MARINE MAMMAL SCIENCE, 22(3): 700–707 (July 2006)

DOI: 10.1111/j.1748-7692.2006.00045.x

DOMOIC ACID CAUSES REPRODUCTIVE FAILURE IN CALIFORNIA SEA LIONS (*ZALOPHUS CALIFORNIANUS*)

ERIN C. BRODIE
FRANCES M. D. GULLAND
DENISE J. GREIG
The Marine Mammal Center,
Marin Headlands, 1065 Fort Cronkhite, Sausalito, California 94965, U.S.A
E-mail: brodiee@tmmc.org

MICHELE HUNTER
Pacific Marine Mammal Center,
20612 Laguna Canyon Road, Laguna Beach, California 92651, U.S.A.

JACKIE JAAKOLA
Marine Mammal Care Center at Fort MacArthur,
3601 South Gaffey Street, San Pedro, California 90731, U.S.A.

JUDY ST. LEGER
SeaWorld of California,
500 SeaWorld Drive, San Diego, California 92109, U.S.A.

TOD A. LEIGHFIELD
FRANCES M. VAN DOLAH
Marine Biotoxins Program, NOAA, National Ocean Service,
Center for Coastal Environmental Health and Biomolecular Research,
219 Fort Johnson Road, Charleston, South Carolina 29412, U.S.A.

Exposure of marine mammals to toxins can influence their survival and reproduction. A number of marine mammal mortality events have been attributed to naturally occurring algal toxin exposure, including deaths of manatees (*Trichechus manatus latirostris*) in Florida due to brevetoxin exposure (O'Shea *et al*. 1991, Bossart *et al*. 1998), Hawaiian monk seals (*Monachus schauinslandi*) due to ciguatoxin (Gilmartin *et al.* 1980), humpback whales (*Megaptera novaeangeliae*) due to saxitoxin (Geraci *et al*. 1989), and California sea lions exposed to domoic acid (DA) (Scholin *et al*. 2000). Although anthropogenic toxins have been shown to reduce reproductive success in marine mammals (Reijnders 1986) and mortality of marine mammals due to exposure to a variety of marine biotoxins is becoming increasingly recognized (Van Dolah 2000), effects of biotoxins on reproduction have not been reported to date. DA is a potent excitatory neurotoxin produced by marine diatoms in the genus *Pseudonitzschia* that can affect humans and marine mammals (Perl *et al*. 1990, Van Dolah *et al*. 2003). Large-scale mortality of California sea lions has occurred after ingestion of prey that fed on toxin-producing algae (Scholin *et al.* 2000). DA-producing algal blooms have increased on the west coast of the United States in

recent years (Van Dolah 2000), yet the effects on sea lion population dynamics are unknown.

Since 1998, hundreds of California sea lions have stranded alive along the California coast showing clinical signs and hematological and serum biochemical changes typical of DA toxicosis (Scholin *et al*. 2000, Gulland *et al*. 2002). In 1998 and 2002, algal blooms occurred during the months immediately prior to the sea lion pupping season (30 May–30 June) (DeLong *et al*. 1973). During these blooms, 209 pregnant adult female sea lions stranded. The animals were in good body condition, with blubber thicknesses between 15 and 50 mm, consistent with those of adult female sea lions in good condition as reported by Ylitalo *et al*. (2005). Symptoms ranged from ataxia, head weaving, and scratching to seizures and coma. The animals were rescued and transported to wildlife rehabilitation centers authorized by the Marine Mammal Health and Stranding Response Program of the National Marine Fisheries Service for further examination and treatment. Animals that stranded in San Diego County were rehabilitated at SeaWorld San Diego, sea lions in Orange County at the Pacific Marine Mammal Center, those from Los Angeles and Ventura Counties at the Marine Mammal Care Center at Fort MacArthur, and those from Santa Barbara, San Luis Obispo, Monterey, and Santa Cruz Counties at The Marine Mammal Center in Sausalito. Rehabilitation was aimed at controlling seizures and maintaining hydration as described by Gulland *et al*. (2002). Pregnancies were identified by transabdominal ultrasonography (Aloka ultrasound machine, model #SSD-900V, Tokyo, Japan), documenting abortions, observing subsequent birth of a pup, or by postmortem examinations. Uterine enlargement with absence of a fetus was also observed at necropsy in eight adult females, suggesting that premature parturition occurred prior to stranding (Table 1). During the care of these animals, clinical improvement was observed in the adults after spontaneous abortion of the pup. Thirteen animals were therefore treated with intramuscular injections of 500 mcg of prostaglandin F2α (Estrumate, Miles, KS) and/or 40 mg dexamethasone (Dexamethasone Sodium Phosphate, Elkins-Sinn, Inc., NJ) to induce abortion.

