RATE OF DNA DEGRADATION IN PYGMY KILLER WHALES FOR THE ESTIMATION OF POST-MORTEM INTERVAL

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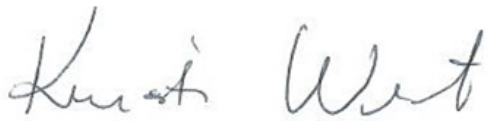
I certify that I have read this thesis and that, in my opinion, it is satisfactory in scope and quality as a thesis for the degree of Bachelor of Science in Global Environmental Science.

THESIS ADVISOR



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For Mamma and Papa

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I am so grateful for my family that cultivated my interest in science and in the beautiful world around me. I would not be here without their inspiration and support. Mamma having been a doctor in Sweden perked my interest in medicine. Like her, I am not faint of heart can merrily take part in necropsies of large mammals like humpback whales. Pappa is a scientist who encouraged my exploration of science, and his calm temperament gave me the patience to spend hundreds of hours pipetting. Finally, I want to thank my love, Tristan, who makes me smile every day no matter if my studies or work have been frustrating.

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ABSTRACT

Marine mammals are vital to the health of ocean ecosystems. To better protect these animals, it is important to understand the threats they face. The University of Hawai‘i Health and Stranding Lab (HSL) maintains an archive of tissues from stranded cetaceans (whales, dolphins, and porpoises) in the North Pacific and conducts research examining health related metrics of these animals. For much of this research, having an estimation of the post-mortem interval (PMI), or the time between death of an animal and the recovery of its carcass, can be useful for identifying the cause of death. However, there is currently no quantative method to determine the PMI in marine mammals. Current methods require observational and qualitative measures to estimate the PMI. Forensic research has utilized the degradation of DNA post-mortem to establish a quantitative tool for estimating PMI. Measuring DNA concentration over time, thereby evaluating DNA degration rate, could be used to develop a model for estimation of the PMI. Archived tissues from pygmy killer whales that were euthanized in a mass stranding event were selected to represent freshly dead animals for this study. From each cetacean, seven tissue types were left to degrade in both a replicated land and water stranding. Tissues were sampled over a period of 28-days and DNA concentrations were measured. The DNA concentrations were evaluated against two intervals of degradation time. The most significant relationships were seen in liver and lung tissues (R^2 =0.877 and 0.81) in the shorter time interval indicating they would be most useful in future estimations of PMI in animals.

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LIST OF ABBREVIATIONS AND SYMBOLS

UH: University of Hawai‘i

HSL: Hawai‘i Stranding Lab

DNA: deoxyribonucleic acid

RNA: ribonucleic acid

PMI: post-mortem time interval

PCR: polymerase chain reaction

ng: nanogram

mg: milligram

µl: microliter

ml: milliliter

ng/mg: nanogram per miligram

rpm: revolutions per minute

°C: Degrees centigrade

1.0 INTRODUCTION

1.1 BACKGROUND

Marine mammals are sentinels of ocean health (Bossart, 2011) with some populations heavily exploited in the past and currently in decline. The International Union for the Conservation of Nature (IUCN) Red List classifies 25% of marine mammals to be threatened with extinction (Davidson, 2011). Marine mammals are threatened by lack of food, overfishing, discarded fishing gear, disease, and more. A cetacean is considered stranded when they are found dead on shore or in the water, or if they are alive on shore, but unable to return to the water (NOAA, 2022). When their carcasses are found, investigation of these stranding incidents provides a means to determine causes of mortality and to evaluate threats faced by marine mammals. Animals do not always strand in public areas, with their carcasses often being found severely decayed in remote areas, and it is often unclear when the animal died. This results in a limited ability to examine temporal factors that may contribute to these deaths.

The date and time of a marine mammal death can be difficult to determine. The post-mortem time interval (PMI) is defined as the time between death and the recovery of a carcass for examination. During this time, tissues begin to degrade, limiting the information that may be obtained from the animal (Shukla, 2017; Zhu et al., 2017). The date and time that a marine mammal stranding is reported is often not the same as the date and time as when the animal died. A deceased marine mammal can drift at sea for days to weeks prior to being reported once it drifts close to shore or lands on an inhabited coastline. No method to establish quantitative estimates of the post-mortem interval

currently exists for use in cetacean stranding investigations. Qualitative estimates of carcass decomposition are commonly recorded by examiners, and the ability to quantitatively estimate the post-mortem interval would provide a valuable tool for investigating the potential causes of stranding events.

1.2 STUDY ANIMAL & ENVIRONMENT

The University of Hawai‘i Health and Stranding Lab (HSL), located at Marine Corps Base Hawai‘i (MCBH), responds to strandings of cetaceans in the Pacific Islands and conducts necropsies for research and archival purposes. During necropsy, internal and external examinations are conducted, and an extensive suite of tissues are preserved for future research. The work performed at the HSL helps to evaluate and identify threats to cetaceans in the North Pacific. In the fall of 2019, a mass stranding event of pygmy killer whales occurred on the island of Maui, where a total of seven animals died in two separate events and over the course of approximately four weeks. During this extended stranding event, pygmy killer whales were euthanized on site, and carcasses were immediately chilled and transported using air cargo services to the HSL for necropsy and tissue sample collections.

The pygmy killer whale is a rare species of oceanic dolphin that is primarily black in color with white coloration on the lips and belly (Figure 1). Pygmy killer whales have one of the lowest densities of all cetaceans observed in the main Hawaiian Islands. This species is ranked as 12th of 13 in abundance when considering delphinid species sighted in Hawaiian waters (McSweeney et al., 2009). Relatively little is known about the species on a global scale, but pygmy killer whales are thought to be generally sighted closer to shore when considering oceanic islands (Baird et al., 2011). Pygmy killer

whales are found world-wide but primarily in tropical waters with an overall range between 40°N and 35°S (Figure 2). Around the Hawaiian Islands, only one stock of pygmy killer whales has been recognized (Baird et al., 2011).

Figure 1. Photograph of a pygmy killer whale off the bow of a research vessel in 2010. Credit to Robin W. Baird of Cascadia Research.

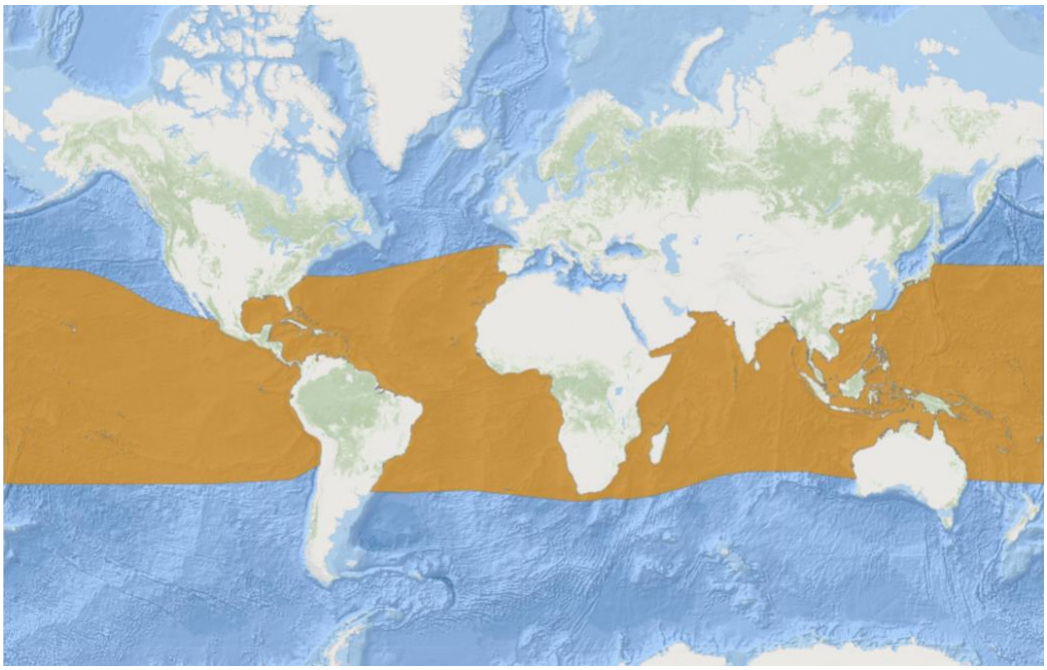

Figure 2. Image of the global distribution of pygmy killer whales (Braulik, 2018)

