The Type B Brevetoxin (PbTx-3) Adversely Affects Development, Cardiovascular Function, and Survival in Medaka (*Oryzias latipes*) Embryos

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Brevetoxins are produced by the red tide dinoflagellate *Karenia brevis*. The toxins are lipophilic polyether toxins that elicit a myriad of effects depending on the route of exposure and the target organism. Brevetoxins are therefore broadly toxic to marine and estuarine animals. By mimicking the maternal route of exposure to the oocytes in finfish, we characterized the adverse effects of the type B brevetoxin brevetoxin-3 (PbTx-3) on embryonic fish development and survival. The Japanese rice fish, medaka (*Oryzias latipes*), was used as the experimental model in which individual eggs were exposed via microinjection to various known concentrations of PbTx-3 dissolved in an oil vehicle. Embryos injected with doses exceeding 1.0 ng/egg displayed tachycardia, hyperkinetic twitches in the form of sustained convulsions, spinal curvature, clumping of the erythrocytes, and decreased hatching success. Furthermore, fish dosed with toxin were often unable to hatch in the classic tail-first fashion and emerged head first, which resulted in partial hatches and death. We determined that the LD_{50} (dose that is lethal to 50% of the fish) for an injected dose of PbTx-3 is 4.0 ng/egg. The results of this study complement previous studies of the developmental toxicity of the type A brevetoxin brevetoxin-1 (PbTx-1), by illustrating *in vivo* the differing affinities of the two congeners for cardiac sodium channels. Consequently, we observed differing cardiovascular responses in the embryos, wherein embryos exposed to PbTx-3 exhibited persistent tachycardia, whereas embryos exposed to PbTx-1 displayed bradycardia, the onset of which was delayed. *Key words:* brevetoxin, development, icthyotoxicity, red tide, sodium channels. *Environ Health Perspect* 111:1920–1925 (2003). doi:10.1289/ehp.6386 available via *http://dx.doi.org/* [Online 22 September 2003]

Brevetoxins are produced by the marine dinoflagellate *Karenia brevis*. *K. brevis* produces lipid-soluble polyether toxins, which are filtered by bivalves that are in turn consumed by humans, resulting in neurotoxic shellfish poisoning (NSP). Common symptoms of NSP include gastrointestinal disorders, perceived temperature reversal, paresthesia of the mouth and extremities, tachycardia, and a lack of motor coordination. Brevetoxins, along with the structurally and functionally related ciguatoxins, elicit these responses by activating voltage-gated sodium channels in nerve, heart, and muscle tissues (Wang and Wang 2002). *K. brevis* produces type A and type B brevetoxins, which differ in the flexibility of their respective backbone structures. Type A brevetoxins include the congeners PbTx-1, -7, and -10, while type B brevetoxins include the congeners PbTx-2, -3, -5, -6, -8, and -9. These different backbone structures result in differing efficacies of the congeners in biologic systems (Trainer et al. 1990).

Fish are highly susceptible to brevetoxins during *K. brevis* blooms (Catterall and Risk 1980). Reports of fish kills have been recorded as far back as 1530 (Taylor 1917). The route of exposure to brevetoxins can be either through ingestion of *K. brevis* cells, followed by absorption of the toxin across the intestine, or absorption of toxin across the gill epithelium from lysed cells in the water (Landsberg 2002). Brevetoxin exposure may also occur at various levels of the food web via trophic transfer (Tester et al. 2000). Exposure of adult fish to brevetoxins can result in death. Fish that survive brevetoxin exposures are able to depurate some of the toxins via the biliary route and other metabolic processes (Kennedy et al. 1992). However, not all of the toxin is eliminated; it can be found in tissues of exposed fish and is able to transfer through the food web (Tester et al. 2000; Washburn et al. 1994). Common symptoms of brevetoxin toxicity in fish include spinal curvature, corkscrew swimming, loss of equilibrium, and convulsions before death (Kennedy et al. 1992; Stuart and Baden 1988).

At nonlethal doses, an orally administered type B brevetoxin (PbTx-3) appears in two tissue compartments in fish: 40% in the liver and nearly 30% in the muscle mass (Washburn et al. 1994). Although brevetoxin sequestered in tissue may not be immediately bioavailable, because of the lipophilic nature of the toxin it can be mobilized during oogenesis (Ungerer and Thomas 1996a). Hence, an indirect route of exposure may exist as the maternal transfer of toxins from the exposed fish to its oocytes. Embryonic and juvenile animals of many genera are often more sensitive to toxicants than their adult counterparts because they are undergoing development at this time (Walker et al. 1996). For this reason, we have decided to analyze the effects of PbTx-3 as it is transferred through the maternal exposure route in finfish.

The maternal transfer of toxins can be mimicked *in vivo* using a modification of the microinjection methods described by Walker et al. (1996). In previous egg microinjection studies with ciguatoxins and a type A brevetoxin (PbTx-1), we determined microinjection to be an effective means of delivering known amounts of toxin to individual fish eggs (Edmunds et al. 1999; Kimm-Brinson and Ramsdell 2001). In these studies, the Japanese rice fish, medaka (*Oryzias latipes*), was used as the experimental animal.

