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NUCLEAR AND MITOCHONDRIAL DNA QUANTITY AND QUALITY EVALUATION OF KEMP'S RIDLEY SEA TURTLE (*LEPIDOCHELYS KEMPII*) BONES AFTER OCEANIC AND TERRESTRIAL ENVIRONMENTAL EXPOSURE

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NUCLEAR AND MITOCHONDRIAL DNA QUANTITY AND QUALITY EVALUATION KEMP'S RIDLEY SEA TURTLE (*LEPIDOCHELYS KEMPII*) BONES AFTER OCEANIC AND TERRESTRIAL ENVIRONMENTAL EXPOSURE

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THESIS ABSTRACT

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TITLE OF THESIS: Nuclear and Mitochondrial DNA Quantity and Quality Evaluation of Kemp's Ridley Sea Turtle (*Lepidochelys kempii*) Bones After Oceanic and Terrestrial Environmental Exposure

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ABSTRACT: Molecular biology techniques for the analysis of nuclear and mitochondrial DNA are routinely used to make species identifications in wildlife forensic science. Species identification is crucial to wildlife forensic science casework, as organisms or parts of organisms must be definitively identified prior to prosecution of the suspect(s). Unfortunately, wildlife remains are not typically discovered in a timely manner, which prolongs the exposure of the organism's DNA to damaging environmental factors. Regardless, it is essential to be able to identify which species the remains are from, such as in forensic investigations regarding sea turtle remains. Kemp's ridley sea turtles (*Lepidochelys kempii*) are critically endangered and are closely related to olive ridleys, a vulnerable species, thus it is especially important to distinguish between these two species even when only skeletal remains are discovered. Bone demineralization typically employs Proteinase K (Pro K) and EDTA to lyse skeletal osteons and release cortical DNA. However, sea turtle bones are more cartilaginous than terrestrial vertebrates. I tested the effectiveness of Collagenase Type II (CTII) against Pro K over a three-year exposure time series using two species, Kemp's ridley sea turtles and domestic cattle (*Bos*

taurus). Evidence had been found demonstrating the effectiveness and improved DNA quality of Collagenase Type II extractions using non-degraded human bone samples. I utilized the Epicentre MasterPure kit for DNA isolation and purification, in triplicate, for each sample. I quantified the samples with the Qubit system, before amplification with genus-specific nuclear primers and species-specific mitochondrial primers, in preparation for cycle sequencing. I was able to evaluate the overall effects supratidal and subtidal environments had on skeletal DNA degradation over a three-year period in both a terrestrial mammal as well as a marine reptile. Mitochondrial DNA (mtDNA) was detected and recovered after exposure to either supratidal or subtidal environments after 424 days in both species. The mtDNA was of high enough quality in both organisms after extraction to make species-specific identifications. After 424 days, DNA recovery became inconsistent in the *L. kempii* samples exposed to subtidal conditions, with failed sequencing results at 664 days and 1152 days for the CTII samples. In the *L. kempii* supratidal samples, species identifications could still be made after 787 days. For the terrestrial mammal, mtDNA species-specific identifications could be made from the samples exposed to supratidal and subtidal environments after 1511 and 1152 days, respectively. Nuclear DNA (nuDNA) was only detected and amplified in the terrestrial *B. taurus* samples and was of high enough quality for genus-specific identifications after 787 days. Additionally, I determined that CTII is comparable to Pro K in most circumstances. However, Pro K produced statistically favorable results for *L. kempii* samples when comparing DNA quantities and while controlling for the effects of exposure length. Pro K also had statistically favorable results for *B. taurus* samples when comparing DNA quantities while controlling for the effects of location, as well as comparing DNA quality and while controlling for the effects of exposure length. DNA recovery for species identification of Kemp's ridley sea turtles is primarily affected by exposure length,

rather than exposure to subtidal or supratidal environments and Pro K is as effective as CTII for mtDNA quantity and quality returns.

KEYWORDS: *mitochondrial DNA, nuclear DNA, species identification, skeletal DNA extraction, Lepidochelys kempii, environmental exposure, wildlife forensic science, marine conservation biology*

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Chapter 1: Introduction

In wildlife forensic science, DNA analyses such as DNA sequencing or qPCR, are used to make species identifications, in conjunction with morphological identifications when possible. These identifications are crucial because an organism or part of an organism must be identified before it can be determined if a crime has been committed to comply with investigative and judicial standards (Ogden et al. 2009). Wildlife crime is a global crisis that involves the trafficking of whole organisms as pets or only their parts as cuisine, traditional medicine, or monetary symbols (SWGWILD 2012). Organizations like the International Union for Conservation of Nature (IUCN) and Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES) monitor the health of these populations and designate which species and/or populations are at risk of extinction. In the United States, wildlife is only protected by law if they are classified as endangered, vulnerable, or threatened species (e.g., the U.S. Endangered Species Act (ESA) or the U.S. Marine Mammals Protection Act). This is how the majority of nations around the world protect the species IUCN and CITES list as endangered, but for marine and migratory species, like sea turtles, the laws protecting these animals change every time they cross a border. The most common reasons that a species becomes listed as endangered or at risk are due to loss of environment, global climate change, or wildlife trafficking. Unfortunately, when an organism is deemed rare or of societal value, it is often exploited for a monetary gain. Wildlife trafficking is reported to produce $53 billion dollars in annual revenue for organized crime and is closely related to other criminal activities, such as drug and firearm trafficking. Wildlife investigations often provide a nexus to other illegal activities and enterprises (Wilson-Wilde 2010, SWGWILD 2012, Barron 2015). In some instances, animals are trafficked as exotic pets (Razkallah et al. 2019, Ribeiro et al. 2019) and in

others, animal parts are coveted as integral components of traditional remedies and cultural medicines (Kumar et al. 2019, Sharma et al. 2019, Summerell et al. 2019). In a majority of wildlife criminal cases, the reliable identification of an organism, or its parts, is integral to the apprehension and prosecution of criminal enterprises and is often achieved through genetic analysis (Cronin et al. 1991, Jun et al. 2011, Fain et al. 2013, Joseph et al. 2014, Wasser et al. 2015, 2018, Ng et al. 2016).

In most wildlife forensic cases, trafficked organisms or their parts can be identified morphologically, but after organisms have been processed, they are frequently stripped of identifiable morphological characteristics and genetic identification becomes a necessity (Tobe & Linacre 2010, Moore & Frazier 2019). For example, 'tortoise shell' ornaments and accessories were seized and genetically identified to be from sea turtle shells (Foran & Ray 2016) and 21 claw samples were seized and genetically confirmed as *Panthera leo* and *Panthera pardus* (Khedkar et al. 2016). DNA markers have even been used to link 38 different ivory seizures back to organized crime operations (Wasser et al. 2015, 2018). Unfortunately, when animal parts are processed for trafficking, DNA degradation can be accelerated by factors such as decomposition, cooking, or chemical baths used to preserve hides and furs (Teletchea et al. 2005, Dawnay et al. 2007). DNA can also be degraded by prolonged exposure to UV light and water, especially saltwater (Armstrong 2014, Eichmiller et al. 2016, Sassoubre et al. 2016, Collins et al. 2018). In other cases, the animal part being trafficked does not contain very much DNA to start with, as is the case with hard tissues like bone. Bone matrices have been shown to protect DNA from some environmental variables, but the environment can often play more of a role in DNA degradation than the age of the remains (Stray & Shewale 2013, Latham & Miller 2019). Therefore, when

bone is exposed to genetically destructive environments, extracting DNA becomes more challenging.

Most genetic identification studies concerning the quality and quantity of DNA obtained from bones after exposure to environmental conditions focus on humans and other terrestrial mammals. However maritime reptiles, such as sea turtles, are heavily trafficked for their meat and eggs. Poachers often leave sea turtle remains disarticulated and abandoned on beaches or in the water after killing the turtles (Moore et al. 2003, Joseph et al. 2014, 2016). These remains are typically not discovered immediately, which prolongs the DNA's exposure to damaging environments, but it is still vital to be able to identify which species the remains represent. In the U.S., all sea turtles are protected, but Kemp's ridleys (*Lepidochelys kempii*) are critically endangered and only extant in the Gulf of Mexico and along the Atlantic coast of the U.S. (https://www.iucn-mtsg.org/, Wibbels & Bevan 2019). However, they are closely related to olive ridleys (*Lepidochelys olivacea*), which has only one subpopulation protected by the U.S. and are commercially harvested around the world (Abreu-Grobois & Plotkin 2008). Both species migrate internationally, but conservation biologists have been able to determine and track subpopulations via mitochondrial DNA markers and sea turtles' natal homing behavior to lay eggs. This has created maternally inherited haplotypes within family groups that can be tracked back to their range of origin (Joseph et al. 2014, Patricio et al. 2017, Frandsen et al. 2020). These kinds of conservation studies largely utilize blood and tissues samples from living sea turtles for DNA extraction, rather than bony tissue, but that does not exclude skeletal remains from use for phylogeographical assignment. This means illegally trafficked turtles or remains can be linked back to their natal population and potential poaching sites can be isolated and monitored. Additionally, individual-specific DNA identifications could be used to link trafficked parts back

to skeletal remains which would show the evidence of intent to harm these protected animals. Or from a conservation standpoint, this means species remains recovered from the water or on beaches and could still be identified and related back to their population of origin. Also, future studies could then utilize animal remains, not just sampling from living individuals, broadening the sampling scope available to the researchers studying these endangered and protected animals. Successful skeletal DNA extractions after prolonged exposure to harsh environments could provide a new tool for conservation research when studying observed phenomenon that result in the demise of an animal, such as after environmental disasters or heavy storms, these identifications could highlight which subpopulations or haplotypes were more severely affected and where future preservation efforts should focus.

The purpose of this study was to 1) determine how long Kemp's ridley sea turtle bones can be exposed to marine or supratidal terrestrial environments before a genetic species identification cannot be determined, and 2) examine the quality of the DNA extracted over the course of a three-year time series by two enzymes, Proteinase K and Collagenase Type II. I used domestic cattle (*Bos taurus)* bones as a terrestrial mammal control specimen for both the length of time viable DNA can be extracted after environmental exposure and the functionality of Proteinase K (Pro K) verses Collagenase Type II (CTII) in bone demineralization. To ensure the DNA I was evaluating and measuring after extraction was from the samples, I designed DNA primers for both organisms. For the Kemp's ridley sea turtle samples, I used nuclear DNA (nuDNA) primers that amplified a species-specific region on the nuclear genome locus pdCM14 and mitochondrial DNA (mtDNA) primers that amplified a species-specific region on mitochondrial NADH dehydrogenase subunit 4 (NADH4) gene. For the domestic cattle samples, I used nuDNA primers that amplified a genus-specific region in the Melanocortin 1 receptor

(MC1R) gene and mtDNA primers that amplified a species-specific region on the mitochondrial cytochrome B (Cyt B) gene. I aimed to evaluate the following hypotheses: 1) the *B. taurus* samples will have higher quantity and quality scores overall than the *L. kempii* samples, 2) the supratidal samples from both species will have higher quantity and quality values than the subtidal samples, and 3) the CTII demineralized samples will demonstrate quantity values (ng/µL) that are comparable to the Pro K demineralized sample values, but the CTII will produce higher consensus quality scores over the time series.

Bone Structure

When utilizing skeletal remains for DNA extraction and identification, a different methodology is required because hard tissues, such as bone, consist of different materials than soft tissues, such as blood or skin. Bone is comprised of an organic and inorganic matrix and cellular structures suspended in that matrix. The organic components are proteins, type I collagen (85%-90%) and non-collagenous proteins (10%-15%), and the inorganic component is a lattice of hydroxyapatite crystals. Bone can then be categorized into two types: cortical and cancellous. Cortical bone is the hard densely packed outer layer, and cancellous is the soft spongey internal layer (Rho et al. 1998). Cortical bone can be structured in two ways: woven bone or lamellar bone. Woven bone is an irregular pattern of lamellae, long spindles of collagen and hydroxyapatite crystals, while lamellar bone is a circular pattern of lamellae-formed units called osteons (Rho et al. 1998). Within each osteon, the lamellae forms a Haversian canal (Figure 1) for blood vessel and veins to run the length of the bone and osteocytes form lacuna, gaps in the bone which house the cells (Rho et al. 1998).

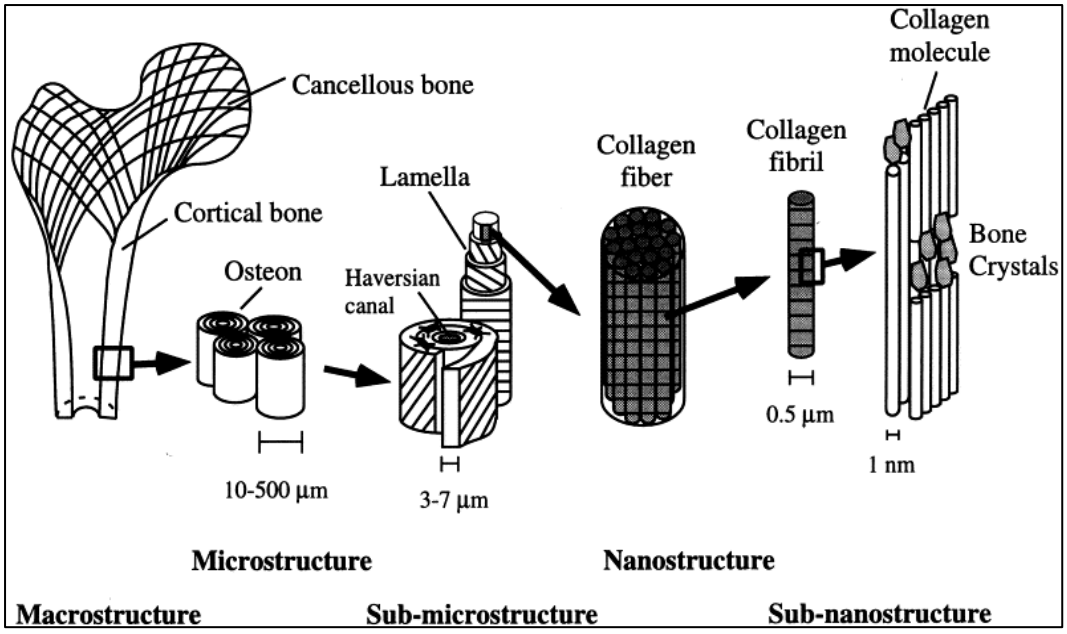

Figure 1. Illustration of the hierarchical structural organization of bone. Cortical and cancellous bone, osteons with Haversian canals, lamellae, collagen fibers, and bone mineral crystals and collagen molecules. Source: Rho et al. (1998)

Osteocytes, osteoblasts, and osteoclasts are the three major cellular structures present in bone. Osteoblasts generate bone and form the bone matrix. Osteoblasts mature into osteocytes after the bone has extended past them, and osteoclasts degrade bone. Osteocytes create canaliculi (Figure 2), a network of small transverse canals, to connect them for waste and resource transportation and are the most abundant of the three cell types (Cadena & Schweitzer 2012). Sea turtles have two morphologically distinct osteocytes: flattened osteocytes and stellate osteocytes (Cadena & Schweitzer 2012). Most of the cellular structures in bone are present in the cortical bone and therefore it is the target sample for DNA extractions (Li 2012).

Hatchling and juvenile sea turtles have a higher ratio of cartilage than bony structures, unlike terrestrial mammals. Through a process called endochondral ossification (cartilage replacement), the cartilage slowly changes into bone until adulthood, but they retain some cartilage throughout their lives (Wyneken 2013). Type II collagen is the primary protein in cartilaginous matrices and in bone matrices, 85-90% of the proteins are Type I collagen. Sea

turtles, therefore, have a higher ratio of both collagen types than other proteins in their skeletal structure, unlike terrestrial mammals.

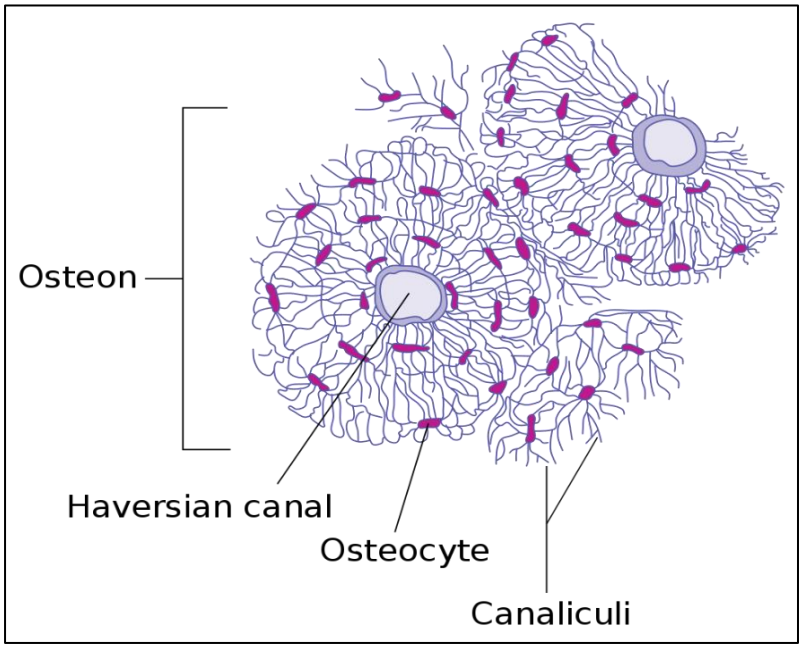

Figure 2. Illustrated diagram of an osteon. Osteocyte canaliculi network highlighted in relationship with the Haversian canal. Source: Gray (1918) colored and modified by Wikimedia Commons and BDB 2006.

Bone Demineralization

In order to extract DNA, the cells and nuclear membranes containing the DNA must be lysed; this can be accomplished by using a detergent to disrupt the lipid bilayers. In bone samples, this becomes more difficult because most bone cells are housed inside the bone matrix. The bone matrix needs to be broken down before cell lysis can begin (Loreille et al. 2007). The matrix is composed of two kinds of proteins, type I collagen and non-collagenous proteins, and hydroxyapatite crystals. Soft tissue DNA extractions rely on Proteinase K (Pro K), a broad-spectrum protease, to degrade unwanted proteins, including nucleases (Jany et al. 1986). In hard tissue DNA extractions, like bone or teeth, a metal chelating reagent, such as

Ethylenediaminetetraacetic acid (EDTA), needs to be utilized in addition to a proteinase to degrade the bone matrix and release the osteocytes into solution (Loreille et al. 2007).

Barrett (2015) demonstrated the effectiveness of Collagenase Type II (CTII), in comparison to Pro K, for hard tissue DNA extractions in human bone samples which had not been exposed to damaging environments. Collagenases, specifically bacterial collagenases, can attack almost all types of collagen and are able to make multiple cleavages at the triple helical regions present in collagen. CTII exhibits greater clostripain activity, which cleaves proteins on the carboxyl peptide bond of arginine, making it optimal to use for bone degradation (http://www.worthington-biochem.com/cls/cat.html). Barrett (2015) validated that CTII demineralization resulted in lower DNA quantities than Pro K samples, but that the extracted nuDNA was of higher quality when sampling from human bone tissues. This increase in DNA quality could be particularly useful in already heavily degraded samples. Less DNA might be obtained from the bone, but it will be of a higher quality and easier to sequence. Inversely, if the goal were to maximize the amount of DNA obtained from a sample, Pro K could be used. Because one enzyme might be more effective than the other, depending on the desired outcome, additional testing is necessary to determine the scenarios under which each enzyme should be used.

Molecular Species Identification

DNA barcoding is an important aspect of species identification in conservation biology and wildlife forensic fields because sometimes identifications need to be made from environmental DNA samples or from wildlife products that no longer possess morphological characteristics (Ogden et al. 2009, Moore & Frazier 2019). Mitochondrial DNA is more durable because of its circular structure and is found in larger quantities than nuclear DNA in all tissues,

rendering it a more reliable source for genetic identification in degraded samples (Latham & Miller 2019). Additionally, mitochondrial marker analyses are the predominant technique utilized for species identifications because these markers are maternally inherited haplotypic coding regions, meaning these regions do not vary significantly between individuals of the same species or population (Hebert et al. 2003). Cytochrome c oxidase subunit 1 (COI), Cyt B, and the control region (D-loop) are all preferred mtDNA regions to sequence when making species identifications (Moore et al. 2003, Fain et al. 2013, Mwale et al. 2017). These sequences are then compared to reference sequences, including the ones found on databases like GenBank, to determine the species identification. Tools and software programs, such as Geneious Prime and BLAST, assist in aligning the sequences to reference samples and searching databases for sequences that closely align to the unknown sample. Other regions can also be sampled, both on nuDNA and mtDNA, by designing custom PCR primers that anneal only to regions found in a specific species or by designing primers that target a region known to contain species-specific variations (Wasser et al. 2015, 2018).

In conservation biology, mtDNA identifications are used to track and monitor the health of target populations. When only using mtDNA to make species identifications, large-scale sampling studies in certain areas, either with mark and recapture sampling techniques or with environmental DNA analyses, can be used to estimate the number of individual present it a local population (Soul 1985, Moritz 1994a, Moritz 1994b, Dimitriou et al. 2017). From year to year, these repeated measurements can be used to track the status of a population, estimate the number of individuals in the population, and record any changes in their geographic range (Epps et al. 2005, Forcina et al. 2018). Certain kinds of mitochondrial variations can be used to identify the haplotypes of the sampled individuals and link them to the other members of their haplotype

through a reference library (Jackson et al. 2013, Patricio et al. 2017, Tabasum et al. 2017, Kheng et al. 2018). Typically, a migratory species, such as sea turtles, can be tracked via their mitochondrial haplotypes. This helps highlight migratory pathways, the current habitat range of the species, and determine the origins of the individuals present in mixed stock populations, like forging grounds (Bowen et al. 1993, Bolker et al. 2007, Bowen & Karl 2007, Duchene et al. 2012, Jones et al. 2018). Haplotype and species identifications can also be made on individuals found in unusual locations or harmful environments as support for observed phenomenon.

After references have been obtained and the unknowns have been aligned and trimmed to only include the target region, it becomes important to generate phylogenetic trees to determine how the unknown genetic sample will group with your known references (Moore & Frazier 2019). Using various molecular evolutionary models, these trees are constructed to demonstrate the similarities or differences present between the branches (i.e., samples or sequences). There are several ways to construct phylogenetic trees including: distance-matrix methods, maximum parsimony methods, and maximum likelihood methods. Distance-matrix models are more simplistic and calculate genetic distances (i.e., proportion of matching bases), but do not account for the causes behind the genetic similarities. Maximum parsimony and maximum likelihood trees both utilize the complete sequence to determine relationships, but maximum likelihood trees generate the most probable configuration of branches, whereas maximum parsimony assesses the fewest number of evolutionary steps required. This means that maximum likelihood trees show the most likely relationship between inputted genetic sequences, given a particular model of evolution. Bootstrapping values are often shown at each node indicating the number of times that branching point was replicated during modeling (Saitou & Imanishi 1989). Phylogenetic trees are constructed using specifically designed computational software, such as

MEGA-X, and are used to show the relationship between the genetic samples. In wildlife forensic casework, conclusions generated from these trees are not referred to as ‘matches’ because these conclusions are based around the current known literature regarding the organism of interest and not a direct comparison to a source (Moore & Frazier 2019).

Chapter 2: Methodology

Sample Collection

DNA was extracted from a time series of Kemp's ridley sea turtle (*Lepidochelys kempii*) and domestic cattle (*Bos taurus*) bone samples to determine how long these samples could be exposed to subtidal or supratidal environments and still be genetically identified, as well as, determined which enzyme, Pro K or CTII, resulted in DNA extractions of a higher quality during demineralization. I used bone samples from two younger male Kemp's ridley sea turtles, approximately one to two years old, both of whom expired naturally due to cold shock of winter onset, and one domestic cow. The Kemp's ridley sea turtle remains were recovered by a NOAA stranding/rehabilitation organization, tissue samples were recovered post-necropsy from the seasonal cold-shock victims, and their remains were used in this educational study with authorization via a NOAA ESA permit. These samples were collected, disarticulated, and prepared as documented by McElreath (2018). In conjunction with the members of the Shoals Marine Laboratory, located on Appledore Island, Maine, these samples were housed in diving bags and secured within modified lobster traps to prevent scavenging. One set of samples was submerged to a depth of 3-9m inside an island cove to protect the traps from storms and strong current waves, and to ensure that the samples would remain submerged regardless of the tide. The other set of samples was located 200m inland. The locations were chosen to represent realistic environments where these marine animal remains would typically be found. Additionally, the location surrounding the Shoals Marine Laboratory is considered a marine sanctuary which further eliminated the risk of commercial or outside interruptions for the study (McElreath 2018).

