Hawaii and Mariana Islands Stranding Analyses

West, K.L.[1,2], Clifton, C.[2], Hofmann, N.[1], Humann, C.[1] and I. Silva-Krott[1]

[1]Hawaii Institute of Marine Biology, University of Hawaii at Manoa, Kaneohe, Hawaii
[2]Human Nutrition Food and Animal Sciences, College of Tropical Agriculture and Human Resources, University of Hawaii at Manoa, Honolulu, Hawaii

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. The purpose of this project is to conduct analyses of historical stranding patterns and causes of mortality that incorporate quantitative estimates of stranding date, genetic identification of species when necessary and advanced diagnostic procedures, including evaluation of nutritional state to categorize past strandings as acute or chronic causes. This report focuses on describing progress associated with conducting genetic species identification for stranding events where an initial species determination was not possible. This data will be used to increase the robustness of stranding data in a historical analysis of temporal and spatial stranding patterns in the Pacific Islands. Additionally, we describe progress towards the screening of archived tissues for the presence of *Brucella* and *Toxoplasma* in stranding cases where infectious disease is suspected. This advanced diagnostic information provides the ability to more thoroughly evaluate potential causes of mortality in stranded animals from the Hawaiian and Mariana Islands.

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE *(DD-MM-YYYY)*	2. REPORT TYPE	3. DATES COVERED *(From - To)*
12-04-2022	Monitoring report	1937 to 2021

4. TITLE AND SUBTITLE
HAWAII AND MARIANA ISLANDS STRANDING ANALYSES

5a. CONTRACT NUMBER
N62473-20-2-0001

5b. GRANT NUMBER

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
Kristi L. West
Cody Clifton
Nicholas Hofmann
Connor Humann
Ilse Silva-Krott

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Hawaii Institute of Marine Biology, University of Hawaii at Manoa, Kaneohe, Hawaii
Human Nutrition Food and Animal Sciences, College of Tropical Agriculture and Human Resources, University of Hawaii at Manoa, Honolulu, Hawaii

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)
Commander, U.S.Pacific Fleet, 250 Makalapa Dr. Pearl Harbor, HI

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSORING/MONITORING AGENCY REPORT NUMBER

12. DISTRIBUTION AVAILABILITY STATEMENT
Approved for public release; distribution is unlimited

13. SUPPLEMENTARY NOTES

14. 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. The purpose of this project is to conduct analyses of historical stranding patterns and causes of mortality that incorporate quantitative estimates of stranding date, genetic identification of species when necessary and advanced diagnostic procedures, including evaluation of nutritional state to categorize past strandings as acute or chronic causes. This report focuses on describing progress associated with conducting genetic species identification for stranding events where an initial species determination was not possible. This data will be used to increase the robustness of stranding data in a historical analysis of temporal and spatial stranding patterns in the Pacific Islands. Additionally, we describe progress towards the screening of archived tissues for the presence of Brucella and Toxoplasma in stranding cases where infectious disease is suspected. This advanced diagnostic information provides the ability to more thoroughly evaluate potential causes of mortality in stranded animals from the Hawaiian and Mariana Islands.

15. SUBJECT TERMS					
Marine mammals, strandings, necropsy, biopsy, DNA, Hawaii Range Complex, Mariana Islands Rangle Complex					
16. SECURITY CLASSIFICATION OF:			**17. LIMITATION OF ABSTRACT**	**18. NUMBER OF PAGES**	**19a. NAME OF RESPONSIBLE PERSON**
			UU	12	Department of the Navy
a. REPORT	**b. ABSTRACT**	**c. THIS PAGE**			**19b. TELEPONE NUMBER (*Include area code)***
Unclassified	Unclassified	Unclassified			808-471-6391

INTRODUCTION

Background: Stranded species identification using genetic tools

Examination of stranding patterns in the Pacific Islands region and how this relates to trends over time and the probability of species specific strandings by location is limited. Published stranding data involves an examination of approximately 200 records of odontocete strandings occurring between 1937 and 2002 in the main Hawaiian Islands (Maldini et al., 2009). Stranding data that has been obtained since 2002 includes well over 200 additional stranding records and stranding data from the Mariana Islands. In other regions of the world, historical stranding patterns have been assessed to describe the probability by location of species specific strandings and the relationship between stranding events and environmental factors (i.e. Norman et al., 2004; Coombs et al., 2019). A similar examination of historical stranding patterns in the Pacific Islands region could be strengthened by conducting species identification using genetic tools where determination of species identification was not possible at the time of stranding. It is possible to apply genetic techniques to distinguish between the species *Kogia breviceps* and *Kogia sima*, two species that are very difficult to distinguish from one another when size range overlaps and/or carcass condition precludes measurements of the dorsal fin. Other potential species that have historically stranded in the Pacific Islands region and are difficult to distinguish include sperm whales and humpback whales, whose carcasses may wash ashore or be found floating in advanced states of decomposition without any species identifying characteristics. Additional records also exist of unidentified odontocete carcasses being discovered in the Pacific Islands region that may represent beaked whales or pilot whales.

