of 120 samples.

For sample processing, frozen samples tubes were thawed at ambient room temperature with open caps for three hours, and then housed in a drying oven at 60°C for a minimum of 48 hours to completely desiccate. These dried samples were pulverized to a uniform powdered consistency.

Lipid extraction methodology involved the submersion of the powdered samples with a 2:1 chloroform to methanol solution for a minimum of 30 minutes. The supernatant was removed with a disposable pipette and discarded, while the sample at the bottom of the tube was retained. This lipid extraction procedure with the 2:1 chloroform: methanol solution was repeated at least three times or until the supernatant was clear and colorless (Fischer-Rush *et al.* 2021). Stable isotope ratios were analyzed using continuous flow isotope-ratio mass spectrometry (CFIRMS) to obtain values of ^{13}C and ^{15}N.

Isotopic data are reported in per mil (‰) and calculated using the following delta (δ) notation calculation:

$$\delta\ (‰) = ((R_{sample} - R_{standard}) / R_{standard}) \times 1000\ ‰$$

where R_{sample} is the ratio of the heavy to light isotope contained in the sample, and $R_{standard}$ is the ratio of the heavy to light isotope in the reference material established by the

International Atomic Energy Agency (IAEA, Vienna, Austria). The universal standard of $\delta^{13}C$ used Vienna Pee-Dee Belemnite (V-PDB (Craig 1957)) and $\delta^{15}N$ used atmospheric air (AIR (Mariotti 1983)), respectively.

In-house secondary standards for BLS (Bovine Liver Standard) and MLS (Muskellunge Muscle Standard) were calibrated against IAEA (International Atomic Energy Agency) standards to normalize the stable isotope values. To assess analytical accuracy, check standards of nicotinamide, N_2 and CH_7 were also analyzed. Furthermore, 5-7% of samples were run with replicates to monitor instrument drift over time. Analytical precision was better than 0.2‰ for $\delta^{13}C$ and 0.3‰ $\delta^{15}N$ following repeated analysis.

d. <u>Statistical analysis</u>

Statistical analyses were performed using R (R Core Team 2013) to assess significant changes in $\delta^{13}C$ and $\delta^{15}N$ for several comparisons. To determine if porcine analogue skin and muscle were enriched as they decompose, the general linear model was used to detect effects of decomposition progression factors on stable isotope data for both tissue types. To determine at what point in the decomposition process these effects become significant, Tukey's Student t-tests (paired samples) compared sampling days for each tissue and isotope. Significance was established at $p < 0.05$.

4.4 Results

a. <u>Weather data</u>

Mean daily temperatures were used to calculate accumulated thermal energy (ADD) over the course of the 12-day experiment (Table 4.1). The mean daily temperature over the entire study period was 12.4°C, ranging from a daily high of 28°C and a low of -1°C. No rainfall occurred over the study period, and humidity ranged from 69-96%.

Table 4.1. Daily weather data for the 12-day study period of six pig (*Sus scrofa*) carcasses left to decompose outdoors in Fredericton, NB, Canada in September 2019.

Day	ADD	Mean Temp °C	Temp range °C	Rainfall mm	Humidity %	Cloud cover
0	0	12.15	10-14	0	80	Light clouds
1	12.15	7.3	4-17	0	82	Clear
2	19.45	3.7	-1-20	0	88	Mostly clear
3	23.15	12.0	2-24	0	80	Mostly clear
4	35.15	16.2	9-23	0	69	Clear
5	51.35	10.0	3-28	0	86	Clear
6	61.35	20.2	17-26	0	89	Mostly cloudy
7	81.55	14.6	11-19	0	96	Overcast
8	96.15	10.8	9-13	0	93	Overcast
9	106.95	14.1	7-21	0	82	Partly cloudy
10	121.05	13.7	9-18	0	80	Mostly clear
11	134.75	14.1	7-20	0	87	Overcast
12	148.85	12.8	4-15	0	83	Partly cloudy

b. <u>TBS data</u>

Visual evidence of decomposition progressed as expected through the fresh, bloat, and active decay stages. Carcasses appeared fresh until days 3-4 when most showed the beginnings of bloat with initial inflation of the abdominal cavity. By day-6 the abdominal cavities showed considerably enlarged abdomens with displacement of the limbs to accommodate the additional abdominal volume, and obvious marbling of superficial blood vessels of dark red, purple, and green discolourations. Blowfly eggs and small larvae were present in the eyes, ears, snout, and mouth. Maggot masses increased in size and activity at day-9, with facial regions showing distinct tissue loss, skin dessication with blackened tissue, and some bone exposure. At the end of the 12-day study, differential decomposition was evident with much of the head region appearing as dry remains or skeletonized, while the rest of the body experienced large maggot masses representative of the active decay stage (Figure 4.4).

Day 0 (0 ADD; fresh) Day 6 (61 ADD; bloat) Day 12 (149 ADD; active decay)

Figure 4.4 Decomposition appearance of pig #4 at sampling days 0, 6, and 12.

Mean TBS for the six porcine analogues showed a uniform increase over time (Figure 4.5). Values increased from a mean TBS of 3 in the day-0 fresh stage to a TBS of 10 during the bloat stage sampled at day-6. As decomposition progressed, pig carcasses observed in the beginning of active decay at day-9 had a mean TBS of 14 and continued to advance through the later active decay stage for the last sampling period at day-12 with a mean TBS of 21.

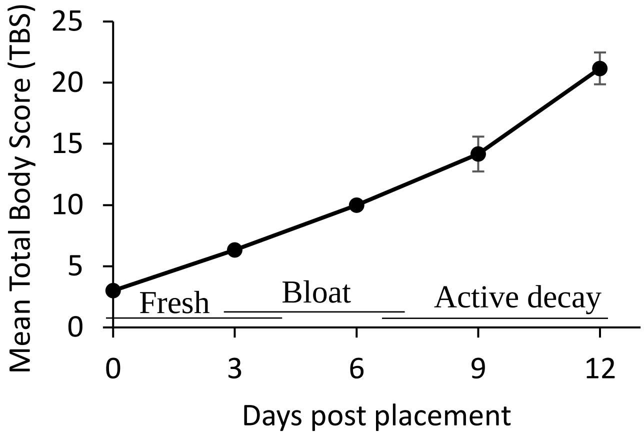

Figure 4.5. Mean TBS total body score (±SE) as per Megyesi *et al*. (2005) for six adult porcine analogues left to decompose outdoors for twelve summer days in Fredericton, New Brunswick, Canada.

c. Stable isotope data analysis

Data for both skin and muscle tissue were analyzed for stable isotopes of carbon and nitrogen contained in replicated samples ($\delta^{13}C_{skin}$, $\delta^{13}C_{muscle}$, $\delta^{15}N_{skin}$, and $\delta^{15}N_{muscle}$). Progression of decomposition, as measured by time in days, was a significant factor only in the model of $\delta^{15}N_{muscle}$ over time (*p<0.001*) and not significant for $\delta^{13}C_{skin}$, $\delta^{13}C_{muscle}$, or $\delta^{15}N_{skin}$.

A series of paired T-tests determined at what points significant increases in tissue stable isotopes were detectable during the decomposition process when compared to fresh day-0 tissues. Results showed no comparative changes for measures of $\delta^{13}C_{skin}$, $\delta^{13}C_{muscle}$, or $\delta^{15}N_{skin}$. The mean $\delta^{15}N_{muscle}$, however, showed significantly higher levels at day-6 (bloat; 4.5‰), and day-9 and day-12 (active decay; 4.7‰ and 4.8‰) when each were compared to day-0 (4.1‰). The difference in mean $\delta^{15}N_{muscle}$ over the study from days 0-12 increased a total of 0.7‰. It is noteworthy that mean $\delta^{15}N_{skin}$ was greater than

$\delta^{15}N_{muscle}$ at the study outset (4.3‰ vs 4.1‰). As decomposition progressed, mean $\delta^{15}N_{muscle}$ was similar to $\delta^{15}N_{skin}$ at day-3 and day-6, and continued to increase whereby $\delta^{15}N_{muscle}$ was higher than $\delta^{15}N_{skin}$ at days 9 and 12. At the end of the experiment, mean $\delta^{15}N_{muscle}$ was ~0.7‰ greater than the mean $\delta^{15}N_{skin}$.

To account for the variability in the starting tissue isotope values between individual porcines, the change in mean isotope values were calculated (Figure 4.7). Mean change in $\delta^{15}N_{muscle}$ became significant at a single point during decomposition when comparing subsequent days 3 and 6, with a mean net increase of ~0.4‰. The largest $\delta^{15}N_{muscle}$ mean change occurred between days 0 and 9 with a net increase of ~0.5‰. Values for mean changes in $\delta^{13}C_{skin}$, $\delta^{13}C_{muscle}$, and $\delta^{15}N_{skin}$ did not reveal any discernable pattern of change over the course of decomposition.

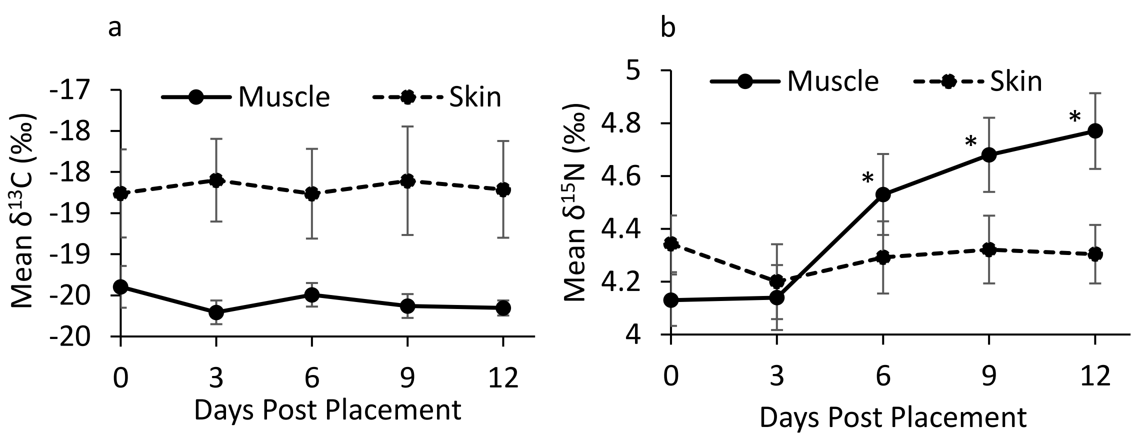

Figure 4.6. Mean (‰±SE) $\delta^{13}C$ (a) and $\delta^{15}N$ (b) of six adult porcine analogues' skin and muscle tissues left to decompose outdoors for twelve days in Fredericton, New Brunswick, Canada. * indicates a significant increase in that sample's $\delta^{15}N$ compared to the day-0 sample.

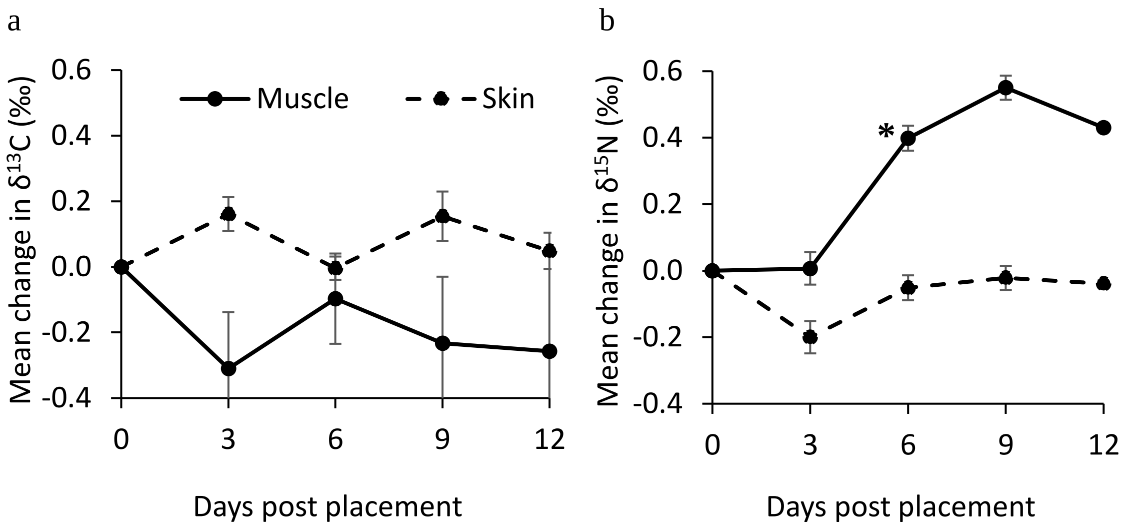

Figure 4.7. Mean change (‰±SE) $\delta^{13}C$ (a) and $\delta^{15}N$ (b) of six adult porcine analogues' skin and muscle tissues left to decompose outdoors for twelve days in Fredericton, New Brunswick, Canada. * Asterisk denotes a significant enrichment in a sampling day's mean change when compared with the previous sampling three days prior (paired T-tests, $p < 0.05$).

