SHORT COMMUNICATIONS

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Green Tree Frog (*Hyla cinerea*) and Ground Squirrel (*Xerospermophilus spilosoma*) Mortality Attributed to Inland Brevetoxin Transportation at Padre Island National Seashore, Texas, 2015

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ABSTRACT: On 16 September 2015, a red tide (*Karenia brevis*) bloom impacted coastal areas of Padre Island National Seashore Park. Two days later and about 0.9 km inland, 30–40 adult green tree frogs (*Hyla cinerea*) were found dead after displaying tremors, weakness, labored breathing, and other signs of neurologic impairment. A rainstorm, accompanied by high winds, rough surf, and high tides, which could have aerosolized brevetoxin, occurred on the morning of the mortality event. Frog carcasses were healthy but contained significant brevetoxin in tissues. Tissue brevetoxin was also found in two dead or dying spotted ground squirrels (*Xerospermophilus spilosoma*) and a coyote (*Canis latrans*). Rainwater collected from the location of the mortality event contained brevetoxin. Mortality of green tree frog and ground squirrel mortality has not been previously attributed to brevetoxin exposure and such mortality suggested that inland toxin transport, possibly through aerosols, rainfall, or insects, may have important implications for coastal species.

Key words: Brevetoxin, green tree frogs, harmful algal bloom, toxin transport.

On 16 September 2015, 30–40 American green tree frogs (*Hyla cinerea*) were found dead or dying at Padre Island National Seashore, Texas, during a coastal red tide (*Karenia brevis*) event. Many affected frogs exhibited labored breathing, tremors, weakness, and loss of righting reflex prior to death. The number of affected frogs was likely higher as carcasses may have been scavenged or not observed in surrounding vegetation. That morning, a rainstorm with high winds, rough surf, and high tides had occurred. Several days later, spotted ground squirrels (*Xerospermophilus spilosoma*) and a coyote (*Canis latrans*) were found nearby that were either dead or exhibiting neurologic impairment signs. Within a day of the tree frog mortality, a spotted ground squirrel carcass was found within 1.75 km of the tree frog mortality location. On 29 September, 1 km from the area of the tree frog mortality, another ground squirrel exhibiting similar clinical signs died within an hour of being found. On the same day, a coyote that exhibited severe neurologic impairment, and was euthanized, was found 9 km from the frog mortality.

Marine mammals, birds, sea turtles, and terrestrial mammals such as coyotes and domestic dogs (*Canis lupus familiaris*) have been involved in mass mortality events from consuming brevetoxin-contaminated fish and biota (Castle et al. 2013). A large brevetoxicosis mortality event involving coyotes and domestic dogs occurred along the park shore following a large *K. brevis* fish kill in 2009 (Castle et al. 2013), but no brevetoxin-attributed deaths have been reported this far inland.

Amphibian brevetoxicosis has not been previously documented. However, given the temporal proximity of the red tide event, the mixed-species mortalities, and the presence of

neurologic signs prior to death, we hypothesized that the tree frogs died from brevetoxin potentially transported in rainwater or aerosols. Brevetoxin concentrations up to 180 ng/m^3 have been measured in aerosols from rough surf but have not been reported in rainwater (Pierce et al. 1989). We aimed to determine the cause of death of the frogs and to measure brevetoxin levels in rainwater.

Fifteen out of 30–40 affected green tree frogs with minimal decomposition were selectively collected. Carcasses were frozen and nine were sent to the US Geological Survey's National Wildlife Health Center (USGS-NWHC; Madison, Wisconsin) for necropsy, diagnostics, and subsequent brevetoxin assessment by Florida Wildlife Research Institute (FWRI, St. Petersburg, Florida). Ground squirrel and coyote carcasses were examined via gross necropsy by park staff and, together with six frogs, were provided to Texas A&M University–Corpus Christi's Center for Coastal Studies (TAMUCC-CCS, Corpus Christi, Texas) for brevetoxin analysis. Body condition was assessed based on the size of the coelomic fat bodies. Tree frog tissues were analyzed for ranavirus (Torrence et al. 2010).

