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Tissue uptake, distribution and excretion of brevetoxin-3 after oral and intratracheal exposure in the freshwater turtle *Trachemys scripta* and the diamondback terrapin *Malaclemys terrapin*

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ABSTRACT

Harmful algal blooms (HABs) occur nearly annually off the west coast of Florida and can impact both humans and wildlife, resulting in morbidity and increased mortality of marine animals including sea turtles. The key organism in Florida red tides is the dinoflagellate *Karenia brevis* that produces a suite of potent neurotoxins referred to as the brevetoxins (PbTx). Despite recent mortality events and rehabilitation efforts, still little is known about how the toxin directly impacts sea turtles, as they are not amenable to experimentation and what is known about toxin levels and distribution comes primarily from post-mortem data. In this study, we utilized the freshwater turtle *Trachemys scripta* and the diamondback terrapin, *Malaclemys terrapin* as model organisms to determine the distribution, clearance, and routes of excretion of the most common form of the toxin, brevetoxin-3, in turtles. Turtles were administered toxin via esophageal tube to mimic ingestion (33.48 μg/kg PbTx-3, 3×/week for two weeks for a total of 7 doses) or by intratracheal instillation (10.53 μg/kg, 3×/week for four weeks for a total of 12 doses) to mimic inhalation. Both oral and intratracheal administration of the toxin produced a suite of behavioral responses symptomatic of brevetoxicosis. The toxin distributed to all organ systems within 1 h of administration but was rapidly cleared out over 24–48 h, corresponding to a decline in clinical symptoms. Excretion appears to be primarily through conjugation to bile salts. Histopathological study revealed that the frequency of lesions varied within experimental groups with some turtles having no significant lesions at all, while similar lesions were found in a low number of control turtles suggesting another common factor(s) could be responsible. The overall goal of this research is better understand the impacts of brevetoxin on turtles in order to develop better treatment protocols for sea turtles exposed to HABs.

1. Introduction

Harmful algal blooms (HABs, Red tides) are increasing in frequency and distribution worldwide (Brand and Compton, 2007; Anderson et al., 2002). These blooms are one of the many threats faced by fish, dolphins, manatees, and sea turtles and they can result in extensive mortalities. HABs typically occur when there is an overload of oceanic nutrients (phosphorus and nitrogen) and an increase in water temperatures; this triggers higher rates of reproduction in dinoflagellates. These single-celled protists rapidly accumulate, and can release toxins into their surrounding environment. The ciguatoxins, saxitoxins and brevetoxins are groups of potent neurotoxins released by different species of dinoflagellates that cause nervous and muscular deficits in exposed animals (Landsberg and Lefebvre, 2014; Fire and Van Dolah, 2012). The brevetoxins are released by the dinoflagellate *Karenia brevis*; brevetoxins bind to and open voltage gated sodium channels (VGSCs) in excitable cells. Brevetoxins specifically bind to site 5 on VGSCs, causing the channels to remain in the open conformation and thus causing continuous depolarization (Poli et al., 1986; Rein et al., 1994). The neurotransmitter glutamate is released and binds to *N*-methyl-D-aspartate (NMDA) receptors, which with the subsequent calcium influx, can trigger intracellular events leading to apoptosis. Brevetoxins can thus affect the neuronal, muscular, and

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cardiac systems directly when present in high enough concentrations (Dechraoui et al., 2006; Dechraoui and Ramsdell, 2003). Symptoms of brevetoxicosis may include ataxia, head bobbing, muscle twitching, partial to complete paralysis, and possibly long-term behavioral changes (Baden and Mende, 1982; Kreuder et al., 2002; Fauquier et al., 2013). Brevetoxins at lower concentrations can depress pulmonary and cardiac function, induce inflammation, (Abraham et al., 2005; Borison et al., 1985; Baden et al., 2005) and suppress immune function (Perrault et al., 2014, 2016; Walsh et al., 2008, 2010). People are impacted by brevetoxins primarily in the form of Neurotoxic Shellfish Poisoning (NSP) from the consumption of contaminated shellfish (Morris et al., 1991; Reich et al., 2015), while asthmatics can be severely affected from inhalation of aerosolized toxins (Fleming et al., 2009).

K. brevis red tides have also been associated with elevated morbidity and mortality in marine animals, including three large dolphin die-offs in the Gulf of Mexico between 1999 and 2006 (Twiner et al., 2012), and mass mortalities in manatees (Bossart et al., 1998; Flewelling et al., 2005), seabirds (Kreuder et al., 2002) and fish (Naar et al., 2007). More than 109 loggerhead sea turtles deaths in 2005 and over 70 sea turtle deaths in 2006 were also attributed to brevetoxin exposure (Fauquier et al., 2013). Juvenile and sub-adult turtles that reside near areas where HABs frequently occur are at high risk for toxin exposure. Not only are animals at risk for toxicity via inhalation (Bossart et al., 1998) and ingestion, but exposure may be greater due to bioaccumulation and biomagnification (Fauquier et al., 2013); seagrasses, crustaceans, and fish accumulate toxins on or in their tissues and may impact animals that consume them, sometimes long after a bloom has occurred (Flewelling et al., 2005; Naar et al., 2007; Perrault et al., 2016). Both loggerheads that prey on filter-feeding invertebrates (Bjorndal, 1996) and seagrass-grazing green sea turtles may thus be vulnerable to the effects of brevetoxin bioaccumulation.