4.5 Discussion

The current study sought to determine if decomposing porcine skin and muscle became enriched over time in $\delta^{13}C$ and $\delta^{15}N$ given the use of porcine analogues in Atlantic Canada forensic taphonomy research. Data showed significantly higher measures of both mean $\delta^{15}N_{muscle}$ and mean change in $\delta^{15}N_{muscle}$ by day-6 when porcines were in the bloat phase of decomposition as compared to day-0 fresh tissues. During bloat, there is considerable production and accumulation of putrefactive gases in the tissues from early decomposition stages. Tissues are subjected to both autolytic breakdown from the cell's own digestive enzymes and exogenous breakdown by the body's own gut microflora in a low oxygen environment of the intestines. The combined effects of these intrinsic degradation processes initiating proteolysis, saccharolysis, and lipolysis in an anaerobic environment leads to marked changes in the cellular integrity and nutrient dynamics of the tissues over time as decomposition progresses (Carter *et al*. 2007).

As bloat continues to advance and tissues enter into the active decay stage of decomposition, tissue changes become more macroscopically evident. Tissues swell and begin to alter in appearance with green discolouration presenting due to accumulation of sulfide gases, with eventual red-purple marbling of superficial blood vessels over time (Gill-King 1997). It follows that obvious visual tissue changes would correlate to changes in tissue stable isotopes, most notably in mean $\delta^{15}N_{muscle}$ as fractionation in the tissues intensifies with decompositional biochemical reactions. As protein in the muscle tissues break down, nitrogen shifts between various forms of nitrogenous compounds including nitrous oxide, ammonia, cadaverine and putrescine, increasing isotopic fractionation within the nitrogen pool (Keenan & DeBruyn 2019). Decomposition accelerates, with the greatest mass loss in a carcass occurring in the active decay phase with peak activity of blowfly larvae feeding (Dawson *et al*. 2020). Results show the first detectable enrichment of mean $\delta^{15}N_{muscle}$ on day-6 of the study when the porcines were in full bloat. Soon thereafter by day-9, porcines progressed into active decay when decomposition peaks in terms of tissue breakdown into liquefied component nutrients. Correspondingly, data in the late bloat/early active decay stage of decomposition yielded the highest mean change in $\delta^{15}N_{muscle}$ over the study; however, this change was relatively small at less than 1‰.

Interestingly, the data also showed a trend that we did not expect when looking at both $\delta^{13}C_{muscle}$ and $\delta^{15}N_{muscle}$. Porcine muscle started the study in the fresh stage with higher mean $\delta^{13}C_{muscle}$ values when compared to $\delta^{15}N_{muscle}$. By day-3, carcasses appeared relatively fresh but were decomposing at the cellular level despite no obvious visual effects. This day-3 sampling was the point when $\delta^{15}N_{muscle}$ increased to be roughly equal to that of $\delta^{15}N_{skin}$. After this point, $\delta^{15}N_{muscle}$ continued to become enriched, eventually becoming significantly higher than the corresponding measures for skin as the carcasses entered bloat. The first chapter of this dissertation yielded $\delta^{13}C$ values remaining higher than corresponding $\delta^{15}N$ for both human skin and muscle over the entire 20-day study; no such transition point occurred where $\delta^{15}N_{muscle}$ exceeded $\delta^{15}N_{skin}$ values in human cadavers. My current porcine study results open a further avenue of enquiry as to whether this point at which $\delta^{15}N_{muscle}$ values eventually exceed the $\delta^{13}C_{muscle}$ in muscle is consistent when applied to porcines of various sizes, decomposing in different conditions, or if $\delta^{15}N_{muscle}$ values can be compared to decomposition-resistant tissues such as hair, hooves/ fingernails, or bone. If such a transition point in decomposition is found when comparing a static value of $\delta^{13}C$ or $\delta^{15}N$ in harder tissues, this would provide additional data on the utility of stable isotopes as quantitative method for estimating PMI in both human remains and porcine analogues. Stable isotope ratios are used for provenancing human remains since bone matrix differs between individuals based on where a person consumes water and what they eat (Bartelink & Chesson 2019, Berg *et al*. 2022). Since mature bone has a very slow turnover rate of several decades, levels of $\delta^{15}N$ in post-mortem bone could act as a constant against which the changing levels of decomposing muscle can be compared to further elucidate patterns and trends in PMI.

This study of porcine analogue skin and muscle throughout the early stages of decomposition shows muscle tissue becomes significantly $\delta^{15}N$ enriched in the active decay stage as predicted. However, there are limitations to how data should be applied to other taphonomic contexts. Porcines in this study were observed and sampled during a single season in the late Atlantic Canadian summer without any precipitation, moderate average temperatures with a large fluctuation in the daily high and lows, and subjected to direct sunlight. Differential decomposition was evident with some carcass trunks entering active decay while the head regions had already skeletonized. Prior works have shown that environmental variables such as temperature, sun exposure and seasonality impact decomposition rate and progression (Meyer *et al.* 2013, Sharanowski *et al.* 2008, Shean *et al.* 1993), As such, future studies should expand on preliminary data and explore the impacts of annual variation and seasonality, including winter and spring with freeze-thaw cycles that will impact both porcine and human remains in Atlantic Canadian biomes. Porcine analogue decomposition in different environments can also be explored including areas of shade, insect and scavenger exclusion with reduced oxygen availability (such as in sealed containers), and interments in water and soil environments. Reduced insect and scavenging will delay decomposition progress, as will reduced oxygen available for putrefactive processes, and may impact the speed and degree to which muscle tissues increase their levels of δ^{15}N when compared to unrestricted outdoor decomposition. Exploring the enrichments of porcine muscle in different climates and interments can further our understanding of how decomposition in porcine analogues in taphonomic research change with varied conditions. This information is especially important given the few taphonomic research facilities globally that permit human body donors. Providing reliable forensic data from porcine analogues is essential to expend our knowledge in stable isotope ratio changes in decomposition as it relates to the post-mortem interval in real-world death investigations, particularly in the under-studied Atlantic Canada region.

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CHAPTER 5 CONCLUSION

This concluding section will summarize and highlight this dissertation's novel contributions to the discipline, present a brief novelty statement about this work, followed by a section on future research.

5.1 Contributions to forensic taphonomy

a. <u>The need for PMI methodologies</u>

Recovery of human remains initiates an investigation into circumstances surrounding death, key being identity of the decedent and how long they have been deceased. Medico-legal death investigations are significantly hampered when correct identity and PMI are not readily available. While thanatochemistry assays exist, their applicability is restricted to limited time frames of hours after death. Current methods of providing investigators with meaningful PMI ranges that can withstand both scientific and legal scrutiny are, unfortunately, scarce. Quantitative methods with robust and repeated testing in a variety of circumstances and locations are needed to supplement other physical evidence and witness statements to establish accurate and precise PMI.

Issues of experimental design and statistical accuracy have risen with PMI equations using multiple variables to estimate time since death. This dissertation was designed to specifically address this need to calculate PMI ranges in forensic taphonomy with accuracy and simplicity. Stable isotopes in soft body tissues and in the underlying grave soils during decomposition were used to evaluate the potential of stable isotope data as an additional quantitative PMI methodology with forensic application. The presented methodology shows that stable isotopes, currently used for forensic provenancing of human remains in hard tissues, can be adapted and expanded into new applications using decomposing soft tissue and soil stable isotope analysis to estimate PMI.

b. <u>New forensic protocols to sample stable isotopes in tissues and soil</u>

Stable isotopes are routinely used by forensic investigators to identify the deceased. Decomposition-resistant tissues such as teeth, bone, hair, and fingernails are analyzed to provenance human remains because these tissues endure into skeletonization. Data obtained from δ^{2}H and δ^{18}O in hard tissues can pinpoint regions where the decedent grew up, lived, and travelled to identify the individual, but stable isotopes are not applied to PMI estimations. As decomposition progresses, soft tissues are degraded over time and should therefore alter their stable isotope ratios, particularly in muscle δ^{15}N.

My research is the first of its kind using soft decomposing tissues to determine when stable isotopes yield a detectable change in the early PMI. This new quantitative methodology was created using human donor remains and gravesoils in Texas. My methodology is simple, straight-forward, requires limited training, and uses few field sampling supplies. Prolonged freezing and storage of samples is easy when delayed processing is needed or access to a stable isotope analysis facility is limited. Using new methodology, data revealed great potential for stable isotopes in soft tissues and soils correlating to PMI ranges.

Forensic taphonomy needs to expand and improve its limited repertoire of quantitative methodologies accurately estimating PMI used in medico-legal death investigations. Muscle and gravesoil measures of δ^{15}N add to our understanding of taphonomic processes and should be further investigated as a tool to aid other quantitative methods of estimating PMI in forensic case work. This avenue of exploration has a wide range of possibilities regarding future research to pinpoint precise aspects of decomposition in a wide range of bio-geoclimatic regions. Ideally, future forensic pathologists could sample muscle from recovered human remains for $\delta^{15}N$ analysis and estimate how long the remains have been decomposing outdoors. This would provide investigators with a useful PMI range to corroborate other evidence such as cell phone data or security camera footage verifying the time of the decedent's last known activity that would stand up to legal and scientific scrutiny. While this single-sample method to estimate PMI does not yet exist, my results contribute a foundation of reference data that supports further research and development along these lines.

c. First of its kind- stable isotope data from Texas human skin, muscle, and gravesoils

I generated unique baseline data for human skin and muscle decomposition, concurrent with the underlying gravesoils impacted by those cadaver-derived nutrients; $\delta^{15}N$ yielded the most dramatic enrichment over time for decomposing muscle and underlying grave soil. Nitrogen is a major constituent of muscle tissue and is naturally present in the environment so its presence can be measured, and changes monitored over time as tissue nutrients liquefy and permeate into the cadaver decomposition island of soil under the remains. Over extended PMIs of months and years, natural processes in the nitrogen cycle eventually reduce nitrogen levels back to ambient levels as it is cycled through nitrogen fixation, nitrification, and ammonification. However, in the early PMI of a few weeks, my data show significant increases in $\delta^{15}N$ that provide a clear, measurable indication of the autolytic and bacterial putrefaction processes occurring in the decomposing body's bloat and active decay stages.

Following a short lag during soft tissue decomposition, δ^{15}N in skeletal muscle liquifies and flows into the cadaver decomposition island's underlying soil. Grave soil δ^{15}N provides the added benefit of being useful when human remains are present at an outdoor scene, but also in cases where the body has been moved, either due to concealment efforts or by scavenging activity. By comparing nearby "control" soil that has not been infused with liquified human nutrients with samples of grave soil, future investigators may be able to determine PMI both in the short and extended periods associated with recovered remains. If such a methodology is refined based on my current work, this would prove to be a powerful forensic tool to estimate PMI even if the body is no longer present at the original deposition site, regardless of how or why the remains are no longer at that location. Currently, no such methodology is available.

