Comprehensive stranding investigations: the application of in-house diagnostics, disease surveillance and research to further understand the timing and cause of strandings

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ABSTRACT

The University of Hawai'i Health and Stranding Lab (HSL), located in Kaneohe Bay, is the only entity in the Pacific Islands region that responds to strandings, conducts necropsy and cause of death investigations, archives tissues and performs research to identify and evaluate threats to Pacific Island cetaceans. The purpose of this project has three objectives: 1) To conduct comprehensive stranding investigations for high priority species through increased capacity for in-house diagnostics; 2) To analyze archived tissues for the presence of two pathogens (circovirus and morbillivirus) and to examine the detectability of *Toxoplasma* positive animals over time and 3) To conduct analyses of historical stranding patterns and causes of mortality that incorporate quantitative estimates of stranding date and advanced diagnostic information. Progress to date includes the development of an operational in-house polymerase chain reaction (PCR) laboratory to screen for known pathogens of concern and the development of tooth aging capabilities at HSL. PCR screening has been conducted on archived tissues representing 20 stranded individuals for circovirus, an emerging disease in cetaceans. The 20 individuals screened for circovirus represent 6 different species. Of these, 35% (7/20) tested positive in at least two of six tissues tested by PCR which was subsequently confirmed by sequencing analysis and represents positive findings in six species. We have also built upon an initial DNA degradation experiment conducted by our laboratory to develop a quantitative tool to estimate the post-mortem interval in stranded specimens that were not fresh dead at the time of stranding discovery. DNA degradation results compared among individuals, tissue types and environmental conditions indicate a significant linear relationship (r^2=0.76) in degradation rate up until 28 days post mortem. These findings can be applied to quantitatively estimate the actual day of death in stranded specimens that may be discovered stranded on a considerably different timescale, leading to increased robustness of temporal stranding analysis in the Pacific Islands region.

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INTRODUCTION

Comprehensive stranding investigations for high priority species through increased capacity for in-house diagnostics

Background

Infectious disease is becoming well recognized as a significant threat to Pacific Island cetaceans. Morbillivirus was first identified in the Pacific Islands region from a Longman's beaked whale (*Indopacetus pacificus*) that stranded in 2010 (West et al., 2013).We have since reported on morbillivirus findings from archived Hawaiian stranding samples (West et al., 2015; Jacob et al., 2016), were involved in new method validation for global surveillance of morbillivirus (Yang et al., 2016) and have contributed to whole genome sequencing in order to describe the unique beaked whale morbillivirus strain (Landrau-Giovanetti et al., 2019). We have most recently discovered another novel strain of morbillivirus in a Fraser's dolphin (*Lagenodelphis hoseii*) that stranded in 2018 that is distinct from both the beaked whale morbillivirus and the Southern hemisphere strains (West et al., Submitted). We have reported other pathogens that threaten Hawaiian cetaceans, including the presence of *Cryptococcus gatti* in a Hawaiian spinner dolphin (*Stenella longirostris*) (Rotstein et al., 2010), *Brucella* cases that involve a neonate sperm whale (*Physeter macrocephalus*) co-infected with morbillivirus (Chernov, 2010; West et al., 2015) and fatal disseminated toxoplasmosis in 3 stranded spinner dolphin deaths where we project based on low carcass recovery rates that this could be representative of the deaths of 60 spinner dolphins (West et al., 2020). We have recently characterized the full genome of the first known cetacean circovirus that was identified from a stranded Longman's beaked whale (Landrau-Giovanetti et al., 2020) as well as four novel cetacean herpes viruses from this region (West at al., 2013; West and Waltzek, unpublished data). Despite describing numerous pathogens that contribute to mortality, the discontinuous coastlines of the Pacific islands combined with dominant currents running towards open ocean lead us to we believe that stranding events in the Pacific Islands region only represent a very small subset of the animals that are actually dying, with most being swept out to sea. Consequently, a true epizootic event may only be detectable with the most concerted efforts. A top priority must be to conduct continued disease surveillance and our prior work highlights the value of thoroughly examining every single carcass so that we understand the potential for disease outbreaks that threaten to decimate small island-associated populations. The development of an operational polymerase chain reaction (PCR) laboratory at the HSL facility for disease screening provides a means for cost effective analysis of stranding samples (both archived and representative of current stranding events) to better understand the impact of disease on cetaceans in the region.