The 12 sets of Kemp's ridley bone fragments and cattle bone fragments, from all parts of the skeleton, used in this experiment were collected over a three-year period. They were sampled from the supratidal and subtidal environments twice a year, once in September and once in May/June, until 2019 when they were only collected once in September. They were shipped overnight in insulted packaging to University of Central Oklahoma for analysis and were stored at to -20 ˚C upon arriving. The bone fragments from each organism were double sealed and labeled. Including the two baseline samples collected at the beginning of the experiment, 26 bone samples were used.

Bone Preparation and Pulverization

A 0.5g portion of- the bone was removed and documented photographically with a ruler at 90˚ to the camera, then the initial mass was documented. The progenitor samples were returned to -20˚C. The primary bone samples were separated and then cleaned according to the DNA Solutions "Bone Demineralization and Isolation Protocol." A Dremel tool was utilized instead of a drill to portion out the thicker and denser domestic cow samples. All appropriate equipment was cleaned using a 10% bleach solution, followed by a 70% isopropanol solution, then treated with UV light for 15 minutes. Approximately 1mm of the surface was removed from each sample via sanding. Each sample was washed using a 5% bleach solution, rinsed 4 times with ultra-pure water, and then washed in absolute ethanol, before being left to dry in a fume hood for at least 12 hours. All surfaces were cleaned using a 10% bleach solution, followed by a 70% isopropanol solution between samples. The 0.5g samples were placed into labeled 2mL tubes and stored at -20˚C.

All appropriate equipment was sterilized and treated with UV light for 15 minutes. The samples were submerged individually into liquid nitrogen until brittle, approximately 20 minutes

for the *L. kempii* samples and 40 minutes for the *B. taurus* samples. Underneath a fume hood, each sample was pulverized with a Bone Morselizer (DDP Medical Supply, St. Petersburg, FL) until desired thickness was reached, less than 2mm in diameter. The pulverized samples were allowed to return to room temperature before being weighed and divided into two portions weighing less than 0.25g and placed into labeled 5mL tubes. In total, 52 samples were generated.

Bone Demineralization

Proteinase K Incubation

Half of each of the pulverized bone samples, approximately 0.25g or less, were prepared according to Barrett's (2015) protocol, but Animal Tissue Lysis buffer (QIAGEN) was replaced with Tissue & Cell Lysis Buffer (TCL Buffer; Epicentre/Lucigen, Wisconsin). The following was added to each sample: 750µL 0.5 M EDTA, 675µL TCL Buffer (Epicentre/Lucigen), 75µL Pro K, and 60µL of 1M DTT. The samples were vortexed thoroughly, for approximately 30 seconds. The samples were placed into a tube agitator at 56˚C for 24 hours. Two reagent blanks (RB-ProK LK and RB-ProK BT) were generated and processed with each sample set, the Kemp's ridley set and the domestic cattle set, for the duration of the experiment. The samples were left to settle for at least 5 minutes. Finally, 300µL of the supernatant was transferred into each labeled 2mL tube, rendering each sample into triplicates, and the remainder was stored at -20˚C.

Collagenase Type II Incubation

Half of each of the pulverized bone samples, approximately 0.25g or less, were prepared according to Barrett's (2015) protocol, but Animal Tissue Lysis buffer (QIAGEN) was replaced with TCL Buffer (Epicentre/Lucigen). First, CTII enzyme was prepared by combining 75mg of CTII enzyme with 250µL of Hanks Balanced Salt Solution (HBSS), allowing the CTII enzyme

to fully dissolve. Another 1.25mL of HBSS was added to bring the final concentration to 50 mg/mL. I added 675µL TCL Buffer (Epicentre/Lucigen) and 75µL 50 mg/mL CTII enzyme to each sample. The samples were vortexed for 30 seconds before being placed into a tube agitator at 37˚C for 5 hours. After incubating, 750µL 0.5 M EDTA and 60µL of 1M DTT was added to each sample, and they were vortexed for another 30 seconds. The samples were returned to a tube agitator at 56˚C for an additional 19 hours. Two reagent blanks (RB-CTII LK and RB-CTII BT) were generated and processed with each sample set, the Kemp's ridley set and the domestic cattle set, for the duration of the experiment. The samples were left to settle for at least 5 minutes. Finally, 300µL of the supernatant was transferred into each labeled 2mL tube, rendering each sample into triplicates, and the remainder was stored at -20˚C.

DNA Isolation

The 156 samples and 12 reagent blanks were prepared, isolated, and purified using the MasterPure™ Complete DNA and RNA Purification Kit (Epicentre/Lucigen) and by following the manufacture's protocol (Epicentre 2012). Samples were cooled to 37˚C and 1µL of 5 µg/µL RNAse A was added, before the samples were vortexed for 5 seconds. They were incubated in a heat block for 30 minutes at 37˚C. Samples were removed from the heat block and placed on ice for 5 minutes. I added 175µL of MPC Protein Precipitation Reagent to the sample and vortexed each tube for 10 seconds. The samples were then centrifuged at 4˚C for 10 minutes at 10,000 xg to pellet the debris. The supernatant was transferred to labeled 2mL tubes. I added 500µL of pure isopropanol to the tubes and inverted them 30-40 times. The samples were then centrifuged at 4˚C for 10 minutes at 10,000 xg to pellet the DNA. The supernatant was discarded, and the pellet washed twice with 70% ethanol. The DNA was resuspended in 100µL of TE buffer and

incubated for 30 minutes at 70˚C to denature any remaining enzymes. All samples were stored at 4˚C.

DNA Quantitation

All 156 samples and 12 reagent blanks were quantified using the Qubit 4 Fluorometer (ThermoFisher Scientific) and the Quant-iT™ PicoGreen™ dsDNA Assay Kit (Invitrogen). The working buffer was generated by combining:10uL of 20X TE buffer with 190uL of ultra-pure water and 1uL of Quant-iT™ PicoGreen® dsDNA reagent, per sample plus three. Two DNA standards were generated: the first with 10uL of ultra-pure water and 190uL of working buffer, the second with 10uL of a 1:10 dilution of Lambda DNA standard and 190uL of working buffer. I added 2uL of each sample to 198uL of the working buffer and they were measured using the dsDNA high-sensitivity assay. All results were documented, and the average was taken between the three replicates of each sample.

PCR Primer Optimization

A PCR optimization test was performed with both the nuDNA and mtDNA primers, using the samples with highest DNA concentration from either of the enzyme protocols. Failsafe buffers A-L (Epicentre/Lucigen) were evaluated for suitable PCR buffer conditions. The nuDNA primers for *L. kempii* and *B. taurus* were generated from locus pdCM14 and MC1R gene, respectively. The mtDNA primers for *L. kempii* and *B. taurus* were generated from NADH dehydrogenase subunit 4 (NADH4) gene and Cyt B gene, respectively (Table 1).

The PCR master mix for the Pro K nuDNA primers contained: 17.1µL DNA-free water, 1.2µL of 10µM forward nuclear primer, 1.2µL of 10µM reverse nuclear primer, 1µL of GoTaq® Hot Start Polymerase (Promega), 1µL of Pro K purified DNA was added if the DNA concentration was greater than 10ng/uL, per buffer A-L. I added 3.5µL of each buffer to the

tubes. If the concentration of DNA was less than 5ng/uL, then 3uL of purified DNA was used and the total amount of water adjusted accordingly to maintain a reaction volume of 25uL. The PCR master mix for the CTII nuDNA primers contained: 17.1µL DNA-free water, 1.2µL of 10µM forward nuclear primer, 1.2µL of 10µM reverse nuclear primer, 1µL of GoTaq Polymerase, 1µL of CTII purified DNA was added if the DNA concentration was greater than 10ng/uL, per buffer A-L. I added 3.5µL of each buffer. The thermocycler (Applied Biosystems, GeneAmp PCR System 9700) was set to run for 30 cycles according to Table 2.

Table 1. Nuclear (Nu) and mitochondrial (Mt) primers for *L. kempii* and *B. taurus*. All selections were made using Geneious Prime primer creation software and were modeled on GenBank references. The primers were then entered into NCBI's Primer BLAST software to confirm species specificity.

	Amplicon Length (bp)	Forward Primers	Reverse Primers
Nu, *L. kempii* pdCM14	298	5' – TTGGGCCCTGGGATTTTACAT – 3'	5' – AACGCAGTCAGTGAACAAGC – 3'
Mt, *L. kempii* NADH4	371	5' – AAGCTCATGTAGAAGCCCCA – 3'	5' – TGTTCGGCTGTGAGTTCGTT – 3'
Nu, *B. taurus* MC1R	265	5' – CCCTTACCCGATTCTTCGCT – 3'	5' – GATGTGAGGGGGTGTGTTGA – 3'
Mt, *B. taurus* Cyt B	284	5' – ACCAGCCTGCTCTTCATCAC – 3'	5' – CGAGAGGTGCAGGAAGAAGG – 3'

The PCR master mix for the Pro K mtDNA primers contained: 17.1µL DNA-free water, 1.2µL of 10µM forward mtDNA primer, 1.2µL of 10µM reverse mtDNA primer, 1µL of GoTaq Polymerase, 1µL of Pro K purified DNA was added if the DNA concentration was greater than 10ng/uL, per buffer A-L. I added 3.5µL of each buffer. If the concentration of DNA was less than 5ng/uL, then 3uL of purified DNA was used and the total amount of water adjusted accordingly to maintain a reaction volume of 25uL. The PCR master mix for the CTII mtDNA primers contained: 17.1µL DNA-free water, 1.2µL of 10µM forward mtDNA primer, 1.2µL of reverse 10µM mtDNA primer, 1µL of GoTaq Polymerase, 1µL of CTII purified DNA was added

if the DNA concentration was greater than 10ng/uL, per buffer A-L. I added 3.5µL of each buffer to the tubes. The thermocycler was set to run for 30 cycles according to Table 2.

Table 2. PCR thermocycler conditions for both *L. kempii* and *B. taurus* nuDNA and mtDNA primers.

	Start	Cycle				Final Extension	Hold
		Denature	Anneal (*L. kempii*)	Anneal (*B. taurus*)	Extension		
Temperature ˚C	94	95	55	55	72	72	4
Time	2 minutes	15 seconds	1 minute	1 minute	20 seconds	10 minutes	∞

A 1% agarose gel was created by mixing 1g of agarose with 100mL of TAE buffer, heating the solution until the agarose powder was fully incorporated. Once the gel had cooled to 50˚C, 4µL of ethidium bromide was added and the solution poured into a gel box with a well-comb in position. The gel was allowed to fully solidify and 4µL of each PCR product was mixed with 2µL of loading dye. Two wells contained 5µL of 1kb allelic ladder. The gel was run at 120V until the dye bands migrated 50% of the length of the gel, approximately 2 hours. Bands were evaluated and photographed using a gel imaging system.

After PCR optimization was achieved, all the replicates and the two reagent blanks were processed and prepped for PCR with one negative control (NC) per PCR master mix generated. Each replicate was cycled through PCR twice, once with the nuDNA primers and once with the mtDNA primers, following the PCR master mix ratios above with the optimal Failsafe buffer added into the PCR mix for all samples. The thermocycler was set to run for 30 cycles according to Table 2. For visualization, 1% agarose gels were generated as detailed above and operated under the same electrophoretic conditions. The gels were analyzed and captured using the same method.

Cycle Sequencing

The PCR products (amplicons) were cleaned up using ExoSAP-IT PCR Product Cleanup Reagent (Applied Biosystems). I added 10uL of the PCR amplicons and 4uL of the ExoSAP-IT to the tubes. The tubes were placed into the thermocycler for 15 minutes at 37˚C and then for 15 minutes at 80˚C, before being stored at 4˚C.

The PCR amplicons generated from the *L. kempii* and *B. taurus* samples were used for the cycle sequencing procedure as detailed in the BigDye™ Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems 2016). The sequencing reaction master mix for the Pro K nuDNA forward primer contained: 4µL DNA-free water, 8µL of BigDye Ready Reaction Mix (RR-100), and 4µL 3µM forward nuDNA per sample. The master mix for the Pro K nuDNA reverse primer was the same except I used 4µL 3µM reverse nuDNA primer instead. I added 4µL of Pro K nuDNA amplicons to both the forward and reverse tubes for each sample. This was repeated for the CTII nuDNA amplicons. The thermocycler was set to run for 30 cycles according to Table 3.

Table 3. Cycle sequencing thermocycler conditions for both the *L. kempii* and *B. taurus* nuDNA and mtDNA primers.

	Start	Cycle				Hold
		Denature	Anneal (*L. kempii)*	Anneal (*B. taurus)*	Extension	
Temperature ˚C	96	96	55	55	60	4
Time	1 minutes	10 seconds	5 seconds	5 seconds	4 minutes	∞

The sequencing reaction master mix for the Pro K mtDNA forward primer contained: 4µL DNA-free water, 8µL of RR-100, and 4µL 3µM forward mtDNA per sample. The sequencing reaction master mix for the Pro K mtDNA reverse primer was the same except I used 4µL 3µM reverse mtDNA primer instead. I added 4µL of Pro K mtDNA amplicons to both the forward and reverse tubes for each sample. This was repeated for the CTII mtDNA amplicons. The thermocycler was set to run for 30 cycles according to Table 3.

The samples were all purified with Performa® Spin Columns (EdgeBio). The gel spin columns were centrifuged at 850 amps for 5 minutes. The columns were transferred to sterile tubes and 20μL of the cycle sequence amplicons was added. They were centrifuged at 850 amps it for 5 minutes and the elute was retained.

A plate map was generated for each of the 96-well plates that were used on the genetic analyzer. I added 10μL of Hi-Di formamide and 10μL of the cycle sequence amplicons to the corresponding well as indicated by the plate map. I added 10μL of DNA standard from the BigDye 3.1v kit into two wells for redundancy. I added 10μL of Hi-Di formamide to a well as a negative control. The plate was sealed and then centrifuged briefly to remove any bubbles or trapped air in the solution. The plate was placed onto the Applied Biosystems 3500 Genetic Analyzer.

The Genetic Analyzer was set up for cycle sequencing data collection with a 36cm 8-capillary array, using POP7 polymer. The instrument protocol was set for Short Sequencing (200-300bp), running module FastSeq36_POP7, with dye set Z. I used following settings modified from default: 2480secs run time, 8.5kV run voltage, 1.2kV injection voltage, and 520secs data delay. The primary analysis protocol was set for BDTv3.1PA_Protocol-POP7 and was unmodified. No secondary analysis protocol was selected.

Analysis

The sequencing results were opened and aligned in Geneious Prime software v2019.2.3 (Kearse et al. 2012), utilizing the Geneious Alignment tool with default settings. The alignments were manually trimmed to exclude the primer regions and the consensus sequences were extracted (Appendix 1). A selection of reference gene regions was collected from GenBank, aligned with my unknowns, and trimmed to the appropriate length for further comparison. These

references were chosen primarily based around the NCBI's reference genomes, all other references were first aligned to the NCBI's reference and then ran through BLAST, while excluding the knowns accession number to confirm the identity of the sequence. Aligned sequences were transferred to MEGA-X software (Kumar et al. 2018) and the Best DNA/Protein model finder was used with default settings to determine the best evolutionary model that fit these sequences. Maximum likelihood trees were estimated with the most appropriate evolutionary model and were generated with bootstrap resampling 1000 times using the MEGA-X software (Kumar et al. 2018). The quality scores of each sample's untrimmed consensus sequence were opened using FASTQC (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/) and then compiled using the MulitQC software (Ewels et al. 2016) to generate phred score graphs for each sequence at every nucleotide (Appendix 2). For each sample, the phred scores were gathered from each sequence and averaged. The averages for each organism were then compared to look for statistical significance using repeated measure ANCOVAs between the quality of the samples using IBM SPSS Statistics software (https://www.ibm.com/products/spss-statistics). The quantity values were also averaged and inputted into SPSS Statistics software and were normalized using $\log_{10}$ for further ANOVA analyses. For both the quality and the quantity data, the two organism's data were pooled to make inter-species comparisons. Using these calculations and comparisons, I anticipated that subtidal samples would have degraded faster than the supratidal samples due to the degenerative properties of saltwater. I also expected Collagenase Type II to be comparable to Pro K in DNA quantity and demonstrate a higher level of DNA quality, especially for the more cartilaginous sea turtle bones.

Chapter 3: Results

DNA Quantitation

The quantitation results were recorded for each triplicate and then averaged, as listed in Table 4. The reagent blanks ran with the *L. kempii* CTII set, the *B. taurus* Pro K set, and the *B. taurus* CTII set had one or more replicates produce low concentration quantity results. Additionally, a majority of sample 091019Te *L. kempii* CTII was lost during incubation; a new bone fragment was cleaned, pulverized, and demineralized. Overall, the *L. kempii* samples produced higher DNA quantity results than the *B. taurus* samples. The averages were than taken for each sample (Figures 3 & 4). The *L. kempii* oceanic samples were consistently lower at every sample period than the terrestrial samples; however, they demonstrated less fluctuation between exposure dates. This trend was consistent with both the Pro K and the CTII data sets. In both the Pro K and CTII terrestrial data sets, the quantity values increased over time until time point 664 and 424 days, respectively, when they began to decrease (Figure 3). Both enzyme treatments for the *B. taurus* oceanic samples were also consistently lower at every sample period than the terrestrial samples, and the oceanic samples demonstrated less fluctuation between exposure dates. The *B. taurus* terrestrial samples also peaked with both enzyme treatments at the 323-day time point (Figure 4).

Table 4. DNA quantities from individual replicates and averages per samples from each of the following data sets: *L. kempii* Pro K, *L. kempii* CTII, *B. taurus* Pro K, and *B. taurus* CTII. All zero scores were entered for Too Low to Count results. The standard deviations (Std Dev) are listed towards the left each average. A majority of sample 091019Te *L. kempii* CTII was lost during incubation and only one allocation was recovered. A new bone fragment was processed for replicates A-C. Reagent Blanks (RB) ran with the *L. kempii* CTII set, the *B. taurus* Pro K set, and the *B. taurus* CTII contained recordable volumes of DNA. All data was measured using a Qubit 4 Fluorometer.

Sample	Exposure (Days)	Replicate	Quantity (ng/µL)				Average Quantity (ng/µL)							
			L. kempii Pro K	*L. kempii* CTII	*B. taurus* Pro K	*B. taurus* CTII	*L. kempii* Pro K	Std Dev	*L. kempii* CTII	Std Dev	*B. taurus* Pro K	Std Dev	*B. taurus* CTII	Std Dev
RB	NA	A	0.000	0.086	0.000	0.118	0.00	0.00	0.07	0.01	0.07	0.12	0.13	0.03
		B	0.000	0.073	0.000	0.166								
		C	0.000	0.060	0.209	0.115								
071616 Baseline	0	A	29.800	11.200	0.113	1.530	32.93	4.20	8.25	2.86	1.07	0.87	1.45	0.10
		B	31.300	8.060	1.830	1.340								
		C	37.700	5.480	1.260	1.480								
091516 Terra	61	A	22.800	13.200	16.200	12.400	22.63	4.55	13.00	0.35	13.79	4.61	11.37	1.05
		B	27.100	13.200	8.480	10.300								
		C	18.000	12.600	16.700	11.400								
092116 Ocean	67	A	1.590	1.790	0.100	0.339	1.69	0.13	2.18	0.85	0.07	0.06	0.33	0.02
		B	1.830	1.600	0.113	0.302								
		C	1.640	3.160	0.000	0.341								
060417 Terra	323	A	21.800	19.600	29.900	14.900	21.40	2.92	22.60	3.75	22.75	14.4	14.27	2.03
		B	18.300	21.400	32.200	15.900								
		C	24.100	26.800	6.140	12.000								
060417 Ocean	323	A	1.770	1.570	0.143	0.764	1.58	0.51	1.79	0.19	0.35	0.18	0.85	0.20
		B	1.960	1.910	0.432	1.080								
		C	1.000	1.890	0.462	0.720								
091317 Terra	424	A	34.400	43.000	5.170	7.700	36.80	2.88	45.67	2.36	7.93	2.63	6.69	0.89
		B	40.000	46.500	10.400	6.350								
		C	36.000	47.500	8.230	6.030								
091317 Ocean	424	A	1.740	3.450	5.640	5.160	2.58	0.82	3.55	0.09	4.27	2.07	5.12	0.17
		B	3.380	3.630	1.890	4.930								
		C	2.630	3.560	5.270	5.270								
051118 Terra	664	A	31.800	24.000	12.900	3.650	46.27	12.6	24.13	0.42	10.42	3.71	3.19	0.43
		B	52.000	23.800	6.150	3.140								
		C	55.000	24.600	12.200	2.790								
051118 Ocean	664	A	2.780	4.540	0.000	0.322	3.43	0.57	4.24	0.29	0.15	0.13	0.27	0.09
		B	3.800	3.970	0.239	0.318								
		C	3.720	4.200	0.201	0.162								
091118 Terra	787	A	17.500	19.500	5.110	3.330	15.87	2.10	22.17	3.71	8.15	2.86	3.60	0.45
		B	16.600	20.600	8.530	3.340								
		C	13.500	26.400	10.800	4.120								
091118 Ocean	787	A	3.240	3.640	0.324	0.396	3.26	0.45	3.58	0.07	0.35	0.12	0.37	0.05
		B	3.720	3.600	0.483	0.315								
		C	2.820	3.500	0.257	0.396								
091019 Terra	1151	A	15.700	29.300	3.600	1.950	15.50	0.20	30.50	1.59	3.27	0.82	2.00	0.08
		B	15.300	32.300	2.330	1.960								
		C	15.500	29.900	3.870	2.100								
091119 Ocean	1152	A	3.540	3.310	0.120	0.499	3.69	1.00	3.94	0.57	0.35	0.40	0.64	0.18
		B	4.760	4.430	0.115	0.571								
		C	2.770	4.070	0.806	0.847								

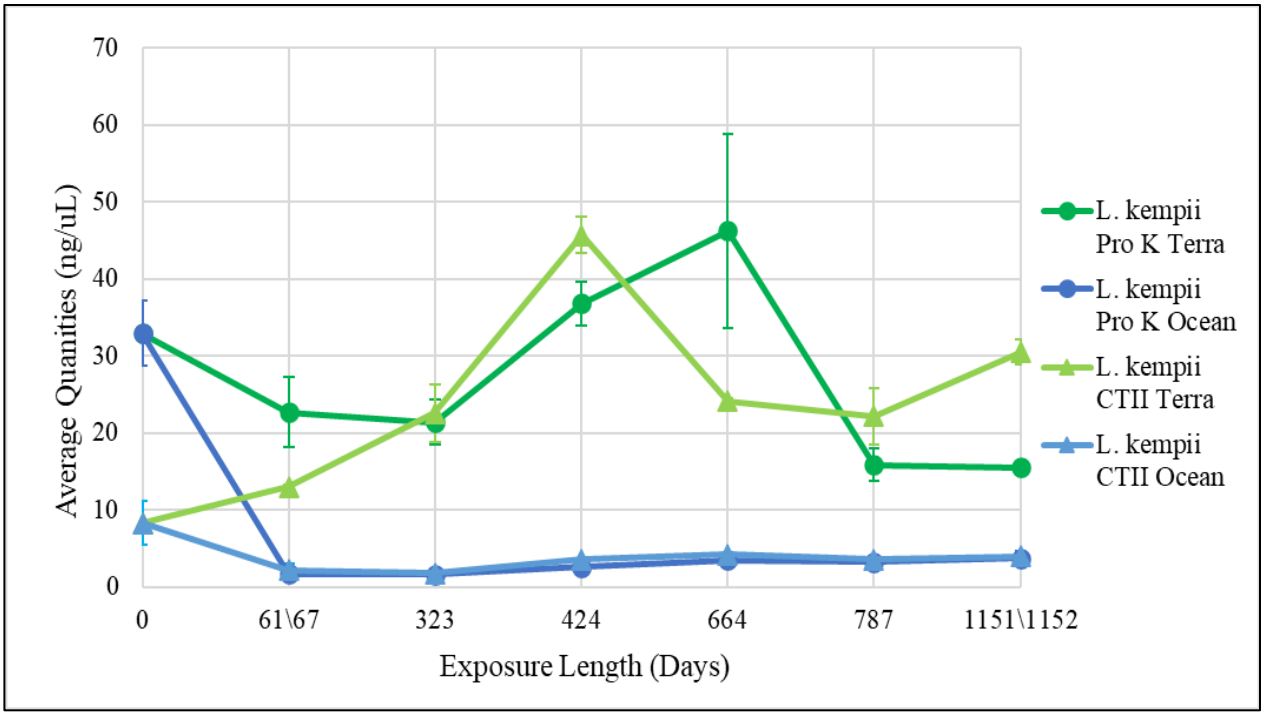

Figure 3. Average DNA quantities recorded for the *L. kempii* data sets. Exposure length is recorded in days along the horizontal axis. 61/67 represents the exposure length for the terrestrial (Terra) samples and the oceanic (Ocean) samples, respectively. This applies to the 1151/1152 listing as well. The average DNA quantities are recorded in ng/uL along the vertical axis. Error bars represent one standard deviation from the reported average.

