DISEASES OF STRANDED PACIFIC ISLAND MARINE MAMMALS

West, K.L., Clifton, C, Humann, C., Traina, S., Landrau-Giovannetti, L. and I. Silva-Krott

Health and Stranding Lab, Human Nutrition Food and Animal Sciences, College of Tropical Agriculture and Human Resources, University of Hawai'i at Mānoa, Honolulu, Hawai'i

ABSTRACT

The University of Hawai'i Health and Stranding Lab located at Marine Corps Base Hawaii (MCBH) 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.

This project focuses on increasing our understanding of infectious diseases in the Pacific Island region by investigating the diseases caused by beaked whale circovirus (BWCV), cetacean morbillivirus (CeMV) and toxoplasmosis infections in cetaceans.

BWCV was initially detected in a Longman's beaked whale a decade after it stranded in Maui in 2010. This case represented the first known circovirus infection in a marine mammal world-wide and led to polymerase chain reaction (PCR) screening of archived tissues and the identification of ten additional host cetacean species across the Pacific Basin. This project applies quantitative polymerase chain reaction (qPCR) technology, and potentially increased testing sensitivity, to investigate the role of viral load estimates in circovirus detectability and in understanding the clinical significance of positive cases. Over the reporting period, we estimated viral load using the number of amplification cycles required to detect positive cases as a proxy for viral load concentration. Using this method of estimation, we categorized prior positive tissues detected by conventional PCR based on qPCR results into high, moderate and low viral load categories. Further work could involve additional effort to quantify viral load as well as larger scale qPCR testing of archived samples in both previously detected cases and from stranded individuals that have not yet been screened for the presence of circovirus.

CeMV is globally recognized as a cause of cetacean mortality with two known strains (Beaked whale morbillivirus and Fraser's dolphin morbillivirus) present in Hawaiian waters. However, no prior study has investigated morbillivirus antibody presence in cetaceans from the central Pacific, which provides a means to determine disease exposure. This project includes initial efforts to validate use of a commercial enzyme immunoassay intended for the agricultural morbillivirus peste des petits ruminants (PPR) tested for use with cetaceans. A suite of tissue samples and fluids collected from a Fraser's dolphin (*Lagenodelphis hosei*) and a Longman's beaked whale (*Indopacetus pacificus*), each previously confirmed to have differing strains of CeMV with severe infection, were tested with the PPR enzyme immunoassay. Antibodies were detected in all tissues and fluids tested for the Fraser's dolphin with the exception of urine and in two of four tissues tested in the Longman's beaked whale. Further testing included serum samples collected from 27 stranded cetaceans that represented ten species with unknown infection status. Ten individuals were seropositive for CeMV antibodies, indicating a 37% exposure rate. Follow-up testing investigated the use of meat juice (the fluid released during the excision of animal tissues) as an

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6. AUTHOR(S)
Kristi L. West
Cody Clifton
Conor Humann
S. Traina
L. Landrau-Giovannetti
Ilse Silva-Krott

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Hawai'i Institute of Marine Biology, University of Hawai'i at Mānoa, Kāne'ohe, Hawai'i
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14. ABSTRACT

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morbillivirus antibody presence in cetaceans from the central Pacific, which provides a means to determine disease exposure. This project includes initial efforts to validate use of a commercial enzyme immunoassay intended for the agricultural morbillivirus peste des petits ruminants (PPR) tested for use with cetaceans. A suite of tissue samples and fluids collected from a Fraser's dolphin (*Lagenodelphis hosei*) and a Longman's beaked whale (*Indopacetus pacificus*), each previously confirmed to have differing strains of CeMV with severe infection, were tested with the PPR enzyme immunoassay. Antibodies were detected in all tissues and fluids tested for the Fraser's dolphin with the exception of urine and in two of four tissues tested in the Longman's beaked whale. Further testing included serum samples collected from 27 stranded cetaceans that represented ten species with unknown infection status. Ten individuals were seropositive for CeMV antibodies, indicating a 37% exposure rate. Follow-up testing investigated the use of meat juice (the fluid released during the excision of animal tissues) as an alternative sample matrix to serum. We collected meat juice samples (n=48) from thawed tissue types in the individual animals where serum was available and had previously been tested for CeMV antibody presence using the PPR assay. Our results suggest that meat juice, which is more often available from stranded animals than serum, is a viable alternative matrix for CeMV antibody detection, but detection rates were lower in meat juice than in serum. False negatives may occur when testing only a single meat juice sample but this can be accounted for in future testing efforts by testing multiple meat juice tissue types from the same individual animal. Toxoplasmosis represents a significant disease threat to Hawaiian marine mammals, with fatally disseminated cases documented in monk seals, three spinner dolphins and a bottlenose dolphin. Cetacean carcass recovery rates in the Hawaiian Islands are extremely low, averaging near 1% (West and Baird, unpublished data). Prior spinner dolphin deaths were likely underestimated in projections of 60 dolphin deaths based on three cases of fatally disseminated toxoplasmosis (Landrau-Giovanetti et al. 2022). An evaluation of cetacean exposure to the *Toxoplasma gondii* parasite has not previously been conducted in the central Pacific. This study included serological diagnosis of *T. gondii* from previously stranded cetaceans to investigate pathogen exposure rate. Serum samples collected from 35 individuals that represent 12 different cetacean species were tested via enzyme immunoassay for the presence of *T. gondii* antibodies. Three individuals tested positive (seropositivity rate of 8.6%). Matching meat juice samples were also tested for *T. gondii* antibody presence in the 35 individuals where serum samples were available and we found consistent results in the meat juice sample matrix. Meat juice samples from a range of tissue types were tested for an additional 159 individual animals where serum was unavailable. Sixteen additional animals were positive in at least one meat juice tissue type, with an overall meat juice exposure rate of 9.7% in individuals tested across multiple species of Hawaiian cetaceans.

