Comprehensive Stranding Investigations for High Priority Species

West, K.L., Clifton, C., Jones, S., Hofmann, N. and I. Silva-Krott

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

ABSTRACT

This project provides financial support for comprehensive stranding investigations in order to obtain increased baseline information about the health of marine mammals. Such support is essential when considering the Pacific Islands region (PIR) where unique geographical challenges exist. The PIR is comprised of isolated islands, spanning over 4 million square miles across the North, South and Western Pacific basins and includes the Hawai'i Range Complex and the Mariana Islands Range Complex. Cetacean stranding investigative efforts for the PIR are centralized at a dedicated stranding facility located at Marine Corps Base Hawai'i (MCBH). This specialized facility houses the University of Hawai'i (UH) Health and Stranding Lab, which plays a critical role as the only organization in the region to conduct cause of death investigations when dolphins and whales strand. This requires established relationships with partners throughout the region and the mounting of an immediate response to each newly reported stranding event that occurs. The UH Health and Stranding Lab conducts extensive necropsy examinations, including histopathology, disease surveillance, and tissue sampling in support of numerous research efforts aimed at better understanding Hawaiian cetaceans. In addition to this project facilitating advanced diagnostics in-house at the Health and Stranding Lab, we also report on progress towards a project option to measure fecal stress and reproductive hormones in Pacific Island cetaceans. An additional project option includes characterizing marine debris ingestion by abundance and mass in previously stranded short-finned pilot whales. Progress associated with the project option in support of the marine debris analyses has been prepared for peer-reviewed publication in the journal Marine Pollution Bulletin. These findings are described separately from the base tasks and fecal hormones option in the attached manuscript draft.

SUMMARY OF STRANDING CASES DURING CALENDAR YEAR 2024

Stranding response, necropsy, and cause of death investigative summaries were provided for 12 stranding events in quarterly reports that occurred during the 2024 calendar year. In each of these cases, obtained specimen samples were given a unique specimen identification number that allows for long-term tracking of any analyses within the Health and Stranding Lab.

In addition to cetacean strandings or other rare events where cetacean specimen samples are obtained and tracked with a unique identifier that reflects the calendar year of the initial report, a number of additional cetacean strandings were confirmed and responses mounted that are not reported as stranding case summaries because of limitations that precluded the necropsy and/or sample collection. Basic stranding information such as location and species are entered into the Marine Mammal Health and Stranding Response Program's National Stranding Database. Verified or unverified stranding reports may not result in the death of an animal and/or the collection of a biological sample. In these types of stranding response scenarios, a case number

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE *(DD-MM-YYYY)*	2. REPORT TYPE	3. DATES COVERED *(From - To)*
Submitted	Monitoring report	2024

4. TITLE AND SUBTITLE
COMPREHENSIVE STRANDING INVESTIGATIONS FOR HIGH PRIORITY SPECIES

5a. CONTRACT NUMBER

5b. GRANT NUMBER

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
Kristi L. West
Cody Clifton
S. Jones
Nicolas Hofmann
Ilse Silva-Krott

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Health and Stranding Lab, Human Nutrition Food and Animal Sciences, College of Tropical Agriculture and Human Resources, University of Hawai'i at Mānoa, Honolulu, Hawai'i

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

This project provides financial support for comprehensive stranding investigations in order to obtain increased baseline information about the health of marine mammals. Such support is essential when considering the Pacific Islands region (PIR) where unique geographical challenges exist. The PIR is comprised of isolated islands, spanning over 4 million square miles across the North, South and Western Pacific basins and includes the Hawai'i Range Complex and the Mariana Islands Range Complex. Cetacean stranding investigative efforts for the PIR are centralized at a dedicated stranding facility located at Marine Corps Base Hawai'i (MCBH). This specialized facility houses the University of Hawai'i (UH) Health and Stranding Lab, which plays a critical role as the only organization in the region to conduct cause of death investigations when dolphins and whales strand. This requires established relationships with partners throughout the region and the mounting of an immediate response to each newly reported stranding event that occurs. The UH Health and Stranding Lab conducts extensive necropsy examinations, including histopathology, disease surveillance, and tissue sampling in support of numerous research efforts aimed at better understanding Hawaiian cetaceans. In addition to this project facilitating advanced diagnostics in-house at the Health and Stranding Lab, we also report on progress towards a project option to measure fecal stress and reproductive hormones in Pacific Island cetaceans. An additional project option includes characterizing marine debris ingestion by abundance and mass in previously stranded short-finned pilot whales. Progress associated with the project option in support of the marine debris analyses has been prepared for peer-reviewed publication in the journal Marine Pollution Bulletin. These findings are described separately from the base tasks and fecal hormones option in the attached manuscript draft.

15. SUBJECT TERMS
Marine mammals, strandings, necropsy, disease, beaked whales, cetaceans, Hawaii Range Complex, Mariana Islands Range Complex, Pacific Islands

16. SECURITY CLASSIFICATION OF:			**17. LIMITATION OF ABSTRACT**	**18. NUMBER OF PAGES**	**19a. NAME OF RESPONSIBLE PERSON**
			UU	14	Department of the Navy
a. REPORT	**b. ABSTRACT**	**c. THIS PAGE**			**19b. TELEPONE NUMBER (*Include area code)***
Unclassified	Unclassified	Unclassified			808-471-6391

is not assigned, and the event is not documented with a unique specimen identification number that is tracked in the Health and Stranding Lab records. When a stranding response effort for a confirmed cetacean stranding event is significant, these events are included in the quarterly reports even if a unique specimen identified is not assigned. An example of one of these types of stranding events occurred on April 24, 2024, when a striped dolphin live stranded at Heeia fishpond on the island of Oʻahu. Data was collected from the free-swimming but weak dolphin through the day by the UH Health and Stranding Lab including respiration rate and activity level. Monitoring of the individual was continued into the early evening when the striped dolphin was observed being predated upon by a shark by our team. No carcass or samples were recovered from this stranding response event.