Background: Increasing our understanding of causes of mortality through disease screening

Infectious disease is becoming well recognized as a significant threat to Pacific Island cetaceans. Morbillivirus was first identified in the Pacific Islands region from a Longman's beaked whale that stranded in 2010 (West et al., 2013), and we have since reported on morbillivirus findings from archived Hawaiian stranding samples (West et al., 2015; Jacob et al., 2016), were involved in new method validation for global surveillance of morbillivirus (Yang et al., 2016) and have contributed to whole genome sequencing in order to describe the unique beaked whale morbillivirus strain (Landrau-Giovanetti et al., 2019). We have most recently discovered an additional novel strain of morbillivirus in a Fraser's dolphin that stranded in 2018 that is distinct from both the beaked whale morbillivirus and the Southern hemisphere strains (West et al., 2021). We have reported other pathogens that threaten Hawaiian cetaceans and have historically caused mortality, including the presence of *Cryptococcus gatti* in a Hawaiian spinner dolphin (Rotstein et al., 2010), *Brucella* cases that involve a neonate sperm whale co-infected with morbillivirus (Chernov, 2010; West et al., 2015) and fatal disseminated toxoplasmosis in 3 stranded spinner dolphin deaths, where we project based on low carcass recovery rates that this could be representative of the deaths of 60 spinner dolphins (West et al., 2020). We have recently characterized the full genome of the first known cetacean circovirus that was identified from a stranded Hawaiian whale (Landrau-Giovanetti et al., 2020) as well as four novel cetacean herpes viruses from this region (West at al., 2013; West and Waltzek, unpublished data). However, much remains unknown about the prevalence of identified diseases among Hawaiian and Mariana Island cetaceans.

Despite describing numerous pathogens that contribute to mortality in the Pacific Islands region, it is difficult to quantify the contribution of each identified pathogen to overall causes of mortality in the region without applying advanced diagnostic techniques. This project supports the polymerase chain reaction

(PCR) analysis of archived tissues for 35 stranding cases where infectious disease is suspected. Specifically, PCR screening focuses on testing for the presence of the pathogens *Brucella* and *Toxoplasma* and the comparison of testing results with necropsy and histopathology findings. Disease screening results contribute to the developing integrated causes of mortality database as part of this overall project, and directly supports a quantitative examination of causes of mortality among cetaceans in the Pacific Islands.

METHODS

Stranded species identification using genetic tools

The selection of previously stranded specimens for genetic analysis

Distinguishing between species identification of *Kogia breviceps* and *Kogia sima* is notoriously difficult. Identification of *Kogia spp.* typically relies on morphological measurements that differ between the two species. If teeth are present in the upper jaw, this is also diagnostic of *K. sima*, but the absence of teeth in the upper jaw could indicate either *K. breviceps* or *K. sima*. The first measurement that can be used diagnostically is total body length (TBL), as *K. breviceps* reach a larger total length than *K. sima*. Individuals with a total body length beyond the maximum reported for *K. sima* (270 cm) are identified as *K. breviceps* when stranding events occur. However, when total body length is less than the maximum for *K. sima*, this single measurement cannot be used to distinguish between the two species. The other useful measurement that is applied when diagnosing species is the dorsal fin height (DFH). *K. sima* typically have a dorsal fin height that is larger in comparison to its total body length where *K. breviceps* typically have a dorsal fin that is smaller compared to the body length. As a general rule, if the dorsal fin height is less than 5% of the total body length, the animal is considered to represent *K. breviceps* and greater than 5% represents *K. sima*.

Due to variation in or a lack of morphological measurements available because of carcass condition at the time of carcass recovery, the individuals with borderline measurements could not be identified on morphometrics alone. Borderline measurements included a DFH:TBL ratio between 4 and 6% where total body length was less than the maximum reported for *K. sima* which indicated a potential overlap in body size. Ten animals were initially selected based on potential overlap in total body length measurements and/or a DFH:TBL ratio that was borderline or considered not definitive as a species diagnostic indicator. Two of the animals had no dorsal fin measurements available. After the examination of each of the *Kogia* individuals that had previously stranded and been assigned a species identification at the time of necropsy based on morphometrics, we decided it would be beneficial to include additional cases in genetic analyses for confirmation of the initial species identification. This effort resulted in conducting genetic identification among *Kogia* strandings for a total of 24 individual animals, 10 of which had borderline measurements and could not be confirmed as either *K. sima* or *K. breviceps* based solely on measurements.