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Toxoplasmosis represents a significant disease threat to Hawaiian marine mammals, with fatally disseminated cases documented in monk seals, three spinner dolphins and a bottlenose dolphin. Cetacean carcass recovery rates in the Hawaiian Islands are extremely low, averaging near 1% (West and Baird, unpublished data). Prior spinner dolphin deaths were likely underestimated in projections of 60 dolphin deaths based on three cases of fatally disseminated toxoplasmosis (Landrau-Giovanetti et al. 2022). An evaluation of cetacean exposure to the *Toxoplasma gondii* parasite has not previously been conducted in the central Pacific. This study included serological diagnosis of *T. gondii* from previously stranded cetaceans to investigate pathogen exposure rate. Serum samples collected from 35 individuals that represent 12 different cetacean species were tested via enzyme immunoassay for the presence of *T. gondii* antibodies. Three individuals tested positive (seropositivity rate of 8.6%). Matching meat juice samples were also tested for *T. gondii* antibody presence in the 35 individuals where serum samples were available and we found consistent results in the meat juice sample matrix. Meat juice samples from a range of tissue types were tested for an additional 159 individual animals where serum was unavailable. Sixteen additional animals were positive in at least one meat juice tissue type, with an overall meat juice exposure rate of 9.7% in individuals tested across multiple species of Hawaiian cetaceans.

INTRODUCTION

Determining cause of death when cetaceans strand is needed to dispel misinformation and ensure accurate public understanding of stranding cause. This is especially relevant in areas that are critical to Navy training and testing operations such as the Mariana and Hawaiian Islands. Prior work of the Health and Stranding Lab has demonstrated that infectious disease poses a significant threat to Pacific Island cetaceans (whales, dolphins and porpoises), with natural disease accounting for 62% of stranded cetaceans that were examined with significant pathology (West et al. In Revision). Circovirus, morbillivirus and toxoplasmosis represent viral and parasitic diseases now known to infect cetaceans in the Pacific Islands, with morbillivirus and toxoplasmosis responsible for cetacean mortalities (West et al. 2013; West et al. 2021; Landrau-Giovanetti et al. 2022).

Studying infectious diseases involves both direct and indirect measures of the presence of pathogens and their impact (Stevenson, 2024). Direct and indirect methods of investigating disease are complementary, improving understanding of the factors that contribute to animal mortality. Direct methods often rely on detection of the pathogen of interest itself by using procedures that directly stain the pathogen in infected tissues, grow the pathogen from tissue cultures, or amplify the minute amounts of genetic material of the disease present in infected tissues through conventional and quantitative PCR. Indirect methods of disease research rely on

detection of an animal's response to an infection. This includes serology, which allows for indirect study of diseases by testing for the presence of disease specific antibodies in the bloodstream that are generated in response to exposure to the pathogen of interest. Specific indirect testing methodologies include agglutination tests, immunofluorescent response, Western blot, and enzyme-linked immunosorbent assays (ELISA).

Extensive cetacean tissues are archived at the Health and Stranding Lab, including samples from 20 species that have stranded in the Pacific Islands region. Tissues from these animals are both frozen and formalin fixed to allow for the application of many different direct and indirect methodologies in both current and future studies. Archived frozen fluids include serum from a small sub-set of individuals and meat juice (the fluid released upon excision of animal tissues, including blood) from a suite of organ tissues such as brain, heart, lung, liver, kidney, spleen and lymph nodes from most fresh dead cetaceans where necropsies have been conducted. Meat juice, sometimes referenced as tissue juice or fluid, can be used as viable sample matrix to investigate the presence of antibodies against infectious disease (e.g., Tryland et al., 2006). A meat juice sample can often be taken from within the body cavity of animals in wildlife or livestock studies during necropsy as the cutting of tissues releases fluids, and this has been shown to be a valuable substitute for serum (Nielsen et al., 1998; Tryland et al., 2006; Simon et al., 2011). Antibody prevalence in other wildlife (e.g., wolverines, caribou) suggests that *Toxoplasma, Brucella* and other diseases' antibody prevalence can be reliably measured from matrices besides blood serum (Bachand et al. 2018; Sharma et al. 2019). Prior research with livestock such as pigs and chicken have also utilized meat juice tissues for the detection of *T. gondii* (Schares et al. 2018; Wallander et al. 2015).

Infectious disease testing at the Health and Stranding Lab has traditionally focused on using PCR to directly detect pathogens present in organ tissues at time of death or in previously frozen tissue samples. Serology testing for pathogen exposure reveals infection rates in populations over time. Currently minimal data is available in scientific literature that details antibody prevalence for any cetacean infectious diseases of concern in the Pacific Islands region. Evaluating the exposure of Hawaiian cetaceans to known pathogens via antibody analysis provides for opportunity to compare exposure rate to active infection rate among species and populations. This will allow us to better understand the impact of known infectious disease threats within populations and improve estimations of survival rates of infected individuals.

In this study, we apply direct methods (PCR/qPCR) to investigate cetacean circovirus and apply indirect methods (serology) in our investigation of exposure to cetacean morbillivirus (CeMV) and *Toxoplasma gondii.* PCR screening for circovirus present in stranded Hawaiian cetaceans will provide insight into circovirus pathology, which is yet unclear through the limited studies conducted (Landrau-Giovannetti et al., 2020; Clifton et al., 2023). Antibody testing of archived specimen tissues containing a mixture of fluids, including blood, provides a means to determine the prevalence of CeMV and *T. gondii* exposure among Hawaiian cetacean species. Such testing could provide valuable information that can be used to evaluate the probability that pathogen exposure leads to death.

