Diseases of Stranded Pacific Island Marine Mammals

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ABSTRACT

The University of Hawaii 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 (dolphins, whales, and porpoises). The work involved in this project focused on increasing our understanding of infectious diseases in the Pacific Island region by investigating circovirus, morbillivirus, and toxoplasmosis infections in cetaceans. The first cetacean circovirus, beaked whale circovirus (BWCV), was recently reported in a Longman's beaked whale (*Indopacetus pacificus*) stranded in Hawaii and represents an emergent disease with unknown population impacts. In other species, circovirus infection may cause mortality or opportunistic co-infection by other pathogens. We report on a targeted surveillance of stranded cetaceans in the Pacific basin where pathological findings suggested disease presence. Archived tissues from individuals stranded between 2000 and 2021 (n=20) were tested by polymerase chain reaction (PCR) for the presence of BWCV. Suspect positive tissue amplicons were confirmed as BWCV through sequencing. Of the screened individuals, seven animals tested positive in one or more tissues, with a single striped dolphin (*Stenella coeruleoalba*) testing positive in all six tissues. The highest rate of detection among positive cases was found in brain and liver tissues (85.7%), followed by spleen tissue (83.3%) and lung tissue (66.7%). These results expand the potential host range for BWCV into six additional odontocete species. New host species include dwarf sperm whales (*Kogia sima*) with BWCV being found in an individual that stranded on Oahu in 2000, predating the initial case of BWCV. The results also broaden the known geographic range of BWCV to Saipan in the Western Pacific and American Samoa in the South Pacific, where stranded Cuvier's beaked whales (*Ziphius cavirostris*) tested positive. In respect to the infectious disease cetacean morbillivirus (CeMV), cetaceans that had previously stranded in the Pacific Island region were screened for the presence of morbillivirus (n=25). Among the tested individuals, a single adult female pygmy killer whale (*Feresa attenuata*) that was a part of a mass stranding event was found to have multiple lymph nodes (n=3) infected with a newly discovered Fraser's dolphin strain of morbillivirus. While significant in the fact that it is only the second animal to be discovered with this strain of CeMV, the infection rate among tested animals (4%) is lower than what was observed in the last screening effort that took place in 2014 (24%). Finally, efforts to investigate the degradation characteristics of *Toxoplasma gondii*, the parasite that can cause fatal toxoplasmosis, were conducted to determine the length of time postmortem that an animal carcass can successfully be tested by PCR for presence of this disease. Findings show that detection rates remain relatively constant in the first two weeks after death, and then drop significantly, with no successful detection at 28 days of degradation. This indicates that there is a benefit to testing animals with moderate to advanced states of decomposition for presence of *T. gondii.* The results

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14. ABSTRACT

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with BWCV being found in an individual that stranded on Oahu in 2000, predating the initial case of BWCV. The results also broaden the known geographic range of BWCV to Saipan in the Western Pacific and American Samoa in the South Pacific, where stranded Cuvier's beaked whales (*Ziphius cavirostris*) tested positive. In respect to the infectious disease cetacean morbillivirus (CeMV), cetaceans that had previously stranded in the Pacific Island region were screened for the presence of morbillivirus (n=25). Among the tested individuals, a single adult female pygmy killer whale (*Feresa attenuata*) that was a part of a mass stranding event was found to have multiple lymph nodes (n=3) infected with a newly discovered Fraser's dolphin strain of morbillivirus. While significant in the fact that it is only the second animal to be discovered with this strain of CeMV, the infection rate among tested animals (4%) is lower than what was observed in the last screening effort that took place in 2014 (24%). Finally, efforts to investigate the degradation characteristics of *Toxoplasma gondii*, the parasite that can cause fatal toxoplasmosis, were conducted to determine the length of time postmortem that an animal carcass can successfully be tested by PCR for presence of this disease. Findings show that detection rates remain relatively constant in the first two weeks after death, and then drop significantly, with no successful detection at 28 days of degradation. This indicates that there is a benefit to testing animals with moderate to advanced states of decomposition for presence of *T. gondii*. The results of this study that investigated three pathogens of concern have significantly increased our understanding of infectious disease in Pacific Island cetaceans while demonstrating that there is still more to be learned about their population level impacts in the central, Western, and South Pacific.

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INTRODUCTION

CIRCOVIRUS

Diseases caused by circoviruses have historically been of concern to the agricultural and pet trade industries, and are one of the many families of pathogens of concern when assessing the effects that emerging diseases can have on global conservation efforts for various species. The family *circoviridae* is composed of the genus *Circovirus* and *Cyclovirus*, though to date the genus *Circovirus* has only been found in vertebrate animals, as opposed to *Cyclovirus,* which can also be found among invertebrates (Breitbart et al., 2017). Circoviruses are small, non-enveloped viruses with a circular genomic structure of single-stranded DNA (Breitbart et al., 2017). In recent years, many novel strains of circoviruses have been discovered, but their pathological significance is not always clear. To be considered a distinct circovirus species, the demarcation threshold for nucleotide similarity is 80%, with anything below that threshold being considered a new species (Breitbart et al. 2017). Many of these new strains have been found in mammals, with recently discovered novel strains being found in pandas, elk, wolverines, bats and the first marine mammal (Dai et al., 2021; Fisher et al., 2020; Bandoo et al., 2021; Landrau-Giovanetti et al. 2020; Lecis et al., 2020). Additionally, novel avian strains have been discovered in multiple species of duck and penguins, the latter of which was connected to an avian feather disorder, demonstrating that pathogenicity is present in these new circoviruses (Wang et al., 2021; Levy et al., 2020).

Circovirus Pathology and Transmission

Circovirus infections do not always result in a pathogenic response, though many of the strains across various species similarly result in negative animal health impacts (Gavier-Widen et al., 2012). Swelling, lesions, and necrosis are often described among species infected with pathogenic strains, including within the brain, lungs, liver, heart, spleen, intestinal, and lymph tissues (Bexton et al., 2015; Rampin et al., 2006; Seo et al., 2014; Woods and Latimer 2000; Yang et al., 2015). Viral inclusion bodies in lymph tissues and lymphoid depletion commonly occur with circovirus infections, which can lead to immune suppression (Mao et al., 2017; Palinski et al., 2017; Yang et al,. 2015). These viruses are frequently associated with respiratory illnesses (Lin et al., 2011; Naya et al., 1999; Seo et al., 2014) and several wasting diseases (Gavier-Widen et al., 2012; Seo et al., 2014; Yang et al., 2015). Circoviruses have also been directly linked to reproductive failure and mortality in fish, birds, and swine, often in newly hatched or young offspring, although this is not always the case (Lorincz et al., 2011; Woods et al., 1993; Yang et al., 2015; Grasland et al., 2013). Even those strains that are not directly pathogenic have the potential to cause negative impacts to their hosts through immunosuppression. Circoviruses have been demonstrated to cause immunosuppression in several species of mammals and birds that allows for co-infections by other viruses and bacterium (Dal Santo et al., 2020; Tregastis et al., 2020; Zhen et al., 2021; Zaccaria et al., 2016).

Circovirus transmission can occur through multiple vectors and between different species. Horizontal transmission has been documented through contact with infected secretions such as feces, urine, and respiratory aerosols (Patterson 2010; Rose et al., 2012). Vertical transmission of these viruses from parent to offspring within a species has been well documented in mammals and has been proposed as the most likely route of transmission for avian circovirus strains (Dong et al., 2016; Wang et al., 2016; Yu et al., 2016; Li et al., 2014). Circoviruses have also been shown to cross from one species to another (Firth et al., 2009; Li et al., 2019; Zhai et al., 2017). Porcine circovirus has been demonstrated to cross between species at the family level with a strain of Porcine Circovirus 2 being able to pass back and forth between swine and buffalo (Zhai et al., 2017). At the Order level, an avian strain of circovirus was recently found in the rainbow bee-eater (*Merops ornatus*), a Coraciiform species, when previously it had been thought to be restricted to Psittaciform and Columbiform birds and it is presumed that circovirus transmitted from one Order of birds to another (Sarker et al., 2015b).

