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Eosinophilia and biotoxin exposure in bottlenose dolphins (*Tursiops truncatus*) from a coastal area impacted by repeated mortality events ☆

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ABSTRACT

Bottlenose dolphins (*Tursiops truncatus*) inhabiting coastal waters in the northern Gulf of Mexico have been impacted by recurrent unusual mortality events over the past few decades. Several of these mortality events along the Florida panhandle have been tentatively attributed to poisoning from brevetoxin produced by the dinoflagellate *Karenia brevis*. While dolphins in other regions of the Florida coast are often exposed to *K. brevis* blooms, large-scale dolphin mortality events are relatively rare and the frequency and magnitude of die-offs along the Panhandle raise concern for the apparent vulnerability of dolphins in this region. We report results from dolphin health assessments conducted near St. Joseph Bay, Florida, an area impacted by 3 unusual die-offs within a 7-year time span. An eosinophilia syndrome, manifested as an elevated blood eosinophil count without obvious cause, was observed in 23% of sampled dolphins. Elevated eosinophil counts were associated with decreased T-lymphocyte proliferation and increased neutrophil phagocytosis. In addition, indication of chronic low-level exposure to another algal toxin, domoic acid produced by the diatom *Pseudo-nitzschia* spp., was determined. Previous studies of other marine mammal populations exposed recurrently to *Pseudo-nitzschia* blooms have suggested a possible link between the eosinophilia and domoic acid exposure. While the chronic eosinophilia syndrome could over the long-term produce organ damage and alter immunological status and thereby increase vulnerability to other challenges, the significance of the high prevalence of the syndrome to the observed mortality events in the St. Joseph Bay area is unclear. Nonetheless, the unusual immunological findings and concurrent evidence of domoic acid exposure in this sentinel marine species suggest a need for further investigation to elucidate potential links between chronic, low-level exposure to algal toxins and immune health.

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1. Introduction

Unusual mortality events (UMEs) of bottlenose dolphins, manifested by a marked increase in the number of carcasses washing ashore, have occurred repeatedly over the past two decades in the northern Gulf of Mexico and specifically along the Florida Panhandle. Some of the events have been tentatively attributed to exposure to brevetoxin (PbTx), produced by the dinoflagellate, *Karenia brevis*. Although definitive evidence of brevetoxicosis as the primary cause of death has been elusive,

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circumstantial evidence points to *K. brevis* as the most likely culprit in at least some of the Florida Panhandle mortality events. A UME spanning 9 months (August 1999–May 2000) was spatially and temporally coincident with *K. brevis* blooms and the associated deaths of fish, sea turtles, invertebrates and sea birds (NOAA, 2004). Early in the event, dolphins primarily stranded in and around St. Joseph Bay (Fig. 1), strandings later spread westward to St. Andrew's Bay and Choctawhatchee Bay, eventually totaling over 150 bottlenose dolphin carcasses.

A subsequent event in the spring of 2004 remained localized to the St. Joseph Bay area and lasted only a single month but still resulted in more than 100 dolphin mortalities (NOAA, 2004). There was a concurrent absence of significant densities of *K. brevis* in the region, but stomach contents sampled from stranded dolphins showed high levels of PbTx, with many of the dolphins stranding with full stomachs (N. Barros, pers. comm.), suggesting an acute toxic effect rather than chronic disease or a lack of food resources as likely causal factors (Flewelling et al., 2005; NOAA, 2004).

Bottlenose dolphin strandings spiked again in the beginning of September 2005 and continued to be elevated through April 2006, with dispersed strandings across much of the Florida Panhandle early in the event, and then a peak of mortalities occurring in spring 2006 in the more western Choctawhatchee Bay. *K. brevis* blooms were noted in the first few months of the event, but then dissipated. Although measured seawater cell counts of *K. brevis* were negligible by March/April 2006 (Naar et al., 2007), the number of bottlenose dolphin strandings was still elevated and PbTx was detected in the stomach contents of many of the recovered carcasses as well as in prey species (MJT unpublished data). This suggests that the dynamics of *K. brevis* blooms are complex and measured cell counts of *K. brevis* may not always correlate with risks to marine wildlife and/or human populations.

It is puzzling as to why *K. brevis* blooms, which have historically been rare in the Panhandle area, would prompt such massive mortalities. Dolphin mortality events of the same frequency or intensity have not been noted in other portions of the Florida coast where *K. brevis* blooms are relatively commonplace. In fact, *K. brevis* blooms have been documented almost annually along the south and central west coast of Florida since the 1940s, but only a few dolphin mortality events have been documented (1946–1947, 1953–1955, 2005) and these were in conjunction with severe and prolonged blooms (TKR unpublished data; Gunter et al., 1948; Steidinger et al., 1972; Steidinger and Joyce Jr., 1973). Why dolphin die-offs have occurred recently along the Florida Panhandle and seem to be related to atypical blooms of *K. brevis*, yet are not seen in southern areas where blooms are frequent, is a driving question for ongoing research (Gaydos, 2007). One potential hypothesis is that dolphins in the Panhandle area differ with regard to prey preference and/or availability, thus creating potential vectors of exposure that are not as likely for bottlenose dolphin populations along the Florida west coast. Alternatively, Panhandle dolphins may be exposed to some yet unknown environmental stressor, rendering them more susceptible to the toxic effects of *K. brevis* and thus more vulnerable to mortality events. It is also possible that the PbTx to which animals were exposed differed qualitatively between events. Understanding the toxin exposure of dolphins and correlated health impacts is important not only for the management of this protected species, but also to elucidate the potential for human health risks.

As part of ongoing research to address these hypotheses, capture–release efforts were conducted in and around St. Joseph Bay to evaluate dolphin health and background toxin exposures. The first health assessment was conducted in April, 2005, one year following the die-off centralized within St. Joseph Bay at approximately the same time of year. The 2005/2006 die-off began approximately 4 months later. A second health assessment effort was conducted in July, 2006 approximately 2 months after the longer term and less localized 2005/2006 UME had subsided. The objectives of the health assessment efforts were to (1) evaluate a number of health and functional immune parameters for dolphins in St. Joseph Bay and compare these health endpoints with those measured from other wild populations and (2) measure baseline exposure of dolphins to the biotoxin PbTx. Levels of domoic acid (DA), produced by the diatom *Pseudo-nitzschia* spp., were also measured in tissues of the sampled dolphins.