Despite recent mortality events and rehabilitation efforts, still little is known about how the toxin directly impacts marine animals, as they are not amenable to experimentation and what is known about toxin levels and distribution comes primarily from post-mortem data. Recent studies on sea turtles have included analyses of brevetoxin levels in blood in animals admitted for rehabilitation (Fauquier et al., 2013; Perrault et al., 2014) and in nesting females (Perrault et al., 2016), and reports of rehabilitation treatment protocols (Manire et al., 2013), but without a better understanding of the initial organ system impacts, toxin distribution and elimination, rate of clearance, and physiological effects due to toxin exposure, it is difficult to devise effective treatment protocols. Since all sea turtles are listed as threatened or endangered however, these concerns cannot be directly addressed as experiments increasing morbidity and mortality, especially in the large number of animals required, would not be permitted. We have therefore developed the freshwater turtle, *Trachemys scripta*, as a model system to investigate brevetoxin's action in the reptilian system, and have shown recently that the toxin acts on nerve cells in turtles in the same manner as has been shown in laboratory studies on mammals (Cocilova and Milton, 2016). *T. scripta* is a well-studied model animal with a great deal known about its physiology, especially neuronal function (Milton and Prentice, 2007), which is a primary target of brevetoxin. And as breath-hold diving turtles with low standard metabolic rates, *T. scripta* will share a similar basic physiology with sea turtles. The goal of this study was to utilize brevetoxin exposed *T. scripta* to determine the initial organ system distribution of the toxin, rates and mechanisms of clearance, and potential histopathological effects. Brevetoxin-3 was utilized in this study because it is a major component of the brevetoxin mixture produced by *K. brevis* (Baden, 1989) and also of brevetoxin-containing aerosols measured along red tide affected beaches (Cheng et al., 2005), making it the most likely candidate to affect wildlife through ingestion or inhalation. By mimicking inhalation and ingestion exposure, and determining organ effects at different time points, we established a basic understanding of brevetoxin impacts; the ultimate goal of this research is to devise treatment strategies that will improve rehabilitation outcomes and reduce deaths caused by red tide exposure in sea turtles. Treatments will be targeted to helping reduce neurological symptoms and to eliminate PbTx-3 more rapidly. We also utilized a second comparative model organism, the diamondback terrapin (*Malaclemys terrapin*), which is an estuarine species and thus may have more in common physiologically with sea turtles than *T. scripta*. Since no significant differences between the freshwater turtle and the terrapin were found, we limited the use of this species as it is listed as protected in Florida.

2. Materials and methods

2.1. Experimental animals

All work was approved by the Florida Atlantic University Institutional Animal Care and Use Committee (IACUC) and all animals were acclimated to the laboratory for two weeks prior to any dosing experiments. Fifty-seven male and female juvenile freshwater turtles (*T. scripta*), approximately 15–20 cm straight carapace length and weighing 0.40–1.10 kg were obtained from a commercial supplier (Niles Biological Inc., Sacramento, CA) and maintained in tanks at room temperature (22 °C ± 3 °C, 50% relative humidity ± 4%) on a 12 h day/night cycle. *T. scripta* turtle tanks were cleaned according to standard husbandry methods and fed (commercial aquatic food, to satiety) 3× weekly. All animals were given individual identification numbers and randomly assigned to an experimental group.

Twelve male diamondback terrapins (*M. terrapin*) approximately 10 cm straight carapace length and weighing 0.16–0.26 kg were captured by hand from a near shore barrier island off of Apalachicola, Florida, under a Florida FWC scientific collecting permit. Only males were collected to reduce impacts on the breeding population. Animals were brought back to Florida Atlantic University where they were maintained in brackish water. Animal tanks were cleaned 3×/week and they were fed frozen shrimp and fish to satiety. All animals were given individual identification numbers and randomly assigned to an experimental group.

2.2. Brevetoxin

Brevetoxin (PbTx-3) was purchased from LKT Laboratories (St. Paul, Minnesota) and was dissolved in ethanol and mixed with 0.9% NaCl saline to a final concentration of 0.05 μg/μl. Turtles were restrained by hand and administered PbTx-3 orally (33.48 μg/kg, 3×/week for two weeks for a total of 7 doses) via esophageal tube to mimic ingestion or by intratracheal instillation (IT) (10.53 μg/kg, 3×/week for four weeks for a total of 12 doses). For intratracheal doses, the toxin was administered ~1.5 cm into the tracheal opening at the base of the tongue, and a rubber bulb and pipette tip were used to inflate the lungs 3 times following instillation of the toxin to mimic inhalation. Appropriate doses were determined using a dosage curve ranging from 22.32 μg/kg to 33.48 μg/kg for oral dosing and 3.12 μg/kg to 13.16 μg/kg for IT exposures; the highest IT dose increased mortality and so the next lower dose was selected, which induced obvious neuronal deficits with low mortality (Table 1). The initial dose curves were based on previous mammalian studies using 18.6 μg/kg for oral exposure and 6.6 μg/kg or lower for intratracheal instillation exposures in rats (Benson et al., 1999; Cattet and Geraci, 1993; Tibbetts et al., 2006). While our initial dose curve started much lower based on differences in metabolic rates between mammals and reptiles, at the

Table 1
Dose curve experiments for IT and oral PbTx-3 exposures in *T. scripta*. IT PbTx-3 doses ranged from 3.12 μg/kg to 13.16 μg/kg with a final concentration of 10.53 μg/kg. Oral PbTx-3 doses ranged from 22.32 μg/kg to 33.48 μg/kg. Behavioral symptoms were noted after each subsequent PbTx-3 exposure. When present, symptoms for IT exposures occurred within minutes post toxin exposure and oral symptoms occurred ~30 min post toxin exposure.

Experimental group	Dose (μg/kg)	Behavioral symptoms
IT	3.12	Alert, responsive to touch, no notable signs of brevetoxicosis
IT	4.68	Alert, responsive to touch, slight movement in the tanks
IT	7.02	Reduced ambulation, mild twitching, slight head bobbing
IT	10.53	Immediate muscle twitching, swimming in circles, head bobbing, partial to complete paralysis, ataxia
IT	13.16	Coma, death
Oral	22.32	Responsive to touch, mild head bobbing, slow movements, mild twitching
Oral	27.95	Responsive to touch, limb twitching, muscular spasms
Oral	33.48	Severe ataxia, head bobbing, partial to complete paralysis, comatose

lower doses no behavioral or pathological effects were observed and tissue concentrations were below the detection limit except in the kidney, liver and feces. Thus, the toxin was increased until noted neurological effects were present. Terrapins were orally dosed (30.13 μg/kg, a 10% decrease due to smaller size) 3×/week for two weeks for a total of 7 doses. Control animals received sham doses of physiological saline solution mixed with ethanol to a final concentration to 0.1% EtOH (Cocilova and Milton, 2016) to mimic the treatment solution, and sham exposures were conducted as in toxin exposures.