1.3 POST-MORTEM TIME INTERVAL

The PMI is a very important tool in forensic medicine and for criminal investigations. More advanced methods to estimate PMI for forensic applications have been investigated for decades (Li et al., 2016). One method used to investigate PMI is through molecular degradation, which most often focuses on the degradation of RNA and DNA molecules. The use of DNA degradation for the estimation of PMI has been studied for the last 40 years (Li et al., 2016). Previous research has established rates of tissue DNA degradation for use in forensic studies to determine the time of death more accurately in humans (Elharoony et al., 2008). After death, DNA breaks down due to damage from nuclease enzymes that break molecular bonds in DNA (Shukla, 2017). DNA degradation can be analyzed using a variety of techniques including polymerase chain reaction (PCR), gel-based techniques, dye staining, blotting techniques,

electrophoresis, and spectroscopy (Mohamed, et al. 2020; Shukla, 2017). An Invitrogen Qubit 4 Fluorometer (Invitrogen, Waltham, Massachusetts), can be used to quantify DNA by detecting fluorescent dyes that bind to DNA. When using a Qubit dsDNA HS Assay Kit, the Qubit solution bonds only to whole double stranded segments of DNA, thus fragmented pieces are not detected in the Qubit fluorometer. While RNA has been found to be much less stable, degrading more quickly post-mortem, it has also been used in recent research to establish reliable PMI data when in a controlled environment and of short-term duration.

Most PMI studies can be separated into two different time intervals: early PMI and late PMI. Early PMI spans hours to days and late PMI spans days to months. Most of the data obtained has focused on early PMI investigations as it has been found to be the most reliable for forensic studies (Li et al. 2016). The accuracy and precision of PMI estimates decrease over longer durations. PMI has been found to be heavily dependent on external factors. As the length of time after death increases, the chance of more external factors influencing for the rate of degradation also increases, thus impacting the accuracy of PMI estimates. The rate of DNA degradation can be highly variable based on a wide range of factors with varying levels of impact. The rate of degradation has been found to vary by species, tissue type, temperature, humidity, UV exposure, pH, disease, genetics, and predation by scavengers (Li et al. 2016). Previous research has found temperature to be one of the most significant sources of variation in DNA degradation rates with higher temperature causing degradation at a faster rate (Williams et al., 2015; Tozzo et al., 2020). The rate of degradation increases with high temperatures, as proteolysis is accelerated by heat (Moore et al., 2015). The effect of temperature is commonly

investigated by assessing the effect of accumulated degree-days on the DNA yield in study samples (Li et al., 2016). Despite this impact, studies have shown that DNA can survive in pig and rabbit tissues up to 101 days in high temperatures and to 138 days in cold temperatures, indicating that it is still a useful molecule for PMI determination (Nazir et al., 2011). Another study utilized burial to determine differences in PMI due to temperature and humidity. However, this study, which only utilized bone DNA, found no correlation between the amount of DNA degradation and PMI (Reza Alaeddini et al., 2011).

1.4 DNA DEGRADATION

Analysis of DNA degradation has been historically used in human forensic studies, with minimal or no intended application to the study of wildlife. Despite this, a large portion of these studies have been done on mice, rats, and pigs, leading to the possibility that the same work can be applied to wildlife. Conservation officers have traditionally used more imprecise PMI estimators than DNA degradation, such as carcass temperature, pupil diameter, and rigor mortis to estimate the time of death, such as in the assessment of white-tailed deer poaching (Hadley et al., 1999). These measures are used to identify if a deer has been poached or had been legally taken during hunting season (Gill & O'Meara, 1965). Utilization of DNA degradation for PMI has broad conservation and natural resource management applications, where time of death might allow for a greater understanding of where and when death occurred, potentially aiding in establishing a body of evidence for cases of illegal wildlife trade. Some protein degradation work has been done with pinnipeds, although the purpose of that research has been to determine the viability of post-mortem tissue in physiological study (Moore et al.,

2015). This research utilizing tissues from pygmy killer whales (*Feresa attenuata)* has the potential to be of great importance in the estimation of PMI, which would contribute to determination of cause of death in stranded cetaceans.

1.5 STUDY PURPOSE

The purpose of this experiment was to establish the degradation rate of DNA in air and water exposure environments to replicate both land and at sea strandings of dead pygmy killer whales. Tissues from freshly euthanized pygmy killer whales were used to establish the rate of DNA degradation over time in both air and water exposure environments. A suite of tissue types from the euthanized pygmy killer whales were sampled at set time intervals from each of the replicated environments, and the DNA concentration quantified using a Qubit fluorometer. The data was then analyzed to examine the relationship when comparing DNA concentration of the air and water exposed tissues with increasing time intervals. We hypothesized that there would be a negative trend line associated with DNA concentration as PMI increased. The rate was expected to vary between tissues and in the air and water exposure environments based on current literature regarding DNA degradation in humans and other animals. It was hypothesized that the air exposure environment representative of a land stranding would have increased DNA degradation, because of experiencing higher environmental temperatures. To determine if the use of DNA degradation rate from pygmy killer whales would be a reliable tool to apply to decomposed carcasses where time of death is unknown, we examined the relationship at a one-week interval versus a month, which was represented in this study by a 28-day time interval. We anticipate that this study will result in more accurate estimations of post-mortem time intervals (PMI) in recovered

stranded marine mammals where time of death is unknown. This is expected to provide important temporal information that will contribute to cause of death investigations for individual animals.

2.0 METHODOLOGY

2.1 STUDY ANIMALS

Two fresh dead pygmy killer whales, KW2019016 and KW2019017, were selected for the DNA degradation experiment based on their similar body condition and age demographics. The individuals were juveniles and of opposite sex. These animals were part of a prolonged mass stranding event that occurred on Maui in 2019, in which 11 pygmy killer whales were stranded (Wu, 2019). These individuals were both euthanized within minutes of one another, giving a known time of death and limiting the amount of tissue degradation that could occur prior to archival preservation. The stranded animals were euthanized on Maui in the morning and promptly put on ice and chilled using air cargo services until they arrived on O‘ahu and were transported to the HSL the same day. The individuals were necropsied on O‘ahu immediately after arriving at the HSL facility, and collected tissues were immediately put on dry ice and then stored at −80 °C for long-term storage.