Information on age is an important component of life history data that is critical to the full interpretation of contaminant burdens or age-related disease conditions among marine mammals. Additionally, shifts in population demographics may serve as a potential early warning indicator of a detrimental population level response to stressors in marine mammals. Odontocete cetaceans, which comprise the majority of cetaceans species documented in the Pacific Islands region, have traditionally been aged by the counting of growth layer groups in histological sections prepared from their teeth. Despite the value in determining the age of stranded cetaceans using teeth, there are few laboratories that offer odontocete tooth aging services on a fee for sample basis. One wildlife laboratory that offers such services for a wide variety of terrestrial species did not generate reliable ages when a set of trial odontocete teeth were submitted for processing and subsequent aging (Baird, personal comm.). Consequently, few teeth from Hawaiian cetaceans have been aged with the exception of a small number of high priority individuals as part of a

collaboration with Southwest Fisheries Science Center. The development of in-house aging capabilities at the HSL facility provides opportunity to obtain age estimates from stranded individuals, contributing to the suite of advanced diagnostic information available for any given case and aiding in the interpretation of the significance of stranding findings.

Screening of archived tissues for the presence of two pathogens (circovirus and morbillivirus) and examining the detectability of Toxoplasma positive animals over time

Background

Knowledge of emerging infectious diseases is critical to understanding the threat that any given disease poses to cetaceans on a population level. The application of Next Generation Sequencing (NGS) technology allows for the detection of emerging viral and bacterial pathogens. A recent NGS application identified a circovirus in archived tissues from a Longman's beaked whale that had previously stranded on the island of Maui which represents the first known case of circovirus in a marine mammal world-wide (Landrau-Giovanetti et al., 2020). The nucleotide similarity of this circovirus to other circovirus species was only 56% and falls below the 80% similarity threshold for consideration as a new species. The full genome of this newly identified marine mammal circovirus has been characterized and named as beaked whale circovirus (BWCV) (Breitbart et al., 2017; Landrau-Giovanetti et al., 2020).

Circoviruses are small, single stranded DNA viruses of the family c*ircoviridae* found in vertebrate animals, with the majority of circovirus species found in mammals (Breitbart et al., 2017). Diseases that are the result of circovirus infections are generally of interest to agriculture and pet trade industries, as these viruses have the potential to cause severely detrimental health effects to their host animals (Gavier-Widen et al., 2012; Seo et al., 2014; Yang et al., 2015). Multiple strains of circovirus have also been directly linked as a cause of death in fish, birds and pigs (Lorincz et al., 2011; Woods et al., 1993; Yang et al., 2015). Two major diseases that result due to the presence of a circovirus infection are psittacine beak and feather disease (PBFD) in birds and postweaning multisystemic wasting syndrome (PMWS) in swine (Fogell et al., 2018; Rose et al., 2012). These wasting diseases suppress the immune systems of the affected animals and can result in diminished or deformed growth, respiratory distress and ultimately mortality (Alarcon et al., 2013; O' Dea et al., 2011; Peters et al., 2014; Sarker et al., 2015).

The recent discovery of circovirus in a stranded marine mammal raises many questions about the prevalence and potential impact of this virus on marine mammal populations. It is currently unknown if the presence of BWCV in the stranded Longman's beaked whale represents a highly unusual finding or is actually a common, but previously unidentified, finding among stranded cetaceans. Circoviruses can cause immunosuppression in other species (Peters et al., 2014; Rose et al., 2012) and it also remains unknown if any of the pathology observed in the stranded Longman's beaked whale was caused by circovirus. This stranded individual was believed to have died from morbillivirus and was tri-infected with herpes virus and circovirus (West et al., 2013; Landrau-Giovanetti et al., 2020), making this a complicated case for evaluating the potential clinical significance of a circovirus infection in marine mammals. Co-infections in cetaceans have been described from several regions of the world but to our knowledge this represents the first report of a tri-infection. For example, co-infection of *Brucella* and morbillivirus was reported in a sperm whale in Hawaii (West et al., 2015), nine striped dolphins were co-infected with morbillivirus and herpesvirus in the Mediterranean (Vargas-Castro et al., 2021), a minke whale was co-infected with *Brucella* and herpesvirus in the United Kingdom (Davison et al., 2021) and morbillivirus and toxoplasmosis was described from a fin whale in Italy (Mazzariol et al., 2012). There is much yet to be

learned about cetacean circovirus and the interplay that this novel disease may have with other cetacean pathogens of concern.

Analyses of historical stranding patterns and causes of mortality that incorporates quantitative estimates of stranding date and advanced diagnostic information.

Background

In order to conduct a robust statistical analysis of historical stranding data, it is necessary to consider the date of stranding in any form of a temporal analysis. The date that a stranding is discovered may be considerably different from the date of death as animals may float dead at sea for some time before initial observation or prior to currents driving these carcasses to the shoreline. Additionally, large areas of coastline in the Hawaiian and Mariana Islands are remote and inaccessible, where dead stranded cetaceans may decompose in place from days to weeks to months before discovery. Certainly, carcass recovery of stranded cetaceans in the remote Pacific Islands is low and close to half of the historical stranding events reported in the past decade represent stranded cetaceans that have undergone some extent of decomposition and were not fresh dead at the time of initial discovery (Fisheries, NOAA database).