From the selected cases, tissues for genetic analysis were chosen based on availability and included kidney, spleen, gingiva, testis, skin, brain, heart, muscle and lung. Only one tissue is required for genetic identification, but multiple tissues were used as needed to increase the likelihood of PCR and sequencing success.

Species Identification by PCR

DNA was extracted from each tissue using Qiagen DNeasy Blood and Tissue Kits (Qiagen, Germantown, Maryland) according to the manufacturer's protocol. To confirm the success of the DNA extraction, the DNA concentration of each extract was determined using Qubit dsDNA Broad-Range Assay Kits and a Qubit 4 fluorometer (Thermo Fisher Scientific, Waltham, Massachusetts).

PCR was conducted for each tissue extract using primers and thermocycler conditions based on Wang et al., 2013. Four primers were used in total: two for PCR amplification and two for Sanger sequencing of the amplified products. PCR primers included Kogia A DLP1.5 forward primer 5' TGTAAAACGGCCACTTCACCCAAAGCTGRARTT 3' and Kogia A DLP4H-fast reverse primer 5' AGCGGGWTRYTGRTTTCACGCGGCATG 3'. Thermocycler conditions included an initial incubation at 98ºC for 50s followed by 35 cycles of denaturation at 95ºC for 10s, annealing at 65ºC for 10s, and extension at 65ºC for 10s, with a final extension at 68ºC for 3 minutes. Amplified products were visualized using gel electrophoresis. Amplified DNA fragments measuring approximately 420bp in size were cut from the gel and further purified using QIAquick PCR and Gel Cleanup Kits (Qiagen, Germantown, Maryland). The DNA from a known *Kogia sima* was used as the positive control throughout the entire PCR screening to ensure the amplification and sequencing was successful.

Cleaned samples were submitted to the University of Hawaii Advanced Studies in Genomics, Proteomics, and Bioinformatics (ASGPB) lab for genetic sequencing. These samples were sequenced once with each primer: Kogia B DLP1.5 5' ACGACGGCCAGTTCACCCAAAGCTG 3' and Kogia B DLP4H-fast 5' AGCGGGTTGCTGGTTTCACGCGGGATG 3'. Sequences were analyzed using NCBI Nucleotide BLAST and match percentage to *K. breviceps* and *K. sima* sequences were used to diagnostically identify specimens to species.

Increasing our understanding of causes of mortality through disease screening

Toxoplasma: Animal and Tissue Selection

In conducting disease screening for the pathogen *Toxoplasma gondii*, 35 animals were selected from prior strandings cases from the University of Hawaii Health and Stranding Lab frozen tissue archive that stranded in Hawaii and the Mariana Islands between 2014 and 2021. Emphasis was placed on screening spinner dolphins (*Stenella longirostris*) and ten individuals were included based on three previously confirmed cases of fatally disseminated toxoplasmosis in this species in Hawaii. Animals involved in mass stranding events were also selected for screen as disease has been associated with these events in other regions (Mazzariol et al., 2017; Garrigue et al., 2016; Vargas-Castro et al., 2020). These cases included five short-finned pilot whales (*Globicephala macrorhynchus*) that mass stranded on Kaua'i in 2017, four pygmy killer whales (*Feresa attenuata*) that stranded on Maui in 2019 and three Fraser's dolphins (*Lagenodelphis hoseii*) that stranded on Oahu in 2021. The remaining animals that were selected for screening were based on histopathology findings indicative of infectious disease or the location of stranding. This included seven striped dolphins (*Stenella coeruleoalba*), two Cuvier's beaked whales (*Ziphius cavirostris)*, one melon-headed whale (*Peponocephala electra*) and one false killer whale (*Pseudorca crassidens*).

A total of five tissues were selected from each of the 35 animals for screening for *T. gondii.* Tissue selection emphasized a focus on the brain followed by remaining tissues that are likely targets for infection. The tissue types selected for screening included brain, lung, liver, spleen and lymph tissue. If

priority tissues were unavailable for an individual, kidney, pancreas, heart or muscle tissue were substituted to ensure that it was possible to screen 5 different tissues from each individual.