Seven tissues were selected from each animal for degradation analysis in this study: cerebrum, blubber, muscle, marginal lymph node, lung, liver, and bone marrow. Approximately 100 mg of each tissue type was set to degrade in triplicate under two different environmental conditions – one condition with air exposure replicating a beach stranding event with the samples in a sealed container placed on a pier and the other a water exposure replicating an aquatic stranding with a sealed sample container suspended

from the same pier and submerged in the ocean for the 28-day duration of the study. Each sample was individually sealed in a plastic sample bag to replicate the tight membranes of an animal's internal anatomy. An initial sample was taken in triplicate from each tissue to develop a baseline DNA concentration (Day 0) for both animals. As tissue degraded, samples were taken on days 1, 3, 5, 7, 10, 14, 21, and 28 from each environmental condition (air and water exposure). In total, 714 samples were taken during this study.

2.3 EXPERIMENTAL PROCEDURE

To establish the degradation rate of DNA under differing environmental conditions, land and at water stranding events were simulated in the study design. The air condition was replicated by storing the tissues in an opaque container on a pier at the HSL facility. The water condition was replicated by using a container suspended from the same pier and submerged in the waters of Kāneʻohe Bay. Each sample container was equipped with two Elitech data logging probes (Elitech Technology, San Jose, California) to record the temperature of the tissues at 15-minute intervals. The probes were monitored throughout the experiment to ensure that they were accurately recording. On each established sampling day, 42 samples were removed from each of their environmental conditions (air and water) at the same time. Once removed, tissue samples were stored in a -80 °C freezer to prevent further DNA degradation prior to DNA extraction. On day 28, the final degradation day, both the samples marked for that day and the probes were removed from the containers.

2.3 LABORATORY PROCEDURE

All the samples were kept in a -80 °C until they were ready to be subsampled. Prior to subsampling, all samples were first thawed at room temperature for 5-10 minutes. Once thawed, tissues were removed from sample bags, and approximately 10 mg were removed from the initial 100 mg samples. The 10 mg subsample was cut up into at least three pieces to facilitate tissue lysis during extraction. Tissue samples were then stored in pre-weighed RNase-free 1.5 ml microcentrifuge tubes and put back into -80 °C to await extraction. They remaining tissues was saved for future use or as reserve.

2.4 EXTRACTION PROCEDURE

DNA was extracted from 10 mg tissue samples using DNeasy Blood and Tissue Kits (Qiagen, Germantown, Maryland). The extraction procedure was performed according to the manufacturer's protocol. Prior to starting extraction procedure, tissues were removed from the -80 °C and thawed for approximately 10 minutes at room temperature. To start the extraction the tissue was first lysed. Buffer ATL (180 µL) was added to the microcentrifuge tube containing the sample. Then, proteinase K (20 µL) was added, and the tube was vortexed for 15 seconds or until all tissue was submerged in the solution. Once vortexed, samples were incubated for a minimum of 4 hours in a 56 °C water bath so that the tissues would lyse. During the incubation, samples were vortexed for 15 seconds every hour. If any samples appeared to be taking a long time to lyse, namely if tissues could be visually seen in the tube, they were vortexed for double the time. If after 4 hours the tissue solution had any unlysed tissues, the homogenized tissues were left to incubate for another hour.

After incubation was complete, Buffer AL (200 µL) was added to the sample and mixed by vortexing for 15 seconds. 200 µL ethanol (96-100%) was then added and vortexed for at least 15 seconds, until the mixture was homogeneous. Once homogenized, the solution was pipetted into a DNeasy Mini spin column that was held in a 2 ml collection tube. The solution was centrifuged at ≥6000 x g (8000 rpm) for 1 minute and the spin column was placed into a new 2 ml collection tube. 500 µL Buffer AW1 was then added before centrifuging at 9000 rpm for two minutes. Once finished, the spin column was placed into a new 2 ml collection tube and 500 µL Buffer AW2 was added before centrifuging at 20,000 x g (14,000 rpm) for three minutes. The spin column was then placed in a 1.5 ml microcentrifuge tube, and 200 µL Buffer AE was pipetted directly onto the DNeasy membrane, which was incubated at room temperature for one minute before centrifuging at 8000 rpm for one minute.

2.5 QUBIT PROTOCOL

Once the extraction was finished, the samples were immediately processed with a Qubit dsDNA HS (double-stranded DNA high sensitivity) kit (Thermofisher Scientific, Waltham, Massachusetts). The Qubit fluorometer was calibrated in accordance with the Qubit manual each day prior to extractions. For DNA concentration readings, Qubit solution was first made daily according to protocol using a ratio of 199 µl of Qubit buffer to 1 µl of reagent. 199 µl of Qubit solution was added to a Qubit tube. Then 1 µl of the finished extraction sample was added. The solution was vortexed for five seconds and then left to incubate at room temperature for two minutes. After incubating, the samples were immediately read in the Qubit. All samples were read within five minutes of incubation. Each sample was measured in the Qubit fluorometer three times for accuracy.

If any measurements fell below 500 ng/ml, extracts were resampled to ensure that there was not an error involving the measurement of DNA concentration.

2.6 DATA ANALYSIS

All data obtained on DNA concentrations was stored in Google Sheets. The three measurements of DNA concentration from the Qubit fluorometer were recorded and averaged. Standard deviation and percent coefficient of variation was calculated for the collected data. The average concentration in ng/ml was then corrected for the original sample tissue weight in mg and results in a final concentration in ng/mg. The results of the rib marrow could not be corrected for the weight of bone that was not lysed with tissue. Because this could not be accounted for, the DNA concentration could not be accurately measured; this data is not included in the results. An average was calculated for each tissue, in each animal (KW201916 vs KW201917), and in each condition (air/water). The averages were grouped by animal and condition. All the averages were then graphed by tissue type in Google Sheets. The results were graphed for each animal and condition with DNA concentration in ng/mg on the Y-axis and days post-mortem on the X-axis. Each graph had a point for each data point and a trendline for each respective tissue. All graphs also had the R^2 value calculated for each line. The results of the R^2 values were then presented in tables with averages for tissues, conditions, and individuals. Temperature data was assesed for variations between conditions. Statistical analyses were performed to assess the significance of DNA concentration vs. animal sampled, stranding condition, tissue type, and post-mortem interval. Data was also examined on a weekly timeframe (first 7 days of the experiment) and a monthy

timeframe (full 28 days of the experiment). The statistical tests were performed using R and included Levene's, Kruskal-Wallis, and Dunn's tests.

3.0 RESULTS

3.1 STATISTICAL ANALYSES

The coefficient of determination, R^2, acts as measure of the relationship between experimental values in this study and is referred to often in these results. The R^2 values are measured from 0-1 with higher values, or values closer to 1, indicating a greater adherence to the experimental trends or stronger relationship between the experimental values. Statistical analyses were performed to assess the significance of DNA concentration when compared to four factors: individual animal, air or water condition, tissue type, and post-mortem duration. Levene's tests were used to assess the homogeneity of variance, and groups were found to be non-homogenous when comparing the DNA concentrations between tissue types and the DNA concentrations according to different post-mortem durations. Kruskal-Wallis tests were then used to compare each of the variables to the DNA concentration, and resultant p-values of less than 0.05 indicated that there was a significant relationship between concentration vs. tissue type and concentration vs. sampling date. In addition to analysis of the relationships between DNA concentration and the variable examined (individual animal, air or water condition, tissue type and post-mortem duration), temperature variation was also considered during the experiment, though no statistical analysis was performed on the temperature probe data.