Studies have examined nitrogen input into gravesoil, specifically with ninhydrin-reactive nitrogen (Carter *et al.* 2008, Van Belle *et al.* 2009), but those works report NRN can be used as a presumptive test to detect the presence of grave soil in a fixed PMI range. There is no published data using NRN to quantify possible PMI, and existing NRN gravesoil studies used rat and porcine carcasses; no such human cadaver data currently exist. Assimilating results from Texas human tissue and human gravesoil studies presented in this dissertation support the potential of δ^{15}N as a PMI tool with quantifiable increases in δ^{15}N during bloat and active decay. However, these data are human tissue-specific. The majority of taphonomic research facilities globally do not permit human donor remains in research. Porcine analogues are the standard experimental proxy and should therefore be subjected to the same methodology if any meaningful forensic information is to be gained when making cross-species comparisons.

d. First of its kind- stable isotope data from porcine analogues in Atlantic Canada

Published research examining taphonomic processes in Atlantic Canada are restricted to one porcine carcass study from Nova Scotia and a few in New Brunswick on forensic entomology. My work is the first to examine porcine analogues specifically for human decomposition in New Brunswick, and the first to use stable isotopes as a quantitative measure of taphonomic progress anywhere.

Using the same methodology developed from my research in Texas with human donor bodies, the porcine analogues decomposed in a predictable manner over the course of the experiment. A distinct elevation of muscle δ^{15}N during the bloat and active decay stages correlated with increased lysosomal autolysis, tissue bacteria, and maggot activity. Decomposition increased through the active decay phase and plateaued as tissue nutrients began to decline and could no longer sustain bacterial and maggot metabolic activity as a food source. Results of the New Brunswick porcine analogue study are specifically compared with the Texas human donor body study detailed below in the next section, with interesting parallels despite two different study species in two different biomes.

e. First of its kind- comparisons of stable isotopes in human and porcine analogue muscle tissues

For both human donor bodies sampled in Texas and porcine analogues in New Brunswick, similar trends emerged despite the differences in species and bio-geoclimatic environments (Figure 5.1). In both human donor and porcine carcasses, increased levels of mean δ^{15}N were detectable at roughly the same sampling time (day-5 for humans and day-6 for pigs). While the absolute values of δ^{15}N were different at deployment (9.5‰ vs

4.1‰), both species' decomposing muscle tissues were significantly increased in the same time frame, corresponding to the bloat and active decay stages when putrefactive

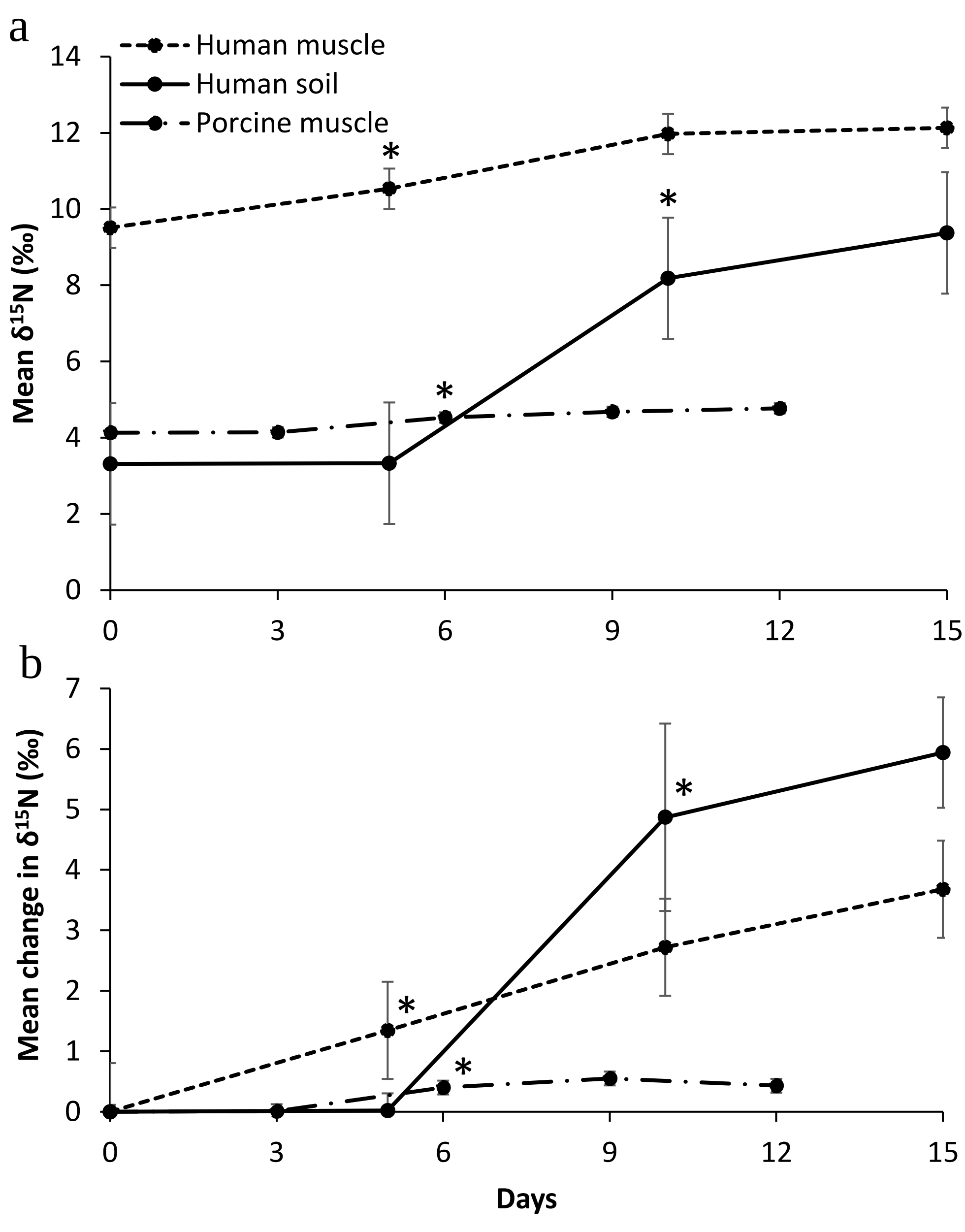

Figure 5.1. Summary graph of human donor muscle, human grave soil, and porcine analogue muscle studies presented in this dissertation showing (a) mean $\delta^{15}N$ (‰) and (b) mean change in $\delta^{15}N$ (‰) over decomposition progression in days. Bars represent standard error of the mean. * denotes the first detection of a significant increase in $\delta^{15}N$ when compared with a sample's corresponding day-0 value ($p<0.05$).

processes exponentially increase. Following this, grave soil under the Texas human body donors also became considerably enriched in $\delta^{15}N$ at sampling day-10 (Figure 5.1a). These mean elevations in $\delta^{15}N$ tell only part of the story, however, as they do not account for the starting value variation for each sample type (human muscle, porcine muscle, grave soil).

A second summary graph using human donor, porcine analogues, and gravesoil data is presented using the mean *change* in $\delta^{15}N$ values over the decomposition process, removing the variability between individuals and examines only the *differences* in the $\delta^{15}N$ over the early PMI (Figure 5.1b). Eliminating the issue of individual variation shows a slightly different but nevertheless compelling result. By examining the mean changes over time, human donor muscle increased by 1.3‰ by day 5-6, while the porcine analogue muscle increased by 0.4‰. Converting these $\delta^{15}N$ increases to a percentage of the mean per mill starting values, human donor muscle increased $\delta^{15}N$ by 13% by day-5, porcine analogue muscle increased 9% by day-6, while gravesoil increased 20% by day-10. When continuing to convert the mean changes over time with the absolute values, human muscle tissues increased in $\delta^{15}N$ by ~10% of the mean absolute value every 5 days when compared to their starting values ay day-0 (23% on day-10, 30% by day-15). Porcine muscle tissue, however, after the sizeable initial enrichment measured at day-6, failed to have any further significant increase in $\delta^{15}N$ over time and plateaued on and after day-9. Porcine analogues are very useful as a human body proxy, but there remain important differences between the decomposition rates and patterns between the two species that require verification with human donor bodies validation studies prior to general forensic application in case work.

Continuing with the second graph illustrating the mean change in δ^{15}N over time, a second interesting phenomenon emerges. At approximately day-7, the mean change in human muscle δ^{15}N is equal to that of the mean change in the gravesoil beneath the remains at ~ 2‰. This crossover may be an important transition point where the amount of δ^{15}N putrefying and escaping the remains during the bloat and active decay stages of decomposition is sufficient to enrich the grave soil to such a degree that they are, for a brief time, equal. This observation raises questions as to whether sampling human muscle tissue concurrent with associated grave soil may, at least to start with, provide investigators an indication whether the remains have been decomposing less than or more than a week, based on comparisons of the mean changes when compared to starting values. While this comparison would be relatively easy to compare gravesoils with nearby control soils, comparing the remains' muscle tissue at discovery with a day-0 sample is impossible. However, ideas for future research may overcome this problem and are worth examining to help refine this methodology.

5.2 Novelty Statement

This dissertation is the first to investigate the forensic utility of changes in stable isotope ratios in soft tissues and underlying grave soil over the course of outdoor decomposition correlated to PMI. Stable isotope ratios are currently used with decomposition-resistant tissues to providence human remains to geographic areas of origin or recent travel, but have not yet been correlated with PMI. My work examines δ^{13}C and δ^{15}N changes in decomposing skin and muscle in humans and their underlying gravesoils in Texas, during the early PMI, followed by a validation study in New Brunswick, Canada using porcine analogues. Mean muscle tissue δ^{15}N showed marked enrichments by days 5-6 in the bloat and active decay stages of decomposition and remained high; elevated gravesoil levels of $\delta^{15}N$ were detected shortly thereafter by day-10. Porcine analogue validation of decomposing skin and muscle tissues in Canada, a vastly different bioclimatic zone than Texas, showed similar elevations in muscle $\delta^{15}N$. These preliminary data provide evidence that stable isotope ratios in soft tissues and cadaver-fluid permeated soil have potential for development into a methodology to objectively quantify PMI for forensic medico-legal investigations.

5.3 Future research

Using the findings of this dissertation as a framework, future studies should expand on stable isotope ratios in forensic taphonomy, with a focus on $\delta^{15}N$ in decomposing muscle and its impact on adjacent grave soil. My dissertation results provide important baseline data that invites expansion and further avenues of exploration for forensic utility of $\delta^{15}N$ in tissues and grave soil with additional focused experimental designs and hypotheses, outlined below.

First, I showed significant results using only nine human donors and six porcine analogue subjects, so validation studies of muscle and grave soil $\delta^{15}N$ are required with increased sample sizes to provide a more statistically robust data set. These repeated studies should be conducted without the use of scavenger exclusion cages to permit natural unencumbered scavenging, an important factor in forensic case work impacting PMI estimates. Second, porcine analogues used in my dissertation were in the 25-35 kg range, and it would be worth repeating that study using larger pig carcasses that weigh closer to adult humans (the mean weight of the donors from Texas was ~ 70 kg). This would remove the mass difference as a decomposition variable, as the smaller pigs in this study decomposed faster than the larger human donors with the pigs showing exposed bony elements in the head and neck region not observed in the human subjects.

Third, in addition to future research incorporating porcine analogues with increased mass, studies should also address the unknown impact of seasonality, particularly in areas like New Brunswick that have very cold winters involving freeze-thaw cycles not encountered in Texas. Freezing does not impact stable isotope ratios over time, but frozen and thawed tissues decompose faster than never-frozen tissues. I hypothesize that an accelerated rate of decomposition may result in similar overall trends of enrichment but with an expedited rate of increase in muscle and gravesoil δ^{15}N compared to tissues decomposing in warmer, never-frozen environments. This will provide much-needed data on decomposition rates and processes in differing biomes, adding to regionally-specific data applications.

Fourth, future research should also consider the impact of clothing on the remains and their effect on decomposition. Recent literature has shown that while most taphonomic studies, whether human or porcine, are conducted on unclothed test subjects, a high proportion of forensic cases involve clothed remains (Appendix C, Spies *et al.* 2020). Clothing can impact the remains by holding moisture, providing shade and shelter for insects, and impacts scavenging- all of which directly impact decomposition rates. Repeating these studies on clothed donors and carcasses would provide a useful and more precise picture of what happens in actual death investigations. These subjects should also be deployed individually at the study site. Current ecological theory suggests that deploying multiple cadavers at once, even if separated by at least 10 m or more, will affect the availability of nutrients entering the local ecosystem and can impact insect and scavenging behaviour and activity (Miles *et al.* 2022). By deploying one test subject at a time, this will more accurately reflect what is seen in forensic case work and further efforts at increasing accuracy with respect to changes in muscle and gravesoil $\delta^{15}N$ changes over the decomposition process.