Four of five frogs necropsied at USGS-NWHC were in fair to good body condition and had moderate to large amounts of insect material in the stomachs; the remaining frog was in poor body condition but also had recently ingested food in the stomach. There were no significant gross or microscopic tissue abnormalities. Tissues from all five frogs, including skin, liver, spleen, and kidney, were negative for ranavirus.

At FWRI, liver, skin, lung, and gastrointestinal tracts of frogs were extracted for brevetoxin as previously described (Naar et al. 2002; Flewelling 2008). Liver was pooled from the five specimens and skin was pooled from two animals to reach a sufficient sample volume for brevetoxin-3 and congeners (PbTx-3 equivalents) quantification using a competitive enzyme-linked immunosorbent assay (ELISA; MARBIONC Development Group, Wilmington, North Carolina, USA). Ultra-performance liquid chromatography (UPLC)-tandem mass spectrometry (Castle et al. 2013) was used to identify the brevetoxin congeners present (specifically PbTx-2, PbTx-3, PbTx-7, PbTx-9, oxidized PbTx-2, cysteine-PbTx-B, and cysteine-PbTx-B-sulfoxide).

The analysis at TAMUCC-CCS of six frogs, livers and muscles from two ground squirrels, liver from one coyote, and rainwater used the Abraxis ELISA kit (Number 520026, Abraxis Inc., Warminster, Pennsylvania, USA). The frogs were rinsed in deionized water, then 70% methyl alcohol was added, and the skin and selected internal organs were dissected and homogenized prior to analysis. The ground squirrel samples were dissected with analysis of liver, skeletal muscle, and gastrointestinal tract and contents, and coyote liver was analyzed from the single animal collected. Rainwater, collected at two locations on 25 September 2015 at an altitude of 90 m and at the dune transition to beach at an altitude of about 900 m from the frog mortality, was analyzed for brevetoxin.

We compared the ELISA results of frog tissues using a *t*-test (α=0.05) to evaluate for differences between the MARBIONC and Abraxis ELISA kits used at the two different labs. No significant differences in brevetoxin concentrations between ELISA test type used were detected so data were pooled (*t*-value 1.86, P=0.11 for skin; t=2.21, P=0.06 for gut and contents). Mean concentrations (SE) of brevetoxin of the skin was 57 ($\pm$6) ng/g and was 87 ($\pm$16) ng/g in gut-intestine. Liver tissue of the 11 frogs averaged 66 ($\pm$20) ng/g (Table 1). Analysis by UPLC showed that only PbTx-3 and the brevetoxin metabolite cysteine-PbTx-B were present in the samples (Table 2).

Brevetoxin levels in two spotted ground squirrels collected at the same time and area as the frogs ranged from 19.3 ng/g in muscle to 493.9 ng/g in liver. Liver from a coyote exhibiting neurologic signs had 92.3 ng/g brevetoxin (Table 1). Two samples of rainwater collected on 25 September from the beach, one directly inland from and the second adjacent to the mortality location, contained 12.3 and 0.6 ng/ml brevetoxin, respectively.

TABLE 1. Concentrations of brevetoxin 3 (PbTx-3) equivalents obtained by enzyme-linked immunosorbent assay testing of tissues from carcasses of American green tree frog (*Hyla cinerea*), ground squirrel (*Xerospermophilus spilosoma*), and one coyote (*Canis latrans*) collected at Padre Island National Seashore, September 2015 following a mortality event caused by brevetoxin putatively aerosolized from a red tide (*Karenia brevis*) bloom.

Species	Sample			PbTx-3 equivalent (ng/g)		
	Tissue	*n*	Mean weight (g)	Mean (SE)	Median	Range
Green tree frog	Skin	10	0.11	57.3 (6.1)	58.8	18.0–92.4
	Gut and contents	11	0.18	86.6 (15.9)	95.4	23.4–165.3
	Liver	7	0.07	65.8 (19.6)	49.2	34.8–181.8
	Skin rinse[a]	6	—[b]	2.3 (0.4)	2.1	1.5–4.0
Ground squirrel	Liver	2	0.27	43.1 (21.3)	—	21.9–64.4
	Skeletal muscle	2	0.38	22.7 (3.4)	—	19.3–26.2
	Gut and contents	1	0.23	—	—	493.9
Coyote	Liver	1	0.34	—	—	92.3

[a] The concentration for skin rinse is given in ng/ml.
[b] (—) Indicates _____.

