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Saxitoxin increases phocine distemper virus replication upon *in-vitro* infection in harbor seal immune cells

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ABSTRACT

Several marine mammal epizootics have been closely linked to infectious diseases, as well as to the biotoxins produced by harmful algal blooms (HABs). In two of three saxitoxin (STX) associated mortality events, dolphin morbillivirus (DMV) or phocine distemper virus (PDV) was isolated in affected individuals. While STX is notorious for its neurotoxicity, immunotoxic effects have also been described. This study investigated the role of STX in altering immune function, specifically T lymphocyte proliferation, in harbor seals (*Phoca vitulina concolor*) upon *in-vitro* exposure. In addition, the study also examined whether exposure to STX could alter the susceptibility of harbor seal immune cells to PDV infection upon *in-vitro* exposure. STX caused an increase in harbor seal lymphocyte proliferation at 10 ppb and exposure to STX significantly increased the amount of virus present in lymphocytes. These results suggest that low levels of STX within the range of those reported in northeast U.S. seals may affect the likelihood of systemic PDV infection upon *in-vivo* exposure in susceptible seals. Given the concurrent increase in morbillivirus epizootics and HAB events in the last 25 years, the relationship between low level toxin exposure and host susceptibility to morbillivirus needs to be further explored.

1. Introduction

Biotoxin-producing harmful algal blooms (HABs) of marine phytoplankton have long been recognized as a source of risk for adverse effects on human health, as well as for adverse effects on the health of wildlife consuming biotoxin-contaminated fish and shellfish (Van Dolah et al., 2001). For humans, exposure to HAB biotoxins has been responsible for loss of income, sickness and discomfort, and even death (Carmichael, 2001; Etheridge, 2010). The potential for exposure to HAB biotoxins in humans and wildlife is now more frequently recognized and concern is growing regarding not only the overt adverse effects with high levels of toxin, but also for low level exposure and their sub-lethal and sub-clinical effects (Van Dolah et al., 2001; Pierce et al., 2005; Levin et al., 2010; Lefebvre et al., 2012; Fauquier et al., 2013; Hiolski et al., 2014).

Saxitoxins (STX) are a class of water soluble neurotoxins produced by three genera of dinoflagellates: *Alexandrium, Pyrodinium* and *Gymnodinium* and several cyanobacteria (Pearson et al., 2010). STX, a potent paralytic toxin, binds to site 1 of voltage dependent sodium and potassium channels (Catterall, 1980), which function in neurotransmission. Blockage of channels prevents depolarization of the membrane resulting in loss of impulse-generation in muscles and peripheral nerves causing paralysis (Kao, 1983), hence, its classification as a potent paralytic toxin (Faber, 2012). The toxin can also block calcium channels, and can prolong the gating of potassium channels in heart muscle cells leading to cardiovascular failure (Su et al., 2004; Llewellyn, 2006; Pearson et al., 2010).

STX can bioaccumulate in the tissues of animals and can be distributed throughout entire food webs, most notably in suspension-feeding shellfish, which are largely resistant to the

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toxin. As STX bioaccumulates in shellfish and other potential vectors, the effects can be devastating to wildlife consuming STX-contaminated prey, leading to acute toxicity and mortality in fish, shore birds, and marine mammals (Nisbet, 1983; Geraci et al., 1989; Anderson and White, 1992; Reyero et al., 1999; Fire et al., 2010, 2012a,b).

Ingestion of STX in humans causes the illness known as paralytic shellfish poisoning (PSP), with affected individuals experiencing tingling of lips, tongue and throat, numbness of the face, vomiting and diarrhea within the first 30 min of ingestion (Llewellyn, 2006). In more severe acute lethal poisoning, loss of motor coordination and respiratory paralysis are evident (Llewellyn, 2006; Pearson et al., 2010). During acute illness, patients diagnosed with PSP had STX levels of 2.8-47 nM in serum and 65–372 nM in urine, with clearance from serum within 24 h (Gessner et al., 1997). Shellfish are often used as indicator species for real time monitoring of STX for human health safety (Etheridge, 2010). The limit of STX imposed for seafood safety is 80 μg STX eq 100 g^{-1} tissue (Etheridge, 2010).