Reported stranding data can be misleading as the date of initial observation does not account for qualitative or quantitative assessment of date of death in cetaceans that are not fresh dead at the time of discovery. The time from death to carcass discovery and subsequent necropsy is also known as the post-mortem interval (PMI). Understanding the PMI has been critical in the determination of time of death in realms outside of marine mammal science and is most well recognized for fine time scale determination used in human forensics with direct application to criminal investigations (Cina, 1994; Di Nunno et al., 1998). Similarly, understanding PMI in marine mammals has the potential to lead to a better understanding of the temporal relationship between anthropogenic and environmental events and date of death, useful in establishing causes that contribute to stranding events.

Determination of the PMI has been the subject of much research in a number of species other than marine mammals. Body rigidity and coloration has historically been used for establishing ranges of time of death in several species, and while easily determined, these morphological characteristics can be subjective and are lacking in quantitative description (Cina, 1994; Di Nunno et al., 1998). Identification of bacterial presence and subject biochemistry have also been used to estimate PMI (Madea, 2015; Madea and Henssge 1990). These metrics have a high margin of error, being directly influenced by the geography, environment and physiological factors present in the subject (Tozzo et al., 2020).

Recent studies have shifted to assessment of subject DNA as a method for determination of the PMI. DNA molecules have been found to remain relatively stable in a number of different animals and their various tissues (Liu et al., 2007). The degradation of these molecules can be assessed over time, showing a linear rate of decay based on the DNA concentration (Boy et al., 2003). PMI determination through DNA degradation analysis has been used in a wide range of human tissues, including muscle, internal organs, teeth, and bones (Mansour et al., 2019; Williams et al., 2015; Li et al., 2011; Alaeddini et al., 2011). The tracking of DNA degradation has been successfully correlated with PMI ranging from hours to weeks in tissues sampled from muscle and internal organs of rats (Xiong et al., 2010; Itani et al., 2011; Zaki et al., 2017), eye tissue and skeletal muscle from rabbits (Liu et al., 2003; Nazir et al., 2011), and muscle and internal organs from pigs (Larkin et al., 2010; Johnson et al., 2002). To our knowledge PMI has not previously been investigated in stranded cetaceans but may provide a reliable means to estimate the date of death as compared to the date of initial observation in stranded cetaceans.

METHODS

Comprehensive stranding investigations for high priority species through increased capacity for in-house diagnostics

Developing the capacity to perform in-house polymerase chain reaction (PCR) diagnostics at the HSL facility involved acquiring instrumentation, protocol development and the validation of positive and negative control samples for selected bioassays. New instrumentation in support of in-house diagnostics included a miniPCR 16 thermocycler unit (miniPCR, Cambridge, Massachusetts). This device allows for the replication of existing protocols and the development of new protocols involved in the amplification of DNA and RNA from viruses and bacteria. Additionally, a Qubit 4 fluorometer (Thermo Fisher Scientific, Waltham, Massachusetts) was installed for the determination of DNA and RNA fragment concentration. Together, these devices allow for the initial establishment of testing protocols.

Protocol development and validation has involved the comparison of PCR reagents and primers in terms of their efficacy, shelf life, and cost, ultimately selecting Thermo Fisher and Invitrogen (Thermo Fisher Scientific, Waltham, Massachusetts) products. Using known positive animals for specific diseases preserved in -80 C freezer archives, the testing of diagnostic protocols have targeted cetacean strains of *Brucella*, circovirus, herpesvirus, morbillivirus and *Toxoplasma*. Development of in-house methodology has involved the testing of published primer sets and the subsequent alteration of melting temperatures and annealing times to work most effectively on our instrumentation. This effort has also included primer design based on information from genome databases and creating original thermocycler protocols to improve upon detectability of selected genes.

Cetacean teeth vary greatly in size, depending on species, and different preparations for the aging of teeth of variable size is needed. For small and medium sized teeth, we developed a method that involved mounting a tooth to glass slides with crystal bond. Once mounted, the tooth was then wet-sanded using 400, 600, 1500, and 1200 grit sandpaper with water coating the sandpaper. The tooth was then heated to release the crystal bond and flipped over to repeat the sanding process on the other side. The sanding and polishing was performed until the section was transparent. Following the development of this initial procedure, a staining process was developed involving decalcification of the teeth using the rapid decalcifying solution Osteomoll, then sectioning of the teeth with a microtome to 20-40μm thick sections. These sections were then stained using hematoxylin, which will darken the layers of dentine within the teeth, before moving on to the aging steps.