Although the Japanese medaka is a freshwater species that would not normally come into contact with marine toxins, it has proven to be an excellent developmental model with well-characterized life stages. Furthermore, medaka are easy to maintain, and breeding can be induced and regulated in these fish (Shi and Faustman 1989). By injecting lipid-soluble marine toxins of known concentrations into medaka eggs, we hope to shed light on the effects of these toxins on the development and survival of finfish. In this study, we hypothesized that PbTx-3 would elicit adverse effects on finfish development and that it

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would negatively affect survival, leading to an overall reduction in larval recruitment.

Materials and Methods

Animals. Breeding sets (six females and four males) of wild-type medaka (*Oryzias latipes*) were obtained from Carolina Biological Supply (Burlington, NC). Fish were housed in 2-gallon aquaria in a balanced salt solution (17 mM sodium chloride, 0.4 mM potassium chloride, 0.2 mM calcium chloride, 0.3 mM magnesium sulfate, 0.24 mM sodium bicarbonate) under a 16:8-hr light:dark cycle. Water temperatures were maintained at 25–28°C and declined approximately 3°C during the dark cycle due to the lack of light. This provided optimal breeding conditions for the fish. Medaka were fed a diet of either Wardley's Spirulina Plus flake food (Aquarium Parts West, Burbank, CA) or live *Artemia*, twice daily.

Eggs were collected from the female fish each morning and inspected for fertilization. Healthy eggs were embedded in 2% agarose dissolved in 12.5% Hank's solution for stabilization during microinjection. Embedding and injections occurred 6–8 hr postfertilization. After injection, each egg was rinsed for approximately 1 min in 12.5% Hanks solution and then transferred to one well in a sterile 24-well plate (Corning; Fisher Scientific, Pittsburgh, PA) containing Yasamoto's solution (133 mM NaCl, 2.7 mM KCl, 2.1 mM $CaCl_2$, 0.2 mM $NaHCO_3$, pH 7.3). Egg trays were maintained under the same light and temperature regimen as the adult fish.

Microinjection tools. Aluminosilicate micropipettes (outer diameter, 1 mm; Sutter Instrument Co. Novato, CA) were pulled using a P-87 Sutter micropipette puller and beveled with a BV-10 Sutter micropipette beveler. Each pipette was coated in Sigmacote (Sigma, St. Louis, MO) to increase durability and reduce clogging. Micropipettes were set in a three-dimensional manipulator (MO-150; Narashige Group, Long Island, NY) and front-loaded with triolein oil (Sigma) vehicle by means of a nitrogen gas picoinjector (PL1-100; Harvard Apparatus, Holliston, MA). All injections were carried out and visualized with the aid of a stereomicroscope (MZ 12; Leica Microsystems, Chantilly, VA), as were developments of the fish embryos. Digital images were captured using an RGB Automaticam (A209; MicroImage Video Systems Co., Boyertown, PA) mounted onto the microscope. Images were enhanced using Flashpoint 128 video frame-grabbing software (Integral Technologies Inc., Indianapolis, IN).

Microinjections. Brevetoxin congeners PbTx-1 and PbTx-3 (Calbiochem, La Jolla, CA) were dissolved in methanol and then added to triolein oil. Once the toxin was added to the oil, the methanol was removed by evaporation under a stream of nitrogen, resulting in a final concentration of 3 µg/µL brevetoxin in oil. PbTx-3 was administered as one of eight doses, wherein each dose covered a 1-ng range from 0 to 6 ± 0.5 ng/egg. A final dose range of 8.5 ± 1.5 ng/egg was also administered. PbTx-1 was administered as a single dose (3.5 ± 0.5 ng/egg), which has previously been demonstrated to elicit toxic effects (Kimm-Brinson and Ramsdell 2001). PbTx-1 injections were administered in order to verify that observations between scientists were consistent and to further compare the effects of these toxins *in vivo*. Following injection, the diameter of each droplet was measured using an ocular micrometer, and the volume was determined based on the diameter of the injected droplet. Because the concentration of the toxin in oil was known to be 3 µg/µL, the amount injected into each individual egg could then be determined. For each dose range of PbTx-3, at least 10 eggs were injected. For the single-dose PbTx-1 group, 30 eggs were injected. The amount of toxin loaded into each egg was determined volumetrically. To confirm that there were no artifactual effects resulting from exposure to the vehicle (triolein oil), 10 eggs were also injected with equivalent volumes of toxin-free oil in parallel to each dose range (Table 1). Finally, eggs were reared without injection to act as a control for the vehicle-injected eggs.

Injected embryos were allowed to grow for 13 days before being removed from the study. The developing fish were monitored for physical abnormalities, bradycardia, tachycardia, hyperkinetic twitches, and hatching success. We analyzed three end points to characterize the extent of the toxic effects of PbTx-3 on embryonic fish development: heart rate, hyperkinetic twitches, and death. Heart rate (beats per minute) was observed and recorded for each embryo on days 4 and 6 after injection. Observations were delayed until 4 days after injection to ensure the development of a strong heart beat. In addition, hyperkinetic twitches were observed on the same days and were recorded as the percentage of time the embryos twitched within a 3-min period. On day 13, we recorded the final status of each fish as hatched, nonhatched, partially hatched, or dead. Medaka normally hatch from the egg tail first, approximately 10 days after fertilization. The presence of partially hatched fish was a result of the embryos emerging head first from the egg

Table 1. Minimum number of eggs injected for each dose with PbTx-3, PbTx-1, or vehicle (triolein oil).

