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Full Length Research Paper

Neurotoxin Prevalence from Stranded Hawaiian Cetaceans

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Abstract

This study assessed the presence of naturally - produced neurotoxins in the muscle, liver, brain, and testes tissues from cetacean strandings. We examined 89 samples from 34 individuals of 13 species of cetaceans; tissues consisted of samples of 29 livers, 26 brains, 26 muscles and 8 testes. *In toto*, 14 samples (16%) tested positive for sodium channel aberration using the N2a bioassay that is commonly used to assess neurotoxin presence. Samples from 6 different species had at least one tissue type that tested positive; liver samples were most frequently positive for neurotoxin. Unexpected positives obtained from cetacean species feeding primarily on deep water squid (sperm whales) or filter feeding in Arctic waters (humpback whales) led to an investigation of the effect of tissue degradation on neurotoxin results. False positives were observed in tissues when extensive tissue degradation had occurred, but false positives were not observed in the fresh tissues characteristic of most of these samples. Neurotoxin activity was detected in fresh tissues from a number of different cetacean species that exhibit very diverse feeding behavior. The presence of neurotoxin activity in Hawaiian cetaceans warrants further investigation.

Keywords: Stranding, cetacean, neurotoxins, ciguatera, marine mammals.

INTRODUCTION

Almost 20 different species of cetaceans inhabit Hawaiian waters but with the exception of the Humpback whale (*Megaptera novaeangliae*) that migrates annually between Alaska and Hawaii and the Hawaiian spinner dolphin (*Stenella longirostris longirostris*) found close to shore on leeward coasts, little is known about many of these species. Fresh dead stranding events provide an opportunity to examine potential threats that may contribute to cause of death in otherwise difficult to study species. For example, tissue samples from stranded cetaceans in Hawaiian waters have recently led to the identification of a number of infectious diseases that can result in mortality for the first time in cetaceans from this region of the world, including morbillivirus, *Brucella* and *Cryptococcus* (Rotstein et al., 2010; West et al., 2013;

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West et al., In Revision). A recent survey of 16 different species of stranded Hawaiian cetaceans measured levels of persistent organic pollutants, biomarker exposure (Bachman et al. 2014; Bachman et al. In Press) and trace elements (Hanson et al., Submitted) to better understand the potential health effects of pollutants on Hawaiian cetaceans. There are a number of threats to cetaceans inhabiting Hawaiian waters that remain poorly understood, including the potential impact of neurotoxins on cetaceans in this region of the world.

It has frequently been speculated that natural marine toxins such as ciguatera might play a role in the deaths of marine mammals found in strandings. Ciguatoxin, for example, is seen in a wide range of aquatic organisms including fish, invertebrates, and marine mammals (Lehane and Lewis, 2000; Bienfang et al., 2008; Bottein et al., 2011; Pawlowiez et al., 2013) in tropical and subtropical environments including the Hawaiian Archipelago. Evidence that such naturally occurring marine toxins are subject to biomagnification up the food chain has raised concerns for mammalian species whose foraging behavior may include feeding on near-shore coral reef species. Though not confirmed analytically at the time, ciguatera was suspected in the death of several monk seals in the 1970's (Gilmartin et al., 1980); more recently, ciguatera was identified in the Hawaiian monk seal (Bottein et al., 2011).

Ongoing programs at two research institutions collaborated to assess the possible role of neurotoxins in the mortality of cetaceans in Hawaiian waters. The Marine Mammal Stranding Program at Hawaii Pacific University responds to any stranding of approximately 20 different cetacean species from diverse ecological niches and conducts cause of death investigations when possible. This program also maintains an archive of frozen and formalin-fixed cetacean tissues (e.g., muscle, liver, testes, brain) from stranded animals. A program to assess the frequency and spatial/temporal distribution of ciguatoxin in carnivorous reef fish (www.fish4science.com) throughout the Main Hawaiian Islands (Bienfang et al., 2012), implemented by the University of Hawaii-Manoa, supported the maintenance of bioassay capabilities for the assessment of sodium channel neurotoxins such as ciguatoxin in marine fauna. Project objectives were to (1) apply the capabilities for assessing sodium channel neurotoxins to analysis of tissues that were available from cetacean strandings in Hawaiian waters to determine if neurotoxin presence was detected and with what frequency and (2) to assess the possibility for bioassay artifacts due to decomposition of these tissues recovered from marine mammal strandings. This represents the first study to examine neurotoxin presence in cetacean tissues from the central North Pacific.