2.3. Tissue and fluid collection

All turtles were euthanized by decapitation and the following tissues and fluids were collected for ELISA assays: kidney, liver, intestines, bile, brain, heart, spleen, lung, trachea, and plasma. Whole blood was collected by exsanguination, feces were removed from the large intestine, and fluids were collected directly from the organ with a syringe post mortem. While the urine, feces and fat were collected for *T. scripta* experiments we were unable to obtain adequate material for these samples from the terrapins. Tissue and fluid samples were collected for both oral and IT post exposure for time points 1 h, 24 h, 48 h, and 1 wk post final PbTx-3 exposure to examine rates of clearance (Table S1). Tissues samples were divided and one part flash frozen in liquid nitrogen and stored at −80 °C. Plasma was extracted from whole blood by centrifugation and the plasma and other fluid samples were frozen at −80 °C. Tissue and fluid samples were sent to Florida Fish and Wildlife Research Institute overnight on dry ice and remained frozen at −80 °C until processing. The remaining solid tissue sample portions were placed in 10% neutral buffered formalin and shipped to Dr. Gregory Bossart for histopathology.

2.4. Brevetoxin analysis

2.4.1. Extraction

A competitive ELISA was used to detect brevetoxins in turtle tissues and biological fluids, as previously described (Fauquier et al., 2013). Solid tissues were thawed prior to extraction and homogenized in the presence of 80% aqueous methanol (4 ml/g tissue). Homogenates were centrifuged for 10 min at 3000 × *g* and the supernatants were transferred to clean 50 ml centrifuge tubes. The pellets were extracted a second time in the same manner and the supernatants were pooled. The combined extracts were then partitioned once with 100% hexane (1:1, v:v), and the methanol fractions were retained at −20 °C until analyzed. Brevetoxin in bile was extracted by liquid-liquid extraction. In 15 ml centrifuge tubes, bile (0.5 ml) was combined with ethyl acetate (1 ml) and vortexed for 1 min. After centrifuging for 5 min at 3000 × *g*, the top ethyl acetate fraction was transferred to a clean 15 ml centrifuge tube, the bottom bile fraction was extracted two more times in the same manner, and the ethyl acetate fractions were combined. Deionized water (3 ml) was then added to the combined ethyl acetate fraction, the sample was vortexed for 1 min, centrifuged for 5 min at 3000 × *g* and the top ethyl acetate fraction was transferred to a clean disposable glass test tube. The ethyl acetate was evaporated to dryness in a Speedvac (Thermo Scientific Savant) and the residue was re dissolved in 1 ml of 80% aqueous methanol. Urine and plasma were not extracted. They were thawed and diluted in phosphate buffered saline (pH 7.4) containing 0.05% Tween 20 and 0.5% gelatin immediate prior to analysis.

2.4.2. ELISA

Brevetoxins and brevetoxin-like compounds were quantified in all samples extracts using a competitive enzyme linked immunosorbent assay (ELISA; Marbionc, Wilmington NC) performed according to Naar et al. (2002) with modifications described in Flewelling (2008). Toxin concentrations were calculated using a PbTx-3 standard curve, and results are reported in PbTx-3 equivalents. The limit of detection as described was approximately 1–2 ng/ml of plasma or urine, 10 ng/ml of bile, and 10 ng/g of feces or tissue.

2.4.3. LC–MS

A subset of samples (primarily feces and some urine) were analyzed for PbTx-3 and two common brevetoxin metabolites for which reference material was available (S-desoxy-BTX-B2 and BTX-B2) using liquid chromatography with tandem mass spectrometry (LC-MS/MS). Prior to analysis, a 0.5 g equivalent of feces extract diluted to 25% methanol or 1 ml of urine was applied to a preconditioned Strata-X cartridge (Phenomenex; 60 mg, 3 ml). The column was washed with 6 ml of 20% methanol and toxins were eluted with 4 mL of 100% methanol. The methanol extract was then evaporated to dryness and re-dissolved in 0.5 ml of 100% methanol. LC-MS/MS analyses were performed using an Acquity UPLC system coupled to a Quattro micro™ API triple quadrupole mass spectrometer as described in Sunda et al. (2013). Toxins were quantified using a 6-point calibration of a mixed standard of pure brevetoxins purchased from Marbionc (Wilmington, NC). Control extracts of feces and urine were spiked with multiple concentrations of PbTx-3 to provide matrix-matched standards for quantifying PbTx-3.

2.5. Histopathology

Multiple tissue sections were collected and fixed in 10% neutral buffered formalin for histopathology. Freshwater turtle (*T. scripta*) samples included the brain, heart, kidney, liver, spleen, lung, trachea and intestine (and some adrenal gland, pancreas, ovary and testicle, where removed with the target tissue) and kidney, liver, lung, trachea, intestine (and some testicle, epididymis and stomach) from diamond backed terrapins (*M. terrapin*). Brain, heart and spleen of *Malaclemys* could not be utilized for histopathology due to their small size; the full tissue was needed for ELISA. Formalin-fixed tissues were routinely processed, embedded in paraffin, sectioned

at 5 μm, and stained with hematoxylin and eosin for examination by light microscopy as previously described (Benson et al., 2005; Bossart et al., 1998; Kreuder et al., 2002). The tissues were initially evaluated blindly followed by a review of the experimental protocol for each group.

2.6. Statistics

Data were analyzed using one-way analysis of variance (ANOVA) followed by Holm-Sidak Test (Holm-Sidak) pairwise comparison test using Sigma Plot 11.0 (Systat Software, Inc, San Jose, California). Samples in which brevetoxin was not measurable were given a value of half the level of detection (2.5 ng per g tissue or ml bile or 0.5 ng per ml fluid) to calculate mean. Significant differences are indicated by asterisks where one asterisk (*) represents $p \leq 0.05$ and three asterisks (***) represents $p \leq 0.001$. PbTx-3 concentrations were compared 1 h post exposures to the 24 h, 48 h, and 1 wk exposures.