The summary cases reported on in 2024 include cetacean strandings where full necropsies or sampling was conducted that are indicated by a unique specimen identification number used for the tracking of sample status. During the 2024 calendar year, the UH Health and Stranding Lab coordinated or conducted stranding responses, necropsy, and sample collection from five of the main Hawaiian Islands including Kauaʻi (3), Lānaʻi (1), Maui (3), Molokaʻi (1), and Oʻahu (1), as well as Midway Atoll (1) and Wake Island (1) (Figure 1). We also conducted multiple responses over several months in 2024 on the North Shore of Oʻahu to bury and move remains of a large sperm whale that initially stranded at the very end of 2023. These responses resulted in sample collections and specimen tracking at the UH Health and Stranding Lab from a confirmed total of 7 species including spinner dolphins (*Stenella longirostris*) (4), sperm whales (*Physeter macrocephalus*) (2), bottlenose dolphin (*Tursiops truncatus*) (1), goose-beaked whale (*Ziphius cavirostris*) (1), humpback whale (*Megaptera novaeangliae*) (1), melon-headed whale (*Peponocephala electra*) (1), and striped dolphin (*Stenella coeruleoalba*) (1) (Table 1). Samples of ambergris were collected on Kauaʻi in February 2024 and are not included in the table or figure. Due to poor carcass condition, animals identified as sperm whales were tested via PCR for genetic confirmation of species. Specimens represented both male and female sexes, as well as various reproductive stages including newborn calves, juveniles and sexually mature adults.

Table 1. Cetacean stranding cases by species in 2024 where samples were collected and tracked by the UH Health and Stranding Lab.

Common Name	Species	Sex	Age Class	Stranding Date	Necropsy Date	Stranding Location
melon-headed whale	*P. electra*	M	A	2/11/2024	2/13/2024	Maui
spinner dolphin	*S. longirostris*	F	C	2/12/2024	2/13/2024	Kaua'i
humpback whale	*M. novaeangliae*	M	C	2/27/2024	2/28/2024	Lana'i
bottlenose dolphin	*T. truncatus*	F	A	4/16/2024	4/16/2024	Midway Atoll
striped dolphin	*S. coeruleoalba*	M	SA	4/19/2024	4/20/2024	Maui
sperm whale	*P. macrocephalus*	U	U	6/14/2024	6/14/2024	Kaua'i
spinner dolphin	*S. longirostris*	F	A	6/20/2024	7/11/2024	Kaua'i
spinner dolphin	*S. longirostris*	U	U	7/9/2024	7/9/2024	O'ahu
goose-beaked whale	*Z. cavirostris*	M	A/SA	7/21/2024	7/21/2024	Wake Island
spinner dolphin	*S. longirostris*	M	C	8/30/2024	9/5/2024	Moloka'i
sperm whale	*P. macrocephalus*	U	U	11/9/2024	11/9/2024	Maui

Case highlights from quarterly reports submitted during the reporting period are included below. These include a fatal vessel strike in a humpback whale calf on the island of Lānaʻi in 2024, a

bottlenose dolphin that stranded on Midway Atoll, a case of fatal hydrocephalus caused by *Brucella* in a striped dolphin on the island of Maui and a Goose-beaked whale stranding at Wake Island in 2024.