Three marine mammal mortality events have been associated with exposure to STX. The toxin, most likely produced by *Alexandrium tamarense*, was implicated in the death of 14 humpback whales (*Megaptera novaeangliae*) off the coast of Cape Cod, MA between November 1987 and January 1988 (Geraci et al., 1989). Although STX was not confirmed in whale samples from this die-off, mouse bioassays conducted on extracts of whale liver, stomach contents and kidney indicated toxic levels. Toxin levels were reported based on the presence of the STX in mackerel sampled where whales were feeding (Geraci et al., 1989). Further studies have also supported the hypothesis for bioaccumulation of the toxin in mackerel as a vector for STX ingestion in these large whales (Haya et al., 1989; Castonguay et al., 1997).

In May and June of 1997, a devastating mortality event occurred in Mediterranean monk seals (*Monachus monachus*) along Cape Blanc on the West African coast, resulting in the death of over 70% of the local population and a third of the worlds' population (Hernandez et al., 1998; Reyero et al., 1999). Among the toxins identified in dead animals, STX was found in seal liver, kidney, muscle and brain tissues, as well as in their fish prey. Adults were disproportionally affected during this mortality event leading to long term changes in population structure of this critically endangered population (Reyero et al., 1999; Aguilar and Lowry, 2013).

The third mass mortality event linked to STX exposure occurred in the southeast Atlantic along the coast of Florida. Landsberg (2002) suggested that the deaths of bottlenose dolphins in the Indian River Lagoon, Florida (2001 and 2002) were linked to STX exposure from the dinoflagellate *Pyrodinium bahamense*. Affected individuals were in poor body condition and had severe skin lesions. In addition, an unlikely prey, puffer fish, was found in their stomachs. The fish were shown to have high levels of STX (Quilliam et al., 2002; Van Dolah et al., 2001).

In the latter two events, morbilliviruses were also implicated as a potential cause of mortality (Osterhaus et al., 1998; Bossart, 2011). Morbillviruses are a genus of enveloped, negative-sense, single stranded non segmented RNA viruses within the subfamily Paramyxovirinae in the Paramyxoviridae family. They are 15,000–16,000 nt in size with a helical nucleocapsid in a herring-bone appearance (Rijks et al., 2012). Morbilliviruses infect a wide host range of terrestrial and aquatic animals globally and their role in domestic and wildlife disease has important consequences for both conservation and economic values. In humans, the most well recognized viruses of the family Paramyxoviridae include measles, Nipah and Hendra virus. The genus Morbillivirus includes measles virus (MeV), canine distemper virus (CDV), peste de petits-ruminants virus, rinderpest virus and the two species of marine mammal morbillviruses: phocine distemper virus (PDV) and the Cetacean morbilliviruses (CeMV): dolphin morbillivirus (DMV), porpoise morbillivirus (PMV) and pilot whale morbillivirus (PWMV) (Soto and Domingo, 2013).

Seals are affected by phocine distemper virus (PDV). This virus was first recognized in 1988 after one of the largest recorded epizootic events in wildlife killed over half the population of harbor seals in Europe (Dietz et al., 1989; Osterhaus and Vedder, 1988). Phocine distemper is extremely contagious between seals, with the virus shed by respiratory, urinary, fecal and ocular routes (Gage, 2013). The virus has a strong affinity for epithelial cells of the respiratory and gastrointestinal mucosa. Disease clinically presents with fever, lethargy, nervous signs, emphysema, serous mucopurulent occulonasal discharge, conjunctivitis and coughing (Kennedy, 1990; Phillipa et al., 2009). Immunosuppresion due to lymphocyte depletion is generally a result of the morbillivirus infection (Beineke et al., 2009), and therefore secondary bacterial infection commonly occurs. As with all disease, the effects of morbillivirus in an infected individual and in populations depend on several factors including the host, the strain, exposure of the host to environmental stressor and immunocompetence of the individual and species. The impact of infectious diseases on the host can be altered by risk factors which may increase host susceptibility.