For teeth from medium to large cetaceans, a tooth was first mounted to a wooden rod. The tooth was then cut into two sections, using a slightly off-center cut, on a water-cooled Gryphon AquaSaw Diamond Band Frag Saw (Bulk Reef Supply, Golden Valley, Minnesota). This off-center cut allows for the larger section to retain all of the surface necessary for the final aging process. This larger section was then wet-sanded using 400, 600, 1000, and 1500 grit sandpaper with a water coating until it was at the centerline of the formerly intact tooth's width. The tooth was then alternated between exposure in dilute formic acid and acetone baths and rinsed with water for three-minute intervals between baths and drying post rinse. This process was repeated to etch the dentine until the separate layers were visible. Once the etching was complete, a #1 soft-graphite pencil was then gently rubbed along the surface of the tooth to enhance the visibility of the dentine layers.

Screening of archived tissues for the presence of two pathogens (circovirus and morbillivirus) and examining the detectability of Toxoplasma positive animals over time

Progress to date focuses on PCR screening of archived stranding samples for the presence of circovirus. From our extensive tissue archives at HSL, 20 stranded individuals that represent eight different cetacean species were selected for PCR screening of archived tissues. Animals were selected based on pathologies identified during necropsy and subsequent cause of death investigations that were similar to the pathologies associated with circovirus in other species, including immune suppression, respiratory illness, and poor body condition (Alarcon et al., 2013; Gavier-Widen et al., 2012; Rose et al., 2012; Yang et al., 2015). For each animal, an initial suite of six tissues was targeted for testing from archived samples stored at - 80C: cerebrum, lung, kidney, liver, spleen and lymph node. For animals that did not have all six of these tissues available, pancreas or adrenal gland tissues were alternatively selected to total six separately analyzed tissue samples for each individual. DNA was extracted from the tissue samples using Qiagen DNeasy Blood and Tissue extraction kits (Qiagen, Germantown, Maryland). Extracted samples were then tested using a Qubit fluorometer to confirm adequate concentrations of the extracted DNA prior to proceeding with PCR testing.

Following extraction, PCR amplification of the extracted DNA was performed using primers designed to select for the BWCV genome, using tissue from the initial circovirus positive Longman's beaked whale as a positive control for in-house PCR screening (Landrau-Giovanetti et al., 2020). Amplified PCR product was then visualized using gel electrophoresis to confirm the correct band size. Bands from the positive control and a subset of the positive cases were excised from the gels and the PCR product was cleaned using Qiaquick PCR and Gel Cleanup Kits (Qiagen, Germantown, Maryland). Cleaned product was then submitted to the University of Hawai'i's Advanced Studies in Genomics, Proteomics, and Bioinformatics laboratory (ASGPB) for Sanger DNA sequencing to allow for sequence comparison to BWCV.

Analyses of historical stranding patterns and causes of mortality that incorporates quantitative estimates of stranding date and advanced diagnostic information.

Two fresh dead spinner dolphins, KW2015013 and KW2019006, were selected for the DNA degradation experiment. Both individuals were qualitatively determined to be fresh dead animals based on internal and external examinations, which included assessment of internal organ coloration and palpation characteristics. From each of these two animals, the tissue types that were selected for DNA degradation included: cerebrum, cerebellum, lung, liver, mesenteric lymph node and hilar/marginal lymph nodes. Subsamples of each tissue type were taken and vacuum-sealed in sample bags to simulate visceral membranes that surround internal organs *in situ*.

Subsamples were then sealed in containers used to establish two separate environmental conditions for the DNA degradation experiment. One set of subsamples were prepared for "Air" treatment by covering the sealed container with a black bag for UV protection. The container was then placed on the UH Health and Stranding Lab facility pier to replicate a beached cetacean carcass experiencing air temperature conditions. Subsamples prepared for the "Water" treatment were similarly sealed in a waterproof container and tethered to the same pier, to replicate the water temperature exposure of a floating carcass.

Small samples of each tissue were taken from each treatment on days 1, 3, 5, 7, 10, and 14 for the initial DNA degradation experiment. Sampling days were selected based on suspected decomposition rate from

past qualitative observations of cetacean carcasses. Sampling days were targeted in effort to increase the likelihood of detecting significant changes in decomposition over a 14-day time period. Preliminary findings from this initial experiment were built upon and extended to double the timeframe of data collection by degrading samples until days 21 and 28. DNA was extracted from approximately 100 mg samples of each tissue using Qiagen DNeasy Blood and Tissue Kits (Qiagen, Germantown, Maryland). A total of six extracts were collected for each treatment on each sampling date. Final DNA concentration of each extract was then determined through the use of a Qubit 4 fluorometer bioassay and reported in ng/µL of DNA. Remaining DNA extracts were then stored at -20 C for future analytics.