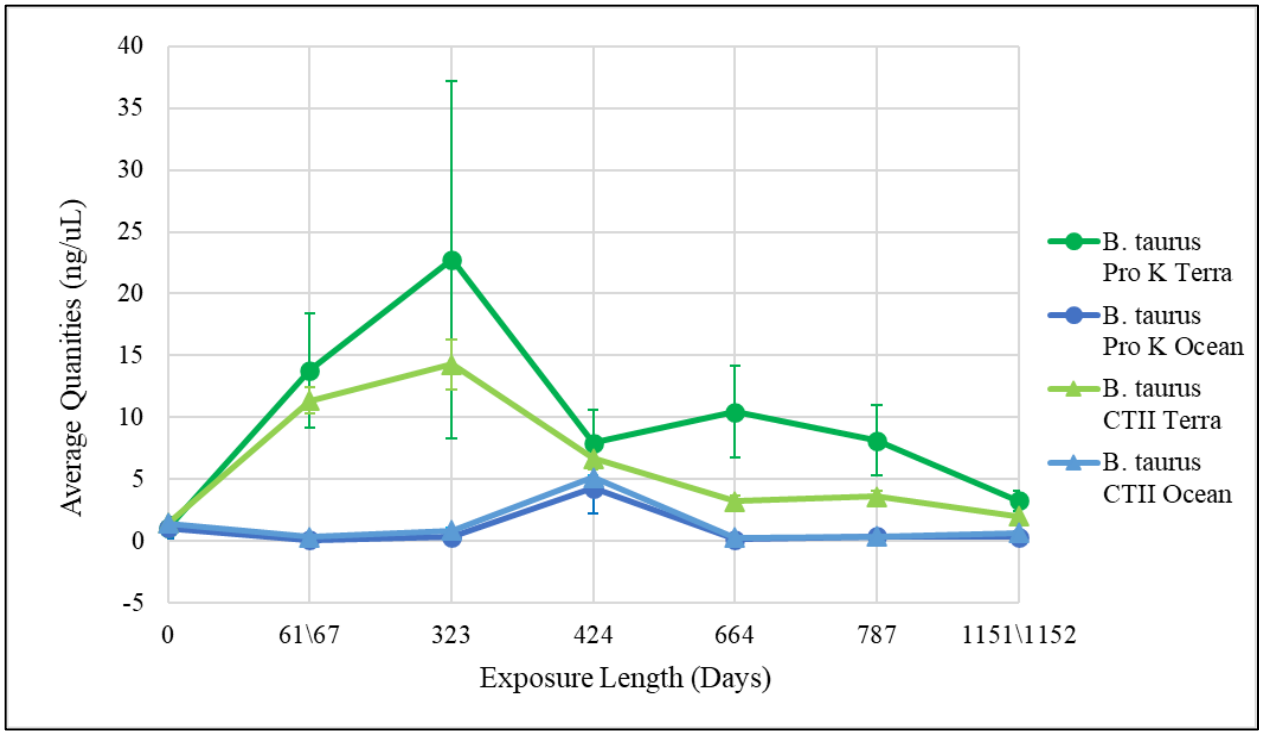

Figure 4. Average DNA quantities recorded for the *B. taurus* data sets. Exposure length is recorded in days along the horizontal axis. 61/67 represents the exposure length for the terrestrial (Terra) samples and the oceanic (Ocean) samples, respectively. This applies to the 1151/1152 listing as well. The average DNA quantities are recorded in ng/uL along the vertical axis. Error bars represent one standard deviation from the reported average.

Several repeated measures ANCOVA tests were used to evaluate the individual data sets, plus the baseline samples (*L. kempii* terrestrial and oceanic amplicons, *B. taurus* terrestrial and oceanic amplicons), using the $\log_{10}$ of the averages acquired from the triplicate samples. The baseline sample of each organism was duplicated so it could serve as the starting point for both locations. For the terrestrial and oceanic *L. kempii* samples, the effect of the enzyme type on DNA quantity was significant (p-value = 0.031). However, exposure length had an effect on the DNA quantity recovered by both enzyme treatments (p-value = 0.028), but the location of the bone samples (terrestrial or oceanic) did not have a significant effect on the DNA quantity recovered by both enzyme treatments (p-value = 0.640) (Table 5a). Looking at the between group effects of exposure length and location on the treatments for the terrestrial and oceanic *L. kempii* samples, exposure length did not account for a significant amount of variation between the groups (p-value = 0.925), but the location of the bone samples did (p-value = 0.000) (Table 5a). Using these p-values, I can conclude that Pro K had a higher mean than CTII in both environments (Table 5b).

For the terrestrial and oceanic *B. taurus* samples, the effect of the enzyme type on DNA quantity was not significant (p-value = 0.085). The exposure length did not have a significant effect on the DNA quantity recovered by both enzyme treatments (p-value = 0.090), but the location (terrestrial or oceanic) of the bone samples did (p-value = 0.001) (Table 6a). The exposure length did not account for a significant amount of variation between groups (p-value = 0.637), but the location of the bone samples did (p-value = 0.002) (Table 6a). Using these p-values, I can conclude that Pro K had a higher mean than CTII in both environments (Table 6b).

Table 6. Repeat measures ANCOVA tables for the *L. kempii* DNA quanity data sets. The α value is 0.05. A) Tests of within-subject constraints. The within subjects analysis comparing the results at each collection date between the ProK and CTII results. The Enzyme row is only comparing the Pro K and CTII without the effects of Exposure (exposure length) and Location (terrarestrial or oceanic). Tests of between-subject effects. The between subjects analysis comparing the effects of Exposure (exposure length) and Location (terrarestrial or oceanic) with the enzyme treatment groups averaged together at each collection date. B) Descriptive statistics. The means and standard deviation recorded and used for the ANCOVA tables. The Pro K averages row is the results from the log10 transformation of the Pro K data, it is then divided by location. The CTII averages row is the results from the $\log_{10}$ transformation of the CTII data, it is then divided by location.

A

Source	Sum of Squares	df	Mean Square	F	p-values	Partial Eta^2
Within-Subjects						
Enzyme	0.173	1	0.173	6.152	0.031	0.359
Enzyme*Exposure Length	0.179	1	0.179	6.379	0.028	0.367
Enzyme*Location	0.007	1	0.007	0.232	0.64	0.021
Error	0.309	11	0.028			
Between-Subjects						
Intercept	9.926	1	9.926	71.56	0.000	0.867
Exposure Length	0.001	1	0.001	0.009	0.925	0.001
Location	4.541	1	4.541	32.74	0.000	0.748
Error	1.526	11	0.139			

B

	Location	Mean	Std Dev	N
Pro K Averages	Terra	1.4034	0.1838	7
	Ocean	0.5673	0.4442	7
	Total	0.9854	0.5431	14
CTII Averages	Terra	1.3224	0.2429	7
	Ocean	0.5477	0.2139	7
	Total	0.9351	0.4582	14

Table 5. Repeat measures ANCOVA tables for the *B. taurus* DNA quanity data sets. The α value is 0.05. A) Tests of within-subject constrasts. The within subjects analysis comparing the results at each collection date between the ProK and CTII results. The Enzyme row is only comparing the Pro K and CTII without the effects of Exposure (exposure length) and Location (terrarestrial or oceanic). Tests of between-subjects effects. The between subjects analysis comparing the effects of Exposure (exposure length) and Location (terrarestrial or oceanic) with the enzyme treatment groups averaged together at each collection date. C) Descriptive statistics. The means and standard deviation recorded and used for the ANCOVA tables. The Pro K averages row is the results from the $\log_{10}$ transformation of the Pro K data, it is then divided by location. The CTII averages row is the results from the $\log_{10}$ transformation of the CTII data, it is then divided by location.

A

Source	Sum of Squares	df	Mean Square	F	p-values	Partial Eta^2
Within-Subjects						
Enzyme	0.068	1	0.068	3.583	0.085	0.246
Enzyme*Exposure Length	0.066	1	0.066	3.462	0.090	0.239
Enzyme*Location	0.348	1	0.348	18.29	0.001	0.624
Error	0.210	11	0.019			
Between-Subjects						
Intercept	1.100	1	1.1	2.502	0.142	0.185
Exposure Length	0.103	1	0.103	0.235	0.637	0.021
Location	7.003	1	7.003	15.93	0.002	0.591
Error	4.837	11	0.44			

B

	Location	Mean	Std Dev	N
Pro K Averages	Terra	0.8381	0.44028	7
	Ocean	-0.3851	0.5776	7
	Total	0.2265	0.80395	14
CTII Averages	Terra	0.6513	0.37421	7
	Ocean	-0.1261	0.44917	7
	Total	0.2626	0.5661	14

Lastly, when comparing DNA quantities of the *L. kempii* and *B. taurus* samples, a one-way ANOVA was used to evaluate the joint data, using the $\log_{10}$ of the averages acquired from the triplicate samples. For both the Pro K and the CTII treatments, the difference between the *L. kempii* and *B. taurus* samples was statistically significant, with p-values of 0.007 and 0.002, respectively (Table 7a). Using these p-values, I can conclude that the greater *L. kempii* (sea turtle) means in both the PK (Pro K) and CT (CTII) rows are significantly larger than the *B. taurus* (cow) means (Table 7b), which supports the visual observations made from Table 4.

Table 7. ANOVA table (A) and descriptive statsistics (B) for the combined *L. kempii* and *B. taurus* DNA quantity data sets. The α value is 0.05. A) The ANOVA results for the combined organism data set for the log10 transformed average quanities. Pro K averages row shows the differences between the *L. kempii* and *B. taurus* Pro K samples. CTII averages row shows the differences between the *L. kempii* and *B. taurus* CTII samples. B) The means and standard deviation recorded and used for the ANOVA tables. The Pro K averages row is the results from the $\log_{10}$ transformation of the Pro K data, it is then divided by organism. The CTII averages row is the results from the $\log_{10}$ transformation of the CTII data, it is then divided by organims.

A

		Sum of Squares	df	Mean Square	F	p-values
Pro K Averages	Between	3.765	1	3.765	8.57	0.007
	Within	11.423	26	0.439		
	Total	15.188	27			
CTII Averages	Between	3.143	1	3.143	11.91	0.002
	Within	6.86	26	0.264		
	Total	10.003	27			

B

	Organism	Mean	Std Dev	N
Pro K Averages	*L. kempii*	0.9854	0.543	14
	B. taurus	0.252	0.7641	14
	Total	0.6187	0.75	28
CTII Averages	*L. kempii*	0.9326	0.4551	14
	B. taurus	0.2625	0.5662	14
	Total	0.5976	0.6087	28

Gel Electrophoresis

For primer optimization, the samples with the highest concentration, or the baselines, were amplified with both the nuclear and the mitochondrial DNA primers for *L. kempii* and *B. taurus* sample sets. The MC1R primers for *B. taurus* produced bands with mild to moderate smearing with all failsafe buffers, except failsafe buffer G, and the generated amplicons were carried through the remainder of the experiment (Figure 5). The pdCM14 primers for *L. kempii* either generated long smears with all 12 failsafe buffers (A-L) or failed to produce bands (Figure

6). The mtDNA primers for *L. kempii* (NADH4) and *B. taurus* (Cyt B) generated bands at the expected lengths and the generated amplicons were carried through the remainder of the experiment, using buffers K and I. All reagent blank triplicates did not generate bands after amplification with the NADH4 primers for *L. kempii* or after amplification with the MC1R and Cyt B primers for *B. taurus*. All mtDNA amplicons for both *L. kempii* (NADH4) and *B. taurus* (Cyt B) were sequenced, forward and reverse, regardless of band intensity. Only *B. taurus* MC1R amplicons were sequenced, forward and reverse, regardless of band intensity.

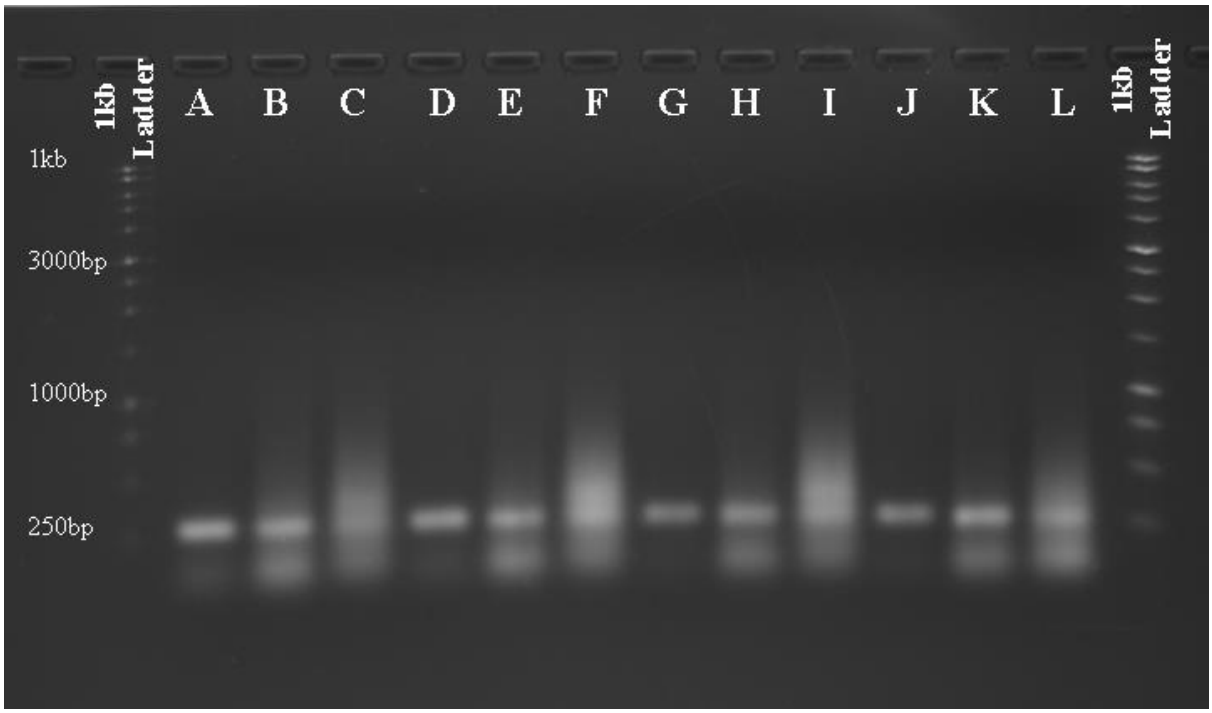

Figure 3. Primer optimization with Pro K 060417Te sample, replicate B. 1% agarose gels post electrophoresis for MC1R *B. taurus* primer optimization. A-L are the different failsafe buffers tested. The 1kb ladder is labeled at key regions. Failsafe buffers A, D, G, and J produced minimally smeared bands at the correct lengths.

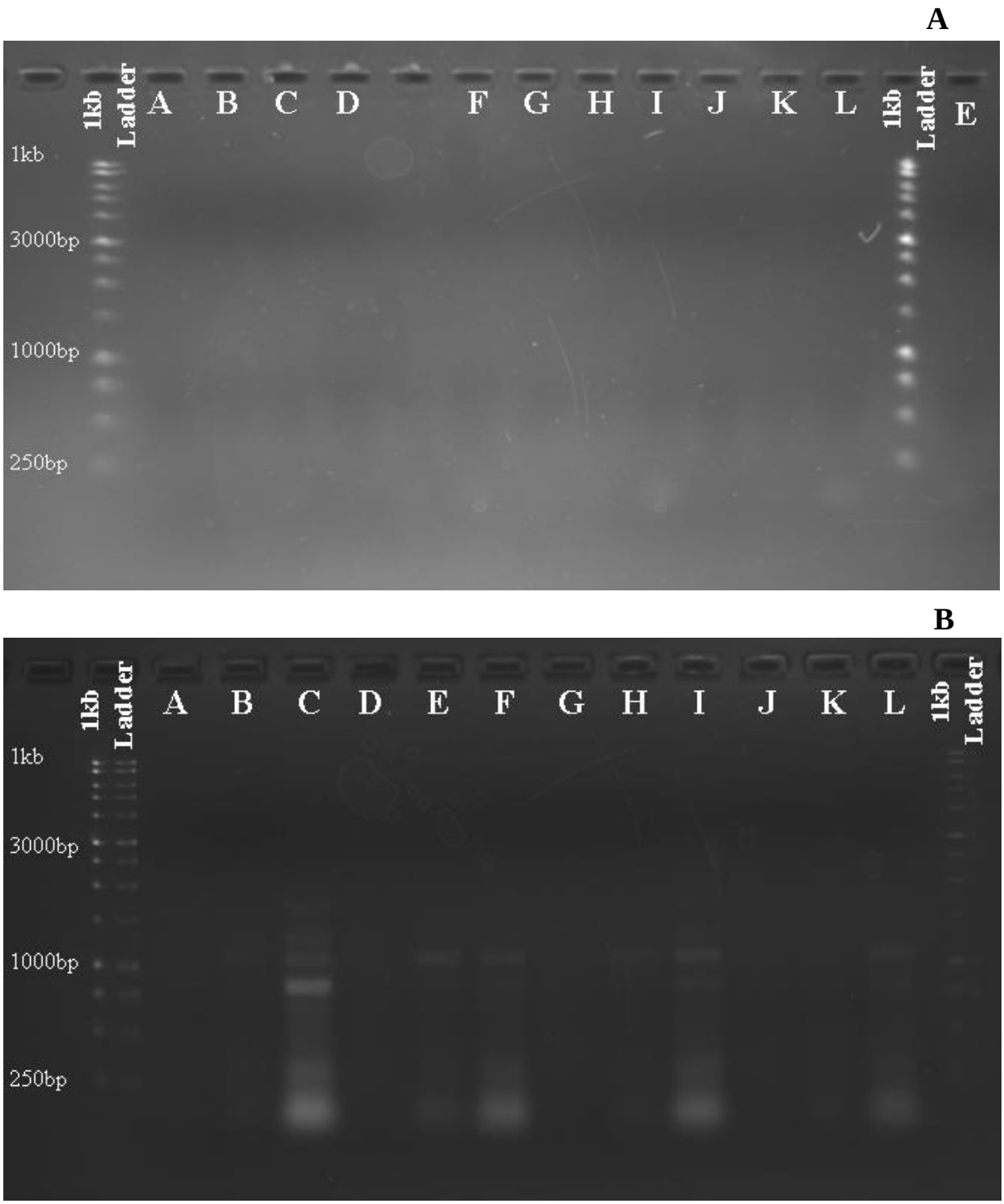

Figure 4. 1% agarose gels post electrophoresis for pdCM14 *L. kempii* primer optimization. A-L are the different failsafe buffers tested. The 1kb ladder is labeled at key regions. A) Primer optimization with *L. kempii* CTII 071616 baseline sample. All buffers failed to produce bands. B) Primer optimization with *L. kempii* Pro K 060417Te sample, replicate B. Failsafe buffers C, E, F, I, and L produced banding, but all the bands were smeared and segmented in the incorrect locations.

DNA Quality

The forward and reverse sequence phred scores for each triplicate were recorded and are provided in Table 8. For the *L. kempii* NADH4 amplicons, the following samples did not generate useable sequence data from all replicates, forward and reverse, to make a consensus sequence: 051118 Oceanic Pro K & CTII, 091019 Terrestrial Pro K & CTII, and 091119 Oceanic CTII. The amplicons were either of too low quality to sequence or absent as detected in the gel images. Figure 7 depicts the average phred scores for each *L. kempii* NADH4 amplicon and a decreasing trend over time can be observed in all sample sets; however, as the exposure length increases, the sample quality becomes more variable between enzyme treatments. All the *B. taurus* Cyt B amplicons produced enough useable sequence data to construct consensus sequences. Only the following *B. taurus* MC1R amplicons produced enough useable sequence data from all replicates, forward and reverse, to make consensus sequences: 091516 Terra Pro K & CTII, 060417 Terra Pro K & CTII, 091317 Terra Pro K & CTII, 051118 Terra CTII, and 091118 Terra Pro K & CTII. All other amplicons were either of too low quality to sequence or absent as detected in the gel images. Figure 8 depicts the average phred scores for each *B. taurus* Cyt B and MC1R amplicon for both enzyme treatments. A downward trend can be observed over time across all sample sets as the exposure length increases, but more of the *B. taurus* samples approach the questionable range (28-20) for phred scores than the *L. kempii* samples.

Table 8. DNA quality scores from individual replicates and averages per samples from each of the following data sets: *L. kempii* NADH4 Pro K, *L. kempii* NADH4 CTII, *B. taurus* Cyt B Pro K, *B. taurus* Cyt B CTII, *B. taurus* MC1R Pro K, and *B. taurus* MC1R CTII. All blanks represent failed sequencing results. The standard deviations (Std Dev) are listed towards the left each average. All reagent blanks did not produce sequencing data and were left out of the table. All sequences were detected by an ABI 3500 genetic analyzer. The sequences were aligned in Geneious software and converted to FASTQ files. The FASTQ files were opened using FASTQC and MultiQC and the phred scores were recorded. A larger version of this table can be found in Appendix 3.