The virus isolated from Mediterranean monk seals was determined to be most similar to an isolated dolphin morbillivirus (DMV) rather than known sequences of phocine distemper virus (PDV) or canine distemper virus (CDV) (Osterhaus et al., 1998). While the morbillivirus was identified and isolated, clinical signs and histopathological findings in monk seal mortalities suggest that STX was the proximal cause of the die-off (Hernandez et al., 1998). Clinical signs observed included horizontal floating, paralysis, lethargy, and lack of motor coordination (Hernandez et al., 1998). In addition, lungs were congested and both the lungs and airways were filled with fluid. Final histopathological diagnosis was drowning caused by paralysis due to poisoning, without evidence of primary viral damage or secondary opportunistic infections in the lungs (Hernandez et al., 1998). These findings led the authors to suggest that the death of affected monk seals was influenced by STX poisoning (Hernandez et al., 1998).

Saxitoxin has yet to be implicated in the mortality of other pinnipeds and the role of STX as a potential contributing factor to the development of infectious disease is unknown (Jensen et al., 2015). The presence of HAB toxins, including STX, was recently correlated to areas where Northeast Atlantic harbor seal populations are declining (Jensen et al., 2015). While STX is notorious for its neurotoxicity, immunotoxic effects have also been described (Pípole et al., 2011; Mello et al., 2013). Voltage-gated sodium channels (VGSC) targeted by STX are widely expressed on lymphocytes and macrophages (Roselli et al., 2006). Normal sodium influx through these channels is necessary for lymphocyte activation and proliferation (Roselli et al., 2006). Several VGSC blockers have been shown to modulate immune response, specifically suppressing Th1-mediated response in favor of the Th2 response in mice (Roselli et al., 2006). In bivalve mollusks, hemocytes (immune cells of invertebrates) have also been identified as a target for the effects of STX exposure, with genes related to immune response being up-regulated upon exposure to STX (Nunez-Acuna et al., 2013; Galimany et al., 2008).

Detrimental health effects observed in additional marine vertebrates may be linked to STX in the Northwest Atlantic. Exposure to STX in North Atlantic Right whales was implicated as a factor for the decrease in reproductive success in this highly endangered species (Reeves et al., 2001; Durbin et al., 2002; Doucette et al., 2006). Harbor and gray seals are also exposed to STX (12–400 ng/g STX equivalents) in the Northwest Atlantic as

these toxins have been detected in feces, bile, urine and gastrointestinal contents (Fire, unpublished data). In addition, STX was recovered from PDV-infected harbor seals and gray seals that died during the 2006 Northeast US Pinniped Unusual Mortality Event (UME) (Fire, unpublished data; Earle et al., 2011; NOAA, 2014). Large-scale HABs and the mortality of farmed salmon directly attributed to STX produced by *Alexandrium* spp. were also spatially and temporally related to the 2006 seal UME (Burridge et al., 2010).

Given the presence of STX during the 2006 UME and the mortality of seals due to PDV, it is unclear whether STX exposure could have contributed to the mortality observed in this die off event. Therefore, the objective of this study was to determine whether STX can cause changes in immune response in harbor seals and assess whether levels of exposure within the range likely encountered by these animals could contribute to an increase in PDV infection. By using an *in-vitro* lymphocyte proliferation assay and RT-qPCR quantification of virus during the course of *in-vitro* infection on immune cells derived from live harbor seals, an assessment of the effects of STX on immune cell modulation and PDV replication was determined.