	0.5 ± 0.5	1.5 ± 0.5	2.5 ± 0.5	3.5 ± 0.5	4.5 ± 0.5	5.5 ± 0.5	6.5 ± 0.5	8.5 ± 1.5
Noninjected	10	10	10	10	10	10	10	10
Vehicle	10	10	10	10	10	10	10	10
PbTx-3	10	10	10	10	10	10	10	10
PbTx-1	0	0	0	30	0	0	0	0

Vehicle injections were volumetric equivalents to PbTx-1 and PbTx-3 doses.

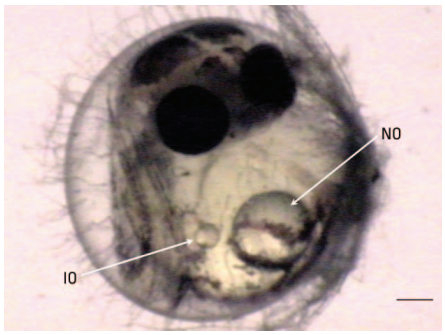

Figure 1. Still frame of a medaka embryo (80×) injected with 3.14 ng PbTx-3 after 6 days of development. Abbreviations: IO, injected droplet; NO, natural oil droplet. Bar = 150 µm.

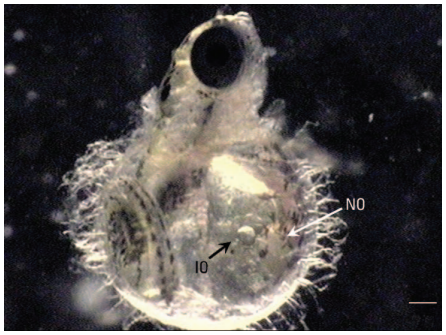

Figure 2. Still frame of a medaka embryo (80×) injected with 3.14 ng PbTx-3. This fish has hatched partially with the head-first orientation and is now trapped by the chorion. Abbreviations: IO, injected droplet; NO, natural oil droplet. Bar = 167.5 µm.

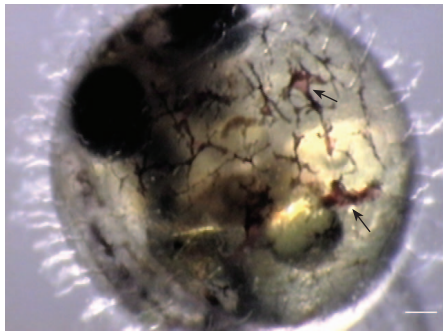

Figure 3. Still frame of a medaka embryo (80×) injected with 2.5 ng PbTx-3. This fish has clumped erythrocytes, which are indicated by arrows along the surface of the yolk. The clotting of blood in fish dosed with toxin causes the heart to beat without observable blood flow. Bar = 100 µm.

and getting trapped in the chorion. Larval survivability was not examined because of complications in rearing the medaka fry.

Data analysis. By measuring the diameter of the injected oil droplets with an ocular micrometer, the volume and thus the concentration of injected toxin could be calculated. From the concentration ranges, we determined the LD_{50} (dose lethal to 50% of the fish) based on the percentage of embryos that died, using Graphpad Prism software (Graphpad Software Inc., San Diego, CA). Heart rate was the most consistently observable and quantifiable response we observed. In analyzing the cardiovascular responses of the embryos, we used a Dunnett's multiple comparisons test to determine if there were significant differences between the different volumes of vehicle injected into the control animals compared with the noninjected eggs. Because no differences were observed in embryos dosed with any volume of nontoxic vehicle, those receiving the highest dose (8.5 ± 1.5 ng/egg) of oil, which we assume would elicit the greatest artifactual response, represented the control group for the eggs injected with toxin. Subsequent statistical analyses compared this vehicle-injected group with eggs treated with different doses of toxin using the Dunnett's multiple comparisons test, as described above. Statistical analyses were conducted using JMP software (SAS Institute Inc., Cary, NC).

Results

Microinjection of varying concentrations of PbTx-3 into fish eggs (Figure 1) resulted in a myriad of adverse effects, including tachycardia, sustained convulsions, and head-first hatching (Figure 2). Clumping of erythrocytes (Figure 3) and spinal curvature occurred more sporadically and were therefore not analyzed statistically. No significant differences existed between the responses of the noninjected embryos and those injected with any of the volumes of vehicle (triolein oil). Therefore, data collected from embryos receiving the highest equivalent dose (8.5 ± 1.5 ng/egg) of vehicle were designated as the control for the PbTx-3 treatments. In eggs dosed with < 1 ng of PbTx-3, the embryos behaved similarly to those injected with vehicle or nothing at all, indicating no adverse effect of the toxin. On embryonic day 6, there was a significant increase in heart rate for doses of PbTx-3 exceeding 1 ng/egg (Figure 4). Over the full range of exposure doses, increases in PbTx-3 resulted in tachycardia. Tachycardia was also observed in the PbTx-3–injected embryos on day 4 (data not shown), following the same dose-dependent patterns as seen on day 6 (Figure 4). This suggests that the increase in heart rate was experienced throughout development. Furthermore, there was no difference in the heart rates of embryos exposed to vehicle and to ≤ l ng PbTx-3, nor were there any differences in the heart rates of embryos injected with varying volumes of vehicle on either day 4 or day 6. In contrast with PbTx-3, single-dose injections of PbTx-1 resulted in a delayed bradycardic response on day 6 (Figure 5). As with previous studies of PbTx-1, we observed no effects of PbTx-1 on heart rate on day 4 (Kimm-Brinson and Ramsdell 2001).