Sample	Exposure (Days)	Quality (Phred Scores)						Average Quality (Phred Scores)											
		L. kempii NADH4 Pro K	*L. kempii* NADH4 CTII	*B. taurus* Cyt B Pro K	*B. taurus* Cyt B CTII	*B. taurus* MC1R Pro K	*B. taurus* MC1R CTII	*L. kempii* NADH4 Pro K	Std Dev	*L. kempii* NADH4 CTII	Std Dev	*B. taurus* Cyt B Pro K	Std Dev	*B. taurus* Cyt B CTII	Std Dev	*B. taurus* MC1R Pro K	Std Dev	*B. taurus* MC1R CTII	Std Dev
071616 Baseline	0	48	46	41	43			46.7	1.0	46.7	1.0	40.3	0.8	30.0	14.2				
		48	46	41	43														
		46	46	41	43														
		46	46	40	17														
		46	48	40	17														
		46	48	39	17														
091516 Terra	61	48	41	41	43	43	44	46.5	1.8	44.0	1.8	39.0	1.9	31.5	12.7	42.3	1.2	43.7	0.5
		48	43	41	43	43	44												
		48	44	40	43	43	44												
		46	45	38	22	43	44												
		45	45	37	21	42	43												
		44	46	37	17	40	43												
092116 Ocean	67	44	47	40	42			41.5	2.3	45.0	3.5	36.7	3.7	27.8	15.2				
		43	47	40	42														
		43	47	40	41														
		41	46	34	15														
		40	45	33	14														
		38	38	33	13														
060417 Terra	323	48	48	41	44	43	44	46.7	1.5	46.5	1.0	40.0	1.7	32.3	12.1	42.8	0.4	43.5	0.8
		48	47	41	43	43	44												
		48	47	41	43	43	44												
		46	46	41	22	43	44												
		45	46	39	22	43	43												
		45	45	37	20	42	42												
060417 Ocean	323	49	47	39	42			44.2	6.2	46.5	0.5	34.5	4.4	29.0	14.3				
		47	47	39	42														
		47	47	35	42														
		46	46	35	17														
		44	46	31	16														
		32	46	28	15														
091317 Terra	424	48	48	44	43	41	43	38.2	10.2	46.8	1.3	34.8	12.5	31.7	12.1	38.8	2.4	41.0	2.3
		47	48	44	43	41	43												
		47	48	43	42	40	42												
		31	46	40	22	39	41												
		30	46	22	21	37	40												
		26	45	16	19	35	37												
091317 Ocean	424	46	44	43	42			38.0	8.0	41.0	2.8	30.0	13.2	27.7	15.0				
		45	43	42	41														
		45	43	41	41														
		31	40	19	15														
		31	39	18	14														
		30	37	17	13														
051118 Terra	664	49	45	43	42		37	40.2	9.0	43.7	1.0	33.3	11.0	29.8	13.3			30.7	6.0
		48	44	43	42		36												
		48	44	43	42		33												
		33	44	29	19		30												
		32	43	22	17		27												
		31	42	20	17		21												
051118 Ocean	664			42	41							26.8	15.2	26.2	14.2				
				42	40														
				38	36														
				13	14														
				13	13														
				13	13														
091118 Terra	787	48	46	44	43	28	41	46.5	1.0	45.3	0.8	37.2	6.8	29.2	13.7	38.0	5.8	31.2	11.5
		47	46	44	41	38	39												
		47	46	42	41	40	37												
		46	45	32	18	42	37												
		46	45	31	16	42	17												
		45	44	30	16		16												
091118 Ocean	787	47	44	43	43			44.5	2.2	36.8	8.6	29.0	14.6	28.8	15.2				
		46	44	42	43														
		45	41	42	42														
		45	40	17	15														
		43	27	15	15														
		41	25	15	15														
091019 Terra	1151			42	44							33.2	9.3	31.5	13.0				
				42	44														
				41	42														
				25	20														
				25	20														
				24	19														
091119 Ocean	1152	47		42	43			44.8	1.6			29.0	12.8	28.7	15.1				
		46		40	42														
		45		39	42														
		45		23	18														
		43		15	14														
		43		15	13														

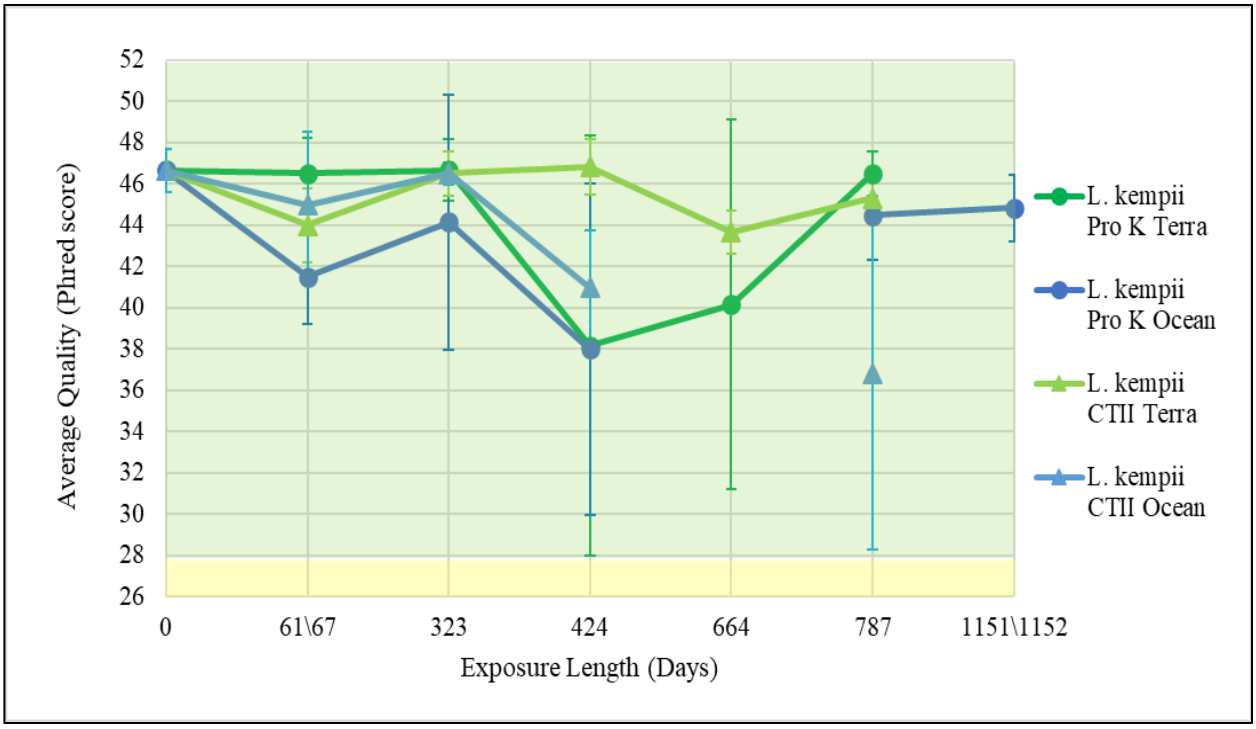

Figure 5. Average phred scores recorded for the *L. kempii* NADH4 data sets. Exposure length is recorded in days along the horizontal axis. 61/67 represents the exposure length for the terrestrial (Terra) samples and the oceanic (Ocean) samples, respectively. This applies to the 1151/1152 listing as well. The average DNA quality scores are recorded along the vertical axis. The background color shows the divisions typical of phred scores quality. The green background indicates "good" phred scores (>28) and yellow indicates "questionable" phred scores (28 to 10). The following samples did not generate useable sequence data: Day 664 Oceanic Pro K & CTII, Day 1151 Terrestrial Pro K & CTII, and Day 1152 Oceanic CTII. Error bars represent one standard deviation from the reported average.

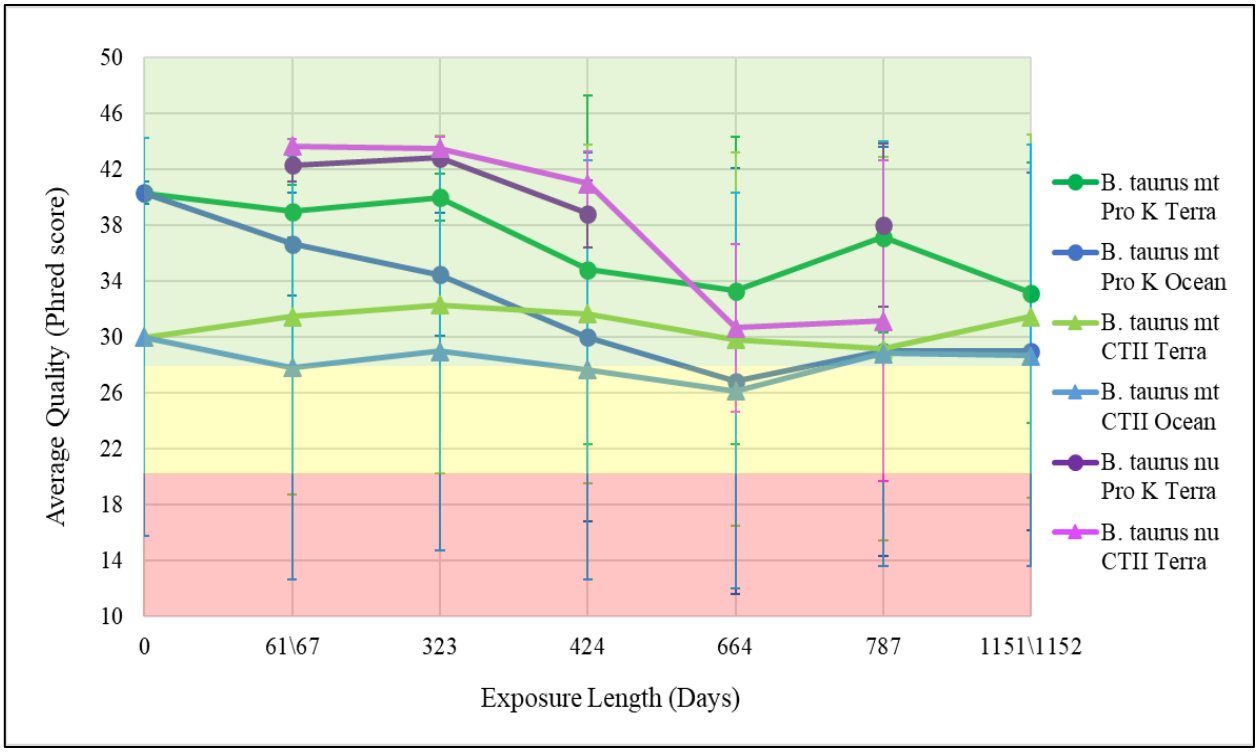

Figure 6. Average phred scores recorded for the *B. taurus* MC1R and Cyt B data sets. Exposure length is recorded in days along the horizontal axis. 61/67 represents the exposure length for the terrestrial (Terra) samples and the oceanic (Ocean) samples, respectively. This applies to the 1151/1152 listing as well. The average DNA quality scores are recorded along the vertical axis. The background color shows the divisions typical of phred scores quality. The green background indicates "good" phred scores (>28), yellow indicates "questionable" phred scores (28 to 10), and red indicates "poor" phred scores (<10). Only the following *B. taurus* MC1R samples produced enough useable sequence data to make consensus sequences: Day 61 Terra Pro K & CTII, Day 323 Terra Pro K & CTII, Day 424 Terra Pro K & CTII, Day 664 Terra CTII, and Day 787 Terra Pro K & CTII. Error bars represent one standard deviation from the reported average.

As with the quantity data, repeated measures ANCOVA tests were utilized to analyze the effects the enzyme treatments had on each sample sets (NADH4 *L. kempii* terrestrial and oceanic, Cyt B *B. taurus* terrestrial and oceanic, MC1R *B. taurus* terrestrial and oceanic), while taking environmental exposure length into account. The baseline sample of each organism was duplicated so it could serve as the starting point for both locations. The missing quality scores for the failed sequences were recorded as zeros for statistical analysis. For the terrestrial and oceanic *L. kempii* NADH4 samples, the effect of the enzyme type on sequence quality was not significant (p-value = 0.279). Neither exposure length (p-value = 0.059) or the location (terrestrial or oceanic) of the bone samples (p-value = 0.238) had a significant effect on the sequence quality

produced by both enzyme treatments (Table 9a). The exposure length accounted for a statistically significant amount of variation regardless of the enzyme treatment (p-value = 0.014), but the location of the bone samples did not (p-value = 0.551) (Table 9a). Using these p-values, I can conclude that Pro K and CTII do not have significantly different means in both environments (Table 9b).

Table 9. Repeat measures ANCOVA tables for the *L. kempii* NADH4 quality data sets. The α value is 0.05. A) Tests of within-subjects contrasts. The within subjects analysis comparing the results at each collection date between the Pro K and CTII results. The Enzyme row is only comparing the Pro K and CTII without the effects of Exposure (exposure length) and Location (terrarestrial or oceanic). Tests of between-subjects effects. The between subjects analysis comparing the effects of Exposure (exposure length) and Location (terrarestrial or oceanic) with the enzyme treatment groups averaged together at each collection date. B) Descriptive statistics. The means and standard deviation recorded and used for the ANCOVA tables. The Pro K averages row is the results from the Pro K data, it is then divided by location. The CTII averages row is the results from the CTII data, it is then divided by location.

A

Source	Sum of Squares	df	Mean Squares	F	p-values	Partial Eta^2
Within-Subjects						
Enzyme	79.898	1	78.898	1.3	0.279	0.105
Enzyme*Exposure Length	274.087	1	274.087	4.45	0.059	0.288
Enzyme*Location	95.773	1	95.773	1.55	0.238	0.124
Error	678.109	11	61.646			
Between-Subjects						
Intercept	25930.262	1	25930.26	72.2	0.000	0.868
Exposure Length	3016.992	1	3016.992	8.400	0.014	0.433
Location	136.037	1	136.037	0.38	0.551	0.033
Error	3950.964	11	359.179			

B

	Location	Mean	Std Dev	N
Pro K Averages	Terra	37.8286	17.05166	7
	Ocean	37.1000	16.5992	7
	Total	37.4643	16.17123	14
CTII Averages	Terra	39.0000	17.24413	7
	Ocean	30.8571	21.36289	7
	Total	34.9286	19.12403	14

Regarding the terrestrial and oceanic *B. taurus* Cyt B samples, the effect of the enzyme type on sequence quality was significant both on its own (p-value = 0.000) and when adjusted for the effects of exposure length (p-value = 0.001). However, the effect of location (terrestrial or oceanic) on sequence quality for each enzyme treatment was not significant (p-value = 0.140) (Table 10a). Both the between-subjects effects of exposure length and location on the enzyme treatments for the *B. taurus* Cyt B samples accounted for a significant amount of variation between the groups with p-values of 0.004 and 0.002, respectively (Table 10a). Using these p-values, I can conclude that Pro K had a significantly higher mean than CTII in both

environments (Table 10b). Due to the minimal sequencing success of the *B. taurus* MC1R fragments, no statistical calculations can be made regarding enzyme effectiveness.

Table 10. Repeat measures ANCOVA tables for the *B. taurus* Cyt B quality data sets. The α value is 0.05. A) Tests of within-subject contrasts. The within subjects analysis comparing the results at each collection date between the Pro K and CTII results. The Enzyme row is only comparing the Pro K and CTII without the effects of Exposure (exposure length) and Location (terrarestrial or oceanic). Tests of between-subjects effects. The between subjects analysis comparing the effects of Exposure (exposure length) and Location (terrarestrial or oceanic) with the enzyme treatment groups averaged together at each collection date. B) Descriptive statistics. The means and standard deviation recorded and used for the ANCOVA tables. The Pro K averages row is the results from the Pro K data, it is then divided by location. The CTII averages row is the results from the CTII data, it is then divided by location.

A

Source	Sum of Squares	df	Mean Square	F	p-values	Partial Eta^2
Within-Subjects						
Enzyme	196.086	1	196.086	74.396	0.000	0.871
Enzyme*Exposure Length	55.659	1	55.659	21.117	0.001	0.658
Enzyme*Location	6.652	1	6.652	2.524	0.14	0.187
Error	28.993	11	2.636			
Between-Subjects						
Intercept	12327.857	1	12327.857	2181.2	0.000	0.995
Exposure Length	72.413	1	72.413	12.812	0.004	0.538
Location	86.594	1	86.594	15.322	0.002	0.582
Error	62.17	11	5.652			

B

	Location	Mean	Std Dev	N
Pro K Averages	Terra	36.829	3.07393	7
	Ocean	32.329	4.92467	7
	Total	34.579	4.58328	14
CTII Averages	Terra	30.857	1.17027	7
	Ocean	28.314	1.21165	7
	Total	29.586	1.74658	14

Lastly, when comparing DNA quality of the *L. kempii* and *B. taurus* samples, a one-way ANOVA was used to evaluate the combined NADH4 and Cyt B data, using the $\log_{10}$ of the averages acquired from the triplicate samples. For both the Pro K and the CTII treatments, the difference between the *L. kempii* and *B. taurus* samples was not statistically significant, with p-values of 0.424 and 0.3556, respectively (Table 11a). Using these p-values, I can conclude that, with both enzymes, the *L. kempii* samples did not have significantly lower means than *B. taurus* samples (Table 11b).

Table 11. ANOVA table and descriptive statsistics for the combined *L. kempii* and *B. taurus* mtDNA quality data sets. The α value is 0.05. A) The ANOVA results for the combined organism data set for the $\log_{10}$ transformed average quanities. Pro K averages row shows the differences between the *L. kempii* and *B. taurus* Pro K samples. CTII averages row shows the differences between the *L. kempii* and *B. taurus* CTII samples. B) The means and standard deviation recorded and used for the ANOVA tables. The Pro K averages row is the results from the $\log_{10}$ transformation of the Pro K data, it is then divided by organism. The CTII averages row is the results from the $\log_{10}$ transformation of the CTII data, it is then divided by organism.

A

		Sum of Squares	df	Mean Square	F	p-values
Pro K Averages	Between	0.118	1	0.118	0.659	0.424
	Within	4.665	26	0.179		
	Total	4.784	27			
CTII Averages	Between	0.218	1	0.218	0.883	0.356
	Within	6.412	26	0.247		
	Total	6.63	27			

B

	Organism	Mean	Std Dev	N
Pro K Averages	*L. kempii*	1.4051	0.59615	14
	B. taurus	1.5351	0.05908	14
	Total	1.4701	0.07955	28
CTII Averages	*L. kempii*	1.264	0.18757	14
	B. taurus	1.4704	0.0069	14
	Total	1.3822	0.09365	28

Species Identification

After aligning each sample's forward and reverse sequences in Geneious bioinformatic software, they were trimmed to remove the primer sites and a consensus was formed for each sample point. For the *L. kempii* samples, green sea turtle (*Chelonia mydas*), olive ridley sea turtle (*Lepidochelys olivacea*), and Kemp's ridley sea turtle NADH4 samples were acquired from GenBank and trimmed to the corresponding regions to serve as reference sequences. The maximum likelihood best fit models of substitutions for the Pro K and CTII data sets were determined in MEGA-X. In both data sets, the Hasegawa-Kishino-Yano (HKY) model received the lowest BIC (Bayesian Information Criterion) scores and was used to generate the maximum likelihood (ML) trees for each set. Figure 9 represents the ML tree for the Pro K *L. kempii* sequences, with bootstrapping scores visible at each node. Figure 10 represents the ML tree for the CTII *L. kempii* sequences, with bootstrapping scores visible at each node. The NADH4 regions isolated from the bone samples with both enzyme treatments grouped exclusively with

the *L. kempii* reference sequences, with the *L. olivacea* references placed as sister taxa. The *L. kempii* clade has strong support with bootstrap scores of 93 and 94 for the Pro K and CTII trees, respectively.

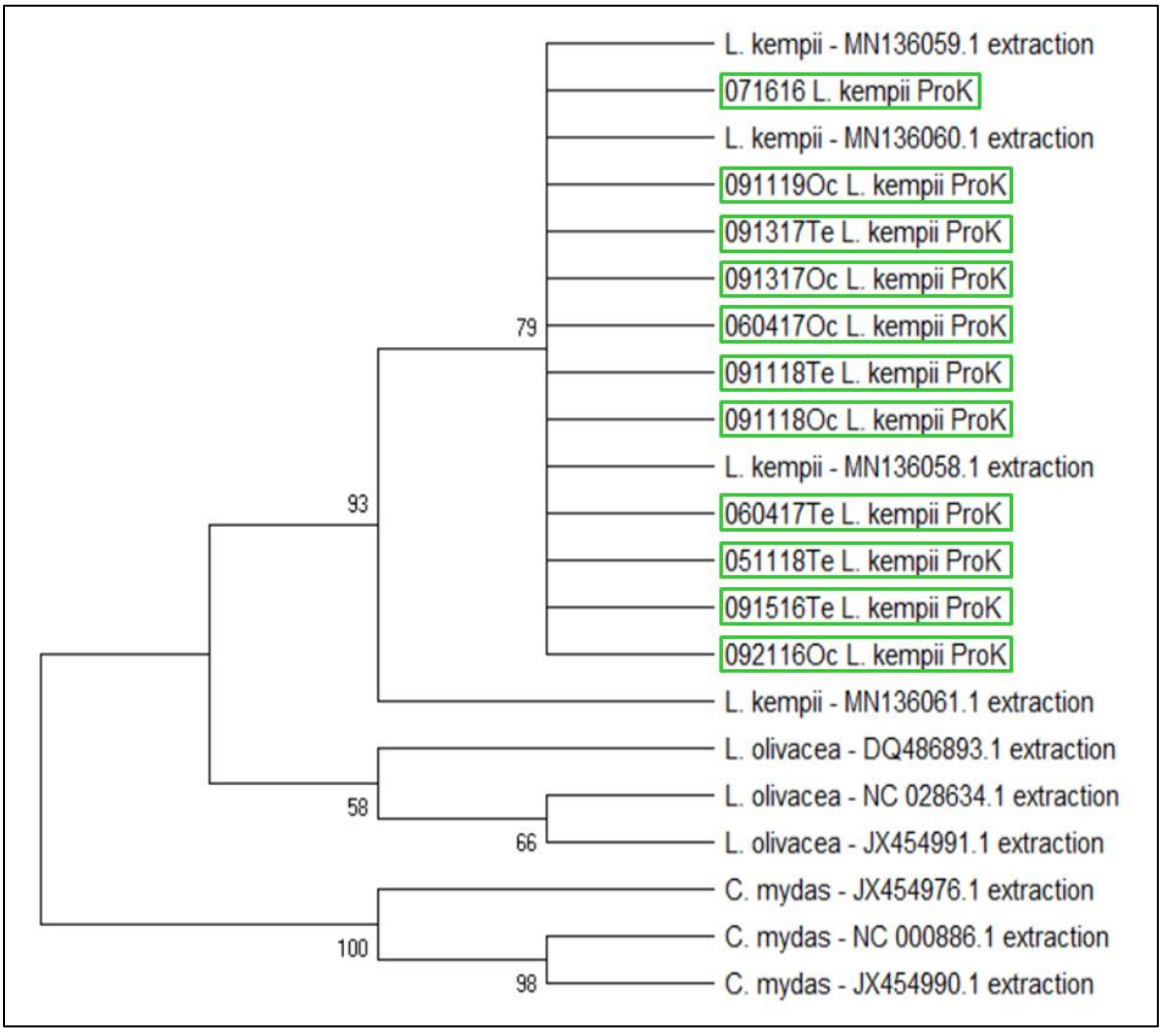

Figure 7. Maximum Likelihood tree for the *L. kempii* NADH4 Pro K consensus data. All sequences generated for this study are outlined in green and start with the sample collection date. The reference sequences start with the organisms' genus and species followed by the GenBank accession number. This tree was consrtucted using the Hasegawa-Kishino-Yano model. Bootstrap values are shown near each node.

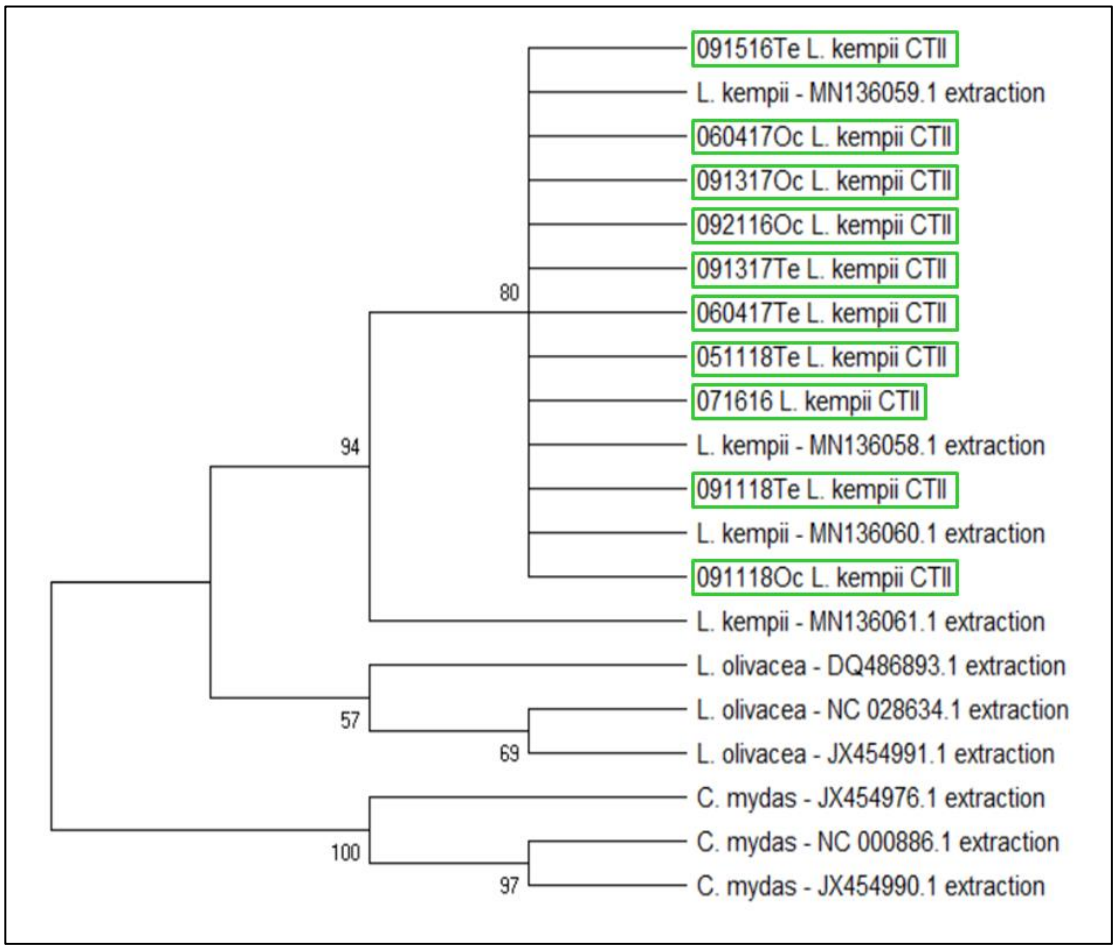

Figure 8. Maximum Likelihood tree for the *L. kempii* NADH4 CTII consensus data. All sequences generated for this project are outlined in green and start with the sample collection date. The reference sequences start with the organisms' genus and species followed by the GenBank accession number. This tree was consrtucted using the Hasegawa-Kishino-Yano model. Bootstrap values are shown near the node.

