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Project Title:
A Multi-Modal Method for Determining the Postmortem Interval in Juvenile Remains and Assessing Skeletal Health

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PURPOSE OF THIS STUDY

In forensic casework, understanding all stages of decomposition is an integral part in establishing time-since-death or the postmortem interval (PMI), assisting with victim identification (e.g. exclusion or inclusion of an individual within a missing person's pool) and case resolution. Research has shown that decomposition can be a highly variable process owing to intrinsic factors such as body mass (e.g. height and weight) of the individual (Matuszewski et al., 2014), or extrinsic factors such as how the body was deposited or local environmental factors (e.g. temperature, soil acidity, insect activity) (Mann et al. 1990; Rodriquez and Bass 1985; Galloway 1997; Meygesi et al. 2005; Carter et al., 2007; Haslam and Tibbett 2009; Voss et al., 2011; Meyer et al. 2013). Estimating the PMI is relatively accurate using early decompositional changes to soft tissue, which typically involve the forensic pathologist evaluating stages of rigor mortis, livor mortis and algor mortis. This is not the case, however, with the evaluation of the later stages of soft tissue decomposition and postmortem changes to the skeleton due to taphonomic agents (Grivas and Komar 2008; Beherensmeyer et al., 1978). Therefore, the estimation of the postmortem interval (PMI) is often the most difficult and generally seen as most inaccurate analysis to perform.

Considering the difficulty with estimating the PMI in adult remains and the dearth of data for decomposition and weathering patterns in juvenile remains, establishing the PMI for juvenile remains is problematic. The leading issue lies in the lack of comparative decomposition studies in varied depositional styles (e.g. different coverings such as plastic bags and blankets) and scientific data regarding the effects of decay on bone mineral density (BMD). While decreased BMD is a product of skeletal weathering due to the loss of organic material in the postmortem environment, intentional starvation and or neglect may also result in lower BMD in a juvenile prior to death. We proposed to develop a regional model for soft and hard tissue decay rates applicable to the broader warm temperate climate region of the Southeastern United States using juvenile and fetal pigs (Sus scrofa) as juvenile and infant human analogs.

Differences in decompositional changes between adults and children are related to overall size as evidenced by a greater surface-to-volume ratio (Morton and Lord 2002) and bone density. The smaller size of juvenile remains contributes to faster decomposition (Morton and Lord 2002, 2006). Several studies have addressed differential bone mineral densities (BMD) in the human skeleton and how overall morphology and bone density affects preservation and degradation of specific skeletal material (Dirrigl 2001, Galloway et al. 1997, Klepinger et al. 1986, Wiley et al. 1997). Bone mineral density is one intrinsic factor impacting survivorship of vertebrate remains (Dirrigl 2001). A major research focus has been the issue of survivability of skeletal elements based on differential bone mineral density and the comparison of these values to elements recovered or overall representation of the elements (Dirrigl 2001, Lam et al. 1999). Pickering (2002) was the first to conduct a systematic, element-by-element comparison of baboon and bovid BMD using dual-energy x-ray absorptiometry or DXA.

The accelerated decomposition process, which can reduce a small child to a skeleton in as little as six days, poses many challenges for law enforcement and medico-legal personnel (e.g. locating remains, establishing time-since-death, and determining cause-of-death). Regional studies on bone weathering in the United States are lacking for both adults and children. Such studies would provide much needed data for postmortem interval estimates of decomposed and skeletonized remains, especially in children homicide cases. Quantitative data is currently not available regarding the amount of bone mineral density loss during the postmortem interval. If bone mineral density loss is not significant in the early postmortem interval than one could argue that low values are due to the skeletal health of the child rather than loss during the postmortem interval. This information is critical in determining if malnutrition/starvation, which could be determined from decomposed and skeletonized remains.

Thus, the goals of this study were:

- *To develop a model to estimate the postmortem interval of skeletal decomposition and bone mineral density changes for juvenile remains in common depositional environments that can be applied regionally to the Southeast United States.*

- *Evaluate microscopic agents that cause changes to bone in the postmortem environment (histotaphonomic changes) and its relationship to macroscopic weathering stages*.

PROJECT DESIGN

Materials

Sample

The use of pigs (Sus scrofa) is an accepted proxy for human decomposition research due to factors such as compositional similarities, the body mass of a pig is greater than 5kg, they are a readily available analog, they are inexpensive and they provide a general eutherian mammalian model for bone anatomy and histology (Cunningham et al. 2011, Morton and Lord 2002, 2006, Spicka et al. 2011, Janjua and Rogers 2008). As in humans, bone density varies by sex and age in animals and appears to follow similar patterns to humans increasing with age (Ioannidou 2003). In order to best approximate the decompositional process of juvenile remains, immature domestic pigs were utilized in this study as proxies for human children.

Pigs underwent euthanasia following an approved protocol established by the North Carolina State University Institutional Animal Care and Use Committee (NCSU IACUC). Fresh killed pigs were be double bagged in knotted plastic bags to prevent colonization by resident flies. Within 2 hours of euthanasia, pigs were scanned for bone mineral density and placed in simulated environments (see *Taphonomic Changes- Bone Mineral Density* section below). Juvenile (immature) pigs, 22 to 55 lbs and fetal pigs, 966 grams to 1055 grams, were used in this study, which is equivalent to ages ranging from 7.5 month old infants to 9 year old children. One pig was placed in four scenarios where the remains of children are commonly found, at the beginning of each of the four seasons. This was repeated for two years. The four scenarios in which carcasses were placed are: 1) outdoors on the surface, 2) buried, 3) outdoors wrapped in a blanket, and 4) outdoors inside a plastic bag. The pigs placed on the surface in outdoor settings were positioned inside cages to prevent loss of data by scavengers.

Environmental data was collected daily from the local Lake Wheeler Field Station weather station, located one-quarter mile from the field site (Dabbs 2010). A total sample of 32 pigs were used in this study (4 pigs per season for two years). There was an additional six fetal pigs placed on the surface with no covering as controls.

Field Site

The study site is on the NCSU Lake Wheeler Field Lab, in Raleigh, NC, a 1500 acre education and research facility located approximately 4 miles south of the NCSU campus. This research facility is unique in that it has a weather station on location that is approximately 0.25 miles from the study site. The site allowed for a 20-foot spacing between depositions to alleviate any cross-contamination.

Data Collection Methods

Taphonomic Changes - Evaluating Soft Tissue Decomposition

Decompositional information was recorded using the Meygesi and co-workers (2005) total body score approach. Each body region (head, trunk, and limbs) was scored separately and the total score was calculated. Comparative data was also collected using the Anderson and VanLaerhoven (1996) stages: fresh, bloated, active, advanced, and dry remains. Fly activity was recorded as presence of adults, eggs, or larvae as well as beetle activity. The remains were systematically observed, data recorded, and photographed daily during early decomposition. All data was collated and stored using Google Sites, which will be made available upon acceptance of publication. All data was collected using the Google Form seen in Appendix 3.

Taphonomic Changes - Evaluating Skeletal Weathering

The environmental effects of decomposition and bone degradation were acquired using onsite weather station data, which recorded relative humidity, temperature, soil moisture, soil temperature and precipitation for the different scenarios. The use of weather station data allowed for environmental effects to be recorded and accounted for unique circumstances such as an unusually wet or dry

season/year. A trained graduate student conducted the observations and photographed remains weekly during late decomposition. All pigs were collected after the two-year cycle with the exposure for first-year pigs ranging from fifteen months to two years. Pigs staged during the second year were exposed from three months to one year. The remains were collected to assess bone weathering stages after the first and second years. Behrensmeyer's (1978) work on skeletal decomposition created a framework for building on our understanding of preservation and decay. Cunningham and co-workers (2011) noticed variable weathering patterns on juvenile pig bones in the Southeast United States. Thus, this study used both scoring methods to establish weathering patterns. In addition, four observers with differential abilities with assessing weathering scored all remains to test potential inter-observer error with each scoring system. The data was collected and collated using a Google Form seen in Appendix 3 for standardization purposes.

Taphonomic Changes - Evaluating Bone Mineral Density

The BMD of each pig was acquired using a Hologic® Dual-energy X-ray absorptiometry (DXA) scanner prior to staging. Following retrieval of the pigs, all juveniles were reconstructed and BMD values were calculated to determine the amount of skeletal degradation for each scenario. Rice was used to simulate soft tissue (Agarwal and Grynpas 2009). This was done in order to develop estimates of skeletal survivorship for different skeletal elements and possible confounding effects when assessing pediatric bone health post-deposition. The study attempted to also scan fetal remains post-deposition, but the scanner was unable to detect the remains after deposition.