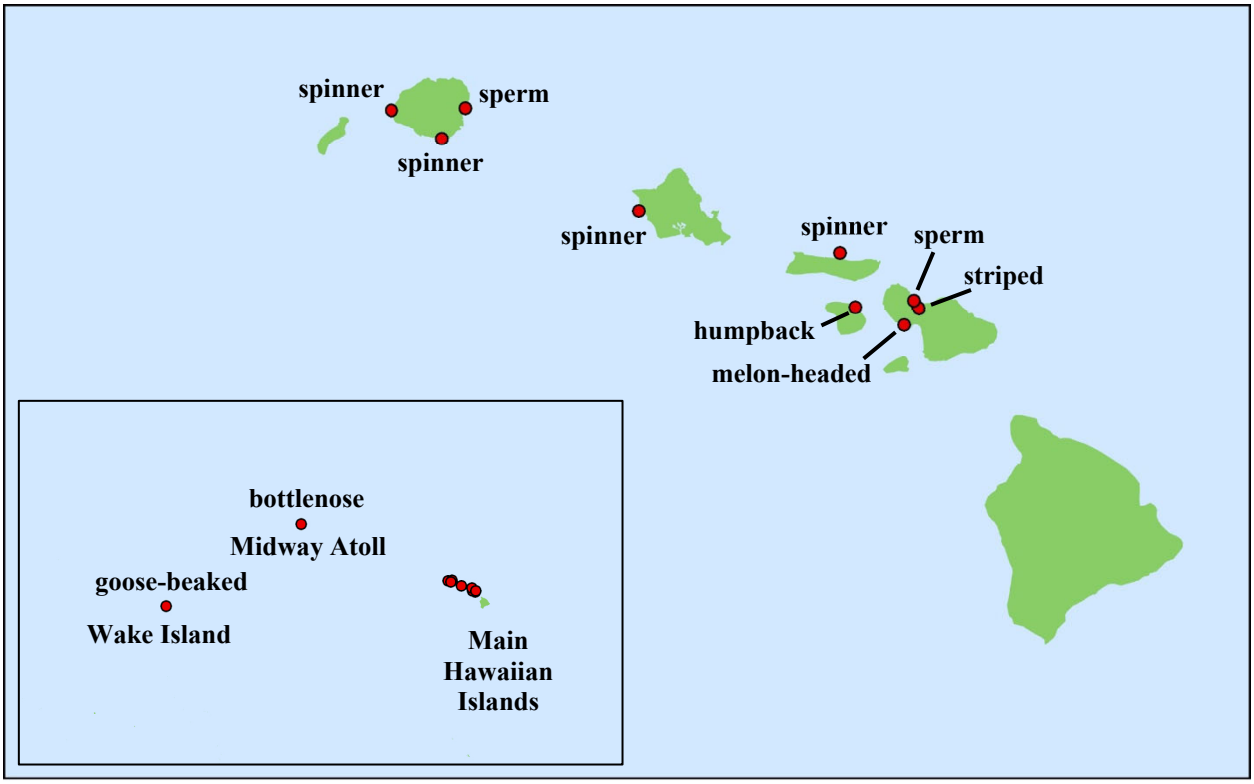

Figure 1. Cetacean stranding locations by species in 2024 across the main Hawaiian Islands, Midway Atoll and Wake Island.

Humpback whale, Megaptera novaeangliae, Lāna'i

On February 27th, 2024, a humpback calf was reported dead on Lāna'i to the Hawaii state Department of Land and Natural Resources. A team from the Health and Stranding Lab traveled to Lāna'i to conduct a necropsy on site as the large size precluded shipping of the carcass to O'ahu. Timely travel options to Lāna'i were limited and the necropsy team left O'ahu around 9:00 pm in order to stay the night on Maui before travelling to Lāna'i the next morning by ferry. Around 9:30 am on February 28th, 2024, the Health and Stranding Lab team arrived on site and began preparing for necropsy.

The external exam began at approximately 10:00 am on February 28th. The animal was identified as a male calf, measured 427 cm in total length and was estimated to be between 1100 and 1300 lbs. The tongue had distinctive frills extending approximately six inches from the tip of the tongue and the umbilicus was only partially healed. The external surface of the animal was intact and in relatively good condition. No abnormalities were noted upon the external examination.

At approximately 11:00 am, the first incision was made and the internal examination began. The internal examination indicated advanced decomposition, and not many organs could be identified.

There was left ventrolateral hemorrhage in the tongue noted that may have been related to positional lividity. The animal was confirmed to be an immature male; however, testes were not positively identified and only the penis was sampled. The stomach had fluid and sparse contents that were collected. The heart was sub-sampled, and tissues were collected in formalin for further evaluation. The skull had a complete comminuted fracture in both left and right supraoccipital bones, extending over an area that measured 18 cm by 10 cm. There were three skeletal shards, measuring 5 cm x 5 cm, 4 cm x 1 cm, and 5 cm x 2 cm. Fractures were observed on the cranial end of both the left and right supraoccipital bones. There were also fractures in the right temporal bone with one break measuring 7 cm. Many internal organs could not be identified and examined due to advanced decomposition, and limited sampling occurred as a result. Histopathology and disease screening results are currently pending but the severe blunt trauma to the skull is believed to be the cause of death in this individual, likely caused by the force of a vessel strike.

Figure 1. Humpback whale stranding on Lānaʻi as a result of skull fractures consistent with a vessel strike.

Bottlenose dolphin, Tursiops truncatus, Midway Atoll, Northwestern Hawaiian Islands

A dolphin was reported as dead stranded at Eastern Island, Midway Atoll on April 16th, 2024. Photographs of the dolphin were obtained by US Fish and Wildlife Service biologists who worked with the UH Health and Stranding Lab remotely to perform a field necropsy examination and collect a suite of samples. The dolphin measured 250 cm in total length and was determined to be a female bottlenose dolphin from the photographs obtained.

US Fish and Wildlife biologists prepared for necropsy the day the dolphin was reported and obtained necropsy instructions by phone and pictorial necropsy guides electronically from the Health and Stranding Lab prior to traveling by boat to Eastern Island to perform the necropsy. Cellular service or the benefit of Whats App or other platforms to facilitate necropsy in real-time was not possible, limiting gross necropsy findings. Photographs of the gross examination during the field necropsy were obtained as well as an extensive suite of samples. Samples included frozen and 10% formaldehyde/seawater fixed samples in some cases, skin, blubber, muscle, heart, right lung, liver, suspected adrenal gland, right kidney, stomach contents, pancreas and reproductive tract. The head was collected and retained frozen. Stomach contents included fish spines and partially digested fish, which were frozen for future species identification.

Figure 2. Bottlenose dolphin stranding at Midway Atoll.