Background: Beaked whale circovirus: Obtaining better understanding of an emerging disease

Circovirus was first identified in marine mammals from a stranded juvenile Longman's beaked whale in Maui, Hawai'i, and this novel circovirus was named beaked whale circovirus (BWCV) (Landrau-Giovanetti et al. 2020). In a prior conventional PCR study with funding support from NAVSEA, our laboratory detected BWCV in 10 additional cetacean host species across the Pacific, including from beaked whale strandings in American Samoa and in the Commonwealth of the Northern Mariana Islands, greatly expanding the known geographical range of this virus (Clifton et al. 2023). These additional cetacean host species include Blainville's beaked whales (*Mesoplodon densirostris*), dwarf sperm whales (*Kogia sima*), false killer whales (*Pseudora crassidens*), Fraser's dolphins (*Lagenodelphis hosei*), Goose-beaked whales (*Ziphius cavirostris*), melon-headed whales (*Peponocephala electra*), short-finned pilot whales (*Globicephala macrorhynchus*), sperm whales (*Physeter macrocephalus*), spinner dolphins (*Stenella longirostris*) and striped dolphins (*Stenella coeruleoalba*).

Despite obtaining sequencing confirmation of circovirus from at least one tissue in each stranded individual deemed positive for circovirus (Clifton et al. 2023), gel electrophoresis results were not always consistent when repeating analysis of the same sample extract in some animals. We believe that this is likely due to inconsistent amplifications that may occur when using traditional PCR if the viral load is low. Gel electrophoresis of traditional PCR allows for qualitative visualization of amplified sequences by separating final genetic products by size in agarose gel using an electric field. Animals are then deemed positive when sequences of a targeted size aggregate, producing a bright band in the agarose gel. Should the starting sample have very low levels of infection, amplification may not be great enough to visualize and thus result in a false negative. To combat this issue, we have implemented protocols using qPCR instrumentation that has a higher degree of sensitivity and allows for quantitative measures of the viral load present. These procedures provide a means to directly determine the viral load in sample extracts that previously yielded inconsistent results. Details of viral loads in circovirus positive animals are anticipated to provide valuable information that allow for better assessments of the clinical impact of the pathogen on individual cetaceans and improve our interpretation of future circovirus test results.

The pathogenicity of BWCV is currently unknown. Circoviruses have long been recognized for their impact on mortality in the pet trade and agricultural industries, causing Psittacine beak and feather disease in parrots, as well as porcine respiratory diseases complex and post-weaning multi-systemic wasting syndrome in pigs (Crowther et al., 2003; Fogell et al., 2018; Rose et al., 2012). Circovirus infections do not always result in a pathogenic response, though many of the strains across various species can cause negative health impacts (Gavier-Widen et al., 2012). Disease due to pathogenic circovirus strains includes necrosis and inflammation in the brain, lung, liver, heart, spleen, intestine and lymph tissues (Bexton et al., 2015; Rampin et al., 2006; Seo et al., 2014; Woods and Latimer, 2000; Yang et al., 2015). These viruses are frequently associated with respiratory illnesses (Chen et al., 2021; Lin et al., 2011; Seo et al., 2014) and several wasting diseases (Gavier-Widen et al., 2012; Seo et al., 2014; Yang et al., 2015). Circoviruses have been directly linked to reproductive failure and mortality in fish, birds, and swine, often in newly hatched or young offspring, although these outcomes can be found in infected juveniles and adults as well (Grasland et al., 2013; Lőrincz et al., 2011; Woods et al., 1993; Yang et al., 2015). There is also potential for indirect negative impacts to infected hosts, with lymphoid depletion observed

in cases of chronic circovirus infections that may indicate immune suppression (Mao et al., 2017; Palinski et al., 2017; Yang et al., 2015). Co-infections by other viruses and bacteria have been documented in both mammals and birds found to be infected with circoviruses (Dal Santo et al., 2020; Lagan Tregaskis et al., 2020; Zaccaria et al., 2016). Of the 16 positive circovirus cases (including the initial Longman's beaked whale) recorded to date, five of those cases have been found to have co-infections present (Clifton et al., 2023; Jacob et al., 2016; Landrau-Giovanetti et al., 2020, 2022; West et al., 2013, 2015, 2021), further supporting the need to systematically examine the pathology as well as better understand the clinical significance of observed viral load in infected cetaceans. The results of this report provide a more detailed view on potential pathology associated with circovirus infections in cetaceans, helping to advance our understanding of the significance of this disease within protected cetacean hosts.

Background: The threat of cetacean morbillivirus to Hawaiian cetaceans

Infectious disease outbreaks have increased substantially in marine mammal populations during the past 30 years (Sanderson et al., 2020; Simeone et al., 2015). Cetacean morbillivirus (CeMV) has emerged as the greatest infectious disease threat to cetacean populations and is the causative agent of severe epizootics in the North and South Atlantic (Groch et al., 2018; Rubio-Guerri et al., 2013). Classical symptoms and pathogenesis of this virus include lymphoid depletion, immunosuppression, pneumonia, secondary infections (e.g., parasites, bacteria, other viruses) and encephalitis. In 2013-2015, over 1600 bottlenose dolphins (*Tursiops truncatus*) died along the Atlantic coast of the United States in an epizootic event caused by CeMV (NOAA Fisheries, 2021).