We report a unique case of brevetoxin-associated green tree frog mortality. Because we do not know the sensitivity of green tree frogs to brevetoxin, we cannot determine whether the brevetoxin levels detected in the frogs were alone sufficient to result in mortality or whether other processes may have contributed. However, the history, neurologic presentation, ELISA results, presence of recently ingested food in the stomach, and lack of other diagnoses support a diagnosis of acute brevetoxicosis. The route of exposure remains unclear. Toxic aerosols can travel up to 4.2 km from the beach and 1.6 km from the coastal shoreline, but concentrations decrease rapidly with increasing distance from shore (Kirkpatrick et al. 2010). All of the carcasses in this study were found 900 m from the beach. Gray tree frogs (*Hyla versicolor*) are a closely related species with a maximum recorded movement distance of 330 m over a period of days to weeks (Johnson et al. 2007). It is unlikely the affected tree frogs would have been able to directly contact seawater or spray and return to their breeding ponds prior to their death.

It is encouraging that comparison of ELISA from two manufacturers yielded results that were not statistically different. A more-formal comparison involving shared paired samples and different laboratories performing both tests would be useful.

Aerosolized brevetoxin alone would be unlikely to be concentrated enough to cause

TABLE 2. Total brevetoxin concentrations (ng/g), reported as brevetoxin 3 (PbTx-3) and cysteine-PbTx-B congeners combined, measured by ultra-performance liquid chromatography tandem mass spectroscopy in tissues of American green tree frogs (*Hyla cinerea*) recovered at Padre Island National Seashore, September 2015 following a mortality event caused by brevetoxin putatively aerosolized from a red tide (*Karenia brevis*) bloom.

Tissue	*n*	Total PbTx-3+cysteine-PbTx-B (ng/g)		
		Mean	Median (SE)	Range
Skin[a]	4	10	14.9 (1.73)	12.4–22.0
Gut and contents	5	22	20.7 (3.18)	12.6–32.3
Liver (pooled)	1	—	—	66.4

[a] Cysteine-PbTx-B was not detected in two of the four skin samples.
[b] (—) Indicates _____.

death in other species (Benson et al. 2005), but the effects of aerosolized or dissolved brevetoxin exposure in amphibians capable of cutaneous respiration are unknown. High levels of brevetoxin were detected in frog gut contents, suggesting toxin was also consumed, potentially in rainwater or in contaminated insects. The route of exposure influences brevetoxin toxicity. Baden and Mende (1982) established an oral acute 24 h 50% lethal dose (LD_{50}) in mice of 520 μg/kg for PbTx-3 and 6,600 μg/kg for PbTx-2, with similar LD_{50} for the two toxins when dosed intravenously and intraperitoneally. Doses as low as 20 μg/kg were toxic in 30 min when administered intravenously to guinea pigs (Franz and LeClaire 1989).

Brevetoxin particles range from 6–10 μm; particles this size quickly accumulate in precipitation and are deposited during rainfall (Andronache 2003). The rainwater samples collected 9 d following frog mortalities had lower brevetoxin levels than did the frog and ground squirrel tissues. Red tide events can be variable in space and time. Higher wind speeds (23–34 km/h compared to 16–25 km/h) and lower total rainfall (0.13 cm of rain compared to 0.36 cm) occurred immediately prior to the mortality event compared to the day in which we sampled rainfall, so we hypothesize brevetoxin rainfall concentrations were likely higher on the day of the mortality event.

More information is needed on brevetoxin impacts to terrestrial fauna, particularly as climate change is predicted to increase the frequency and severity of red tides. Until now, terrestrial fauna mortalities are known from the consumption of brevetoxin-killed fish. The spotted ground squirrels and coyote we tested had tissue brevetoxin levels consistent with those reported during a previous canid die-off (Castle et al. 2013). Coyotes are known to feed on red tide–killed fish, but the ground squirrel diet is typically herbaceous and they are not known to frequent beaches. Microcystins in freshwater systems can be transported in riparian food webs as emerging aquatic insects are consumed by insectivorous birds and other predators. Attention to the long-distance inland transport of toxin may be critical for managing brevetoxin events (Moy et al. 2016).

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