2. Materials and methods

2.1. Saxitoxin

Saxitoxin dihydrochloride (NRC CRM-STX-f, Lot no. 20110316, NRC Canada Institute for Marine Biosciences, Halifax, Nova Scotia, Canada), designed for both bioassay use and analytical calibration, was used in STX exposure experiments. STX stock solution was suspended in 0.5 ml of 3 mM HCl.

2.2. Seals and blood sampling

Blood samples from wild-captured harbor seals were collected as part of an ongoing population assessment by the Northeast Fisheries Science Center (NEFSC) of the National Marine Fisheries Service (NMFS). Blood samples from stranded seals were collected prior to their release into the wild from the University of New England Marine Animal Rehabilitation and Conservation Program (UNE MARC) (Table 1). RNA from peripheral blood mononuclear cells (PBMCs) was processed as stated below to detect infection in PBMCs prior to use in the infection experiment. Blood was collected from the extradural vein or hind flipper and collected into sterile Vacutainer tubes containing sodium heparin (Becton Dickson, Franklin Lakes, NJ), shipped to the University of Connecticut, Storrs CT, overnight on cold packs and processed within 24 h.

2.3. PBMC isolation

PBMCs were collected from heparinized blood by Ficoll density gradient centrifugation. To compare blood leukocyte proportions prior to and after PBMC isolation, erythrocytes from a subsampled one ml aliquot of blood were first lysed using 0.15 M ammonium chloride lysing buffer solution (Brosseau et al., 1999) at room temperature (25 °C). Briefly, 9 ml of lysing buffer solution was added to 1 ml of blood and rocked gently for 2 min. The sample was washed twice at 220 × *g* for 10 min with Hanks Balanced Salt Solution (HBSS, Life Technologies, Grand Island, NY). Blood PBMC isolation was diluted 1:1 in HBSS, placed 1:1 over Ficoll-Paque PLUS 1077 (Amersham Biosciences, Uppsala, Sweden) and centrifuged at 990 × *g* for 45 min. The interface layer was isolated using a sterile transfer pipette and washed 10:1 in HBSS at 220 × *g* for 10 min. Erythrocytes were lysed twice using lysing buffer. Leukocytes were re-suspended in HBSS and washed twice with HBSS at 220 × *g* for 10 min each. The PBMCs were counted and the viability of cells was assessed using the exclusion dye trypan blue. The proportion of leukocyte sub-populations was assessed by FACS flow cytometry and the PBMCs were cryopreserved in ice cold fetal bovine serum (Hyclone, Logan, UT) with 10% DMSO (Sigma, Sigma-Aldrich, St. Louis, MO).

2.4. Lymphocyte proliferation

Cyropreserved PBMCs from seven wild-captured harbor seals were thawed in a 37 °C water bath, washed twice and re-suspended in complete Dulbeco's Modified Eagle Medium (DMEM). This medium was supplemented with 1 mM sodium pyruvate, 100 μM nonessential amino acids, 25 mM HEPES, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin (all from Gibco BRL, Grand Island, NY), along with 10% fetal bovine serum (Hyclone, Logan, UT). PBMCs were plated (1×10^6 cells/ml final concentration, 100 μl per well) in triplicate in 96 well flat bottom tissue culture plates (Falcon, Becton Dickinson, Franklin Lakes, NJ). Cells from the same individual seal were exposed to STX *in-vitro* at 0.1, 1, 10, 100, 500 ppb or media only (0 ppb). Concentrations of STX were selected based on levels measured in fecal, gut, bile and urine samples from seals in the northeast U.S. (Fire, pers. comm.). As stock STX was dissolved in 0.1 N HCl, all cells in control wells were given equal concentrations of HCl (8% of total volume or 0.06 mM) in complete DMEM media. Cells were incubated at 37 °C with 5% CO_2 for a total of 66 h with the T cell mitogen concanavalin A (Con-A) (Sigma, St Louis, MO). Con-A was used at the suboptimal concentration of 0.1 μg/ml, as suboptimal concentrations of mitogens have shown to increase the sensitivity of detection of immunotoxicity while maintaining specificity (Mori

Table 1
Seal blood samples. Seals from wild capture harbor seals were collected as part of an ongoing population assessment by the National Marine Fisheries Service Northeast Fisheries Science Center (NEFSC). Blood samples from stranded seals were collected from seals before release back into the wild from the University of New England Marine Animal Rehabilitation and Conservation Center Program (UNE MARC). "NA" = not available.