Statistical analyses of DNA concentrations measured during the 28-day DNA degradation experiment included a three-way analysis of variance to investigate potential interactions between treatment conditions, tissue types and degradation over time. There were no significant differences in total DNA concentration between tissue types, treatment type and time. Data from individuals and tissue types were then combined for each day of sampling. Linear regression analysis was used to analyze the relationship between total DNA concentration and time for all samples in the air treatment group, the water treatment group and both treatment groups combined. Statistical analyses were conducted using R software.

RESULTS

Comprehensive stranding investigations for high priority species through increased capacity for in-house diagnostics

Protocols for the in-house testing for the presence of circovirus, morbillivirus, and *Toxoplasma* have been developed and validated through the use of tissues from archived animals that represent known positives and negatives based on prior analysis at outside laboratories. Herpesvirus protocols are in early stages of development, having selected and conducted initial testing of published primer sets.

Counting of the growth layer groups evident within the sectioned teeth was conducted to determine the success of our methodology development protocols. Layers of dentine appeared as light and dark growth rings, running the length of the tooth. The central layer is not counted as this represents current growth. The outer layer is also not included in the count as this layer is formed before birth and is known as the neonatal line. Mounted sectioned teeth were analyzed using a dissecting microscope by counting each layer group, and then recording the number of layers as the calculated age for the assessed individual.

The tooth of a false killer whale (*Pseudorca crassidens*) that stranded in 2010 was used to validate the described methods for in-house aging of medium to large sized cetacean teeth (Figure 1). This animal was initially determined to be 22 years of age by collaborators at the Southwest Fisheries Science Center. Following the described in-house preparation methods, our laboratory aged this individual at 20-22 years old, suggesting a small margin of error when aging teeth at the HSL facility.

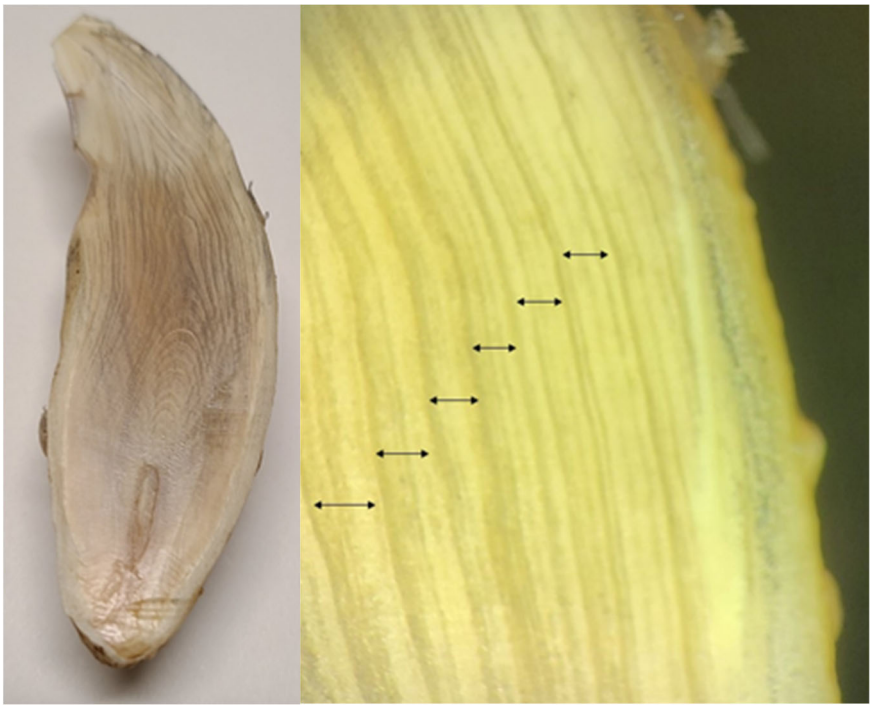

Figure 1: Acid etched tooth from KW2010019, a false killer whale that stranded in 2010. This tooth has been cut, sanded, acid etched and marked with graphite. The graphite marks the growth layer groups as the unmarked, lighter bands. Each arrow represents a growth layer group, representing a year of growth.