Reproductive failure as a result of abortion, premature parturition, or death of pregnant female sea lions was observed in 209 intoxicated adult females admitted

Table 1. Types of reproductive failure attributed to domoic acid toxicosis observed in pregnant stranded California sea lions.

Strand date	Induced abortion	Abortion	Premature live birth	Dead *in utero*	Postmortem evidence of pregnancy	Number of offspring lost
May 1998	0	9	3	16	0	28
February 2002	0	1	0	0	1	2
March 2002	0	3	0	4	0	7
April 2002	7	59	19	18	4	107
May 2002	6	30	12	13	3	64
June 2002	0	0	0	1	0	1
Total	13	102	34	52	8	209

to rehabilitation centers in California in 1998 and 2002 (Table 1). Of these females, 108 died. The other 101 animals survived after aborting or giving birth prematurely, and were released. Postmortem examinations were performed within 24 h of death. A subset of tissues from 29 adult animals was examined histologically, and lesions consistent with DA toxicity (neuronal atrophy and necrosis in the hippocampus or dentate gyrus, Silvagni *et al*. 2005) were observed. No evidence of infectious agents or inflammation was detected in 26 fetuses examined histologically.

Urine, amniotic fluid, gastric fluid, or fecal samples were analyzed from 65 adult sea lions and fetuses for DA by the NOAA Marine Biotoxins Program in Charleston, South Carolina. Urine and amniotic fluid were analyzed directly without extraction, whereas fetal gastric fluid samples were primarily liquid and were centrifuged at approximately 3,000 × g for 10 min then filtered through a 0.45-μm nylon filter prior to analysis. A receptor binding assay (RBA) for DA was first used to screen samples for DA. All samples were treated with glutamate decarboxylase to remove endogenous glutamate prior to RBA analyses. The DA RBA uses membrane preparations containing a high density of glutamate (GluR6) receptors, the pharmacological target of DA. The total DA-like activity in each sample was measured using baculovirus expression, and was performed according to the methods of Van Dolah *et al*. (1997).

Samples showing DA-like activity were further analyzed by liquid chromatography with tandem mass spectrometry (LC-MS) for both DA confirmation and quantification. Triplicate analyses of a 5-μL sample were subjected to liquid chromatography utilizing an Agilent 1100 HPLC coupled to an ABI-SCIEX API-4000 triple quadruple mass spectrometer. Separations were conducted utilizing a gradient (5%–95%) flow of methanol/water with the addition of 0.1% trifluoroacetic acid at a flow rate of 2.5 mL/min over a monolithic support C18 (Merck Chromolith SpeedROD RP-18e, 50 × 4.6 mm, Merck KgaA, Darmstadt, Germany). The methanol and water was of HPLC grade from Burdick and Jackson (Muskegon, MI). In each case the entire flow of the chromatography system was directed to a Turbo V^{TM} ESI source. Source conditions, including temperature and gas flows, were adjusted to provide maximum ion intensity for flow injected DA at the flow rate appropriate for the use of the column used in this study. The nominal mass for the parent ion of DA is 312 m/z. Fragment ions characteristic of DA were obtained at 266 Da (loss of carboxyl group from DA during mass spectrometry) and 248 Da (loss of water from 266 m/z). Quantification of DA was carried out using the 266-Da fragment ion by comparison with a standard curve of log dilutions of the domoic acid certified standard reference from NRC Canada (0.01–10 μg/mL).

DA was confirmed by LC-MS in 5 maternal urine samples, 16 amniotic fluid samples, 4 fetal urine samples, and 8 fetal gastric fluid samples tested from 65 adults and fetuses. All of the fetal urine samples, 17 fetal gastric fluid samples and 24 amniotic fluid samples were positive for DA using RBA (Table 2). All samples were collected during the last trimester of pregnancy. Equivalent adult/pup fluid samples from each pair of animals were not available for testing as some amniotic sacs were ruptured at the time of collection, some fetuses had no urine, and others had insufficient gastric contents for testing. In the five adult/pup pairs of animals for which both maternal urine and fetal fluids were available for testing, maternal urine

Table 2. Domoic acid results (number of positive samples/total number tested) using RBA and LC-MS from all samples tested (includes fetal samples without matched maternal samples).