3.2 INDIVIDUAL

The statistical analysis did not indicate statistically significant differences between individuals when compared to DNA concentrations. Observing Figure 3 and Figure 5 the two individuals have visually similar graphs. Both KW2019016 and KW2019017 have low starting concentrations and shallow slopes for blubber, muscle, and cerebellum. However, there are some differences like the starting concentration of liver being much lower for KW2019017. In addition, during the 7-day timeline the R^2 values of tissues liver, lung, and lymph node are consistently higher in KW2019017 regardless of stranding environment. Overall, the difference between the individuals appears minimal, and tissues from each animal appeared to degrade in a similar fashion. Between individuals, tissue types had similar starting concentrations, similar slopes, and similar R^2 values (Figure 3 & 5 : Table 1-2).

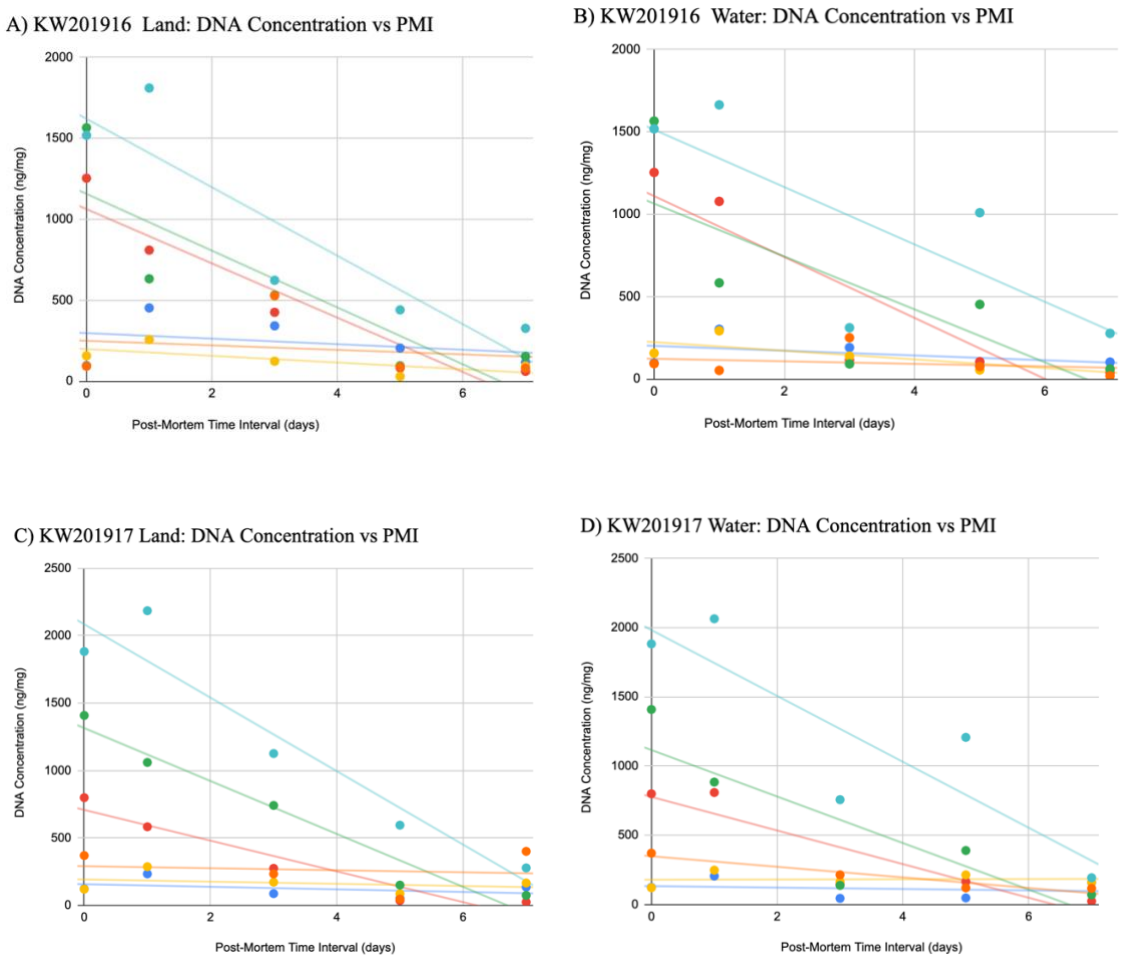

Figure 3: This panel contains four graphs that display the relationship between DNA concentration and post-mortem time interval that spans 7-days. Graphs A and B are from the pygmy killer whale identified as KW201916 and graphs C and D are from are from the pygmy killer whale identified as KW201917. Graphs A and C display tissues in the replicated land stranding while graphs B and D display tissues in the replicated water stranding. On each graph six tissues are displayed with respective colors (lymph node= light blue, liver= red, muscle= yellow, lung = green, cerebrum = orange, blubber =dark blue) along with a trendline

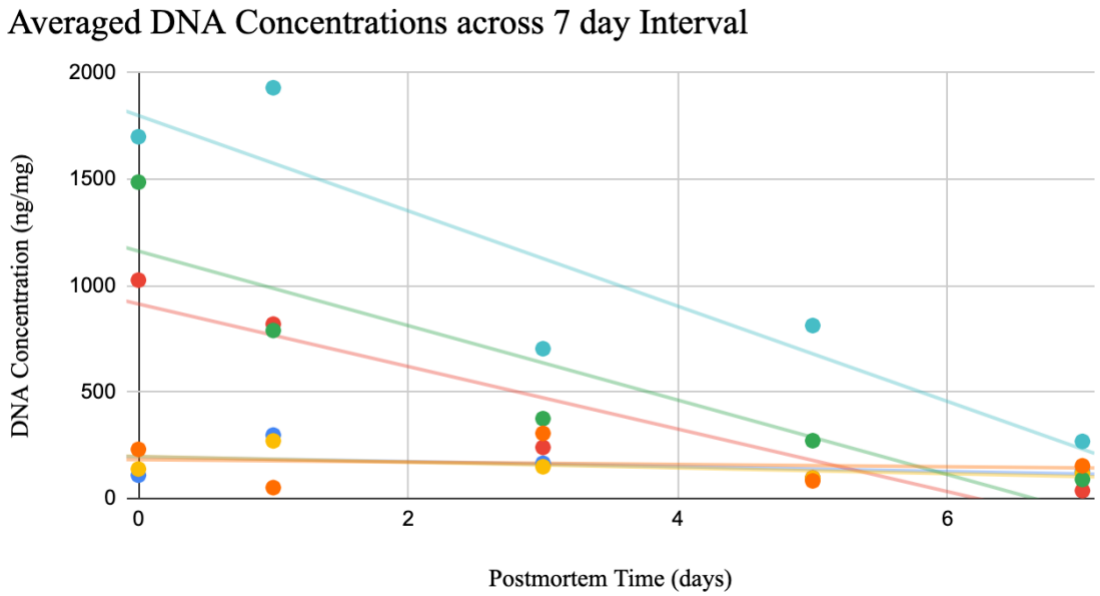

Figure 4: This graph displays the relationship between averaged DNA concentration and post-mortem time interval across 7-days. These results are from the pygmy killer whale identified as KW201917 with tissues in the replicated water stranding. Six tissues are displayed with respective colors (lymph node= light blue, liver= red, muscle= yellow, lung = green, cerebrum = orange, blubber =dark blue) along with a trendline.

