A NOTE ON THE DETECTION OF THE NEUROTOXIN DOMOIC ACID IN BEACH-STRANDED *DOSIDICUS GIGAS* IN THE SOUTHERN CALIFORNIA BIGHT

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ABSTRACT

The first occurrence of the neurotoxin domoic acid (DA) in Humboldt squid (*Dosidicus gigas*) during a toxic *Pseudo-nitzschia* bloom in the Southern California Bight is reported. Bloom levels of cells within the *Pseudo-nitzschia delicatissima* group were detected on 6 July 2009 at 4 nearshore collection sites in the Southern California Bight (Scripps Pier, Newport Pier, Goleta Pier and Sterns Wharf). Particulate DA was detected in all of these locations, except for Newport Pier. Stranded Humboldt squid were found south of the Scripps pier 5 days after the toxic bloom was detected. DA was measured using ELISA and low DA concentrations were detected in the stomach or mantle tissue of the stranded specimens. Stomach content analysis indicated that possible DA vectors to Humboldt squid included both pelagic (Pacific hake, *Merluccius productus*, and Pacific sardine, *Sardinops sagax*) and nearshore (pile surfperch, *Damalichthys vacca*, and shiner surfperch, *Cymatogaster aggregata*) fish species. Although low DA levels were detected in stranded squid specimens, neurological symptoms of DA toxicity were not observed and low DA concentrations alone may not have been the cause of the strandings. Further studies should focus on DA toxic effects in *D. gigas* to verify whether this pelagic predator can be affected by a toxin frequently detected in pelagic ecosystems influenced by the California Current System.

INTRODUCTION

The Humboldt squid *Dosidicus gigas* is a large nerito-oceanic squid and an important link between lower trophic levels and apex predators in the pelagic food web. *D. gigas* is an opportunistic predator of small meso-pelagic, pelagic and demersal fish, crustaceans and squid (Markaida and Sosa-Nishizaki 2003; Field et al. 2007) and common prey for billfish, sharks, pinnipeds, and toothed whales (Olson and Watters 2003; Ruiz-Cooley et al. 2004; Vetter et al. 2008). *D. gigas* is endemic to the eastern Pacific Ocean between 30°N and 20–25°S and 140°W (Nigmatullim et al. 2001) and can cover great horizontal distances within this range at speeds up to 30 km d^{-1} (Gilly et al. 2006). Poleward excursions have been reported in both hemispheres, with significant range expansions taking place over the past decade (Zeidberg and Robison 2007; Field et al. 2007; Alarcón-Muñoz et al. 2008).

Large-scale (ranging from dozens to thousands of individuals) beach stranding events of *D. gigas* have taken place both historically and recently along the Eastern Pacific rim, particularly in the fringes of the squid's range and during periods of episodic high abundance (Mearns 1988; Alarcón-Muñoz et al. 2008). Beach strandings frequently result in flurries of short-term media attention and speculation into the causes of mortality, often with minimal scientific consultation. The frequency and range of reporting on these events has spiked over the past five to ten years along the west coast of the USA and Canada (fig. 1). Among the most significant strandings in recent years include events in July 2002 in La Jolla, CA; October 2003 in Carmel, CA; October 2004 in Westport, WA; January 2005 in Los Angeles and Newport Beach, CA; March 2005 in Oceanside, CA; October 2008 in Westport, WA; July 2009 in La Jolla, CA (samples reported in this manuscript); September 2009 in Westport, WA, Seaside, OR and Vancouver Island, British Columbia (J. Field, unpublished data). The reasons for *D. gigas* mortality and strandings remain unknown.