Collected samples were transferred to the Health and Stranding Lab following necropsy for further examination and sub-sampling. The frozen head was examined in the laboratory and no obvious signs of prior fishery interactions were noted around the mouth. The sound channel (left lower jaw) had a pooling of blood and was red tinged. Jaw bones were intact, and teeth looked normal. The brain was autolytic when thawed for further examination and sub-sampling in the laboratory. The majority of the heart was collected at necropsy and frozen and examination of the left ventricle that formed the apex of the heart and valves were normal. No gross abnormalities were noted upon inspection of liver, pancreas, blubber and muscle. Reproductive organs were preserved intact. The stranded bottlenose dolphin was determined to represent a mature female, with evidence of multiple prior ovulations based on examination of the uterus and ovary.

Formalin fixed tissues were labeled as skin, blubber, right lung, liver, right adrenal, right kidney and pancreas. Histological examination of tissues revealed sections of skin and blubber, heart, brain and intestinal tract. The section of brain has a smattering of inflammatory cells around meningeal blood vessels exhibiting mild endothelial hypertrophy.

Disease screening efforts included polymerase chain reaction (PCR) testing of multiple tissue types for morbillivirus, *Toxoplasma* and *Brucella*; all tissues tested negative for the presence of these pathogens. Circovirus screening was also conducted and positive in the liver which was confirmed with sequencing. This expands our host species and geographic locations of beaked whale circovirus cases to bottlenose dolphins and Midway Atoll.

Tooth aging was also conducted for this individual bottlenose dolphin. Counting of growth layer groups estimated the age of animal at 17 years.

Striped dolphin, Stenella coeruleoalba, Maui

The sub-adult, male striped dolphin in moderate (normal) body condition was necropsied at the UH Health and Stranding Lab at Marine Corps Base Hawaii within 24-48 hours of dead stranding on April 19, 2024, on the island of Maui. During the external examination, approximately 12 individual whale lice were removed from the eyelids. The skin of the rostrum was peeled back and the rostral end of the right mandibular bone exposed. The rostral end of the maxilla had exposed connective tissue. A partially healed skin cut in the shape of the number one, estimated at 11 cm long x 0.3 cm wide (long arm) and about 4 cm by 0.3 cm (short arm) was observed on the side of the head behind the left eye. An S-shaped skin cut, about 15 cm in length on the left side was noted in the mid-body location and likely occurred post-mortem. A round scar, 5 cm in diameter was noted on the right side (healed cookie cutter scar). A deviation of the dorsal spine to the left, about 22.5º, that was located near or at the precaudal-caudal boundary was observed. A detailed examination of the vertebral column of this individual will be carried out once the post-cranial skeleton has been fully flensed and cleaned.

Significant gross pathological changes included lymphadenopathy throughout the body, evident in the prescapular, thoracic and abdominal lymph nodes. Enlarged lymph nodes were bulging and tan-brown to mottled dark red on cut surface.

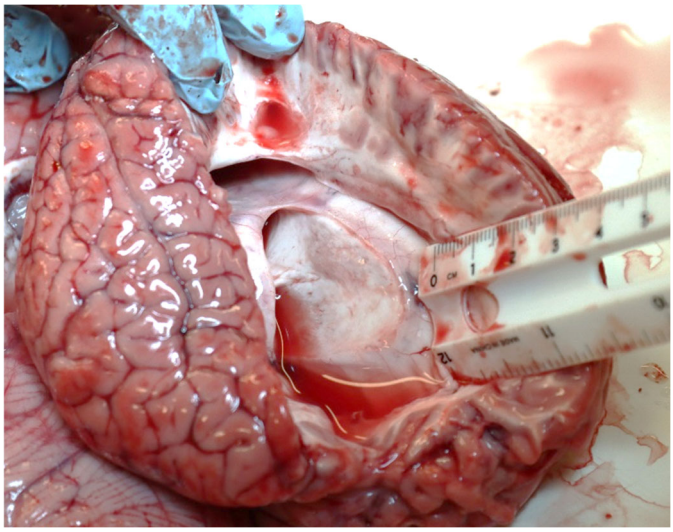

Figure 3. Hydrocephalus in a stranded striped dolphin. Fluid was observed in the ventricles of the brain.

Cerebrospinal fluid (CSF) collection from the atlanto-occipital joint yielded 12 ml of slightly cloudy, white/gray fluid. Similar fluid spilled upon the opening of the skull to remove the brain. In-situ inspection of the brain revealed a flattened appearance of the normally domed dorsal surfaces of the cerebral hemispheres and cerebellum. Lateral cerebral ventricles, the third and fourth ventricle and cerebellar ventricular extensions were moderately dilated. The white matter seemed more prominent and the grey matter was thin and irregular in the cortex of the hemispheres. Choroid plexi in ventricles were of normal size. The pituitary was normal in size.

The left and right lung had scant edema in bronchi. Lung parenchyma was spongy throughout and reddish/brown on cut surface. Pulmonary surfaces showed irregular depressed darker areas, with sizes ranging between 0.5 – 3 cm which is suggestive of atelectasis. The left lung had a smattering of dark punctate areas on the left caudal surface, and over the dorsum. A firm nodule, approximately 1-2 mm in diameter was near the caudal border and presumed as a parasitic granuloma. The lateral portion of the cranial lobe was undeveloped and this is consistent with past cases where this typically occurs in the left lung.

The thyroid was prominent and associated with multiple tan nodules, approximately 2-3mm in diameter, that were presumed parathyroid glands.