Taphonomic Changes - Evaluating Diagenesis

Histological thick sections were prepared from a femur from each of the pigs (n=32) used in the study. Preparation of the histological samples followed published methods (Frost, 1958; Maat et al., 2001; Goldschlager et al., 2010). The samples will be embedded in plastic resin to preserve the sample and ensure sample integrity during slide preparation. One-millimeter thick sections will be produced using a *Buehler Isomet 1000* saw with a 15 HC (high concentration) diamond-edged blade. Each thick-

section wafer will be ground to a final thickness of 75-50 µm on a Buehler™ variable-speed grinding unit with a diamond disc. Each thin-section will be mounted on a glass slide with cover slip using SECUREMOUNT mounting media. The following information was recorded on each slide: 1) slide identifier, 2) element name, 3) element side, and 4) anatomical orientation. One thick section per bone will be produced for 32 pigs (32 midshaft femoral thick sections).

Histological sections were evaluated using a standard brightfield light (it produced better results than the recommended polarized light) in order to assess the degree of diagenetic change and the Histological Index (HI) was employed as described by Hedges and Millard (1995). The HI (also referred to as the Oxford Histological Index) assigns a value from 0 to 5 to summarize the degree of diagenetic change to bone. Through the use of the index one can quantify the amount of porosity and histological integrity of the bones for the selected sample sites.

Applying the Accumulated Degree Day Model to Estimate PMI

To statistically predict known insect succession we applied the degree day model presented in Michaud and Moreau (2009, 2011). Four pigs per season were monitored for specific environmental conditions as well as regional weather station data. The degree day model was calculated from decomposition rates, and accumulated degree-days. Specifically the degree day index will be calculated from the environmental data, decomposition stage and degree-day accumulation

$$ADD_{total} = \sum_{t=1}^{n} [\{T_{min} + T_{max})/2] \qquad (1)$$

where T_{min} and T_{max} represent the daily minimum and maximum air temperature and *t* represents time and *n* represents the number of days (Michaud and Moreau 2009, 2011).

Data Analysis Methods

Statistical analyses were performed using a time series analysis that accounts for time between observations that can identify significant changes in quantified observations. The time series analysis utilizes an autoregressive integrated moving average (ARIMA) that incorporates a longitudinal mixed

effects model. All statistical analyses were performed using JMP Pro 12.1. A mixed random coefficients model, which is useful for analyzing repeated measures was used to examine the relationship between the dependent (ADD) and independent variables (TBS, daily temperature, daily precipitation, soil temperature, soil moisture, and deposition). These results were presented at the 2017 American Academy of Forensic Sciences, which are under preparation for publication in the Journal of Forensic Sciences.

A paired t-test was used to test whether BMD changed over time from initial deposition until collection. Surface and buried remains were tested separately. The scanner could not pick up the fetal remains. Thus, only the juveniles were tested. A correlation coefficient was used to determine strength of the relationship between between weathering data and the Oxford histological index using Excel.

Results

The surface juvenile remains showed a significant seasonal pattern in days for decomposition with the summer juvenile reaching a TBS of 26 in eight days (p-value=0.0001), the fall juvenile reaching a TBS of 28 in 11 days (p-value=0.0006), and the winter juvenile reaching a TBS of 27 in 79 days (p-value=0.0090). These TBS values correspond with more than half the remains being skeletonized. The variables analyzed showed significant associations between TBS and ADD for summer, fall, and winter (p-values=0.0023, 0.0030, and 0.0022, respectively). The blanket fetal remains showed significant seasonal changes that mirror those seen in the juvenile remains. The summer fetal remains reached a TBS of 27 in seven days (p-value=0.0001), in the fall they reached a TBS of 29 in 10 days (p-value=0.0004), and in the winter they reached a TBS of 27 in 79 days (p-value=0.0001). The variables analyzed showed significant associations between TBS and ADD for summer, fall, and winter (p-values=0.0023, 0.0300, and 0.0024, respectively). The bagged fetal remains for summer and fall showed a similar decomposition patterns not related to seasonal deposition with the summer bagged fetal remains reaching a TBS of 26 in nine days (p-value=0.0004), and in the fall reaching a TBS of 27 in six days (p-value=0.0001). These results can be viewed in the presentation from the American Academy of Forensic Scientists in 2015 in Appendix 2.

The paired t-test showed a significant difference in BMD in juveniles between initial deposition and final recovered remains for both surface (t statistic = 4.0; df = 7; p-value = 0.005) and buried (t statistic = 5.5; df = 7; p-value = 0.0009) remains. The correlation coefficient showed a weak association between the Oxford histological index and TBS and ADD (0.243 and 0.202, respectively). There was a moderate association between ADD and TBS (0.58), however.

SCHOLARLY PRODUCTS

Publications

Ross, Ann and Amanda Hale. "A Macroscopic and Microscopic Approach to Decomposition of Child-Sized Remains." In production.

Hale, Amanda R. and Ann H. Ross. The Impact of Freezing on Bone Mineral Density: Implications for Forensic Research. Journal of Forensic Sciences.doi: 10.1111/1556-4029.13273.

Presentations

Ross, Ann and Amanda Hale. "Decomposition of Child-Sized Remains in Different Depositions." 69th Annual Meeting of the American Academy of Forensic Sciences, New Orleans, LA. Poster Presentation.

Hale, Amanda and Ann Ross. "An Innovative Look at the Postmortem Interval and its Role in Juvenile Decomposition." 67th Annual Meeting of the American Academy of Forensic Sciences, Orlando, FL. Podium Presentation.

Ross, Ann, Amanda Hale, and Kenda Honeycutt. "Taphonomic Impact of Depositional Environment for Juvenile and Infant Remains." 10th Annual Meeting Forensic Anthropology Society of Europe, Heidelberg, Germany. Podium Presentation.

Hale, Amanda and Ann Ross. "The Impact of Freezing on Bone Mineral Density." 10th Annual Meeting Forensic Anthropology Society of Europe, Heidelberg, Germany. Podium Presentation.

IMPLICATION FOR CRIMINAL JUSTICE POLICY AND PRACTICE

To date, a regional multifactorial standard accounting for the early postmortem period and the later bone weathering stages is lacking for juvenile remains. To our knowledge this is the first attempt to

examine PMI for a two-year period along with seasonal variations using bone mineral and histological data. The development of regionally specific bone weathering standards has tremendous implications for more accurate PMI estimates that could impact case solvability and produce a much more informative assessment of unidentified human remains, particularly juvenile homicides.

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APPENDIX II - SCHOLARLY PRODUCTS PRODUCED

Journal of Forensic Sciences Technical Note

Presentation with ARIMA

https://docs.google.com/a/ncsu.edu/presentation/d/1hBHDv3FkA0OSkE5jcmiMe1cVXloG2sQvZBKy71Y-9tc/edit?usp=sharing

APPENDIX III - GOOGLE FORMS

Decomposition Form

https://docs.google.com/forms/d/e/1FAIpQLScYZWAyJlFkHLeJIgKaEqQwJkXRhRVg65vbnZxSEYyWgeCsdg/viewform?usp=send_form

Weathering Form

https://docs.google.com/a/ncsu.edu/forms/d/e/1FAIpQLSePlKPpOF1hxLIQmtOnPqrnhGx9KNxcd-hWeanpxbarxYye9g/viewform?usp=send_form

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TECHNICAL NOTE

PHYSICAL ANTHROPOLOGY

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The Impact of Freezing on Bone Mineral Density: Implications for Forensic Research*

ABSTRACT: It is common for researchers using animal or human remains for scientific study to freeze samples prior to use. However, effects of freezing on bone macro- or microstructure are relatively unknown. The research objective of this study was to determine whether freezing could potentially bias experimental results by analyzing changes in bone mineral density (BMD) with the freezing of remains over time. Eight fetal pigs were scanned to determine their initial BMD before freezing. Three piglets underwent a freeze-thaw cycle to assess the effects of the freezing process. Four piglets were frozen and scanned weekly for 20 weeks to assess freezing over time. The overall average between the fresh initial scan and final frozen scan was significantly different ($p < 0.001$). Per contra, the final thawed BMD scans did not differ from the initial fresh scan ($p = 0.418$). Thus, completely thawed remains are recommended for experimental studies.

KEYWORDS: forensic science, forensic anthropology, bone mineral density, freezing, experimental studies, longitudinal analysis

Many clinical and research studies in forensic science use animal cadavers (e.g., pigs and rats) as a proxy for human tissue (1–3). For example, many forensic anthropological studies of decomposition (4–6), experimental trauma, and biomechanical analyses (7–9) employ animal cadavers for human analog research purposes. Nonetheless, due to availability and insufficient supplies, animal cadavers, particularly carrion animals, may require periodic freezing in order to preserve test material prior to experimentation or deposition. This is a particularly salient point as most experimental studies within forensic anthropology are performed to assess the effects of modification in medicolegal contexts on fresh bone (1,2,5). Therefore, if freezing significantly alters remains prior to experimental testing, it could impact their validity when applied to cases of medicolegal significance.