	Maternal urine	Fetal feces	Fetal urine	Fetal gastric fluid	Amniotic fluid
RBA	6/6	0/1	6/6	17/25	24/40
LC-MS	5/5	0/0	4/6	8/8	16/20

levels were higher than fetal fluid levels by at least an order of magnitude. DA was detectable in fetal fluids up to 8 d after the adult female stranded (the latest date she could have ingested DA) (Table 3).

To investigate other potential causes of premature parturition, sera from a subset of aborting females and fetuses were tested for antibodies to *Leptospira interrogans* vars. *pomona*, *grippotyphosa*, *icterohemorrhagica*, *canicola*, *bratislava*, and *hardjo*; *Brucella* spp.; Caliciviruses; and *Chlamydophila psittaci* spp. Tests were performed at the California Animal Health and Food Safety Laboratory (Davis, CA), the National Veterinary Services Laboratories (Ames, IA), and the Oklahoma Animal Disease Diagnostic Laboratory (Stillwater, OK) as described by Burek *et al*. (2003). All serological test results to bacteria were negative (12 tested for *Leptospira* spp., 22 for *Brucella* spp., 5 for *Chlamydophila psittaci*), and nine had low titers of 1:8 to calicivirus serotype 1, suggesting previous exposure to this virus but no active infection (Burek *et al*. 2003).

These data indicate that DA can cause reproductive failure in California sea lions through mortality of pregnant females, abortion and premature parturition of pups. Whether the effects of DA on the fetus are direct or indirect is unclear, as are the mechanisms involved in these effects. Presence of DA in amniotic and fetal fluids shows that DA crosses the placenta of intoxicated adult female sea lions. The mode of transfer is unknown but may involve an active process, as DA is water soluble (Falk *et al*. 1991). The presence of DA in the different fluids suggests that DA in fetal blood is excreted through fetal urine into the amniotic fluid. There it would be diluted and swallowed by the fetus, enter the stomach, and reenter the fetal blood stream. Thus the fetus and amniotic fluid may act as a reservoir of DA initially ingested by pregnant females, ultimately prolonging fetal tissue exposure after ingested DA has been excreted in maternal urine. The presence of DA in the fetal fluids up to a week after the female sea lions stranded is interesting, as DA is typically cleared within hours from the blood stream of exposed sea lions (Gulland 2000). Once in the fetal blood stream, DA may act as it does in adult animals, binding to kainite and alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) subclasses of glutamate receptor causing excitation (Van Dolah *et al*. 2003).

The effects of DA exposure on the developing sea lion fetus are unknown. Experimental studies on pregnant mice suggest *in utero* exposure of fetal mice may cause postnatal hippocampal damage (Dakshinamurti *et al*. 1993). Intraperitoneal administration of DA at $\geq$0.5 mg/kg body weight per day to pregnant rats caused a reduction in the number of live fetuses at term, and a dose-dependent increase in the number of fetuses with visceral or skeletal anomalies (Khera *et al*. 1994). In another

Table 3. Levels of domoic acid from adult/pup samples.

Date adult stranded	Date adult sampled	Date pup sampled	Sample identification	Maternal urine		Fetal urine		Fetal gastric fluid		Amniotic fluid	
				RBA	LC-MS	RBA	LC-MS	RBA	LC-MS	RBA	LC-MS
4/24/2002	5/28/2002	4/25/2002	zc 0253b	+	10			+	1.5		
4/16/2002	4/17/2002	4/24/2002	CSL 5197	+	261	+	38				
4/16/2002	4/17/2002	4/21/2002	CSL 5199	+	61			+	15		
4/18/2002	4/21/2002	4/21/2002	CSL 5201							+	12
4/18/2002	4/20/2002	4/18/2002	CSL 5206	+	251					+	5
4/18/2002	–	4/30/2002	CSL 5207			+	11				
4/20/2002	4/30/2002	4/21/2002	CSL 5208			+	7			+	4
4/29/2002	4/29/2002	5/4/2002	CSL 5217	+	65			+	6		
4/29/2002	–	5/3/2002	CSL 5218			+	<LOD	<DL	4		
5/3/2002	–	5/4/2002	CSL 5222			+		+	0.5		
5/5/2002	–	5/6/2002	CSL 5224			+	<LOD	<DL	3		
5/5/2002	–	5/5/2002	CSL 5225					+	1.2	+	
5/5/2002	–	5/7/2002	CSL 5226					+	1.2	+	34