3. Results

3.1. Brevetoxin exposure causes neurological and muscular deficits

All experimental animals exposed to final concentrations of PbTx-3 either orally or intratracheally exhibited muscular and neurological symptoms consistent with brevetoxicosis including head bobbing, muscle twitching, ataxia, swimming in circles, partial to complete paralysis, penile prolapse in males, edema, and in some cases apparent coma. The onset of symptoms in the oral experimental groups began ~30 min post PbTx-3 exposure and in the intratracheally exposed animals ~2–5 min post PbTx-3 exposure; symptoms were similar and did not become more or less severe with subsequent doses. Clinical signs were recorded every hour for the first 8 h following each exposure and daily thereafter. The clinical neurological symptoms declined over a 24 h period and lethargy were the primary symptom that persisted for longer than 24–48 h as well as decreased appetite for the duration of the experiment. Animals that were unresponsive for up to 4 h following PbTx-3 exposure were euthanized (Table S2). The terrapin experimental groups exhibited clinical signs consistent with those observed in *T. scripta*. Animals exposed to lower doses of PbTx-3 showed reduced symptoms of brevetoxicosis (Table 1). No behavioral changes were observed in sham treated animals.

3.2. PbTx-3 distributes to all organ systems, with the highest concentrations in the detoxification and excretion systems

PbTx-3 was distributed to all *T. scripta* organs systems sampled in both oral and IT treatment groups. By 1 h post oral toxin exposure concentrations were highest in the detoxification and excretion systems: the kidney, liver, and feces (Table 2). IT exposed animals had the highest PbTx-3 concentrations in the trachea, liver, and bile 1 h post toxin exposure (Table 3). Lower PbTx-3 concentrations were found in the brain, lung, fat, urine, intestine and plasma for all animal groups. The oral exposure animals showed higher initial toxin levels overall in their tissues and fluids as significantly higher concentrations had to be administered orally to achieve the same symptoms compared to the IT experiments. By 24 h post exposure, all tissues and fluids other than the feces and bile averaged less than 55 ng/g (ng/ml) of toxin. The toxin similarly distributed to all organ systems in *M. terrapin*, although toxin levels there were highest in the kidneys, and the liver concentrations were similar to other organs including the brain, spleen, and heart (Table 4).

Table 2
Distribution of PbTx-3 in *T. scripta* tissues and fluids post oral (33.48 μg/kg) exposure. Parenthesis indicates the range of PbTx-3 found in each tissue (ng/g) or fluid (ng/ml). Intestines were analyzed with contents. Significant values were determined based on initial toxin concentrations at 1 h post exposure and comparing them to 24 h, 48 h and 1 wk time points. <LD values for sham control animals are not included in statistics. Samples in which brevetoxin was not detectable were given a value of half the level of detection (2.5 ng per g tissue or ml bile or 0.5 ng per ml fluid) to calculate mean. $n \geq 4$. *** Indicates $p \leq 0.001$ and * indicates $p \leq 0.05$ using a one-way AVOVA.

PbTx-3 ng/g tissue or ng/ml fluid (mean ± SEM)				
Organ	1 h	24 h	48 h	1 wk
Kidney	145.7 ± 24.9 (89.4–231.3)	52.1 ± 27.5* (2.5–149.9)	13.6 ± 3.8*** (2.5–26.0)	9.5 ± 3.2*** (2.5–17.7)
Urine	5.9 ± 1.5 (1.7–14.5)	7.7 ± 1.9 (4.3–17.1)	2.3 ± 0.6 (0.5–3.2)	8.6 ± 6.8 (0.5–35.4)
Liver	177.5 ± 19.7 (127.6–256.6)	45.5 ± 23.6* (2.5–120.0)	7.5 ± 3.1*** (2.5–16.8)	2.5 ± 0.0*** 2.5
Intestines	26.0 ± 7.3 (11.0–59.2)	13.7 ± 6.9 (2.5–33.0)	2.5 ± 0.0 2.5	2.5 ± 0.0 2.5
Bile	49.3 ± 7.2 (11.6–86.3)	72.1 ± 22.4 (27.7–222.7)	77.5 ± 30.3 (2.5–148.0)	34.5 ± 10.1 (2.5–57.3)
Feces	192.9 ± 80.0 (81.1–430.2)	220.0 ± 123.8 (2.5–367.6)	155.8 ± 90.0 (26.0–417.9)	159.4 ± 115.5 (2.5–613.7)
Brain	71.9 ± 8.1 (40.5–98.0)	27.9 ± 16.1* (2.5–79.4)	2.5 ± 0.0*** 2.5	2.5 ± 0.0*** 2.5
Heart	61.4 ± 4.8 (47.1–74.1)	19.6 ± 11.2*** (2.5–57.6)	2.5 ± 0.0*** 2.5	2.5 ± 0.0*** 2.5
Spleen	55.4 ± 6.5 (41.1–76.6)	21.1 ± 11.7* (2.5–57.1)	3.7 ± 1.2*** (2.5–8.7)	2.5 ± 0.0*** 2.5
Lung	36.5 ± 5.8 (22.1–59.7)	21.4 ± 11.6 (2.5–50.6)	2.5 ± 0.0* 2.5	2.5 ± 0.0* 2.5
Trachea	23.3 ± 3.8 (10.1–38.9)	12.6 ± 6.2 (2.5–30.3)	2.5 ± 0.0*** 2.5	2.5 ± 0.0*** 2.5
Plasma	12.6 ± 1.5 (6.2–19.4)	3.1 ± 1.3*** (0.5–11.5)	0.5 ± 0.1*** (0.5–0.8)	0.5 ± 0.0*** 0.5
Fat	62.1 ± 11.3 (37.5–113.9)	23.7 ± 13.8* (2.5–70.1)	6.6 ± 4.1*** (2.5–23.1)	2.5 ± 0.0*** 2.5

Table 3
Distribution of PbTx-3 in *T. scripta* tissues and fluids post intratracheal instillation (10.53 μg/kg). Parenthesis indicates the range of PbTx-3 found in each tissue (ng/g) or fluid (ng/ml). Intestines were analyzed with contents. Significant values were determined based on initial toxin concentrations at 1 h post exposure. <LD values for sham control animals are not included in statistics. Samples in which brevetoxin was not detectable were given a value of half the level of detection (2.5 ng per g tissue or ml bile or 0.5 ng per ml fluid) to calculate mean. $n \geq 4$. *** Indicates $p \leq 0.001$ and * indicates $p \leq 0.05$ using a one-way ANOVA.