Very few studies have focused on the effect of freezing cadavers prior to placing them in experimental contexts (10). Some histological studies have examined changes in the cellular matrix after freezing, with findings suggesting that freezing changes the appearance of the cellular matrix, but did not affect the overall ability to distinguish tissue types (11–14). For decomposition studies, Micozzi (15) found that animal cadavers frozen prior to study began initial decay via aerobic (or outside in) decomposition, rather than anaerobic decomposition or putrefaction (or inside out) as is typical in fresh cadavers. This occurs because the internal structures will take additional time to thaw and begin the decay process. Interestingly, the study also found disarticulation at joints occurred more rapidly in prefrozen cadavers than those freshly deposited. Bone mineral density (BMD) is most commonly assessed in studies investigating the mechanical properties of bone (16). These studies have typically measured BMD on frozen specimens due to the difficulty in harvesting fresh bone (16). However, due to required experimental parameters in forensic decomposition, biomechanics, and trauma studies, this is not a feasible option. Testing the efficacy of scanning frozen or thawed remains, Wähnert et al. (16) found that BMD was significantly higher in frozen human femora than in the thawed specimens. In addition, they found that measured BMD and bone mineral content (BMC) differed depending on the type of bone being measured (i.e., cortical vs. trabecular). The bone shaft, which has greater cortical area, exhibited a significant decrease in measures after thawing, while the trabecular regions of interest (ROI) or Ward area of the femoral neck showed the largest increase in BMD and BMC after thawing (16). Thus, they recommend that experiments measuring BMD from frozen specimens should be performed at constant temperatures on all scans for accurate results. Similarly, Lee and Jasiuk (17) found a significant difference in BMD measures between frozen and fresh remains and that those changes affected Young's modulus but not ultimate strength. Their results indicate that long-term freezing weakens bone and its mechanical properties due to the formation and expansion of ice crystals (17). An additional factor is the temperature at which remains are frozen. Kang et al. (18)efound that decompositional enzymes are still active at temperatures above −20°C and continue to destabilize the organicee matrix. This is an important consideration when storing remainsee for use in taphonomic or diagenetic studies. The paucity of studies investigating effects of freezing on bone is surprising asee BMD is the primary underlying cause of bone diagenesis andee changes in BMD may have an impact on decomposition, trauma,ee and biomechanics (7,19,20). Freezing can slow or inhibit decomposition (21) and can produce cellular damage and degradationee

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of hard tissues. When studies are concerned with microdecomposition of bone, diagenetic analyses are one of the only methods to distinguish natural postmortem processes from human-mediated activities such as funerary practices (5). For example, diagenetic change in stable isotopes illustrates the exchange of minerals with surrounding soils and varies considerably by the type of hard tissue, the extent of the surface exposure, the integrity of the collagen structure, and the actual skeletal element (22,23). The process of diagenesis accounts for much of the differences seen in BMD as this is a common site of calcium mineral substitution with magnesium, barium, and other elements (5). Thus, the measureable diagenetic effect is the change in bone mass, which in turn affects BMD. As histotaphonomic studies are often concerned with the effects of diagenesis, understanding how the freezing of samples for storage affects BMD could impact the application of these techniques.

Experimental trauma studies employing remains frozen in either experimental or natural settings should also be concerned about possible changes in BMD. Many studies have utilized nonhuman models for assessing trauma patterns due to the difficulty in acquiring human remains for destructive purposes (24–26). As referenced above, freezing of specimens for storage purposes prior to testing is problematic in trauma studies due to the unique biomechanics of bone (27). For example, Brown and Cruess (28) found that formation of ice crystals causes the loss of moisture in bone, broadening the tissue, and causing structural damage. In addition, trauma studies that utilize outdoor settings to test both trauma and decomposition effects (29) have reported difficulties in assessing trauma due to freeze-thaw cycles that occur in natural environments. These data suggest that freezing has degradative effects on bone that can disrupt natural properties that need to be assessed for study purposes.

The aim of this study was to examine the impact of freezing on BMD measurements and to test body mass and mineral density loss in frozen remains as a function of time. This study was designed to determine whether freezing is an acceptable method for preservation and storage when conducting experimental studies or measuring BMD.

Materials and Methods

A sample of eight fetal pigs was obtained from the North Carolina State University (NCSU) swine farm; all eight piglets were approximately two to five pounds in weight (average = 2.97 ± 1.10 lbs). The fetal remains received were the result of stillborn births and were collected fresh by the swine facility staff immediately following farrowing. All piglets were gathered from the same treatment group to minimize maternal nutritional effects.

The initial BMD scans and body mass measurements were performed on the same day that the pigs were obtained from the swine facility. Fresh body mass was measured with an Uline® Industrial platform floor scale. The piglets were split between two research components. Three piglets were used to examine BMD changes due to a single freeze-thaw cycle regardless of time frozen. Four of the piglets were used to monitor weekly BMD levels while frozen over 20 weeks. One piglet (FS-2) was placed fresh at an open-air site as a control. However, because this piglet was scavenged, additional scans could not be performed. Following the initial BMD scan, the remaining seven piglets were wrapped in industrial freezer paper to prevent freeze-drying and then placed in a Kenmore® chest freezer (7.2 cu. ft.) at 15°F (−9.4°C). The three piglets (FS-6, FS-7, FS-8) involved in the freeze-thaw component of the research were assessed daily until they reached an internal temperature of 15°F (−9.4°C). This occurred within five days. Once the three piglets were frozen (15°F), a single BMD scan was performed. After scanning, they were then placed at an open-air site to allow them to reach ambient temperature. Three days were required for the piglet's internal temperature to reach average ambient temperature of 77°F (25°C). The remains were then recovered for the final thawed scan. This procedure allowed for the comparison that concurrently freezing and thawing specimens might have on BMD.

For four piglets (FS-1, FS-3, FS-4, and FS-5), each subsequent scan occurred once a week for 20 weeks; between scans, the four pigs were returned to the freezer. The piglets were exposed to ambient temperature for approximately five minutes during the weekly scanning process, which did not allow time for thawing to occur. After 20 weeks, the remains were thawed at a room temperature of 68°F (20°C) for a final thawed scan.

Scanning Protocol

All scans were performed by dual X-ray absorptiometry (DXA) on a Hologic® QDR Discovery 4500W. This system utilizes a constant X-ray source that produces fan-beam dual-energy radiation over a wide range of transmitted intensities. Precision is ensured by quality control, which entails scanning a spine phantom (30). The spine phantom is constructed of hydroxyapatite molded from a cadaver spine (L1–L4), which is encased in epoxy resin to simulate soft tissue. The spine phantom was scanned daily to assess deviations of the measurements.

The Hologic® QDR Discovery 4500W software performs calculations of the differential attenuations of the photon energies and presents data in the form of bone mineral content (BMC) (g) and BMD (g/cm^2) (31). BMC is the measure of hydroxyapatite in grams for the total scan area, while BMD accounts for the cross-sectional area of the scan being analyzed. The Hologic® DXA Apex software version 12.4.3 assesses the predetermined regions to provide a BMC/BMD measurement (32). Each piglet was scanned using a 40-inch table length to achieve a more accurate reading in the smaller specimens. The entire piglet was scanned to assess the global BMD of the whole body. Because of their small size, they occupied one region of interest or ROI within the software and thus, the BMD estimate did not need to be averaged over several regions to obtain the global BMC and BMD, which increased the accuracy of the study as the coefficient of variation has been shown to be 0.5% using one region of interest (32). The DXA is considered the gold standard method for measuring BMD as it is most commonly used in clinical settings to ascertain bone fragility and porosity in living individuals (30,33). In addition, it has been validated by Clasey et al. (34) for whole juvenile pigs when estimating survivorship of skeletal elements.

Statistical Analysis

Paired sample *t*-tests were used to identify any significant changes in BMD between each weekly scan and the initial scan in order to evaluate overall BMD loss and body mass of each piglet and to test for differences between frozen or thawed specimens. Descriptive statistics such as the standard error, standard deviation, and range of all the scans for each pig were derived (Table 1).

TABLE 1—*Body mass, initial BMD, final BMD, BMD change, BMC change, and descriptive statistics for all 20 scans (initial to final frozen) of first four frozen piglets are reported. Standard error, standard deviation, and range were calculated for all 20 scans in JMP 11.0.*

ID	Body Mass (lbs.)*	Initial BMD (g/cm^2)	Final Frozen BMD (g/cm^2)	Final Thawed BMD (g/cm^2)	Total BMD Change (g/cm^2)	Total BMC Change(g)	Standard Error	Standard Deviation	Range
FS-1	5	0.618	0.437	0.654	−0.181	45.78	0.015041	0.067267	0.235
FS-3	4.8	0.604	0.443	0.595	−0.161	43.47	0.016686	0.074624	0.245
FS-4	2.6	0.544	0.371	0.535	−0.173	21.55	0.021222	0.094906	0.377
FS-5	2.3	0.762	0.344	0.561	−0.418	19.11	0.026788	0.119800	0.427

*Body mass is fresh initial.