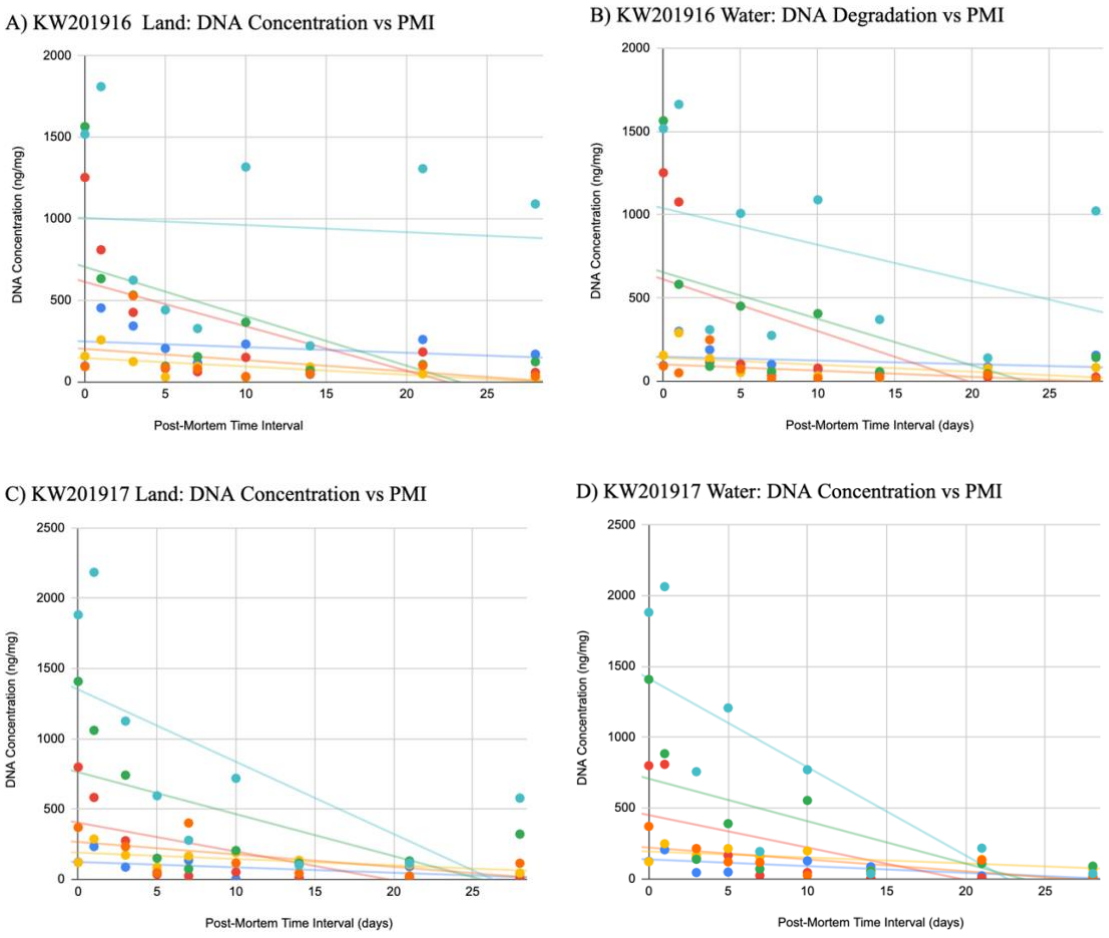

Figure 5: This panel contains four graphs that display the relationship between DNA concentration and post-mortem time interval that spans 28-days. Graphs A and B are from the pygmy killer whale identified as KW201916 and graphs C and D are from the pygmy killer whale identified as KW201917. Graphs A and C display tissues in the replicated land stranding while graphs B and D display tissues in the replicated water stranding. On each graph six tissues are displayed with respective colors (lymph node= light blue, liver= red, muscle= yellow, lung = green, cerebrum = orange, blubber =dark blue) along with a trendline.

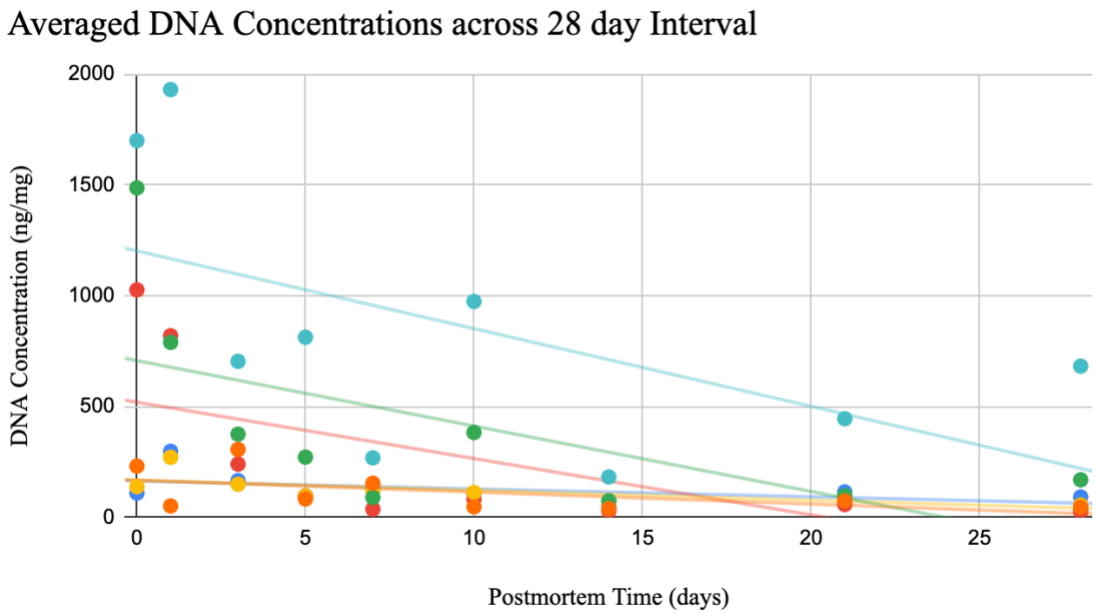

Figure 6: This figure displays the relationship between averaged DNA concentration and post-mortem time interval across 28-days. These results are from the pygmy killer whale identified as KW201917 with tissues in the replicated water stranding. Six tissues are displayed with respective colors (lymph node= light blue, liver= red, muscle= yellow, lung = green, cerebrum = orange, blubber =dark blue) along with a trendline.

Table 1: R^2 Values by Tissue Type and Animal across 7-day PMI

	KW201916		KW201917		
	Land	Water	Land	Water	Average
Liver	0.892	0.792	0.913	0.822	**0.855**
Lymph	0.806	0.586	0.915	0.768	**0.769**
Lung	0.725	0.567	0.952	0.729	**0.743**
Muscle	0.501	0.597	0.092	0.002	**0.298**
Cerebrum	0.036	0.061	0.019	0.917	**0.258**
Blubber	0.103	0.201	0.161	0.043	**0.127**
Average	**0.511**	**0.467**	**0.509**	**0.547**	
Animal Average	**0.488**		**0.528**		**0.508**

Table 1: Displays the R^2 of each tissue and the right column displays averages of each tissue type across the weeklong interval. The two bottom rows show the averaged R^2 of each animal in each condition. The bottom right corner also displays the R^2 of all the tissues averaged.