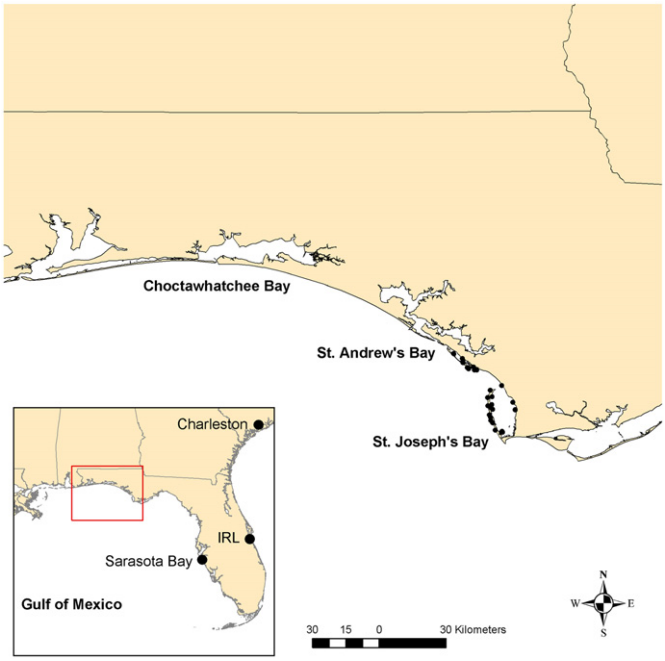

Fig. 1. Map of Florida Panhandle. Black circles represent locations where dolphins were sampled.

2. Materials and methods

2.1. Sampling

Bottlenose dolphin health assessments were conducted during April 18–28, 2005 and July 17–28, 2006 in and around St. Joseph Bay, FL (Fig. 1). Methods for the capture–release health evaluation generally followed those described by Wells et al. (2004). Dolphin groups that included young calves (seemingly <2 yr) were avoided. Dolphins were captured by encircling them with a net and restraining them by hand. Adult female dolphins were examined via ultrasound to determine reproductive condition; pregnant females had blood drawn and were then released. Non-pregnant females and males had blood drawn and were weighed and hoisted aboard a processing boat for complete physical examination, skin assessment, morphometric measurement, diagnostic ultrasound and collection of urine, feces, blowhole swab sample, gastric fluid, and blubber samples. Standardized data collection forms developed for dolphin health assessment studies in Sarasota, FL and other NOAA studies along the southeast coast, were used for physical examination and skin assessment. This facilitated comparison with other dolphin study sites. Freezebrands and VHF tags were also applied to enable post-capture follow-up (Balmer et al., 2008).

2.2. Age class determination

A single tooth was extracted under local anesthesia from most individuals and age was determined by reading dentinal and cemental growth layer groups (GLGs) (Hohn et al., 1989). A tooth was not obtained from some dolphins due to their presumed young age ($n=3$), or pregnancy status ($n=3$). Also, two dolphins were

released prior to tooth extraction due to veterinary concerns about the animals' stress response. Dolphins were classified into age/length classes based on the following criteria (McFee and Hopkins-Murphy, 2002; Schwacke et al., 2009):

Adult: ≥10 GLGs, or if GLGs not determined then ≥240 cm
Subadult: ≥2 and <10 GLGs, or if GLGs not determined then ≥200 and <240 cm
Calf: <2 GLGs, or if GLGs not determined then <200 cm.

2.3. Hematology and clinical blood chemistry

Blood collection for hematology and clinical blood chemistry followed basic protocols described in Schwacke et al. (2009). Briefly, blood samples for hematology were drawn from the ventral fluke vasculature using a 19-gauge butterfly catheter (Becton–Dickinson (BD), Franklin Lakes, New Jersey, USA). Samples were collected into a Vacutainer tube with EDTA as an anticoagulant (BD #6452) and immediately stored in an onboard cooler. Blood chemistry samples were collected in a 10-mL serum separator tube, slowly inverted several times and then immediately refrigerated in an onboard cooler for at least 20 min before being centrifuged. Serum and whole blood samples were sent overnight each evening to the Animal Health Diagnostic Laboratory (AHDL) at Cornell University, Ithaca, New York. At AHDL, hematology was performed using an ADVIA 120 Hematology System (Bayer Diagnostics, Tarrytown, New York, USA) and serum chemistries were analyzed using a Hitachi 917 Chemistry Analyzer (Roche Diagnostics Corporation, Indianapolis, Indiana, USA). Leukocyte subgroup counts were determined manually by microscopic examination of modified Wright-stained blood smears (Bayer Healthcare, Tarrytown, New York, USA).

Clinicopathologic values were compared with reference intervals derived from other wild bottlenose dolphin populations (Schwacke et al., 2009); abnormal cases were indicated when the individual's value was outside of the established reference interval.

2.4. Biotoxin analysis

Urine, blood and/or fecal material were analyzed for both DA and PbTx.

Urine was collected by inserting a sterile, lubricated catheter in the urethra and collecting urine into a 50 mL centrifuge tube. An 8 Fr × 22 cm urinary catheter (Tyco Healthcare Group, Mansfield, Massachusetts, USA) was used for males and a 14 Fr × 18 cm urinary catheter (Rusch Medical Supplies, Duluth, Georgia, USA) was used for females. Sterile gloves were worn for the procedures and every effort was made to obtain a sterile sample.

When dolphin defecated during the physical examination, a sample of feces was caught in a 50 mL centrifuge tube. If a fecal sampled could not be recovered in this manner, an autoclaved, lubricated tygon fecal tube (1/8 in I.D., 1/4 in O.D., wall thickness: 1/16″, Saint-Gobain Performance Plastics, Aurora, Ohio, USA) cut to 15 cm long, sanded on the end and attached to 10 cm^3 syringe was passed into the anus to extract a fecal sample.