To test the significance of the dependent variables (e.g., BMD and mass) when associated with time, a longitudinal mixed effects model with time as an influencing factor was employed (24). The longitudinal mixed effects model for y_i of n_i observations introduces time as a fixed effect over the series (β) with an associated slope as shown in equation (1), where x_i represents the vector of known observations for β, Z_i is the vector of observations for the random effects (b_i), and e_i represents the vector of random error terms (35–37):

$$y_i = x_i\beta + Z_i b_i + e_i \quad (1)$$

The time series separates the data by equal time lags giving weight to the association of time and the variable examined. An autoregressive covariance structure was used in this study to decrease the time lags by one per observation as there is one week between all observations. Autoregressive integrated moving average (ARIMA), a longitudinal mixed effects model type, was chosen to test the time series for significance. ARIMA requires input for time lags between observations and covariance structure. These orders are designated: (p) as the autoregressive order, (d) as the differencing order, and (q) as the moving average order (37). A nonseasonal model with p = 1, d = 1, and q = 0 was chosen as there was only one variable for time difference and one time lag between observations. All statistical methods were performed using JMP Pro 11.0 (38).

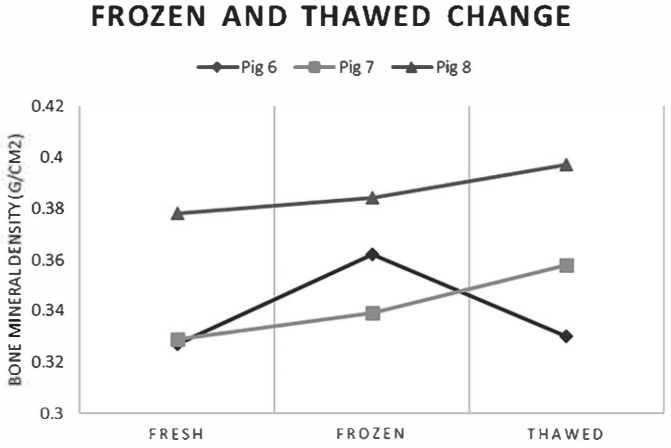

FIG. 1—*Graph illustrating the consistency in BMD from fresh to defrosted for all three piglets.*

TABLE 2—*Statistical results for the mean BMD change in the three piglets from the fresh-frozen, frozen-thawed, and overall fresh-thawed changes.*

Comparison	Mean BMD Difference (g/cm^2)	*t*-Ratio	df	*p*-Value (α = 0.05)
Fresh frozen	0.015	1.731	3	0.226
Frozen thawed	0.018	2.516	3	0.128
Fresh thawed	0.002	0.159	3	0.888

Results

Thawed Analysis

The masses of the three piglets used in phase two of this study are 1.668, 1.758, and 2.659 g, respectively (FS-6, FS-7, and FS-8). Figure 1 illustrates the minimal change observed between fresh, frozen, and thawed conditions. There were minimal body mass associations with no statistically significant relationships (Table 2). However, these remains were only frozen for five days before being thawed, for a total eight-day cycle.

Frozen Analysis

Table 1 reports descriptive statistics including the initial BMD measure, final frozen BMD measure, final thawed, and total BMD change for each piglet from the 20-week freezing protocol. An initial fluctuation of BMD after freezing is illustrated in Fig. 2 over scans one to four. A plateau in BMD is evident between scans five and six as the BMD readings remain relatively low until the final thawed scans where there is a significant BMD increase in all four pigs. Over the 20 weeks, the final frozen BMD scans were significantly different relative to their individual initial scans ($p < 0.001$). Conversely, the final thawed BMD scans did not significantly differ from the initial fresh scans ($p = 0.418$).

The body mass of the frozen remains is also presented in Table 1 and illustrates an apparent negative association with the frozen BMD loss readings. In other words, the results of this study found a greater change in BMD readings as body mass decreased. The relationship between body mass and BMD is unclear. However, this may relate to the crystallization of soft tissues during freezing. As the DXA machines incorporate body mass into the analysis of BMD, this is an interesting observation when considering possible method artifacts when using frozen remains. This association can also be observed in the change of BMC for each piglet. BMC drastically increased over the series of scans even while bone mineral area remained consistent. The association of change in BMD ($p = 0.011$) and BMC ($p = 0.020$) with body mass was also statistically significant suggesting body mass may be an indirect influencing variable on the instrument's ability to scan frozen tissue (21).

A significant change in BMD from the initial fresh scan was first observed in scan six at 34 days. Table 3 shows that the BMD loss between scans five and six increased on average across specimens suggesting this is the interval where freezing

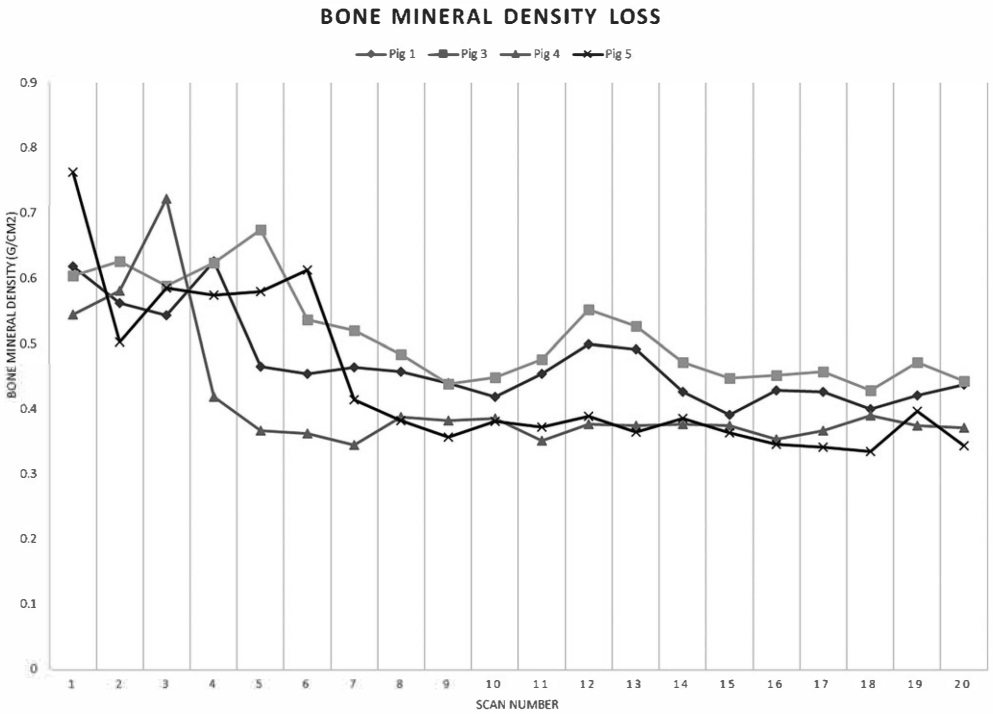

FIG. 2—*Chart illustrating BMD loss over 20 weeks with final thawed scan. Each piglet had a statistically significant difference between the final and initial scans* ($p < 0.001$).

TABLE 3—*Statistical results for the scan difference between the initial scan and scans five, six, twenty, and twenty-one. Scan six was the first to show significant difference in BMD readings.*

Scan Comparison	Days	Mean BMD Difference	*t*-Ratio	df	*p*-Value ($\alpha = 0.05$)
Scans 5-1	27	−0.111	−1.828	3	0.165
Scans 6-1	34	−0.141	−5.592	3	**0.011**
Scans 20-1 (final frozen thawed)	132	−0.233	−3.779	3	**0.033**
Scans 21-1 (final thawed fresh)	139	−0.0958	−0.936	3	0.418

began to impact the BMC/BMD readings. However, for FS-5, there was an increase in BMD between scans five and six, with a significant loss between scans six and seven ($p < 0.001$). This is substantiated by the paired *t*-test results showing significant change for BMD between scans six and one, while on average, no changes for BMD were found between scans five and one. The overall average between scan twenty (final frozen) and one (initial fresh) for all piglets is also reported in Table 3. According to the paired *t*-tests, the change over the entire 132 days was significant when compared to the initial scans for each piglet ($p = 0.033$). However, the final thawed scans did not significantly differ from the initial fresh scans.

The time series analysis detected which scan was significantly different relative to the initial scan. Each piglet was plotted for BMD reading versus days in Fig. 3. All four piglets show the area of statistically significant separation around 30–34 days. However, both FS-1 and FS-3 show a second area of significant change. This may be related to an increased duration of limb retraction or time to thoroughly freeze as these two are twice the mass of piglets four (FS-4) and five (FS-5). In addition, both appear to have plateaued at a higher overall BMD frozen reading than the smaller two. The above results demonstrate that effects of freezing on BMD readings are not evident until around 30 days.