Table 2: R^2 Values by Tissue Type and Animal across 28-day PMI

	KW201916		KW201917		
	Land	Water	Land	Water	Average
Liver	0.378	0.362	0.437	0.425	**0.401**
Muscle	0.409	0.223	0.376	0.392	**0.350**
Lung	0.358	0.297	0.341	0.372	**0.342**
Cerebrum	0.123	0.288	0.319	0.45	**0.295**
Lymph	0.005	0.136	0.425	0.589	**0.289**
Blubber	0.068	0.071	0.252	0.437	**0.207**
Average	**0.224**	**0.230**	**0.358**	**0.444**	**0.314**
Animal Average	**0.227**		**0.401**		

Table 2: Displays the R^2 of each tissue and the right column displays averages of each tissue type across the monthlong interval. The two bottom rows show the averaged R^2 of each animal in each condition. The bottom right corner also displays the R^2 of all the tissues averaged.

3.3 AIR AND WATER ENVIRONMENTAL CONDITIONS

Analysis did not find the effect of the air or water condition (stranding type) to be significantly different. Visually comparing the DNA concentration, the overall trendlines look similar, but the slopes of the air condition (land replicated stranding) are steeper (Figure 3 & 5). The difference between the air and water exposure conditions was more notable between some of the specific tissue types examined. The most interesting observation was that the R^2 values were generally higher in the water stranding; however,

the tissues with the highest R^2 values (liver, lung, and lymph node) were highest in the land stranding (Table 1 & 2). When comparing the effects of the conditions between animals, it is also notable that for KW2019017 the water had the higher R^2 values for both the shorter and longer time interval (Table 1 & 2). Whereas for KW2019016, at the 28-day timespan, the R^2 value was higher for the water stranding, but in the 7-day timespan the land had a higher R^2 value (Table 1 & 2).

3.4 TISSUE TYPE

The most visually apparent differences in the figures are the differences between tissue types. Statistical analysis confirmed that there was a statistically relevant difference between tissue types. Comparing the values of the 7-day and 28-day intervals the tissues were much more reliable on the shorter timescale. The highest to lowest average R^2 values for tissues was not consistent when comparing the 7-day and 28-day interval. Both liver and lung were consistently among the three highest. Lymph node was on the lower end of the R^2 spectrum on the 28-day interval, but within the 7-day interval it was the second best performing (Table 3). The average R^2 of all tissues was 0.505 for the 7-day interval and 0.344 for the 28-day interval. Across both timescales liver had the highest average R^2 value with an average of 0.641 (Table 3). Blubber and cerebrum consistently had the lowest R^2 values with averages of 0.196 and 0.155 respectively (Table 3). The average slope of the tissues, which indicates how quickly something degraded, ordered steepest to shallowest is lymph node, lung, liver, cerebrum, muscle, liver, and blubber (Figures 3-6). While the lung, liver, and muscle both have higher slopes and R^2 values, lymph node has the highest slope but lowest R^2 value. Analyzing the R^2 values in conjunction with their coefficient of variability, liver and lung are the most reliable

tissues. The two are among the highest in R^2 values and unlike lymph node, which also has a high R^2, the two have a much smaller coefficient of variability. Liver and lung are also the only tissue types whose trendlines have a consistent negative slope in each figure (Figures 3-6).

Table 3: R^2 Values Comparing 7-day Averages & 28-day Averages

	Blubber	Liver	Muscle	Lung	Cerebrum	Lymph	Average
7-day Interval	0.164	0.877	0.325	0.81	0.023	0.831	**0.505**
28-day Interval	0.228	0.404	0.469	0.372	0.286	0.307	**0.344**
Average	**0.196**	**0.6405**	**0.397**	**0.591**	**0.1545**	**0.569**	**0.425**

Table 3: Displays the average *R^2 of each tissue in the 7-day and 28-day interval. The last column displays averages for the intervals and the bottom row displays average of each tissue type.*

3.5 POST-MORTEM TIME INTERVAL

The statistical analysis confirmed that the post-mortem time interval had a significant effect on DNA concentration. Negative trendlines can be seen visually in all the graphs, though the data points show that there is variability (Figures 3-6). In addition, the average R^2 values (0.508 for the 7-day PMI and 0.314 for the 28-day PMI) indicate that much of the variability in DNA concentration is not explained with its relationship to PMI (Table 1-3). Examining the difference between the two timespans, the post-mortem interval across one week was much more reliable than the month-long duration (Table 1-3). The slope of the trendline is highly variable between tissues with an average decrease

of 33.6 ng/mg DNA concentration per day (Figures 3-6). Comparing Figures 4 and 6 the slopes for the weeklong duration are steeper in all tissues, except the cerebrum. This is a result of many of the slopes plateauing after 7 days.

3.6 TEMPERATURE PROBES

The temperature was recorded every 15 minutes across the 28-days in both the simulated land and water stranding. The data was put into Google Sheets and two graphs were made, one for the replicated land stranding and one for the water. Results from the temperature probe reported that the replicated land stranding had an average temperature of 27.1 °C and 61% humidity (Figure 5). The temperature fluctuated by 21.3 °C with a low of 20.4 °C and a high of 41.7 °C (Figure 5). The water stranding had an average temperature of 26.0 °C (Figure 6). The temperature fluctuated with a low of 22.2 °C and a high of 28.2 °C. (Figure 6). The average temperature was 4.9 °C colder in the water stranding and the temperature fluctuated only 6 °C as compared to 21.3 °C in the land stranding.

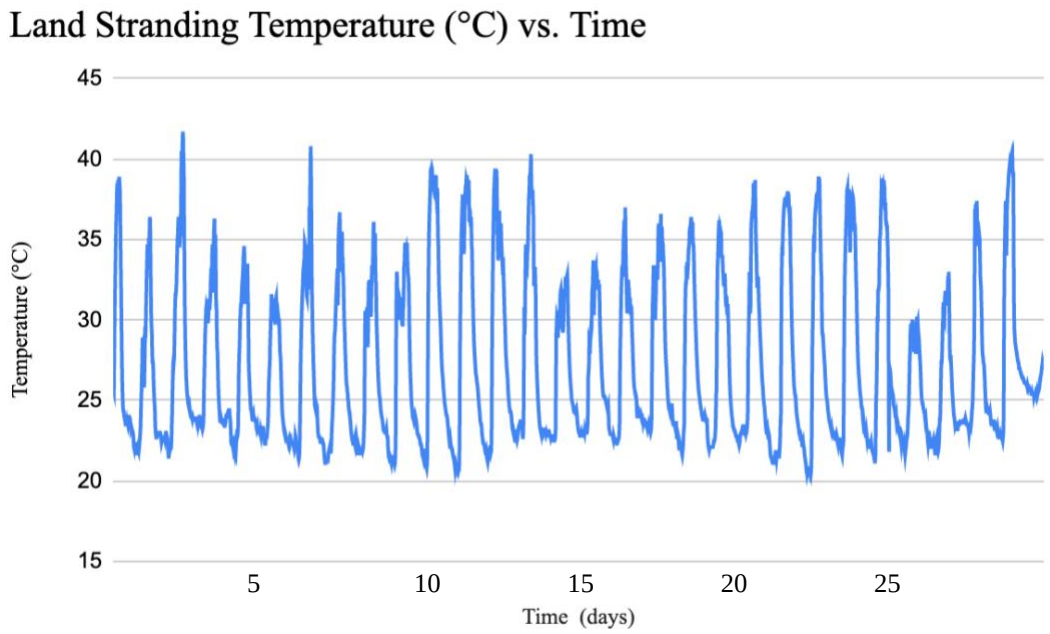

Figure 7: Displays the temperature experienced by the tissues in the replicated land stranding.