Impact of Circovirus on Swine and Birds

Porcine circovirus (PCV) is one of the most widely studied subsets of circoviruses. Until recently, PCV1 and PCV2 were thought to be the only strains of this virus, with PCV1 being non-pathogenic and PCV2 pathogenic, making PCV2 of greater concern to the agricultural industry (Alarcon et al., 2013). Several diseases are associated with PCV2 infections, including postweaning multisystemic wasting syndrome (PMWS), porcine dermatitis and nephropathy syndrome (PDNS), and porcine respiratory diseases complex (PRDC), among others (Rose et al. 2012). These diseases have severe health impacts on swine, such as reduced growth, respiratory distress, necrotizing pneumonia and reproductive disorders (Alarcon et al., 2013; O' Dea et al., 2011). Co-infections by other pathogens in addition to circovirus have been shown to exacerbate the underlying conditions caused by PCV, such as in the case of *Mycoplasma hyponeumoniae*, which is a known cause of pneumonia in swine (Seo et al., 2014). The continued search for novel diseases has led to recent relevant developments within PCV, with the discovery of PCV3 and its connection to cardiac-related abnormalities and systemic organ inflammation (Phan et al., 2016), and more recently the discovery of PCV4 in 2020, although efforts to determine the pathological significance of PCV4 remain ongoing (Zhang et al., 2020).

Avian circoviruses pose a potential threat to both the agriculture industry and to species that are targeted by conservation efforts. Psittacine beak and feather disease (PBFD) is a circovirus-caused disease that has been found in both captive and wild parrot populations and is ultimately fatal (Fogell et al., 2018). This wasting disease results in a suppressed immune system and leads to the deformation and loss of a bird's plumage, as well as deformation of the beak, which makes eating difficult and adds to the factors that contribute to mortality (Peters et al., 2014; Sarker et al., 2015a). Similarly, pigeon circovirus (PiCV) can cause young pigeon disease syndrome (YPDS), which while not always fatal, does have a mortality rate estimated at greater than 50% in infected racing pigeons (Raue et al., 2005). PiCV infections result in lymphoid depletion, which causes immune suppression and development of lesions that allow for the growth of other bacterial and viral co-infections, including strains of herpesvirus (Pendl & Tizard 2016). Other pathogenic strains of avian circovirus exist that cause similar wasting effects, including those found in ducks (Li et al., 2014) and canaries (Rampin et al., 2006). Efforts to study novel viral presence in chickens has led to the discovery of the first known chicken circovirus (CCV), which has recently been linked to

gastrointestinal abnormalities (Li et al., 2019). While the overall pathology of CCV is not clearly known, chicken anemia virus of the circovirus related genus *Gyrovirus* has been shown to have wasting effects similar to other avian circoviruses (Crowther et al., 2003; Li et al., 2014).

Discovery of the First Whale Circovirus

Using Next Generation Sequencing, characterization of the full genome of the first known marine mammal circovirus was identified in a Longman's beaked whale (*Inodopacetus pacificus*) that stranded in Maui, Hawaii in 2010, that is now recognized as Beaked Whale circovirus (BWCV) (Landrau-Giovanetti et al., 2020). Longman's beaked whales have been described as the second most poorly known of all whale species, with fewer than 20 strandings having occurred world-wide (Kobayashi et al., 2021). Genetic identification as a distinct species only occurred in the last two decades (Dalebout et al., 2003). Prior to this, the species was known only from their skulls (Azzaroli 1968; Longman 1926) without knowledge of what the animal looked like until the relatively recent matching of photographs (Dalebout et al., 2003; Pittman et al., 1999). Longman's beaked whale population estimates and geographic range remain poorly known. Stranding events have occurred in both the North and South Pacific, as far east as Taiwan and west as South Africa (Kobayashi et al., 2021). The potential threats that this species may face are poorly understood, including the population level impact of infectious disease such as morbillivirus, despite positive cases in stranded Longman's beaked whales in New Caledonia and the individual stranded in Maui, Hawaii (West et al., 2013: Garrigue et al., 2016). The stranded Maui, Hawaii individual was co-infected with both morbillivirus and herpes virus at time of death, confounding the ability to determine if the pathology observed can be attributed to the presence of circovirus that was later discovered in archived tissues from this same individual (Landrau-Giovanetti et al., 2020; West et al., 2013). Necropsy and histopathology findings from the Longman's beaked whale infected with BWCV included pathology that is common to pathogenic circoviruses in other species, including lymphoid depletion and pulmonary edema but these may also be associated with the co-infections also described in this individual (West et al., 2013). Circoviruses in other species have been shown to have an immunosuppressive effect, and this may also be at play in marine mammals that are infected with circovirus and other pathogens (Pendl & Tizard 2016; Seo et al., 2014).

Sequencing of the full beaked whale circovirus (BWCV) genome allowed for the development of DNA primers and subsequent PCR amplification of the viral DNA to test multiple tissues for BWCV presence in the infected Longman's beaked whale (Landrau-Giovanetti et al., 2020). Spleen, muscle, left ventricle, mesenteric lymph node, scapular lymph node, mediastinal lymph node, left adrenal, liver, lung, cerebrum, and cerebellum were all positive for BWCV in this Longman's beaked whale by PCR (Landrau-Giovanetti et al., 2020). To our knowledge this represents the only marine mammal tested by PCR to date for the presence of circovirus. The goal of this investigation was to better understand the prevalence of BWCV among stranded marine mammals in the Pacific Island region. We conducted PCR screening for BWCV presence in archived tissues of previously stranded cetaceans that represent a wide diversity of species from stranding locations in the central, Western and South Pacific Ocean basins.

MORBILLIVIRUS

Cetacean morbillivirus (CeMV) has emerged as the greatest infectious disease threat to cetacean populations world-wide and is the causative agent of severe epizootics in the North and South Atlantic (Groch et al., 2018; Rubio-Guerri et al., 2013). CeMV is a linear negative-sense single-stranded, enveloped RNA virus. CeMV replicates in the lymphoid tissue before dissemination and infection of other cell types and organ systems. Currently, there are 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 seven years, four additional strains have been described, two from Western Australia and Brazil, and two from Hawai'i (Groch et al., 2014; Stephens et al., 2014; West et al., 2013; West et al., 2021). 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, 2019).

Cetacean Morbillivirus in the Pacific Islands

Morbillivirus was first identified in the Pacific Islands as the beaked whale morbillivirus strain (BWMV) from a Longman's beaked whale that stranded in Maui 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 BWMV strain found in Hawaiian waters and how this compares globally (Landrau-Giovanetti et al., 2015). We have most recently discovered another novel morbillivirus in a Fraser's dolphin that stranded in 2018 on Maui that is surprisingly distinct from both the BWMV and the Southern hemisphere strains (West et al., 2021). 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-Giovanetti, 2019; West et al., 2021). It is also distinct from all other recognized strains, meeting the unofficial species demarcation threshold of 80.3% dissimilarity for consideration as a new morbillivirus species (Landrau-Giovanetti, 2019; West et al., 2021).