Screening of archived tissues for the presence of two pathogens (circovirus and morbillivirus) and examining the detectability of Toxoplasma positive animals over time

During PCR testing, detection was not initially consistent when conducting a single replicate, so multiple PCR replicates were performed. To date, all tissues listed in Table 1 have been analyzed at minimum twice, with intended third replicates still to be run for select tissues for seven individuals. The screening tests for the twenty selected animals detected the presence of BWCV in seven of the individuals, across six different species, indicated in gray in Table 1. Cleaned and amplified products from each of the positive test case animals, as well as samples from the control animal (Longman's beaked whale), were sequenced and analyzed in BLAST. For the control sample, the brain PCR product was 96.4% similar and the lung PCR product was found to be 97.3% similar to the beaked whale circovirus genome. Percent similarity for the test samples are indicated by the numbers in the cells of each sequenced tissue in Table 1 and range between 97.1% and 99.4% similarity. The most commonly positive tissues across the seven positive cases were the brain (n=6) and the liver (n=6). The striped dolphin (*Stenella coeruleoalba*) (stranded 5/2/2020) was the only animal to test positive in all six tissues, though the spinner dolphin (stranded 10/14/2008) tested positive in all tissues that were available for testing in the sample archive (brain, kidney, and liver).

Table 1. PCR results for the amplification of tested tissues. Numbers in parentheses indicate the percent similarity the amplified product had to the beaked whale circovirus genome in BLAST when compared post sequencing. Positive cases are highlighted in gray. nt = not tested

Species	Stranding Date	Location	Brain	Lung	Kidney	Spleen	Liver	Lymph Node	Pancreas	Adrenal
Kogia sima	8/31/2000	Kailua, Oahu	+	+ (97.8)	-	+ (98.8)	+	nt	nt	-
Stenella longirostris	10/14/2008	Kailua-Kona, Hawaii	+	nt	+ (97.2)	nt	+	nt	nt	nt
Ziphius cavirostris	8/23/2011	Saipan	-	+	+	+ (97.7)	+ (97.2)	+	nt	nt
Ziphius cavirostris	3/23/2015	Merizo, Guam	-	-	-	-	-	-	nt	nt
Ziphius cavirostris	5/6/2015	American Samoa	+ (99.4)	-	+	+	-	-	nt	nt
Ziphius cavirostris	2/15/2016	South Point, Hawaii	-	-	-	-	-	nt	-	nt
Globicephala macrorhynchus	10/13/2017	Kalapaki Beach, Kauai	-	-	-	-	-	nt	-	nt
Globicephala macrorhynchus	10/13/2017	Kalapaki Beach, Kauai	-	-	-	-	-	-	nt	nt
Globicephala macrorhynchus	10/13/2017	Kalapaki Beach, Kauai	nt	-	-	-	-	-	nt	nt
Lagenodelphis hoseii	2/7/2018	Ukumehame Beach, Maui	+ (97.1)	-	-	-	+ (97.2)	-	nt	nt
Peponocephala electra	4/9/2019	Kailua, Oahu	+ (97.4)	+ (97.8)	-	+	+	+	nt	nt
Feresa attenuata	8/29/2019	Sugar Beach, Maui	-	-	-	-	-	-	nt	nt
Feresa attenuata	8/29/2019	Sugar Beach, Maui	-	-	-	-	-	-	nt	nt
Feresa attenuata	8/29/2019	Sugar Beach, Maui	-	-	-	-	-	-	nt	nt
Feresa attenuata	8/29/2019	Sugar Beach, Maui	-	-	-	-	-	-	nt	nt
Feresa attenuata	8/29/2019	Sugar Beach, Maui	-	-	-	-	-	-	nt	nt
Feresa attenuata	8/29/2019	Sugar Beach, Maui	-	-	-	-	-	-	nt	nt
Feresa attenuata	8/29/2019	Sugar Beach, Maui	-	-	-	-	-	-	nt	nt
Stenella coeruleoalba	5/2/2020	Sugar Beach, Maui	+ (98.3)	+	+	+	+	+	nt	nt
Stenella coeruleoalba	6/19/2020	Kaaawa, Oahu	-	-	-	-	-	-	nt	nt

Analyses of historical stranding patterns and causes of mortality that incorporates quantitative estimates of stranding date and advanced diagnostic information.

Regression models were generated for the 14-day and 28-day trials (Figure 2). These findings indicate that the relatively longer time scale of 28 days accounts for greater variability within the datasets, with the best fit regression being within the "Air" condition dataset that extends to 28 days ($r^2 = 0.856$). When considering both the 14-day and 28-day tissue degradation time frames, the DNA concentration decreased in a linear fashion over time. This effect was apparent in all tissues, regardless of the environmental condition ($P < 0.001$) based on the three-way analysis of variance. Pairwise multiple comparison procedures using the Holm-Sidak method were applied. Significant differences were found in the amount of degradation that occurred between tissue types that were tested under "Air" environmental conditions, with higher rates of degradation occurring in the mesenteric lymph nodes, when compared to the other five tissues tested (P range <0.001 to 0.006). Significant differences were only apparent between "Air" and "Water" conditions in the cerebellum ($P=0.038$) and the mesenteric lymph node ($P < 0.001$), while the remaining tissues had no significant difference in results.