Fifth, I recommend comparing the $\delta^{15}N$ measured in decomposing muscle tissue with that of the same human donor body's hard tissue. My work shows a trend over time with respect to how the $\delta^{15}N$ ratios change over decomposition, but it is impossible to use a lone sample taken at the time of remains recovery and use that single value to estimate PMI. I am keenly interested in taking not only muscle tissue samples, but also a sample of decomposition-resistant tissue such as hair, fingernails, or even teeth or bone if possible. By having a static value of $\delta^{15}N$ in hard tissue with little to no change over time and comparing it to what I now know about the enrichment of $\delta^{15}N$ in muscle over time, it is vital to determine the relationship between these soft and hard tissues at the time of death. Correlations between the constant hard tissue $\delta^{15}N$ ratio and how the soft tissue $\delta^{15}N$ ratio changes in an individual over the decomposition process will provide much-needed data that, if verified, merits further development of $\delta^{15}N$ ratios as not only markers of decomposition occurrence, but can standardize the methodology to be specific to that individual set of remains given the variability of human $\delta^{15}N$ derived from dietary protein. Quantifying changes in muscle $\delta^{15}N$ in relation to hard tissue constant values gives forensic taphonomists and death investigators additional analysis that furthers our understanding of the complex processes and rates involved with human decomposition.

Sixth, while my study sampled soil from Texas, increases in other grave soil types' $\delta^{15}N$ including clay, loam, sand, and combinations thereof should be quantified.

This relates back to regionally-specific taphonomic data as soil particulate size, organic content, porosity, and microbe populations play a role in initial and changing values of $\delta^{15}N$. As cadaver-derived fluids move into grave soil, permeability, water saturation, and biochemical activity of the soil will impact not only $\delta^{15}N$ levels over time, but also its horizontal and vertical permeation in the cadaver decomposition island around the remains. With the great variation in biomes and soil types found globally, grave soil edaphic properties need their own special focus in individual locations. From a forensic taphonomy lens, results from additional soil types should be interpreted with respect to local laws pertaining to taphonomic research; this is entirely dependent on whether additional study sites permit human donor remains use or if solely porcine analogues are used, given the disparity between muscle tissue values on day-0 for each species. Even in regions where human remains are prohibited from research, porcine analogue studies will provide important data that can then be validated using human remains in other facilities.

Seventh and lastly, it would be very interesting to examine human muscle $\delta^{15}N$ enrichment rates over the course of indoor decomposition. Most forensic taphonomy studies are conducted in outdoor field sites, but a significant volume of forensic case work involves human remains recovered indoors. Human decomposition indoors is markedly different from outdoors as remains found in dwellings, for example, experience a more stable thermal and humidity environment, shelter from direct sunlight and precipitation, and no/little insect activity or scavenging. Elucidating both the indoor and outdoor environments with respect to muscle $\delta^{15}N$ in tandem would be a handy and useful tool to obtain dependable PMI data.

In conclusion, my work found elevated δ^{15}N in human muscle, human gravesoil, and porcine muscle, most notably in the early PMI during the active decay stage. These preliminary results are encouraging and provide proof-of-concept validation for further development. However, several factors and variables need examination before an accurate and rigorous methodology based on muscle δ^{15}N can be presented and used reliably in forensic investigations. In conjunction with other PMI estimation methods currently available, future refinement of the utility of muscle and grave soil δ^{15}N ratio changes over the course of decomposition merits additional study to fill gaps in our understanding of taphonomic agents and in our ability to dependably quantify PMI in recovered human remains with medicolegal ramifications.

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<u>Appendix A</u>-Total Body Score (TBS) = Summation of points for Head & Neck + Trunk + Limbs (Megyesi *et al.* (2005).

<u>I. HEAD AND NECK</u>

A. Fresh

(1 point) 1. Fresh, no discoloration

B. Early decomposition

(2 points) 1. Pink-white appearance with skin slippage and some hair loss.

(3 points) 2. Gray to green discoloration: some flesh is still relatively fresh.

(4 points) 3. Discoloration and/or brownish shades particularly at edges, drying of nose, ears, and lips.

(5 points) 4. Purging of decompositional fluids out of eyes, ears, nose, mouth, some bloating of neck and face may be present.

(6 points) 5. Brown to black discoloration of flesh.

C. Advanced Decomposition

(7 points) 1. Caving in of flesh and tissue of eyes and throat

(8 points) 2. Moist decomposition with bone exposure less than one half of that area being scored.

(9 points) 3. Mummification with bone exposure less than one half of that area being scored.

D. Skeletonization

(10 points) 1. Bone exposure of more than half of the area being scored with greasy substances and decomposition tissue.

(11 points) 2. Bone exposure of more than half the area being scored with desiccated or mummified tissue.

(12 points) 3. Bones largely dry, but retaining some grease.

(13 points) 4. Dry bone.

II. TRUNK

A. Fresh

(1 point) 1. Fresh, no discoloration

B. Early decomposition

(2 points) 1. Pink-white appearance with skin slippage and marbling

(3 points) 2. Gray to green discoloration: some flesh relatively fresh.

(4 points) 3. Bloating with green discoloration and purging of decompositional fluids.

(5 points) 4. Post-bloating following release of abdominal gases, with discoloration changing from green to black.

C. Advanced decomposition

(6 points) 1. Decomposition of tissue producing sagging of flesh; caving in of the abdominal cavity.

(7 points) 2. Moist decomposition with bone exposure less than one half that of the area being scored.

(8 points) 3. Mummification with bone exposure of less than one half that of the area being scored.

D. Skeletonization

(9 points) 1. Bones with decomposed tissue, sometimes with body fluids and grease still present.

(10 points) 2. Bones with desiccated or mummified tissue covering less than one half of the area being scored.

(11 points) 3. Bones largely dry, but retaining some grease.

(12 points) 4. Dry Bone

III. LIMBS

A. Fresh

(1 point) 1. Fresh, no discoloration

B. Early Decomposition

(2 points) 1. Pink-white appearance with skin slippage of hands and/or feet.

(3 points) 2. Gray to green discoloration; marbling; some flesh still relatively fresh.

(4 points) 3. Discoloration and/or brownish shades particularly at edges, drying of fingers, toes, and other projecting extremities.

(5 points) 4. Brown to black discoloration, skin having a leathery appearance.

C. Advanced Decomposition

(6 points) 1. Moist decomposition with bone exposure less than one half that of the area being scored.

(7 points) 2. Mummification with bone exposure of less than one half that of the area being scored.

D. Skeletonization

(8 points) 1. Bone exposure over one half the area being scored, some decomposed tissue and body fluids remaining.

(9 points) 2. Bones largely dry, but retaining some grease.

(10 points) 3. Dry bone.

Appendix B- COMPARATIVE DECOMPOSITION RATES OF PIGLETS BURIED IN MANURE: A PILOT STUDY AND IMPLICATIONS FOR FORENSIC TAPHONOMY.

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Kelly Miles (2019): Comparative decomposition rates of piglets buried in manure: a pilot study and implications for forensic taphonomy, *Canadian Society of Forensic Science Journal*, DOI: 10.1080/00085030.2019.1664972.

ABSTRACT

Comparative decomposition rates of piglet carcasses were obtained when interred in air, soil, horse manure or pig manure to test the belief that pig manure expedites soft tissue decomposition in forensic cases. Data do not support this claim. Results show initial rapid rates of decomposition variables in air, but piglets buried in soil, horse manure and pig manure caught up to the exposed piglets' decomposition rates by the end of the twelve-day experiment. Both piglet tissues and manure have low C/N ratios (<20:1) as opposed to the optimal composting C/N ratio of 30:1 to yield accelerated decomposition. Limitations of this study are addressed with suggestions for future studies with forensic relevance.

INTRODUCTION

Forensic taphonomy studies the decomposition processes of human remains. As such, advanced decomposition suggests a longer interval between the discovery of the remains and the time of death, referred to as the post-mortem interval (PMI). However, the environmental conditions to which a cadaver is exposed can alter the rate of decomposition either by speeding or slowing tissue decay. It is well established that environments with high temperatures and high humidity yield accelerated decomposition rates, while colder and drier environments usually prolong decomposition [1–3].

A key variable directly impacting decomposition rates is the method of interment. Taphonomists acknowledge that exposed cadavers generally decompose fastest, followed by submerged bodies, and then buried remains. This is due to buried bodies having limited oxygen availability for aerobic taphonomic processes, as well as hindering insect and scavenger feeding on the soft tissue remains [3]. Casper's Law states that buried bodies should take roughly eight times as long to decompose to the same degree as a body left on the ground [4], but this long-held general rule is invalid when universally applied across climates and soil types. For example, we now know that acidic soils can result in decomposition that occurs three times faster when compared to alkaline soils [5], and soils with small particle sizes inhibit oxygen flow into and out of the tissues, which directly impacts decomposition rates [6]. Forensically, it is impossible to simply apply Casper's Law to surface remains to get a reliable indicator of decomposition progress with buried remains.

Clandestine burials are sometimes used for body disposal in forensic cases when the perpetrator wishes to hide the remains and prevent discovery. In theory, burying a body will hinder its immediate detection, but will paradoxically slow down the decomposition process, ultimately extending the time in which soft tissues can be recovered. Despite this, there is a widely held belief that burying a body in pig manure will accelerate its decomposition, and this has been relayed to this author by both law enforcement and forensic pathologists. Placing deceased animal carcasses in pig manure piles is a common agricultural disposal practice on farms and is officially termed swine mortality composting [7].

This method of body disposal has also been used in forensic cases for nefarious purposes. Perhaps most famously, convicted Canadian multiple-murderer Robert Pickton murdered and dismembered his estimated 50 victims [8]. The remains were then fed to his pigs or left to decompose in the various manure piles on his family's pig farm and were ultimately skeletonized at discovery. Other forensic cases report human remains recovered from not only pig, but cow and horse manure as well [9–11]. Despite the persistence of this belief that burying remains in pig manure expedites decomposition, there are no published forensic studies on the effect of this method of cadaver disposal. This experiment, therefore, set out to test the null hypothesis that burial in pig manure does not significantly expedite the rate of soft tissue decomposition when compared to other burial media.

METHODS

Piglets

Piglets (*Sus scrofa domesticus*) were obtained from a private pig farmer in Sussex, New Brunswick, Canada. Pigs are the standard experimental proxy where human cadavers are not available or permitted for taphonomic research [12,13]. Piglets were either stillborn or died very recently after birth and had not yet suckled. For uniformity, piglets were selected with an estimated mass in the 1–2 kg range, with actual masses ranging from 0.836–2.122 kg, excluding very small and very large piglets. These piglets were immediately frozen and stored at -20 °C. Piglets were thawed in a 4 °C walk-in refrigerator for 48 hours prior to placement at the field site. Each piglet was randomly assigned an identification number for reference and a toe tag was fastened to the back right leg. Piglet data was recorded for total mass, neck circumference, waist Circumference and total volume. Piglet volume was measured by displacement of water in a 10 L bucket.

Location

The experimental site was in Charter's Settlement, just outside Fredericton, NB on private rural property. The site is a boreal-forested environment with oak, birch, maple, spruce, and pine trees, with groundcover including grasses, mosses and associated leaf litter. The area was approximately 50% shaded and 50% exposed to direct sunlight. Daily temperatures (high and low) were obtained from the Charter's Settlement Environment Canada weather station data.

MATERIALS

Sixteen piglets were divided into groups of four for each treatment for each trial.
Four piglets were assigned to a treatment: control; buried in soil; buried in horse manure; or buried in pig manure. Control piglets were left exposed on the ground surface and not covered with any burial media. The three piglet treatments involving burials each used 10 L of the respective burial material in a bucket. An estimated 3 L was placed on the Ground to provide a 2 cm base layer of the respective burial material in each of four quadrants of a 1 m^2 plot. One piglet was placed in each quadrant of the base layer, with the remaining 7 L placed over the piglet in a uniform 5–6 cm layer to ensure complete

and consistent thickness. Soil treatments used local adjacent soil to the experimental site, fresh horse manure was donated from a nearby horse farm, and fresh pig manure was obtained from the same pig farm that provided the piglet cadavers. Each of the four treatment groups of four piglets was then covered with a wood-framed cage measuring 1 m x 1 m x 0.5 m covered on five sides with 1-inch chicken wire, with the unwired side placed on the ground; this prevented scavenging from affecting tissue loss and confounding decomposition rates. Each treatment group under an exclusion cage was located at least 4 m away from an adjacent treatment cage.