Disease screening for CeMV has not been conducted on stranded cetaceans in Hawaii since 2013 (Jacob et al., 2016). Since that time, the University of Hawaii Health and Stranding Laboratory has maintained an archive of ultracold stranded cetacean tissues that can be used to better understand the prevalence of the newly discovered Fraser's dolphin morbillivirus and the persistence of BWMV in Pacific Island cetaceans. Screening of these tissues will contribute to a better understanding of not only the prevalence of the two CeMV strains previously identified from Hawaiian waters but would also provide insight into the clinical significance and population level impacts.

TOXOPLASMOSIS

Toxoplasmosis Impact and Transmission

Fatally disseminated toxoplasmosis has been identified as a threat to cetaceans from a number of locations around the world. Toxoplasmosis has been documented as the cause of death in Maui dolphins and Hector's dolphins from New Zealand, Indo-Pacific humpback dolphins from Australia, in a Bryde's whale in Brazil and in several delphinids and a fin whale in the Mediterranean Sea (Roe et al., 2013; Mazzariol et al., 2012; Diaz-Delgado et al., 2020; Jardin and Dubey, 2002; Profeta et al., 2015). *Toxoplasma* has also been detected by PCR in several stranded cetacean species in the Philippines and in Canadian beluga whales (Obusan et al., 2019; Iqbal et al., 2018). Studies of *Toxoplasma* antibody prevalence have been conducted in stranded cetaceans from other regions of the world where this parasite represents a significant health risk in order to better understand exposure. Serology based studies indicate that *T. gondii* infection is frequent in at least 3 dolphin species (striped dolphins, bottlenose dolphins and common dolphins) in the Mediterranean Sea (Bigal et al., 2018; Cabezon et al., 2004; Di Guardo et al., 2011). Additionally, 11.5% of recently investigated Russian beluga whales were found to be positive for *Toxoplasma* antibodies (Alekseev et al., 2017).

Toxoplasmosis, caused by the parasite *Toxoplasma gondii*, was first described as a cause of Hawaiian spinner dolphin mortality more than 30 years ago (Migaki et al., 1990). The parasite has recently re-emerged as a threat to Hawaiian cetaceans after determining fatally disseminated toxoplasmosis as cause of death in stranded spinner dolphins in 2015 and again in April of 2019. *T. gondii* has recently been recognized as a significant threat to other endangered wildlife in Hawaii including the Hawaiian monk seal (*Neomachus schauinslandi)* and the nēnē goose (*Branta sandvicensis)*. *T. gondii* is a unique parasite due to the resiliency of its oocytes, which are able to survive for long periods of time, especially in water, without a host (Lindsay and Dubey 2009). The vector for this parasite is cat feces, and sexual reproduction of the parasite occurs only in their main host *Felidae* (Conrad et al., 2005). In Hawaii specifically, the main transporter of this disease is typically feral cats, but it is prevalent in domestic cats as well (Dabritz et al., 2006). *T. gondii* enters the ocean via runoff, and from there can infect near-shore marine species such as the Hawaiian spinner dolphin (Conrad et al., 2005). Once the oocyte infects an intermediate host through ingestion, the parasite manifests in the form of cysts in the individual, and the parasite is not able to be contracted by another organism unless ingestion of that cyst occurs.

Toxoplasmosis Detection in Decomposing Cetaceans

Traditionally, stranded cetacean carcasses recovered in a state of moderate to advanced decomposition are not tested for the presence or absence of *T. gondii* or any other disease agents due to the likely degradation of genetic material required for detection. However, the resiliency of *T. gondii* oocytes suggests that evidence of *T. gondii* DNA may still be detectable in cetacean tissues that are in a state of moderate to advanced decomposition. To test this experimentally, tissue samples from fresh dead, known *T. gondii* infected cases, could be exposed to various environmental conditions and allowed to degrade naturally. These samples could then be periodically assessed for tissue quality and *T. gondii* detectability in order to determine how

viable samples may be for disease investigations. If it is possible to detect positive *T. gondii* via PCR at varying states of decomposition, this would greatly increase the scientific information that can be gained from decomposed cetaceans and would allow for greater surveillance of *T. gondii* among recovered stranded cetaceans.

METHODS

CIRCOVIRUS

BWCV Animal Selection

To investigate the presence of circovirus among cetacean species throughout the Pacific Ocean basin, tissues were selected from 20 previously stranded individuals for screening of BWCV. The individuals were selected from the University of Hawaii Health and Stranding Laboratory's tissue archive from cetaceans that have stranded in Hawaii and other regions of the Pacific since 1998. Animals selected for this study were targeted based on species identification as a beaked whale species, pathological findings associated with infectious disease, the confirmed presence of other diseases, or individuals from mass stranding events as disease has been linked to mass strandings in other regions (Mazzariol et al., 2017; Garrigue et al., 2016; Vargas-Castro et al., 2020) Individuals selected for testing included four Cuvier's beaked whales (*Ziphius cavirostris*) due to the initial circovirus case in a Longman's beaked whale. Three short-finned pilot whales *(Globicephala macrorhynchus)* that mass stranded in 2017 were selected, as well as seven pygmy killer whales (*Feresa attenuata)* that stranded during a prolonged mass stranding event in 2019. The remaining animals selected included a dwarf sperm whale (*Kogia sima),* a melon-headed whale (*Peponocephala electra)*, and several species of dolphin to represent a broad assessment of BWCV presence among the 20 cetacean species that regularly inhabit the region (Baird 2016).

BWCV Detection by PCR

Tissues selected for testing from each individual targeted samples of brain, lung, kidney, spleen, liver, and lymph tissue. When these specific tissues were unavailable, other available tissue types were substituted when possible, to total the testing of six different tissues from each individual. A spinner dolphin that stranded on 10/14/2008 had only three available tissues to test, one sample being "brain," without the ability to denote if it is cerebrum or cerebellum. Additionally, brain and lung tissues from the single historically positive Longman's beaked whale were used as a positive control in all assays. DNA was extracted from tissue samples using DNeasy Blood and Tissue kits (Qiagen, Germantown, Maryland) and quantity of DNA was confirmed using a Qubit 4 fluorometer (Thermo Fisher Scientific, Waltham, Massachusetts). Utilizing traditional PCR, each tissue extract was run in triplicate to test for the presence of BWCV. The PCR protocols were developed using primers and thermocycler settings adapted from Landrau-Giovanetti et al. 2020. The BWCV forward primer 5' CTTCAGATTCCCCGTCAAGA 3' and BWCV reverse primer 5' GTCTCCCCACAATGGTTCAC 3' were used with an initial denaturation at 94°C for five minutes, 40 cycles of denaturing at 94°C for 30 seconds, annealing at 56°C for 30 seconds, and extension at 72°C for 30 seconds, with a final extension step at 72°C for five minutes.

Amplified products were visualized through gel electrophoresis, with bands indicating presence of BWCV expected at 400bp in 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). Amplified DNA was sequenced at the Advanced Studies in Genomics, Proteomics, and Bioinformatics lab at the University of Hawaii. Sequencing results were processed using NCBI Nucleotide BLAST.

MORBILLIVIRUS

CeMV Animal Selection

To investigate the presence of morbillivirus among cetacean species throughout the Pacific Ocean basin, tissues were selected from 25 previously stranded individuals for screening of CeMV presence. The individuals were selected from the University of Hawaii Health and Stranding Laboratory's tissue archive from cetaceans that have stranded in Hawaii and other regions of the Pacific since 2014. Animal selection for this study prioritized individuals confirmed to have other diseases, pathological findings associated with infectious diseases, or those from mass stranding events. Individuals with evidence of infectious diseases selected for testing included a melon-headed whale (*Peponocephala electra*), three spinner dolphins (*Stenella longirostris*), one humpback whale (*Megaptera novaeangliae*), and one false killer whale (*Pseudorca crassidens*). Seven pygmy killer whales (*Feresa attenuata)* from a mass stranding event in 2019 and three Fraser's dolphins (*Lagenodelphis hoseii*) from a mass stranding event in 2021 were also selected. The remaining animals selected from recent strandings between 2018 and 2021 included six striped dolphins (*Stenella coeruleoalba*), two Cuvier's beaked whales (*Ziphius cavirostris*), and one pygmy sperm whale (*Kogia breviceps*).