Urine samples were prepared for analysis by centrifugation (≥300*g* for ≥5 min) and filtration (0.45 or 0.22 μm). Urine samples were analyzed for DA using an enzyme-linked immunosorbent assay (ELISA) (Biosense, Bergen, Norway) and/or liquid chromatography/mass spectrometry (LC/MS) according to the methods of Maucher and Ramsdell (2005) and Wang et al. (2007), respectively. PbTx concentrations in urine samples were analyzed by LC/MS following C18 solid phase extraction (Plakas et al., 2002; Twiner et al., 2007). Blood samples collected via blood collection cards were extracted and analyzed for DA (Maucher and Ramsdell, 2005) and PbTx (Fairey et al. 2001; Maucher et al., 2007). Blood samples from 2005 were also subjected to DA analysis by LC/MS (Wang et al., 2007). In 2006, fecal samples were extracted for PbTx according to the method of Plakas et al. (2002) and analyzed by receptor binding assay (Twiner et al., 2007; Van Dolah et al., 1994). DA in feces was extracted by homogenizing with four volumes of 50% methanol, removal of the solids by centrifugation (3000*g* for 5 min) and filtration (0.45 μm) of the supernatant extract. DA analysis was performed using LC/MS (Wang et al., 2007).

2.5. Functional immune assays

Functional immune assays were added for the 2006 samples. Blood was collected in sodium heparin Vacutainer tubes, kept cool, wrapped and shipped overnight on ice packs to the University of Connecticut for immunological testing. For the assessment of lymphocyte proliferation, peripheral blood mononuclear cells (PBMCs) were isolated by density gradient centrifugation on Ficoll (Amersham Biosciences, Uppsala, Sweden) for 35 min at 900*g*. PBMCs were then resuspended in complete Dulbecco's Modified Eagle Medium, washed three times, and cell counts and viability were determined using Trypan blue and light microscopy.

For the evaluation of phagocytosis, erythrocytes were lysed using NH_4Cl. Briefly, 45 mL of NH4Cl were added to 5 mL of blood, gently rocked for 3 min, and then centrifuged for 10 min at 250*g*. The leukocytes were washed twice in Hank's Balanced Salt Solution (HBSS), resuspended in HBSS, and cell counts and viability were determined using Trypan blue and light microscopy.

Mitogen-induced lymphocyte proliferation was evaluated as previously described (Levin et al., 2008). Briefly, lymphocytes were incubated with mitogens for 66 h in flat-bottom 96-well plates at 37 °C with 5% CO_2. The mitogens chosen included two T-cell mitogens (concanavalin A or Con-A, and phytohemagglutinnin A or PHA) and a B-cell mitogen (lipopolysaccharide or LPS). Mitogens were added at optimal as well as suboptimal concentrations in order to allow for the detection of subtle changes, however only results from the experiments with suboptimal concentrations are reported here. Lymphocyte proliferation was evaluated by the incorporation of bromo-deoxyuridine (BrdU), a non-radioactive analog of thymidine, into the nucleus of proliferating cells and further detection with a monoclonal antibody and a colorimetric enzymatic reaction (Boehringer Mannheim Corp., Indianapolis, Indiana, USA) using an ELISA plate reader. A stimulation index was calculated as the percent increase in proliferation in the mitogen stimulated cells as compared to the unstimulated cells.

Flow cytometry was used to measure phagocytosis following methods described by (DeGuise et al., 1995). Briefly, peripheral blood leukocytes were incubated at 37 °C under agitation, and 1 μm diameter fluorescent latex beads (Molecular probes, Eugene, Oregon, USA) were added. After 60 min, free beads were separated using centrifugation on a 3% bovine serum albumin cushion, cells resuspended in 0.5% formalin, and phagocytosis evaluated using flow cytometry. Results were reported as the proportion of cells which engulfed 2 or more beads.

Blood samples were also collected from 6 dolphins sampled in June 2006 from Sarasota Bay, Florida as part of regular health assessment efforts conducted by Chicago Zoological Society. Protocols for blood collection were standardized between the St. Joseph Bay and Sarasota Bay health assessments to ensure comparability. Identical functional immune assays were performed on the Sarasota Bay samples in order to have a basis for comparison with the St. Joseph Bay immunological assays.

Spearman rank order correlation coefficients were calculated to examine the relationships between immunological indices and leukocyte differential counts (neutrophils, lymphocytes, monocytes, eosinophils). Immunological indices were compared between the Sarasota Bay dolphin samples and the St. Joseph Bay dolphin samples using a multivariate analysis of variance (MANOVA) with dolphin length included as a continuous covariate. The MANOVA was followed by univariate tests for each immune index. Homogeneity of variances was confirmed using a Levene's test.

3. Results

Twelve dolphins were sampled in and around St. Joseph Bay during April, 2005 and 18 dolphins were sampled during July, 2006. Two 2005 dolphins were resampled in 2006 resulting in 28 unique individuals. Seventeen (61%) were classified as adults, 7 (25%) as subadults and 4 (14%) as calves.

Comparison of clinicopathologic values with established reference ranges (Schwacke et al., 2009) classified 7 eosinophilia cases (*i.e.*, absolute eosinophil count above the 97.5th percentile). These cases represent nearly one quarter (23%) of the sampled dolphins and include all age and sex classes (1 calf, 1 subadult, 5 adults). A similar proportion of individuals (21%) showed elevated lactate dehydrogenase (LDH). A *G*-test of independence with a Yate's correction for continuity (Sokal and Rohlf, 1995) was conducted to examine whether the cases of eosinophilia were associated with the cases with elevated LDH. Fifty seven percent (4/7) of the eosinophilia cases also demonstrated elevated LDH, a statistically significant association ($G=4.06$, $p<0.05$). As a follow-up, mean eosinophil count and total white blood cell count (WBC) for all sampled St. Joseph Bay dolphins were compared with published values from other wild dolphin populations (Fair et al., 2006; Goldstein et al., 2006) (Fig. 2).