CeMV is a linear negative-sense single-stranded, enveloped RNA virus. CeMV genome size ranges between 15-16 kbp with a diameter size of 150- 300 nm and consists of six proteins: nucleocapsid protein, phosphoprotein, matrix protein, fusion glycoprotein, haemagglutinin glycoprotein, and RNA-dependent RNA polymerase. CeMV replicates in the lymphoid tissue before dissemination and infection of other cell types and organ systems. Historically, there were three genetically distinct recognized strains: porpoise morbillivirus (PMV), pilot whale morbillivirus (PWMV) and dolphin morbillivirus (DMV) (Domingo et al., 1992; Kennedy et al., 1988; Taubenberger et al., 2000). These three strains were first described in two harbor porpoises (*Phocoena phocoena*) stranded in Ireland (Kennedy et al., 1988), a long-finned pilot whale (*Globicephala melas*) stranded in New Jersey, USA (Taubenberger et al., 2000) and from striped dolphins (*Stenella coeruleoalba*) stranded during an outbreak in the Mediterranean Sea (Domingo et al., 1992). In the last ten years, four additional strains have been described, one from Western Australia, one from Brazil, and two from Hawai'i (Groch et al., 2014; Stephens et al., 2014; West et al., 2013, West et al. 2021). The two Hawaiian strains are surprisingly distinct from each other. The partial Fraser's dolphin morbillivirus *L gene* sequence only indicated 76% similarity when compared to the beaked whale morbillivirus previously described from Hawai'i (Landrau-Giovannetti, 2019; West et al., 2021). It is also distinct from all other recognized strains. This meets the unofficial species demarcation threshold of greater than 80.3% dissimilarity from other recognized strains, including beaked whale morbillivirus, for consideration as a new morbillivirus species (Landrau-Giovannetti, 2019; West et al., 2021).

Despite finding active CeMV infections by PCR in at least 13 Hawaiian cetacean species (Jacob et al., 2016; Landrau-Giovannetti, 2019; West et al., 2013, 2021) exposure to CeMV has not been previously investigated. Exposure to disease is assessed by serology to determine antibody prevalence and these techniques have been applied to understand CeMV exposure and implied immunity in regions outside of the central Pacific (Van Bressem et al., 2014). A high exposure rate determined through antibody testing of Fraser's dolphins (*Lagenodelphis hosei*) off of Argentina, Brazil and the Gulf of Mexico suggests that morbillivirus is likely endemic in Fraser's dolphins in the Southwestern Atlantic and Gulf of Mexico region, which could have relevance to the novel Fraser's dolphin morbillivirus recently described from Hawai'i (Van Bressem et al., 2021; West et al., 2021). No CeMV antibody testing of Hawaiian cetaceans has been conducted to date, but such testing would allow for insight into implied immunity and the ability to examine infection rate as a function of exposure rate.

Background: Toxoplasma Serology Testing to Evaluate Disease Exposure

Toxoplasmosis is the most significant disease threat facing endangered Hawaiian monk seals (*Monachus schauinslandi*) in the main Hawaiian Islands, responsible for the deaths of at least 15 known seals. Fatally disseminated toxoplasmosis has also been determined as the cause of death in three individual spinner dolphins (*Stenella longirostris*) that have previously stranded in Hawaiian waters and was responsible for the death of a stranded bottlenose dolphin in 2023 (Migaki et al. 1990; Landrau-Giovannetti et al. 2022; West, unpublished data). We previously projected that the three confirmed cases of fatally disseminated toxoplasmosis in spinner dolphins equates to the deaths of at least 60 spinner dolphins in Hawaiian waters but believe this is an underestimate based on recent calculations of extremely low carcass recovery rates (West and Baird, unpublished data). Despite toxoplasmosis being identified as a significant threat to Hawaiian marine mammals, no information is available on the estimated exposure rate of any Hawaiian cetaceans to *T. gondii*. Researchers have conducted studies of *T. gondii* antibody prevalence in stranded cetaceans from other regions of the world where this parasite poses a significant health risk in order to better understand its role in mortality. Serology based studies indicate that *T. gondii* infection is frequent in at least three dolphin species (striped dolphins, bottlenose dolphins and common dolphins (*Delphinus delphis*)) in the Mediterranean Sea (Bigal et al. 2018; Cabezon et al. 2004; Di Guardo et al. 2011). In Russian beluga whales (*Delphinapterus leucas*), 11.5% were positive for *Toxoplasma* antibodies (Alekseev et al. 2017). Additionally, positive antibody titers were evident among a number of stranded cetaceans in the Philippines, including the Fraser's dolphin, spotted dolphin (*Stenella attenuata*), rough-toothed dolphin (*Steno bredanensis*), Bryde's whale (*Balaenoptera brydei*) and pygmy killer whale (*Feresa attenuata*) (Obusan et al. 2019).

METHODS

This project applies direct methods (PCR/qPCR) to investigate the emerging disease BWCV, and applies indirect methods (serology) in our investigation of exposure to cetacean morbillivirus (CeMV) and *Toxoplasma gondii* (Table 1).

Circovirus Viral Load Estimation Using qPCR

During the reporting period we focused on estimating viral load levels from individual tissues from stranded cetaceans previously determined as positive for circovirus presence using conventional PCR (Clifton et al. 2023). Brain and lung samples from the first BWCV case were used as positive controls during screening. DNA was extracted from each sample using Qiagen DNeasy Blood and Tissue Kits (Qiagen, Germantown, Maryland) according to the manufacturer's protocol. The DNA concentration of each extract was quantified using Qubit dsDNA Broad-Range Assay Kits and a Qubit 4 fluorometer (Thermo Fisher Scientific, Waltham, Massachusetts).