For the *B. taurus* samples, domestic yak (*Bos grunniens*), banteng (*Bos javanicus*), and domestic cow Cyt B samples as well as, domestic yak (*Bos grunniens*), domestic water buffalo (*Bubalus bubalis*), common eland (*Tragelaphus oryx*), and domestic cow MC1R samples were acquired from GenBank and trimmed to the corresponding regions to serve as reference sequences. The maximum likelihood best fit models of substitutions for the Pro K and CTII data sets were determined in MEGA-X. For the Cyt B Pro K data set, the HKY model received the lowest BIC score, whereas for the Cyt B CTII data set, the Hasegawa-Kishino-Yano with

assumed site evolutionarily invariable (HKY+I) model received the lowest BIC score. These models were used to generate the ML trees for each set. Figure 11 represents the ML tree for the Pro K *B. taurus* Cyt B sequences, with bootstrapping scores visible at each node. Figure 12 represents the ML tree for the CTII *B. taurus* Cyt B sequences with bootstrapping scores visible at each node The Cyt B regions isolated from the bone samples with both enzyme treatments were grouped exclusively with the *B. taurus* reference sequences and have strong support with bootstrap of 99 and 100 for Pro K and CTII trees, respectively.

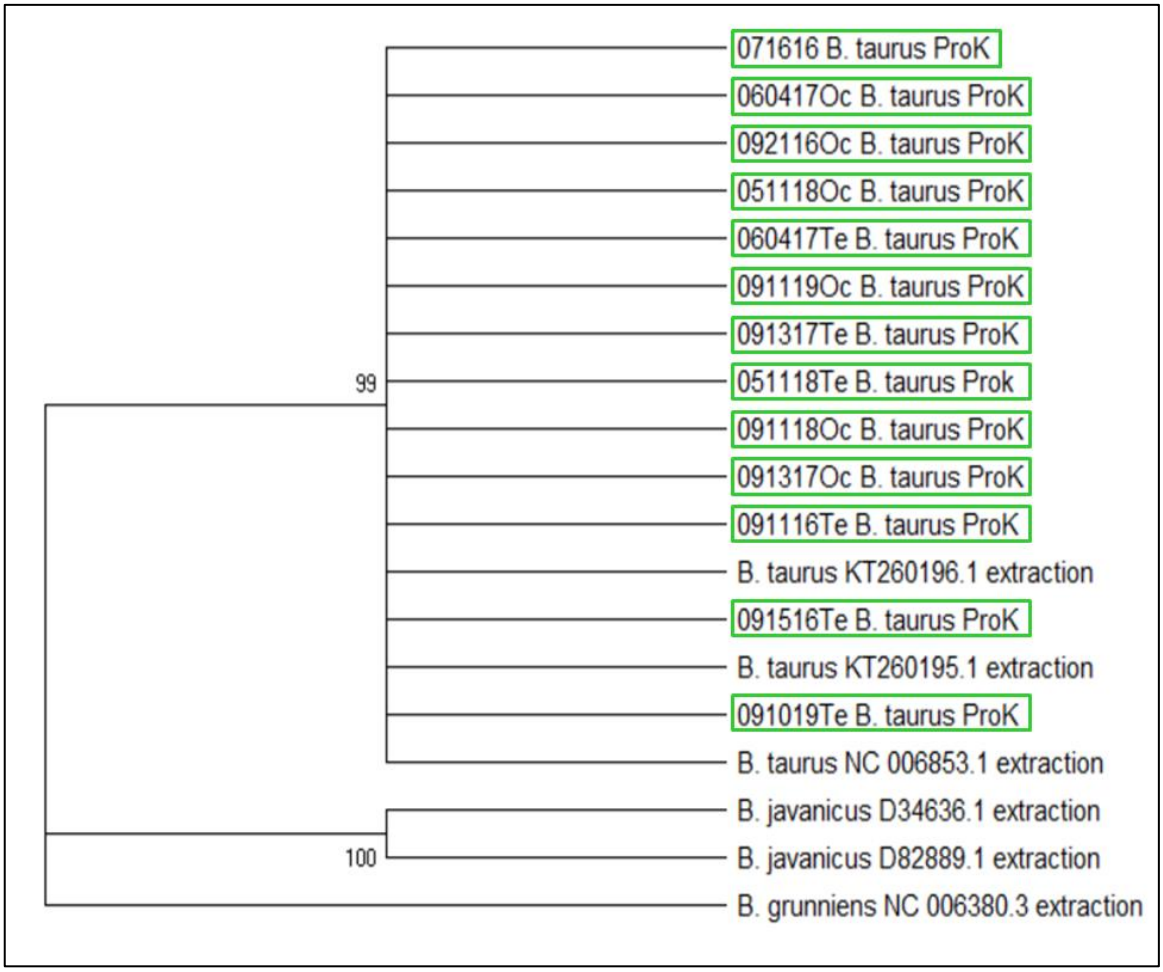

Figure 9. Maximum Likelihood tree for the *B. taurus* Cyt B Pro K consensus data. All sequences generated for this project are outlined in green and start with the sample collection date. The reference sequences start with the organisms' genus and species followed by the GenBank accesssion number. This tree was consrtucted using the Hasegawa-Kishino-Yano model. Bootstrap values are shown near each node.

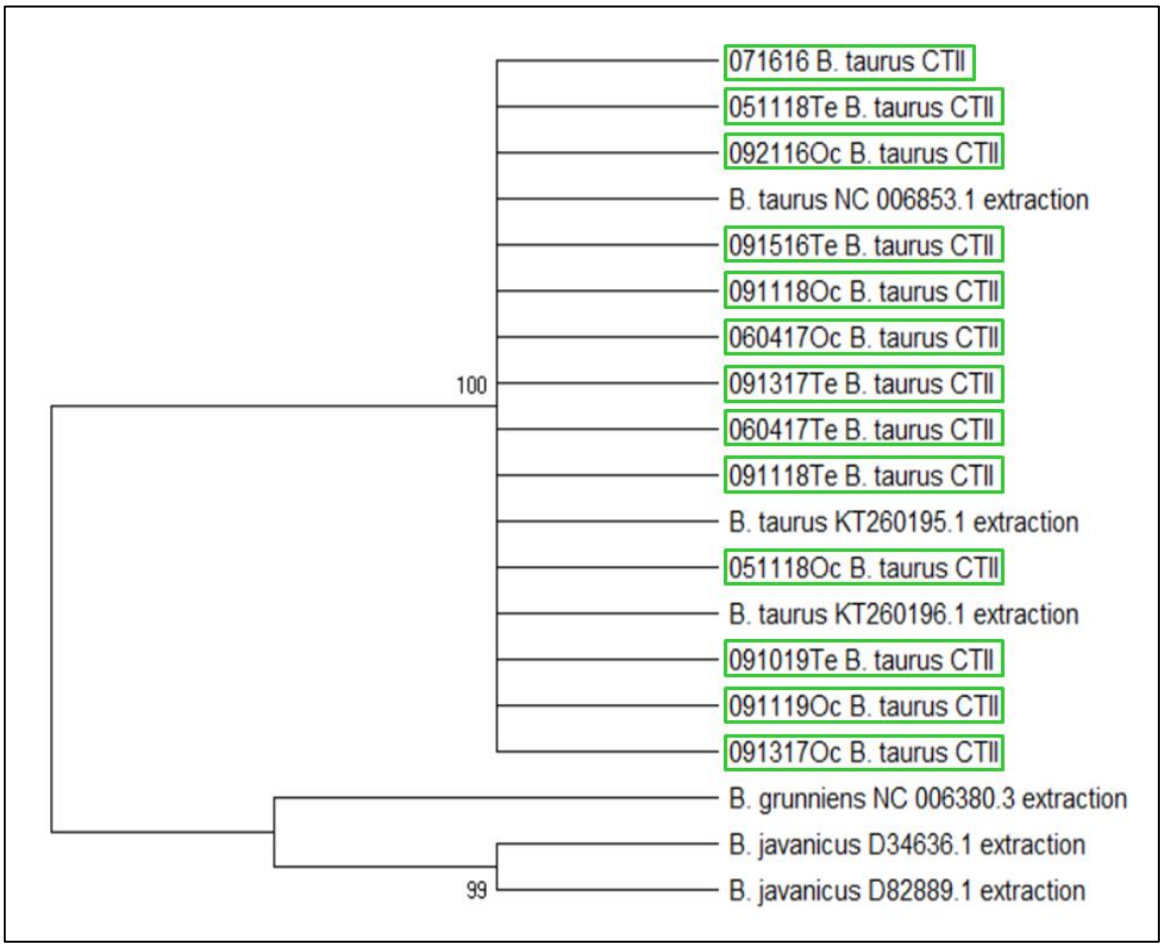

Figure 10. Maximum Likelihood tree for the *B. taurus* Cyt B CTII consensus data. All sequences generated for this project are outlined in green and start with the sample collection date. The reference sequences start with the organisms' genus and species followed by the GenBank accession number. This tree was consrtucted using the Hasegawa-Kishino-Yano model with assumed site evolutionarily invariable. Bootstrap values are shown near each node.

In both MC1R data sets, the Tamura 3-parameter (T92) model received the lowest BIC score and was used to generate the ML trees. Figure 13 represents the ML tree for the Pro K *B. taurus* MC1R sequences, with bootstrapping scores visible at each node. Figure 14 represents the ML tree for the CTII *B. taurus* MC1R sequences, with bootstrapping scores visible at each node. In both instances, the MC1R regions isolated from the bone samples were grouped exclusively with the *B. taurus* reference sequences and have strong support with bootstrap scores of 86 and 90 for the Pro K and CTII trees, respectively.

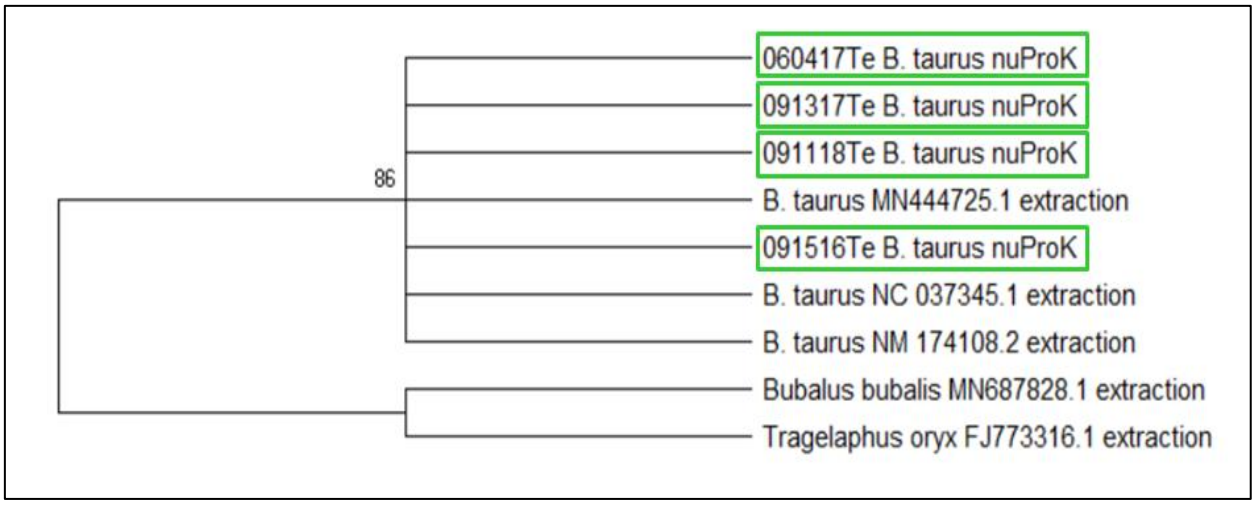

Figure 13. Maximum Likelihood tree for the *B. taurus* MC1R Pro K consensus data. All sequences generated for this project are outlined in green and start with the samples collection date. The reference sequences start with the organisms' genus and species followed by the GenBank accession number. This tree was consrtucted using the Tamura 3-parameter model. Bootstrap values are shown near each node.

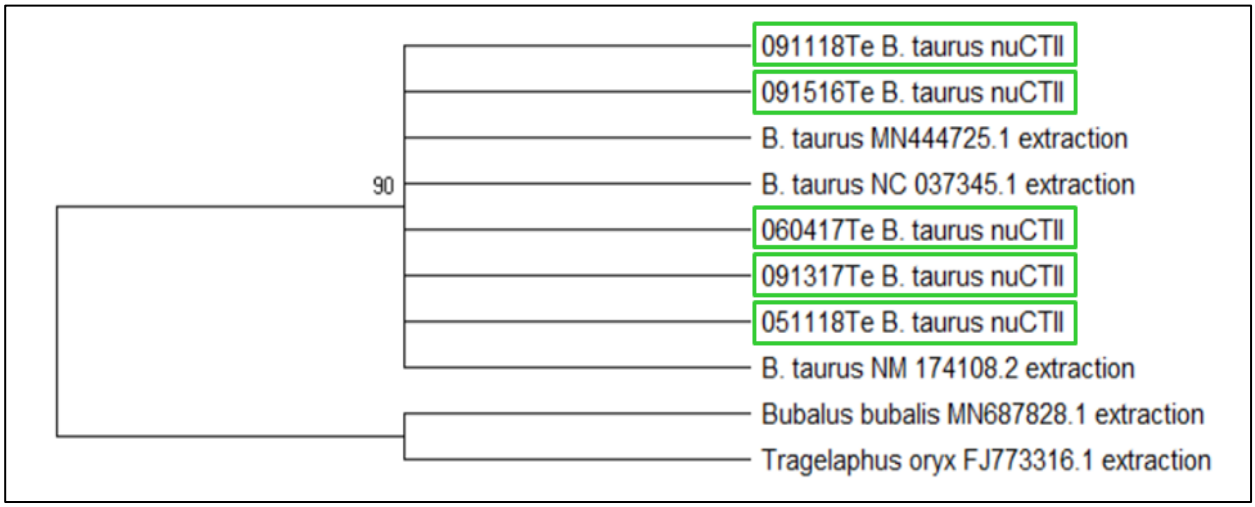

Figure 14. Maximum Likelihood tree for the *B. taurus* MC1R CTII consensus data. All sequences generated for this project are outlined in green and start with the sample collection date. The reference sequences start with the organisms' genus and species followed by the GenBank accession number. This tree was consrtucted using the Tamura 3-parameter model. Bootstrap values are shown near each node.

Chapter 4: Discussion

Kemp's ridley sea turtles are critically endangered, but share many genetic and morphological similarities to olive ridleys, a sister taxon (Wibbels & Bevan 2019). Olive ridleys are considered the most abundant sea turtle species in the world and thus not deemed a protected species in many countries (Abreu-Grobois & Plotkin 2008). This means it is critical to forensic wildlife communities to be able to tell these two species apart, even when only presented with degraded parts or portions of skeletal remains. Advances in research conducted on human remains identification can serve as a foundation for techniques and procedures that can be used in forensic wildlife science. For example, Barrett (2015) studied the difference in effectiveness between two enzymes, Pro K and CTII, during skeletal demineralization and DNA extraction.

My goal was to determine how long Kemp's ridley sea turtle bones could be exposed to marine or supratidal terrestrial environments before a genetic species identification could not be achieved. Additionally, I expanded upon Barrett (2015) research into an applied scenario that forensic wildlife biologist could encounter by examining the potential differences in the quality of the DNA extracted over the course of a three-year time series by two enzymes, Proteinase K and Collagenase Type II. My initial hypotheses were the following: 1) the *B. taurus* samples will have higher quantity and quality scores overall than the *L. kempii* samples, 2) the supratidal samples from both species will have higher quantity and quality values than the subtidal samples, and 3) the CTII demineralized samples will demonstrate quantity values (ng/µL) that are comparable to the Pro K demineralized sample values, but the CTII will produce higher consensus quality scores over the time series.

Regarding the DNA quantities extracted from the *L. kempii* and *B. taurus* bones, four key observations can be made from Figures 3 and 4: 1) the terrestrial samples had larger

DNA quantities than the oceanic samples, 2) there was a peak in DNA quantity around one year of terrestrial exposure in both organisms, 3) the variability within each oceanic sample was smaller than the terrestrial samples, and 4) the *L. kempii* samples had higher averages than the *B. taurus* samples. The first observation was expected due to the reported detrimental effects salt water has on DNA (Armstrong 2014, Eichmiller et al. 2016, Sassoubre et al. 2016, Collins et al. 2018), but the second observation was not. This increase in the amount of DNA extracted around one year of exposure, under one year for *B. taurus* and closer to two years for *L. kempii*, could be due to several reasons, such as cortical thickness variation between time points, non-source-sample contamination, or increased demineralization efficiency on more degraded samples. The importance of cortical thickness has been well documented in identifying human skeletal remains which might be an explanation for the fluctuation seen here (Barrett 2015, Latham & Miller 2019). With a limited number of irregularly shaped bone fragments per collection point for the *L. kempii* samples, it was not possible to control for this factor in this experiment. As seen in the reagent blank data (Table 6), several of the controls had low level DNA contamination; it was later confirmed that this DNA was not from the samples after PCR amplification and visualization with gel electrophoresis resulted in no bands. Additionally, after cycle sequencing, the reagent blanks did not produce sequence data. A real-time PCR assay for both *L. kempii* and *B. taurus* would negate the effects of this outside source contamination. However, the efficiency of bone demineralization on terrestrial exposed bone samples is not well understood due to a lack of research sampling skeletal remains at regular intervals in natural environments.

The smaller amounts of variation detected within the oceanic samples is likely due to the lower volume of DNA extracted overall. This decreased the potential for sampling bias when I split the demineralized volume of each sample into triplicate. Lastly, and surprisingly, the *L.*

kempii samples had higher DNA quantities than the *B. taurus* samples, despite having thinner bones. This difference might have been due to the variation of cortical thickness in combination with the manual pulverization technique. The outsides of the thicker *B. taurus* bone samples were pulverized first, making up more of the total weight for each sample, rather than the thinner *L. kempii* bone samples which needed to be completely crushed to reach the required weight. Furthermore, the uniformity of the pulverized bone samples was difficult to control for while using the bone morselizers.

Despite only having seven samples per treatment group, ANCOVA analyses are sufficiently robust to detect significant differences. My data supported statistically significantly larger quantity averages of the Proteinase K demineralization treatment in *L. kempii* samples, but not in *B. taurus* samples. The type of environmental exposure, supratidal or subtidal, played a significant role in the overall quantity of DNA extracted, regardless of the enzyme treatment used in both organisms, with supratidal samples having the higher averages. The type of environment also influenced the quantity of *B. taurus* DNA extracted, with Pro K producing better results, but this was not seen in the *L. kempii* samples. Exposure length, however, was not a significant factor effecting DNA quantity regardless of enzyme treatment used in both organisms. Exposure length did influence the effects of the enzymes on DNA quantity for the *L. kempii* samples, with Pro K producing better results. Interestingly, the *L. kempii* samples had significantly higher DNA quantities with both enzyme treatments than the *B. taurus* samples. This information has been summarized in Table 12. In any case, for a forensic or conservation applications, this data strongly supports extracting DNA from skeletal remains located in supratidal environments with Pro K, to generate results with the highest concentrations of DNA.

Table 12. DNA quantity summary of significance for all data sets.

		Significance for *L. kempii*?	Significance for *B. taurus*?
Effects of Environmental Location		Yes	Yes
Effects of Exposure Length		No	No
Enzyme	Pro K	Yes	No
	CTII	No	No
Enzyme effected by Environmental Location	Pro K	No	Yes
	CTII	No	No
Enzyme effected by Exposure Length	Pro K	Yes	No
	CTII	No	No

DNA quality can be a more important factor for wildlife forensic cases and conservation biology research than quantity. It is critical to have enough template DNA of high enough quality for primers to bind and amplify the DNA, especially if the analysts or researchers are utilizing cycle sequencing rather than massively paralleled sequencing. From the mtDNA quality data, Figures 7 and 8, two important observations can be made: 1) the oceanic environment did not play a significant role in the quality of the mtDNA region NADH4 that was amplified from the *L. kempii* samples, but it did effect the quality of the Cyt B regions amplified from the *B. taurus* samples, and 2) overall there was no significant difference in the quality of the mtDNA between both organisms. Despite previously held opinions regarding the detrimental effects of saltwater on skeletal DNA, this data shows that even with a lower volume of extracted DNA the oceanic *L. kempii* had comparable mtDNA quality scores with the higher concentration terrestrial samples. This could be due to several environmental reasons, primarily the consistent lower oceanic temperatures and mediated fluctuations in seasonal climatic extremes when compared to the terrestrial environment of Appledore Island, Maine. Additionally, oceanic environments typically have less UV light penetration through the water, reduced oxygen levels, and more limited bacterial decomposer community abundance/diversity than terrestrial environments.

Furthermore, the missing oceanic time points at Day 664 brought down the oceanic averages, but the difference between environmental exposure for *L. kempii* NADH4 sequences was still not significant, unlike the continuous time series for *B. taruus* Cyt B sequences. This could be evidence that sea turtle bones might better protect the mtDNA from oceanic environments than terrestrial mammal bones.

Despite the notable differences between cortical thickness there was no statistical difference in mtDNA quality between the organisms, but only *B. taraus* samples generated usable nuDNA. This difference between nuDNA success was because of the *L. kempii* pdCM14 primer failure. Due to the nature of the database-centric primer construction method, these primers annealed to homologous regions of the *L. kempii* nuclear genome as indicated by Figure 6b. In future research, other primer locations will need to be chosen and optimized. A hypothetical explanation for the disparity between the nuDNA availability could also be due to cortical bone thickness and density. The *B. taurus* samples were larger and thicker than the *L. kempii* samples, all of which were from younger males who had more cartilaginous bone, and the importance of cortical thickness in DNA extractions has been supported numerous times with human remains (Barrett 2015, Latham & Miller 2019). Additionally, cortical variation between these two organisms could explain why the *B. taurus* samples continued to have amplifiable DNA throughout the three-year time series, while a majority of the *L. kempii* samples failed to produce sequencing results from the September 2019 samples.

Something to be noted is the dip in quality, or the lack of data, in both organisms for the oceanic samples of May 2018 (Figures 8 & 9). The following was indicated on all samples received from the Shoals Marine Laboratory for that set: the subtidal lobster cages were exposed to air for one to two months. However, this drop in quality did not follow through to the

September 2018 samples and beyond, therefore it is hard to say if this change between environments influenced the DNA quality long term.

Inversely to the quantity data, the mtDNA quality averages for *B. taurus* Cyt B amplicons were significantly larger for Pro K treatments, but there is not statistical significance between the enzyme treatments in *L. kempii* NADH4 amplicons. The type of environmental exposure, supratidal or subtidal, played a significant role in the overall quality of mtDNA extracted, regardless of the enzyme treatment for *B. taurus* Cyt B amplicons, with supratidal samples having the higher averages. Location did not have an influence on the *L. kempii* NADH4 amplicons. However, the environmental exposure did not significantly affect one enzyme treatment over another in both organisms. Exposure length had a strong effect on the quality of mtDNA extracted regardless of the enzyme in both the *L. kempii* NADH4 and the *B. taurus* Cyt B amplicon sets. Exposure length only affected *B. taurus* Cyt B quality regarding enzyme treatments, favoring Pro K, but this was not seen in the *L. kempii* samples. Despite the *L. kempii* samples having significantly higher DNA quantities with both enzyme treatments, there was no statistical difference in mtDNA quality between organisms. This information has been summarized in Table 13.

Without *L. kempii* pdCM14 results, it is difficult to make conclusions regarding the effectiveness of one enzyme over the other for nuDNA extractions, but with the *B. taurus* MC1R results, it appears that samples in the subtidal environment contained less amplifiable nuDNA than ones in the supratidal environment. Also, anecdotally, exposure length appears to influence nuDNA quality over time in the supratidal environment. With regards to wildlife forensic casework and conservation biology field studies, this data supports the continued use of Pro K for demineralization and highlights the known effect of prolonged environmental exposure on

DNA degradation, but demonstrates that mtDNA extractions are still possible regardless of prolonged exposure to marine environments.