Physical examinations and skin assessments showed no remarkable pattern. Healing or healed lesions from traumatic events such as predator or human interactions were noted in both 2005 (4/12) and 2006 (3/14). Dolphin pox, a relatively common and usually benign disease for wild dolphins (Van Bressem et al., 1999) was also noted in half of the animals examined in 2005 (6/12) and in just over a third of the animals examined in 2006 (5/14). In 2006, ulcerative oral lesions (2/14), genital papilloma (1/14) and dermatitis (1/14) were also noted. The observed

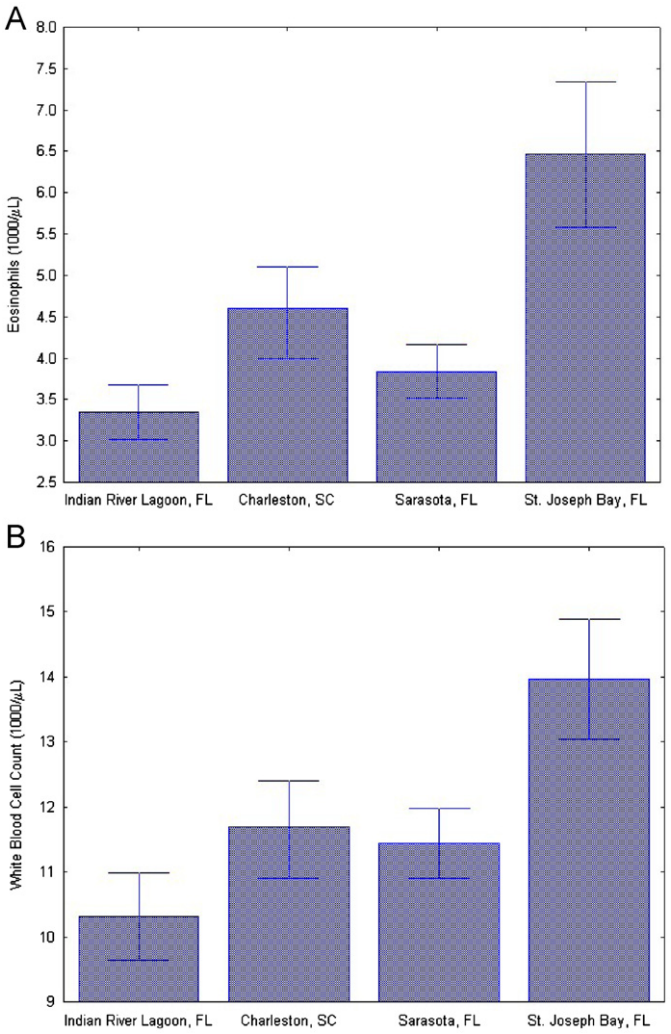

Fig. 2. Comparison of eosinophil count (A) and total white blood cells (B) in dolphins sampled from St. Joseph Bay with values reported from wild dolphins in other geographic locations. Bars represent means and whiskers represent 95% confidence intervals. Data sources for Indian River Lagoon, FL and Charleston, SC are Goldstein et al. (2006) and Fair et al. (2006), respectively. Sarasota mean and 95% confidence intervals were computed from data reported in Schwacke et al. (2009).

prevalence of infectious lesions (14%) was not considered unusual and was comparable to prevalence seen in other studies of free-ranging dolphins (Reif et al., 2008).

Fecal cytology found nematode eggs or larval nematodes in 2/7 samples in 2005 and 1/7 samples in 2006. None of these findings were considered unusual and were consistent with findings from other dolphin health assessments.

3.1. Biotoxin analysis

In 2005, PbTx was not detected in either urine ($N=7$) or blood ($N=12$) of the sampled dolphins (Table 1). PbTx was not detected in urine collected in 2006 ($N=12$), but very low levels (≤ 1.1 ng/mL) were detected in 78% of blood samples.

DA was found in urine samples in both years. DA was higher in urine versus blood and was detected in 43% of urine samples in 2005 and 29% of urine samples in 2006. The highest DA concentration measured in 2005 was 201 ng/mL and in 2006 was 28.6 ng/mL, both in urine.

3.2. Immunological assays

Functional immune assays were added in the 2nd year of health assessments in an effort to assess relationships with the observed eosinophilia. For dolphins sampled in the 2nd year, eosinophil count was found to have a significant positive correlation with neutrophil and monocyte phagocytosis indices, and a significant negative correlation with T-cell mitogen proliferation indices, calculated as the increase in proliferation in the mitogen stimulated cells as compared to unstimulated cells (Table 2, Fig. 3). The only functional immune index that did not appear to be significantly related to eosinophil count was the B-cell proliferation index (LPS mitogen). No significant correlation was determined between functional immune indices and lymphocyte, monocyte, or neutrophil counts (Table 2).

Samples from St. Joseph Bay, 2006 were grouped as eosinophilia cases ($n=4$) or non-cases ($n=14$) prior to comparison with samples from Sarasota dolphins ($n=6$). The MANOVA indicated a significant overall effect for group ($p=0.001$), but the effect of length was not significant ($p=0.58$). Therefore, length was removed from the model. Univariate analyses indicated that the neutrophil phagocytosis index differed among groups ($p=0.004$) but the effect of group was not statistically significant for any of the other immune indices ($p>0.050$, all groups). For neutrophil phagocytosis index, a Dunnett's test using Sarasota as the control group was conducted as a follow-up analysis (Fig. 4A). The Dunnett's test indicated that neutrophil phagocytosis was significantly greater for St. Joseph Bay cases as compared to Sarasota ($p=0.002$), but differences between Sarasota and St. Joseph Bay non-cases were not statistically significant ($p=0.064$). While differences among groups in T-cell lymphocyte proliferation indices were not statistically significant (PHA $p=0.144$, Con-A $p=0.226$), the trend was opposite of that for neutrophil phagocytosis index, *i.e.*, Con-A and PHA indices were highest for samples from Sarasota and lowest for St. Joseph Bay cases (Fig. 4B).

4. Discussion

An eosinophilia syndrome (ES), defined by elevated blood eosinophil count without obvious cause and often accompanied by elevated LDH, was seen in a large proportion of dolphins sampled in and around St. Joseph Bay. The ES was found in dolphins sampled from both years, indicating a likely chronic, or at least recurrent, condition. Blood eosinophilia is often associated with parasitosis, specifically related to helminthic infections, although protozoal, bacterial and viral etiologies have also been reported (reviewed in Tefferi, 2005). Concurrent alterations of blood chemistry are common with some tissue-invasive parasites and LDH is specifically considered to be of diagnostic value for infection with trichinella (Boczon et al., 1981; Piergili-Fioretti et al., 2005). While infection with trichinella, a muscle-invasive nematode acquired by carnivorous mammals through ingestion of infected meat, has been reported in marine mammals, cases have been largely restricted to arctic regions with only a single case involving a cetacean (Forbes, 2000). A trichinella vector affecting bottlenose dolphins in the Gulf of Mexico that could lead to such a high prevalence of infection is unlikely.