CeMV Detection by PCR

Tissues selected for testing from each individual targeted samples of brain, lung, kidney, spleen, liver, spinal cord, and lymph tissue. When these specific tissues were not available, other available tissues were substituted to result in a total of twelve different tissues being tested for each individual (Table 3). Brain tissue from a known CeMV positive animal was used as a positive control (West et al., 2021). RNA was extracted from the tissues using RNeasy Plus Mini Kits (Qiagen, Germantown, Maryland) and quantity of RNA was confirmed using a Qubit 4 fluorometer (Thermo Fisher Scientific, Waltham, Massachusetts). Utilizing reverse transcription polymerase chain reaction (RT-PCR), each tissue extract was run to test for the presence of CeMV. The RT-PCR protocols were developed using primers and thermocycler settings adapted from Barrett et al., 1993 and recommended procedures from the RT-PCR master mix manufacturer's protocols (Thermo Fisher Scientific, Waltham, Massachusetts). The CeMV forward primer 5' ATGTTTATGATCACAGCGGT 3' and CeMV reverse primer 5' ATTGGGTTGCACCACTTGTC 3' were used with an initial denaturation at 95ºC for 15 minutes, 35 cycles of denaturation at 94ºC for 30 seconds, annealing at 50ºC for 30 seconds, and extension at 72ºC for 60 seconds, followed by a final extension at 72ºC for 10 minutes. Amplified products were visualized through gel electrophoresis, with bands indicating presence of CeMV expected at 450bp in size.

Positive products were cleaned using QIAquick Gel Extraction Kits (Qiagen, Germantown, Maryland) and then sequenced at the Advanced Studies in Genomics, Proteomics, and Bioinformatics lab at the University of Hawaii. Sequencing results were processed using NCBI Nucleotide BLAST.

TOXOPLASMOSIS

T. gondii Animal Selection and Establishing Environmental Conditions

Two fresh dead spinner dolphins (*Stenella longirostris*), KW2015013 and KW2019006, were selected for this DNA degradation and *T. gondii* detection experiment. These 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. Additionally, both cases were determined to have wide-spread *T. gondii* infections at time of death through PCR analyses. From each of these two animals, the tissue types that were selected for environmental degradation included the following: 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 MCBH 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 lowered into the waters of Kaneohe Bay, Hawaii, and tethered to the same pier to replicate the water temperature exposure of a floating cetacean carcass.

Detection of T. gondii in Degraded Samples

Samples of each tissue were taken from each treatment on days 1, 3, 5, 7, 10, and 14 for the initial degradation experiment. Preliminary findings from this initial experiment were built upon and extended to double the timeframe of data collection by degrading and sampling tissues up to 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 (one per tissue type) on each sampling date. The final DNA concentration of each extract was determined using Qubit dsDNA Broad-Range Assay Kits and a Qubit 4 fluorometer (Thermo Fisher Scientific, Waltham, Massachusetts).

Utilizing PCR, each tissue extract was run to test for the presence of *T. gondii*. The PCR protocols were developed using primers and thermocycler settings adapted from Silva et al., 2009. The *T. gondii* primers Ext-JS4 5' CGAAATGGGAAGTTTTGTGAAC 3' and Ext-CT2b 5' TTGCGCGAGCCAAGACATC 3' were used with an initial denaturation at 94°C for 5 minutes followed by 40 cycles of denaturation at 94°C for 60 seconds, annealing at 60°C for 60 seconds, and extension at 72°C for 60 seconds, with a final extension at 72°C for seven minutes. Amplified products were visualized through gel electrophoresis, with bands indicating presence of *T. gondii* expected at 500bp in size.

RESULTS

CIRCOVIRUS

Of the 20 animals that were tested in this study, seven animals were positive for BWCV in one or more tissues (Table 1). Tissues that had amplified product sequenced for genetic confirmation are noted in Table 1. In total, eleven amplified products were sequenced, resulting in an average percent similarity to the BWCV reference genome of 97.8% (range 97.1% to 99.4%). Among these cases, brain and liver tissues were the most consistently positive tissue types (85.7% positivity rate), followed by spleen tissue (83.3% positivity rate) and lung tissue (66.7% positivity rate). BWCV was also detected in lower frequencies in kidney, liver, and spleen tissue of positive cases. Adrenal tissues were negative for BWCV in two cases that were considered BWCV positive based on results from other tissues screened. A striped dolphin (stranded 5/2/2020) was the only animal to test positive in all six tested tissues, though a spinner dolphin (stranded 10/14/2008) tested positive in all tissues that were available for testing in the sample archive (brain, kidney, and liver).

The positive cases were detected among six different species that have stranded in the Pacific Islands region since 2000, bringing the total to seven known cetacean species where BWCV was detected, when including the initial Longman's beaked whale (Table 2). New species where BWCV was detected in the current study included Cuvier's beaked whales (n=2), dwarf sperm whales (n=1), spinner dolphins (*Stenella longirostris)* (n=1), striped dolphins *(Stenella coeruleoalba)* (n=1), Fraser's dolphins *(Lagenodelphis hosei)* (n=1), and melon-headed whales (n=1). Animals were tested from multiple age classes, and positive cases were found in four adults (out of 12 animals), two sub-adults (out of six animals), and one calf (out of two animals). The majority of these positive cases occurred in animals stranded in the main Hawaiian islands (n=5), but the BWCV positive Cuvier's beaked whales stranded in American Samoa and Saipan.

MORBILLIVIRUS

From the 25 animals that were screened in this study, CeMV positive tissues were found in a single individual, resulting in a 4% positivity rate among tested individuals (Table 3). An adult female pygmy killer whale that stranded as part of a prolonged mass stranding event on Maui in 2019 was positive for CeMV in the hilar, mesenteric, and right marginal lymph nodes. This indicated a positivity rate of 25% in this pygmy killer whale case when considering the total number of tissues tested from this individual. Sequencing analysis performed in BLAST on amplified PCR product from the positive animal's hilar lymph node revealed high similarity (87.8%) to the novel Fraser's dolphin morbillivirus strain that was recently described (West et al. 2021). The CeMV sequence obtained from the pygmy killer whale was also similar to DMV strains of CeMV (84.2-86.8%), though with lower percent similarity to the referenced genome (query coverage) than the Fraser's dolphin strain (10% vs 65%). The partial sequence from the pygmy killer whale obtained during this work was dissimilar when compared to the partial BWMV strain previously described from Hawaiian waters.

TOXOPLASMOSIS

Over the course of this study, 206 tissues samples were tested for presence of *T. gondii*. For the first eight timepoints, 24 tissues were tested at each point (12 "Air" condition, 12 "Water" condition). Due to sample availability, the remaining two timepoints were only sampled from the "Air" condition (nine tissues for Day 21, five tissues for Day 28). Across the 28-day duration of the experiment, *T. gondii* detection rates ranged from 0% (Day 28) to 62.5% (Day 2 and Day 10)

Table 1. Animal tissues tested for the presence of beaked whale circovirus.