PbTx-3 ng/g tissue or ng/ml fluid (mean ± SEM)				
Organ	1 h	24 h	48 h	1 wk
Kidney	38.6 ± 4.4 (21.8–52.6)	10.8 ± 2.9*** (2.5–29.4)	14.7 ± 4.5*** (2.5–28.5)	4.8 ± 1.4*** (2.5–9.1)
Urine	5.9 ± 2.1 (2.0–13.9)	5.3 ± 2.0 (1.1–13.5)	10.1 ± 8.5 (1.1–43.9)	2.0 ± 0.5 (1.0–2.7)
Liver	44.8 ± 3.5 (36.3–57.3)	10.5 ± 3.2*** (2.5–23.3)	9.1 ± 4.4*** (2.5–24.4)	4.2 ± 1.7*** (2.5–12.5)
Intestines	8.0 ± 1.6 (2.5–13.1)	2.5 ± 0.0 2.5	3.6 ± 1.1* (2.5–8.0)	2.5 ± 0.0* 2.5
Bile	36.1 ± 4.3 (21.8–45.5)	34.4 ± 5.9 (23.3–80.3)	38.8 ± 6.2 (28.5–61.7)	13.1 ± 1.2* (9.6–16.7)
Feces	39.2 ± 11.1 (15.2–8.9)	31.2 ± 8.1 (9.8–82.8)	42.4 ± 15.4 (16.6–100.7)	28.7 ± 14.8 (2.5–71.3)
Brain	30.2 ± 3.0 (22.6–40.7)	6.7 ± 3.2*** (2.5–21.7)	4.5 ± 2.0*** (2.5–12.3)	2.5 ± 0.0*** 2.5
Heart	20.0 ± 1.2 (17.1–23.8)	4.1 ± 1.6*** (2.5–11.8)	3.9 ± 1.4*** (2.5–9.5)	2.5 ± 0.0*** 2.5
Spleen	22.2 ± 2.2 (16.7–31.4)	3.6 ± 1.1*** (2.5–12.4)	7.6 ± 3.2*** (2.5–18.0)	4.2 ± 1.7*** (2.5–12.8)
Lung	20.0 ± 6.0 (2.5–52.2)	3.1 ± 0.6* (2.5–8.2)	3.5 ± 1.0 (2.5–7.7)	2.5 ± 0.0* 2.5
Trachea	363.3 ± 106.4 (72.5–805.1)	11.1 ± 2.7* (2.5–24.7)	11.9 ± 4.5* (2.5–24.2)	10.7 ± 3.6* (2.5–26.2)
Plasma	4.8 ± 1.0 (3.5–5.1)	1.4 ± 0.5*** (0.5–5.3)	1.3 ± 0.5* (0.5–2.5)	0.6 ± 0.1*** (0.5–1.0)
Fat	15.2 ± 4.0 (2.5–26.7)	6.4 ± 2.9 (2.5–25.7)	2.5 ± 0.0 2.5	2.5 ± 0.0 2.5

Table 4
Distribution of PbTx-3 in *Malaclemys terrapin* tissues and fluids post oral (30.13 μg/kg) exposure. Parenthesis indicates the range of PbTx-3 found in each tissue (ng/g) or fluid (ng/ml). Intestines were analyzed with contents. Significant values were determined based on initial toxin concentrations at 1 h post exposure. <LD values for sham control animals are not included in statistics. Samples in which brevetoxin was not detectable were given a value of half the level of detection (2.5 ng per g tissue or ml bile or 0.5 ng per ml fluid) to calculate mean. $n = 2$–4 samples per time point. *** Indicates $p \leq 0.001$ and * indicates $p \leq 0.05$ by a one-way ANOVA.

PbTx-3 ng/g tissue or ng/ml fluid (mean ± SEM)			
Organ	1 h	24 h	1 wk
Kidney	76.1 ± 19.5 (37.2–96.7)	7.5 ± 2.6* (2.5–11.1)	2.5 ± 0.0* 2.5
Liver	32.8 ± 3.5 (26.0–37.2)	2.5 ± 0.0*** 2.5	2.5 ± 0.0*** 2.5
Intestines	14.1 ± 5.6 (7.8–25.4)	2.5 ± 0.0 2.5	2.5 ± 0.0 2.5
Bile	17.7 ± 4.4 (12.0–26.4)	19.0 ± 1.8 (16.8–22.6)	10.7 ± 1.5 (9.2–12.1)
Brain	41.0 ± 9.8 (21.4–51.9)	2.5 ± 0.0* 2.5	2.5 ± 0.0* 2.5
Heart	38.3 ± 8.7 (21.3–50.2)	2.5 ± 0.0* 2.5	2.5 ± 0.0* 2.5
Spleen	37.9 ± 14.0 (12.5–60.8)	2.5 ± 0.0 2.5	2.5 ± 0.0 2.5
Lung	13.8 ± 2.8 (8.4–17.2)	2.5 ± 0.0* 2.5	2.5 ± 0.0* 2.5
Trachea	9.4 ± 3.6 (2.5–14.8)	2.5 ± 0.0 2.5	2.5 ± 0.0 2.5
Plasma	11.3 ± 1.4 (8.5–12.6)	0.63 ± 0.1* (0.5–0.9)	0.5 ± 0.0* 0.5

3.3. PbTx-3 clears from tissues 24–48 h post exposure

PbTx-3 rapidly clears from each organ system over a period of 24–48 h for both oral and IT exposures, and in both *T. scripta* and *M. terrapin*, which coincides with the decline in clinical symptoms over the same time period. The highest PbTx-3 concentrations in *T. scripta* were initially present in the liver and kidney 1 h after oral exposure while bile levels rose from 24 h post exposure to 48 h, though the increase was not statistically significant (Fig. 1A). Similarly, kidney and liver levels were high following IT exposures but rapidly declined (Fig. 1B). By 1 wk, PbTx-3 in the tissues and fluids were near or below the ELISA detection limits for both oral and IT exposures. The results for both routes of exposure are consistent, reflecting a rapid decrease in toxin concentration over time with increases in the bile and feces; despite high initial toxin levels in the kidney, urine levels remained relatively low. Though the rapid appearance by 1 h of elevated levels in the bile and feces resulted in further increases being not statistically significant, the results clearly suggest that removal via the bile and feces is the primary route of excretion, though in two animals where LC-MS/MS was run, the amount of PbTx-3 present in the urine was greater than that in the feces.