Table 13. MtDNA quality summary of significance for all data sets

		Significance for *L. kempii*?	Significance for *B. taurus*?
Effects of Environmental Location		No	Yes
Effects of Exposure Length		Yes	Yes
Enzyme	Pro K	No	Yes
	CTII	No	No
Enzyme effected by Environmental Location	Pro K	No	No
	CTII	No	No
Enzyme effected by Exposure Length	Pro K	No	Yes
	CTII	No	No

When evaluating the ML trees generated by the consensus sequences found in Appendix 1, *L. kempii* NADH4 species identification can still be made before at least 442 days of continuous subtidal exposure, with the potential of extraction and identification after 1152 days. *Lepidochelys kempii* NADH4 species identification can still be made before at least 787 days of continuous supratidal exposure, with the potential for extraction and identification after further environmental exposure. Regarding *B. taurus*, Cyt B species identification can still be made after 1151 or 1152 days of continuous supratidal or subtidal exposure, respectively. This highlights the viability of skeletal DNA extraction for conservation biology research needs, showing that mtDNA identifications can still be made despite prolonged environmental exposure. This greatly expands the potential study field for future conservation efforts by reducing the need for tissue or blood sampling from living sea turtles and the extensive permitting requirements associated with live-capture endangered species research. Studies driven by observational data regard turtle death rates can use these same techniques and link the deaths back to their populations of origin.

Regarding the effectiveness of Pro K versus CTII, the two enzymes are sometimes comparable, but more often the Pro K demineralization treatment produced higher DNA quantities and higher quality mtDNA sequences. With the difference in expense and the marginal evidence showing support for an increased effectiveness of Pro K, I support the continued use of Proteinase K as the primary enzyme for skeletal demineralization and cell lysis.

Overall, when comparing the effects of prolonged subtidal or supratidal exposure to a marine reptile and a terrestrial mammal, the oceanic environmental exposure negatively impacts the amount of recoverable DNA in both organisms and prolonged exposure negatively effects the quality of the recoverable DNA in all samples. For the *L. kempii* samples, the quality of the extracted DNA was not strongly impacted by the oceanic environment, but additional factors played a role in the decrease in DNA quality over time. Despite having thicker bones, the DNA quality of the *B. taurus* samples were negatively impacted by oceanic exposure and by prolonged exposure in both environments. Further research into the variations detected between marine reptile and terrestrial mammal skeletal DNA availability and quality is needed to pinpoint the specific cause or causes responsible. Testing specific bones in different sea turtle species for both nuclear and mitochondrial DNA robustness could outline one such source of variation. Additionally, comparing the DNA extracted from the skeletal remains of marine mammals that have been exposed to these environmental conditions, while controlling for bone thickness, would help isolate the differences between oceanic environmental exposure in maritime animals versus terrestrial ones. Finally, expanding the scope of this study both linearly, by continuing to sample the remains at the Marine Shoal Laboratory, and horizontally, by expanding the number of organisms in each species study group, would significantly strengthen the reliability of the trends I found.

Chapter 5: Conclusion

Wildlife forensic science is a rapidly growing field, and with that growth comes the requirement to support the field with scientific literature and research. Human forensic science and conservation biology have laid the groundwork for hundreds of scientific techniques, but without supplementary studies demonstrating the effectiveness, or lack thereof, for those techniques on wildlife in forensic applications, then the industry cannot use them. Inversely, expanding upon wildlife forensics research problems opens new avenues and techniques for conversation biology to explore that would not normally be considered feasible. Studies, such as this one, expand the foundational knowledge of the both communities and serve as a starting point for future research and development.

I was able to evaluate the overall effects supratidal and subtidal environments had on skeletal DNA degradation over a three-year period in both a terrestrial mammal as well as a marine reptile. The terrestrial mammal samples contained enough amplifiable nuclear DNA for genus identification after 664 days of supratidal exposure, with variable results after 787 days. The inaccessibility of nuDNA in environmentally damaged sea turtle skeletal remains means future research and methodologies should focus on identifying these organisms with mitochondrial DNA. More research is also required to evaluate DNA retention in more cartilaginous remains, like those typically found in younger sea turtles.

Mitochondrial DNA degradation occurred more slowly in both species and was recovered from both organisms after exposure to either supratidal or subtidal environments for 424 days. The NADH4 and Cyt B regions were of high enough quality after extraction to make species-specific identifications. After 424 days, the NADH4 sequence quality became variable in the marine reptile samples exposed to subtidal conditions, but in the supratidal conditions species

specific identifications could still be made after 787 days. For the terrestrial mammal, Cyt B species-specific identifications could be made from the samples exposed to supratidal and subtidal environments after 1511 and 1152 days, respectively. This timeline shows that successful species identification can still be made from environmentally degraded sea turtle remains, like those that might be found in wildlife forensic casework. Often wildlife forensic DNA samples are of poor quality or have been exposed to environments that are harmful to DNA, but we have shown that identifications are still possible for juvenile sea turtle remains left submerged in marine water or left on the beach after two years of exposure. This would allow for continued prosecution of turtle poaching and genetic cataloging of endangered species for conservation efforts. Furthermore, with the rising popularity and affordability of massively paralleled sequencing, mitochondrial haplotypes utilizing full mitochondrial genomes could be identified to link poached or trafficked individuals to specific populations for further monitoring and protective measures (Patricio et al. 2017, Frandsen et al. 2020).

Many female sea turtles, including Kemp's ridleys, take part in natal homing, which means the same females return year after year to the beaches where they hatched to lay their eggs and their female hatchlings will do the same. This preserves the maternally inherited mitochondrial genomes and the subsequent haplotypes associated with specific locations. Conservation biologists, such as Patricio et al. (2017), have demonstrated that these haplotypes can be tracked via mixed-stock sampling at foraging grounds, creating population groups that can be monitored and protected as a whole. For wildlife forensic biologists, these haplotypic groups can be utilized for identifying the origin of the poached sea turtles they encounter. Recently, Frandsen et al. (2020) demonstrated that using complete Kemp's ridley mitochondrial genomes can delineate between haplotypes even further and with more accuracy. Altogether,

understanding more about the efficacy of sampling DNA from degraded sea turtle remains can further the reach wildlife forensic investigations might have in identifying and then protecting the most vulnerable sea turtle populations from further poaching.

Solely within conservation biology, this time series represents a unique look at skeletal DNA recovery from degraded samples of an endangered species in two common habitats where Kemp's ridley sea turtle remains might be discovered. Typically, haplotypic and species identifications are made from soft tissue samples or from environmental DNA, but this research shows that remains can be utilized as well. This means individuals that died of natural causes could be linked to their population of origin, furthering the understanding of migratory habits for certain species. For example, if the two juvenile sea turtles that died as a result of seasonal cold-shock used in this study had not been discovered until after significant degradation had taken place, their remains could still be used to determine their natal origin and further observations could be made regarding why these turtles remained in the northern part of their range during the winter. This is only one such example demonstrating the utility of successfully extracting DNA from animal remains. Additionally, with the push in conservation biology towards massively paralleled sequencing, even fragmented genomes could still be analyzed and used for species identifications; which further highlights the importance of being able to successfully extract DNA from remains found in unfavorable environments.

Additionally, I expanded upon Barrett (2015) research regarding the comparative effectiveness of Proteinase K and Collagenase Type II for human skeletal DNA extraction and preservation, by evaluating those enzymes in two animal species after exposure to damaging environmental factors. Between both species, Kemp's ridley sea turtles and domestic cows, the enzymes were often comparable in extracted DNA quantity and mtDNA amplicon quality.

However, in the few instances when they were not, Proteinase K was always indicated to have more statistically significant results.

Furthering the literary scientific support for wildlife forensic techniques and conservation biology procedures is essential to the prosecution of wildlife traffickers and the continued preservation of endangered species, like the Kemp's ridley sea turtle. Because of their rarity, monetary value or status signifiers have been attached to endangered animals, their parts, and their consumption, making them internationally trafficked placeholders for monetary exchanges for other illegal activities. As well as expanding what kinds of samples can be utilized by conservation research, studies like this one are important to the future protection of wildlife and to the continued prevention of illegal trafficking.

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Appendix 1: Consensus Sequences

Appendix 1. List of consensus sequences obtained from all *L. kempii* and *B. taurus* samples with both CTII and Pro K over the exposure time series. Each entry is listed with the sample collection date, the environmental location of the sample (Te = Terrestrial, Oc = Oceanic, B = Baseline), the source animal, the enzyme used for demineralization, and the gene each region is from. NADH4 and Cyt B are both mtDNA and MC1R is nuDNA Each sample was aligned and trimmed to exclude the primer sites using the Geneious Prime bioinformatics software. All sequences are listed 5' to 3' and have the number of sources sequences used to make each consensus is listed.

Name	# Sources	Sequence (5' -> 3')	Length
071616 B *L. kempii* CTII NADH4	6	ATTGCAGGCTCAATAATCCTAGCCGCAGTATTACTAAAATTAGGAGGATATGGCATTATC CGCATTACAATAATGCTAAACCCCCTATCAAAAACACTCTCCTACCCTTTCATGGTACTC GCATTATGAGGAGTAATCATAACTAGTTCTATTTGCTTACGACAAACAGATCTAAAATCA TTAATCGCCTACTCATCAGTAAGCCACATAGGACTAATCATCGCCGCAACACTAACACAA ACCCAATGAGCCTACACCGGCGCAATTACACTTATAATTGCCCATGGCTTAACGTCATCA ATACTCTTTTGCCTAGCCAACACAAATTACG	331
091516Te *L. kempii* CTII NADH4	6	ATTGCAGGCTCAATAATCCTAGCCGCAGTATTACTAAAATTAGGAGGATATGGCATTATC CGCATTACAATAATGCTAAACCCCCTATCAAAAACACTCTCCTACCCTTTCATGGTACTC GCATTATGAGGAGTAATCATAACTAGTTCTATTTGCTTACGACAAACAGATCTAAAATCA TTAATCGCCTACTCATCAGTAAGCCACATAGGACTAATCATCGCCGCAACACTAACACAA ACCCAATGAGCCTACACCGGCGCAATTACACTTATAATTGCCCATGGCTTAACGTCATCA ATACTCTTTTGCCTAGCCAACACAAATTACG	331
092116Oc *L. kempii* CTII NADH4	6	ATTGCAGGCTCAATAATCCTAGCCGCAGTATTACTAAAATTAGGAGGATATGGCATTATC CGCATTACAATAATGCTAAACCCCCTATCAAAAACACTCTCCTACCCTTTCATGGTACTC GCATTATGAGGAGTAATCATAACTAGTTCTATTTGCTTACGACAAACAGATCTAAAATCA TTAATCGCCTACTCATCAGTAAGCCACATAGGACTAATCATCGCCGCAACACTAACACAA ACCCAATGAGCCTACACCGGCGCAATTACACTTATAATTGCCCATGGCTTAACGTCATCA ATACTCTTTTGCCTAGCCAACACAAATTACG	331
060417Te *L. kempii* CTII NADH4	6	ATTGCAGGCTCAATAATCCTAGCCGCAGTATTACTAAAATTAGGAGGATATGGCATTATC CGCATTACAATAATGCTAAACCCCCTATCAAAAACACTCTCCTACCCTTTCATGGTACTC GCATTATGAGGAGTAATCATAACTAGTTCTATTTGCTTACGACAAACAGATCTAAAATCA TTAATCGCCTACTCATCAGTAAGCCACATAGGACTAATCATCGCCGCAACACTAACACAA ACCCAATGAGCCTACACCGGCGCAATTACACTTATAATTGCCCATGGCTTAACGTCATCA ATACTCTTTTGCCTAGCCAACACAAATTACG	331
060417Oc *L. kempii* CTII NADH4	6	ATTGCAGGCTCAATAATCCTAGCCGCAGTATTACTAAAATTAGGAGGATATGGCATTATC CGCATTACAATAATGCTAAACCCCCTATCAAAAACACTCTCCTACCCTTTCATGGTACTC GCATTATGAGGAGTAATCATAACTAGTTCTATTTGCTTACGACAAACAGATCTAAAATCA TTAATCGCCTACTCATCAGTAAGCCACATAGGACTAATCATCGCCGCAACACTAACACAA ACCCAATGAGCCTACACCGGCGCAATTACACTTATAATTGCCCATGGCTTAACGTCATCA ATACTCTTTTGCCTAGCCAACACAAATTACG	331
091317Te *L. kempii* CTII NADH4	6	ATTGCAGGCTCAATAATCCTAGCCGCAGTATTACTAAAATTAGGAGGATATGGCATTATC CGCATTACAATAATGCTAAACCCCCTATCAAAAACACTCTCCTACCCTTTCATGGTACTC GCATTATGAGGAGTAATCATAACTAGTTCTATTTGCTTACGACAAACAGATCTAAAATCA TTAATCGCCTACTCATCAGTAAGCCACATAGGACTAATCATCGCCGCAACACTAACACAA ACCCAATGAGCCTACACCGGCGCAATTACACTTATAATTGCCCATGGCTTAACGTCATCA ATACTCTTTTGCCTAGCCAACACAAATTACG	331

Name	# Sources	Sequence (5' -> 3')	Length
091317Oc *L. kempii* CTII NADH4	6	ATTGCAGGCTCAATAATCCTAGCCGCAGTATTACTAAAATTAGGAGGATATGGCATTATCCGCATTACAATAATGCTAAACC CCCTATCAAAAACACTCTCCTACCCTTTCATGGTACTCGCATTATGAGGAGTAATCATAACTAGTTCTATTTGCTTACGACA AACAGATCTAAAATCATTAATCGCCTACTCATCAGTAAGCCACATAGGACTAATCATCGCCGCAACACTAACACAAACCCAA TGAGCCTACACCGGCGCAATTACACTTATAATTGCCCATGGCTTAACGTCATCAATACTCTTTTGCCTAGCCAACACAAATT ACG	331
091118Te *L. kempii* CTII NADH4	6	ATTGCAGGCTCAATAATCCTAGCCGCAGTATTACTAAAATTAGGAGGATATGGCATTATCCGCATTACAATAATGCTAAACC CCCTATCAAAAACACTCTCCTACCCTTTCATGGTACTCGCATTATGAGGAGTAATCATAACTAGTTCTATTTGCTTACGACA AACAGATCTAAAATCATTAATCGCCTACTCATCAGTAAGCCACATAGGACTAATCATCGCCGCAACACTAACACAAACCCAA TGAGCCTACACCGGCGCAATTACACTTATAATTGCCCATGGCTTAACGTCATCAATACTCTTTTGCCTAGCCAACACAAATT ACG	331
091118Oc *L. kempii* CTII NADH4	6	ATTGCAGGCTCAATAATCCTAGCCGCAGTATTACTAAAATTAGGAGGATATGGCATTATCCGCATTACAATAATGCTAAACC CCCTATCAAAAACACTCTCCTACCCTTTCATGGTACTCGCATTATGAGGAGTAATCATAACTAGTTCTATTTGCTTACGACA AACAGATCTAAAATCATTAATCGCCTACTCATCAGTAAGCCACATAGGACTAATCATCGCCGCAACACTAACACAAACCCAA TGAGCCTACACCGGCGCAATTACACTTATAATTGCCCATGGCTTAACGTCATCAATACTCTTTTGCCTAGCCAACACAAATT ACG	331
071616 B *L. kempii* Pro K NADH4	6	ATTGCAGGCTCAATAATCCTAGCCGCAGTATTACTAAAATTAGGAGGATATGGCATTATCCGCATTACAATAATGCTAAACC CCCTATCAAAAACACTCTCCTACCCTTTCATGGTACTCGCATTATGAGGAGTAATCATAACTAGTTCTATTTGCTTACGACA AACAGATCTAAAATCATTAATCGCCTACTCATCAGTAAGCCACATAGGACTAATCATCGCCGCAACACTAACACAAACCCAA TGAGCCTACACCGGCGCAATTACACTTATAATTGCCCATGGCTTAACGTCATCAATACTCTTTTGCCTAGCCAACACAAATT ACG	331
091516Te *L. kempii* Pro K NADH4	6	ATTGCAGGCTCAATAATCCTAGCCGCAGTATTACTAAAATTAGGAGGATATGGCATTATCCGCATTACAATAATGCTAAACC CCCTATCAAAAACACTCTCCTACCCTTTCATGGTACTCGCATTATGAGGAGTAATCATAACTAGTTCTATTTGCTTACGACA AACAGATCTAAAATCATTAATCGCCTACTCATCAGTAAGCCACATAGGACTAATCATCGCCGCAACACTAACACAAACCCAA TGAGCCTACACCGGCGCAATTACACTTATAATTGCCCATGGCTTAACGTCATCAATACTCTTTTGCCTAGCCAACACAAATT ACG	331
092116Oc *L. kempii* Pro K NADH4	6	ATTGCAGGCTCAATAATCCTAGCCGCAGTATTACTAAAATTAGGAGGATATGGCATTATCCGCATTACAATAATGCTAAACC CCCTATCAAAAACACTCTCCTACCCTTTCATGGTACTCGCATTATGAGGAGTAATCATAACTAGTTCTATTTGCTTACGACA AACAGATCTAAAATCATTAATCGCCTACTCATCAGTAAGCCACATAGGACTAATCATCGCCGCAACACTAACACAAACCCAA TGAGCCTACACCGGCGCAATTACACTTATAATTGCCCATGGCTTAACGTCATCAATACTCTTTTGCCTAGCCAACACAAATT ACG	331
060417Te *L. kempii* Pro K NADH4	6	ATTGCAGGCTCAATAATCCTAGCCGCAGTATTACTAAAATTAGGAGGATATGGCATTATCCGCATTACAATAATGCTAAACC CCCTATCAAAAACACTCTCCTACCCTTTCATGGTACTCGCATTATGAGGAGTAATCATAACTAGTTCTATTTGCTTACGACA AACAGATCTAAAATCATTAATCGCCTACTCATCAGTAAGCCACATAGGACTAATCATCGCCGCAACACTAACACAAACCCAA TGAGCCTACACCGGCGCAATTACACTTATAATTGCCCATGGCTTAACGTCATCAATACTCTTTTGCCTAGCCAACACAAATT ACG	331

Name	# Sources	Sequence (5' -> 3')	Length
060417Oc *L. kempii* Pro K NADH 4	6	ATTGCAGGCTCAATAATCCTAGCCGCAGTATTACTAAAATTAGGAGGATATGGCATTATCCGCATTACAATAATGCTAAAC CCCCTATCAAAAACACTCTCCTACCCTTTCATGGTACTCGCATTATGAGGAGTAATCATAACTAGTTCTATTTGCTTACGA CAAACAGATCTAAAATCATTAATCGCCTACTCATCAGTAAGCCACATAGGACTAATCATCGCCGCAACACTAACACAAACC CAATGAGCCTACACCGGCGCAATTACACTTATAATTGCCCATGGCTTAACGTCATCAATACTCTTTTGCCTAGCCAACACA AATTACG	331
091317Te *L. kempii* Pro K NADH4	6	ATTGCAGGCTCAATAATCCTAGCCGCAGTATTACTAAAATTAGGAGGATATGGCATTATCCGCATTACAATAATGCTAAAC CCCCTATCAAAAACACTCTCCTACCCTTTCATGGTACTCGCATTATGAGGAGTAATCATAACTAGTTCTATTTGCTTACGA CAAACAGATCTAAAATCATTAATCGCCTACTCATCAGTAAGCCACATAGGACTAATCATCGCCGCAACACTAACACAAACC CAATGAGCCTACACCGGCGCAATTACACTTATAATTGCCCATGGCTTAACGTCATCAATACTCTTTTGCCTAGCCAACACA AATTACG	331
091317Oc *L. kempii* Pro K NADH4	6	ATTGCAGGCTCAATAATCCTAGCCGCAGTATTACTAAAATTAGGAGGATATGGCATTATCCGCATTACAATAATGCTAAAC CCCCTATCAAAAACACTCTCCTACCCTTTCATGGTACTCGCATTATGAGGAGTAATCATAACTAGTTCTATTTGCTTACGA CAAACAGATCTAAAATCATTAATCGCCTACTCATCAGTAAGCCACATAGGACTAATCATCGCCGCAACACTAACACAAACC CAATGAGCCTACACCGGCGCAATTACACTTATAATTGCCCATGGCTTAACGTCATCAATACTCTTTTGCCTAGCCAACACA AATTACG	331
051118Te *L. kempii* Pro K NADH4	6	ATTGCAGGCTCAATAATCCTAGCCGCAGTATTACTAAAATTAGGAGGATATGGCATTATCCGCATTACAATAATGCTAAAC CCCCTATCAAAAACACTCTCCTACCCTTTCATGGTACTCGCATTATGAGGAGTAATCATAACTAGTTCTATTTGCTTACGA CAAACAGATCTAAAATCATTAATCGCCTACTCATCAGTAAGCCACATAGGACTAATCATCGCCGCAACACTAACACAAACC CAATGAGCCTACACCGGCGCAATTACACTTATAATTGCCCATGGCTTAACGTCATCAATACTCTTTTGCCTAGCCAACACA AATTACG	331
091118Te *L. kempii* Pro K NADH4	6	ATTGCAGGCTCAATAATCCTAGCCGCAGTATTACTAAAATTAGGAGGATATGGCATTATCCGCATTACAATAATGCTAAAC CCCCTATCAAAAACACTCTCCTACCCTTTCATGGTACTCGCATTATGAGGAGTAATCATAACTAGTTCTATTTGCTTACGA CAAACAGATCTAAAATCATTAATCGCCTACTCATCAGTAAGCCACATAGGACTAATCATCGCCGCAACACTAACACAAACC CAATGAGCCTACACCGGCGCAATTACACTTATAATTGCCCATGGCTTAACGTCATCAATACTCTTTTGCCTAGCCAACACA AATTACG	331
091118Oc *L. kempii* Pro K NADH4	6	ATTGCAGGCTCAATAATCCTAGCCGCAGTATTACTAAAATTAGGAGGATATGGCATTATCCGCATTACAATAATGCTAAAC CCCCTATCAAAAACACTCTCCTACCCTTTCATGGTACTCGCATTATGAGGAGTAATCATAACTAGTTCTATTTGCTTACGA CAAACAGATCTAAAATCATTAATCGCCTACTCATCAGTAAGCCACATAGGACTAATCATCGCCGCAACACTAACACAAACC CAATGAGCCTACACCGGCGCAATTACACTTATAATTGCCCATGGCTTAACGTCATCAATACTCTTTTGCCTAGCCAACACA AATTACG	331
091119Oc *L. kempii* Pro K NADH4	6	ATTGCAGGCTCAATAATCCTAGCCGCAGTATTACTAAAATTAGGAGGATATGGCATTATCCGCATTACAATAATGCTAAAC CCCCTATCAAAAACACTCTCCTACCCTTTCATGGTACTCGCATTATGAGGAGTAATCATAACTAGTTCTATTTGCTTACGA CAAACAGATCTAAAATCATTAATCGCCTACTCATCAGTAAGCCACATAGGACTAATCATCGCCGCAACACTAACACAAACC CAATGAGCCTACACCGGCGCAATTACACTTATAATTGCCCATGGCTTAACGTCATCAATACTCTTTTGCCTAGCCAACACA AATTACG	331