Species	Common Name	Stranding Date	Positive Tissues	Not Detected
Kogia sima	Dwarf sperm whale	8/31/2000	Cerebrum, lung[1], spleen[1], liver	Kidney, adrenal
Stenella longirostris	Spinner dolphin	10/14/2008	Brain, kidney[1], liver	-
Ziphius cavirostris	Cuvier's beaked whale	8/23/2011	Lung, kidney, spleen[1], liver[1], mediastinal LN	Cerebrum
Ziphius cavirostris	Cuvier's beaked whale	3/23/2015	-	Cerebrum, lung, kidney, spleen, liver, lung LN
Ziphius cavirostris	Cuvier's beaked whale	5/6/2015	Cerebrum[1], kidney, spleen	Lung, liver, lung LN
Ziphius cavirostris	Cuvier's beaked whale	2/15/2016	-	Cerebrum, lung, kidney, spleen, liver, pancreas
Globicephala macrorhynchus	Short-finned pilot whale	10/13/2017	-	Cerebrum, lung, kidney, spleen, liver, pancreas
Globicephala macrorhynchus	Short-finned pilot whale	10/13/2017	-	Cerebrum, lung, kidney, spleen, liver, marginal LN
Globicephala macrorhynchus	Short-finned pilot whale	10/13/2017	-	Lung, kidney, spleen, liver, adrenal, marginal LN
Lagenodelphis hoseii	Fraser's dolphin	2/7/2018	Cerebrum[1], liver[1]	Lung, kidney, spleen, mesenteric LN
Peponocephala electra	Melon-headed whale	4/9/2019	Cerebrum[1], lung[1], spleen, liver, marginal LN	Kidney
Feresa attenuata	Pygmy killer whale	8/29/2019	-	Cerebrum, lung, kidney, spleen, liver, marginal LN
Feresa attenuata	Pygmy killer whale	8/29/2019	-	Cerebrum, lung, kidney, spleen, liver, marginal LN
Feresa attenuata	Pygmy killer whale	8/29/2019	-	Cerebrum, lung, kidney, spleen, liver, marginal LN
Feresa attenuata	Pygmy killer whale	8/29/2019	-	Cerebrum, lung, kidney, spleen, liver, marginal LN
Feresa attenuata	Pygmy killer whale	8/29/2019	-	Cerebrum, lung, kidney, spleen, liver, marginal LN
Feresa attenuata	Pygmy killer whale	8/29/2019	-	Cerebrum, lung, kidney, spleen, liver, marginal LN
Feresa attenuata	Pygmy killer whale	8/29/2019	-	Cerebrum, lung, kidney, spleen, liver, marginal LN
Stenella coeruleoalba	Striped dolphin	5/2/2020	Cerebrum[1], lung, kidney, spleen, liver, marginal LN	-
Stenella coeruleoalba	Striped dolphin	6/19/2020	-	Cerebrum, lung, kidney, spleen, liver, marginal LN

[1] PCR product was sequenced with a percent similarity to the BLAST BWCV reference genome of greater than 97%

Table 2. Stranding dates, locations, sex, age class, and co-infections of beaked whale circovirus positive animals.

Species	Common Name	Stranding Date	Stranding Location	Sex	Age Class	Coinfections
Kogia sima	Dwarf sperm whale	8/31/2000	Kailua, Oahu	Male	Adult	
Stenella longirostris	Spinner dolphin	10/14/2008	Kailua-Kona, Hawaii	Female	Adult	
Ziphius cavirostris	Cuvier's beaked whale	8/23/2011	Saipan	Male	Subadult	
Ziphius cavirostris	Cuvier's beaked whale	5/6/2015	American Samoa	Male	Calf	
Lagenodelphis hoseii	Fraser's dolphin	2/7/2018	Ukumehame Beach, Maui	Male	Adult	Morbillivirus[3]
Peponocephala electra	Melon-headed whale	4/9/2019	Kailua, Oahu	Male	Adult	
Stenella coeruleoalba	Striped dolphin	5/2/2020	Sugar Beach, Maui	Male	Subadult	
Indopacetus pacificus (positive control)	Longman's beaked whale	3/22/2010	Hana, Maui	Male	Subadult	Morbillivirus[1], herpesvirus[2]

[1] *West et al., 2013*

[2] *Landrau-Giovanetti et al., 2020*

[3] *West et al., 2021*

Table 3. Animal tissues tested for the presence of cetacean morbillivirus.