A second effect of PbTx-3 on fish development was observed in the form of sustained convulsions or seizures. These were quantified as the percentage of time (3 min) spent in hyperkinesis. Twitching was observed and recorded on the same days as heart rate; however, only data from day 6 are presented (Figure 6). Similarly to the heart rate effects, no twitches were observed in fish administered < 1 ng PbTx-3 or any of the controls. For all other doses, twitch duration decreased with increasing concentrations of PbTx-3 in a dose-dependent fashion. However, as doses of toxin increased, so did early deaths within each treatment group, which may have added variability to the recordings at our higher doses.

Finally, death was monitored as the percentage of each treatment group that died after 13 days of embryonic development, thus indicating the effects of the toxin on hatching success. One of the adverse effects of brevetoxin is that it causes the fish to hatch head first (Figure 3), whereas a healthy fish will hatch tail first. Many fish that break through the chorion head first are unable to fully remove themselves from the egg, becoming trapped, or some may experience tearing of the skull or eyes. Therefore, fish that were only able to hatch partially from the egg were classified as dead. Because recordings of deaths also provided a dose-dependent response, we were able to determine an LD_{50} for PbTx-3 of approximately 4.0 ng/egg (Figure 7). Although death was observed in some embryos in each of the three treatment groups (noninjected, vehicle injected, and PbTx-3 injected), for reasons ranging from fungal infection to poor egg health, a clear dose-dependent response was observed in the medaka as concentrations of injected PbTx-3 increased.

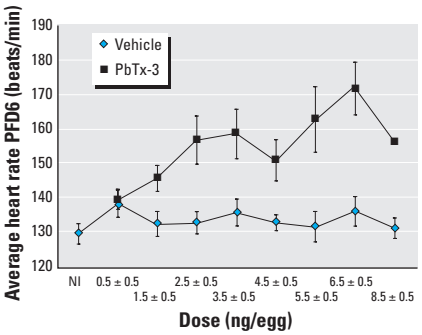

Figure 4. Comparative analysis of heart rate on postfertilization day 6 (PFD6; beats/minute ± SD) measured at each dose range for fish injected with PbTx-3 or volumetric equivalents of vehicle. NI, noninjected eggs. n = 10 at the time of injection for all dose groups. A Dunnett's multiple comparisons test was used to test for significant differences.

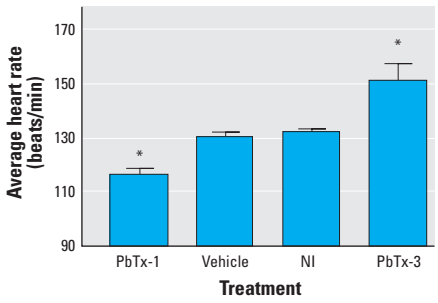

Figure 5. Comparative analysis of heart rate (beats/minute ± SE) for eggs injected with PbTx-1, vehicle, or PbTx-3, or noninjected eggs (NI), on postfertilization day 6. PbTx-1 injections resulted in bradycardia, whereas PbTx-3 injections resulted in tachycardia. n = 10 at the time of injection for all dose groups except PbTx-1, for which n = 30.
*Significantly different from the control (Dunnett's multiple comparisons test).

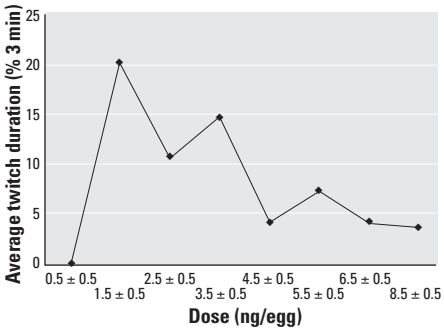

Figure 6. Sustained convulsions observed on postfertilization day 6 in fish injected with PbTx-3. Convulsions were quantified as the percentage of a 3-min time interval during which twitching was observed. No vehicle-treated fish displayed the twitch behavior. n = 10 at the time of injection for each dose group.

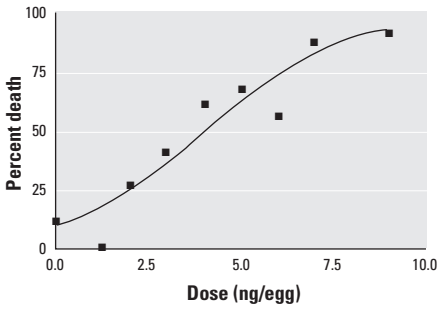

Figure 7. Dose response of medaka mortality expressed as a percentage for each PbTx-3 dose at the end of the 13-day developmental period. Vehicle-treated embryos are represented as a dose of 0 ng/egg. LD_{50} = 4 ng/egg.