Data collection

Data were collected at three-day intervals from day 0–12, as all piglets regardless of treatment reached the dry remains stage of decomposition by the twelfth day. On each sampling day, one piglet in each trial was uncovered and removed from its respective burial treatment and measures taken for its mass, volume and neck and waist circumferences. To account for the variation in piglet sizes at the beginning of the experiment, these measures were then converted to a percentage difference as compared to that piglet's initial measurements on the day of field placement.

In addition to these measures, piglets were visually scored on their respective sampling days based on the Total Body Score (TBS) system from Megyesi et al. [14]. This system assigns points for a sequence of established visual criteria over the course of decomposition for the head, trunk and limbs separately, which are then summed for a total TBS score ranging from 3 for a completely fresh body to 35 for complete skeletonization. The TBS scores were assigned by two data collectors and averaged for the official TBS data. Once the physical measurements and TBS data were recorded, each piglet was removed from the experiment; each piglet was sampled (and thus disturbed) only once.

This experimental set-up provided one replicate of data, and the entire protocol was repeated for a total of four replicate trials for each treatment conducted in June and July. Each replicate was performed in a fresh area that was not previously subjected to any soil disturbance or manure exposure to prevent possible contamination effects between replicates.

RESULTS

Throughout the experimental trials, environmental temperatures ranged between a high of 25 °C and low of 10 °C, with an overall mean daily temperature of 17.8 °C. Data showed expedited decomposition for exposed piglet cadavers in the early phase of the experiment, but the rates of decomposition of the buried treatments caught up to that of the exposed treatment by the end of each replication. As time progressed and decomposition advanced, mean percentage values for mass, volume, neck and waist circumferences decreased (Figures 1–4). The control piglets (exposed, no burial materials) showed a faster decrease for all four variables measured in the early decomposition period from days 0–6 and sometimes 0–9, however by day 9 and especially day 12, the rate of change for the burial treatments (soil, horse manure, pig manure) generally caught up to the rates of the control piglets. At day 6, percent mass, percent volume, and neck and waist circumferences were statistically lower for the unburied control treatment when compared to other treatments (Figures 1–4).

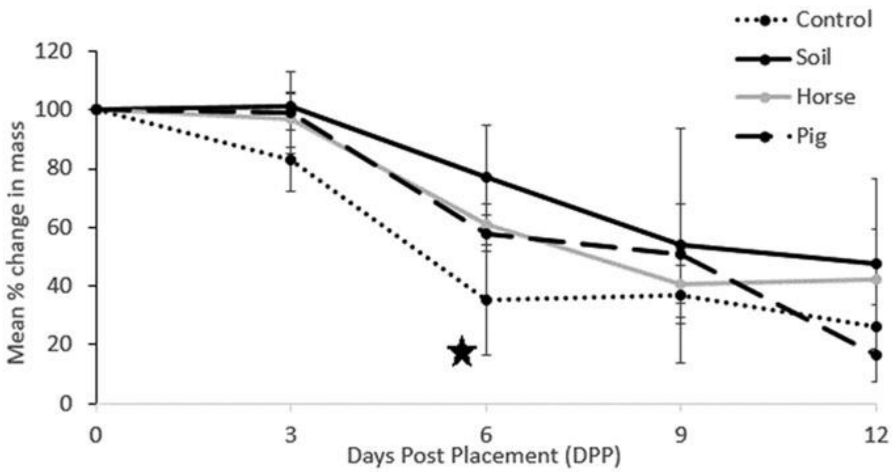

Figure 1. Mean percentage loss (±SE, n=4) of total piglet mass over time measured in days post placement at the field site (DPP) when buried in one of four treatments (control- exposed, buried in soil, buried in horse manure, or buried in pig manure). The black star * indicates sampling days when the control treatment piglets lost significantly more % mass when compared to piglets in other treatments (p<0.05).

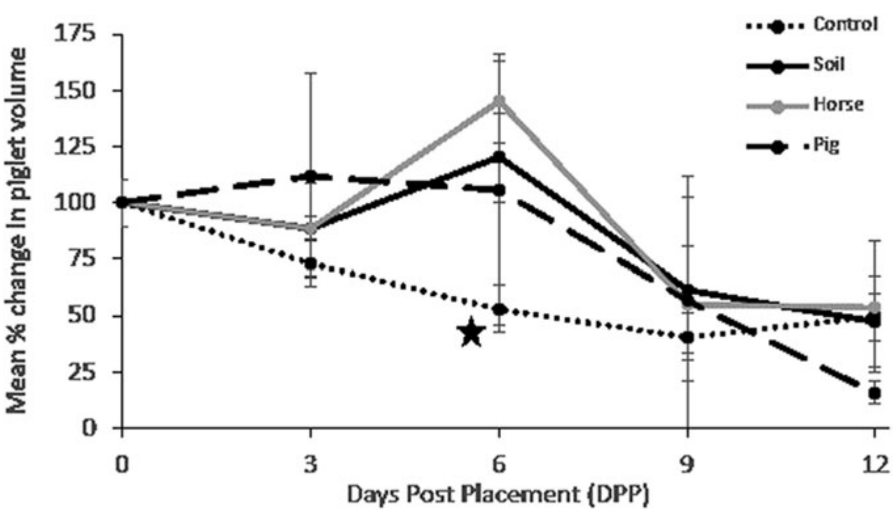

Figure 2. Mean percentage loss (±SE, n=4) of total piglet volume over time measured in days post placement at the field site (DPP) when buried in one of four treatments (control- exposed, buried in soil, buried in horse manure, or buried in pig manure). The black star $indicates sampling days when the control treatment piglets lost significantly more % volume when compared to piglets in other treatments (p<0.05).

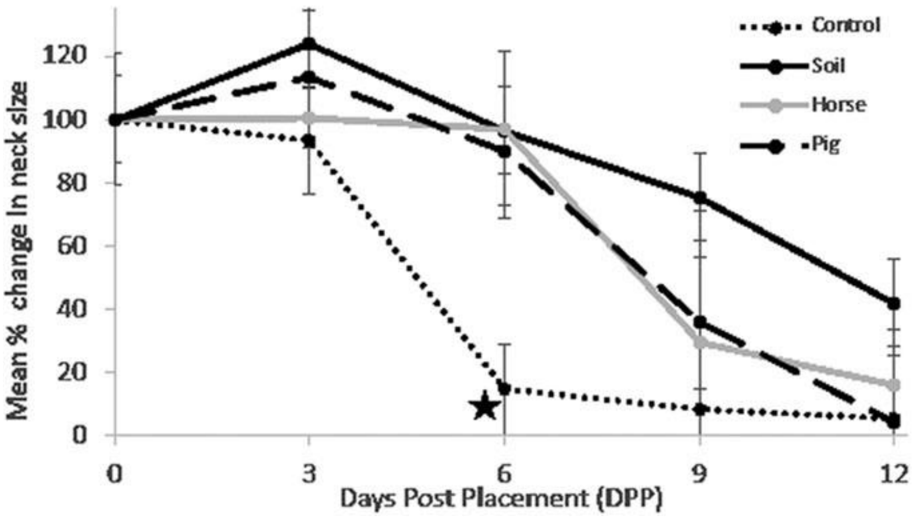

Figure 3. Mean percentage loss (±SE, n=4) of piglet neck circumference over time measured in days post placement at the field site (DPP) when buried in one of four treatments (control, exposed, buried in soil, buried in horse manure, or buried in pig manure). The black star indicates sampling days when the control treatment piglets lost significantly more % neck circumference when compared to piglets in other treatments ($p<0.05$).

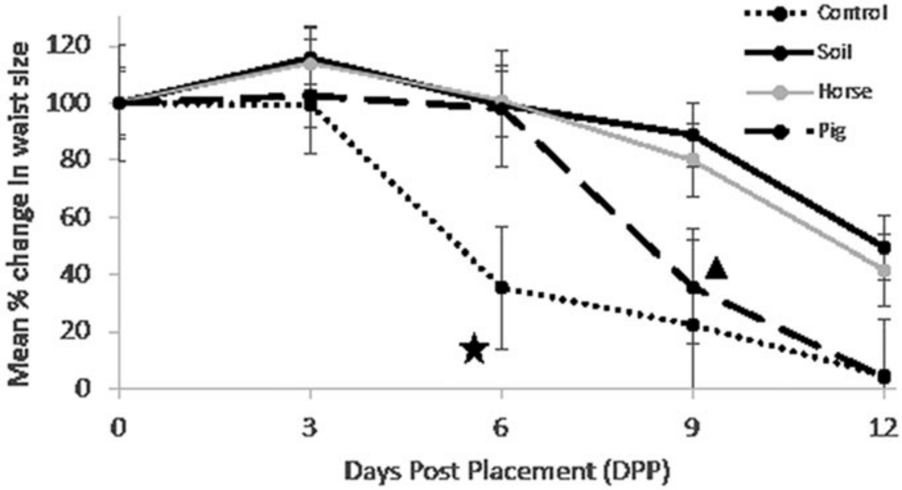

Figure 4. Mean percentage loss (±SE, n=4) of piglet waist circumference over time measured in days post placement at the field site (DPP) when buried in one of four treatments (control, exposed, buried in soil, buried in horse manure, or buried in pig manure). The black star * indicates sampling days when the control treatment lost significantly more % neck circumference when compared to the other treatments ($p<0.05$). The black triangle indicates sampling days when piglets in the pig manure treatment lost significantly more % waist circumference compared to piglets in the horse manure or soil treatments ($p<0.05$).

Focusing on the change in piglet volume over time (Figure 2), data for the horse manure and soil burial media show a net increase as compared to the starting day of the experiment, while in the pig manure treatment, piglet volume slightly increased compared to the start of the experiment. Similar increases in volume were not seen in the control piglets, most likely because the uncovered piglets completed the bloat phase quickly between sampling days 0 and 3 and were not detected on day 3 specifically. Interestingly, there were no significant differences between the pig manure and horse manure burial treatments throughout the experiment, with the sole exception of day 9 for waist circumference (Figure 4).

The soil burial treatment generally showed the slowest losses for mass, volume, and neck and waist circumference, as well as the slowest changes in TBS, but this was not significantly different from the pig or horse manure burials (Figures 1–5). TBS scores generally increased over time, as expected, but no significant differences were found between any of the treatments (including the exposed control) for any of the days sampled (Figure 5).

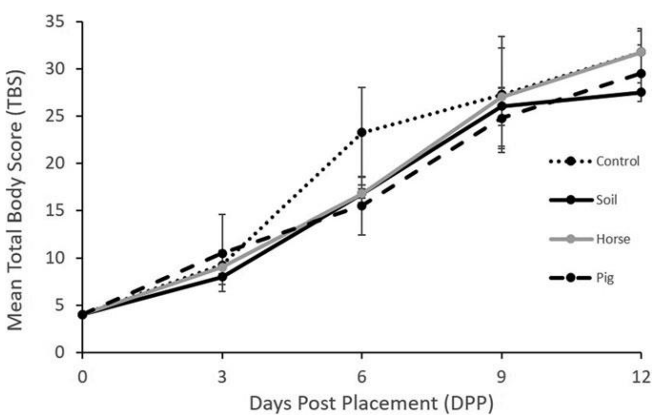

Figure 5. Mean Total Body Score (Megyesi et al. (2005) (±SE, n=4) changes over time measured in days post placement at the field site (DPP) when buried in one of four treatments (control, exposed, buried in soil, buried in horse manure, or buried in pig manure).