METHODS

Sampling Protocol

Tissue samples examined in this study were obtained from cetacean strandings in Hawaiian coastal waters between 1997 and 2010. Hawaii Pacific University stranding activities operate under a Stranding Agreement from National Marine Fisheries Service to respond to strandings, conduct necropsy examinations, investigate cause of death and archive tissue samples. Upon notification of stranding events, the HPU stranding program initiated an immediate response where necropsy examinations were typically conducted within 24 hours of notification and tissue samples stored at -80□C. Carcass freshness was described according to codes established by Geraci and Lounsbury (2005) wherein stranded animals are graded as alive (Code 1), fresh carcasses in good condition (Code 2), carcasses with organs intact and evidencing decomposition (Code 3), and carcasses showing advanced decomposition (Code 4). Samples addressed in this study were thus coded according to their decomposition status of tissues <2 days as "Fresh" (i.e., Code 1 & 2), 2-10 days as "Moderate" (i.e., Code 3) and >10 days as "Advanced" (i.e., Code 4).

This study investigated 31 stranded individuals which represented 13 different species having diverse feeding strategies. A total of 89 samples for N2a bioassay included frozen aliquots of liver, brain, testes, and muscle collections.

Neurotoxin Analyses

The N2A bioassay for assessing changes in sodium channel activity generally followed the protocols given by Manger et al. (1993), Manger et al. (1995a), and Dickey et al. (1999), and subsequently evaluated analytically and validated by Bottein Decharoui et al. (2005); Canete and Diogene (2009); Chan et al. (2011); O'Toole et al. (2012); and Pawlowiez et al. (2012). This bioassay relies on the addition of ouabain and veratridine, which potentiate a depolarization of cellular membranes. This effect is used to cause a sodium channel disruption as would be caused by the presence of toxins such as ciguatoxin in tissue extracts. These cetacean samples were pre-processed for application to the N2a bioassay using the CREM (ciguatoxin rapid extraction method) protocol developed by Lewis et al. (2009) to eliminate potential interference from high fat contents. Within the CREM process, samples are homogenized in methanol/hexane (3:1), centrifuged, the methanol phase filtered and subjected to reverse phase solid phase extraction cleanup using 900mg C18 SPE cartridges (Alltech Prevail Maxi-Clean). These samples were admixed with 1 M

NaCl and chloroform prior to a normal phase cleanup on a Silica SPE cartridge (Silica Plus, Waters), evaporated and subsequently reconstituted in methanol prior to testing with the neuroblastoma bioassay.

The bioassay involves preparation of a cell solution of ~ 200,000 neuroblastoma cells mL-1 using the CCL-131 cell line (ATCC, Manassas, VA) in RPMI-1640 cell medium supplemented with 5% fetal bovine serum, 2 mM glutamine, 1 mM sodium pyruvate, 50 µg/mL streptomycin, and 50 units/mL penicillin. Cells are plated with 100 µL per well at a density of ~ 20,000 cells per well in the inner 60 wells of 96-well plates. One hundred microliters of phosphate-buffered saline (PBS) was added to the 36 perimeter wells to maintain humidity. The 96-well plates were then placed in an incubator at 37˚C with 5% CO2 enriched and humidified air and allowed to acclimate overnight. The following day, 500 µL of methanol was added to the vials containing the tissue extracts, and the vials were placed in an ultrasonic bath for 3-5 minutes. Plates were admixed with a 4X range of extract dosages, using ten wells per concentration. Five wells of each concentration also contain 0.3 mM ouabain and 5 µM veratridine, referred to hereafter as O/V. Ten additional wells containing cells-only controls (i.e., no sample and no O/V) were used to represent uninhibited cell growth. Another ten wells, containing cells plus O/V, were used to represent the baseline decrease in cell viability due to the addition of these two chemicals. Additionally, one plate was dosed with 310 pM per well of P-CTX-1 and neuroblastoma cells (R. J. Lewis, University of Queensland) as a positive control. Well volumes were brought to 200 µL using RPMI medium, and plates placed in the incubator overnight. The next day 20 µL of CellTiter 96 Aqueous One Solution (Promega, Inc., Madison, WI) was added to each well. This solution contains a tetrazolium compound that is bioreduced by metabolically active cells to produce a colorimetric response. Plates were incubated for one hour to allow for color development (used to measure cell viability) and then read on a Multiskan MCC/340 Eliza plate reader at 492 nM. Absorption values were analyzed to identify significant differences between the means of controls and samples. Evaluation for possible cytotoxicity of the extracts to neuroblastoma cells is done as described above using wells containing only neuroblastoma cells and fish extracts (i.e., no O/V); only when cytotoxicity is not evident do the interpretive analyses proceed to assessment for aberrant sodium channel activity. Extracts are deemed to be non-cytotoxic when the means between wells with sample extracts and the control wells are not different ($p>0.05$). Non-cytotoxic samples are analyzed for sodium channel disruption by comparison of readings from wells containing cells plus extract, plus O/V versus the control wells plus O/V to determine if significant decreases in cell metabolic activity are evident.