The primary focus of this paper is to explore the hypothesis that *D. gigas* can be exposed to domoic acid (DA) during toxic *Pseudo-nitzschia* algal blooms. Investigation of this hypothesis may help understand whether or not domoic acid poisoning (DAP) can be considered a contributing factor to *D. gigas* mortality and strandings. DA is a neurotoxin produced by several species of the diatom *Pseudo-nitzschia* (Moestrup and Lundholm 2007) and has caused mass mortality of marine mammals and
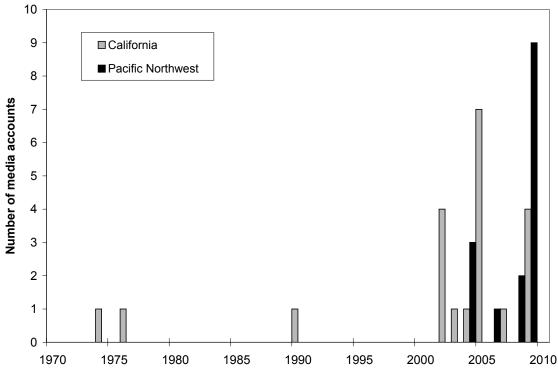
Figure 1.   Frequency of media reports of stranding events in California and the Pacific Northwest over the past forty years.

birds (Work et al. 1993; Scholin et al. 2000). However, determining that DAP is the cause of death in marine animal strandings is difficult. Studies that have come to that conclusion used a combination of observations such as (1) DA detection in hundreds of specimens in question and/or in their prey items; (2) DA detection in the water along with high concentration ($<10^4$ cells L^{-1}) of DA-producing cells; and (3) observations of typical DA neurological symptoms (i.e., seizures, ataxia, head weaving, and stereotypic scratching) and (4) histopathology to show lesion in hippocampus brain region characteristic of DA poisoning (Work et al. 1993; Scholin et al. 2000; Gulland et al. 2002).

Accordingly, pelagic predators have been detected with DA, but DA toxicity effects and whether or not DA could cause the death of these animals have not been verified. For example, North Atlantic right whales (*Eubalaena glacialis*), pygmy sperm whales (*Kogia breviceps*) and dwarf sperm whales (*Kogia sima*) found stranded along the U.S. Atlantic coast were tested positive with low DA levels (Fire et al. 2009; Leandro et al. 2010), but DA toxicity symptoms were not determined in these studies and thus DA could not be related to the cause of stranding. However, Leandro et al. (2010) hypothesized that the observed long-term exposure of North Atlantic right whales to DA may perhaps enhance mortality due to other well-documented factors in their populations since it has been found that chronic DA exposure can impair navigational abilities of other marine mammals such as

sea lions (Goldstein et al. 2007). Furthermore, high DA levels have been found in feces and prey of blue whales (*Balaenoptera musculus*) and humpback whales (*Megaptera novaeangliae*) during a toxic *Pseudo-nitzschia* bloom in Monterey Bay (California, USA) (Lefebvre et al. 2002). Although DA toxicity was not observed, the high DA doses that these whales were exposed to could lead to DA neurotoxicity effects (Lefebvre et al. 2002).

Additionally, DA has been detected in cephalopods such as market squid (*Loligo opalescens*), cuttlefish (*Sepia officinalis*) and common octopus (*Octopus vulgaris*) after feeding on DA-contaminated prey items (Costa et al. 2004; 2005; Bargu et al. 2008). *D. gigas* could be exposed to DA through a variety of vectors. Humboldt squid are active predators of small pelagic fish such as northern anchovies (*Engraulis mordax*) and Pacific mackerel (*Scomber japonicus*) (Markaida and Sosa-Nishizaki 2003; Markaida 2006; Field et al. 2007), which have been previously identified as DA vectors to marine mammals and birds (Sierra-Beltran et al. 1998; Lefebvre et al. 1999). Krill are also a potential vector of DA, as they are a prey item of *D. gigas* (Field et al. 2007) and have been found to acquire DA (Bargu et al. 2003). Furthermore, Pacific hake consume both krill and northern anchovies (Buckley and Livingston 1997; Mackas et al. 1997), and are a key prey item of *D. gigas* in California waters (Field et al. 2007).