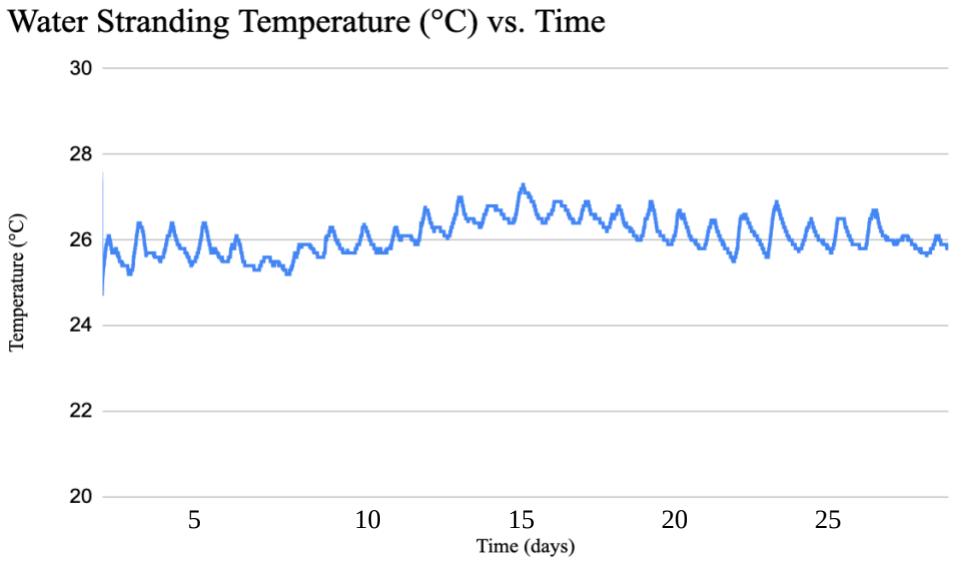

Figure 8: Displays the temperature experienced by the tissues in the replicated water stranding.

4.0 DISCUSSION

The findings from this study indicated that DNA concentration decreased according to post-mortem time interval over a 28-day period in each tissue type examined. However, the results of the experiment indicated high amounts of variation. The R^2 values also indicated variability in the strength of the relationships between DNA concentration and post-mortem time interval which was found to be dependent on tissue type. Specific tissue types generally indicated stronger relationships than other tissues, with liver, lung and lymph node having the most significant decreases in DNA concentration over time. Comparison of the two timeframes indicated that the strongest relationship between decreasing DNA concentration and post mortem time was in the shorter weeklong timeframe. The R^2 values for the liver, lung, and lymph node tissues were all over 0.8, when only considering a 7-day time span.

As expected, every tissue did generally display degradation in concentration and had a negative trendline. However, the R^2 values yielded in the experiment imply that DNA degradation cannot be used exclusively to give reliable estimates of PMI. While this research gives us DNA degradation results for pygmy killer whales, other marine mammals may degrade at either less or greater rates. This research can help to provide an approximation of the PMI; however, the findings suggest that accurate approximation may be improved with exploration of other methods.

4.1 INDIVIDUALS

The results of the statistical analyses found that the differences in DNA concentration between individuals was not statistically significant. However, the experiment was limited in scope, assessing only two individuals. The demographic

similarities of these animals (juvenile pygmy killer whales) could impact any differences that may exist in degradation effects between differing sexes and age classes. Regional variations may also exist, depending on how overall body composition differs between animals of the same species in different geographic areas. To utilize DNA degradation for research with other species new trendlines will have to be established for each respective species. Ultimately, it would be useful to expand this study into pygmy whales from different demographic areas and other species to better understand the variations that may exist in the degradation of cetacean carcasses.

4.2 IMPACT OF TISSUE TYPE ON DNA DEGRADATION

The findings of this study demonstrate that the various tissue types examined have large differences in both the concentration of DNA and the rate at which they degrade. The statistical analysis also confimed that the differences between tissue types was statistically significant. Figures 3-6 demonstrate that each tissue type had a similiar trendline. The tissues in order of highest to lowest starting DNA concentration are lymph node, lung, liver, cerebrum, muscle, liver. The slope observed for each of the tissue types is an indicator of how rapidly the tissue degraded. The tissues in order from steepest to shallowest slopes are lymph node, lung, liver, cerebrum, muscle, liver. This is the same order as the starting DNA concentration which reflects a correlation between starting DNA concentration and the rate at which the DNA degrades. While higher starting DNA concentrations may suggest that the tissue should end with the highest DNA over the time period examined, this was not supported by the results. Tissues (liver and lung) which had higher starting concentrations had lower DNA concentrations than tissues (blubber and muscle) that started low near the end of the 28-day long interval (Figures 3-6).

While the average DNA concentration among tissue types was similar between the stranding conditions and animal there was large variation between replicates. Inconsistency among tissues may have played a role in this observed variability between replicates. Large variations in the starting DNA concentrations among the various tissues were observed which may suggest that different parts of the same tissue may degrade at different rates. Several of the tissues used had differences within the tissue type that could be observed grossly. For example, lung samples might contain large bronchioles and liver samples might contain blood vessels. In addition, there could have been trouble with the lysis of some tissues. If tissues were not properly cut into small enough pieces during subsampling, they may not have fully lysed. By preventing the complete lysis of the tissue, the DNA concentration can be erroneously low. Indeed, there were instances in the laboratory where the tissues did not fully lyse within the 4 hours. In those instances, additional vortexing and incubation was conducted. However, it is possible that other samples were not fully lysed within the 4 hours but that these were not able to be distinguished grossly.

4.3 IMPACT OF TEMPERATURE ON DNA DEGRADATION

The results of the experiment refuted the hypothesis that the air exposure condition designed to replicate a land stranding would result in DNA degradation at a faster rate due to increased temperature. While this general trend was suggested by a steeper slope in the tissues exposed to the air versus water condition, it was not found to be statistically significant. Prior research on DNA degradation in humans that studied the effects of temperature found that DNA degraded at a faster rate in warmer temperatures Williams et al., 2015 ; Tozzo et al 2020). While our study on pygmy killer whales did not

find this to be statistically significant it is possible that the difference between temperatures of the air and water conditions were not extreme enough to produce an effect. The difference in temperature only averaged 4.9 °C in our study, but this difference would likely be greater in regions with higher latitudes.

Temperature was recorded in the experiment utilizing a temperature probe vacuum sealed in plastic and kept in same respective container as the tissues exposed to air and water conditions. However, in a real stranding it is likely that the deeper tissues would be insulated from temperature changes compared to exterior tissue types. Therefore, the interior tissue types exposed to air and water in our study likely endured greater temperature fluctuation than would be expected to occur in a real stranding. In addition, the container which replicated an at sea stranding through water exposure was submerged under a foot of water near shore. In an actual at sea stranding the animal might be variably buoyant with different parts exposed to the air above water. One study of 14 whales found that all initially sank but later floated due to increased buoyancy from gases produced during decomposition (Moore et al., 2020). As a result, the whales could experience a colder temperature while they are sunk at lower depths in the water column. Future studies might isolate the effect of temperature and utilize more extreme temperature differences to better establish its influence on degradation in cetaceans. While this may be less influential to strandings in Hawai‘i where the difference in seasonal temperature is minimal, this may not be the case when considering strandings in higher latitudes or locations that experience greater temperature extremes.