Decomposition trials

To assess the likelihood that development of septicemia might cause artifacts in the neurotoxin analyses, tissue samples of a variety of marine organisms were subjected to a decomposition trial. Tissues included muscle and liver samples from the spotted dolphin (Stenella attenuata) and killer whale (Orcinus orca). All tissue samples were fresh at the beginning of the experiment and confirmed to be negative for neurotoxin presence. Dolphin and whale samples were obtained by necropsy of recently (<24hr) deceased animals. Samples (~60 gram) were placed in a container covered with a fine mesh lid and placed under a canopy to protect from heavy rain; samples were misted lightly with water every other day. Samples were allowed to decompose outside (T=23-27 °C) over a period of 28 days. On Days 0, 1, 3, 7, 14, 21, and 28, triplicate 2 gram subsamples were taken from each of the tissue types, and subjected to CREM solid phase extraction in preparation for neurotoxin analysis as described above.

RESULTS

The study examined 89 samples from 34 individuals that represent 13 species of cetaceans. These tissue samples consisted of 29 liver samples, 26 brain samples, 26 muscle samples and 8 testes samples (Table 1). Of this total, 14 samples (16%) tested positive for sodium channel neurotoxin behavior, and samples from 6 different species had at least one tissue type that tested positive. *In toto*, positive neurotoxin activity was found in samples of 7 livers, 4 muscles, and 3 brains; neurotoxin activity was detected in none of the 8 samples of testes.

Evidence of positive neurotoxin activity in tissues of *Megaptera novaeangliae* and *Physeter macrocephalus*, raised interest in the possibility for analytical artifacts from septicemia due to tissue degradation. This concern arose because of greater decomposition for these specimens, and the fact that neither *M. novaeangliae* nor *P. macrocephalus* derive a substantial portion of their nutrition directly from the coral reef environment that is habitat for the causative algae (i.e., *Gambierdiscus spp*.) of neurotoxins such as ciguatoxin.

To investigate the potential for false positive signals from decomposed cetacean tissue, muscle samples from a whale (*Orcinus orca*) and a dolphin (*Stenella attenuata*) that initially tested negative for neurotoxins were subjected to repeated analyses over 28day time period (Table 2). Bioassay results showed no evidence of a change to a positive signal over a 3 day period, which is the time frame where collection occurred for most of the cetacean samples examined in this study. One of the liver samples tested positive after 7 days, and both a muscle and a liver sample tested positive after 21 days.

Table 1. Results of neurotoxin analyses of various tissues from 89 samples collected from cetaceans that stranded between 1997 and 2010 from Hawaiian coastal waters. N2a bioassay results are presented as number of positive neurotoxin samples / total number of samples analyzed for each species and sample type.

Species	Common name	Liver	Muscle	Brain	Testes
Stenella coeruleoalba	Striped dolphin	2/7	0/7	2/7	0/1
Tursiops truncatus	Bottlenose dolphin	1/1	0/0	0/1	0/0
Megaptera novaeangliae	Humpback whale	2/3	2/3	0/2	0/0
Steno bredanensis	Rough toothed dolphin	1/1	0/0	0/1	0/0
Stenella longirostris	Spinner dolphin	1/6	1/6	1/4	0/1
Physeter macrocephalus	Sperm whale	0/0	1/1	0/0	0/0
Kogia breviceps	Pygmy sperm whale	0/2	0/1	0/1	0/1
Feresa attenuata	Pygmy Killer whale	0/3	0/2	0/3	0/2
Kogia sima	Dwarf sperm whale	0/2	0/1	0/2	0/1
Orcinus orca	Killer whale	0/1	0/1	0/1	0/1
Indopacetus pacifica	Beaked whale	0/2	0/2	0/2	0/1
Stenella attenuata	Spotted dolphin	0/1	0/2	0/2	0/0

Table 2. Degradation experiment shows number of positive neurotoxin samples recorded after each of seven time intervals of exposure. Three replicate N2a bioassay analyses were conducted at each time interval.