The heart was unremarkable upon gross examination. The liver was dark brown, uniform firm and the central sinuses appeared distended. The pancreas had multiple sharp delineated dark red hemorrhages next to pale brown glandular tissue. The forestomach contained scant sand and mucous, a few small, thin nematodes that were presumed *Anisakis*, and a row of small ulcers near the cardia. The glandular stomach was devoid of contents. The gastrointestinal tract contained greenish, foamy material. A section of large intestine, about 15 cm long and 15 cm anterior to the anus was markedly thickened and filled with nematodes, up to 10 cm in length. Worms were buried in the thickened mucosa and could not easily be removed, which is suggestive of Acantocephalus, genus *Bolbosoma*.

No abnormalities were noted during the gross examination of the spleen, adrenal gland and kidneys. The testes were immature based on measurements of the length, width and height. The urinary bladder was empty and the mucosa covered with scant light tan colored mucus.

Based on the gross examination, the hydrocephalus and cloudy characteristics of the CSF, combined with markedly enlarged lymph nodes suggested that the animal succumbed to an infectious disease, possibly meningoencephalitis.

Brucella screening by PCR of CSF and lymph nodes was positive. Histological examination of the brain revealed non-suppurative subacute meningoencephalitis. Further molecular analysis and sequencing of the *Brucella ceti* strain isolated from the CSF was conducted in collaboration with collaborator Dr. Michael Norris, faculty in the microbiology department at the University of Hawai‘i. Isolation of the pathogen was possible using Biosafety Level 3 facilities at the University of Hawai‘i at Manoa John H. Burns medical school. Sequencing data indicated a sub-type 26 of *Brucella ceti* in the striped dolphin that has not previously been identified from the central Pacific. This case represents the first documentation of a second strain of *Brucella ceti* in Hawaiian cetaceans, in addition to the sub-type 27 identified from a neonate sperm whale that stranded in 2011. We anticipate that this case will represent a significant milestone in our efforts to better characterize brucellosis in striped dolphins from this region of the world. An ancillary histological finding was a cluster of epithelial cells with granular basophilic cytoplasm in the thyroid consistent with a benign hyperplasia or an adenoma of the parathyroid.

Goose-beaked whale, Ziphius cavirostris, Wake Island

On the evening of July 20th, 2024, a dead stranded beaked whale was reported on Wake Island. Dr. Kristi West (UH Health and Stranding Lab Director) worked remotely with Stefan Kropidlowski, Deputy Superintendent of the Pacific Remote Islands National Monument, US Fish and Wildlife Service, who was on Wake Island at the time of the stranding as part of a rat eradication project. Several images of the dead beaked whale were sent using Whats App. The animal was moderately decomposed (Code 3), the carcass was intact and species was unknown from photographs. A plan was generated for a small team on Wake Island, led by Stefan Kropidloswki, to conduct a rudimentary field necropsy. Dr. West provided instructions by phone

and electronic pictoral guides prior to necropsy as there was no cellular or internet service at the site of the stranding. The animal was located at least a mile in hiking distance from any personnel at Wake Island.

Figure 4. Stranded Goose-beaked whale at Wake Island.

A basic field necropsy was conducted on site the following day and photographs were obtained as the carcass was opened and samples of recognizable organs were collected. Stomach contents were not observed but these may have been missed by the necropsy team. Kidneys were not observed but the advanced decomposition and inability to use remote video platforms during the real-time necropsy such as Whats App limited the ability to identify this organ and examine the kidneys for signs of the renal parasite, *Crassicauda* spp.

Samples were frozen and stored until shipment to O'ahu on July 26th, 2024. On July 27th, 2024, the samples were defrosted, further examined, sub-sampled and identified to tissue type when possible. Eight bags of tissue were sent, with five bags being labeled with the presumed organ identification and three bags were labeled as unknown. Gross examination of submitted tissues and organs confirmed skin, eye and heart. All chambers of the heart and heart valves were present and no abnormalities were observed upon gross examination. Histological slides of skin, blubber and skeletal muscle did not show any significant abnormalities. The section of heart had multiple gas bubbles disrupting the tissue and bacterial colonies in and around blood vessels consistent with advanced decomposition of the organ. A section of liver showed overall architecture of the organ but cellular details could not be discerned due to autolysis.

A sub-set of frozen samples of liver, muscle, pancreas and unknown organ tissue that was collected during the Wake Island beaked whale necropsy were sent to a specialized analytical National Wildlife Research Center laboratory in Fort Collins, Colorado in order to test for the presence of the rodenticide brodifacoum. This testing effort was conducted in collaboration with the US Fish and Wildlife Service and the United States Department of Agriculture in light of the rat eradication project at Wake Island that was temporally correlated with the beaked whale stranding event. Testing of liver, heart muscle, pancreas and unknown organ tissue from the necropsied beaked whale were all negative for the presence of brodifacoum based on an analytical detection limit of 1.5 ng/g. Each of the tissues was analyzed in triplicate using dSPE and LC-MS/MS methodology with positive and negative brodifacoum rat liver used as control samples in the analysis.

DNA was extracted from tissue samples for PCR testing in order to conduct species and sex identification. The species of the stranded whale was confirmed as the Goose-beaked whale,

Ziphius cavirostris, and the sex as male. Tooth presence was not confirmed by photographs of the mouth and we suspect that this individual represents an immature specimen. PCR testing for presence of *Brucella, Toxoplasma,* herpesvirus and circovirus DNA in the limited number of collected tissues for disease testing was negative. Morbillivirus screening of the collected tissues from the Wake Island beaked whale is pending.