Detection of Toxoplasma by PCR

Deoxyribonucleic acid (DNA) was extracted from each tissue using Qiagen DNeasy Blood and Tissue Kits (Qiagen, Germantown, Maryland) according to the manufacturer's protocol. To confirm the success of the DNA extraction, the DNA concentration of each extract was determined using Qubit dsDNA Broad-Range Assay Kits and a Qubit 4 fluorometer (Thermo Fisher Scientific, Waltham, Massachusetts).

PCR was used for each tissue extract 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 1 minute, annealing at 60°C for 1 minute, and extension at 72°C for 1 minute, with a final extension at 72°C for 7 minutes. Gel electrophoresis was used to visualize the amplified products, with visible bands indicating presence of *T. gondii* expected at about 500bp in size.

Positive and negative controls were used throughout the disease screening process as confirmation of the results. Nuclease-free water was used as the negative control and was used specifically to detect any potential contaminations in the PCR. Cerebrum from a known infected spinner dolphin that died of fatally disseminated toxoplasmosis was used as the positive control throughout the entire screening process. Our positive control animal stranded on Hawaii Island in 2015 and died of *T. gondii.* Prior screening of a large suite of tissues from this individual for *Toxoplasma* by PCR were positive.

Tissues that showed suspected positive bands were further processed for confirmation by genetic sequencing. The amplified products were cleaned using Qiagen QIAquick PCR and Gel Cleanup Kits (Qiagen, Germantown, Maryland). These samples were then submitted to the University of Hawaii Advanced Studies in Genomics, Proteomics, and Bioinformatics lab for genetic sequencing. Obtained Sequences were analyzed using National Center for Biotechnology Information (NCBI) Nucleotide Basic Local Alignment Search Tool (BLAST) to determine if matches were present with *Toxoplasma gondii* sequencing data.

Brucella: Animal and Tissue Selection

The animal and tissue selection process for this study is currently on-going. To date, 21 of the proposed 35 test animals have been selected (Table 1). These include animals involved in a mass stranding (pygmy killer whales stranded in 2019), as well as multiple striped dolphins that stranded over a time-scale of less than a month. Additionally, a full-term humpback whale fetus that stranded on Molokai in 2020 was selected for testing as several strains of *Brucella* have been linked to abortions and other reproductive abnormalities. The remaining animals that have been selected thus far include several species of dolphin and dwarf and pygmy sperm whales. The initially targeted tissues for testing include brain, lung, kidney, liver, and lymph nodes. Potential substitutions of tissue types depended on sample availability and histopathology findings available for individual animals. In-house tissue samples from cases previously confirmed at outside diagnostic laboratories were used as both positive and negative controls for all amplifications.

Table 1. Results of tissue analyses conducted for the presence of *Brucella*

Species	Common Name	Stranding Date	Detected Tissues	Not Detected
S. bredanensis	Rough-toothed dolphin	7/11/2000	Lung, spleen	Kidney
K. sima	Dwarf sperm whale	8/31/2000	Brain, liver	Kidney, lung, spleen
S. longirostris	Spinner dolphin	9/30/2007	Brain	Kidney, lung, spleen
S. longirostris	Spinner dolphin	10/14/2008	-	Kidney, brain, liver
P. crassidens	False killer whale	11/7/2015	-	Cerebrum, lung
L. hoseii	Fraser's Dolphin	2/7/2018	Cerebrum, liver, lung, mesenteric LN	L Kidney, Spleen
F. attenuata	Pygmy Killer Whale	8/29/2019	Cerebrum[1]	Lung
F. attenuata	Pygmy Killer Whale	8/29/2019	-	Lung
F. attenuata	Pygmy Killer Whale	8/29/2019	Marginal LN	Brain, Lung
F. attenuata	Pygmy Killer Whale	9/24/2019	Lung	Brain, marginal LN
F. attenuata	Pygmy Killer Whale	9/24/2019	Brain	Lung, marginal LN
P. crassidens	False Killer Whale	12/6/2019	-	Lung, liver, mediastinal LN, cerebrum
M. novaeangliae	Humpback whale	1/22/2020	Aortic LN[1], lung, liver	Umbilicus, kidney, thymus, mesenteric LN
S. coeruleoalba	Striped Dolphin	5/2/2020	R marginal LN	Hilar LN, mesenteric LN, R lung, meninges, cerebrum
Z. cavirostris	Cuiver's Beaked Whale	10/20/2020	-	Cerebrum, kidney, L lung, liver, L hilar LN, spleen
K. breviceps	Pygmy sperm whale	12/23/2020	-	Cerebrum, liver, R lung, hilar LN
L. hoseii	Fraser's Dolphin	2/8/2021	-	Cerebrum, lung, liver, spleen
K. breviceps	Pygmy sperm whale	3/11/2021	-	Cerebrum, L lung, L kidney, liver, L adrenal, diaphragmatic LN
S. coeruleoalba	Striped dolphin	3/18/2021	Cerebrum[1], R lung, spleen	Liver, R kidney, L marginal LN
S. coeruleoalba	Striped Dolphin	3/24/2021	Cerebrum, lung	Liver, kidney, spleen, L marginal LN
S. coeruleoalba	Striped Dolphin	4/13/2021	Cerebrum[1], L lung, spleen	L kidney, liver, L marginal LN