RECOMMENDATIONS

Screening of archived tissues for the presence of two pathogens (circovirus and morbillivirus) and examining the detectability of Toxoplasma positive animals over time

While conducting PCR testing for the emerging disease circovirus, we observed that PCR gel electrophoresis results were not consistent when repeating analysis of the same sample extract. Due to the potential for inconsistent results, instead of analyzing each sample extract one time as originally planned, the number of replicates run for each sample was increased to three. Amplified DNA from suspect positive bands were sequenced and analyzed before confirming any individual animal as positive for BWCV. Although we are not certain of the explanation for the inconsistent results observed among repeated analysis of sample extracts, we believe that it is likely that the DNA template used resulted in inconsistent amplifications when a low viral DNA load was present. To test this hypothesis future efforts will focus on the use of RT-qPCR instrumentation to conduct circovirus testing which has increased sensitivity and quantitatively measures the viral load present. This will provide a means to determine if sample extracts that yielded inconsistent circovirus results using traditional PCR have a lower viral load than sample extracts that were consistently positive when replicates of the same sample extract were repeatedly measured. We do not currently have a RT-qPCR instrument at the HSL facility but we have requested short-term access to such instrumentation located at the UH Mānoa campus to analyze a small subset of our sample extracts for circovirus.

The inconsistency in results observed when conducting circovirus testing leads to increased likelihood of reporting false negatives as opposed to false positives. To mitigate the potential to report false negative results, our testing protocol was modified from the testing of single sample extracts to the testing of sample extracts in triplicate. The potential for the reporting of false positives is unlikely but could be confirmed with independent methodology such as in situ hybridization techniques as was conducted for the Longman's beaked whale in addition to full genome sequencing (Landrau-Giovanetti et al., 2020). Sanger sequencing results give a high level of confidence in confirming positive cases when the percent similarity of the isolate is compared to the BWCV genome and is found to be the same percent similarity or greater than the positive control sample (above 96%). This was the case for each of the positive individuals listed in Table 1. The control sample isolate submitted for sequencing with our unknown test isolates is from

the same whale host where the full BWCV genome was initially derived and submitted to the NCBI database.