FECAL HORMONES OPTION

Over the past year we have conducted experimental trials associated with the development of laboratory research protocols to reliably measure fecal hormone concentrations in Hawaiian cetaceans. This effort has focused on a number of important steps to be able to accurately measure fecal hormone concentrations across species and to provide the necessary foundation for meaningful interpretation of future measurements obtained from the feces of free-swimming cetaceans in Hawaiian waters and elsewhere.

When conducting endocrinology studies of cetaceans, it is necessary to conduct technical validations for each new species of interest and for each new sampling matrix. Our laboratory efforts to date have primarily focused on false killer whales, pygmy sperm whales and pilot whales. Specifically, we have focused on conducting technical validations, comparison of the performance of commercially available enzyme immunoassay kits with cetacean fecal samples, and the measurement of steroid hormones in individual animals to evaluate the biological relevance of obtained results.

Technical validation steps for each hormone of interest by species and sample matrix are required to test assay repeatability and reliability prior to the testing of hormone concentrations from unknown samples. Parallelism trials are one of the necessary technical validations required to assess fecal matrix effects by generating serial dilutions of each animal's extracted hormones. Spike recoveries are another important validation test for accuracy that is conducted to ensure that the extraction procedure is removing all hormone present in the sample, and is typically conducted for low, medium and high concentrations of any given hormone of interest.

We have completed parallelism and spike recovery technical validations for the reproductive hormone progesterone and stress hormone cortisol for pooled fecal samples from false killer whales and pygmy sperm whales. Fecal samples were first extracted and analyzed for progesterone concentrations using methods adapted from Lemos et al. 2020. Fecal samples were directly collected from the intestines of both animals, in the case of the stranded individuals that were the focus of our initial trials no filtration or centrifugation steps were necessary to remove saltwater contaminants. Approximately 10 g of total feces from a number of individuals were collected in glass scintillation vials and freeze dried prior to extraction. Dried samples were homogenized and three 100 mg replicates were weighed into glass vials for each animal. The next step involved the addition of 1.5 mL of 90% methanol that was added to each vial and then mixed on a shaker plate at 500 rpm for 30 minutes. Vials were then centrifuged at 2200 rpm for 20 minutes. The liquid layer was then pipetted off each pellet and into new glass scintillation vials, before being dried using a Speedvac vacuum concentrator. Samples were then resuspended in 1.5 mL of diluted assay buffer provided in each enzyme immunoassay (EIA) kit and frozen at -80C until analysis.

Progesterone parallelism testing was conducted with the Arbor Assays commercial progesterone EIA kit (progesterone kit # K025-H5; https://www.arborassays.com) by generating serial dilutions of each animal's extracted hormones. Seven dilutions were made of varying concentrations for progesterone: 1:2 – 1:32. Diluted samples demonstrated parallelism with the standard curve, with dilutions falling within the curve's range for each species, indicating that there is negligible impact by the matrix on measured response (Figure 5).