	Fresh			Moderate	Advanced		
	Day 0	**Day 1**	**Day 3**	**Day 7**	**Day 14**	**Day 21**	**Day 28**
Spotted dolphin Muscle	0	0	0	0	0	0	0
Spotted dolphin Liver	0	0	0	1	0	1	1
Killer whale Muscle	0	0	0	0	0	3	0
Killer whale Liver	0	0	0	0	0	0	0

Table 3. Decomposition Quality Status of various sample types (e.g., liver, muscle, brain) that showed positive (+) neurotoxin activity. All samples collected between 1997 and 2010 from Hawaiian coastal waters. See Methods section for details on Quality nomenclature. ns = no sample taken of that tissue type.

Species	Common name	Liver	Muscle	Brain	Quality
Stenella coeruleoalba	**Striped dolphin**	+	-	+	Fresh
Stenella coeruleoalba	**Striped dolphin**	+	-	+	Fresh
Stenella longirostris	**Spinner dolphin**	-	+	ns	Fresh
Stenella longirostris	**Spinner dolphin**	+	ns	+	Fresh
Tursiops truncatus	**Bottlenose dolphin**	+	ns	-	Fresh
Steno bredanensis	**Rough toothed dolphin**	+	ns	-	Fresh
Megaptera novaeangliae	**Humpback whale**	+	+	ns	Fresh
Megaptera novaeangliae	**Humpback whale**	+	+	-	Moderate
Physeter macrocephalus	**Sperm whale**	ns	+	ns	Advanced

Of the 14 cetacean tissues that showed positive neurotoxicity, 11 were graded as “fresh” in quality, i.e., sampled from strandings within a <2day time frame (Table 3); positive muscle and liver samples from one of the humpback whales ranked “moderate” in quality, and a positive muscle sample from the sperm whale came from a carcass quality condition that ranked as “advanced” deterioration (Geraci and Lounsbury, 2005).

DISCUSSION

The current study represents the first investigation into neurotoxin presence in stranded cetaceans from the central Pacific. Neurotoxins such as ciguatoxin are among the most potent natural toxins known; the ingestion of sub-picogram (i.e., 10^{-13}g) concentrations of toxin in seafoods have been known to cause clinical symptomologies in humans (Dickey, 2008; Lehane and Lewis, 2000). Ciguatoxin may also represent a potential threat to cetaceans who utilize near shore, subtropical waters for foraging. The cause behind cetacean stranding is often unknown, and the ability to detect the presence of neurotoxins in cetacean tissue samples is the first step to understanding if naturally occurring marine toxins may play a role in cetacean mortality in this region of the Pacific.

There are several marine neurotoxins (e.g., ciguatoxin, brevetoxin, saxitoxin) that produce symptomologies via perturbations of the voltage-gated sodium channels (Catterall, 1985; Manger et al., 1994) and thus would signal positive via the N2a bioassay applied here. Of these, ciguatera poisoning is the most prominent, and the form with a history in the region. Only clupeotoxism (Halstead, 1967; Randall, 2005), whose vector is small pelagic fish (e.g., sardines, herrings, anchovies), occupy habitat/biotope in the region that might intersect with cetacean feeding, but it is not known whether clupeotoxin would test positive under the N2a bioassay. Ciguatera occurs pantropically throughout the world within the 35 °N – 35 °S latitudinal band (Lehane and Lewis, 2000; Bienfang et al., 2008; Dickey, 2008; Dickey and Plakas, 2010). Ciguatera has a long history within the Hawaiian Archipelago (Banner et al., 1960; Banner and Helfrich, 1964; Anderson et al., 1982; Hokama and Yoshikawa-Ebesu, 2001; Bienfang et al., 2008). Ciguatera poisoning is caused by the ingestion of coastal biota that have accumulated naturally-occurring ciguatoxins that are produced by dinoflagellates of the genus *Gambierdiscus spp*. Ciguatoxins are a powerful suite of lipid-soluble polyether compounds (Yasumoto et al., 1977; Holmes, 1998; Chinain et al., 2010; Roeder et al., 2010) that enter the coral reef food web when herbivorous fish graze on macroalgae in the littoral zone, and inadvertently ingest the *Gambierdiscus spp*. and the ciguatoxins contained within. The grazing of these dinoflagellates by herbivorous fish and subsequently by carnivores begins processes of biomagnification up the food web (Bienfang et al., 2013).