The metabolic pathways and products of brevetoxin in turtles are not fully characterized, and analytical standards are unavailable even for known metabolites; however LC-MS/MS analyses were done on a subset of samples to further examine metabolism and excretion. Samples analyzed were primarily feces, covering a range of post-intratracheal exposure time points (24 h to 1 month). For four turtles exposed orally ($n = 2$) or by intratracheal instillation ($n = 2$), both urine and feces collected within 24 h of exposure were analyzed. Of PbTx-3 and the two common brevetoxin metabolites monitored for, only PbTx-3 was observed. Brevetoxin is known to metabolize extensively in some animals, but the lack of analytical standards prevented us from identifying more metabolites. However, the degree of metabolism of PbTx-3 by *T. scripta* can be inferred by calculating the difference between toxin concentrations measured by ELISA and levels of PbTx-3 quantified by LC–MS/MS

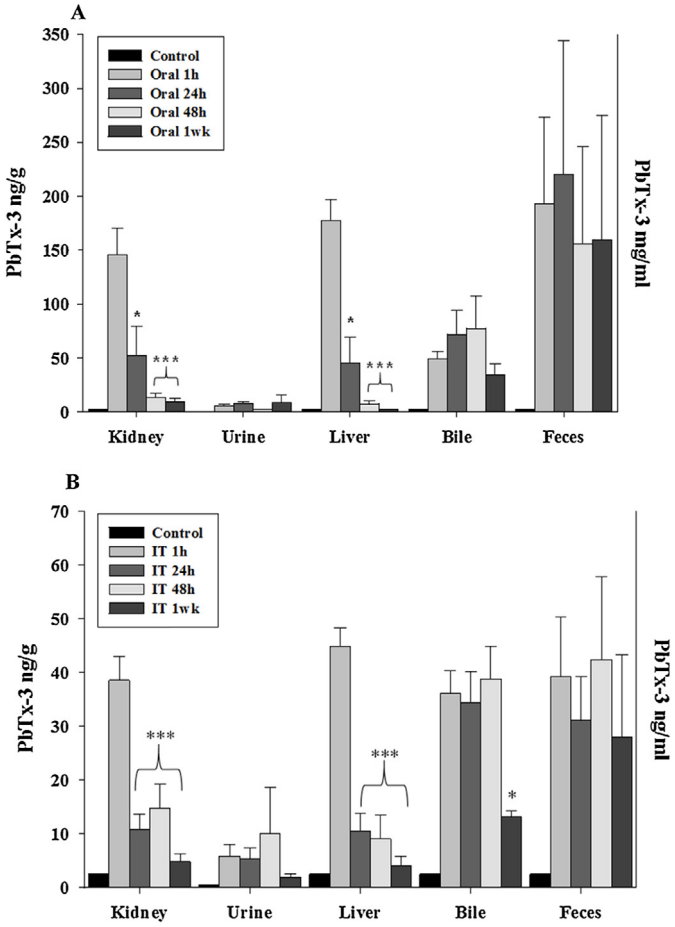

Fig. 1. Distribution and concentrations of brevetoxin (ng PbTx-3 eq. per g or ml) in the excretion tissues and fluids of *T. scripta* over 1 week post oral exposures (A) and following intratracheal instillation (B). Error bars represent standard error of the mean and significance was determined using a one-way ANOVA where *** $p \leq 0.001$ and * $p \leq 0.05$ compared to 1 h post-exposure samples, $n \geq 4$. Samples in which brevetoxin was not detectable were given a value of half the level of detection (2.5 ng per g tissue or ml bile or 0.5 ng per ml fluid) to calculate mean. Control (sham) exposures were all <LD.

analysis. In turtles exposed via intratracheal instillation, after 24 h, the majority of the toxin eliminated through the feces was still in the form of PbTx-3. At 48 h and one week post exposure, PbTx-3 was either not detected or accounted for a minority (19–47%) of the toxin concentration measured by ELISA. After two weeks, PbTx-3 was mainly not detectable. In paired urine and feces samples, PbTx-3 accounted for the highest proportion of the toxin measured in the urine (37–70%) compared to the feces (6–13%), suggesting toxin cleared through the feces had metabolized more extensively.

In *Malaclemys*, PbTx-3 was also cleared out of nearly all tissues within 24 h post toxin administration (Table 4). Toxin levels fell below ELISA assay detection limits (<LD) within a week. Results show significant changes in the liver, kidney, brain, heart, lung and plasma from 24 h to a 1 wk compared with the 1 h post exposures.

3.4. Histopathological findings were similar between oral and intratracheal experimental groups

The *T. scripta* PbTx-3 exposure studies demonstrated a similar pattern of pathologic findings in both IT and orally exposed turtles, involving primarily inflammatory changes of the respiratory tract (trachea and lungs) and to a lesser extent the small intestine, meninges and endocardium/myocardium. Importantly, similar lesions were found in some control animals. Other findings

such as acute tubular necrosis of the kidney and pancreatic inflammation occurred occasionally in animals but could not be attributed specifically to brevetoxin exposure.

Parasites were common in both control and experimental *T. scripta* and included helminths consistent with spirorchid trematode ova and acanthocephalans. The spirorchid trematode ova were the most commonly observed parasites but occurred in varying numbers by tissue and case. The acanthocephalans were always present in intestinal lumina and were not usually associated with a host inflammatory response. In contrast to the findings in *T. scripta*, the respiratory and enteric lesions observed in *M. terrapin* were usually minimal to mild and probably did not result in any significant organ compromise.