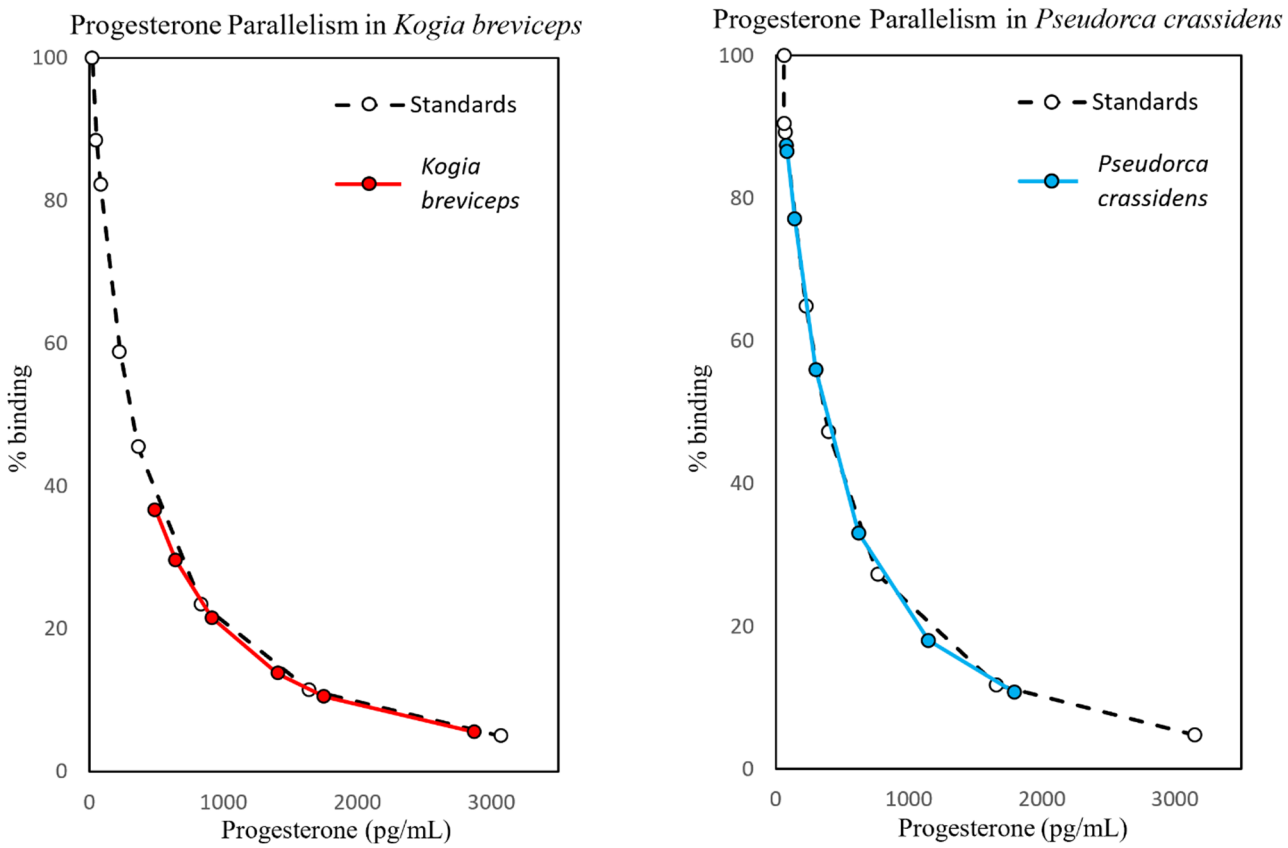

Figure 5. Progesterone parallelism serial dilution results for two species of cetacean.

Sample dilutions were viable at above 50% binding in false killer whales, which is the ideal dilution for accurate measurement of hormone concentrations. Pygmy sperm whale dilutions were only viable up to around 40% binding, potentially requiring re-run with dilution due to high concentration. Intra-assay CVs met quality control requirements, with values below 10%. Progesterone concentrations in the pygmy sperm whale pooled sample was generally higher in concentration than the false killer whale pooled samples, which is likely reflective of individual reproductive status that comprised the pool.

Spike recovery validation testing was similarly conducted from pooled fecal extracts from pygmy sperm whales and false killer whales. Spike recovery values within the 70 to 130% range are generally considered as acceptable technical validation tests (Hunt et al. 2022). Spike recovery data averaged within this acceptable range at 120% for the pygmy sperm whale validations (Table 1) and an average of 116% for the false killer whale validations (Table 2). The low spike recovery data for the pygmy sperm whale as an individual value was higher than generally accepted when

conducting technical validations. All of the false killer whale values (low, medium and high) were within the accepted range when considered as individual values.

Table 1. *Kogia breviceps* progesterone spike recovery

	Spike (ng)	% Recovery
High spike sample	480	100.83
Medium spike sample	240	121.75
Low spike sample	120	138.94
Average Recovery		120.51

Table 2. *Pseudorca crassidens* progesterone spike recovery

	Spike (ng)	% Recovery
High spike sample	480	101.08
Medium spike sample	240	123.20
Low spike sample	120	124.50
Average Recovery		116.26

Cortisol parallelism testing was conducted with the Arbor Assays commercial cortisol EIA kit (cortisol kit # K003-H5; https://www.arborassays.com) by generating serial dilutions of each animal's extracted hormones. Six dilutions were made of varying concentrations for cortisol: 1:2 – 1:64. Similar to progesterone, the diluted samples displayed parallelism with the standard curve, with dilutions falling within the curve's range for each species, indicating that there is negligible impact by the matrix on measured response (Figure 6). Sample dilutions were viable at above 50% binding in both animals, which is the ideal dilution for accurate measurement of hormone concentrations. Intra-assay CVs met quality control requirements, with values below 10%. Similar to progesterone, cortisol concentrations in the pygmy sperm whale pool was generally higher in concentration than false killer whale pooled samples.

Cortisol spike recovery validations carried out as part of this study are also considered acceptable for method validation (Hunt et al. 2022). Spike recovery data averaged within this acceptable range at 109% for the pygmy sperm whale validations (Table 3) and an average of 101% for the false killer whale validations (Table 4). The pygmy killer whales had to be re-run at 1:10 dilution for their spike recovery due to values reading initially too high, potentially due to reproductive status of sampled animals, unlike the false killer whale where spike recovery was successful with neat

samples. After adjustment, all values (low, medium and high) for both pygmy killer whales and false killer whales were within the accepted range when considered as individual values.