As might be expected, positive samples for neurotoxins in our study occurred most often in liver tissues. This is consistent with the biochemical behavior of lipid-soluble ciguatoxin that vertebrate physiology tends to remove from the blood stream and preferentially sequester in the lipid-rich tissues such as in the livers of fishes (Vernoux et al., 1985; Swift and Swift, 1993; O'Toole et al., 2012; Bienfang et al., 2014). Such piscine metabolites as ciguatoxin are ingested/assimilated by high-level carnivores via grazing on near-shore fish communities. The residence time of ciguatoxin within an animal is driven by the relative rates of toxin ingestion versus the rates for toxin excretion, and are thus highly variable. Longer toxin residence times within the lipid-rich liver tissues are thought to account for both higher frequency of neurotoxin and higher concentrations relative to muscle tissues. This would also mean that ingestions of near-shore macrobiota would elicit positive signals from livers for protracted periods relative to blood or muscle tissues.

Our results indicated aberrant sodium channel activity in 16% of the samples tested which represented 6 of 13 species that were tested. The occasional appearance of positive neurotoxin results for species (e.g., *M. novaeangliae*, *P. macrocephalus)* without a clear connection to near-shore macrobiota brought attention to the possibility for analytical artifacts from septicemia due to tissue degradation. In most cases the egregious positive samples came from muscle and livers of individuals that were subsequently found to be graded as moderately or advanced decomposed (Table 3). In the decomposition experiment, none of the samples of muscle or liver tissues of *S. attenuata* or *O orca* changed from negative to positive neurotoxin signals over the first three days of the experiment, i.e., the time period reflecting most samples (Table 2). It was only after extended (7-28d) periods in the decomposition trial that samples produced positive signals. The degradation experiment results confirm that false positives using the N2a bioassay can be elicited with increasing decomposition. Additionally, these findings suggest that neurotoxin positives in cetacean tissues obtained by the N2a bioassay should be interpreted with caution and that further work is warranted to understand the mechanism behind the occurrence of false positives.

The decomposition trials demonstrated a clear relationship between extensive decomposition and the occurrence of false positives in this study. However, the majority of the cetacean tissues screened for neurotoxin presence using the N2a bioassay were collected from fresh carcasses within a 48 hour time period post mortem. No tissues in the degradation experiment elicited false positives during the first 7 days of the trial, suggesting that this factor was not responsible for the positive findings in the fresh cetacean tissue samples. Several of the cetacean species where fresh tissue samples indicated a positive result utilize nearshore coastal habitats and it is reasonable that feeding behaviors include grazing upon near-shore fish biomass. For example, the Hawaiian spinner dolphin rests and sleeps in shallow bays around the Hawaiian islands in the morning hours and heads off shore for nighttime foraging on the deep scattering layer to eat small mesopelagic squid and fish (Dolar et al. 2003). Prey identification from the stomach contents of a Hawaiian spinner dolphin

indicated that 61 of 64 beaks present (Clarke and Young, 1998) were identified as the mesopelagic squid, *Abralia trigonura* that migrates to the upper water column to feed at night (Young, 1978). The diet of Hawaiian striped, bottlenose and rough-toothed dolphins, other smaller cetacean species that also tested positive using the N2a bioassay, in Hawaiian waters has not been described but these species have been sighted during vessel surveys within a few miles of shore around the islands (Baird et al. 2003). Tissues from a fresh humpback whale tested positive using the N2a assay but the North Pacific humpback population feeds only in Alaska during the summer months and fasts while breeding and calving in Hawaiian waters during the winter. This finding, combined with the results of our decomposition trials, strongly suggest the need for further work, specifically use of LC-MS/MS analytical techniques to confirm that the ciguatoxin molecular structure is in fact consistent with the aberrant sodium channel activity measured in the fresh tissues of Hawaiian cetaceans.

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