Our PCR protocol (Clifton et al. 2023) was adapted for qPCR using a QuantStudio 3 Real-Time PCR System (Applied Biosystems, Thermo Fisher Scientific, Waltham, Massachusetts). Nuclease-free water was used as a negative control to assess primer dimerization and result quality. All traditional PCR products and qPCR products with positive amplification curves were examined by gel electrophoresis on a 1 % agarose gel.

Visible bands at 400bp in size indicated the likely presence of BWCV. Products from all suspected positive cases were prepared for DNA sequencing using QIAquick PCR and Gel Cleanup Kits (Qiagen, Germantown, Maryland) and sequenced using the reverse primers at the Advanced Studies in Genomics, Proteomics, and Bioinformatics lab at the University of Hawai‘i. DNA sequences were analyzed using BioEdit Sequence Alignment Editor and NCBI BLAST nucleotide database.

Morbillivirus: Antibody Testing for Morbillivirus Exposure

Biological samples were tested for the presence of CeMV antibodies using the commercially available ID Screen® PPR Competition enzyme immunoassay (EIA) diagnostic kit (PPRC-4P-O58, Innovative Diagnostics, Grabels, France). This EIA was developed for use with serum or plasma for detection of peste des petits ruminants (PPR), a disease caused by a morbillivirus that is of concern to the agricultural industry and infects camels, goats, sheep and swine. To our knowledge, this diagnostic kit has not previously been investigated for potential use in detecting antibodies to CeMV. Currently no commercially available EIA kits have been validated for detection of morbilliviruses in meat juice samples, however other products produced by Innovative Diagnostics have been validated for use with meat juice. Our study acts as a validation for both detection of CeMV antibodies using a broad array EIA and the use of meat juice as a sample matrix.

We began investigating the application of this diagnostic kit for the detection of CeMV antibodies by the deliberate selection of samples for experimental testing. We initially selected two individuals with different strains of CeMV that had known and severe CeMV infection to test the kit's effectiveness at CeMV antibody detection. All available tissues and body fluids that could be tested were utilized to understand the potential and limitations associated with use of the PPR kit for CeMV antibody detection. Archived samples of cetacean serum obtained from previously stranded animals were also selected for testing (n=27) with the PPR antibody diagnostic kit. Additionally, meat juice samples from a variety of tissues (kidney, liver, lung, lymph node, spleen) (n=59) were collected for sample tissue type validation and for direct comparative testing with

serum samples obtained from the same individual animals. All sample types were diluted 1:2 with "Dilution Buffer 13" prior to analysis, per the manufacturer's instructions for serum samples. The PPR kit manufacturer instructions provide two sets of directions, one with a 45-minute incubation at 37 ℃ (sheep, goat, and swine) and the other with an 18-hour incubation step at 21 ℃ (camelids). Kit validations were conducted following both methods. Post-validation samples were incubated following the methods intended for camelid samples, utilizing an 18-hour incubation step at room temperature.

Plates were read at 450 nm on a microplate reader (Biotek HTX Synergy, Agilent Technologies, Santa Clara, California, USA) using Gen5 analysis software. Final results were determined based on protocol competition percentage (S/N%) ranges for sheep, goat, and camelid samples and are as follows: Positive: S/N% ≤ 50%; Doubtful: 50% < S/N% ≤ 60%; Negative: S/N% > 60%.

Toxoplasma gondii: Antibody Testing for Toxoplasma Exposure

Previous validations have indicated that meat juice is a reliable matrix for detection of *T. gondii* antibodies in other livestock and wildlife species (Bachand et al. 2018; Schares et al. 2018; Sharma et al. 2019; Wallander et al. 2015). Meat juice was collected from multiple tissue types, including adrenal, brain, heart, kidney, lung, liver, and muscle, as well as a variety of lymph node types (anal, hilar, marginal, mediastinal, mesenteric and prescapular). In addition to meat juice, samples of serum and aqueous humor were analyzed when available. Samples were diluted (meat juice 1:2, serum and aqueous humor 1:10) using solutions from a commercially available EIA kit (TOXOS-MS-2P-I59, Innovative Diagnostics, Grabels, France) and analyzed in duplicate per the manufacturer's protocol. Bovine serum positive controls were analyzed in each assay, in addition to the kit's internal controls, to ensure accuracy of the results. Optical density was read at 450 nm (Biotek HTX Synergy, Agilent Technologies, Santa Clara, California, USA). S/N% were determined for final results and categorized as follows: Negative: S/N% ≤ 40%; Doubtful: 40% < S/N% < 50%; Positive: S/N% ≥ 50%.

RESULTS

Circovirus Viral Load Estimation Using qPCR

Final output by qPCR instrumentation is a digital graph that shows the amplification of sequences over time, where peaks indicate successful amplification and the presence of the target disease. This has the potential to identify disease in samples where amplification was not detected until near the end of the testing procedure. In these cases, traditional PCR would not have produced high enough concentrations of amplified sequences to be visualized by gel electrophoresis, resulting in a false negative. In our study, the critical threshold (Ct), or the cycle where exponential amplification of the target DNA is detected, was used to estimate broad levels of viral DNA. Samples where high concentrations of viral DNA are present would achieve amplification relatively quickly, and thus have a lower Ct. A higher Ct would indicate samples started with small amounts of viral DNA, requiring additional replication to reach detectable levels. Using this general concept, viral load was estimated into three categories: high, moderate and low (Table 2). Tissues that have been previously confirmed as either positive or negative for BWCV were tested in triplicate using qPCR and the Ct was noted for each sample. The Cts were grouped based on

overlapping values across replicates which determined high viral load between Ct of 26 to 27; moderate viral load between Ct of 28 to 29, and low viral load with a Ct of 30 or more. Thirteen positive tissues, three negative tissues, two positive controls and one negative control were tested in triplicate to evaluate the point of detectable amplification. Both positive controls and one positive tissue reported all Cts between 26 and 27. Two positive tissues reported all Cts between 28 and 29. Ten of the positive tissues reported Cts greater than 29. The negative control and three negative tissues reported no Cts.