DA effects in cephalopods have not been confirmed. Only a few studies focused on the DA effects in invertebrates, mostly shellfish, and the results are conflict-

ing (Maeda et al. 1987; Jones et al. 1995a; b; Dizer et al. 2001; Blanco et al. 2006; Liu et al. 2007a, b, 2008). However, *D. gigas* might be susceptible to DA neurotoxicity effects. DA is structurally similar to glutamic acid, a neurotransmitter in central nervous systems (CNS) of mammals (Nakajima et al. 1985). Such a similarity allows DA to bind to the same receptors of glutamic acid and trigger a cascade of molecular reactions inducing neuronal degradation, and consequently, DA neurotoxicity effects (Pulido 2008). Evidence indicates that DA binds with high affinity to 2 glutamate receptor subtypes: kainic acid and AMPA receptors (Hampson et al. 1992); a third receptor subtype, NMDA, is a co-participant in inducing DA neurotoxicity effects (Pulido 2008). These receptors are concentrated in the hippocampus of mammals (Foster et al. 1981; Debonel et al. 1989; Scallet et al. 1993), a brain region responsible for memory and spatial navigation and thus, hippocampus lesions are common in mammals and humans after exposure to specific DA doses (Teitelbauum et al. 1990; Gulland et al. 2002). Cephalopods have the largest brains of any invertebrate with a complexity analogous to those of vertebrates (Messenger 1996), and it has been suggested that the arrangement of neurons in the vertical lobe of octopus is involved in memory and it has structural similarities to the vertebrate hippocampus (Boycott and Young, 1950; Young, 1965). Moreover, glutamic acid also serves as a neurotransmitter in invertebrates (Messenger, 1996) and all 3 subtypes of glutamate receptors (i.e., kainic acid, NMDA and AMPA receptors) have been detected in central and peripheral nervous systems of cephalopods (Evans et al. 1992; Messenger, 1996, Garcia 2002; Lima et al. 2003; Di Cosmos et al. 2004). The fact that cephalopods have highly developed CNS and similar glutamate receptors as mammals potentially indicate that Humboldt squid may be susceptible to DA neurotoxicity effects.

The goal of this study was to ascertain whether beach stranded *D. gigas* found in two different locations in San Diego (California, USA) were exposed to DA during the summer of 2009. Our approach was to (1) measure DA in stomach content and lining as well as in mantle tissue of 5 stranded *D. gigas*, (2) analyze surface water particulate DA in locations within 300 km of the stranding site, and (3) examine the stomach contents of the stranded individuals to identify possible DA vectors.

METHODS

Water Sample Collection

Surface (~1 m) seawater samples were collected weekly from five pier sampling stations as part of the Southern California Coastal Ocean Observing (SCCOOS) Harmful Algal Bloom Monitoring Program
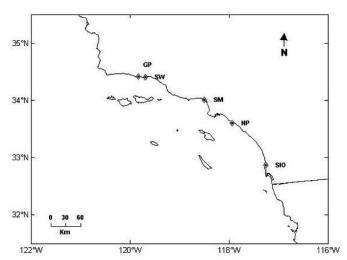

Figure 2.   Locations of samples collected for DA analysis and *Pseudo-nitzschia* identification and quantification: Scripps Institution of Oceanography Pier (SIO), Newport Pier (NP), Santa Monica Pier (SM), Stearns Wharf (SW), Goleta Pier (GP). Stranded squid were found between 3km and 6km south of the SIO pier.

(fig. 2). Samples were collected with surface bucket and provided subsamples for DA analysis and *Pseudo-nitzschia* identification and quantification. Cross–contamination of samples was avoided by acid washing the sampling gear and bucket with dH_2O 3 times after sample collections and 3 times with seawater before sample collections.