Discussion

This study provides insight into the developmental toxicity of one of the most prevalent brevetoxins likely to accumulate in fish (Plakas et al. 2002; Tester et al. 2000). Although *K. brevis* cells are able to produce several brevetoxin congeners, PbTx-2 and PbTx-3 are the most common (Plakas et al. 2002; Poli et al. 1986). Therefore, certain congeners are more likely to accumulate in fish than others, thus limiting potential embryonic exposure to only a few brevetoxin subtypes. Additionally, this investigation of both type A and type B brevetoxins in the same experimental model permits an *in vivo* analysis of our recent study in which cloned heart sodium channels showed strong affinity for type A brevetoxins over type B brevetoxins (Bottein Dechraoui and Ramsdell 2003). Consequently, we sought to determine if the sensitivity of cardiac sodium channels for brevetoxin congeners translates into the differential cardiovascular effects observable in embryonic sodium channel subtypes *in vivo*. PbTx-3 adversely affected development and survival in the medaka via a myriad of effects. However, the distinct cardiovascular responses to type A and type B brevetoxins observed in our embryos (delayed bradycardia and consistent tachycardia, respectively) provided the most insight as to the differing efficacies of these brevetoxin congeners on finfish development.

The toxins most commonly produced by *K. brevis* are the unstable aldehyde PbTx-2 and its stable alcohol PbTx-3. In purified samples from log phase laboratory cultures, PbTx-2 is the predominant toxin, and PbTx-3 is present in approximately one-third the concentration of PbTx-2 (Poli et al. 1986). In shellfish tissue, PbTx-3 remains largely intact, whereas PbTx-2 is rapidly converted to PbTx-3 and cysteine conjugates (Plakas et al. 2002). Studies of cockle (*Austrovenus stutchburyi*), mussel (*Perna canaliculus*), and Pacific oyster (*Crassostrea gigas*) tissues in New Zealand after an NSP event indicated that the predominant brevetoxin forms in the tissue were metabolites of PbTx-2, including PbTx-3, brevetoxin-B_1, cysteine conjugates, and fatty acid conjugates (Ishida et al. 1995; Morohashi et al. 1999; Murata et al. 1998). Differences were observed in brevetoxin metabolite composition between the mollusks wherein oysters contained more PbTx-3 and cysteine conjugates and cockles contained brevetoxin-B_1 (Ishida et al. 1995). However, some of the toxins found in cockle tissues were more water soluble due to conjugation and would therefore be less likely to accumulate in fatty ovarian tissue or eggs. Adult fish exposed to brevetoxins during a harmful algal bloom would be exposed mostly to PbTx-2 and PbTx-3 (Poli et al. 1986). The brevetoxin types that would most likely mobilize into the oocytes of those fish would include the metabolites of PbTx-2 in the forms of PbTx-3 and, probably to a lesser extent, more polar cysteine conjugates. Our investigation of the developmental effects of PbTx-3 in finfish is therefore relevant in that this brevetoxin congener is likely to preferentially partition to the egg.

The differential accretion of brevetoxins and their metabolites observed in mollusks suggests that some unknown brevetoxin congeners may accumulate in fish tissues. Liver tissue analysis of marine mammals exposed to *K. brevis* blooms by radio immunoassay indicated high levels of PbTx-3, and lesser amounts of a brevetoxin metabolite that coeluted with PbTx-2 (Van Dolah et al. 2003). However, the brevetoxin metabolites identified by Dickey et al. (1999) and Poli et al. (2000) in shellfish extracts were not found. Future studies are needed to determine which brevetoxin congeners accumulate in finfish tissues and, as relates to this study, those that accumulate in ovarian tissue and eggs.

Natural blooms of *K. brevis* can be directly associated with reduced larval recruitment due to mortality of the adult fish population associated with the bloom (Riley et al. 1989; Warlen et al. 1998). This reduction in the adult population could then lead to an indirect delay in reproductive activity or fecundity after a red tide event. A secondary indirect effect of brevetoxin exposure, as demonstrated by our microinjection studies, is a decrease in the numbers of fish growing to reproductive age as they are impacted by maternally transferred toxins during oogenesis. The accumulation of lipid-soluble contaminants such as *o,p′*-DDT has been demonstrated in finfish (Ungerer and Thomas 1996a, 1996b). These materials usually collect in muscle tissue and viscera, including ovaries. They can be mobilized during oogenesis and can therefore be transferred to the oocytes. Once the lipophilic materials are in the oocyte, following a fertilization event, the developing embryo will absorb them as part of its food source. During this time, the embryos of oviparous species are confined to the internal environment designated by the egg's chorion, wherein the fish are nourished by an oil-laden yolk sack around which it develops. Lipophilic contaminants such as DDT and polychlorinated biphenyls have been demonstrated to accumulate in eggs, and the amounts are proportional to the contaminant body burden levels of the adult fish (Miller 1993). Maternally transferred organic contaminants have been linked to population declines of lake trout, where strong correlations have been determined to exist between egg content of organic contaminants and hatching and fry survival (Mac et al. 1993). These effects have been experimentally replicated by egg microinjection of *o,p′*-DDT (Edmunds et al. 2000). This model can also be applied to mimic the maternal transfer of natural toxins such a ciguatoxins and brevetoxins (Edmunds et al. 1999; Kimm-Brinson and Ramsdell 2001).