Only one of the dolphins diagnosed with eosinophilia was found to have any indication of parasitic infection; nematode eggs were noted in fecal samples from X03, who also demonstrated an elevated eosinophil count (9.2 thous/mL). Larval nematodes were noted in two other fecal cytologies, but neither of these dolphins concurrently demonstrated elevated eosinophils (4.6

Table 1
Summary of biotoxin concentrations from dolphin samples. Eosinophilia and elevated lactate dehydrogenase (LDH) cases are indicated. Blanks indicate that eosinophil and LDH measures were within normal limits.

Dolphin identifier	Year	Sex	Age class	Eosinophilia/LDH cases	Domoic acid			Brevetoxin		
					Urine (ng/mL)	Feces (ng/g)	Blood (ng/mL)	Urine (ng/mL)	Feces (ng/g)	Blood (ng/mL)
X00	2005	F	Calf		na	na	< dl	na	na	< dl
X01	2005	F	Adult		51.1	na	< dl	< dl	na	< dl
X02	2005	M	Adult	↑LDH, ↑eosinophils	< dl	na	< dl	< dl	na	< dl
X03	2005	F	Subadult	↑eosinophils	na	na	< dl	na	na	< dl
X04	2005	M	Adult	↑LDH, ↑eosinophils	201	na	1.4	< dl	na	< dl
X05	2005	F	Adult	↑LDH	< dl	na	< dl	< dl	na	< dl
X05	2006	F	Adult		< dl	na	< dl	< dl	na	< dl
X06	2005	M	Calf		< dl	na	< dl	< dl	na	< dl
X06	2006	M	Calf		< dl	na	< dl	< dl	na	0.9
X07	2005	F	Subadult		na	na	< dl	na	na	< dl
X08	2005	M	Adult	↑LDH	< dl	na	< dl	< dl	na	< dl
X09	2005	F	Adult		na	na	< dl	na	na	< dl
X10	2006	M	Adult		2	42	na	na	< dl	1
X11	2005	F	Adult		6.3	na	< dl	< dl	na	< dl
X12	2006	M	Adult		< dl	na	< dl	< dl	na	< dl
X13	2005	F	Subadult		na	na	< dl	na	na	< dl
X14	2006	M	Adult		< dl	na	< dl	< dl	na	1
X15	2006	F	Calf		1.9	na	< dl	< dl	na	0.8
X16	2006	M	Adult		< dl	< dl	na	na	< dl	0.8
X17	2006	F	Adult	↑eosinophils	na	na	na	na	na	< dl
X18	2006	M	Subadult		< dl	1.2	< dl	< dl	< dl	< dl
X19	2006	F	Adult		na	na	na	na	na	1.1
X20	2006	M	Adult		28.6	na	< dl	< dl	na	0.9
X21	2006	F	Subadult		na	na	na	na	na	0.8
X22	2006	M	Adult	↑LDH, ↑eosinophils	< dl	na	< dl	< dl	na	0.8
X23	2006	F	Subadult		0.5	0.8	< dl	< dl	< dl	0.7
X24	2006	M	Subadult		< dl	< dl	< dl	< dl	< dl	0.6
X25	2006	F	Adult		< dl	na	< dl	< dl	na	0.8
X27	2006	F	Calf	↑eosinophil	na	na	na	na	na	0.9
X29	2006	F	Adult	↑LDH, ↑eosinophils	< dl	< dl	< dl	< dl	< dl	0.7

dl = detection limit, na = not analyzed.

Table 2
Spearman rank correlation coefficients for differential counts and functional immune indices. Concavalin A (Con-A) and phytohemagglutinnin A (PHA) are mitogens that were used to induce T-lymphocyte proliferation. Lypopolysaccharide (LPS) was used as a mitogen to induce B-cell proliferation. T-lymphocyte proliferation indices, as well as neutrophil and monocyte phagocytosis indices showed significant correlation with eosinophil count.

	Neutrophil phagocytosis	Monocyte phagocytosis	Con-A proliferation	PHA proliferation	LPS proliferation
Neutrophils	0.00	−0.17	0.01	0.02	−0.03
Lymphocytes	−0.24	−0.09	0.19	0.16	0.12
Monocytes	0.06	−0.06	0.16	0.06	0.15
Eosinophils	**0.73**	**0.58**	**−0.70**	**−0.70**	−0.14

Values in bold indicate significant correlations at $p < 0.05$.

and 5.8 thous/mL). These findings were not considered unusual as similar results have been determined in health assessments of other free-ranging dolphin populations (RAV unpublished data). In addition, necropsies of carcasses retrieved during the preceding (NOAA, 2004) and subsequent (DCR unpublished data) UME events found no signs of excessive parasitism in dolphins from this area.

In humans, blood eosinophilia has also been reported with underlying non-infectious causes, such as drugs, toxins, or allergic disorders (reviewed in Tefferi, 2005). In this study, indications of toxin exposure were found for the St. Joseph Bay dolphins. Specifically, measurable levels of DA were found in urine, feces and/or blood samples, in both sampling years. In addition, DA was determined to be present at low levels in blood, urine and stomach contents of several dolphins analyzed as part of the 2004 UME investigation (MJT unpublished data). High densities of *Pseudo-nitzschia* spp. were noted in the area during the 2004 event as well as for many months during the 2005/2006 event (C. Heil, pers. comm.).

On the U.S. west coast, DA produced by blooms of the diatom *Pseudo-nitzschia* spp. has been associated with recurrent mortality and morbidity events of another marine mammal, the California sea lion (*Zalophus californianus*) (Bejarano et al., 2008; Scholin et al., 2000). Furthermore, elevated eosinophil counts have been reported in California sea lions affected by the DA exposure and clinically evaluated (Gulland et al., 2002). Along with an acute toxicosis in sea lions, a neurological syndrome resulting from chronic exposure to sub-lethal DA exposures has also been described (Goldstein et al., 2007) and the chronic cases are also often found to exhibit eosinophilia. Furthermore, degenerative cardiomyopathy has been associated with both acute and chronic DA exposure (Zabka et al., 2009) and such conditions could lead to elevated LDH levels. DA levels found in dolphin urine from this study (< dl—201 ng DA/mL urine) were lower than levels measured in California sea lions diagnosed with acute toxicosis (10–3720 ng DA/mL urine) but were similar to concentrations measured from sea lions exhibiting chronic neurological effects (2–110 ng DA/mL urine) (Goldstein et al., 2007).