Species	Common Name	Stranding Date	Positive Tissues	Not Detected
Stenella longirostris	Spinner Dolphin	8/11/2014	-	Cerebellum, Cerebrum, Left Kidney, Left Lung, Liver, Muscle, Right Lung, Spinal Cord, Spleen, Anal LN, Left Marginal LN, Mesenteric LN
Stenella coeruleoalba	Striped Dolphin	5/4/2016	-	Cerebellum, Cerebrum, Left Kidney, Left Lung, Liver, Right Lung, Spleen, Colonic LN, Left Hilar LN, Left Marginal LN, Left Prescapular LN, Mesenteric LN
Stenella longirostris	Spinner Dolphin	2/26/2018	-	Cerebellum, Cerebrum, Left Kidney, Left Lung, Liver, Right Lung, Spinal Cord, Spleen, Diaphragmatic LN, Left Hilar LN, Left Marginal LN, Mesenteric LN
Kogia breviceps	Pygmy Sperm Whale	7/3/2018	-	Cerebellum, Cerebrum, Left Kidney, Left Lung, Muscle, Right Kidney, Spinal Cord, Spleen, Prescapular LN, Retroperitoneal LN, Sublumbar LN
Stenella coeruleoalba	Striped Dolphin	8/28/2018	-	Cerebellum, Cerebrum, Left Atrium, Left Kidney, Left Lung, Left Ventricle, Liver, Pancreas, Right Lung, Aoritc Chain LN, Mesenteric LN, Pulmonary LN
Ziphiuscavirostris	Cuvier's Beaked Whale	1/17/2019	-	Cerebellum, Cerebrum, Left Kidney, Left Lung, Liver, R Adrenal, Right Kidney, Right Lung, Spleen, Colonic LN, Mesenteric LN, Splenic LN
Stenella longirostris	Spinner Dolphin	4/3/2019	-	Cerebellum, Cerebrum, Left Kidney, Left Lung, Liver, Muscle, Right Lung, Spinal Cord, Spleen, Aortic LN, Left Hilar LN, Right Hilar LN
Peponocephala electra	Melon-headed Whale	4/9/2019	-	Cerebellum, Cerebrum, Left Lung, Liver, Right Kidney, Right Lung, Spinal Cord, Spleen, Left Hilar LN, Left Marginal LN, Left Prescapular LN, Mesenteric LN
Feresa attenuata	Pygmy Killer Whale	8/29/2019	-	Cerebrum, Cerebellum, Left Lung, Right Lung, Right Kidney, Liver, Spleen, Left Adrenal, Left Marginal LN, Prescapular LN, Mesenteric LN, Axillary LN
Feresa attenuata	Pygmy Killer Whale	8/29/2019	-	Cerebrum, Cerebellum, Left Lung, Right Lung, Right Kidney, Liver, Spleen, Spinal Cord, Right Hilar LN, Marginal LN, Mesenteric LN, Left Submandibular LN
Feresa attenuata	Pygmy Killer Whale	8/29/2019	-	Cerebellum, Cerebrum, Left Lung, Right Lung, Right Kidney, Liver, Spleen, Spinal Cord, Left Adrenal, Hilar LN, Left Marginal LN, Right Prescapular LN
Feresa attenuata	Pygmy Killer Whale	8/29/2019	Hilar LN, Mesenteric LN, Right Marginal LN	Cerebellum, Cerebrum, Left Lung, Liver, Right Kidney, Right Lung, Spleen, Left Marginal LN, Scapular LN
Feresa attenuata	Pygmy Killer Whale	8/29/2019	-	Cerebellum, Cerebrum, Left Kidney, Left Lung, Liver, Muscle, Spleen, Thymus, Colonic LN, Left Marginal LN, Left Prescapular LN, Mesenteric LN
Feresa attenuata	Pygmy Killer Whale	9/24/2019	-	Cerebellum, Cerebrum, Left Kidney, Left Lung, Liver, Pancreas, Right Lung, Left Hilar LN, Mediastinal LN, Prescapular LN, Right Marginal LN, Right Submandibular LN
Feresa attenuata	Pygmy Killer Whale	9/24/2019	-	Cerebellum, Cerebrum, Left Lung, Liver, Right Kidney, Right Lung, Spinal Cord, Spleen, Colonic LN, Left Prescapular LN, Mesenteric LN, Right Marginnal LN
Pseudorca crassidens	False Killer Whale	12/6/2019	-	Cerebellum, Cerebrum, Left Kidney, Left Lung, Liver, Right Lung, Spinal Cord, Spleen, Left Scapular LN, Mesenteric LN, Right Marginal LN
Megaptera novaeangliae	Humpback Whale	1/22/2020	-	Bladder, Internal Umbilicus, Left Kidney, Left Lung, Liver, Muscle, Right Kidney, Right Lung, Thymus, Aortic LN, Diaphragmatic LN, Mesenteric LN
Stenella coeruleoalba	Striped Dolphin	5/2/2020	-	Left Kidney, Left Lung, Liver, Right Lung, Spleen, Anal LN, Aortic LN, Colonic LN, Hilar LN, Mesenteric LN, Prescapular LN, Right Marginal LN
Stenella coeruleoalba	Striped Dolphin	6/19/2020	-	Cerebellum, Cerebrum, Left Kidney, Left Lung, Liver, Right Lung, Spleen, Aortic LN, Colonic LN, Left Marginal LN, Left Prescapular LN, Liver, Mesenteric LN
Ziphius cavirostris	Cuvier's Beaked Whale	10/20/2020	-	Cerebellum, Cerebrum, Left Kidney, Left Lung, Liver, Muscle, Right Kidney, Right Lung, Spleen, Left Hilar LN, Left Marginal LN, Right Diaphragmatic LN
Stenella coeruleoalba	Striped Dolphin	3/18/2021	-	Cerebellum, Cerebrum, Left Lung, Liver, Right Kidney, Right Lung, Spleen, Anal LN, Aortic LN, Left Marginal LN, Left Prescapular LN, Mesenteric LN
Stenella coeruleoalba	Striped Dolphin	4/13/2021	-	Cerebellum, Cerebrum, Left Kidney, Left Lung, Liver, Right Kidney, Right Lung, Spinal Cord, Spleen, Left Marginal LN, Left Prescapular LN, Mesenteric LN
Lagenodelphis hoseii	Fraser's Dolphin	12/5/2021	-	Cerebellum, Cerebrum, Left Kidney, Left Lung, Liver, Right Kidney, Right Lung, Spinal Cord, Spleen, Left Marginal LN, Left Prescapular LN, Mesenteric LN
Lagenodelphis hoseii	Fraser's Dolphin	12/6/2021	-	Cerebellum, Cerebrum, Left Kidney, Left Lung, Liver, Right Kidney, Right Lung, Spinal Cord, Spleen, Left Marginal LN, Left Prescapular LN, Mesenteric LN
Lagenodelphis hoseii	Fraser's Dolphin	12/6/2021	-	Cerebellum, Cerebrum, Left Kidney, Left Lung, Liver, Right Kidney, Right Lung, Spinal Cord, Spleen, Left Marginal LN, Mesenteric LN, Right Prescapular LN

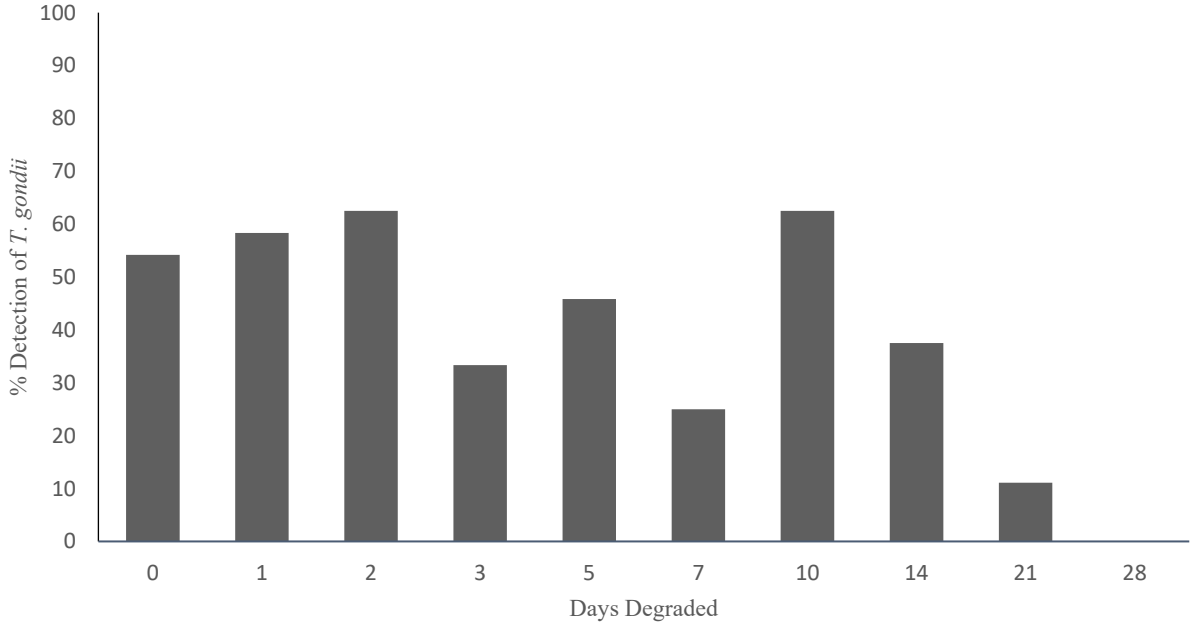

Figure 1. Rates of detection in *T. gondii* positive tissues during environmental degradation

(Figure 1). A one-way ANOVA test was conducted and indicated significant differences in detection rates across the duration of this study (P=0.00454). Fluctuations in *T. gondii* detection rates occurred between Day 2 and Day 10, with a consistent decline in detection rates occurring after Day 10. The parasite was not detected in the limited number of samples tested that represented 28 days postmortem.

DISCUSSION

CIRCOVIRUS

Findings from this study have significantly increased our understanding of BWCV in marine mammals, including the expansion of known host species, the geographic range of BWCV presence, and providing further insight into when this disease may have emerged in the central Pacific. Our results suggest 35% prevalence of BWCV among the 20 individual cetaceans tested, and indicates that despite being named beaked whale circovirus, this recently identified circovirus species can infect a number of other host cetacean species. The current study confirms the discovery of BWCV in six new cetacean species in addition to the initial Longman's beaked whale case and includes individuals of diverse age and sex classes. These newly detected infections of BWCV were found in an additional species from family Ziphiidae (beaked whales), which includes the initial Longman's beaked whale, as well as in animals from the families Delphinidae (oceanic dolphins) and Kogiidae (dwarf and pygmy sperm whales). This suggest that this virus is wide-spread across odontocete species in the central Pacific, with infections likely in a greater number of cetacean species than we report. Of 8 cetacean species tested in the current study, only 2 species were found to be negative for BWCV. The two BWCV negative species in the current study were pygmy killer whales and short-finned pilot whales, but in both cases the only

individuals tested that represent these species were part of mass stranding events. It would be valuable to test additional individuals involved in solitary stranding events that are represented by the species where BWCV was not found as well as additional odontocete and mysticete species that have not been tested for BWCV to date. Much is yet to be learned about the extent of BWCV presence among the approximately 20 species of cetaceans that are considered resident to the central Pacific.