Discussion and Conclusions

The fluctuations in BMD readings between weeks two and six are consistent across all piglets analyzed in the long-term assay. Thus, there is an overall effect on BMD readings throughout the freezing process with the final thawed scans showing a significant increase in BMD. As DXA has been shown to have 99% accuracy when measuring soft tissue composition in clinical settings, these changes may be confounded by the soft tissue changes occurring during the freezing process (39) and confirm findings by Wähnert and co-workers (16) that DXA measurements should be performed on thawed specimens. These fluctuations in X-ray attenuation should also be considered when conducting diagenetic analyses as changes in BMD have been identified as the most influential variable when there are changes in bone mass (5). However, the resulting increase in BMD readings in the final thawed specimens suggests that this may be related to measurement error rather than diagenetic change. In addition, BMD is a measurement of bone mineral content/area and the scanner could be inaccurately including the frozen tissue as bone area while diluting the bone mineral content. The results of this study suggest that freezing may not permanently alter the microstructure of bone; and thus, does not influence the amount of mineral remaining. However, the results do indicate that completely thawed remains should be employed in experimental studies rather than frozen remains to avoid introducing instrument error and to obtain accurate readings.

The cessation of fluctuation in the observations is marked by a significant BMD loss between scans five and six relative to the initial scan with the remaining readings showing a steady

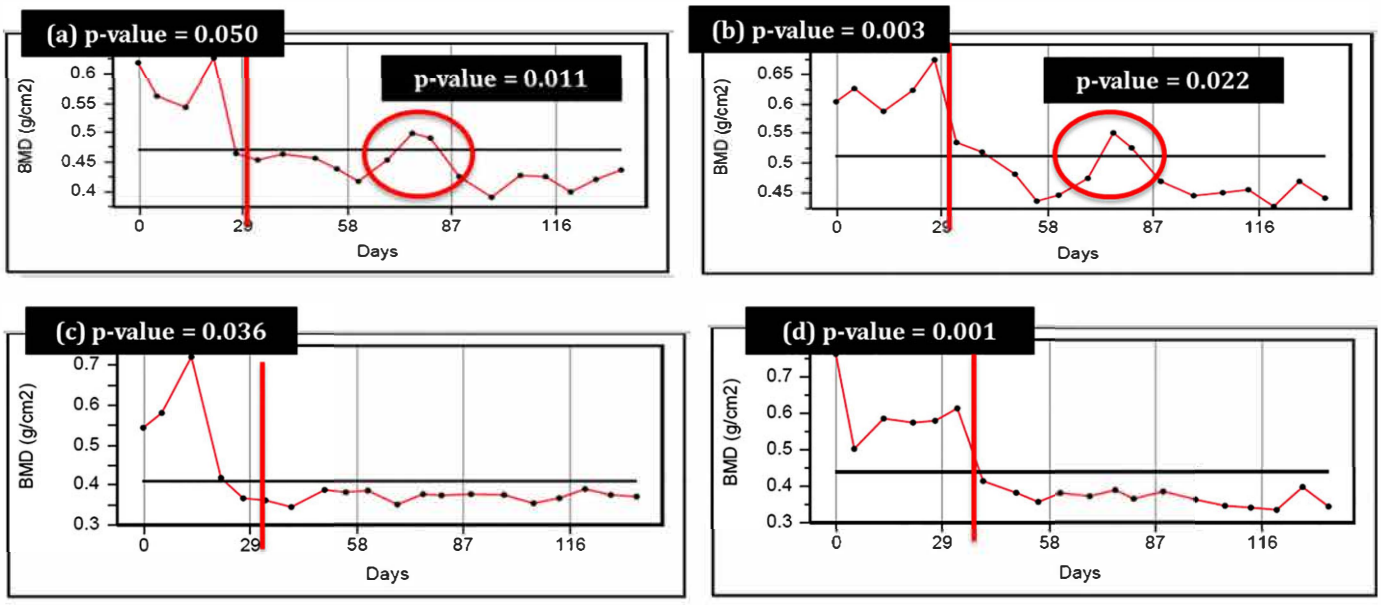

FIG. 3—*ARIMA graphs for each pig: (a) FS-1 shows significant change after days 30–34 with a second significant peak around day 60; (b) FS-3 shows significant change also around day 34 with a second significant peak between days 60–70; (c) FS-4 shows significant change around days 29–30; (d) FS-5 shows significant change at approximately days 34–40. [Color figure can be viewed at wileyonlinelibrary.com].*

decline after an initial increase in pigs three and four. This increase may be due to the interference introduced by the crystallization of soft tissue or related to the reliance on area by the DXA protocol (32). As the remains freeze, they naturally retract and *area* is a dependent factor in the calculation of BMD, which would impact its computation. In addition, this may be related to the increase in BMD seen for FS-6 and FS-7 after thawing because area would increase again. The histological structure and mineralization differences between human and animal remains should also be considered as a factor for experimental studies as this was not examined here and could potentially affect how bone freezes (40). Because BMD is an important variable in the reaction of bone to applied forces, experimental trauma studies should consider how the effects of freezing will affect the trauma patterns observed (41).

This study expounds how error can be introduced if specimens are not properly thawed prior to use in experimental studies. This change does not appear to be significant until days 30–34 for all specimens observed in this study. Interestingly, body mass appears to be a significant factor with larger individuals displaying less apparent loss over time. However, the small sample size does not allow for further interpretation. This may also have more of an impact when measuring adults due to increased magnitude and variability in body mass as BMD in adults is influenced by genetic factors, physical activity, and nutritional habits (32). Fetal remains like those used in this study are primarily subject only to genetic and ontogenetic factors (42). In addition, fetal remains have less cortical bone than adult remains and this may impact the overall study when making extrapolations to the use of freezing in adult bones. Cortical bone is more prone to microcracking than trabecular bone, and this could be influenced by the freezing process (15); thus, fetal bone may show less drastic change between freezing and thawing measurements than adult specimens.

Further research is needed to ascertain how error is introduced after freezing (i.e., the difference due to decreasing area from body retention or does the X-ray attenuation of clinical scanners have difficulty discerning frozen soft tissue from bone). However, based on these results, it is recommended that frozen remains should be completely thawed prior to use in experimental studies involving bone.

Acknowledgment

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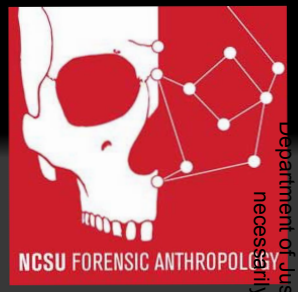

An Innovative Look at the Postmortem Interval and its Role in Juvenile Decomposition

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FORENSICSCIENCES INSTITUTE

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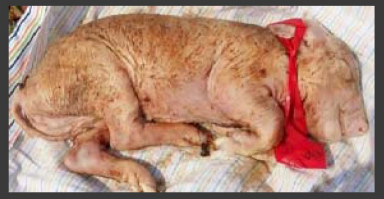
Estimation of the Postmortem Interval (PMI) in Forensic Anthropology

- Emphasis on post-skeletonization
- Variability across environments and depositional contexts
- Multi-disciplinary perspective
- Lack of quantitative taphonomic variables

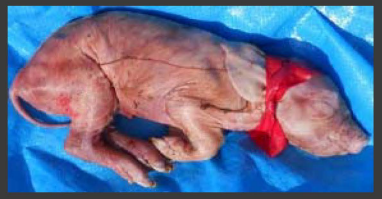
Does size matter?

- Most studies have focused on adult decomposition
- Smaller animals have been used in decomposition research, but they have differences related to hair and skin composition
- Spicka et al. (2011)



4.2 lbs.
1.9 kg

53.4 lbs.
24.2 kg



3.8 lbs.
1.7 kg

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Research Objectives

- Develop PMI model in the Southeastern US for juveniles using innovative statistical analyses
- Evaluate the effects of seasonality on decomposition
- Evaluate differences between depositional contexts and carcass size within the same environment

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Materials

- 8 Juvenile *Sus scrofa* as proxy for human juvenile remains up to 9 years old (35-50 pounds) (Stokes et al. 2013)

- 16 Fetal *Sus scrofa* as proxy for human neonatal remains (4-6 pounds)

NC State University Lake Wheeler Field Site

Field site on 1st day of study

- Deposition Dates ($T_{average}$):
  - Summer 2013/2014 (24.6°C)
  - Fall 2013/2014 (15.0°C)
  - Winter 2013-14/2014-15 (11.6 C)
  - Spring 2014/2015 (23.4°C)
- Weather station located <1 mile from field site to collect (Dabbs 2010):
  - daily temperature
  - daily precipitation
  - relative humidity
  - soil temperature
  - soil moisture

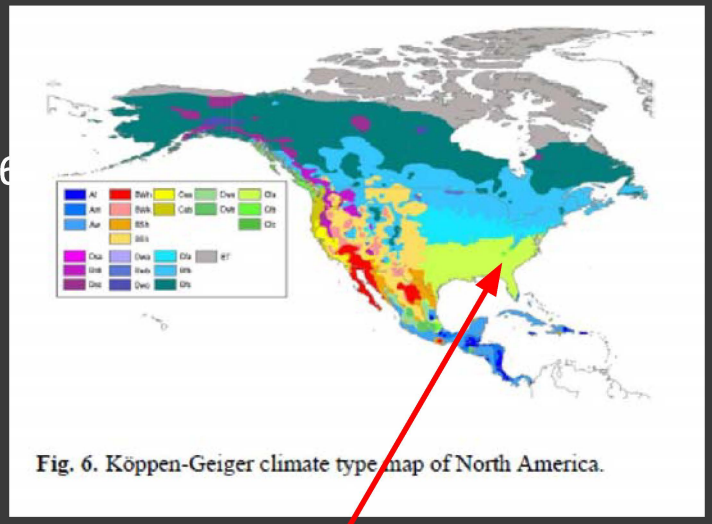


Fig. 6. Köppen-Geiger climate type map of North America.