The Detection of Morbillivirus Antibodies in Hawaiian Cetaceans

Testing of Hawaiian cetacean samples for CeMV antibody detection included an effort to apply the commercially available PPR EIA developed for use with camels, goats, sheep and swine to cetaceans. We began initial testing of the PPR EIA for potential application to cetacean samples by first evaluating the kit's performance in our laboratory using samples collected from PCR confirmed CeMV positive cases. We prepared a suite of available samples from the Longman's beaked whale that signified our initial discovery of beaked whale morbillivirus (BWMV) and the stranded Fraser's dolphin in which we discovered Fraser's dolphin morbillivirus (FDMV) (West et al. 2013; West et al. 2021). The rationale of focusing on these two individuals is that each animal had severe disease that is well described in the scientific literature at the time of death, and we anticipate that an immune response occurred with the production of CeMV antibodies. The selection of these two individuals also provided a means to theoretically test the effectiveness of the PPR kit against two very distinct strains of morbillivirus that are now known to infect Hawaiian cetaceans (West et al. 2013; Jacob et al. 2016; West et al. 2021). We also included samples from an animal that tested negative via PCR for morbillivirus infection to act as a negative control. The PPR kit manufacturer instructions provide two sets of directions, one that involves an additional 18-hour incubation step that is recommended for successful detection of antibodies in camelid samples. Our initial testing in-house of the PPR kit performed better with the 18-hour incubation step (as opposed to warmer, faster incubations recommended for goat, sheep and swine samples) and we therefore followed this method when testing samples from unknown cases for CeMV antibody presence.

The commercial EIA was successful in detection of CeMV antibodies (Table 3). In the Longman's beaked whale, meat juice from four tissue types (lung, kidney, lymph node and spleen) were tested, with CeMV antibodies being detected in only the lung and lymph node. In the Fraser's dolphin, cerebral spinal fluid (CSF), aqueous humor, and meat juice from the lung, spleen, lymph node and kidney all tested positive. Urine from the Fraser's dolphin was negative. The negative control animal was negative for CeMV antibodies in serum and four additional meat juice tissue types that had been negative for morbillivirus by previous PCR testing. These results suggest that the PPR kit can be used to effectively detect morbillivirus antibodies in individual dolphins when meat juice from a variety of tissues or other fluids like CSF or aqueous humor are tested when serum is unavailable. These findings also assume that an individual animal is considered positive for CeMV antibodies if detected in at least one tissue tested, even if other tissue types from the same individual are negative upon testing.

Archived serum samples from previously stranded cetaceans represent a small sub-set of necropsy samples collected by the Health and Stranding Lab. In the majority of cases, serum from

necropsied individuals is not available due to carcass degradation. Follow-up testing focused on evaluating consistency in results within animals where both serum and meat juice samples were available. We tested serum samples from 27 previously stranded cetaceans that represent a range of species (Table 3). In 22 cases, matched meat juice samples from two tissue types were also tested. In four cases one comparative meat juice sample was tested. In a single case, cerebrospinal fluid, milk and urine were tested alongside serum. When considering only the serum results, 10 of the 27 individuals were positive for morbillivirus antibodies using the PPR kit, indicating a 37% CeMV exposure rate among Hawaiian cetaceans. Additionally, in no cases where serum results were negative did matched meat juice samples yield a positive result, which increases our confidence in the results obtained by the PPR kit when applied to cetacean meat juice samples. In eight of the ten seropositive cases, meat juice samples were also positive in one or two of the additional tissue types tested. In two cases where serum results were positive, all meat juice samples were negative. Based on our findings, we consider an animal to be positive for CeMV antibodies if they are detected in at least one tissue tested, even if other tissue types from the same individual are negative upon testing. Meat juice from a maximum of two tissue types were tested per individual in this study, but meat juice from additional tissue types is available from all study cases for future testing. Additional meat juice tissue types will be tested for all serum positive cases during follow up laboratory efforts. This will improve our understanding of the influence tissue type has on detecting CeMV antibodies in meat juice.

The Detection of Toxoplasma gondii Antibodies in Hawaiian Cetaceans

We have made significant progress in measuring *Toxoplasma gondii* antibody exposure in stranded cetacean serum and meat juice samples. The data presented is a combination of antibody presence results for a sub-set of individual archived tissues supported by the current award and another sub-set of individual cetaceans supported by another source of funding. We have tested 35 individual animals representing 12 cetacean species for *T. gondii* antibody presence in serum and found 3/35 positive individuals, which suggests an exposure rate of 8.6%. In the 35 cases where serum was available for testing, matched meat juice was tested from one to fifteen tissue types in addition to serum. In all 35 cases, serum results and meat juice samples results were consistent within the individual animals. In three cases, serum was positive and a range from three to fourteen matching meat juice samples that were tested were also positive. The remaining 32 cases had negative serum results and a range of one to thirteen meat juice tissue type samples for each individual were also negative.

In addition to our testing of matched serum and meat juice tissue types from 35 individual cetaceans that had previously stranded, we tested meat juice from animals with known health histories. Over the project duration, two cetaceans housed under human care that were seropositive for *T. gondii* died and were necropsied by the Health and Stranding Lab. In one of the cetaceans, thirteen meat juice tissue types were collected and all were positive and in agreement with serum results. In the other dolphin, ten meat juice tissue types were collected and nine were positive, with only the muscle meat juice tissue type testing negative.