[1]Positive sequencing result for *Brucella spp.*

Primer Selection and Brucella Detection

Multiple genes from the *Brucella* genome were initially selected for assessment in order to develop a testing protocol with high specificity for detecting infections, while reducing the likelihood of false positives. Primers from the published literature and in-house primer design were tested for BCSP-31, OMP2b, and IS711 gene sequences, in both singular and nested formats. A final selection was made of primers from published literature that targeted a 150 bp sequence from the IS711 gene: Forward – 5' TACCGCTGCGAATAAAGCCAAC 3' and Reverse – 5' TGAGATTGCTGGCAATGAAGGC 3' (Wu et al., 2014).

DNA was extracted from each tissue using Qiagen DNeasy Blood and Tissue Kits (Qiagen, Germantown, Maryland) according to the manufacturer's protocol. To confirm the success of the DNA extraction, the DNA concentration of each extract was determined using Qubit dsDNA Broad-Range Assay Kits and a Qubit 4 fluorometer (Thermo Fisher Scientific, Waltham, Massachusetts). Final extracts were frozen at -20°C prior to amplification. PCR protocol steps were also adapted from Wu et al., 2014, which in brief were an initial denaturation of 10 minutes at 95°C and then 40 cycles of denaturing for 10 seconds at 95°C and 30 seconds of annealing at 60°C.

All amplified products were visualized using gel electrophoresis to determine suspected positive cases. Bands were cut from suspect positive gels and cleaned using Qiagen QIAquick PCR and Gel Cleanup Kits (Qiagen, Germantown, Maryland). These samples were then submitted to the University of Hawaii Advanced Studies in Genomics, Proteomics, and Bioinformatics lab for final genetic sequencing. These sequences were then analyzed using the NCBI database's Nucleotide BLAST online tool for potential matches with *Brucella ceti* and *Brucella pinnipedialis*.

RESULTS

Stranded species identification using genetic tools

Species Confirmation of All Cases

Using genetic sequencing, the species diagnostics were confirmed for 22 of the 24 total individuals analyzed. Our genetic sequencing results indicated that 17 of the 22 specimens (77.3%) were identified genetically as the same species they were identified as morphologically. A single specimen was genetically identified as the opposite species as indicated by species diagnostics based on morphological information.

We had difficulty conducting species identification through genetic sequencing efforts for 2 of the 24 cases examined and species identification remains unknown for these two individuals. One individual has a body length measurement just over the *K. sima* maximum and an overlapping Dorsal Fin Height: Total Body Length (DFH: TBL) value. This case has few available tissues due to its remote stranding location and the extracted tissues did not produce interpretable results. The other is below the *K. sima* maximum and has no available measurements for the dorsal fin. This case similarly has few available tissues due advanced decomposition and sequencing attempts were unsuccessful.

Increasing our understanding of causes of mortality through disease screening

Toxoplasma Disease Screening Results

Our findings from the screening of 35 individuals for the pathogen *Toxoplasma* indicated low detection rates among the selected animals. All 35 cases tested showed negative results among the tissues tested (Table 2). Some suspect positives were submitted for sequencing based on gel electrophoresis but the sequencing results did not suggest infection by *T. gondii*. The positive control was sequenced alongside all unknown samples to serve as technique confirmation.

Table 2. Results of tissue analyses conducted for the presence of *Toxoplasma gondii*