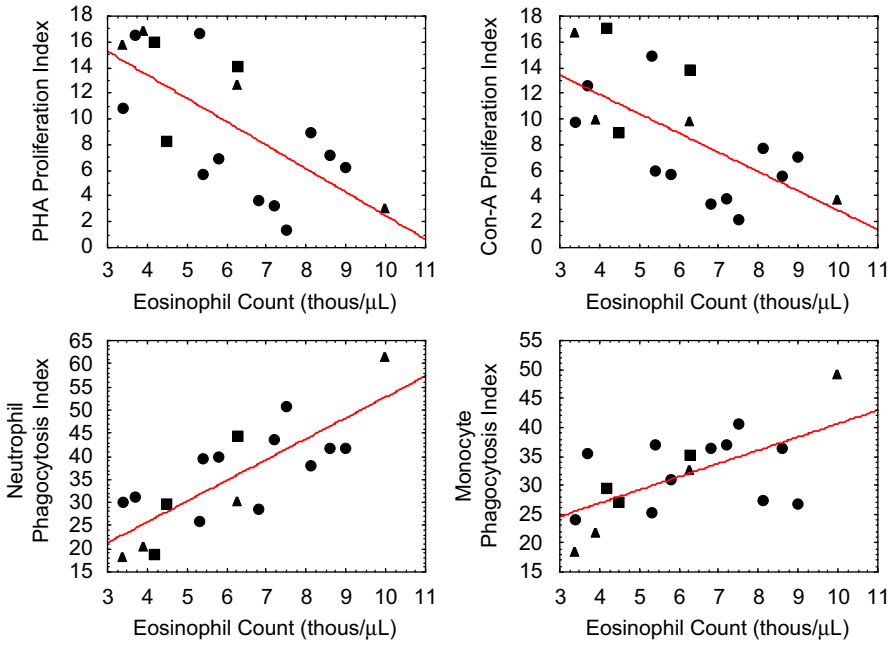

Fig. 3. Correlation matrix for eosinophil count and functional immune indices. Triangles represent calves, squares represent subadults and circles represent adults.

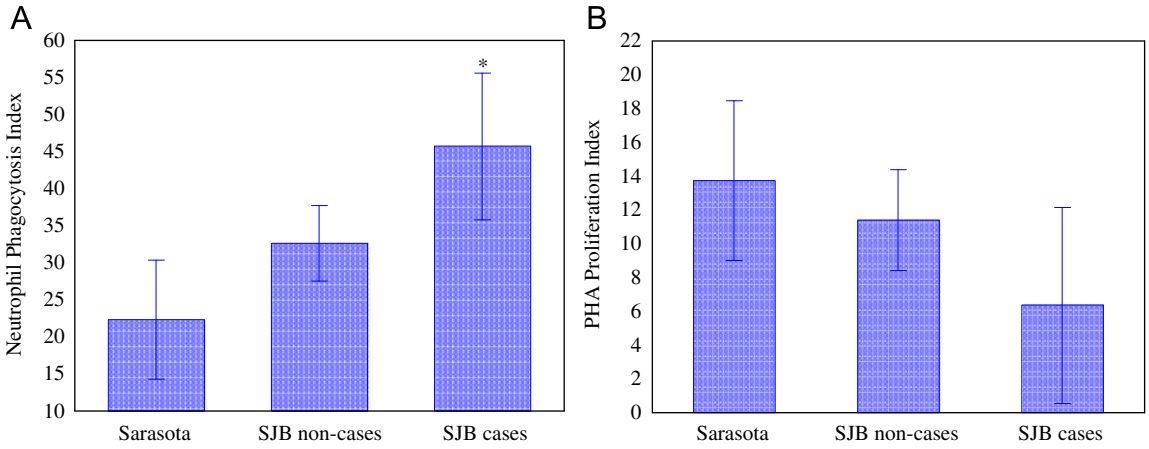

Fig. 4. Comparison of mean neutrophil phagocytosis (A) and con-A proliferation indices (B) between Sarasota Bay samples, St. Joseph Bay (SJB) non-cases and St. Joseph Bay cases. * indicates groups that differed significantly from control group (Sarasota). For neutrophil phagocytosis index, St. Joseph Bay cases differed significantly from Sarasota ($p=0.002$) but differences between St. Joseph Bay non-cases and Sarasota were not statistically significant ($p=0.064$). The univariate F-test indicated no difference among groups for phytohemagglutinnin A (PHA) proliferation ($p=0.144$).

A definitive cause and effect relationship between eosinophilia and exposure to DA could not be determined in this study. Unfortunately, urine samples could not be obtained from every sampled dolphin and 3 of the dolphins later determined as eosinophilia cases did not have concurrent urine samples for DA analysis (Table 1). Of the remaining 4 eosinophilia cases for which DA in urine was measured, one had the highest DA measurement (201 ng/mL), but DA was not detected in urine from the other three. Even if DA measurement data had been available for all animals, the rapid elimination of DA makes the interpretation of measures from cross-sectional studies such as this one difficult. In experimental studies of rats and monkeys, DA elimination rates have been determined to be on the order of hours (Suzuki and Hierlihy, 1993; Truelove and Iverson, 1994), and DA in the urine of stranded California sea lions was completely eliminated within 3 days (Bejarano et al., 2007). In contrast, the development of eosinophilia involves production of cytokines which then stimulate the production and maturation of eosinophils in the bone marrow. In mammalian systems, an eosinophil spends approximately 8 days maturing in the bone marrow before migrating into the blood-stream (Wilkins et al., 2005). Therefore, by the time eosinophilia is manifested, the DA is unlikely to be detectable if the exposure is not continuous.