Cfa = Temperate, without dry season, hot summer (Peel et al. 2007)

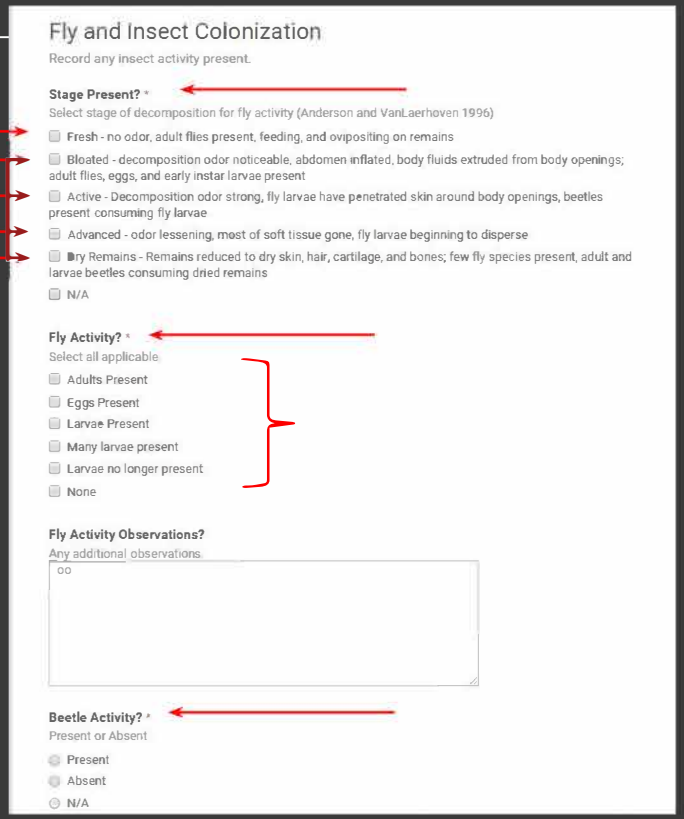
Latitude: 35.72816 Longitude: -78.67981

Data Collection - Decomposition

- Meygesi et al. (2005)
  - Head Decomposition Score
  - Trunk Decomposition Score
  - Limb Decomposition Score
  - Total Body Score
- Additional observations were noted

Data Collection – Comparative and Entomological Data

- Anderson and VanLaerhoven (1996) Stages
  - Fresh
  - Bloated
  - Active
  - Advanced
  - Dry Remains
- Fly Activity – Scored for adults, eggs, and larvae
- Beetle Activity



AutoRegressive Integrated Moving Average (ARIMA) – Time Series Model

- Longitudinal mixed effects model
  - $y_i = X_i\beta + Z_i b_i + e_i;$
- p=1, d=1, q=0;
  - Where *p* is the autoregressive order and *d* is the differencing order, and *q* is the moving averaging order
- ADD Model
  - ADD = m(TBS) + A & L stage* + FlyActivity
- Days Model
  - Days = m(TBS) + A & L stage* + FlyActivity

* Anderson and VanLaerhoven (1996) Stages

JMP Pro 11.0

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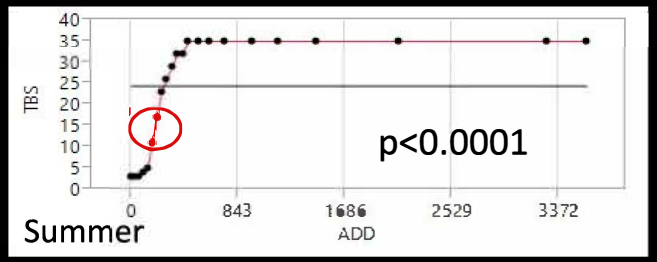
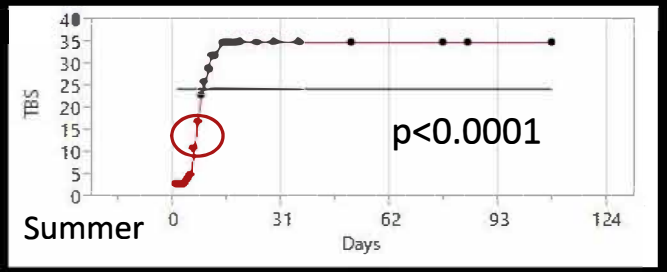
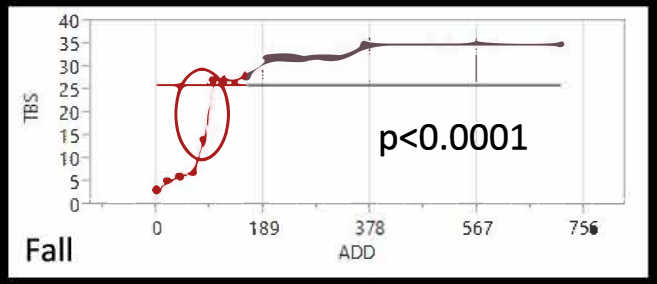
Bagged Remains

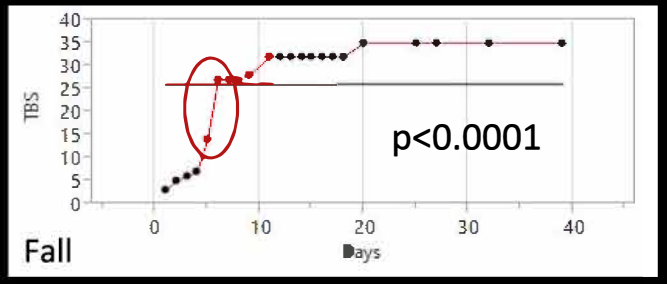


TBS
p=0.0004
A&L Stage*
p=0.2618
Fly Activity
p=0.8876

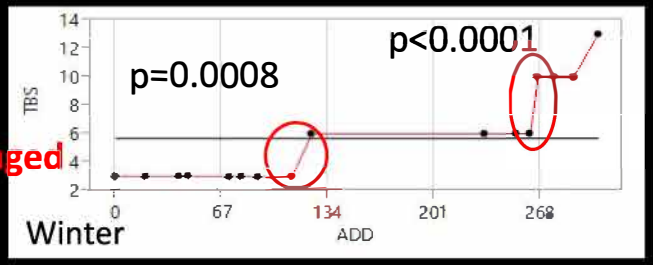




TBS
p=0.1975
A&L Stage*
p=0.6230
Fly Activity
p=0.0231

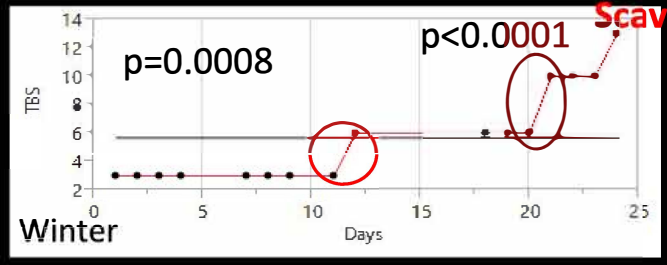


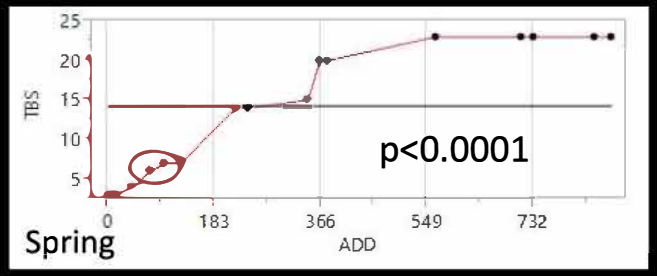
Scavenged



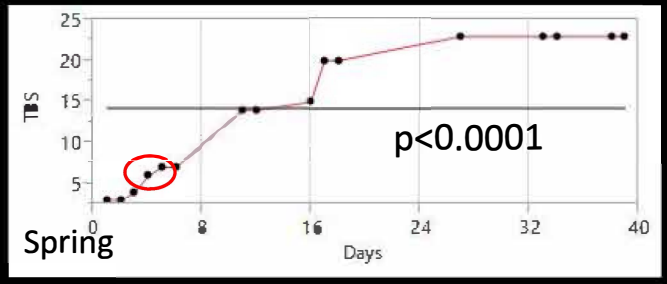
TBS
p=0.2298
A&L Stage*
p=0.3073
Fly Activity
p=0.9292