Brevetoxins have distinct effects on cardiac sodium channels and have been reported to have complex cardiovascular effects when administered to mammals (Borison et al. 1985; Johnson et al. 1985). Heart development occurs quickly in medaka, and weak cardiac contractions are visible after 40 hr of development. A fully functional heartbeat is evident by 46 hr, and circulation occurs approximately 8 hr later. Previous studies have demonstrated effects of sodium channel activators such as brevetoxins and ciguatoxins on embryonic fish heart rates (Edmunds et al. 1999; Kimm-Brinson and Ramsdell 2001). As demonstrated by Bottein Dechraoui and Ramsdell (2003), type B brevetoxins have a lower affinity for cardiac sodium channels than type A brevetoxins. In contrast to our results with PbTx-3, tachycardia was not observed during development of medaka embryos dosed with the type A brevetoxin, PbTx-1 (Kimm-Brinson and Ramsdell 2001). However, a delayed bradycardic response was observed on day 6 in the PbTx-1–treated fish (Figure 5). The time lag observed in cardiovascular effects of the two brevetoxin subtypes suggests independent activation of the sympathetic and parasympathetic nervous system by each toxin. The effects of each respective toxin on heart rate in fish embryos are complicated due to the nature of a developmental model being used *in vivo*. Cardiovascular responses to brevetoxins have been observed in rats, cats, and dogs thus far, but very few aquatic models, and no marine models, have been tested (Borison et al. 1985; Edmunds et al. 1999; Johnson et al. 1985; Kimm-Brinson and Ramsdell 2001; Templeton et al. 1989). The results of these mammalian studies suggest that brevetoxins elicit a Bezold-Jarisch effect in which delayed bradycardia is observed (Johnson et al. 1985; Poli et al. 1990). Delayed bradycardia in these animals is triggered by direct vagal nerve inputs to the heart, which would arise from the activation of cholinergic vagal efferents of the parasympathetic nervous system. Mammalian studies indicated the occurrence of delayed paroxysmal ventricular tachycardia, or delayed arrhythmic tachycardia (Poli et al. 1990). However, our medaka embryos appeared to maintain consistently elevated heart rates throughout development. This suggests that the tachycardic effects of PbTx-3 are elicited via another pathway, such as indirect inputs from adrenergic efferents in the sympathetic nervous system. However, medaka heart rates were not monitored for several hours at a time, so it is possible that their tachycardic responses were not constant. Additional microinjection studies of PbTx-2

and its metabolites, and their effects on heart rate in fish embryos may help to further explain the differences between type A and type B brevetoxins observed in our *in vitro* and *in vivo* assays.

Analyses of the expression of sodium channel subunits during development have demonstrated that structural modifications are required for the generation of mature skeletal and cardiac voltage-gated sodium channels in several models. The precursor forms of sodium channels undergo several posttranslational alterations and are eventually assembled into functional channels (Catterall 1992; Schmid and Guenther 1998). Expression of these channels can be regulated during development by many factors, including enervation and denervation of muscle fibers and coexpression of β subunits with their α-subunit counterparts. It is therefore possible that the sodium channels transfected into our human embryonic kidney cell line (Bottein Dechraoui and Ramsdell 2003) differed structurally from those found in medaka embryonic tissues. Functional differences in sodium channels depend on the channel isoform and the tissue type in which the channel is found. The mode of action of sodium channel activators such as brevetoxins and ciguatoxins in developing fish models has not yet been determined. However, the toxins are likely able to elicit their suite of effects on a variety of sodium channel types as they become expressed or down-regulated during the developmental process (Renaud et al. 1982).

Hyperkinetic twitching and spinal curvature are commonly observed symptoms resulting from exposure to a voltage-gated sodium channel activator such as brevetoxin or ciguatoxin in medaka embryos (Edmunds et al. 1999; Kimm-Brinson and Ramsdell 2001). Although the observed twitching response may be due to direct activation of nerve cells, spinal defects may be the result of secondary responses to persistent activation of these cells by the toxin. Similar responses have been observed in the estuarine fish *Fundulus heteroclitus* when exposed to acetylcholinesterase inhibitors, and in mammalian exposures to anthropogenic toxins such as pyrethroids and chlorinated hydrocarbons (Karen et al. 1998; Ray 1991; Smith 1991). Products of both of these compounds are used commercially as insecticides. The inhibitory compounds (acetylcholinesterase inhibitors) can be deleterious to developing embryos because acetylcholine plays a substantial role in neuronal development and differentiation (Bigbee et al. 1999; Brimijoin and Koenigsberger 1999). Inhibition of acetylcholinesterase activity results in the constant activation of the acetylcholine receptor, which induces muscle cells to use cellular calcium to induce persistent contractions. The reallocation of calcium during development to muscular contraction can result in decreased bone integrity, which can in turn translate into spinal and cranial defects such as the curvatures observed in our medaka studies (Edmunds et al. 1999; Karen et al. 1998; Kimm-Brinson and Ramsdell 2001). Pyrethroids and chlorinated hydrocarbons are sodium channel toxins that elicit their effects as a secondary response to neuronal excitability. Type I pyrethroid and DDT toxicity involves a progressive development of whole body tremors, uncoordinated twitching of the dorsal muscles, and hyperexcitability (Ray 1991; Smith 1991). Furthermore, poisoning is associated with spinal and brainstem excitability. Persistent activation of the sodium channel in a developing vertebrate can potentially have similar effects wherein hyperkinetic twitching represents the muscular contractions and tremors. Spinal and cranial defects were regularly observed in medaka embryos exposed to PbTx-1 and ciguatoxin (CTX-3C), which are both more potent than PbTx-3. The decreased occurrence of spinal curvature in medaka embryos exposed to PbTx-3 may be another example of the differential affinity of brevetoxin subtypes for sodium channels.