4.4 IMPACT OF PMI DURATION

The overall relationship between PMI and DNA concentration was found to be statistically significant. Evaluating the difference in R^2 values, the relationship was also found to be stronger in the 7-day duration in comparison to the 28-day duration (Table 2 and 3). The average R^2 value for the 7-day assessment was 0.508 whereas the 28-day assessment was 0.314. Liver and lung, the tissues which showed the strongest relationship were over twice as strong in the 7-day versus 28-day timeline. While the relationship between PMI and DNA concentration was significant, variations like significant differences between replicates was found. In addition, there were several occurrences in which a greater PMI had a higher DNA concentration than an earlier PMI. It is impossible for the DNA concentration to increase after death, thus indicating laboratory error or variation in DNA concentration between tissue samples.

Findings indicating that the shorter time interval was stronger corroborate previous research that found shorter post-mortem time intervals to yield more reliable results (Li et al., 2016). If time-spans are most accurate in a short time interval, future research that finds DNA concentration in correspondence with a longer time interval should regard estimates made using the DNA concentration to be less reliable. Still the results of this experiment show that important data can be taken from carcasses that have already endured weeks of degradation.

4.5 POTENTIAL IMPACTING FACTORS

4.5.1 IMPACT OF UV and SALINITY DAMAGE

Research has found UV damage to increase DNA degradation, and salinity to increase the decomposition of marine mammal organs (Li et al., 2016 ; Booth & Thomas, 2021). While the water exposure condition replicated the decreased temperature experienced by animals stranded in the water, it did not account for any damage from UV or salinity. Neither exposure condition was designed to affect our study as the samples were vacuum sealed in plastic and contained in an opaque container. Damage from UV radiation should only cause damage to exterior organs like the skin. Skin was not used in our study as preliminary studies found it to be a difficult tissue to lyse. The effects of salinity can have damaging effects on marine mammals like bottlenose dolphins (Booth & Thomas, 2021). In a study of live dolphins, the skin barrier gradually degraded when exposed to low salinity. The low salinity caused infections that also led to the decompensation of adrenal and renal systems (Booth & Thomas, 2021). Our study did not consider the potential impact of salinity on degradation rates, these could be factors to consider in future studies.

4.5.2 IMPACT OF PHYSICAL DAMAGE

Marine animals that strand on land often undergo physical damage as they reach the shoreline. Many of the marine mammals that are brought to the HSL have undergone large amounts of damage to their skin. This can be the result of the animals coming across the coral reef or rocks and rolling in the waves at the shoreline. Damage occurring from waves can be referred to as surf maceration. In addition, the carcass can undergo

damage during the retrieval of the carcass. In some cases, the carcass needs to be pulled out from the water, off the reef, or up a steep incline. Water strandings would likely undergo less physical damage. However, marine mammals stranded low on the shore may be brought back to sea from high tides. In addition, both land and sea may receive damage from decomposition and scavenging. This physical damage can cause difficulties in estimating PMI using observational methods (Moore et al., 2020). It is possible that the physical damage can also alter the DNA degradation rates in some tissues by making them more exposed to environmental conditions. Physical impact from surf maceration could cause DNA degradation in tissues. Studies on traumatic brain injuries in mice found DNA fragmentation and presumed apoptosis in the cerebral cortex and cerebellum (Skotak, 2019). It may be valuable to consider physical damage on DNA degradation rates in cetaceans during future studies.

4.6 QUALITATIVE TOOLS FOR PMI

The findings suggest that the more prudent method of PMI estimation for cetaceans continue to be observational and qualitative measures, due to the variability of the research findings. Observational methods are especially prudent if the animal has been dead for over 7 days and if liver and lung tissues cannot be collected. This can be determined by evaluating several signs of degradation. Bloating and the visible deterioration of the carcass can be used as an indicator of decomposition (Ascobans & Accobams, 2019). One significant issue with qualitative assessment is the presence of surf maceration, which is the result of a carcass rolling across the shoreline in the surf, which can exacerbate degradation. Surf maceration can cause the loss of skin of an animal in addition to deflating the bloating associated with decomposition by destruction

of the body cavity (Moore et al., 2020). Forensic entomology, which involves insects collected around the carcass, can also be applied for longer PMI durations by counting the visible number of insects that the carcass has accumulated. However, entomology is less reliable for shorter PMI durations and if the animal was first stranded at sea before stranding on shore. Due to the exterior tissues having more exposure to external damage, a more reliable assessment can be done by examining internal tissues (Ascobans & Accobams, 2019).

4.7 APPLICATIONS

The utilization of DNA degradation for the post-mortem time interval would provide a quantitative tool for estimating PMI. For any application of DNA degradation for the estimation of post-mortem interval, a variety of tissues would generally provide higher accuracy. However, the current study suggests that certain tissues are much better than others for this purpose. The best tissues to use based on our results would be the liver and lung which were among the highest R^2 values across both the 7-day and 28-day interval. If these tissues are used along with other tissues this would give the most reliable estimation of PMI. In addition, if the results of the DNA extraction place the PMI to be within one week, the results would be more reliable based on the comparison of the 7-day and 28-day interval. The R^2 values were much higher in the one-week time span. The results of the DNA degradation ideally could be used in tandem with qualitative measures to best estimate PMI in carcasses where this is unknown.

4.8 FUTURE STUDIES

Future research in this field can explore the results between additional cetacean species to determine if there are differences in the rate of degration. Research can also be done to see if the degradation of RNA, proteins, or other molecules might prove to be a more reliable indicator of PMI. While the findings of this research did not find the difference between a replicated land and sea stranding to be significant, it could be the case that in other regions of the world there is a significant difference. It would also aid this research if a method can be applied to to determine whether a marine mammal sunk before floating, as well as how long they were sunk. In the future it would be useful to know which variables have the highest influence on degradation rate and whether there is a greater difference from various water vs land strandings, vs if the water stranding included a period of submersion prior to floating. In the case that the 10 mg tissue samples were not sufficiently homogenous, investigation is warranted to determine if a larger tissue sample could be homogenized to yield more consistent results. Methods to homogenize tissues have found that the homogenization can cause damage to molecules (Stanley, 2021). This could cause lower yields of DNA concentration which should be further explored as future work.

5.0 CONCLUSION

Human pressure on marine mammals is only increasing and there is significant likelihood that it will be compounded by climate change. In order to best protect these animals we must understand their threats and establishing the PMI is an important aspect of this. By establishing the PMI, the death can be better connected to various environmental or sonar events that could be factors in the stranding. This experiment

attempted to utilize the forensic tool of analyzing degradation of DNA for the establishment of a PMI in pygmy killer whales. This experiment also shows the benefit of preservation of stranded cetacean tissues, with these tissues having been utilized in the simulation of strandings on land and in water.

Evaluating the results the trendline of DNA concentration to PMI failed to provide a reliable quantitative tool for use of PMI estimation. It is possible that the trendline could help to narrow down the timeline of a stranding with an unknown PMI, but observational techniques remain the best tool for estimating the PMI. While the average R^2 values were low the trendline was still found to be statistically significant. With more studies in the future it might be possible to use the DNA degradation to estimate PMI in marine mammals.

Indeed, there are a significant number of variables that were not controlled for in the experiment. These variables which could influence degradation rates include mechanical damage (such as surf maceration), UV, salinity, bacterial infection, and more extreme temperature differences. In conclusion, while the statistical analyses results show that the DNA concentration to PMI is statistically significant, the majority of the R^2 values are too low for the trendline to be used as a reliable tool. DNA degradation can support other observational methods of estimation if lung and liver tissues are utilized.

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