Regardless of the initial trigger for the eosinophilia syndrome, a Th2 immune response is likely involved (MacDonald et al., 2002; Wilkins et al., 2005). The Th2 response initiates cytokine production associated with the promotion of eosinophil growth and differentiation important to "humoral immunity" which is effective against extracellular pathogens such as parasites and toxins. The fact that a Th2 response supports a humoral response and inhibits cell-mediated immunity (O'Garra, 1998) could explain the negative correlations between eosinophil count and T-cell proliferation indices. Although not well defined in delphinids, in humans interleukin-3 (IL-3), interleukin-5 (IL-5) and granulocyte-macrophage colony stimulating factor (GM-CSF) produced by T-lymphocytes can stimulate eosinophil generation

in the bone marrow (Sanderson, 1992). IL-5 is the most specific for the eosinophil lineage; IL-3 and GM-CSF can simulate other hematopoietic lineages (Sanderson, 1992). It would be interesting to determine whether the T-lymphocyte proliferation and neutrophil and monocyte phagocytosis observed in bottlenose dolphins in this study were directly caused by the eosinophilia or whether they resulted from a cytokine balance that prompted the eosinophilia. It would also be interesting to determine if exposure to DA in bottlenose dolphins can cause such changes in cytokine balance, eosinophilia and changes in immune functions, since in vivo and in vitro exposure to DA in mice has recently been reported to alter immune functions (Levin et al., 2008).

The significance of the observed eosinophilia syndrome to the recurrent UMEs in the St. Joseph Bay area is not clear. If sustained and severe, it is conceivable that organ damage could result from eosinophil infiltration, a defining characteristic for hypereosinophilia syndrome in humans (Tefferi, 2005). Such organ damage could obviously influence mortality rates, but eosinophil infiltration was not reported from necropsies of dolphins recovered from any of the mortality events. However, analysis of samples from subsequent strandings has produced at least one case of eosinophilic myocarditis (DCR unpublished data).

While a direct association between the observed immune perturbations and the recurrent UMEs cannot be made, it is conceivable that the eosinophilia syndrome, its cause(s), or the associated immune perturbations, could create an increased vulnerability to other challenges. If the hypereosinophilia results from a cytokine balance favoring a Th2 response, which also inhibits cell-mediated (Th1) immunity, it is possible that the disease susceptibility of affected dolphins to intra-cellular pathogens may be increased, as is the case in humans and mice who suffer more severe and disseminated cases of leprosy when their cytokine balance is tilted towards a Th2 compared to less severe and localized disease when the cytokine balance is tilted towards a Th1 response (Kobayashi et al., 1997; Sieling and Modlin, 1994). Since the innate immune system represents the first line of defense against pathogens, it is likely that changes in phagocytosis may also be associated with changes in susceptibility to infectious challenges.

5. Conclusions

Marine mammals and people share the same coastal waters and both eat at the top of the marine food web. In this regard, marine mammals have been proposed as sentinels to provide early warning of human health risks posed by a changing ocean environment (Fleming et al., 2006). Investigations of marine mammal mortalities have provided critical insights into the mechanisms for pathogenicity of algal toxins (Goldstein et al., 2007), as well as alerted the research community to previously unknown toxin vectors, *e.g.*, the investigation of the 2004 dolphin UME documented the accumulation of PbTx in live fish and revealed a new mechanism for its spread through marine food webs (Flewelling et al., 2005). The observed eosinophilia from this study, potentially an effect of chronic exposure to low levels of an algal toxin, but at least an indication of environmental stress on a coastal mammal species, has important implications. If these are truly effects of a chronic algal toxin exposure, it has gone unreported by other monitoring strategies. *Pseudo-nitzschia* spp. and DA have previously been reported in the northern Gulf of Mexico (Pan et al., 2001), but DA concentrations have not raised significant health concerns. If lower levels of algal toxin, ingested chronically over time, can increase susceptibility to infectious disease or other toxins, then dolphins in the Florida Panhandle could again be a warning of previously unknown risks.

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References

Balmer, B., et al., 2008. Seasonal abundance and distribution patterns of common bottlenose dolphins (*Tursiops truncatus*) near St. Joseph Bay, Florida, USA. J. Cetacean Res. Manage. 10, 157–167.

Bejarano, A., et al., 2008. Demographics and spatio-temporal signature of the biotoxin domoic acid in California sea lion (*Zalophus californianus*) stranding records. Mar. Mammal Sci. 24, 899–912.

Bejarano, A.C., et al., 2007. Exposure assessment of the biotoxin domoic acid in California sea lions: application of a bioenergetic model. Mar. Ecol.—Prog. Ser. 345, 293–304.

Boczon, K., et al., 1981. The diagnostic value of enzymatic and immunological tests in human trichinellosis. Tropenmed. Parasitol. 32, 109–114.

DeGuise, S., et al., 1995. Immune functions in beluga whales (*Delphinapterus leucas*): evaluation of phagocytosis and respiratory burst with peripheral blood leukocytes using flow cytometry. Vet. Immunol. Immunopathol. 47, 351–362.

Fair, P., et al., 2006. Hematology, serum chemistry, and cytology findings from apparently healthy Atlantic bottlenose dolphins (*Tursiops truncatus*) inhabiting the estuarine waters of Charleston, South Carolina. Aquat. Mammals 32, 182–195.

Fairey, E.R., et al., 2001. Biomonitoring brevetoxin exposure in mammals using blood collection cards. Environ. Health Perspect. 109, 717–720.

Fleming, L.E., et al., 2006. Oceans and human health: emerging public health risks in the marine environment. Mar. Pollut. Bull. 53, 545–560.

Flewelling, L.J., et al., 2005. Red tides and marine mammal mortalities. Nature 435, 755–756.

Forbes, L.B., 2000. The occurrence and ecology of *Trichinella* in marine mammals. Vet. Parasitol. 93, 321–334.

Gaydos, J., 2007. Bottlenose Dolphins and Brevetoxins: A Coordinated Research and Response Plan. NOAA Fisheries, Silver Spring, Maryland.

Goldstein, J.D., et al., 2006. Hematologic, biochemical, and cytologic findings from apparently healthy Atlantic bottlenose dolphins (*Tursiops truncatus*) inhabiting the Indian River Lagoon, Florida, USA. J. Wildl. Dis. 42, 447–454.

Goldstein, T., et al., 2007. Novel symptomatology and changing epidemiology of domoic acid toxicosis in California sea lions (*Zalophus californianus*): an increasing risk to marine mammal health. Proc. R. Soc. B—Biol. Sci. 275, 267–276.