DISCUSSION

The purpose of this experiment was to determine if the comparative rates of piglet decomposition are dependent on the interment medium of the surrounding burial material; specifically, did burial in pig manure significantly hasten decomposition as measured by mass, volume, circumferences of the neck and waist, and TBS. Data do not support this theory. In fact, there were no overall differences between the treatments whether they were buried in manure or soil, and only temporary accelerated decomposition was observed with the unburied piglets. Therefore, I accept my null hypothesis that burial in pig manure does not significantly expedite the rate of soft tissue decomposition when compared to other burial media.

Casper's Law was a forensically important rule that states buried bodies should take roughly eight times longer to reach the same state of decomposition as a body left unburied. The data yielded from the piglets in the current experiment did not adhere to this law; control (surface exposed) piglets certainly did not take an eighth of the time to decompose to the same point as any of the buried piglets. One reason for this is Casper's Law does not take into account the nature of various burial media. We now know that characteristics of the medium, including particle size, water saturation, pH and microbial populations influence the rate of decomposition that occurs in a burial environment [6,15]. It has been reported that acidic and alkaline soils have predominantly more fungal microbes, while soils in the neutral pH range of 5.5–7.5 have more bacterial communities present [16]. I suggest that at least part of the reason for pig manure burial's expected accelerated decomposition comes from two ecological assumptions. First, manure from omnivorous pigs has more nitrogen content than manure from cow or horse herbivores

[17]. Second, the addition of nitrogen is thought to enhance the decomposition activity of microbes on organic materials. An influx of nitrogen has been demonstrated to increase microbial biomass as well as microbial activity in soils adjacent to a decomposing cadaver [18,19]. However, decomposition of tissues with a low C/N ratio, such as skin and muscle, are not significantly impacted by overall influxes of nitrogen in their surroundings [18], which was found in the current experiment.

In forensic cases, criminal behaviour related to body disposal relies on getting rid of the remains to avoid arrest. Perpetrators want to hide the remains from visual detection, but also avoid other issues such as foul odours and attraction of scavengers to the remains. However, my data show that simply covering the tissues with pig manure did not have an appreciable effect on the comparative rates of tissue reduction. When considering the common farm practice of composting dead animal carcasses, a more careful examination of the best composting practices reveals that it is not simply a matter of placing the carcasses in a pile of pig manure and letting time and nature do the work. Having an understanding of the carbon and nitrogen of the material being decomposed is key.

Composting experts agree that in order to produce an aerobically produced compost, gardeners must consider the C/N ratio of the composting material. Compost piles with insufficient N will not see an increase in temperature due to the microbial activity that speeds up decomposition. However, compost with an overabundance of N will see the temperature rise to levels that can harm or kill the microbes in the compost pile and cease decomposition altogether [19]. The C/N ratio should ideally be calculated between 25–30:1 for efficient composting, and can be adjusted by the addition of either green vegetation like grass to lower the C/N, or by adding brown plant materials like wood chips, tree bark or dead leaves to increase the C/N. By taking these factors into account, with recommendations that a good compost pile should aim to have 60% moisture, it is easy to see that simply adding pig manure to soft tissues will not result in faster decomposition. In fact, pig carcasses have a low C/N ratio of 14:1, so they necessitate the addition of brown plant materials like sawdust, shredded paper or straw in conjunction with regular turning of the compost pile to re-introduce oxygen to ensure effective decomposition [7,19]. The current experiment illustrates this: simply adding pig manure to swine carcasses will not speed the microbial metabolism of the soft tissues, but will in fact retard decomposition, similar to that of soil burial because it restricts the flow of oxygen over the remains.

The data showed no significant acceleration in the soft tissue decomposition rates of piglets among the treatments, but there are limitations to this experiment that should be addressed. First, this pilot project did not set about to calculate quantitative rates of decomposition but to simply compare the rates of decomposition across treatments. Second, piglets were used as experimental subjects. At the time of writing, there are not yet any forensic science research facilities in Canada that permit donated human remains to be used for taphonomic experimentation, so pig surrogates must be used. Acquiring fully developed pigs of the sizes recommended by forensic researchers (>23 kg, [18]) are restricted by their costs and availability in this region, so newborn, non-suckling piglets were used instead. This was done to obtain comparative results only as a basis for discussion, with future piglet studies having more application to child remains as opposed to adult remains. The piglets were also frozen and then thawed prior to experimentation.

Freeze-thaw cycles are known to impact decomposition not only in the rate of tissue breakdown but also how the tissues are broken down [20]. In this experiment all piglets were frozen at the same temperature and thawed in the same manner for consistency. Future experiments should be designed with modifications of the current research in four ways: using large adult pigs instead of piglets; using pigs that have not been previously frozen; increasing the number of replicates to elevate statistical power; and adding compost treatments containing green and brown vegetation to the existing manure-only treatments. Obtaining manure-only burial data that can be compared with manure and vegetation compost data should provide a clearer picture of how pig manure truly does or does not impact the comparative rates of soft tissue decomposition and if it plays a role in forensic taphonomy with legal implications.

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Appendix C- A REVIEW OF EXPERIMENTAL DESIGN IN FORENSIC TAPHONOMY: MOVING TOWARDS FORENSIC REALISM

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ABSTRACT

Forensic taphonomy as a discipline requires standardization to satisfy Daubert criteria for scientific data to be admissible in court. In response, there has been a shift towards quantification of methodology and estimating the post-mortem interval. Despite these advances, there are still biases and limitations within the discipline not explicitly addressed in the early stages of experimental design nor in final published works. In this article, unresolved debates with respect to the conductance and reporting of forensic taphonomic research are reviewed, beginning with the nature of experimental cadavers, human or animal analogues and their body size, and second, the forensic realism of experimental setups, specifically with respect to caging, clothing and number of carcases. Pigs, albeit imperfect, are a good model to gain a general idea of the trends that may be seen in humans in subsequent validation studies in facilities where human donors are available. To date, there is no consensus among taphonomists on the extent of the effect that body mass has on decomposition progression. More research is required with both human cadavers and non-human analogues that builds on our current knowledge of forensic taphonomy to answer these nagging questions. This will enable the discipline to make the reliable assumption that pigs and donor decomposition data can be applied to homicide cases. A suite of experimental design aspects is suggested to ensure systematic and standardized data collection across different biogeoclimatic circumstances to identify and quantify the effects of potential confounding variables. Such studies in multiple, varied biogeographic circumstances with standardized protocols, equipment and carrion will facilitate independent global validation of patterns. These factors are reviewed to show the need for adjustments in experimental design to ensure relevance and applicability of data within locally realistic forensic situations. The initiation of a global decomposition data network for forensic taphonomists is recommended.

KEY POINTS

- Pigs are a valuable, albeit imperfect, proxy for human decomposition studies.
- There are few or conflicting data on effects of carcase size, carrion ecology, exclusion cages and scavengers.
- We recommend single, clothed, uncaged carcases for baseline research to reflect regionally specific forensic casework.

KEYWORDS
Forensic sciences; forensic taphonomy; decomposition; Daubert standard; exclusion cages; clothed pigs; carrion biomass; scavenging

INTRODUCTION

Forensic taphonomy is the study of human decomposition to determine circumstances and time-of death, which has become a focus of both experimental research and retro-spective analysis of forensic cases for the past 40 years [1]. Early attempts to document and report decompositional changes were limited to qualitative observations in soft tissue changes, but these were subjective and introduced observer variability when multiple people described visual signs of putrefaction. Taphonomy was in its infancy, and there emerged a need to standardize the data to reduce inter-observer variability. This became even more important with the Daubert standard in trial law [2]. The Daubert standard [3] is a legal rule of evidence that governs the acceptance of expert testimony from a witness who is qualified as an expert by knowledge, skill, experience, training or education as long as four criteria are met: the expert's knowledge will help understand the evidence and facts, the testimony is based on sufficient facts or data, the testimony is the product of reliable principles and methods, and the expert in question has reliably applied the principles and methods to the facts of the case to render an expert opinion. As forensic evidence is being used in more trials, Daubert has increased the threshold that experts must meet to provide evidence to the court to eliminate "junk science" having significant legal implications for both prosecutors and defendants. Decomposition evidence is one type of forensic evidence that plays a role in homicide cases, and standardization of decompositional data began to emerge in taphonomic research, in part to satisfy Daubert criteria. Henssge and Madea [4] built upon this further by proposing four criteria for any postmortem interval (PMI) estimation method to gain practical relevance: (1) quantitative measurement of study variables, (2) mathematical description of the method, (3) taking into account influencing factors quantitatively and (4) declaring the precision and proof of precision on independent materials. In response to this, forensic taphonomic research has seen a massive shift towards quantification of methodology and the subsequent PMI estimation methods. Despite the advances this shift has facilitated in our understanding of human decomposition, there are still biases and limitations within the discipline not explicitly addressed in the early stages of experimental design nor in final published works, leading to ongoing contestation of numerous aspects of experimental design. In this article, we present two sections that review unresolved debates with respect to the conductance and reporting of forensic taphonomic research: the nature of experimental cadavers, specifically whether they are human or animal analogues, and the size thereof; and second, the forensic realism of experimental setups, specifically where caging, clothing and number of carcases are concerned. These factors need to be more fully understood and addressed before taphonomic results are reported to ensure relevance and applicability of data within locally realistic forensic situations.

I. The nature of experimental carcases

a. Human versus animal analogues

Following the advent of human taphonomic facilities, donated human bodies swiftly overtook animal analogues as the preferred model for studying decomposition. This is particularly true where the development of models for estimating the PMI in human forensic cases is concerned. The logic is that the results from such studies are more directly applicable to forensic cases than data derived from research on non-human models. This argument remains to be proven and there are several issues with it, most recently summarized by Matuszewski *et al.* [5]. As a start, there are biographic disparities between the donor population for experimentation and the general population to which results are meant to be extrapolated, namely age, body mass and underlying medical conditions that may impact decomposition cycles. Related to this is the inability to control sample biographics of donors leading to a lack of uniformity in the experimental sample, reducing statistical rigour and inferential power. Uneven supply poses logistical problems for actualistic research, primarily generation of a sufficient sample size and necessitating considerable investment in storage capabilities. Perhaps most conspicuous, however, are the legal and ethical challenges which prohibit taphonomic research using donated human remains in most countries. In locations where it is possible, these facilities are strictly bound to tightly controlled and transparent ethical contracts with the donor or the donor's family. It is for these reasons there are few research facilities that can accommodate human cadavers, which is a limiting factor to obtaining forensically applicable data. Globally, there are only 10 anthropological research facilities in four countries (USA, Australia, Canada and the Netherlands) that permit use of donated human remains for taphonomic investigation. Several countries have proposed the creation of similar facilities, including the UK and India, but proponents have struggled to overcome existing legal restrictions and public resistance. Most forensic taphonomists are, thus, constrained by an inability to use human cadavers as research specimens and must instead use animal analogues.

Animal analogues in biomedical research have been used for over a century with mammals as the most frequently used subjects, including mice, rats, cats, dogs, rabbits, and primates. Ethical considerations in medical trials are a principal concern, particularly in surgical and drug trials. It is standard protocol for medical researchers to first use a mammalian model and then proceed to human trials following rigorous safety protocols and peer review. The safety of human patients is of paramount importance. However, animal care guidelines for research must also factor in the guiding principles of the three Rs: reduction, refinement and replacement, such that animal experimentation must be justified, ethically sound and not take place when other methodologies exist that can reliably simulate the animal outcomes [6]. The ethics of using animal models as human analogues in forensic research is the focus of a recent article by Mole and Heyns [6]. The authors summarize the ethical questions of using human research subjects if there are suitable animal model species available, and the use of animal models at all if other alternatives are available. Taking a closer literature survey at peer-reviewed forensic research from 2012 to 2016, 35.3% used rat models and 29.3% used pigs. However, a breakdown of forensic research reveals that taphonomic and PMI studies made up 24.2% of all forensic results, which were almost entirely reported using pigs as human proxies.

Where forensic taphonomic research is concerned, pigs (*Sus scrofa domesticus*) have emerged as a preferred experimental subject over other mammalian models for several reasons. Pigs are significantly easier to use as taphonomic subjects because they are already domestically raised in agricultural farms and harvested for meat by the general public, so their use avoids issues of human ethical concerns as well as avoiding running afoul of local legislation that prohibits the use of humans in non-medical research. The question needs to be addressed as to whether pigs and humans are sufficiently biologically similar to give meaningful decomposition data that can be applied across species, as has been assumed for many years. These are discussed below regarding their appropriateness as biological analogues for decomposition studies based on ecological theory. The topics discussed are the anatomical similarities and differences, validation studies between humans and pigs and carcase size.