Scavenged





TBS
p=0.2660
A&L Stage*
p=0.5384
Fly Activity
p=0.7778



*Anderson and VanLaerhoven (1996)

Bagged Remains

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Summer

5/27/2013

TBS = 11

5/28/2013

TBS = 17

Fall

9/17/2013

TBS = 14

9/18/2013

TBS = 26

Winter

12/15/2013

TBS = 3

12/16/2013

TBS = 6

Spring

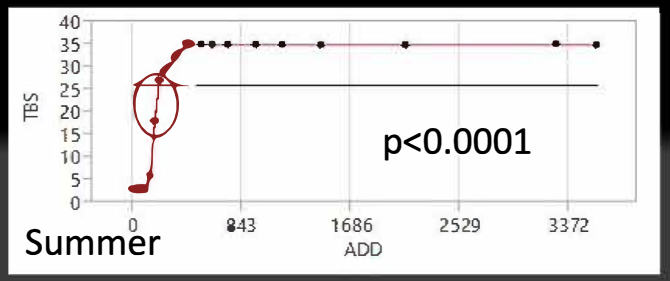
04/03/2013

TBS = 5

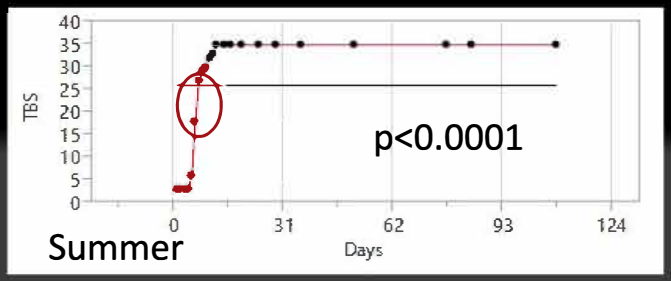
04/04/2013

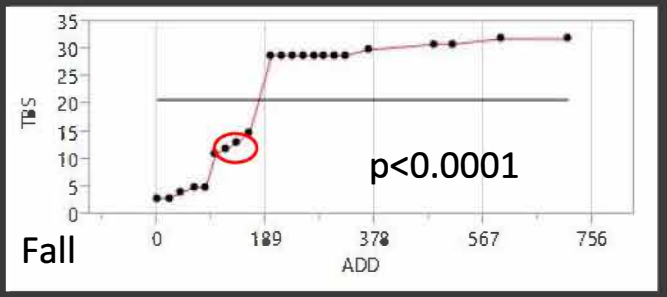
TBS = 7

Blanket Remains

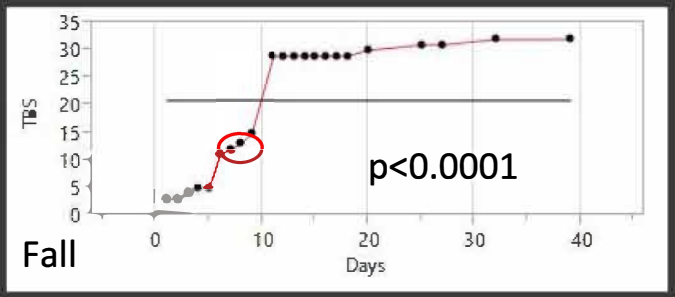


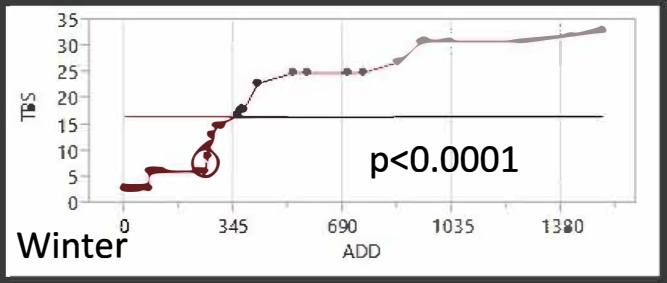
TBS
p=0.0025
A&L Stage*
p=0.1674
Fly Activity
p=0.3106



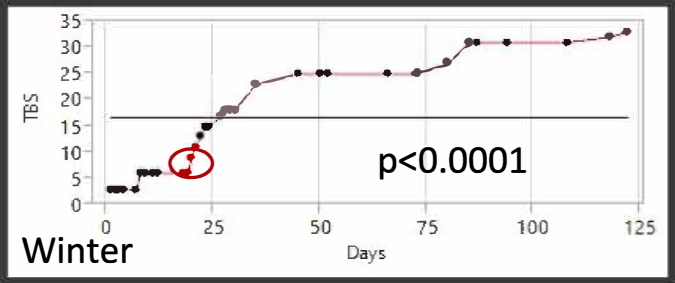


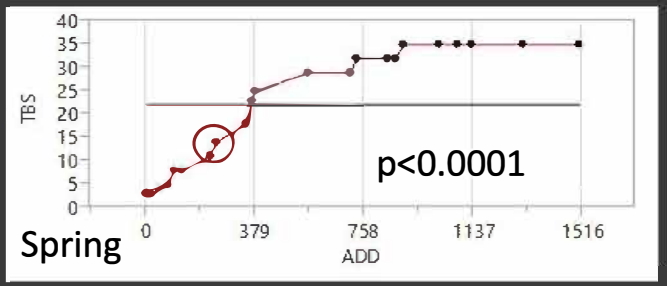
TBS
p=0.9481
A&L Stage*
p=0.7806
Fly Activity
p=0.3695



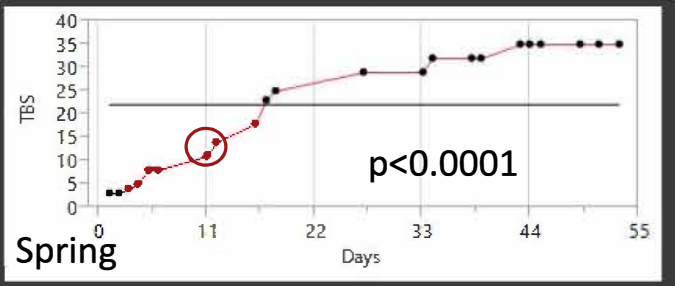


TBS
p=0.5030
A&L Stage*
p=0.4111
Fly Activity
p=0.6616





TBS
p=0.3473
A&L Stage*
p=0.7799
Fly Activity
p=0.4737



*Anderson and VanLaerhoven (1996)