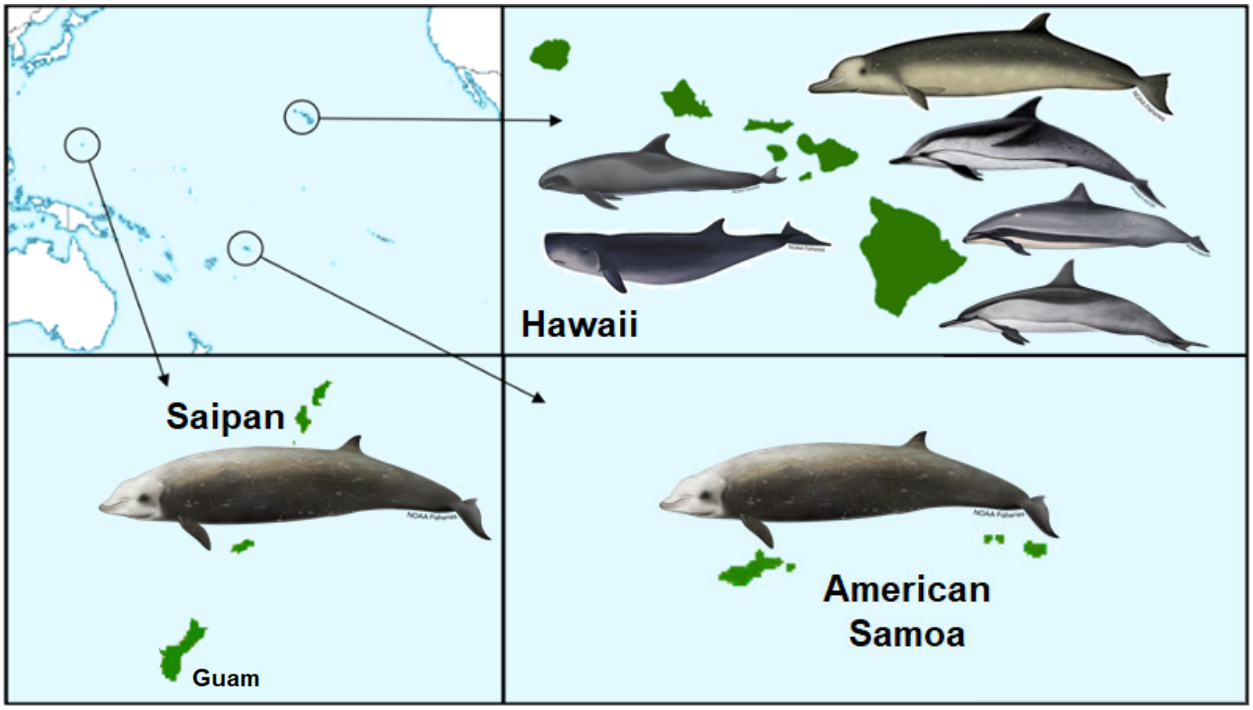

Figure 2. Stranding locations throughout the North and South Pacific of BWCV positive animals.

In terms of geographic scope, our findings indicate that BWCV is a much more wide-spread virus than the isolated stranding location on Maui, Hawaii indicated by the initial Longman's beaked whale case. Our results suggested that BWCV is present in cetacean hosts across the Pacific basin, including the central, Western and South Pacific (Figure 2). Infected individuals were identified across the Hawaiian archipelago, with strandings of BWCV positive delphinids and a dwarf sperm whale occurring on the islands of Hawaii, Maui, and Oahu. Our results also indicated positive circovirus findings in five out of six tissues tested from a Cuvier's beaked whale stranded in Saipan in 2011 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 that stranded in American Samoa in 2015 tested positive for BWCV in three of six tissues tested. We believe that it is likely that the identified BWCV positive cases across the central, Western and South Pacific represent the same strain of BWCV described in the Longman's beaked whale from Maui because of the high percent similarity between the isolates obtained in this study and the BWCV reference genome, which exceeded 96% similarity for each of the BWCV animals identified in the current study (Table 1). The high similarity between isolates is somewhat surprising considering the distance of greater than 6,000 km between locations of the identified BWCV positive animals. These findings signify a high probability that this virus is even more broadly disseminated among

additional cetacean species and locations across the Pacific Ocean basin. With such a high prevalence rate among the 20 animals tested, combined with the greatly expanded geographic scope identified, future work should include testing of marine mammals from other locations in both the Pacific and other ocean basins to determine if this virus is present in marine mammals globally or if it is limited to isolated regions of the Pacific or the entire Pacific Ocean basin.

Another significant finding from this work is the ability to further investigate the emergence of BWCV in the central Pacific. The initial discovery of BWCV occurred 10 years after the stranding of the Longman's beaked whale in 2010 where the stranding cause was attributed to morbillivirus (West et al., 2013; Landrau-Giovanetti et al., 2020). Our screening of archived tissues detected BWCV in 4 of 6 tested tissues in a dwarf sperm that stranded in 2000 off the island of Oahu, which indicates that BWCV had emerged in the central Pacific at least 10 years prior to the initial case and 20 years prior to its discovery (Landrau-Giovanetti et al., 2020). Other infectious diseases recently described from the Pacific Islands region have also indicated a date discrepancy between their discovery and the possible timeframe of emergence. In the case of beaked whale morbillivirus, this strain of morbillivirus was first identified from a stranding in 2010 but was later found in a humpback whale that stranded in 1998, indicating that it had been present in the region for at least 12 years prior to its discovery (West et al., 2013; Jacob et al., 2016). It is currently unknown when BWCV emerged in the central Pacific but this study demonstrates its persistence for over a 20 year period that dates back to the year 2000.

Circoviruses have been demonstrated to have a broad range of effects on their hosts, many times associated with severe impacts to the brain and lungs (Bexton et al., 2015; Seo et al., 2014). Additionally, swollen lymph tissue and lymphocyte depletion are common occurrences with infections of several strains of circoviruses (Mao et al., 2017; Palinski et al., 2017; Yang et al., 2015). The potential for similar impacts when BWCV infections occur exists. The three most commonly infected tissues among positive cases in this study were the brain, lungs, and lymph tissues. Co-infection by other pathogens is also common with other circoviruses (Yang et al., 2015). Cetacean morbillivirus, a potentially fatal disease, was found in two of the BWCV cases from the Pacific Islands, including the initial Longman's beaked whale case, as well as a Fraser's dolphin infected with a novel morbillivirus strain (West et al., 2013; West et al., 2021). The initial BWCV Longman's beaked whale also had a novel herpesvirus, making this it case a tri-infection (Landrau-Giovanetti et al., 2020; West et al., 2015).

The current study did not address the pathological significance of BWCV and instead focused on a PCR screening effort to investigate prevalence. Some of the BWCV positive cases had clear disease-related pathologies, particularly in the individuals with confirmed co-infections. Conditions such as enlarged lymph nodes and lymphocyte depletion occurred among some of the infected cases, as well as signs of respiratory infection and wasting of body condition (Landrau-Giovanetti et al., 2020; West et al., 2021). Future work should focus on investigating the clinical significance of such a high prevalence rate of BWCV in Pacific Island cetaceans and a dedicated effort to tease apart the pathologies observed in co-infected individuals.