DA in Seawater

Particulate DA concentrations were measured by filtering 200 mL of seawater onto GF/F Whatman filters. Filters were stored frozen at −80°C until shipped in dry ice and analyzed within 1 week to 5 months of sample collection, using Rapid Enzyme-Linked Immunosorbent Assay (ELISA) obtained from Mercury Science, Inc. (Durham, NC27713) at the University of Southern California following Schnetzer et al. (2007). The detection limit for the ELISA assay on water samples was 0.02 ng mL^{-1} (ppb).

Toxic *Pseudo–nitzschia* Identification and Quantification

Abundances of two size class categories of the genus *Pseudo-nitzschia*, *seriata* group (frustule width > 3 μm) and *delicatissima* group (frustule width < 3 μm), were determined from settling 10–50 mL of seawater preserved with 4% formaldehyde (Uthermöhl 1958). Cells were categorized under an inverted light microscope. DA–producing *Pseudo-nitzschia* species are found in both of these groups (Hasle and Syvertsen 1997; Moestrup and Lundholm 2007).

Humboldt Squid Sample Collection

Stranded *D. gigas* were collected on 11 July 2009 at La Jolla Shores beach and on 12 July 2009 at La Jolla

TABLE 1
Domoic acid content (µg g^{-1} or ppm) of *Dosidicus gigas* specimens collected in La Jolla, CA, on 11–12 July 2009.

Tissues analyzed for domoic acid (DA)	Mean	SD	Median	Range	Number of replicates	Date Collected	Individual Size (cm)	Sex
Stomachs	0.27	0.05	0.3	0.2–0.3	3	11-Jul-09	na	na
	nd	nd	nd	nd	3	11-Jul-09	na	na
	nd	nd	nd	nd	3	11-Jul-09	na	na
	nd	nd	nd	nd	3	12-Jul-09	63	Female
	nd	nd	nd	nd	3	12-Jul-09	63	Female
Mantle tissue	0.43	0.05	0.4	0.4–0.5	3	11-Jul-09	na	na
	nd	nd	nd	nd	3	11-Jul-09	na	na
	nd	nd	nd	nd	3	11-Jul-09	na	na
	nd	nd	nd	nd	3	11-Jul-09	na	na

Cove. The stranding event was relatively small; only five animals were found in sufficiently good condition to be sampled. Based on media reports and conversations with beachgoers, we concluded that the animals stranded in the early morning of 11 July, and that they were alive at the time of stranding. At the time of dissection, they were dead, most likely through a combination of asphyxiation and partial predation by seagulls. We estimate that the animals dissected on 11 July had been dead for 6–8 hrs, while those dissected on 12 July had been dead for 24–30 hrs. The mantles were in sufficiently good condition that dorsal mantle length (DML) could be measured. Stomachs and samples of mantle tissue (approximately 2×2×2 cm) were removed and frozen at −20°C, then moved to −80°C within 48 hrs. All samples were kept frozen for 3–4 months until stomach content observations and DA analysis could be performed.

Stomach Content and DA Analysis of Humboldt Squid

The stomach contents of the five squid sampled for DA were evaluated as described in Field et al. (2007). Following these observations, DA was analyzed in stomach contents and lining (hereafter referred to as stomach) and mantle tissue using ELISA obtained from Mercury Science, Inc. (Durham, NC27713). Stomach and mantle tissue samples were weighed and each sample was homogenized with a hand-held tissue homogenizer (Tissue Mixer, model PNF2110, Fisher Scientific). Aliquots of 4 g were removed from the homogenized samples and 16 mL of 50:50 MeOH:Nanopure was added. Samples were then sonicated with a Sonifier cell disruptor (Model W185D, Branson Sonic Power) and centrifuged for 20 min at 3800 rpm ($1698 \times g$). The supernatant were filtered through a 3 µm polycarbonate filter. The filtrate was diluted at 1:100 and 1:1000 in the buffer solution provided in the ELISA kit and aliquoted in 3 replicates for each sample (table 1). Samples diluted at 1:1000 were below the detection limit of 0.1 ng mL^{-1} (ppb) in the ELISA kit, but samples in the 1:100 dilution were within the detection limit. The diluted samples were used in the ELISA plates following the protocol accompanying the kit. An EMax Precision Microplate Reader (Model E10968, Molecular Devices) was used to measure absorbance at 450 nm. Final concentrations of DA in squid samples are expressed as µg DA g^{-1} wet tissue mass.