Humans and pigs have numerous anatomical similarities of interest to researchers. Both species are monogastric omnivores with distinct small and large intestines and the ability to adapt to varying amounts of dietary plants and meat, unlike rabbit obligate herbivores or cat obligate carnivores. These anatomical similarities are not limited to the digestive systems of pigs and humans. Humans and pigs share integumentary similarities in the amount of hair, or lack thereof, as compared to other hairy or fur-covered mammals. In fact, pig skin has so many anatomical similarities to human skin that they are now routinely used in providing skin grafts for burn patients [7]. Literature shows that pigs and humans also have similar immunological systems and responses to infectious diseases [8–10]. Due to these similarities, pigs have been identified as test subjects for human medical research; they have been extensively used as a tissue source for living human medical advances such as xenotransplantation of heart valves for the past 50 years, bone in dentistry repair and pancreatic islet cells for treatment of type 1 diabetes [11–13]. Yet despite the numerous similarities between human and pig anatomy, there are differences that should be considered when using pigs as proxies for human research for forensic taphonomy studies. Pig and human digestive systems do differ in some respects. In contrast to a human's average intestinal length of 7.5 m, pigs have 23 m of intestine. Humans and pigs also have very different digestive vasculature, lymphoid tissues, coiling patterns within the abdominal cavity and bacterial loads [14]. Since decomposition is highly reflective of autolysis of tissues as well as endogenous bacterial activity from the gut microflora, the potential exists for patterns and rates of decomposition between human and porcine models to differ.

One tissue type that is important during decomposition is adipose tissue. There are similarities between human and porcine deposition of fat tissues in the body for subcutaneous fat around the abdomen, and omental fat around the heart and kidneys [15, 16]. Both humans and pigs have an average body fat of around 20% with variation between individuals likely [15, 17]. Complex triglycerides stored as body fat will eventually break down into glycerol and fatty acid components. However, closer examination of the composition of pig and human fat reveals specific differences at the molecular level. Dietary fats have some influence on human fatty tissues and are mostly triglycerides predominated by unsaturated fatty acids that include oleic, linoleic and laurel acids. Pig fat triglycerides, by comparison, are mostly saturated fatty acids such as stearic acid [18]. While the same groups of fat molecules were present in both species, their relative proportions within the respective adipose tissues are significantly different. Given both similarities and differences between humans and pigs, researchers using pig proxies ideally need to have those data replicated with human cadavers to either support or refute the porcine results to be valid in forensic cases.

b. Taphonomic anatomical validation studies between pigs and humans

Despite the existence of only 10 forensic research facilities that can use human cadavers for decomposition research, the data gleaned from these experiments are of significance, particularly those validating porcine results with human subjects. Interestingly, these results have not shown consistent results with pig decomposition paralleling human data. One study examined differences in decomposing adipose tissues between the two species.

Pig fat not only decomposed faster than human fat, but after 30 days human fat has a mineral content of mostly potassium and sodium, while decomposed pig fat contained more potassium and magnesium [18].

Muscle tissue, in addition to fat, is another tissue of taphonomic importance in humans. Lab studies on small amounts (1.5 g) of skeletal muscle tissue buried in soil were assessed for decomposition differences between humans, cow, lamb and pigs [19]. No single animal analogue's muscle was a reliable surrogate for human muscle, however, lamb, not pig, muscle produced most similar results to human. In a separate study, forensic entomologists found no difference in the growth and developmental rates of blow flies when reared in the lab whether fed human muscle tissue or minced pork [20]. It should be noted that both experiments were conducted in controlled lab conditions, which may not be realistic of whole carrion in the field.

Whole animal proxies dominate forensic taphonomic literature, due to the complexity of biochemical reactions occurring in a decomposing body as a full entity. But again, these studies show mixed results regarding pig versus human decomposition. Forensic entomology has had the greatest success in using pigs as human proxies when sampling and validating the action of forensically important insects. Schoenly and Hall [21] undertook a series of studies in the USA to validate the use of pigs as human analogues for the purposes of increasing scientific rigour for forensic entomology in legal cases. They found that forensically important species of insects colonize human and pig remains similarly with no significant differences found until after skeletonization occurred. They did find differences between size classes of pigs, with greater parallels between humans and pig size of over 50 lbs (23 kg). These results were later confirmed by Schoenly *et al.* [22] when testing four entomological sampling methods with human and pig carcases. They found slight, but not statistically significant, preference of the insects to colonize humans compared to pigs.

A more recent entomological study from Australia used both humans and pigs to compare decomposition rates and odour profiles for training cadaver-detection dogs [23]. They found that pigs decomposed differently and more quickly than humans. They also noted that insect activity on the pigs occurred over the entire carcase resulting in a more consistent mass loss down to bony remains. Insect activity on the human remains, on the other hand, had initial colonization in the orifices of the face and groin. Because of this, as the pigs progressed to more uniformly skeletonized remains, humans displayed mummified front torsos while the back of the torso continued into active decay with more tissue loss. Entomological experimentation not only has the advantage of similarity between pigs and humans with insects present at each cadaver but have indirectly provided information on differences in actual decomposition patterns and rates between the two species as carrion.

In other studies, pigs have been found to be close approximations to human decomposition, but with important differences. Connor *et al.* [14] noted several disparities between the decomposition of both test subjects. Pigs were found to have a more homogenous decomposition as compared to the high variability in the human donors. One reason put forth for this difference was the heterogeneity in human diets as compared to the more uniform diets provided to the pigs at a rearing facility. A second significant difference between pigs and humans was seen during the bloat stage of decomposition. During bloat, as the name suggests, abdominal gasses accumulate during progression of autolytic cellular enzyme activity and gut microbe metabolism. During bloat, 60% of the pig carcases experienced abdominal rupturing due to the increase in abdominal pressure, while none of the human cadavers' abdomens ruptured. The authors hypothesize that the most likely cause of this rupture seen in pigs is due to differences between digestive anatomies. As previously noted, pigs have triple or more of the intestinal length compared to humans. When factoring in length with the highly coiled pattern of the intestines within the abdomen, pigs have an exponentially higher surface area for gut microflora to produce postmortem putrefactive gases to levels of pressure sufficient to split muscle and skin.

Independently of Connor *et al.* [14], the University of Tennessee's forensic taphonomic research group conducted two concurrent sets of experiments with simultaneous deployment of human, pig and rabbit carrion to not only determine decomposition rates between the three species, but to look at the effects of scavenging on different test subjects [24, 25]. They found that rabbits decomposed fastest in the spring, while pigs decomposed fastest in the summer. Humans were more variable in their decomposition patterns and rates and were more likely to mummify, while pigs and rabbits did not mummify during the experiments. Discriminant analysis of decomposition rates found that rabbits formed one cluster, while pigs and humans formed another cluster. This first aspect to the study concluded that while pigs were more similar to humans than rabbits, pigs were still different enough from human decomposition to merit further study [24].

The second aspect of the study examined scavenging, which is a vastly understudied aspect of forensic taphonomy, as it is well understood that scavenging can be a significant contributor to decomposition rates where more than half of tissue mass loss is a direct result of scavenging activity [25]. Data showed not only differences between humans, pigs and rabbits for scavenger type, but this varied by season as well. In Tennessee, raccoons were the dominant scavenger that preferentially grazed on humans over rabbits and pigs, and this effect was most significant in the winter season when food resources are low. Winter scavenging patterns on humans focussed on the limbs, while scavenger activity on pigs was concentrated on the snout and abdomen. This is theorized to be due to differences in human and pig muscle tissue distribution and skin toughness. Interestingly, wildlife ecology studies show raccoons prefer muscle as a protein source in their diets [26] that was seen with scavenging on human cadavers. During the summer season, only some of the human remains were scavenged, while none of the rabbits or pigs were scavenged at all. However, video and photographic data for raccoon scavenging activity on pig and rabbit carcase show raccoons played with the animal remains by poking pig and rabbit carcases and pulled out clumps of rabbit fur. Only when the human remains had no tissue remaining did the raccoons then select the pig and rabbits as food. This difference in preferential scavenging on human remains over proxy species raises interesting questions that require further investigation.

The resounding conclusion from the studies of Connor *et al.* [14], Dautartas *et al.* [24] and Steadman *et al.* [25] was that the rates and processes of decomposition do, indeed, differ between pigs and humans, with that of humans being more variant. Thus, only humans may be used to directly model human decay. However, this did not diminish the value of animal models in taphonomic research, which, as the authors of these studies explain, lies in their ability to facilitate the establishment of baseline data on decomposition and/or ecological processes where none previously exist and human bodies are not available for use in this type of research [14, 24–28]. These seemingly conclusive settlements to the debate have, however, recently been shown to be flawed. In their recent rebuttal, Matuszewski *et al.* [5] point out that the Connor *et al.* [14] and Dautartas *et al.* [24] studies failed to adequately account for confounding variables, notably the influence of variant carcase size, close inter-carcase distances which may have led to cross contamination of attendant necrophagous insect communities, and inter-annual effects (specifically in Connor *et al.* [14]). Furthermore, they highlight that both studies incorrectly applied the TBS scale for quantifying decomposition progression and did so without any supporting measure (e.g. periodic percentage weight loss). Moreover, both studies conducted inappropriate statistical analyses, with Connor *et al.* [14] selecting inadequate models, not reporting 95% confidence intervals and not allowing pig and human carcases to progress to the same extent of decay, while Dautartas *et al.* [24] committed an error of temporal pseudo-replication (see Michaud *et al.* [29] for detailed explanation of pseudo-replication and other common statistical processing errors).

The aforementioned illustrates the current difficulties associated with quantifiably accounting for sources of variance and error as previously emphasized by Henssge and Madea [4] and Matuszewski *et al.* [5] suggest that it is not possible to recommend a universal analogue for human cadavers in decomposition studies yet. Moreover, they emphasize that abandoning non-human analogues is not currently viable due to the myriad of challenges and restrictions currently limiting use of donated human cadavers for experimental taphonomic research and stress the need for further research to address these shortcomings.

Are pigs a perfect analogue in forensic taphonomy? Perhaps not. There are many unknowns with respect to the differences between what is evidenced by actual forensic cases as compared to donor cadavers or pig analogues. However, we do not believe we should throw out the baby with the bathwater, so to speak, and stop using pigs as human proxies. Preliminary research on pigs demonstrably provides a good way of getting a general idea of the trends that might be seen in humans in subsequent validation studies in facilities where human donors are available.

c. Carcase size

The use of myriad species as animal models gives rise to another pressing question: does the size of the carcase(es) used influence the rate and/or pattern of decay? This is not a new problem, but as Matuszewski *et al.*'s [5] analysis highlights, is as-yet unresolved. The first research objectively investigating this question, albeit for the purposes of assessing possible differences in carrion insect communities, was that of Kneidel [30]. Through comparisons of his own work with previous taphonomic research, he highlighted that carcases greatly disparate in size decompose at different rates and with different patterns. They also appeared to host different invertebrate assemblages. Subsequent work by Hewadikaram and Goff [31] indicated that minor variation in carcase size (e.g. ±10 kg) did not significantly influence the rate of decay or the colonizing invertebrate populations. A trend emerged for using carcases of 23 kg of weight [32, 33] to model human decomposition, but this was soon contested [34]. The reason was highlighted by Kneidel [30] almost 15 years prior: a larger carcase takes longer to decompose than smaller ones. This is due to more biomass and the surface area-to-volume ratio (and, thus, the area available to decomposers for consumption) changes with increased body size. Thus, a 23 kg carcase is not going to decompose at the same rate or with the same pattern as a 60 kg carcase, in any environment. This has been proven in numerous contemporary studies and is an essential consideration when designing and interpreting results of taphonomic research [5, 35–38]. Interestingly, it seems this is irrelevant when there is an absence of insect decomposers [34], which rarely occurs in reality. In the absence of a standard for forensic taphonomic research, Matuszewski *et al*. [5] recommend that carcases larger than 30 kg should be used. The question will, however, remain unanswered until more variables are quantified and considered to disentangle the confounding effect of carcase mass (i.e. size).