Future efforts should also 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 should 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 beneficial to test all archived tissues available for that individual 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. While traditional PCR is effective at detecting viral sequences and ideal for a broad screening effort, it is limited in its capability to investigate concentrations of viral load or provide insight into clinical manifestations. Additional methods would need to be employed to address questions concerning viral load concentration and the clinical significance of BWCV. Quantitative PCR provides a specialized technique that allows for a determination of the level of infection in tissues tested. Full utilization of this technology would require development of probes specific to this viral genome in addition to the primers used for replication to determine the viral load within tissues. Additionally, secondary confirmatory testing that focuses on cellular effects would aid in describing the pathological significance of BWCV. *In situ* hybridization (ISH) binds viral DNA specific probes to infected tissues, labeling them by chemical or radioactive means, which allows for visual confirmation of infection. Through microscopy, identification of inclusion bodies, as well as the level of infection and impact to the infected cells can be investigated. Immunohistochemistry (IHC) provides another potential method to better understand BWCV. Similar to ISH, IHC is used to visualize viral infection through staining and microscopy, though IHC procedures focus on labeling antigens within infected cells. This would require the development of BWCV associated antibodies through in vivo injection and antibody isolation from the tissues of those test animals, as current commercially available antibodies for other strains of circovirus do not appear to exhibit enough cross reactivity to be successful in BWCV IHC staining. Implementation of such available methodology would allow for a detailed analysis BWCV infections and their clinical significance. Much remains to be learned about the clinical significance of this emerging cetacean disease with suggested wide-spread presence over time, among cetacean species and across geographic locations throughout the Pacific.

MORBILLIVIRUS

Our findings indicated low levels of infection among the 25 individuals screened for CeMV that had stranded in Hawaiian waters since 2014. However, an individual pygmy killer whale that stranded as part of a prolonged mass stranding event on Maui in 2019 was positive for CeMV in three of twelve tissues tested. Sequencing results from this positive case indicated closest genetic similarity to the novel and very recently described Fraser's dolphin morbillivirus strain from the Fraser's dolphin that also stranded off of Maui in 2018 (West et al., 2021). This signifies only the second known individual to be infected with the novel Fraser's morbillivirus strain of CeMV. This also confirms the presence of Fraser's dolphin morbillivirus in another species of Hawaiian cetacean, which suggests that this strain is not limited to Fraser's dolphins but is transmissible among cetacean species. This raises concern for cetacean populations in Hawaiian waters, especially those with an already low number of breeding individuals, such as the endangered main Hawaiian Islands insular false killer whale population. Transmission of a novel morbillivirus strain among Hawaiian cetaceans has the potential to have devastating population level impacts.

We expect that the infection level in the positive pygmy killer whale screened in this study represents a low-level infection based on the 25% positivity rate of the tissues tested. Previous findings from Hawaiian cetaceans suggest that wide-spread systemic infection exist in individuals

where all tested tissues have been positive, such as in the initial Longman's beaked whale where BWMV was first characterized, as well as in the Fraser's dolphin where Fraser's dolphin morbillivirus was described (West et al., 2013; West et al., 2021). The low positivity rate among tissues tested in the pygmy killer whale, combined with an absence of major pathological findings in this individual, lead us to believe that the CeMV infection was unlikely to have been the cause of death in this animal. It is possible that the positive CeMV finding indicates that this virus may have played a role in the prolonged mass stranding of pygmy killer whales in 2019. A total of seven individuals were known to have died in this prolonged event that occurred over several weeks, and all were screened for morbillivirus, with only one of the seven individuals positive for CeMV. However, this does not discount the possibility of CeMV infection in other pygmy killer whales associated with this mass stranding event that may have died where carcasses were not recovered, or where debilitation had occurred. This was an unusual mass stranding event both because of the prolonged time period that a group of pygmy killer whales was monitored in the same location following the initial stranding event and because these individuals were not the same animals as those that were initially stranded and refloated. This leaves unanswered questions in regards to the scope of this prolonged mass stranding event that persisted over several weeks as well as the cause.

Findings from this work did not have as high a rate of infection as expected, based on a higher positivity rate in Pacific Island cetaceans that were previously screened for morbillivirus (Jacob et al., 2016). This discrepancy in positivity rates when comparing CeMV screening efforts may be the result of several factors. The Jacob et al., 2016 study assessed a much larger pool of animals (n=62) that stranded between 1998 and 2014, with findings indicating an infection rate of 24% among animals tested (Jacob et al., 2016). The current CeMV screening results indicate a study that is smaller in scope (n=25). The current study also targeted individuals involved in mass strandings, as CeMV has been shown to result in mass mortality events among cetaceans (Mazzariol et al., 2017). Mass stranded individuals comprised nearly half of the individuals tested (n=10), resulting in the screening of only 15 individuals associated with solitary stranding events. In comparison to the Jacob et al. 2016 study, no mass stranded animals were screened, reflecting a difference in the breadth of stranding events investigated for morbillivirus between the two screening efforts. Additionally, the current study focused on a time period of 2014 to 2021, and a significantly lower positivity rate may indicate less viral spread of morbillivirus among Hawaiian cetaceans during this time, when compared to the results that represent the time period of 1998 to 2014 Further work should include screening of additional animals from solitary stranding events occurring between 2014 and the present to better understand if cetacean morbillivirus infections in Hawaiian waters have actually declined or if the differences in results between the two screening efforts are an artifact of targeted individuals and the lower number of individuals screened in the current study.

TOXOPLASMOSIS

Though not initially proposed as a part of this project, the opportunistic sampling of degraded tissues after Day 14 was beneficial to understanding the time point postmortem where detection of this parasite drops significantly. In this investigation, there appears to be significant decline in detectability of *T. gondii* after Day 10, dropping significantly lower after Day 14 to its lowest

levels at Day 21 and no detectability at Day 28. This indicates that testing for *T. gondii* in stranded cetacean tissues is likely to be most successful if conducted within two weeks of an animal's death.

Throughout the first two weeks of this study, detection rates of *T. gondii* fluctuated between 33.3% and 63.5%. This variation is most likely due to the heterogeneity of the infection within the tissues, as the *T. gondii* oocysts are not likely to be evenly distributed. However, the consistent drop after day 10, and lack of detection in a limited number of samples tested on day 28 suggests that there is value in testing decomposed carcasses for *T. gondii* until at least three weeks postmortem.

While the results of this project are significant, this represents a simulated experiment with some limitations. For example, the samples for this study were taken from small portions of the original organ, using plastic bags to simulate tissue membranes surrounding the sample. These would not have the same diffusive properties of a true membrane, and the larger surface area to volume ratio of the samples would potentially impact other factors, such as heat retention of the samples, when compared to the entire organ *in situ*. Additionally, environmental temperature could be a significant variable. Seasonal fluctuations could impact the results, depending on the time of year that the study was performed. Temperature could also be affected by the experimental set up, causing variation from true environmental conditions due to factors like coloration of material and composition of the sample containers, potentially impacting what temperatures the simulated stranded animals would experience. Despite these limitations, the results show a clear degradation trend – DNA degradation appears to be consistent for an almost two-week period before a drop off at the end of the study at 28 days. Better understanding *Toxoplasma* presence and detection is critical to mitigating the detrimental results of infections among marine mammals, and this work contributes important knowledge towards evaluation of this pathogen and future testing efforts.

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