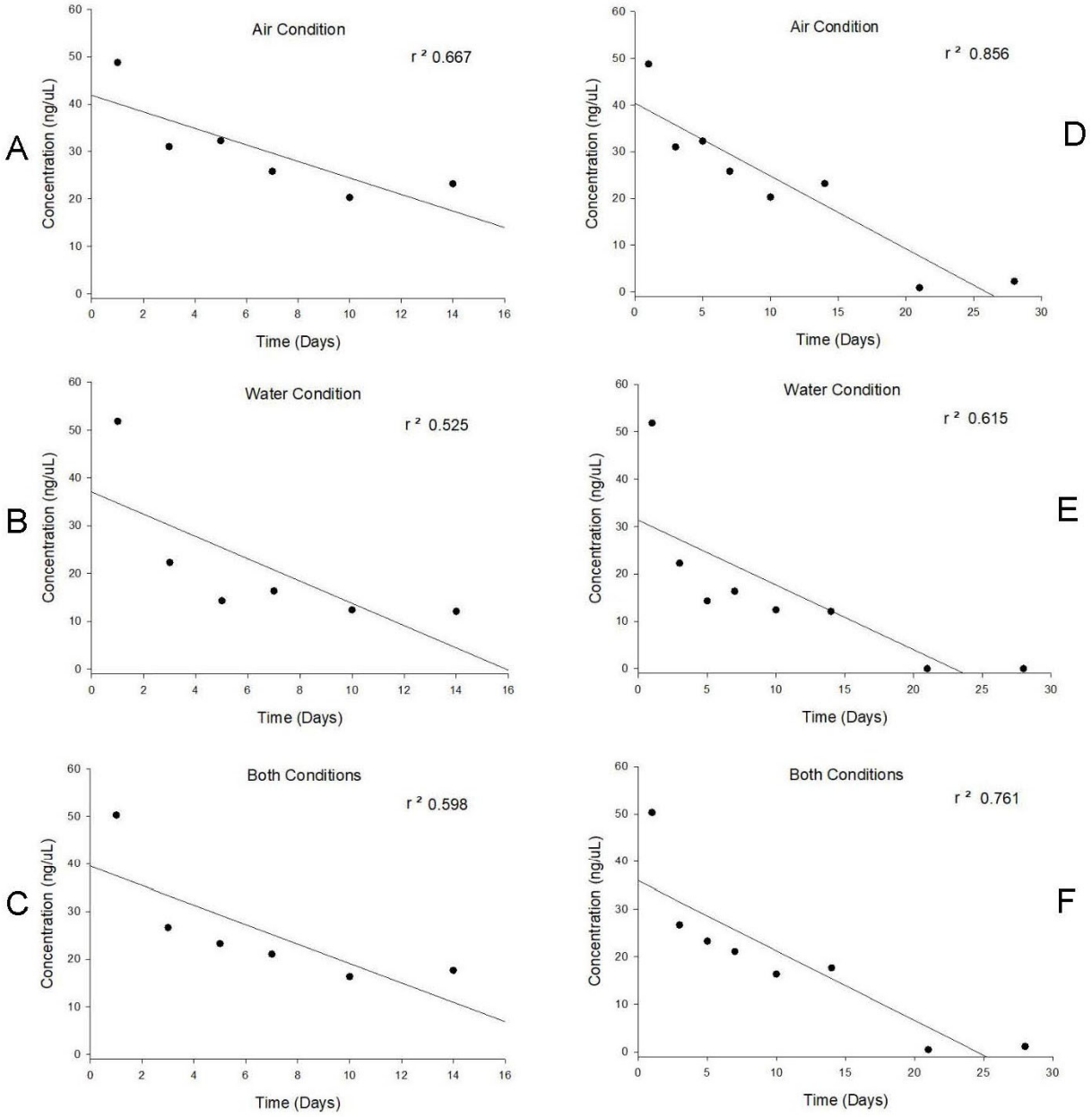

Figure 2. Graphs A, B, C: Regression analysis of tissues degraded under Air condition, Water condition, and the average of both conditions during the 14 day trial period. Graphs D, E , F: Regression analysis of tissues degraded under Air condition, Water condition, and the average of both conditions during the 28 day trial period.

Our findings suggest a 35% prevalence of circovirus presence among the 20 individual cetaceans tested and indicates that despite being named beaked whale circovirus, the recently identified circovirus species can infect a number of other host cetacean species. Our work confirms the presence of BWCV for the first time in six additional cetacean species that include dwarf sperm whales (*Kogia sima)*, Cuvier's beaked

whales (*Ziphius cavirostris*), spinner dolphins, Fraser's dolphins, striped dolphins, and melon-headed whales (*Peponocephala electra*). It is likely that circovirus infects even a greater number of cetacean species than we report being that only two species tested were circovirus negative in all tissues. The animals negative for circovirus were the species pygmy killer whales (*Feresa attenuata*) and short-finned pilot whales (*Globicephala macrorhynchus*) where in both cases the individuals tested were part of mass stranding events. It would be valuable to test additional stranded animals represented by these species and other species that have not been investigated to date.

Other significant findings of this work are discoveries related to the scope of BWCV in both time and space. Despite only very recently reporting on BWCV for the first time in a Longman's beaked whale that stranded in 2010, the initial finding of BWCV in this individual occurred many years after the stranding and attributing cause of mortality in this case to morbillivirus (West et al., 2013; Landrau-Giovanetti et al., 2020). Our findings indicate that the discovery of this virus from a whale stranded in 2010 does not preclude it from emerging undetected at an earlier date. A dwarf sperm whale that stranded on Kauai in 2000 was positive for circovirus in four of six tissues tested. This demonstrates that although undetected to the marine mammal community for two decades, BWCV has been present in Hawaiian cetacean hosts for at least 21 years.

In terms of geographic scope, our findings demonstrate that BWCV is not isolated to a small geographic area near Maui, Hawaii where it was initially described from the stranded Longman's beaked whale host. Instead, our screening results indicated positive circovirus findings in five out of six tissues tested from a Cuvier's beaked whale stranded in Saipan in 2011, a species that was included in a recent temporal analysis of beaked whale strandings in the Mariana Islands (Simonis et al., 2020). Additionally, a young Cuvier's beaked whale stranded in American Samoa in 2015 also tested positive for BWCV in three of six tissues tested. Other positive animals stranded across the Hawaiian archipelago include cases on the islands of Kauai, Maui and Oahu. These findings suggest that BWCV is widespread across the Pacific basin and infects cetaceans in the central North Pacific, the Western Pacific, and South Pacific waters.

Future work should include increased sample sizes to better establish the breadth of infection in archived and newly stranded animals. The current study focused on six tissues per animal, but this could be expanded to additional tissues to assess the extent of systemic infection. In individuals that test positive in at least one tissue it would be useful to test all archived tissues for BWCV. This would allow for a more detailed analysis of the distribution of the pathogen among tissues. Additionally, it is critical to begin to investigate the potential pathogenicity of BWCV, especially in light of the high prevalence rate of 35% described by this work. Antibody staining may provide a means for the detection of inclusion bodies and increase the understanding of the potential effects of a BWCV infection. Much remains to be learned about the clinical significance of this emerging cetacean disease with suggested widespread presence over time, among cetacean species and across geographic locations throughout the Pacific.

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