In total, we have tested meat juice samples from a total of 194 stranded cetaceans. Meat juice tissue types tested range between 2-14 tissues. There were 16 cases where we found positive meat juice but did not have serum available for testing. This totals 19/194 positive individuals, indicating an

overall positivity rate of 9.7%. Tissue types tested include the adrenal gland, brain, heart, kidney, liver, lung, muscle, spleen and many different lymph nodes. The three most tested tissues were kidney (n=142), liver (n=164), and lung (n=118) resulting in positivity rates of 12% (kidney), 7.3% (liver), and 10.2% (lung). When considering positive and negative results among all of the tissues tested in animals that were positive in at least 1 tissue type, over 70% of the tissue types tested were positive. If we found at least one tissue type to be positive from an individual animal we considered this animal to be positive regardless of results from other tissue types tested.

DISCUSSION

Circovirus Viral Load Estimation Using qPCR

In this reporting period, we attempted to develop a reliable system of estimating viral load in tissues where BWCV was previously detected. Our qPCR procedure involves a 40 cycle test which means a maximum Ct value of 40. High viral load was assigned to the two positive controls and one tissue with Cts between 26 and 27 as these were the lowest Ct values measured. Moderate viral load was assigned to the two positive tissues with Cts between 28 and 29, as they were not equal to the positive controls. Low viral load was assigned to remaining 10 positive tissues with Cts of 30 to 36. There was more variation among the triplicates within the 'low' samples (Table 2). The use of thresholds to determine viral load may vary between assays and laboratory protocols, and methods among specific studies may not be applied to general qPCR tests (Segales and Sibila, 2022). The next step will be to develop a standard specific to BWCV that will produce standard curves following qPCR testing.

Serology Testing and Meat Juice as a Sample Matrix for Investigating Infectious Disease

To our knowledge, there are only two prior cetacean studies to date that have investigated the presence of infectious disease antibodies among meat juice samples (Blanchet et al., 2014; van de Velde et al., 2016). One of these studies used meat juice from only the muscle tissue of harbor porpoises and did not compare matched serum from the same individuals (van de Velde et al., 2016). The other study focused on methodology and suggested that false positives may occur when testing meat juice for *T. gondii* that relies specifically on agglutination as opposed to other methods such as ELISA (Blanchet et al., 2014). Our ELISA data for the detection of both *T. gondii* and CeMV antibodies provides the first seroprevalence data for cetaceans stranded in Hawai'i. Using serum samples, we were limited to serum availability from only 27-35 previously necropsied cetaceans representing a wide variety of species (10 species for CeMV, 12 species for *Toxoplasma gondii* testing). In some cases when blood samples are collected during necropsy, clotting has already occurred and serum separation is not possible. This makes the potential for using meat juice for antibody detection a desired alternative, with the potential for more widespread applicability with the meat juice sample matrix to better understand disease exposure in stranded cetaceans.

Meat juice was investigated for the detection of both *T. gondii* and CeMV antibodies as part of this work. We had different opportunities to examine the usefulness of meat juice as an antibody testing matrix between the two diseases, and have conducted more extensive testing of meat juice to date for *T. gondii* (Traina et al. 2024). Necropsies of seropositive cetaceans under human care

and matched serum and meat juice testing for *T. gondii* indicated positive results in all meat juice samples that were matched with positive serum samples in the same individual. The only exception was a negative muscle meat juice sample from one of the dolphins that died under human care. In CeMV, four meat juice sample tissue types in addition to aqueous humor and cerebral spinal fluid were positive in the dolphin that died of Fraser's morbillivirus and two of four meat juice tissue types were positive in the Longman's beaked whale with fatal CeMV (Table 3). This suggests that meat juice is a viable sample matrix when no serum is available for detecting seropositivity for both *T. gondii* and CeMV, but that testing of meat juice among several tissue types may be necessary to ensure that testing of a single meat juice sample does not yield a false negative result and/or underestimate disease exposure rates.

Our CeMV antibody detection among meat juice tissue types was not consistent in the Longman's beaked whale that died of morbillivirus. Additionally, in a number of the individuals tested for CeMV with serum that was matched to one or two meat juice tissue sample types from the same individual, meat juice tissue types were sometimes inconsistent between positive and negative results within the same individuals. However, with respect to the detection of antibodies to both diseases investigated as part of this work, we did not have any cases of positivity in meat juice where serum tested negative for antibody presence. This suggests that the testing of meat juice tissue types is not likely to yield false positive results. Past wildlife or livestock-focused studies have collected meat juice from specific tissues with variable results. Juices collected from diaphragm, heart, tongue, tonsils, lymph nodes, and muscle samples have been utilized in disease studies for a variety of species (Bachand et al., 2018; Bachand et al., 2019; Glor et al., 2013; Meemken et al., 2014; Schares et al., 2018; Sharma et al., 2019; Viana et al., 2020; Wallander et al., 2015; Yonemitsu et al., 2018). High detectability of antibodies has been noted in juice samples collected from the heart and lymph nodes for studies on *Salmonella* and *Toxoplasma gondii* (Wallander et al., 2015; Viana et al., 2020), while diaphragm juices were more effective for a study focused on Japanese encephalitis and hepatitis E virus (Yonemitsu et al., 2018).

Meat juice is not a homogenous mixture, as levels of hemoglobin, hematin, and immunoglobulin can vary greatly across samples, influencing antibody detectability (Wallander et al., 2015). Additionally, the water-holding capacity of tissues varies by tissue and drops after death, and therefore the time of death and type of tissue influences how dilute a sample of meat juice may be at the time of sampling (Nielsen et al., 1998; Wallander et al., 2015). It is unlikely that this played a role in our CeMV meat juice results as all cases were in good enough condition to provide a serum sample, although analysis of particularly desiccated carcasses in follow-up studies may impact findings. We recommend that future cetacean meat juice testing include a number of tissue types from the same individual to increase the probability of detecting antibody presence and to account for a tendency to underestimate exposure rates when only testing a single meat juice sample from any given individual. Additionally, caution should be taken when interpreting results related to stranded animals with signs of advanced decomposition.