Gulland, F.M., et al., 2002. Domoic acid toxicity in Californian sea lions (*Zalophus californianus*): clinical signs, treatment and survival. Vet. Rec. 150, 475–480.

Gunter, G., et al., 1948. Catastrophic mass mortality of marine mammals and coincident phytoplankton bloom on the west coast of Florida, November 1946 to August 1947. Ecol. Monogr. 18, 309–324.

Hohn, A., et al., 1989. Growth layers in teeth from free-ranging, known-age bottlenose dolphins. Mar. Mammal Sci. 5, 315–342.
Kobayashi, K., et al., 1997. Decreased expression of cytokines that induce type 1 helper T cell/interferon-gamma responses in genetically susceptible mice infected with *Mycobacterium avium*. Clin. Immunol. Immunopathol. 85, 112–116.
Levin, M., et al., 2008. Immunomodulatory effects of domoic acid differ between in vivo and in vitro exposure in mice. Mar. Drugs 6, 636–659.
MacDonald, A.S., et al., 2002. Immunology of parasitic helminth infections. Infect. Immun. 70, 427–433.
Maucher, J.M., et al., 2007. Optimization of blood collection card method/enzyme-linked immunoassay for monitoring exposure of bottlenose dolphin to brevetoxin-producing red tides. Environ. Sci. Technol. 41, 563–567.
Maucher, J.M., Ramsdell, J.S., 2005. Ultrasensitive detection of domoic acid in mouse blood by competitive ELISA using blood collection cards. Toxicon 45, 607–613.
McFee, W.E., Hopkins-Murphy, S.R., 2002. Bottlenose dolphin (*Tursiops truncatus*) strandings in South Carolina, 1992–1996. Fishery Bull. 100, 258–265.
Naar, J.P., et al., 2007. Brevetoxins, like ciguatoxins, are potent ichthyotoxic neurotoxins that accumulate in fish. Toxicon 50, 707–723.
NOAA, 2004. Interim report on the bottlenose dolphin (*Tursiops truncatus*) Unusual Mortality Event along the Panhandle of Florida March–April 2004. NOAA Fisheries Office of Protected Resources, 36 pp.
O'Garra, A., 1998. Cytokines induce the development of functionally heterogeneous T helper cell subsets. Immunity 8, 275–283.
Pan, Y., et al., 2001. *Pseudo-nitzschia* sp. cf. *pseudodelicatissima*—a confirmed producer of domoic acid from the northern Gulf of Mexico. Mar. Ecol.—Prog. Ser. 220, 83–92.
Piergili-Fioretti, D., et al., 2005. Re-evaluation of patients involved in a trichinellosis outbreak caused by *Trichinella britovi* 15 years after infection. Vet. Parasitol. 132, 119–123.
Plakas, S.M., et al., 2002. Confirmation of brevetoxin metabolism in the Eastern oyster (*Crassostrea virginica*) by controlled exposures to pure toxins and to *Karenia brevis* cultures. Toxicon 40 9p.
Reif, J.S., et al., 2008. Evaluation and comparison of the health status of Atlantic bottlenose dolphins from the Indian River Lagoon, Florida, and Charleston, South Carolina. J. Am. Vet. Med. Assoc. 233, 299–307.
Sanderson, C.J., 1992. Interleukin-5, eosinophils, and disease. Blood 79, 3101–3109.
Scholin, C.A., et al., 2000. Mortality of sea lions along the central California coast linked to a toxic diatom bloom. Nature 403, 80–84.
Schwacke, L., et al., 2009. Hematologic and serum biochemical reference intervals for free-ranging common bottlenose dolphins (*Tursiops truncatus*) and variation in the distributions of clinicopathologic values related to geographic sampling site. Am. J. Vet. Res. 70.
Sieling, P.A., Modlin, R.L., 1994. Cytokine patterns at the site of mycobacterial infection. Immunobiology 191, 378–387.
Sokal, R., Rohlf, F., 1995. Biometry. W.H. Freeman and Company, New York.
Steidinger, K., et al., 1972. The effects of *Gymnodinium breve* toxin on estuarine animals. In: Martin, D.F., Padilla, G.M. (Eds.), Marine Pharmacognosy. Academic Press, New York, pp. 179–202.
Steidinger, K., Joyce, E.A. Jr., 1973. Florida red tides. Florida Department of Natural Resources Marine Research Laboratory Education Series, no. 17, 26 pp.
Suzuki, C.A., Hierlihy, S.L., 1993. Renal clearance of domoic acid in the rat. Food Chem. Toxicol. 31, 701–706.
Tefferi, A., 2005. Blood eosinophilia: a new paradigm in disease classification, diagnosis, and treatment. Mayo Clin. Proc. 80, 75–83.
Truelove, J., Iverson, F., 1994. Serum domoic acid clearance and clinical observations in the cynomolgus monkey and Sprague-Dawley rat following a single i.v. dose. Bull. Environ. Contam. Toxicol. 52, 479–486.
Twiner, M.J., et al., 2007. Extraction and analysis of lipophilic brevetoxins from the red tide dinoflagellate *Karenia brevis*. Anal. Biochem. 369, 128–135.
Van Bressem, M., et al., 1999. A review of virus infections of cetaceans and the potential impact of morbilliviruses, poxviruses and papillomaviruses on host population dynamics. Dis. Aquat. Organisms 38, 53–65.
Van Dolah, F.M., et al., 1994. Development of rapid and sensitive high throughput pharmacologic assays for marine phycotoxins. Nat. Toxins 2, 189–196.
Wang, Z., et al., 2007. Determination of domoic acid in seawater and phytoplankton by liquid chromatography-tandem mass spectrometry. J. Chromatogr. A 1163, 8.
Wells, R.S., et al., 2004. Bottlenose dolphins as marine ecosystem sentinels: developing a health monitoring system. EcoHealth 1, 246–254.
Wilkins, H.J., et al., 2005. Hypereosinophilic syndrome: an update. Am. J. Hematol. 80, 148–157.
Zabka, T.S., et al., 2009. Characterization of a degenerative cardiomyopathy associated with domoic acid toxicity in California sea lions (*Zalophus californianus*). Vet. Pathol. 46, 105–119.