RESULTS & DISCUSSION

DA was detected in the stomach of one *D. gigas* specimen and in the mantle tissue of one specimen found stranded in La Jolla beach (table 1). Mantles and stomachs were not labeled individually, so it is unknown whether the specimen with DA in its mantle tissue also had DA in its stomach. Low particulate DA concentration, just above the detection limit, was recorded in surface waters at Scripps Pier 5 days before the stranding occurred (fig. 3A). Scripps Pier is located 3 to 5 km north of the stranding locations, well within documented swimming speeds for *D. gigas* of 30 km day^{-1} (Gilly et al. 2006). Particulate DA was also detected on Goleta Pier and Stearns Wharf, but those are located >30 km north of the stranding location (fig. 2 and 3A). DA levels at these three sites peaked on 6 July 2009 and tapered off to below detection levels within one to three weeks. Peak DA concentrations for July occurred at the same time as an increase in the abundance of cells within the *Pseudo-nitzschia delicatissima* group was observed (fig. 3C). At these 3 locations, *P. delicatissima* group densities ranged from 5.6×10^4 to 5.4×10^5 cells L^{-1}, which are typical bloom levels (i.e. $>10^3$ cells L^{-1}). Moreover, the abundance of cells within the *Pseudo-nitzschia seriata* group never exceeded 9.1×10^2 cells L^{-1} at the Scripps Pier. This suggests that *Pseudo-nitzschia* species from the *delicatissima* group were probably responsible for the DA production at that time.

Squid stomach analyses indicated the presence of Pacific sardine, shiner surfperch (*Cymatogaster aggregata*), and pile surfperch (*Damalichthys vacca*). DA has been detected in Pacific sardine and other surfperch species (i.e. rainbow surfperch) within 7 days of the detection of DA in surface waters (Mazzillo et al. 2010), suggesting that these fish found in the stomachs of *D. gigas* could
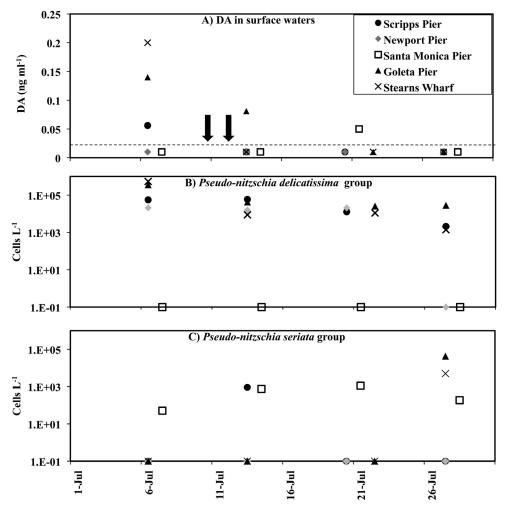
Figure 3. Particulate domoic acid (DA) levels (a), and cell density of *Pseudo-nitzschia delicatissima* group (b) and *Pseudo-nitzschia seriata* group (c) for 1–30 July 2009 from surface waters (1m) at five sites along southern the California coastline: Scripps Pier (●), Newport Pier (♦), Santa Monica Pier (□), Goleta Pier (▲), and Sterns Wharf Pier (×). Arrows in panel (a) indicate collection dates for squid analyzed here: horizontal dashed line indicates analytical detection limit.

have been the DA vectors. Other prey items and potential DA sources included Pacific hake, topsmelt silversides (*Atherinops affinis*) and an unidentified species of *Gonatus* squid, future work should include the examination of these species during periods of algal blooms. Macroalgae and sand were also observed in all stomachs, suggesting other behaviors that are not typical of the class cephalopoda.