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Table 1. Methods and sample types used for investigation of beaked whale circovirus (BWCV), cetacean morbillivirus (CeMV) and *Toxoplasma gondii*.

Pathogen	Methodology	Sample Type
BWCV	PCR/qPCR	Internal organ tissues
CeMV	EIA	Serum and Meat juice
Toxoplasma gondii	EIA	Serum and Meat juice

Table 2. The critical threshold (Ct) for confirmed BWCV positive tissues run in triplicate used to estimate amount of viral DNA present in the sample. PC - Positive Control; NC - Negative Control; Und. - Ct undetermined.

ID	Tissue	Avg Ct	Ct 1	Ct 2	Ct 3	Estimated viral load
PM-3464	Tracheobronchial LN	26.611	26.453	26.451	26.928	High
IP-4830	Brain (PC)	26.852	26.856	27.079	26.620	High
IP-4830	Brain (PC)	27.387	27.535	27.369	27.257	High
LH-6050	Brain	28.581	28.516	28.690	28.537	Moderate
SL-6326	Brain	29.347	29.346	29.437	29.259	Moderate
SL-2287	Brain	30.935	31.423	31.253	30.130	Low
MD-2108	Lung	31.579	32.009	31.877	30.851	Low
SC-4690	Brain	32.444	31.484	32.945	32.903	Low
ZC-1099	Spleen	34.035	34.436	33.801	33.868	Low
ZC-1099	Liver	34.518	35.275	34.529	33.750	Low
15337	Brain	34.784	36.550	33.166	34.636	Low
PC-4290	Brain	35.101	36.137	32.836	36.330	Low
ZC-2247	Kidney	35.497	34.043	36.392	36.056	Low
ZC-2247	Spleen	35.751	35.766	36.721	34.766	Low
PE-1540	Brain	35.816	34.842	36.898	35.709	Low
-	H2O (NC)	Und.	Und.	Und.	Und.	No Detection
GM-8865	Brain	Und.	Und.	Und.	Und.	No Detection
FA-2937	Brain	Und.	Und.	Und.	Und.	No Detection
SC-1142	Brain	Und.	Und.	Und.	Und.	No Detection

Table 3. Animals tested for presence of cetacean morbillivirus (CeMV) antibodies.

Animal ID	Species	Common Name	Positive tissues	Not detected
SC-9044	*Stenella coeruleoalba*	Striped dolphin	-	liver, lung, serum
SC-9645	*Stenella coeruleoalba*	Striped dolphin	serum	kidney, lung
TT-2810	*Tursiops truncatus*	Bottlenose dolphin	-	kidney, liver, serum
TT-4860	*Tursiops truncatus*	Bottlenose dolphin	serum	liver, lung
PE-7757	*Peponocephala electra*	Melon-headed whale	kidney, serum	lung
ZC-1099	*Ziphius cavirostris*	Cuvier's beaked whale	-	lung, serum
MN-9582	*Megaptera novaeangliae*	Humpback whale	-	liver, lung, serum
SL-1047	*Stenella longirostris*	Spinner dolphin	-	lung, serum
SC-9180	*Stenella coeruleoalba*	Striped dolphin	kidney, liver, serum	-
KS-8901	*Kogia sima*	Dwarf sperm whale	serum	kidney, liver
SC-8788	*Stenella coeruleoalba*	Striped dolphin	kidney, liver, serum	-
SC-4110	*Stenella coeruleoalba*	Striped dolphin	kidney, liver, serum	-
SA-3674	*Stenella attenuata*	Pantropical spotted dolphin	-	liver, lung, serum
SC-1774	*Stenella coeruleoalba*	Striped dolphin	liver, lung, serum	-
PE-6633	*Peponocephala electra*	Melon-headed whale	-	kidney, lung, serum
PE-1572	*Peponocephala electra*	Melon-headed whale	-	kidney, liver, serum
KB-9355	*Kogia breviceps*	Pygmy sperm whale	-	liver, lung, serum
MN-5816	*Megaptera novaeangliae*	Humpback whale	-	kidney, lung, serum
FA-6436	*Feresa attenuata*	Pygmy killer whale	-	kidney, liver, serum
FA-9529	*Feresa attenuata*	Pygmy killer whale	-	liver, serum
FA-9519	*Feresa attenuata*	Pygmy killer whale	-	kidney, liver, serum
FA-6599	*Feresa attenuata*	Pygmy killer whale	-	cerebrospinal fluid, milk, serum, urine
LH-2901	*Lagenodelphis hosei*	Fraser's dolphin	kidney, liver, serum	-
MN-3534	*Megaptera novaeangliae*	Humpback whale	-	kidney, serum
PE-7548	*Peponocephala electra*	Melon-headed whale	liver, lung, serum	-
KB-4289	*Kogia breviceps*	Pygmy sperm whale	-	liver, lung, serum
SL-4594	*Stenella longirostris*	Spinner dolphin	-	liver, lung, serum
Pos. control	*Indopacetus pacificus*	Longman's beaked whale	lung, mediastinal LN	kidney, spleen
Pos. control	*Lagenodelphis hosei*	Fraser's dolphin	aqueous humor, cerebrospinal fluid, kidney, lung, mesenteric LN, spleen	urine

LN = lymph node

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