All values in ng/mL.
<LOD = below LC-MS limit of detection of 0.5 ng/mL.
<DL = below RBA detection limit of 10 ng/mL.
+ = DA-like activity in RBA.

study, subcutaneous injection of pregnant rats on gestation day 13 (0–1.2 mg/kg) did not result in clinically evident toxicity in the pups, in terms of survival or weight gain, but did result in alterations in locomotor activity and sex-related differences in spatial learning in pups during postnatal weeks 4–8 (Levin *et al*. 2005). In sea lions, the concentrations of DA found in fetal fluids are at least an order of magnitude lower than those found in maternal urine. However, as the blood-brain-barrier is incompletely developed in neonates (Xi *et al.* 1997), the dose present in fetal fluids is likely readily bioavailable to neuronal tissues. In addition, less efficient renal clearance in the fetus and recirculation of cleared DA from the amniotic fluid may result in greater sensitivity of the fetus to low internal doses of DA compared to adults.

Alternatively, DA-induced fetal death may be the indirect consequence of anoxia resulting from maternal seizures, although this latter mechanism may not explain the premature birth of live fetuses that subsequently died after birth. The mechanisms involved in inducing these effects require detailed histological examination of fetuses, as well as more focused studies on dynamics of DA transfer from mother to fetus.

There are multiple causes of reproductive failure in mammals, the majority of which are undiagnosed (Williams and Barker 2001). These data from over 200 stranded sea lions suggest that biotoxins may be one of these causes. Reproductive failure due to premature parturition has been well recognized in California sea lions at San Miguel Island, California, and the role of organochlorines and infectious agents in its etiology explored (DeLong *et al*. 1973, Gilmartin *et al.* 1976). Although mean total DDT levels in analyzed tissues of early parturient sea lions were higher than in similar tissues from sea lion females giving birth to full term fetuses, the role of DDT in causing the reproductive failure is unclear due to confounding effects of sea lion age, duration of pregnancy for transfer of contaminants to the fetus, and exposure to infectious agents (Gilmartin *et al.* 1976, O'Shea and Brownell 1998). *Leptospira* spp. and caliciviruses are well documented causes of abortion in other mammals (Williams and Barker 2001) and have been demonstrated in sea lions aborting on San Miguel Island (Smith *et al*. 1974, Gilmartin *et al.* 1976). Thus the relative importance of organochlorine exposure and endemic infectious agents in causing reproductive failure in sea lions remains unclear. DA has only been recognized in marine mammals off California since 1998, so its relative importance in causing premature parturition was not investigated in earlier studies. It is not possible to retrospectively investigate the role DA exposure might have played in the reproductive failure of sea lions in the 1970s and 1980s, as suitable fluids for analysis for DA are not available. However, the ability of DA to cross the placenta, and the extent of reproductive failure in stranded sea lions with symptoms of DA toxicity, suggests that the effects of DA exposure could further complicate the etiology of premature parturition and abortion in sea lions on rookeries. This highlights the value of archiving tissues and fluids for future retrospective studies, as our knowledge of marine mammal health threats improves and technological advances allow the recognition of novel infectious agents and toxins. Further studies are needed to elucidate the relative importance of each factor, as well as effects of El Niño associated changes in nutritional status, and potential as yet unidentified factors, in causing reproductive failure in California sea lions.

ACKNOWLEDGMENTS

We thank the staff and volunteers at the various rehabilitation centers affiliated with the California Marine Mammal Stranding Network for all of their time and energy working with the animals, and Joe Cordaro and John Heyning for their coordination of investigations during the DA events. Samples were collected under National Marine Fisheries Scientific Research Permit No. 932-1489-00, and funding was provided by the National Marine Fisheries Program through the John H. Prescott Marine Mammal Rescue Assistance Program, NOAA Oceans and Human Health Program, and the Marine Mammal Unusual Mortality Event Emergency Fund.

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Received: 12 May 2005
Accepted: 7 November 2005