4. Discussion

Brevetoxin metabolism and its physiological impacts are difficult to characterize in sea turtles due to their status as threatened or endangered animals, thus comparative model systems such as the freshwater turtle and diamondback terrapin must be used in order to determine toxin impacts in living organisms under controlled laboratory conditions. Results of this study suggest that turtles are resistant to PbTx-3, as the doses required either by oral or IT administration were significantly higher than those reported effective in rat studies. Our initial toxin dose curve was based on mammalian studies where dosages of 18.6 μg/kg (oral) and 2.6–6.6 μg/kg administered intratracheally had significant impacts in rats (Benson et al., 2005, 1999; Cattet and Geraci, 1993; Leighfield et al., 2014; Tibbetts et al., 2006). While we expected to need less drug due to the lower reptilian standard metabolic rate (typically about 1/10th of mammalian basal metabolic rates), this proved not to be the case as final doses were approximately 2× higher for oral exposures and up to 5× higher in IT exposures in the turtles. In mammals, the common clinical signs of brevetoxicosis were not commonly present post-low dose PbTx exposure, other than lethargy however, PbTx was shown to distribute systemically to all organs at detectable levels (Cattet and Geraci, 1993). At higher doses in rats (50–100 μg/kg) the animals did experience head bobbing, ataxia, and depression (Templeton et al., 1989). By contrast, while PbTx-3 exposure at the lower doses (3.12 μg/kg) in our turtle experiments also did not result in clinical signs; PbTx distribution to tissues was below the limit of detection 24 h post-administration. Similarly, we recently reported that *T. scripta* neurons in cell culture are also resistant to PbTx-3, with EC_{50}'s from 16- to ~26-fold higher than reported in rats (Cocilova and Milton, 2016). In this related *in vivo* portion of the study, though, the required effective doses were only 60–80% higher per dose than has been used in rat studies, though also administered repeatedly over 2–4 weeks to mimic the longer-term exposures that could be expected to occur in the wild. Higher or more frequent doses resulted in increased mortality. This data suggests that the turtles have more resistance or greater rates of clearance, or have other protective mechanisms to cope with toxin exposure. And even though PbTx-3 binding affinity is similar between rats and turtles the maximum binding capacity for the turtles is about 1/3 that of a rat; the lower density of sodium channels however do not result in differences in oxygen consumption (Edwards et al., 1989).

Once an appropriate sublethal dose was determined that resulted in evident neuronal and muscular deficits, PbTx-3 administration to *T. scripta* resulted in similar clinical symptoms as have been documented in laboratory studies, in wildlife studies (Kreuder et al., 2002) and at rehabilitation facilities where sea turtles were known to have been exposed to brevetoxin (Fauquier et al., 2013; Manire et al., 2013). The most severe neurological deficits for both IT and oral routes of exposure were observed 1–6+ h after each PbTx-3 exposure and diminished over a 24 h time period, but animals remained somewhat lethargic for the duration of the 2–4 week exposure regime. Symptoms were similar after each administration and did not intensify with successive doses. The similarity in symptoms is unsurprising as PbTx-3 acts on *T. scripta* neurons by binding to and opening voltage-gated sodium channels, triggering depolarization and over-excitation (Cocilova and Milton, 2016), as also occurs in the mammalian brain (Berman and Murray, 1999, 2000) and is likely to act in a similar manner in muscle tissue.

Once administered, PbTx-3 distributes widely to all organ systems sampled in both *T. scripta* and *Malaclemys*, as occurs in rats (Benson et al., 1999; Poli et al., 1990) and mice (Tibbetts et al., 2006), with the liver and kidney showing the highest immediate concentrations. Studies in laboratory animals (Benson et al., 1999), fish (Washburn et al., 1994), necropsied manatees (Bossart et al., 1998) and dolphins (Fire et al., 2008; Twiner et al., 2012) similarly show that brevetoxin concentrates into the detoxification and excretory systems, especially the liver. Cattet and Geraci, (1993) likewise demonstrated in rats that brevetoxin is metabolized by the liver and excreted through the feces and urine. Interestingly, they commented that in rats, the higher concentrations of brevetoxin in the liver could be due to toxin accumulation via both the hepatic and portal circulation and state that it may not be directly metabolized by the liver, such that the urinary system was the main route of excretion (Cattet and Geraci, 1993). In our study, the toxin was rapidly cleared out of the system over a 24–48 h period of time. While levels were initially high in the kidney and liver, they decreased rapidly in both tissues. But while urine toxin levels remained low throughout the experiment, increased toxin concentrations in the bile and feces over time suggest that in turtles at least this is the main route of excretion. There were significant decreases of PbTx-3 concentrations from the 1 h to 24 h post exposure times in most organs including the kidney, liver, brain, heart and spleen for both oral and IT exposures (Table 3). Toxin concentrations were further reduced in most tissues within 48 h and largely not detectable by 1 wk post-exposure. This rapid clearance was somewhat surprising as it is similar to the rapid clearance reported in mammalian studies; in a study by Benson et al. (1999), ^{3}H-PbTx-3 administered intratracheally in rats was rapidly cleared from the lung, liver and kidneys with only 20% of the toxin remaining in the tissues by 7 days post exposure (Benson et al., 1999). In other studies on ectotherms, the toxin was cleared far more slowly; in mullet exposed to brevetoxin-containing seawater, blood toxin levels decreased by only 50% over 5 days (Woofter et al., 2005), in Gulf toadfish levels were still high in the bile after 96 h, and in stranded sea turtles plasma levels took up to 80 days to decline to below detection limits (Fauquier et al., 2013).

Marine animals, however, are exposed to a diverse array of brevetoxins and brevetoxin metabolites present in *K. brevis* blooms, which may also accumulate in prey and on plants. These can vary in potency and result in different rates of tissue uptake and elimination (Leighfield et al., 2014). Concentrations in necropsied wild animals exposed to *K. brevis* blooms can also range much higher than we observed here; in sharks and rays from the Gulf coast of Florida, toxin levels ranged from below the limit of detection up to 27,760 ng PbTx-3 eq/g in the liver tissue (Flewelling et al., 2010). In stranded sea turtles, liver PbTx-3 ranged from <LD up to 1006 ng PbTx-3 eq/g (in a dead stranded hawksbill turtle *Eretmochelys imbricata*), although the mean liver toxin levels in the three species of sea turtle sampled were not that different from the means reported in this study, ranging from 131–297 ng PbTx-3 eq/g (Fauquier et al., 2013). Toxin levels in the liver, kidney, spleen and lung reported in this study were also similar to those reported in necropsied bottlenose dolphins that stranded in 2004–2005 (Twiner et al., 2011) and in 2007–2008 (Fire et al., 2008).