Name	# Sources	Sequence (5' -> 3')	Length
071616 B *B. taurus* CTII Cyt B	6	TTCCATTTTATCCTTCCATTTATCATCATAGCAATTGCCATAGTCCACCTACTATTCCTCCACGAAACAGGCTCCAAC AACCCAACAGGAATTTCCTCAGACGTAGACAAAATCCCATTCCACCCCTACTATACCATTAAGGACATCTTAGGGGCC CTCTTACTAATTCTAGCTCTAATACTACTAGTACTATTCGCACCCGACCTCCKCGGAGASCCAGATAACTACACSSCA GSCAATCCAS	244
091516Te *B. taurus* CTII Cyt B	6	TTCCATTTTATCCTTCCATTTATCATCATAGCAATTGCCATAGTCCACCTACTATTCCTCCACGAAACAGGCTCCAAC AACCCAACAGGAATTTCCTCAGACGTAGACAAAATCCCATTCCACCCCTACTATACCATTAAGGACATCTTAGGGGCC CTCTTACTAATTCTAGCTCTAATACTACTAGTACTATTCGCACCCGACCTCCTCGGAGACCCAGATAACTACACCCCA GCCAATCCAC	244
092116Oc *B. taurus* CTII Cyt B	6	TTCCATTTTATCCTTCCATTTATCATCATAGCAATTGCCATAGTCCACCTACTATTCCTCCACGAAACAGGCTCCAAC AACCCAACAGGAATTTCCTCAGACGTAGACAAAATCCCATTCCACCCCTACTATACCATTAAGGACATCTTAGGGGCC CTCTTRCTAATTCTAGCTCTAATACTACTAGTACTATTCGCACCCGACCTCCTCGGAGASCCAGATARCTACACSSCA GSCAATCCAS	244
060417Te *B. taurus* CTII Cyt B	6	TTCCATTTTATCCTTCCATTTATCATCATAGCAATTGCCATAGTCCACCTACTATTCCTCCACGAAACAGGCTCCAAC AACCCAACAGGAATTTCCTCAGACGTAGACAAAATCCCATTCCACCCCTACTATACCATTAAGGACATCTTAGGGGCC CTCTTACTAATTCTAGCTCTAATACTACTAGTACTATTCGCACCCGACCTCCTCGGAGACCCAGATAACTACACSCCA GSCAATCCAS	244
060417Oc *B. taurus* CTII Cyt B	6	TTCCATTTTATCCTTCCATTTATCATCATAGCAATTGCCATAGTCCACCTACTATTCCTCCACGAAACAGGCTCCAAC AACCCAACAGGAATTTCCTCAGACGTAGACAAAATCCCATTCCACCCCTACTATACCATTAAGGACATCTTAGGGGCC CTCTTNCTAATTCTAGCTCTAATACTACTAGTACTATTCGCACCCGACCTCCKCGGAGASCCAGATAACTACACSSCA GSCAATCCAS	244
091317Te *B. taurus* CTII Cyt B	6	TTCCATTTTATCCTTCCATTTATCATCATAGCAATTGCCATAGTCCACCTACTATTCCTCCACGAAACAGGCTCCAAC AACCCAACAGGAATTTCCTCAGACGTAGACAAAATCCCATTCCACCCCTACTATACCATTAAGGACATCTTAGGGGCC CTCTTACTAATTCTAGCTCTAATACTACTAGTACTATTCGCACCCGACCTCCTCGGAGACCCAGATAACTACACCCCA GSCAATCCAC	244
091317Oc *B. taurus* CTII Cyt B	6	TTCCATTTTATCCTTCCATTTATCATCATAGCAATTGCCATAGTCCACCTACTATTCCTCCACGAAACAGGCTCCAAC AACCCAACAGGAATTTCCTCAGACGTAGACAAAATCCCATTCCACCCCTACTATACCATTAAGGACATCTTAGGGGCC CTCTTRCTAATTCTAGCTCTAATACTACTAGTACTATTCGCACCCGACCTCCKCGGAGASCCAGATAACTACACSSCA GSCAATCCAS	244
051118Te *B. taurus* CTII Cyt B	6	TTCCATTTTATCCTTCCATTTATCATCATAGCAATTGCCATAGTCCACCTACTATTCCTCCACGAAACAGGCTCCAAC AACCCAACAGGAATTTCCTCAGACGTAGACAAAATCCCATTCCACCCCTACTATACCATTAAGGACATCTTAGGGGCC CTCTTACTAATTCTAGCTCTAATACTACTAGTACTATTCGCACCCGACCTCCTCGGAGASCCAGATAACTACACSCCA GCCAATCCAC	244

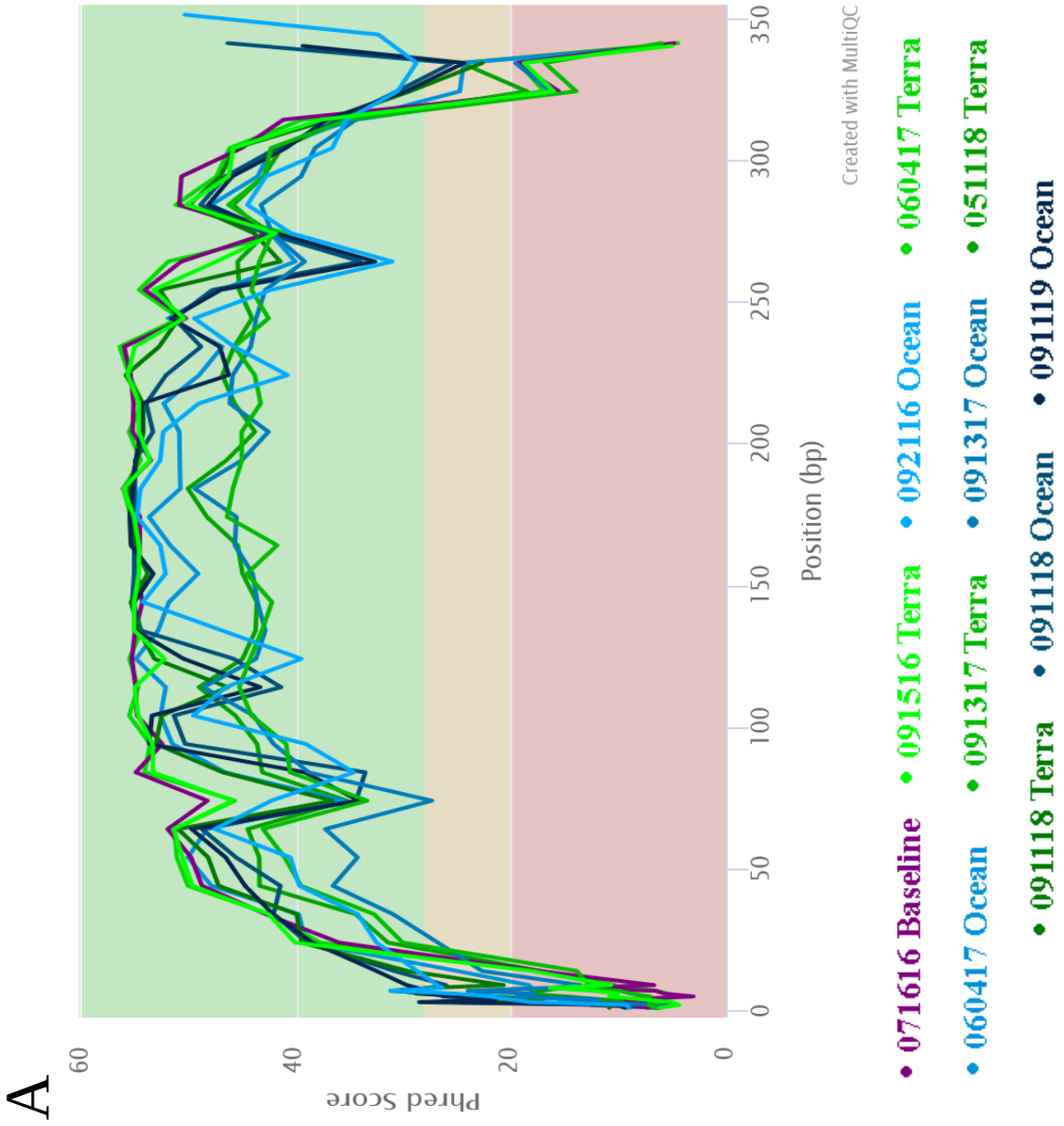
Name	# Sources	Sequence (5' -> 3')	Length
051118Oc *B. taurus* CTII Cyt B	6	TTCCATTTTATCCTTCCATTTATCATCATAGCAATTGCCATAGTCCACCTACTATTCCTCCACGAAACAGGCTCCAACAACC CAACAGGAATTTCCTCAGACGTAGACAAAATCCCATTCCACCCCTACTATACCATTAAGGACATCTTAGGGGCCCTCTTACT AATTCTAGCTCTAATACTACTAGTACTATTCGCACCCGACCTCCKCGGAGASCCAGATAACTACACSSCAGSCAATCCAS	244
091118Te *B. taurus* CTII Cyt B	6	TTCCATTTTATCCTTCCATTTATCATCATAGCAATTGCCATAGTCCACCTACTATTCCTCCACGAAACAGGCTCCAACAACC CAACAGGAATTTCCTCAGACGTAGACAAAATCCCATTCCACCCCTACTATACCATTAAGGACATCTTAGGGGCCCTCTTRCT AATTCTAGCTCTAATACTACTAGTACTATTCGCACCCGACCTCCTCGGAGASCCAGATAACTACACSCCAGSCAATCCAS	244
091118Oc *B. taurus* CTII Cyt B	6	TTCCATTTTATCCTTCCATTTATCATCATAGCAATTGCCATAGTCCACCTACTATTCCTCCACGAAACAGGCTCCAACAACC CAACAGGAATTTCCTCAGACGTAGACAAAATCCCATTCCACCCCTACTATACCATTAAGGACATCTTAGGGGCCCTCTTRCT AATTCTAGCTCTAATACTACTAGTACTATTCGCACCCGACCTCCKCGGAGASCCAGATARCTACACSCCAGSCAATCCAS	244
091019Te *B. taurus* CTII Cyt B	6	TTCCATTTTATCCTTCCATTTATCATCATAGCAATTGCCATAGTCCACCTACTATTCCTCCACGAAACAGGCTCCAACAACC CAACAGGAATTTCCTCAGACGTAGACAAAATCCCATTCCACCCCTACTATACCATTAAGGACATCTTAGGGGCCCTCTTACT AATTCTAGCTCTAATACTACTAGTACTATTCGCACCCGACCTCCTCGGAGASCCAGATAACTACACSCCAGCCAATCCAS	244
091119Oc *B. taurus* CTII Cyt B	6	TTCCATTTTATCCTTCCATTTATCATCATAGCAATTGCCATAGTCCACCTACTATTCCTCCACGAAACAGGCTCCAACAACC CAACAGGAATTTCCTCAGACGTAGACAAAATCCCATTCCACCCCTACTATACCATTAAGGACATCTTAGGGGCCCTCTTRCT AATTCTAGCTCTAATACTACTAGTACTATTCGCACCCGACCTCCTCGGAGASCCAGATAACTACACSCCAGSCAATCCAS	244
071616 B *B. taurus* Pro K Cyt B	9	TTCCATTTTATCCTTCCATTTATCATCATAGCAATTGCCATAGTCCACCTACTATTCCTCCACGAAACAGGCTCCAACAACC CAACAGGAATTTCCTCAGACGTAGACAAAATCCCATTCCACCCCTACTATACCATTAAGGACATCTTAGGGGCCCTCTTACT AATTCTAGCTCTAATACTACTAGTACTATTCGCACCCGACCTCCTCGGAGACCCAGATAACTACACCCCAGCCAATCCAC	244
092116Oc *B. taurus* Pro K Cyt B	9	TTCCATTTTATCCTTCCATTTATCATCATAGCAATTGCCATAGTCCACCTACTATTCCTCCACGAAACAGGCTCCAACAACC CAACAGGAATTTCCTCAGACGTAGACAAAATCCCATTCCACCCCTACTATACCATTAAGGACATCTTAGGGGCCCTCTTACT AATTCTAGCTCTAATACTACTAGTACTATTCGCACCCGACCTCCTCGGAGACCCAGATAACTACACCCCAGCCAATCCAC	244
091516Te *B. taurus* Pro K Cyt B	9	TTCCATTTTATCCTTCCATTTATCATCATAGCAATTGCCATAGTCCACCTACTATTCCTCCACGAAACAGGCTCCAACAACC CAACAGGAATTTCCTCAGACGTAGACAAAATCCCATTCCACCCCTACTATACCATTAAGGACATCTTAGGGGCCCTCTTACT AATTCTAGCTCTAATACTACTAGTACTATTCGCACCCGACCTCCTCGGAGACCCAGATAACTACACCCCAGCCAATCCAC	244
060417Te *B. taurus* Pro K Cyt B	9	TTCCATTTTATCCTTCCATTTATCATCATAGCAATTGCCATAGTCCACCTACTATTCCTCCACGAAACAGGCTCCAACAACC CAACAGGAATTTCCTCAGACGTAGACAAAATCCCATTCCACCCCTACTATACCATTAAGGACATCTTAGGGGCCCTCTTACT AATTCTAGCTCTAATACTACTAGTACTATTCGCACCCGACCTCCTCGGAGACCCAGATAACTACACCCCAGCCAATCCAC	244

Name	# Sources	Sequence (5' -> 3')	Length
060417Oc *B. taurus* Pro K Cyt B	7	TTCCATTTTATCCTTCCATTTATCATCATAGCAATTGCCATAGTCCACCTACTATTCCTCCACGAAACAGGCTCCAACAA CCCAACAGGAATTTCCTCAGACGTAGACAAAATCCCATTCCACCCCTACTATACCATTAAGGACATCTTAGGGGCCCTCT TACTAATTCTAGCTCTAATACTACTAGTACTATTCGCACCCGACCTCCTCGGAGACCCAGATAACTACACCCCAGCCAAT CCAC	244
091317Te *B. taurus* Pro K Cyt B	6	TTCCATTTTATCCTTCCATTTATCATCATAGCAATTGCCATAGTCCACCTACTATTCCTCCACGAAACAGGCTCCAACAA CCCAACAGGAATTTCCTCAGACGTAGACAAAATCCCATTCCACCCCTACTATACCATTAAGGACATCTTAGGGGCCCTCT TACTAATTCTAGCTCTAATACTACTAGTACTATTCGCACCCGACCTCCTCGGAGACCCAGATAACTACACCCCAGCCAAT CCAC	244
091317Oc *B. taurus* Pro K Cyt B	6	TTCCATTTTATCCTTCCATTTATCATCATAGCAATTGCCATAGTCCACCTACTATTCCTCCACGAAACAGGCTCCAACAA CCCAACAGGAATTTCCTCAGACGTAGACAAAATCCCATTCCACCCCTACTATACCATTAAGGACATCTTAGGGGCCCTCT TACTAATTCTAGCTCTAATACTACTAGTACTATTCGCACCCGACCTCCKCGGAGACCCAGATAACTACACSCCAGSCAAT CCAS	244
051118Te *B. taurus* Pro k Cyt B	6	TTCCATTTTATCCTTCCATTTATCATCATAGCAATTGCCATAGTCCACCTACTATTCCTCCACGAAACAGGCTCCAACAA CCCAACAGGAATTTCCTCAGACGTAGACAAAATCCCATTCCACCCCTACTATACCATTAAGGACATCTTAGGGGCCCTCT TACTAATTCTAGCTCTAATACTACTAGTACTATTCGCACCCGACCTCCTCGGAGACCCAGATAACTACACCCCAGCCAAT CCAC	244
051118Oc *B. taurus* Pro K Cyt B	6	TTCCATTTTATCCTTCCATTTATCATCATAGCAATTGCCATAGTCCACCTACTATTCCTCCACGAAACAGGCTCCAACAA CCCAACAGGAATTTCCTCAGACGTAGACAAAATCCCATTCCACCCCTACTATACCATTAAGGACATCTTAGGGGCCCTCT TRCTAATTCTAGCTCTAATACTACTAGTACTATTCGCACCCGACCTCCKCGGAGASCCAKATARCTACACSSCAGSCAAT CCAS	244
091116Te *B. taurus* Pro K	6	TTCCATTTTATCCTTCCATTTATCATCATAGCAATTGCCATAGTCCACCTACTATTCCTCCACGAAACAGGCTCCAACAA CCCAACAGGAATTTCCTCAGACGTAGACAAAATCCCATTCCACCCCTACTATACCATTAAGGACATCTTAGGGGCCCTCT TACTAATTCTAGCTCTAATACTACTAGTACTATTCGCACCCGACCTCCTCGGAGACCCAGATAACTACACCCCAGCCAAT CCAC	244
091118Oc *B. taurus* Pro K Cyt B	6	TTCCATTTTATCCTTCCATTTATCATCATAGCAATTGCCATAGTCCACCTACTATTCCTCCACGAAACAGGCTCCAACAA CCCAACAGGAATTTCCTCAGACGTAGACAAAATCCCATTCCACCCCTACTATACCATTAAGGACATCTTAGGGGCCCTCT TRCTAATTCTAGCTCTAATACTACTAGTACTATTCGCACCCGACCTCCTCGGAGASCCAGATAACTACACSCCAGSCAAT CCAS	244
091019Te *B. taurus* Pro K Cyt B	6	TTCCATTTTATCCTTCCATTTATCATCATAGCAATTGCCATAGTCCACCTACTATTCCTCCACGAAACAGGCTCCAACAA CCCAACAGGAATTTCCTCAGACGTAGACAAAATCCCATTCCACCCCTACTATACCATTAAGGACATCTTAGGGGCCCTCT TACTAATTCTAGCTCTAATACTACTAGTACTATTCGCACCCGACCTCCTCGGAGACCCAGATAACTACACCCCAGCCAAT CCAC	244
091119Oc *B. taurus* Pro K Cyt B	6	TTCCATTTTATCCTTCCATTTATCATCATAGCAATTGCCATAGTCCACCTACTATTCCTCCACGAAACAGGCTCCAACAA CCCAACAGGAATTTCCTCAGACGTAGACAAAATCCCATTCCACCCCTACTATACCATTAAGGACATCTTAGGGGCCCTCT TRCTAATTCTAGSTCTAATACTACTAGTACTATTCGCACCCGACCTCCTCGGAGACCCAGATAACTACACCCCAGCCAAT CCAC	244

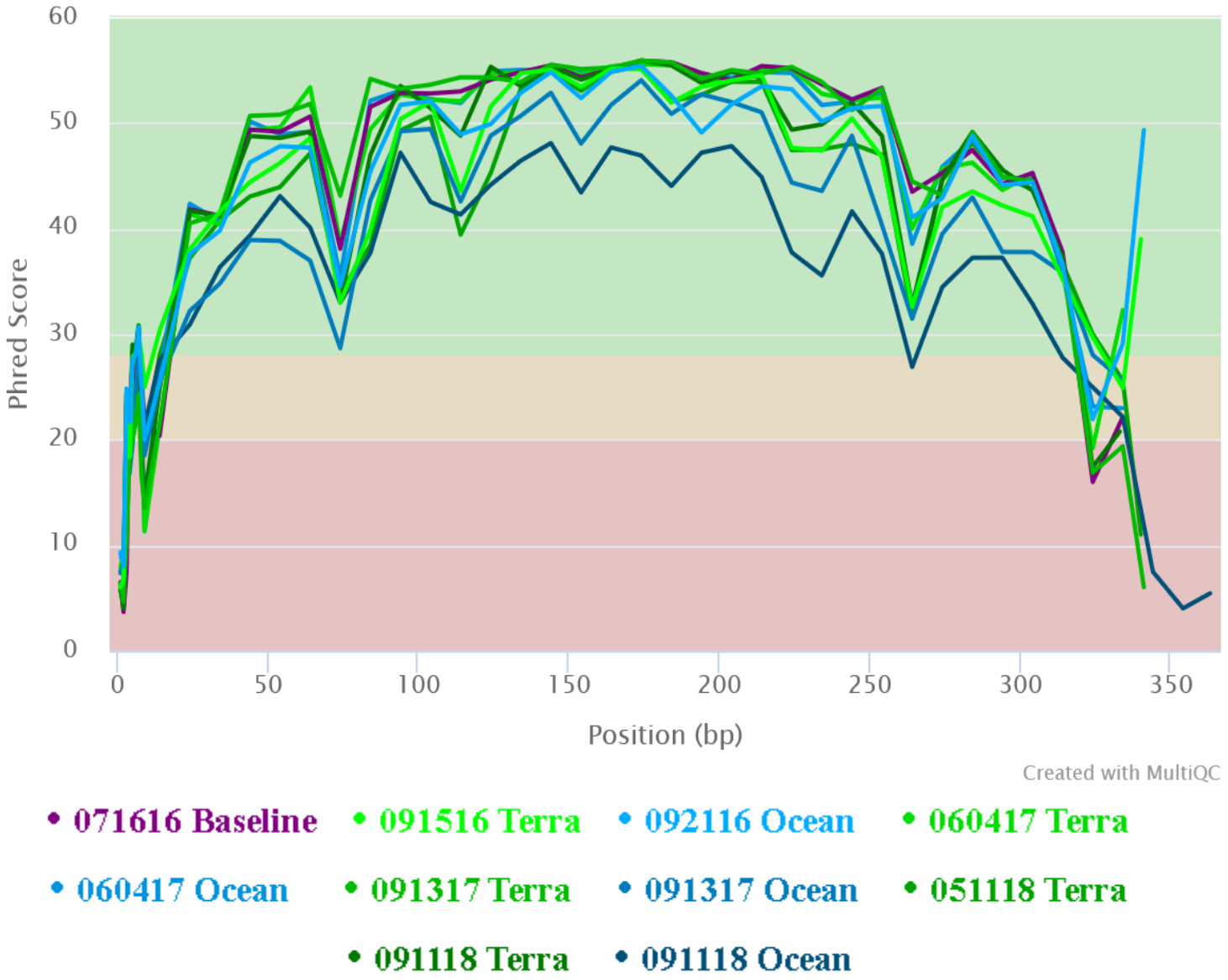
Name	# Sources	Sequence (5' -> 3')	Length
091516Te *B. taurus* CTII MC1R	6	CTACTACAACCACAAGGTCATCCTGCTGTGCCTCGTTGGCCTCTTCATAGCTATGCTGGCCCTGATGGCCGTCCTCTAC GTCCACATGCTGGCCCGGGCCTGCCAGCATGCCCGGGGCATTGCCCGGCTCCAGAAGAGGCAGCGCCCCATTCATCAGG GCTTTGGCCTCAAGGGCGCTGCCACCCTCACCATCCTGCTGGGCGTCTTCTTCCTCTGCTGGGGCC	224
060417Te *B. taurus* CTII MC1R	6	CTACTACAACCACAAGGTCATCCTGCTGTGCCTCGTTGGCCTCTTCATAGCTATGCTGGCCCTGATGGCCGTCCTCTAC GTCCACATGCTGGCCCGGGCCTGCCAGCATGCCCGGGGCATTGCCCGGCTCCAGAAGAGGCAGCGCCCCATTCATCAGG GCTTTGGCCTCAAGGGCGCTGCCACCCTCACCATCCTGCTGGGCGTCTTCTTCCTCTGCTGGGGCC	224
091317Te *B. taurus* CTII MC1R	6	CTACTACAACCACAAGGTCATCCTGCTGTGCCTCGTTGGCCTCTTCATAGCTATGCTGGCCCTGATGGCCGTCCTCTAC GTCCACATGCTGGCCCGGGCCTGCCAGCATGCCCGGGGCATTGCCCGGCTCCAGAAGAGGCAGCGCCCCATTCATCAGG GCTTTGGCCTCAAGGGCGCTGCCACCCTCACCATCCTGCTGGGCGTCTTCTTCCTCTGCTGGGGCC	224
051118Te *B. taurus* CTII MC1R	6	CTACTACAACCACAAGGTCATCCTGCTGTGCCTCGTTGGCCTCTTCATAGCTATGCTGGCCCTGATGGCCGTCCTCTAC GTCCACATGCTGGCCCGGGCCTGCCAGCATGCCCGGGGCATTGCCCGGCTCCAGAAGAGGCAGCGCCCCATTCATCAGG GCTTTGGCCTCAAGGGCGCTGCCACCCTCACCATCCTGCTGGGCGTCTTCTTCCTCTGCTGGGGCC	224
091118Te *B. taurus* CTII MC1R	6	CTACTACAACCACAAGGTCATCCTGCTGTGCCTCGTTGGCCTCTTCATAGCTATGCTGGCCCTGATGGCCGTCCTCTAC GTCCACATGCTGGCCCGGGCCTGCCAGCATGCCCGGGGCATTGCCCGGCTCCAGAAGAGGCAGCGCCCCATTCATCAGG GCTTTGGCCTCAAGGGCGCTGCCACCCTCACCATCCTGCTGGGCGTCTTCTTCCTCTGCTGGGGCC	224
091516Te *B. taurus* Pro K MC1R	6	CTACTACAACCACAAGGTCATCCTGCTGTGCCTCGTTGGCCTCTTCATAGCTATGCTGGCCCTGATGGCCGTCCTCTAC GTCCACATGCTGGCCCGGGCCTGCCAGCATGCCCGGGGCATTGCCCGGCTCCAGAAGAGGCAGCGCCCCATTCATCAGG GCTTTGGCCTCAAGGGCGCTGCCACCCTCACCATCCTGCTGGGCGTCTTCTTCCTCTGCTGGGGCC	224
060417Te *B. taurus* Pro K MC1R	6	CTACTACAACCACAAGGTCATCCTGCTGTGCCTCGTTGGCCTCTTCATAGCTATGCTGGCCCTGATGGCCGTCCTCTAC GTCCACATGCTGGCCCGGGCCTGCCAGCATGCCCGGGGCATTGCCCGGCTCCAGAAGAGGCAGCGCCCCATTCATCAGG GCTTTGGCCTCAAGGGCGCTGCCACCCTCACCATCCTGCTGGGCGTCTTCTTCCTCTGCTGGGGCC	224
091317Te *B. taurus* Pro K MC1R	6	CTACTACAACCACAAGGTCATCCTGCTGTGCCTCGTTGGCCTCTTCATAGCTATGCTGGCCCTGATGGCCGTCCTCTAC GTCCACATGCTGGCCCGGGCCTGCCAGCATGCCCGGGGCATTGCCCGGCTCCAGAAGAGGCAGCGCCCCATTCATCAGG GCTTTGGCCTCAAGGGCGCTGCCACCCTCACCATCCTGCTGGGCGTCTTCTTCCTCTGCTGGGGCC	224
091118Te *B. taurus* Pro K MC1R	5	CTACTACAACCACAAGGTCATCCTGCTGTGCCTCGTTGGCCTCTTCATAGCTATGCTGGCCCTGATGGCCGTCCTCTAC GTCCACATGCTGGCCCGGGCCTGCCAGCATGCCCGGGGCATTGCCCGGCTCCAGAAGAGGCAGCGCCCCATTCATCAGG GCTTTGGCCTCAAGGGCGCTGCCACCCTCACCATCCTGCTGGGCGTCTTCTTCCTCTGCTGGGGCC	224

Appendix 2: Consensus Sequence Quality Scores per Nucleotide

Appendix 2. FastQC: mean quality scores charts with individual nucleotide resolution from the consensus sequences generated from the *L. kempii* and *B. taurus* samples with both CTII and Pro K over the exposure time series. The nucleotide position is recorded along the horizontal axis. The average DNA quality scores are recorded along the vertical axis. The background color shows the divisions typical of phred scores quality. The green background indicates "good" phred scores (>28), yellow indicates "questionable" phred scores (28 to 10), and red indicates "poor" phred scores (<10). The line shades represent the length of exposure, lighter being shorter exposure and darker being longer exposure. The line colors represent the location of the sample, green indicates terrestrial, blue indicates oceanic, and purple indicates baseline. A) FastQC: Mean Quality Scores NADH4 mtDNA *L. kempii* samples demineralized with Pro K. B) FastQC: Mean Quality Scores NADH4 mtDNA *L. kempii* samples demineralized with CTII. C) FastQC: Mean Quality Scores Cyt B mtDNA *B. taurus* samples demineralized with Pro K. D) FastQC: Mean Quality Scores Cyt B mtDNA *B. taurus* samples demineralized with CTII. E) FastQC: Mean Quality Scores MC1R nuDNA *B. taurus* samples demineralized with Pro K. F) FastQC: Mean Quality Scores MC1R nuDNA *B. taurus* samples demineralized with CTII.