Species	Stranding Date	Body Condition Code	*Toxoplasma gondii* Presence	Tissues Tested
S. longirostris	3/10/2014	2	Negative	Brain, Left Lung, Liver, Spleen, Right Marginal LN
S. longirostris	6/11/2014	4	Negative	Brain, Left Atrium, Left Lung, Right Lung, Left Prescapular LN
Z. cavirostris	5/6/2015	2	Negative	Brain, Left Lung, Liver, Spleen, Lung LN
S. longirostris	6/4/2016	4	Negative	Brain, Left Lung, Liver, Muscle, Right Lung
G. macrorhynchus	10/13/2017	2	Negative	Brain, Left Lung, Liver, Pancreas, Spleen
G. macrorhynchus	10/13/2017	2	Negative	Brain, Left Lung, Liver, Spleen, Marginal LN
G. macrorhynchus	10/13/2017	2	Negative	Left Lung, Liver, Right Adrenal, Spleen, Marginal LN
G. macrorhynchus	10/13/2017	2	Negative	Brain, Left Lung, Liver, Spleen, Mediastinal LN
G. macrorhynchus	10/13/2017	2	Negative	Brain, Left Lung, Liver, Spleen, Marginal LN
S. longirostris	12/5/2017	1	Negative	Brain, Left Lung, Liver, Spleen, Left Marginal LN
S. longirostris	12/26/2017	2	Negative	Brain, Left Lung, Liver, Spleen, Right Marginal LN
L. hoseii	2/7/2018	2	Negative	Brain, Left Lung, Liver, Spleen, Mesenteric LN
S. longirostris	2/26/2018	2	Negative	Brain, Left Lung, Liver, Spleen, Right Marginal LN
S. coeruleoalba	8/28/2018	2	Negative	Brain, Left Lung, Liver, Left Kidney, Aortic Chain LN
Z. cavirostris	1/17/2019	1	Negative	Brain, Left Lung, Liver, Spleen, Mesenteric LN
P. electra	4/9/2019	2	Negative	Brain, Left Lung, Liver, Spleen, Left Marginal LN
S. longirostris	8/11/2019	2	Negative	Brain, Left Lung, Liver, Spleen, Mediastinal LN
S. longirostris	8/16/2019	2	Negative	Brain, Left Lung, Liver, Spleen, Mesenteric LN
S. coeruleoalba	8/21/2019	2	Negative	Brain, Left Lung, Liver, Spleen, Left Marginal LN
F. attenuata	8/29/2019	1	Negative	Brain, Lung, Liver, Spleen, Left Marginal LN
F. attenuata	8/29/2019	1	Negative	Brain, Lung, Liver, Spleen, Right Marginal LN
F. attenuata	8/29/2019	1	Negative	Brain, Lung, Spleen, Liver, Left Marginal LN
F. attenuata	8/29/2019	1	Negative	Brain, Lung, Liver, Spleen, Left Marginal LN
P. crassidens	12/6/2019	2	Negative	Brain, Left Kidney, Left Lung, Liver, Spleen
S. coeruleoalba	5/2/2020	2	Negative	Brain, Right Lung, Liver, Spleen, Right Marginal LN
S. coeruleoalba	6/19/2020	2	Negative	Brain, Left Lung, Liver, Spleen, Left Marginal LN
L. hoseii	2/8/2021	1	Negative	Brain, Left Lung, Liver, Spleen, Left Marginal LN
S. coeruleoalba	3/18/2021	2	Negative	Brain, Right Lung, Liver, Spleen, Left Marginal LN
S. coeruleoalba	3/24/2021	1	Negative	Brain, Lung, Liver, Spleen, Left Marginal LN
S. coeruleoalba	4/13/2021	3	Negative	Brain, Left Lung, Liver, Spleen, Left Marginal LN
S. longirostris	9/12/2021	3	Negative	Brain, Left Lung, Liver, Spleen, Left Marginal LN
S. longirostris	10/30/2021	3	Negative	Brain, Left Lung, Liver, Pancreas, Right Marginal LN
L. hoseii	12/6/2021	2	Negative	Brain, Left Lung, Liver, Spleen, Left Marginal LN
L. hoseii	12/7/2021	2	Negative	Brain, Left Lung, Liver, Spleen, Left Marginal LN
L. hoseii	12/7/2021	3	Negative	Brain, Left Lung, Liver, Spleen, Left Marginal LN

The tissue type that most commonly tested *Brucella* positive at this stage in the study appears to be the brain, with a 73% positivity rate among the positive individuals. The second most likely tissue type screened to test positive is lung tissue at 54%. Genetic sequencing has been conducted for four of the suspect positive cases to date (Table 1). Infection was detected in tissues dating back to 2000 in a stranded rough-toothed dolphin, indicating the presence of *Brucella* in stranded cetaceans from Hawaiian waters from a timespan that exceeds 20 years.

FUTURE WORK

Future work will focus on completing genetic sequencing efforts for the remaining two *Kogia* specimens where initial efforts were not successful. To date, PCR and sequencing attempts have only focused on available soft tissues, and bone extraction will be attempted to confirm species identification in these two cases. We have also received primers obtained from the scientific literature and are developing an in-house testing protocol with known humpback and sperm whales to test unknown stranding soft tissues that could represent either humpback or sperm whales in an advanced state of decomposition. We have another two cases that we suspect represent beaked whales and a pilot whale specimen that will also be genetically sequenced in the coming months.