To date there is no consensus among taphonomists on the extent that body mass has on decomposition progression. More research is required with both human cadavers and non-human analogues that builds on our current knowledge of forensic taphonomy to answer these nagging questions so the discipline can reliably make the assumptions that pigs and donor decomposition data can be applied to homicide cases. If not directly applicable, it might be possible to use existing experimental data and adjust it by the use of regionally specific correction factors, thus, increasing the usability of existing data to individual regions. In the worst-case scenario, currently published donor data may be refuted when tested against taphonomic subjects that more closely represent the homicide victims seen in a given locale and new avenues of experimental subjects are investigated. The ultimate goal of forensic taphonomy research is to replicate, using the best available methodologies, what is found in forensic casework to yield accurate and precise date for death investigations. The next section of this article outlines what those current practices are, and whether a fine-tuning of existing protocols can improve our decomposition results.

II. Forensic realism

a. To cage or not to cage?

Differences in decomposition patterns of humans and pigs are not limited to the endogenous activity of the carcase's own tissues or the mass/size thereof. The majority of decompositional experiments, whether human or mammal proxy, use exclusion cages. These are typically metal frames of sufficient size to cover the body with wire mesh on five sides to exclude scavengers from accessing the tissues, while at the same time permitting natural access to sunlight, wind, precipitation and small insects. The motivation for this varies and is linked to each study's objectives. Studies seeking to determine the effects of specific variables on the decay process need to control for as many variables as possible, and excluding scavenging is the easiest way to control for it [39–52]. Scavenging is not a uniform occurrence in decomposition scenarios, so some researchers exclude it when establishing baseline data. In such studies, it is often not possible to catalogue all variables within the decomposition ecosystem and determine their interactions. It may be prudent to control for scavenging as a confounder of findings for the preliminary research. The results of such studies cannot, however, be extrapolated to forensic scenarios unless it can be proven that the remains in question were not scavenged. This is often difficult to do, especially where small scavengers are involved, and their artefacts are not obvious. The same applies to remains that are skeletonized and there is no evidence of scavenging on the bone (even if there may have been on the soft tissue).

However, recent research is suggesting that natural scavenging by a variety of animals is considerably underappreciated as an occurrence in decomposition scenarios and is a significant factor accelerating the rates of carrion decomposition [26, 53–58]. As such, the question needs to be asked whether decomposition experiments that use exclusion cages do, in fact, relate to natural decomposition found in forensic cases that are subject to scavenging activity. This matter was not expressly addressed in the literature until Spies et al. [56] published a pilot study demonstrating that scavenging by the Cape grey mongoose (*Galerella pulverulenta*)— a small mammal not previously considered a significant scavenger—could accelerate the rate of decomposition of uncaged carcases by sixfold compared to a caged carcase in the same habitat. The Steadman *et al.* [25] study, for the first time, provided solid evidence that PMI estimates are significantly impacted when using previously established models where human cadavers are concerned— human PMI estimates are overestimated and pig PMI estimates are underestimated when compared to estimates calculated from studies where scavengers were excluded. It should be noted that terrestrial vertebrate scavenging activity is not restricted to raccoons and mongooses; the literature reports such species as coyotes, bears, opossum, turkey vultures, domesticated dogs, red fox and carrion crows, among others, having taphonomic impacts on human and proxy remains [54–63]. This list is expected to grow as human settlements encroach more and more onto natural habitats and animals become more synanthropic.

It is, thus, important to interpret the data generated in studies where scavengers are excluded through a biased lens, with acknowledgement that the rates and/or patterns of decay observed may be altered by small and/or large vertebrate scavenging. Where caging is implemented but not explicitly necessary, it is prudent to permit scavenger access in follow-up studies aimed at building upon baseline data, as several authors have recommended [46, 64–66]. Alternatively, researchers planning future research of the decomposition ecosystem could work the potential effects of scavenging into their research designs. This may be accomplished by adapting data collection techniques to maintain successful data collection in spite of the disturbance effect of scavenging or leaving at least one carcase uncaged to determine if there are any small vertebrate scavengers in the ecosystem that may need to be studied in greater detail. Baseline studies of indigent fauna pre-carrion deposition may also illuminate the potential for scavenging and help researchers plan accordingly. In doing so, greater strides will be made towards the collective goal of much of the forensic taphonomic and forensic entomological literature: an improvement of our understanding of the decomposition ecosystem with a view to applying the results in forensic scenarios to aid with case resolution.

b. To clothe or not to clothe?

Clothing can impact the rate of decomposition, depending on the materials, number of layers and the degree of coverage of the clothing worn. For example, we know that natural fabrics decompose more quickly on a body than synthetic materials [67] and natural fabrics are shown to accelerate adipocere formation on bodies in aqueous environments [68]. However, findings on the importance of clothing are inconsistent.

Kelly et al. [45] reported that clothed and wrapped pig carcases in South Africa decomposed differently than unclothed and unwrapped carcases, particularly from an entomology perspective. Fabric-clothed pigs were observed to have eggs laid on them at the same time as unclothed pigs, but clothed pigs had larger maggot masses under the clothing likely due to the fabric retaining moisture and offering protection from direct sunlight. Similar results have been reported by Cahoon [69] and Miller [70] in Tennessee, and later by Voss *et al.* [71] in Australia. Matuszewski *et al.*[38], however, examined body mass in conjunction with clothing in Poland and how they interact to impact decomposition and reported clothing to be a minor factor impacting soft tissue loss to the advanced decay stage. Card *et al.* [72] went further, concluding from their research that the presence of clothing had no practical impact on decomposition. Interestingly, there has even been work done on how biochemical aspects of decomposition impact the breakdown of cadaver-associated textiles as an initial step to using crime scene clothing as a possible source of PMI data [73].

Few studies have examined the impact of clothing on scavenging (which, by extension, would affect decomposition). Carson *et al.* [74] anecdotally found that clothing presented no barrier where bear scavengers were concerned. Conversely, Cahoon [68] makes anecdotal mention of the fact that clothing inhibited animal (raccoon) activity where it covered the body, with Young *et al.* [58] reporting similar findings with foxes. However, none of these studies had attempted to quantify the effect of clothing as a barrier to scavengers. Spies *et al.* [57] sought to address this shortcoming by comparing decomposition of clothed and unclothed carcases in the same environment and season, with quantification of scavenger activity benchmarked against carcase mass loss over time. They demonstrated that the progression of decomposition was closely associated with scavenging by Cape grey mongoose and discovered that these scavengers had a clear preference for unclothed carcases. This suggests that studies that seek to describe the effects of scavenging on decomposition but do not involve clothed carcases may be inaccurate. Accordingly, they recommended that previous studies investigating scavenging activity be interpreted with greater caution, and study designs re-evaluated for future research to include clothed remains. The rationale behind this recommendation is reinforced by the fact that most forensic cases recovered are in a clothed state.

c. One or many carcases?

One of the recommendations of Spies *et al*.'s [57] study was that since mongoose exhibited a preference for unclothed over clothed pig carcases, further research on the local scavenger behaviour was required with a single clothed carrion option—more representative of real-world forensic scenarios—to validate this pattern. Further impetus for this research is that carrion ecology investigations have demonstrated that multi-carcase deployments increase carrion biomass above baseline levels, with as-yet unquantified influences on the decomposition ecosystem [75]. Accordingly, a follow-up study by du Toit *et al*. [76] deployed a single clothed pig carcase in the same habitat and equivalent season to the Spies *et al.* [57] study, with quantification of scavenger activity and progression of decomposition (as percentage weight loss over time). The results speak for themselves: the single carcase experienced a 400% increase in scavenging activity and hit 75% mass loss in only 83 days, compared to Spies *et al.*'s [57] multiple clothed carcases that never reached the 75% mass loss milestone, even after 113 days of exposure. A clear change in pattern of scavenging behaviour was noted, with the single carcase experiencing more visits, longer scavenger hours, longer multi-scavenger visit durations and a shorter decomposition cycle. The authors suggested that using a single carcase produced results that were more forensically applicable. However, they recognized the need to repeat the experiment temporally and across varied circumstances to verify the pattern. Moreover, their specific study site lacks baseline data on scavenging occurrence and activity precarrion deposition, meaning the probability and/or extent of scavenger habituation following successive years of carrion deposition cannot be reliably determined. Habituation concerns have been recognized in carrion ecology research [77–79] but have been largely ignored in the forensic taphonomic literature. This is an area requiring urgent attention by the forensic taphonomic research community, given the legal need to quantitatively take influencing factors into account [5], the important role scavengers are increasingly recognized to play in forensic death scenarios, and because of the demonstrable effect altering the amount of carrion biomass may have on scavenging behaviour.

CONCLUSION

Having examined the available literature on the use of animal analogues—pigs in particular—in place of human cadavers, the size of carcases (human or non-human), whether they are caged or clothed, and how many are deployed in each experimental circumstance, it is clear that there are still gaps in our understanding of how these aspects of experimental design influence research outcomes. What elements of experimental design should be planned when investigating forensic taphonomy with forensic implications given what we know? We propose a suite of systematic baseline studies for different biogeoclimatic circumstances aimed at identifying and quantifying the effects of potential confounding variables. Pigs of at least 30 kg should be used to most closely imitate human tissues for first instance (or baseline) research. An initial baseline observation study of regional fauna (vertebrate and invertebrate) should be undertaken to facilitate understanding of how carrion deposition influences the local ecosystem. Where potential scavengers are identified, the subsequent study design should include scavenger monitoring. A single clothed uncaged carcase should then be deployed. Thereafter, multiple clothed carcases may be deployed to determine if the pattern of decomposition changes. Conductance of such studies in multiple, varied biogeographic circumstances with standardized protocols, equipment and carrion will facilitate independent validation of patterns. Human cadaver validation studies may then follow to complete the transition of study findings to forensic usability. This means, though, that we need more forensic taphonomy research facilities globally and researchers should be actively lobbying government and the public to create and fund these much-needed areas of regional specialization to increase our pool of data on human decomposition. Once we have these concrete results, we can begin to tease out the seemingly innumerable factors that impact decomposition to create a more precise, accurate and appropriate system of creating PMI estimates based on decomposition data. As the data for these types of comparisons begin to accumulate and comparisons made between similar geographical areas, it would be advantageous to initiate a global decomposition data network for researchers to share results, collaborate and gain initial information on decompositional patterns that can be modified and refined to have meaningful localized applications on a wide range of individuals, causes of death, interment types and recovery locations and conditions.

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Curriculum Vitae

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Universities Attended (with dates and degrees obtained):

University of New Brunswick, 2024, Doctor of Philosophy, Interdisciplinary Studies

Nova Scotia Community College, 2006, Diploma in Funeral & Allied Health Services

Memorial University, 2004, Master of Science in Aquaculture

Dalhousie University, 1997, Master of Marine Management

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Publications:

Miles, KL, Gibbon VE, Hayden B. Forensic taphonomy and stable isotopes: δ^{13}C and δ^{15}N enrichment patterns in human skin and muscle during outdoor decomposition. *(In prep.)*

Miles, KL, Gibbon VE, Hayden B. Human Cadaver decomposition islands and forensic taphonomy: gravesoil δ^{13}C and δ^{15}N enrichment patterns in short (30 day) and extended (900 day) post-mortem intervals. *(In prep.)*

Miles, KL, Gibbon VE, Hayden B. Forensic taphonomy, pig proxies, and stable isotopes: changes in pig skin and muscle δ^{13}C and δ^{15}N during the early post-mortem interval in Atlantic Canada. *(In prep.)*

Miles KL, Finaughty DA, Gibbon VE. 2020. A review of experimental design in forensic taphonomy- moving toward forensic realism. *Forensic Sci Res* 5(4): 249-259.

Miles KL. 2019. Comparative decomposition rates of piglets buried in manure- a pilot study and implications for forensic taphonomy. *Can J Forensic Sci* 52(4): 174-183.

Conference Presentations:

2023 Atlantic Student Research Journal, Reaching Higher- Sparks of Synergy Keynote Speaker: "The role of stable isotopes in forensic case investigations identification of the deceased and estimating post-mortem interval (PMI)."

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