Interestingly, brain concentrations at 1 h post-administration were higher than most organs other than the excretory systems, but there was no evidence for neurotoxicity as evidenced by histopathologic examination despite clear neurological symptoms. While it has been shown that brevetoxin localizes to the rodent cerebellum after a single injection (Bourdelais et al., 2004) and is cytotoxic in both rat cerebellar granular cells (Berman and Murray, 1999) and turtle neurons *in vitro* (Cocilova and Milton, 2016), a lack of neuropathology despite clinical symptoms has likewise been noted in rats (Benson et al., 2005). In general, the clinical signs observed in this study are consistent with acute brevetoxicosis as described in other species (Van Dolah et al. 2003; Fauquier et al., 2013). Some of the histopathological features detected, including edema in the trachea and lung of PbTx exposed turtles, have also been reported in other wildlife studies. PbTx target organ specificity with respiratory inflammation, pulmonary hemorrhage and nonsuppurative meningitis was reported in Florida manatees (*Trichechus manatus latirostris*) with inhalational brevetoxicosis (Bossart et al., 1998; Bossart et al., 2002, 2004), and mortality-associated inhalational brevetoxicosis is attributed to acute agonal cardiovascular collapse (i.e., acute shock associated with aerosolized intoxication). It is suspected that in manatees, brevetoxicosis may initiate the release of inflammatory mediators that culminate in fatal toxic shock (Bossart et al., 1998). Similar pathologic findings were also reported in cormorants (*Phalacrocorax auritus*) with suspected brevetoxicosis (Kreuder et al., 2002). Additionally, PbTx impacts the respiratory tract in humans acting as an asthma trigger (Fleming et al., 2009).

Brevetoxin exposure may also have significant implications for immune function in loggerhead sea turtles (Walsh et al., 2010), Florida manatees (Walsh et al., 2005), laboratory rats (Benson et al., 2005) and was investigated as well in conjunction with this study (Walsh et al., in review). Related PbTx-associated immunologic perturbations resulting in secondary bacterial infection (postulated in this study) cannot be ruled out as another pathologic mechanism for this biointoxication, although lesions observed in *M. terrapin* were usually minimal which could reflect a species variation, a dose dependent effect, or represent underlying health status (*Malaclemys* were wild caught, rather than obtained from a commercial vendor). Importantly, however, in this study the frequency of lesions varied within experimental groups with some turtles having no significant lesions at all, while similar lesions were found in a low number of control turtles suggesting that another common factor(s) could be responsible for the observations. The infestation of both control and experimental animals with acanthocephalans and spirorchid trematodes confounded interpretation of the pathologic, which together with the presence of lesions in control and experimental groups made it difficult to distinguish results from background and thus limited the utility of the pathology data. The parasites are not uncommon in this species (Aho et al., 1992; Divers et al., 2010).

Lesion severity did generally tend to decrease with increased time post PbTx exposure suggesting that increased time from PbTx oral exposure is associated with less frequent and severe lesions. A notable exception from this temporal trend was a single *T. scripta* turtle in the 48 h post exposure group that had a severe pneumonia with a concurrent meningitis and tracheitis; this animal was one of several over the course of the investigation that were euthanized when it became apparent that they were not recovering from the toxin and would not survive the duration of the experiment (Table S2). The animals were still analyzed for tissue toxin levels; some of these turtles sacrificed 24 h or 48 h post exposure showed higher toxin concentrations in their tissues than animals in the 1 h post exposure group, suggesting an inability to quickly clear the toxin. Notably, the toxin concentrations in the bile and feces of animals that were euthanized early were lower than any other treatment groups (data not shown). While anecdotal to some extent, this suggests that poor health strongly impacts ability to clear toxin, and may explain why PbTx levels in the plasma of loggerhead sea turtles in rehabilitation centers following toxic algal blooms in 2005 and 2006 took over 5–80 days to clear (Fauquier et al., 2013); in that study there was also no correlation between the amount of toxin present in the plasma and whether or not they survived. In the wild, red tides could thus trigger a "vicious circle" of alterations to neural and muscular activity that result in poorer body condition, which in turn makes the animal less able to excrete toxin, impacts the immune system, and results in further impacts to health status.

Questions still remain as to how much of the toxin turtles in the wild are exposed to and the quantity of the toxin they take in, since evidence from this study indicates that turtles can excrete the toxin rapidly and do so via the same mechanisms as occur in mammals. Bioaccumulation and biomagnification are both likely to play a role in brevetoxicosis in marine animals. Brevetoxin levels reported in the stomach contents and tissues of stranded bottlenose dolphins from a 2004 mortality event during a non-bloom period were similar to results reported in animals following algal blooms (Fire et al., 2008). Similarly, a dolphin and manatee mass mortality in Florida in 2002 was related to brevetoxins in fish and on seagrasses, though the mortality event occurred after the dinoflagellate bloom when water toxin levels were low (Flewelling et al., 2005). A recent study has also reported brevetoxin present in the blood of nesting female sea turtles even when an algal bloom was absent (Perrault et al., 2016). Toxins in the water column and in lower trophic organisms may result in nearly continuous exposure for marine animals in areas prone to HAB outbreaks, such as the near annual blooms now occurring in the Gulf of Mexico. Even if the exposure does not result in immediate mortalities, they may be more susceptible to disease or to other additional stressors (Perrault et al., 2014; Walsh et al., 2015, 2010).

The ultimate goal of this research is to design appropriate treatments targeted to sea turtles exposed to red tides, with a more complete understanding of the distribution, clearance, and effects of brevetoxin in turtles. Current treatments are aimed at supportive care for exposed animals and dehydration if edema is present (Fauquier et al., 2013; Manire et al., 2013). Potential treatment strategies are aimed at clearing the toxin out of the system more quickly and also drawing out the toxin from the tissues by creating an enlarged intravascular lipid pool (Cocilova et al., manuscript in prep). Brevenal, an antagonist of VGSCs that is also produced by *K. brevis*, may also be a possible therapy for brevetoxin toxicity (Bourdelais et al., 2005; Sayer et al., 2006), though few studies have utilized it *in vivo*.

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