Blanket Remains

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Summer

5/27/2013

TBS = 18

5/28/2013

TBS = 27

Fall

TBS = 12

9/19/2013

TBS = 13

9/20/2013

TBS = 6

12/23/2013

TBS = 9

12/24/2013

Winter

TBS = 11

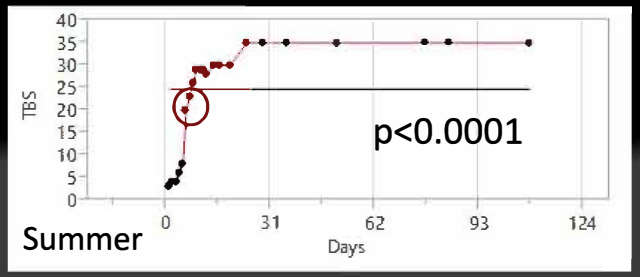
4/09/2014

TBS = 14

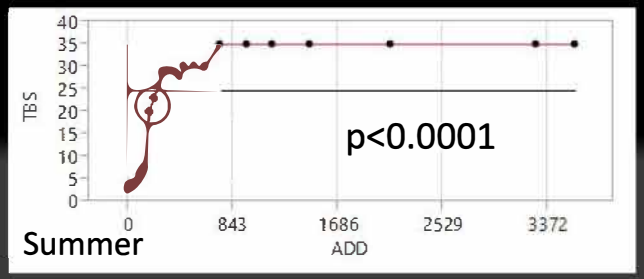
4/10/2014

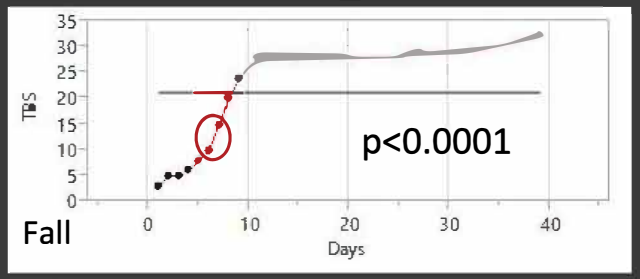
Spring

Surface Remains

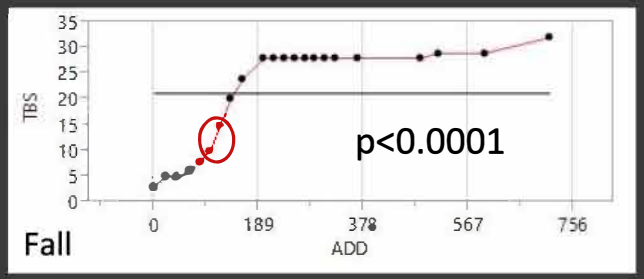


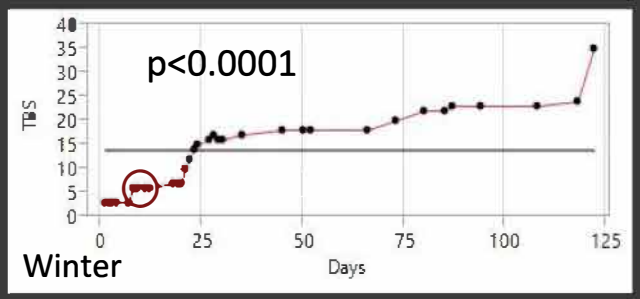
TBS
p=0.3954
A&L Stage*
p=0.8643
Fly Activity
p=0.0046



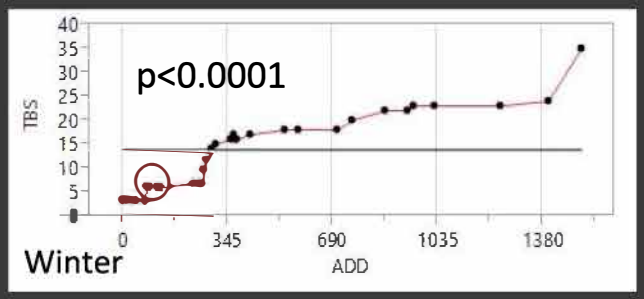


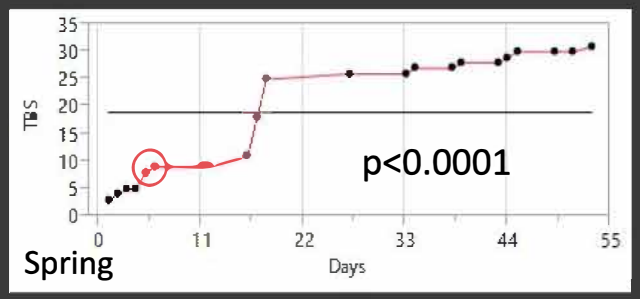
TBS
p=0.0006
A&L Stage*
p=0.3445
Fly Activity
p=0.2386



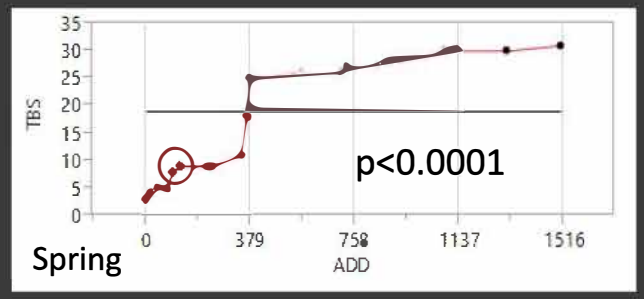


TBS
p=0.5464
A&L Stage*
p=0.6319
Fly Activity
p=0.5364





TBS
p=0.0273
A&L Stage*
p=0.3444
Fly Activity
p=0.1892



*Anderson and VanLaerhoven (1996)

Surface Remains

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Summer

5/27/2013

TBS = 20

5/28/2013

TBS = 23

Fall

TBS = 10

9/18/2013

TBS = 15

9/19/2013

Winter

TBS = 7

12/23/2013

TBS = 10

12/24/2013

Spring

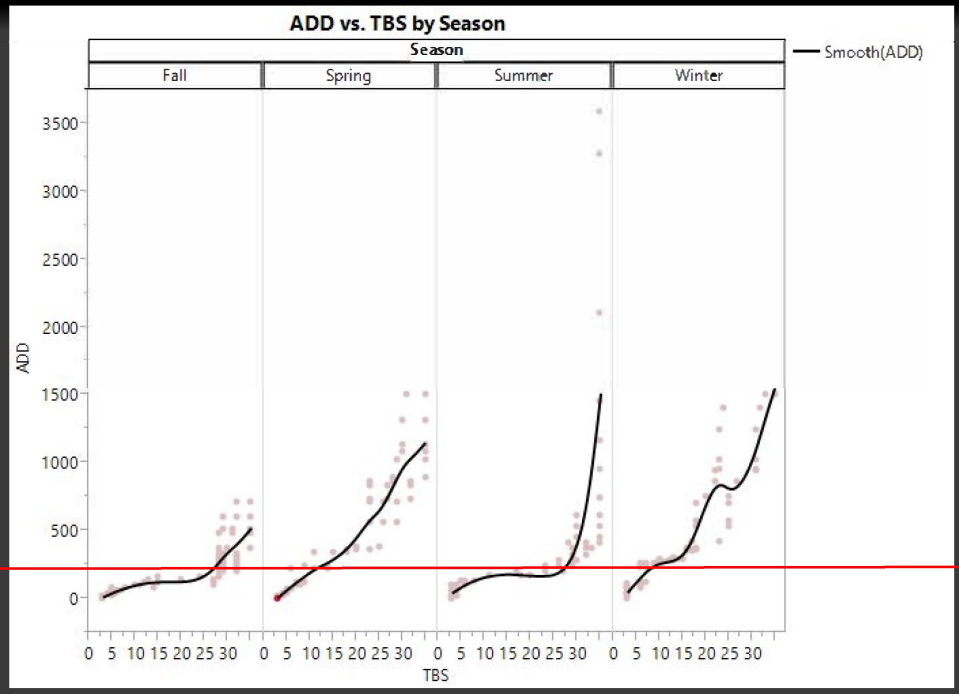
TBS = 6

4/09/2014

TBS = 9

4/10/2014

Season	Bag		Blanket		Surface	
	Day	ADD	Day	ADD	Day	ADD
Summer	7	203.7	7	203.7	7	203.7
Fall	6	101.2	8	138.5	7	119.7
Winter	9/13	111.1/266.7	12	261.9	13	266.7
Spring	6	125.5	8	241.9	8	241.9



Discussion

- Greater resolution of the time series model
- ADD standardized variable can be substituted for time in decomposition analyses (Michaud and Moreau 2011).
- ADD was not always statistically significant with TBS
- Significant areas of change and decompositional stage transitions
- Seasonal differences support Meyer et al. (2013)

Future Considerations

- Addition of a fetal control on the surface
  - Begun in Winter 2013, but both were scavenged
  - Included in second year of analysis
- Investigate the relationship with other climatic variables collected
  - Preliminary results show significance of soil temperature and soil moisture in addition to temperature
- Develop PMI estimation equation for Cfa climates in the southeastern United States

Acknowledgments

- This grant was funded by the National Institute of Justice Grant No. 2012-DN-BX-K049.

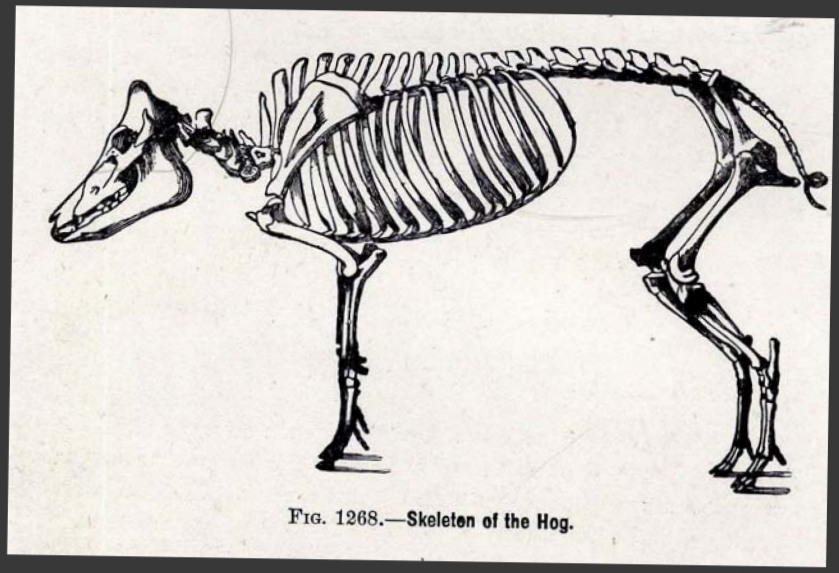


FIG. 1268.—Skeleton of the Hog.