Here we showed that *D. gigas* can be exposed to DA by preying on pelagic and nearshore species when toxic *Pseudo-nitzschia* blooms are detected. However, linking the Humboldt squid strandings themselves to domoic acid poisoning (DAP) is difficult. First, DA was not detected in all stranded individuals, indicating that there could be other reasons for their death. Most of the observed prey items are frequently, if not exclusively, found in shallow water, and the nearshore species are unusual targets for *D. gigas*, further demonstrating the opportunistic feeding strategy of this species. The pres-

ence of sand and algae in the stomachs also supports the hypothesis that these animals were actively foraging in shallow waters. If this is an atypical habitat for them to exploit, they may have become disoriented and accidentally swam onshore.

Second, we cannot determine from this study whether DA was absorbed from the stomach into the bloodstream and interacted with the squid nervous system via blood causing DAP, although we have evidence that DA was transferred to the mantle tissue. We measured low DA values in the squid stomach (table 1) and DA absorption from the gastrointestinal tract to the bloodstream is known to be minimal in vertebrates (Iverson et al. 1989; Truelove et al. 1997; Lefebvre et al. 2001). The range of DA values detected in the stomach and mantle tissue of stranded squid is within the lower limit range of DA detected in body fluids of DAP stranded California sea lions. Urine and serum of California sea lions were found to contain 0.03–3.72 μg DA mL^{-1} and 0.17–0.20

µg DA mL^{-1}, respectively (Scholin et al. 2000). However, Scholin et al. (2000) also measured DA levels >6,000 times higher in fecal material, and observed common DAP neurological symptoms in addition to the detection of DA, making the DAP diagnosis in these marine mammals more certain.

Although DAP was implicated in mass mortality of marine birds and sea lions (Work et al. 1993; Scholin et al. 2000), the causes of other marine mammal strandings remain largely unknown (Bogomolni et al. 2010), and the same appears to be true of Humboldt squid strandings. Even though we report for the first time *D. gigas* exposure to DA, our results cannot be implicated as the cause of death of the stranded squid since we do not know whether the low DA concentrations measured were absorbed from the squid gastrointestinal tract into the blood and interacted with squid CNS at enough concentrations to cause neurotoxicity effects. Nevertheless, it is quite possible that *D. gigas* could be exposed to higher DA levels than those observed here, since higher DA levels have been previously detected in the waters off the coast of California and in the prey of *D. gigas* (Trainer et al. 2000; Busse et al. 2006; Anderson et al. 2009; Mazzillo et al. 2010). Additionally, cephalopods have the similar glutamate receptor sites as mammals, and as DA binds to these sites it triggers toxicity which indicates that *D. gigas* can potentially be susceptible to DA neurotoxicity effects. However, juvenile leopard sharks (*Triakis semifasciata*) appear not to be affected by DA even though it possesses kainic acid–type glutamate receptors (Schaffer et al. 2006). Thus, *D. gigas* susceptibility to DA toxicity is possible, but remains an unanswered question. Laboratory studies designed to verify DA toxicity in squids could be performed using the California market squid (*Doryteuthis opalescens*) or Atlantic longfin squid (*Doryteuthis pealei*) as models since *D. gigas* cannot be kept in captivity for more than 10 days. Laboratory experiments should include observations of squid behavioral response to DA doses, measurements of DA uptake, as well as quantification of DA in brain tissue to evaluate whether or not DA toxicity occurs in squids and thus could responsible for *D. gigas* mass strandings. Sampling larger *D. gigas* stranding events would also be desirable, as our sample size for this study was limited by the small number of stranded individuals.

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