The activational effects of brevetoxins on voltage-gated sodium channels during development can influence the channels' ability to set off transcription and posttranslational modifications (Schmid and Guenther 1998). These molecular processes may be crucial to the differentiation and development of nerve tissues. Therefore, a neurotoxin such as PbTx-3 could negatively affect development and embryonic survival because it disrupts the differentiation process. Based on the results of our three end points (timed convulsions, heart rate, and death), PbTx-3 adversely affects embryonic fish development and is responsible for the decreased numbers of fish surviving into subsequent age classes. Clumping erythrocytes and morphologic abnormalities in embryos dosed with PbTx-3 may be secondary cardiovascular and nervous system responses to the activation of voltage-gated sodium channels during development. However, more studies on the mode of action of these channel activators in developing models are needed. Many of the effects observed in our medaka embryos dosed with PbTx-3 are not seen in adult fish afflicted with brevetoxin poisoning. For this reason, it is important to understand the effects of such toxins on an ecologic scale, not only on adult fish but also on their offspring.

References

Bigbee JW, Sharma KV, Gupta JJ, Dupree JL. 1999. Morphogenic role for acetylcholinesterase in axonal outgrowth during neural development. Environ Health Perspect 107(suppl 1):81–87.

Borison HL, McCarthy LE, Ellis S. 1985. Neurological analysis of respiratory, cardiovascular and neuromuscular effects of brevetoxins in cats. Toxicon 23(3):517–452.

Bottein Dechraoui MY, Ramsdell JS. 2003. Type B brevetoxins show tissue selectivity for voltage-gated sodium channels: comparison of brain, skeletal muscle, and cardiac sodium channels. Toxicon 41(7):919–927.

Brimijoin S, Koenigsberger C. 1999. Cholinesterase in neural development: new findings and toxicologic implications. Environ Health Perspect 107(suppl 1):59–64.

Catterall WA. 1992. Cellular and molecular biology of voltage-gated sodium channels. Physiol Rev 72(4):S15-S48.

Catterall WA, Risk M. 1980. Toxin T4–6 from *Ptychodiscus brevis* (formerly *Gymnodinium breve*) enhances activation of voltage-gates sodium channels by veratridine. Mol Pharmacol 19:345–348.

Dickey R, Jester E, Granade R, Mowdy D, Montcreiff C, Rebarchik D, et al. 1999. Monitoring brevetoxins during a *Gymnodinium breve* red tide: comparison of sodium channel specific toxicity and the mouse bioassay for determination of shellfish extracts. Nat Toxins 7:157–165.

Edmunds JSG, McCarthy RA, Ramsdell JS. 1999. Ciguatoxin reduces larval survivability in finfish. Toxicon 37:1827–1832.

———. 2000. Permanent and functional male-to-female sex reversal in d-rR strain medaka (*Oryzias latipes*) following egg microinjection of *o,p′*-DDT. Environ Health Perspect 108:219–224.

Ishida H, Nozawa A, Totoribe K, Muramatsu N, Nukaya H, Tsuji K, et al. 1995. Brevetoxin B_1, a new polyether marine toxin from the New Zealand shellfish, *Austrovenus stutchburyi*. Tetrahedron Lett 36(5):725–728.

Johnson GL, Spikes JJ, Ellis S. 1985. Cardiovascular effects of brevetoxins in dogs. Toxicon 23(3):505–515.

Karen DJ, Draughn R, Fulton M, Ross P. 1998. Bone strength and acetylcholinesterase inhibition as endpoints in chlorpyrifos toxicity to *Fundulus heteroclitus*. Pestic Biochem Physiol 60:167–175.

Kennedy CJ, Schulman LS, Baden DG, Walsh PJ. 1992. Toxicokinetics of brevetoxin PbTx-3 in the gulf toadfish, *Opsanus beta*, following intravenous administration. Aquat Toxicol 22:3–14.

Kimm-Brinson KL, Ramsdell JS. 2001. The red tide toxin, brevetoxin, induces embryo toxicity and developmental abnormalities. Environ Health Perspect 109:377–381.

Landsberg JH. 2002. The effects of harmful algal blooms on aquatic organisms. Rev Fish Sci 10:113–390.

Mac MJ, Schwartz TR, Edsall CC, Frank AM. 1993. PCBs in Great Lakes lake trout and their eggs: relations to survival and congener composition 1979–1988. J Great Lakes Res 19:752–765.

Miller MA. 1993. Maternal transfer of lipophilic contaminants in salmonines to their eggs. Can J Fish Aquat Sci 49:1405–1413.

Morohashi A, Satake M, Naoki H, Kaspar HF, Oshima Y, Yasumoto T. 1999. Brevetoxin B4 isolated from greenshell mussels *Perna canaliculus*, the major toxin involved in neurotoxic shellfish poisoning in New Zealand. Nat Toxins 7(2):45–48.