Additional advanced diagnostic efforts will focus on the completion of disease screening efforts for *Brucella*. Extracted tissues have been prepared for PCR and will be screened to ensure that a minimum of 5 tissues are examined from the 21 individuals examined to date. We will also select an additional 14 individuals where infectious disease is suspected for extraction and PCR and we will consider species diversity as a component of the targeted individuals. We will also be comparing necropsy and histopathology findings for each individual with *Brucella* positive results. Our examination to date of necropsy and histopathology findings for confirmed and suspect *Brucella* positive individuals suggests a high prevalence of meningitis and pneumonia. Genetic sequencing has been conducted for four of the positive cases (Table 1). Future work will involve genetic sequencing efforts to confirm each of the suspect *Brucella* positive cases identified as part of this work.

Time of death analysis is also underway using archived tissues from cetaceans that were not fresh dead at the time of the first stranding observation. DNA concentrations have been compared among tissues to determine those most representative of the post mortem interval in a controlled DNA degradation experiment. Select tissue analysis will be completed in the coming weeks and calibrated according to the DNA degradation rate in order to estimate date of death for past individual strandings. An assessment of nutritional state at the time of stranding has involved measurement of blubber histology metrics from blubber collected at necropsy from past strandings. The quantitative data generated is currently being evaluated with qualitative observations of body condition at necropsy for individual stranding cases in order to categorize past stranding events as acute or chronic in cause.

ACKNOWLEDGEMENTS

Funding for this project was provided by Commander, United States Pacific Fleet. We would also like to acknowledge the Prescott Grant program for support of stranding response, necropsy and the collection of archived and current samples that support this project. This work was conducted under NOAA National Marine Fisheries Service Permit #18786. Special thanks goes to Jana Phipps and the volunteers at the Health and Stranding lab for sample collection and preservation, as well as to the numerous stranding volunteers throughout the Hawaiian Islands and Pacific island territories that assisted in the collection of samples during remote stranding events and necropsy.

Suggested citation:

West, K.L., Clifton, C., Hofmann, N., Humann, C. and I. Silva-Krott. (2022). *Hawaii and Mariana Islands Stranding Analyses* (Marine Species Monitoring Program Annual Report). United States Navy.

Literature Cited

Chernov, A. (2010). Identification of *Brucella ceti* in a Hawaiian Cetacean. M.S. Marine Science. Hawaii Pacific University. Master's Thesis.

Coombs, E.J., Deaville, R., Sabin, R.C., Allan, L., O'Connell, M., Berrow, S., Smith, B., Brownlow, A., Doeschate, M.T., Penrose, R., Williams, R., Perkins, M.W., Jepson, P.D. & Cooper, N. (2019). What can cetacean stranding records tell us? A study of UK and Irish cetacean diversity over the past 100 years. *Marine Mammal Science, 35*(4), 1527-1555.

Garrigue, C., Oremus, M., Dodémont, R., Bustamante, P., Kwiatek, O., Libeau, G., Lockyer, C., Vivier, J. C., & Dalebout, M. L. (2016). A mass stranding of seven Longman's beaked whales (*Indopacetus pacificus*) in New Caledonia, South Pacific. *Marine Mammal Science*. *32*(3), 884–910. https://doi.org/10.1111/mms.12304

Jacob, J.M., West, K.L., Levine. G., Sanchez, S., & Jensen, B.A. (2016). Initial characterization of the novel beaked whale morbillivirus in Hawaiian cetaceans. *Diseases of Aquatic Organisms. 117*, 215-227.

Landrau-Giovanetti, N. (2019) Detection and characterization of emerging pathogens in stranded cetaceans. University of Florida, PhD dissertation.

Landrau-Giovannetti, N., Subramaniam, K., Brown, M.A., Ng, T.F.F., Rotstein, D.S., West, K., Frasca, S., & Waltzek, T.B. (2020). Genomic characterization of a novel circovirus from a stranded Longman's beaked whale (*Indopacetus pacificus*). *Virus Research*. *277*, 197826. https://doi.org/10.1016/j.virusres.2019.197826

Maldini, D., Mazzuca, L., & Atkinson, S. (2009). Odontocete Stranding Patterns in the Main Hawaiian Islands (1937–2002): How Do They Compare with Live Animal Surveys?. *Pacific Science*. *59*, 55-67.