B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50
40
30
20
10
0
Phred Score
0
50
100
150
200
250
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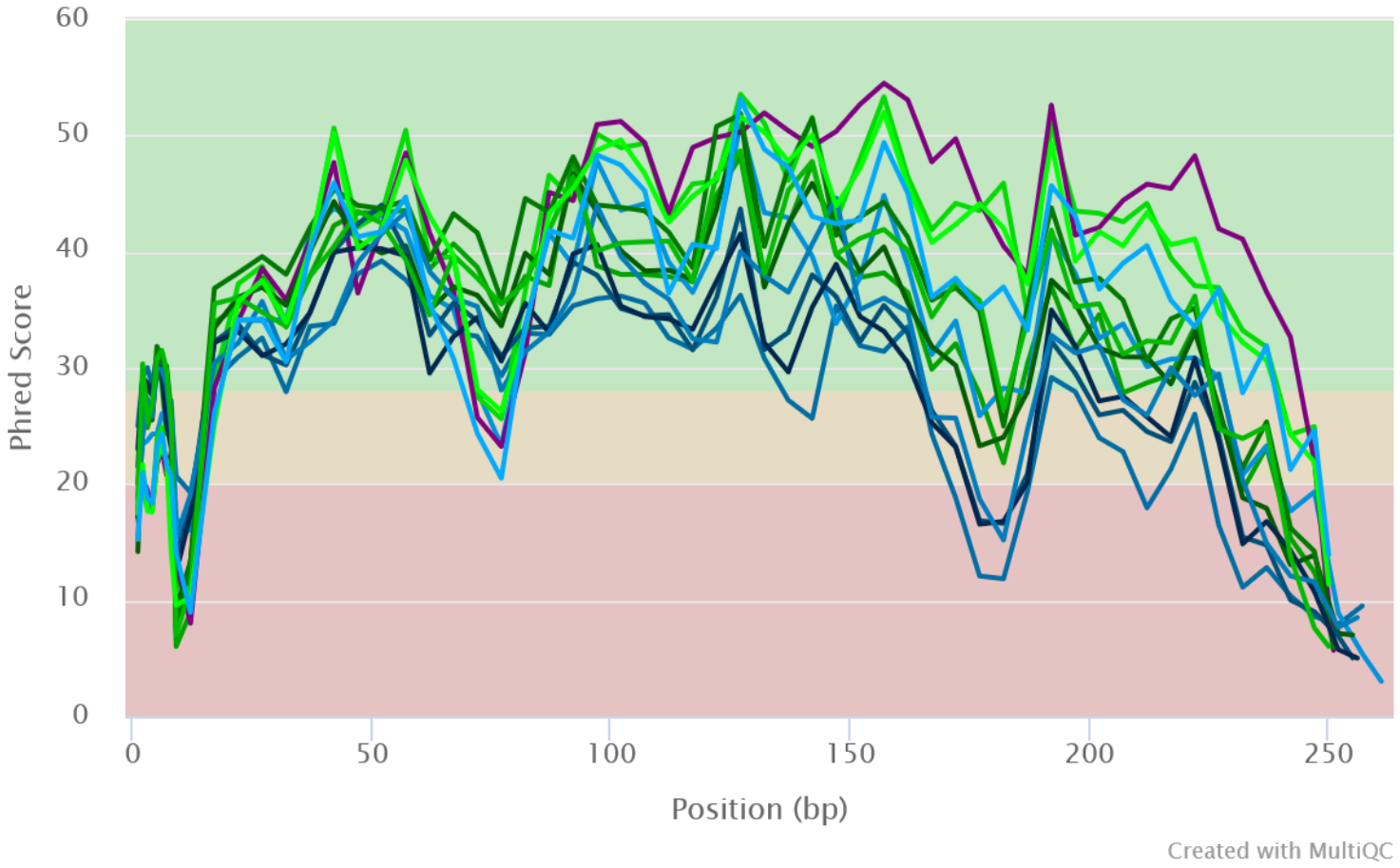
350
Position (bp)
Created with MultiQC
• 071616 Baseline
• 091516 Terra
• 092116 Ocean
• 060417 Terra
• 060417 Ocean
• 091317 Terra
• 091317 Ocean
• 051118 Terra
• 091118 Terra
• 091118 Ocean

C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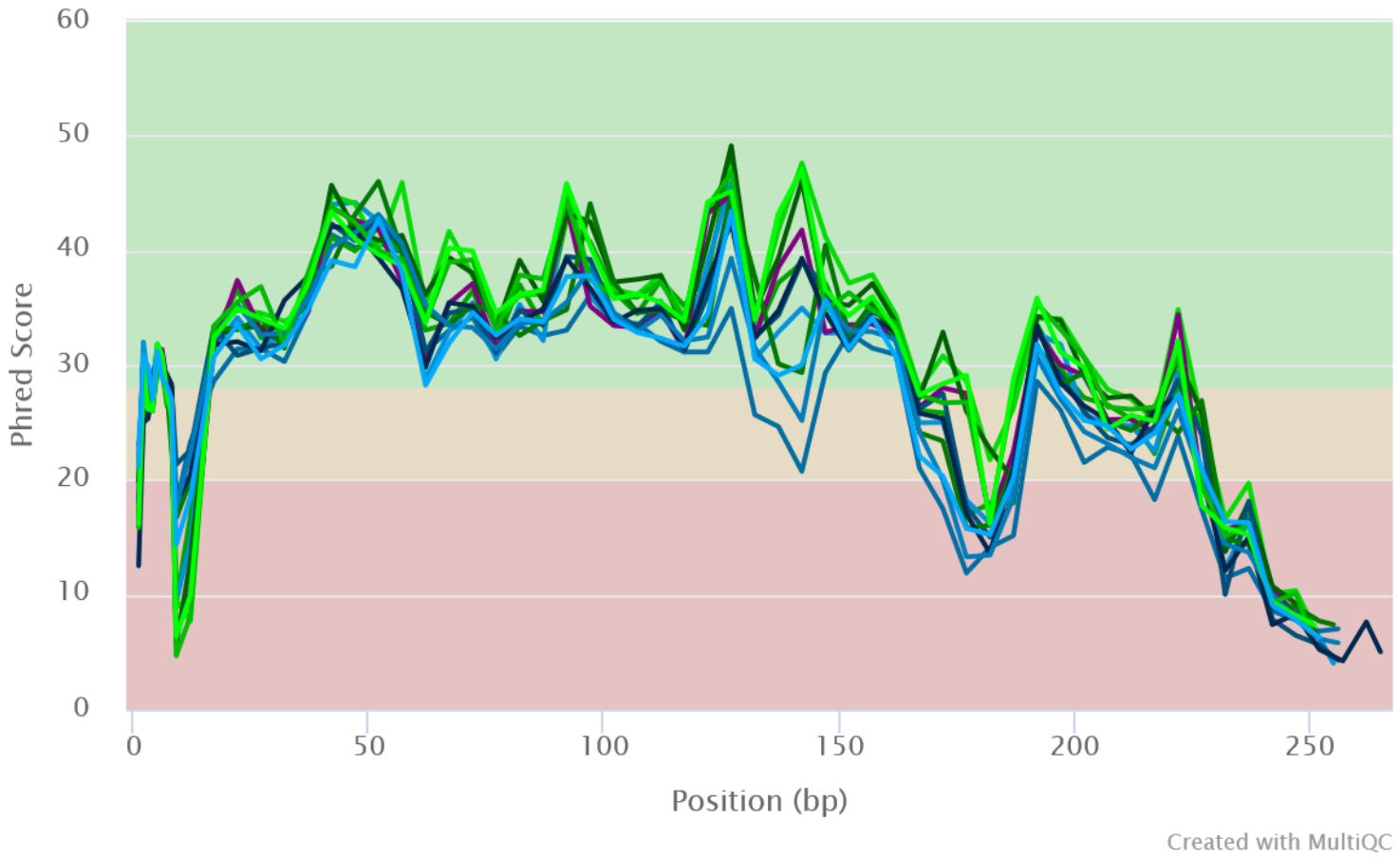
• 071616 Baseline
• 091516 Terra
• 092116 Ocean
• 060417 Terra
• 060417 Ocean
• 091317 Terra
• 091317 Ocean
• 051118 Terra
• 051118 Ocean
• 091118 Terra
• 091118 Ocean
• 091019 Terra
• 091119 Ocean

D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250
Position (bp)
Created with MultiQC
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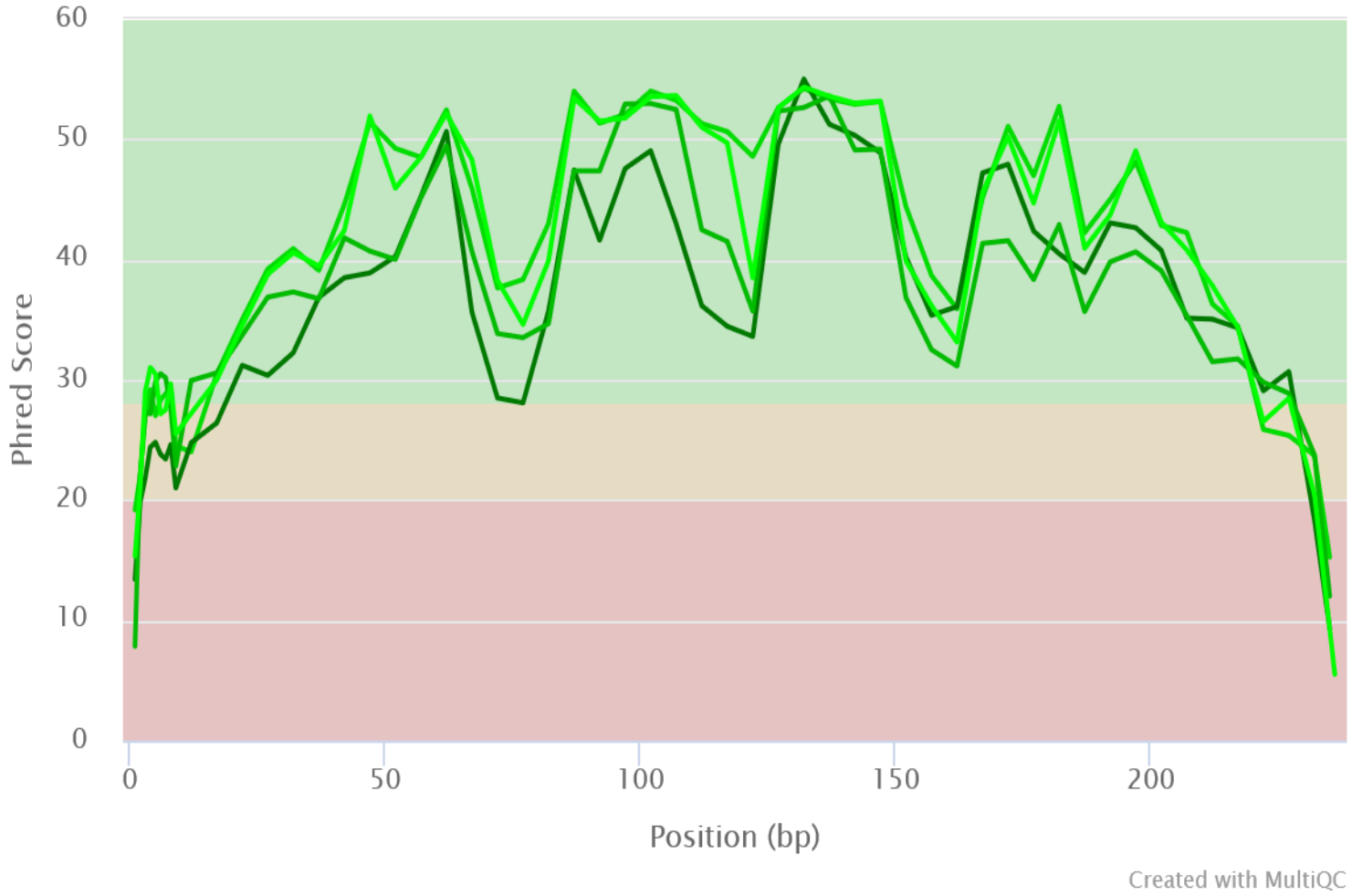
• 091516 Terra
• 092116 Ocean
• 060417 Terra
• 060417 Ocean
• 091317 Terra
• 091317 Ocean
• 051118 Terra
• 051118 Ocean
• 091118 Terra
• 091118 Ocean
• 091019 Terra
• 091119 Ocean

E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50
40
30
20
10
0
Phred Score
0
50
100
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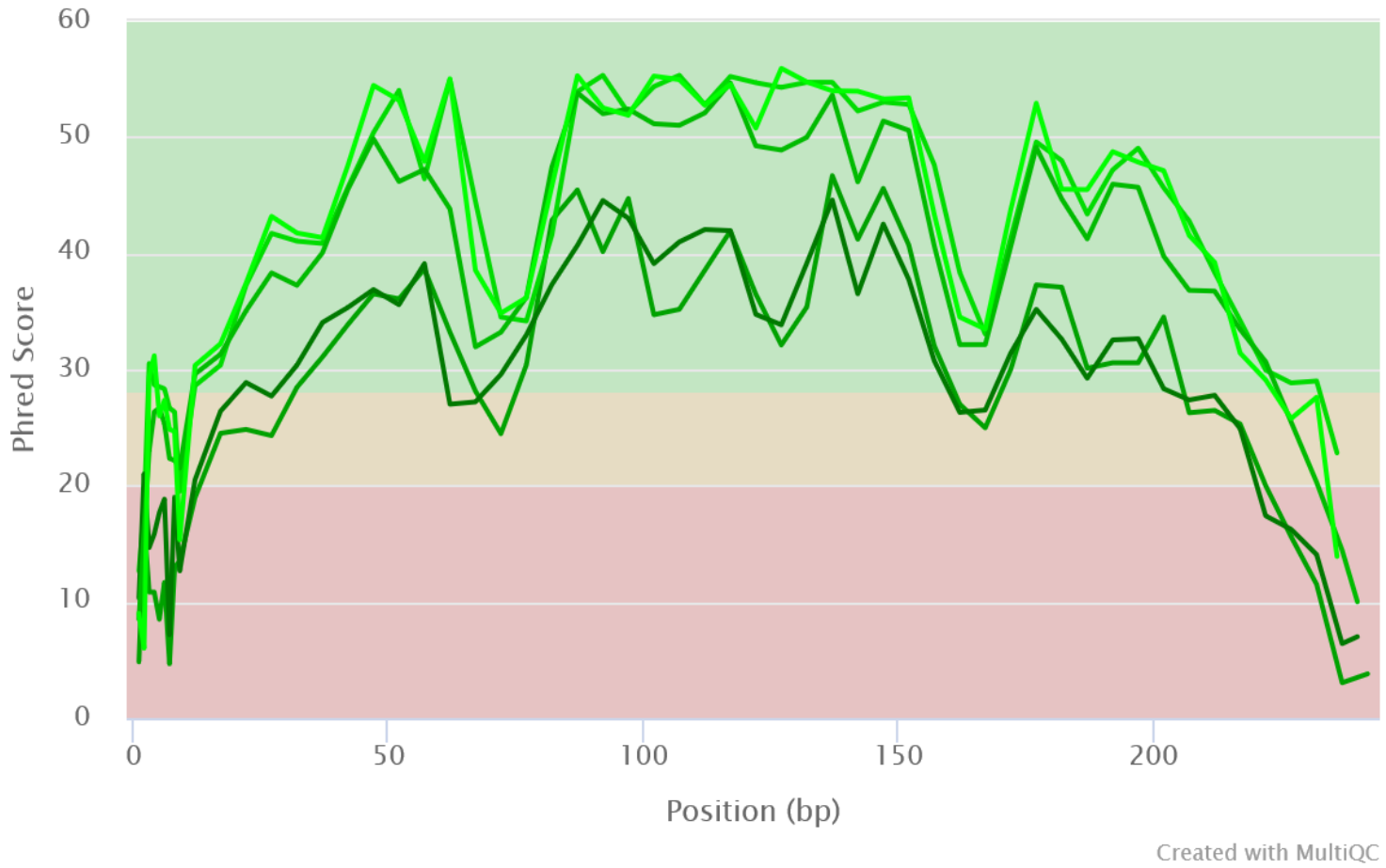
200
Position (bp)
Created with MultiQC
• 091516 Terra
• 060417 Terra
• 091317 Terra
• 091118 Terra

F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10
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Phred Score
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Position (bp)
Created with MultiQC
• 091516 Terra
• 060417 Terra
• 091317 Terra
• 051118 Terra
• 091118 Terra

Appendix 3: Quality Scores Table

Appendix 3. Duplication of Table 8 on page 31. DNA quality scores from individual replicates and averages per samples from each of the following data sets: *L. kempii* NADH4 Pro K, *L. kempii* NADH4 CTII, *B. taurus* Cyt B Pro K, *B. taurus* Cyt B CTII, *B. taurus* MC1R Pro K, and *B. taurus* MC1R CTII. All blanks represent failed sequencing results. The standard deviations (Std Dev) are listed towards the left each average. All reagent blanks did not produce sequencing data and were left out of the table. All sequences were detected by an ABI 3500 genetic analyzer. The sequences were aligned in Geneious software and converted to FASTQ files. The FASTQ files were opened using FASTQC and MultiQC and the phred scores were recorded.

Sample	Exposure (Days)	Quality (Phred Scores)						Average Quality (Phred Scores)									
		L. kempii NADH4 Pro K	*L. kempii* NADH4 CTII	*B. taurus* Cyt B Pro K	*B. taurus* Cyt B CTII	*B. taurus* MC1R Pro K	*B. taurus* MC1R CTII	*L. kempii* NADH4 Pro K	Std Dev	*L. kempii* NADH4 CTII	Std Dev	*B. taurus* Cyt B Pro K	Std Dev	*B. taurus* Cyt B CTII	Std Dev	*B. taurus* MC1R Pro K	Std Dev	*B. taurus* MC1R CTII	Std Dev
071616 Baseline	0	48	46	41	43			46.7	1.0	46.7	1.0	40.3	0.8	30.0	14.2				
		48	46	41	43														
		46	46	41	43														
		46	46	40	17														
		46	48	40	17														
		46	48	39	17														
091516 Terra	61	48	41	41	43	43	44	46.5	1.8	44.0	1.8	39.0	1.9	31.5	12.7	42.3	1.2	43.7	0.5
		48	43	41	43	43	44												
		48	44	40	43	43	44												
		46	45	38	22	43	44												
		45	45	37	21	42	43												
		44	46	37	17	40	43												
092116 Ocean	67	44	47	40	42			41.5	2.3	45.0	3.5	36.7	3.7	27.8	15.2				
		43	47	40	42														
		43	47	40	41														
		41	46	34	15														
		40	45	33	14														
		38	38	33	13														
060417 Terra	323	48	48	41	44	43	44	46.7	1.5	46.5	1.0	40.0	1.7	32.3	12.1	42.8	0.4	43.5	0.8
		48	47	41	43	43	44												
		48	47	41	43	43	44												
		46	46	41	22	43	44												
		45	46	39	22	43	43												
		45	45	37	20	42	42												
060417 Ocean	323	49	47	39	42			44.2	6.2	46.5	0.5	34.5	4.4	29.0	14.3				
		47	47	39	42														
		47	47	35	42														
		46	46	35	17														
		44	46	31	16														
		32	46	28	15														
091317 Terra	424	48	48	44	43	41	43	38.2	10.2	46.8	1.3	34.8	12.5	31.7	12.1	38.8	2.4	41.0	2.3
		47	48	44	43	41	43												
		47	48	43	42	40	42												
		31	46	40	22	39	41												
		30	46	22	21	37	40												
		26	45	16	19	35	37												

091317 Ocean	424	46	44	43	42			38.0	8.0	41.0	2.8	30.0	13.2	27.7	15.0				
		45	43	42	41														
		45	43	41	41														
		31	40	19	15														
		31	39	18	14														
		30	37	17	13														
051118 Terra	664	49	45	43	42		37	40.2	9.0	43.7	1.0	33.3	11.0	29.8	13.3			30.7	6.0
		48	44	43	42		36												
		48	44	43	42		33												
		33	44	29	19		30												
		32	43	22	17		27												
		31	42	20	17		21												
051118 Ocean	664			42	41							26.8	15.2	26.2	14.2				
				42	40														
				38	36														
				13	14														
				13	13														
				13	13														
091118 Terra	787	48	46	44	43	28	41	46.5	1.0	45.3	0.8	37.2	6.8	29.2	13.7	38.0	5.8	31.2	11.5
		47	46	44	41	38	39												
		47	46	42	41	40	37												
		46	45	32	18	42	37												
		46	45	31	16	42	17												
		45	44	30	16		16												
091118 Ocean	787	47	44	43	43			44.5	2.2	36.8	8.6	29.0	14.6	28.8	15.2				
		46	44	42	43														
		45	41	42	42														
		45	40	17	15														
		43	27	15	15														
		41	25	15	15														
091019 Terra	1151			42	44							33.2	9.3	31.5	13.0				
				42	44														
				41	42														
				25	20														
				25	20														
				24	19														
091119 Ocean	1152	47		42	43			44.8	1.6			29.0	12.8	28.7	15.1				
		46		40	42														
		45		39	42														
		45		23	18														
		43		15	14														
		43		15	13														