Murata K, Satake M, Naoki H, Kaspar HF, Oshima Y, Yasumoto T. 1998. Isolation and structure of a new brevetoxin analog, brevetoxin B2, from greenshell mussels from New Zealand. Tetrahedron 54:735–742.

Plakas SM, El Said KR, Jester ELE, Granade HR, Musser SM, Dickey RW. 2002. Confirmation of brevetoxin metabolism in the eastern oyster (*Crassostrea virginica*) by controlled exposures to pute toxins and to *Karenia brevis* cultures. Toxicon 40:721–729.

Poli MA, Mende TJ, Baden DG. 1986. Brevetoxins, unique activators of voltage-sensitive sodium channels, bind to specific sites in rat brain synaptosomes. Mol Pharmacol 30:129–135.

Poli MA, Musser SM, Dickey RW, Eilers PP, Hall S. 2000. Neurotoxic shellfish poisoning and brevetoxin metabolites: a case study from Florida. Toxicon 38:981–993.

Poli MA, Templeton CB, Pace JG, Hines HB. 1990. Detection, metabolism, and pathophysiology of brevetoxins. In: Marine Toxins: Origin, Structure and Molecular Pharmacology (Hall S, Strichartz G, eds). Woods Hole, MA:American Chemical Society, 176–199.

Ray DE. 1991. Pesticides derived from plants and other organisms. In: Classes of Pesticides (Hayes WJ Jr, Laws ER Jr, eds). Handbook of Pesticide Toxicology, Vol 2. San Diego, CA:Academic Press, 585–593.

Renaud JF, Romey G, Lombet A, Lazdunski M. 1982. Developmental properties of the fast NA^+ channel in embryonic cardiac cells using neurotoxins. Toxicon 20(1):17–25.

Riley CM, Holt SA, Holt J, Buskey EJ, Arnold CR. 1989. Mortality of larval red drum (*Sciaenops ocellatus*) associated with a *Ptychodiscus brevis* red tide. Contrib Mar Sci 31:137–146.

Schmid S, Guenther E. 1998. Alterations in channel density and

kinetic properties of the sodium current in retinal ganglion cells of the rat during *in vivo* differentiation. Neuroscience 85(1):249–258.

Shi M, Faustman EM. 1989. Developmental and characterization of a morphological scoring system for medaka (*Oryzias latipes*) embryo culture. Aquat Toxicol 15:127–140.

Smith AG. 1991. Chlorinated hydrocarbon insecticides. In: Classes of Pesticides (Hayes WJ Jr, Laws ER Jr, eds). Handbook of Pesticide Toxicology, Vol 2. San Diego, CA:Academic Press, 731–745.

Stuart AM, Baden DG. 1988. Florida red tide brevetoxins and binding in fish brain synaptosomes. Aquat Toxicol 13:271–280.

Taylor HF. 1917. Mortality of Fishes on the West Coast of Florida. Report of the U.S. Commissioner of Fisheries. Washington, DC:Government Printing Office.

Templeton CB, Poli MA, LeClaire RD. 1989. Cardiorespiratory effects of brevetoxin (PbTx-2) in conscious, tethered rats. Toxicon 27(9):1043–1049.

Tester PA, Turner JT, Shea D. 2000. Vectorial transport of toxins from the dinoflagellate *Gymnodinium breve* through copepods to fish. J Plankton Res 22(1):47–61.

Trainer VL, Edwards RA, Szmant AM, Stuart AM, Mende TJ, Baden DG. 1990. Marine Toxins: Origin, Structure, and Molecular Pharmacology (Hall, S, Strichartz GR, eds). Washington, DC:American Chemical Society, 166–175.

Ungerer JR, Thomas P. 1996a. Role of very low density lipoproteins in the accumulation of *o,p´*-DDT in fish ovaries during gonadal recrudescence. Aquat Toxicol 35:183–195.

———. 1996b. Transport and accumulation of organochlorines in the ovaries of Atlantic croaker (*Micropogonias undulatus*). Mar Environ Res 42(1–4):167–171.

Van Dolah FM, Doucette GJ, Gulland FMD, Rowles TL, Bossart GD. 2003. Impacts of algal toxins on marine mammals. In: Toxicology of Marine Mammals (Vos JG, Bossart GD, Fournier M, O'Shea TJ, eds). London/New York:Taylor & Francis, 247–269.

Walker MK, Zabel EW, Åkerman G, Balk L, Wright P, Tillit DE. 1996. Fish egg injection as an alternative exposure route for early life stage toxicity studies. Description of two unique methods. In: Techniques in Aquatic Toxicology (Ostrander G, ed). Boca Raton, FL:CRC Press, 41–72.

Wang SY, Wang GK. 2002. Voltage-gated sodium channels as primary targets of diverse lipid-soluble neurotoxins. Cell Signal 5623:1–9.

Warlen SM, Tester PA, Colby DR. 1998. Recruitment of larval fishes into a North Carolina estuary during a bloom of the red tide dinoflagellate, *Gymnodinium breve*. Bull Mar Sci 63:83–95.

Washburn BS, Baden DG, Gassman NJ, Walsh PJ. 1994. Brevetoxin: tissue distribution and effect on cytochrome P450 enzymes in fish. Toxicon 32(7):799–805.