Mazzariol, S., Centelleghe, C., Di Provvido, A., Di Renzo, L., Cardeti, G., Cersini, A., Fichi, G., Petrella, A., Di Francesco, C. E., Mignone, W., Casalone, C., & Di Guardo, G. (2017). Dolphin Morbillivirus Associated with a Mass Stranding of Sperm Whales, Italy. *Emerging Infectious Diseases. 23*(1), 144–146.

Norman, S.A., C.E. Bowlby, M.S. Brancato, J. Calambokidis, D. Duffield, P.J. Gearin, T.A. Gornall, M.E. Gosho, B. Hanson, J. Hodder, S.J. Jeffries, B. Lagerquist, D.M. Lambourn, B. Mate, B. Norberg, R.W. Osborne, J.A. Rash, S. Riemer & J. Scordino. (2004). Cetacean strandings in Oregon and Washington between 1930 and 2002. *Journal of Cetacean Research and Management. 6*(1), 87–99.

Rotstein, D., West, K.L., Levine, G., Lockhart, S., Raverty, S., Morshed, M., & Rowles, T. (2010). Cryptococcus gattii VG1 in a spinner dolphin (*Stenella longirostris*) from Hawaii. *Journal of Zoo and Wildlife Medicine*. *41*, 181-183.

Silva, M. S. A., Uzêda, R. S., Costa, K. S., Santos, S. L., Macedo, A. C. C., Abe-Sandes, K., & Gondim, L. F. P. (2009). Detection of Hammondia heydorni and related coccidia (Neospora caninum and Toxoplasma gondii) in goats slaughtered in Bahia, Brazil. *Veterinary Parasitology*, *162*(1–2), 156–159.

Vargas-Castro, I., Crespo-Picazo, J.L., Rivera-Arroyo, B., Sánchez, R., Marco-Cabedo, V., Jiménez-Martínez, M.Á., Fayos, M., Serdio, Á., García-Párraga, D., & Sánchez-Vizcaíno, J.M. (2020). Alpha- and gammaherpesviruses in stranded striped dolphins (*Stenella coeruleoalba*) from Spain: first molecular detection of gammaherpesvirus infection in central nervous system of odontocetes. *BMC Veterinary Research*. *16*(1), 288–288. https://doi.org/10.1186/s12917-020-02511-3

West, K.L., Levine, G.A., Jacob, J., Jensen, B.A., Sanchez, S., Colegrove, K., & Rotstein. D. (2015). Co-infection and vertical transmission of *Brucella* and morbillivirus in a neonatal sperm whale. *Journal of Wildlife Diseases. 51*(1), 227-232.

West, K.L., Sanchez, S. Rotstein, D., Robertson, K.M., Dennison, S., Levine, G., Davis, N., Schofield, T.D., Potter, C.W., & Jensen, B.A. (2013). A Longman's beaked whale (*Indopacetus pacificus*) strands in Maui, Hawaii with first case of morbillivirus in the Central Pacific. *Marine Mammal Science. 29*(4), 767-776.

West, K.L., Silva-Krott, I.U., Landrau-Giovanetti, N., Levine, G., Rostein, D., Su, C., & Waltzek, T.B. (2020). Toxoplasmosis in Hawaiian spinner dolphins. Hawaii Conservation Conference, September 2020. Abstracts.

West, K.L., Silva-Krott, I.U., Landrau-Giovannetti, N., Rotstein, D., Saliki, J., Raverty, S., Nielson, O., Popov, V., Davis, N., Walker, W., & Waltzek. (2021). Novel morbillivirus in a rare Fraser's dolphin (*Lagenodelphis hosei*) stranding from Maui, Hawai'i. *Nature Scientific Reports. 11*, 15986.

Wang, Y.-T., Chou, L.-S., Lo, C., & Yang, W.-C. (2013). Rapid Species Identification of Morphologically Similar Cetacean Species Kogia sima and K. breviceps by High-resolution Melt Analysis. *Pakistan Journal of Zoology*, *45*(1), 273–277.

Wu, Q., McFee, W. E., Goldstein, T., Tiller, R. V., & Schwacke, L. (2014). Real-time PCR assays for detection of Brucella spp. and the identification of genotype ST27 in bottlenose dolphins (Tursiops truncatus). *Journal of Microbiological Methods*. *100*, 99-104. https://doi.org/10.1016/j.mimet.2014.03.001

Yang, W.C., Wu, B.J., Sierra, E., Fernandez, A., Groch, K.R., Catao-Dias, J.L., West, K.L., Chan, K.W. (2016). A simultaneous diagnosis and genotyping method for global surveillance of cetacean morbillivirus. *Nature Scientific Reports.* 6, 30625. doi:10.1038/srep30625.