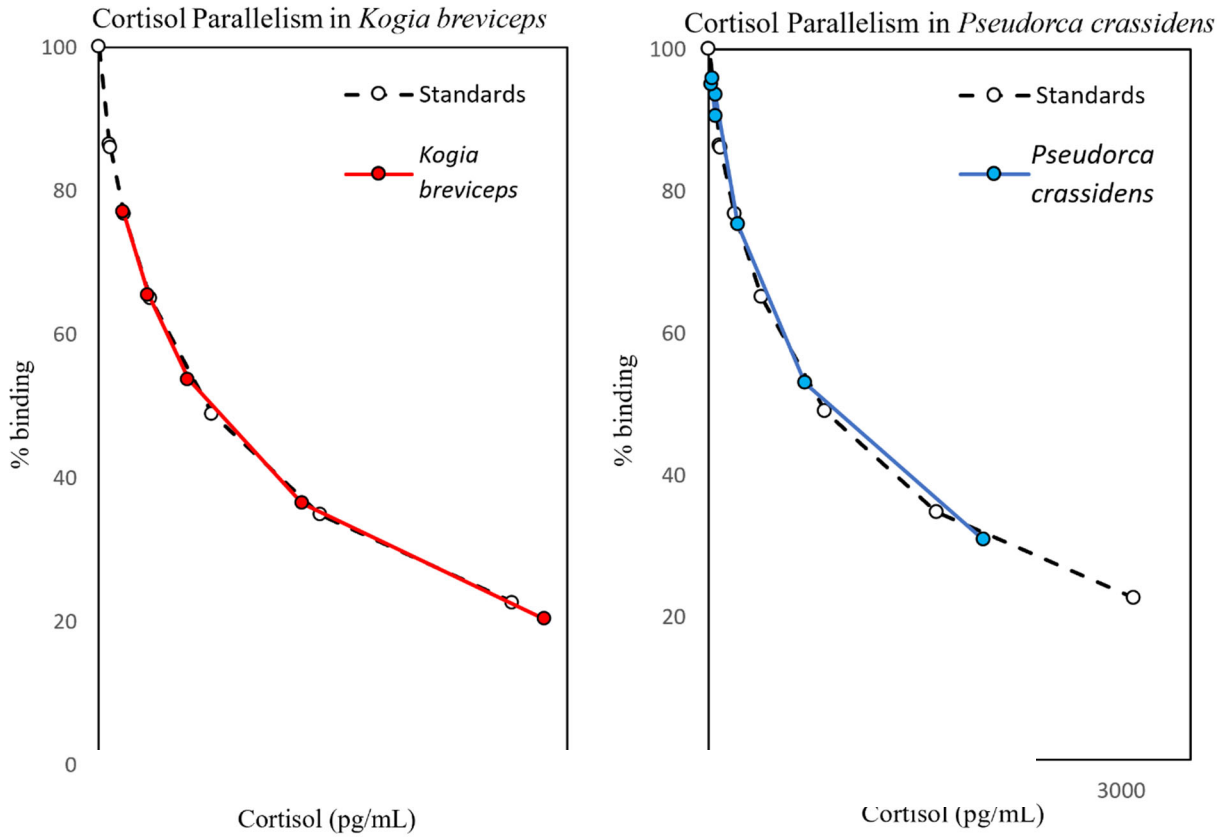

Figure 6. Cortisol parallelism serial dilution results for two species of cetacean.

Table 3. *Kogia breviceps* cortisol spike recovery

	Spike (ng)	% Recovery
High spike sample	480	88.14
Medium spike sample	240	127.51
Low spike sample	120	111.13
	Average Recovery	108.93

Table 4. *Pseudorca crassidens* cortisol spike recovery

	Spike (ng)	% Recovery
High spike sample	480	103.32
Medium spike sample	240	112.82
Low spike sample	120	88.28
	Average Recovery	101.47

After completing successful technical validations for progesterone and cortisol in pooled fecal samples from pygmy sperm whales and false killer whales, we proceeded with fecal analyses of these hormones from a limited number of individual animals. We specifically focused on measurement of individual fecal hormones from three female and one male pygmy sperm whale to test for detectability across individuals and to conduct a preliminary assessment of biological relevance. Cortisol and progesterone concentrations were detected successfully in the individual fecal samples from each of the four pygmy sperm whales tested (Table 5). Specifically, we anticipated that fecal progesterone could be used to address the question of biological relevance of obtained values by comparing concentrations from the one pregnant female in our data set to the other individuals. Progesterone was significantly higher in the fecal progesterone of the pregnant female, with the exact concentration higher than the assay standard curve, and known to exceed a minimum measured value of 20,030 ng/g. All other samples from the individual male and female pygmy sperm whales did not surpass a fecal progesterone concentration of 2,855 ng/g. This strongly suggests that fecal progesterone concentrations in pygmy sperm whales can be used to detect pregnancy and supports high biological relevance in the in-house fecal assays validated as part of this project for pygmy sperm whales and false killer whales. The results of our initial progesterone trials indicate that the methods described above are anticipated to be successful in the assessment of fecal samples from multiple cetacean species.

Table 5. Progesterone and cortisol concentrations in stranded *Kogia breviceps*

Animal ID	Sex	Status	Stranding Date	Progesterone (ng/g)	Cortisol (ng/g)
KW2014002	Female	Adult	2/5/2014	1494.1	143.7
KW2015011	Male	Adult	7/31/2015	2855.1	156.3
KW2021005	Female	Subadult	3/11/2021	157.4	40.1
KW2022009	Female	Pregnant adult	6/4/2022	> 20029.8	119.9

ACKNOWLEDGEMENTS

Funding for this project was provided by Commander, United States Pacific Fleet, Environmental Readiness Division. 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 allow for advanced diagnostics. This work was conducted under NOAA National Marine Fisheries Service Permit #24359. Special thanks goes to the volunteers at the Health and Stranding Lab for necropsy assistance, 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., Jones, S., Hofmann, N. and I. Silva-Krott, I. (2024). *Comprehensive stranding investigations for high priority species* (Marine Species Monitoring Program Annual Report). United States Navy.

LITERATURE CITED

Hunt, K.E., Buck, C.L., Ferguson, S.H., Ajo, A.F., Heide-Jorgensen, M.P., Matthews, C.J.D.. (2022). *Male bowhead whale reproductive histories inferred from baleen testosterone and stable isotopes.* Integrative Organismal Biology, Volume 4, Issue 1. 10.1092/iob/obac014

Lemos, L.S., Olsen, A., Smith, A., Chandler, T., Larson, S., Hunt, K., L.G. Torres. (2020). *Assessment of fecal steroid and thyroid hormone metabolites in eastern North Pacific gray whales*. Conservation Physiology, Volume 8. 10.1093/conphys/coaa110