Source	ID	Sex	Length (cm)	Age class	Stranding/capture location	Assay	PDV titers	PBMC PDV
UNE MARC	MARC10-096	Male	72	Yearling	Cape Nedick, ME	STX	Negative	Negative
UNE MARC	MARC10-093	Female	86	Weanling	Moody Beach, Wells, ME	STX	Negative	Negative
UNE MARC	MARC10-086	Male	86	Weanling	Hampton Beach, NH	STX	Negative	Negative
UNE MARC	MARC10-094	Female	82	Weanling	Cape Elizabeth, ME	STX	Negative	Negative
UNE MARC	MARC10-099	Male	95	Yearling	Prout's Neck, Scarborough ME	STX	Negative	Negative
NEFSC	30	Female	NA	Adult	Chatham, MA	LP	NA	Negative
NEFSC	47	Male	NA		Mark Island, Rockland, ME	LP	1:32	Negative
NEFSC	19	Male	133	Adult	Rockland, ME	LP	NA	Negative
NEFSC	27	Female	NA	Adult	Chatham, MA	LP	NA	Negative
NEFSC	22	Female	94	Adult	PB Dix Island, ME	LP	NA	Negative
NEFSC	12	Female	168	Adult	Chatham, MA	LP	1:128	Negative
NEFSC	32	Male	NA	Adult	Chatham, MA	LP	NA	Negative

et al., 2006). Lymphocyte proliferation was evaluated as the incorporation of 5-bromo-2′-deoxyuridine (BrdU), added for the last 18 h of incubation, and detected with a monoclonal antibody and a colorimetric enzymatic reaction (Cell Proliferation ELISA BrdU (colorimetric), Roche Diagnostics GmbH, Mannheim, Germany) per manufacturer's instructions using an ELISA plate reader (Multiskan EX v 1.0) at 690 nm with a reference wavelength of 450 nm. Stimulation index was calculated as the ratio of the measured optical density between stimulated and unstimulated cells.

2.5. Infection of seal PBMCs with PDV following STX exposure

Cryopreserved cells from five stranded pre-release seals were thawed in a 37 °C water bath and washed twice in pre-infection exposure supplemented DMEM. This medium included Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12, HEPES (DMEM/F-12, HEPES, Gibco BRL, Grand Island, NY) supplemented with 200 μg/ml penicillin/streptomycin, 0.25 μg/ml Fungizone and with 10% cosmic calf (CCS) fetal bovine serum (Hyclone), hereafter referred to as pre-infection DMEM. Before infection with PDV, cells were pre-exposed to 10 ppb STX and 0.1 μg/ml Con-A for 72 h. Briefly, cells were plated in quadruplicate (5×10^4 cells per well) in 96-well round-bottom plates (Falcon, Becton Dickinson, Lincoln Park, NJ). Cells to be infected (PDV+, STX+, PDV+, STX−) and non-infected control cells (PDV−, STX−), were incubated in two separate incubators to prevent viral contamination at 37 °C with 5% CO_2 for 72 h (Siebelink et al., 1992, Visser et al., 1993). Cells were then washed, with plates centrifuged for 5 min at 500 × *g*, medium replaced, cells pipetted to mix, and further kept for 24 h in medium with 10% CCS and 100 IU/ml human recombinant IL-2 (IL-2hr) (Sigma) to activate IL-2 receptors as previously described (Siebelink et al., 1992). Plates were then centrifuged for 5 min at 500 × *g* and media removed for subsequent infection at a multiplicity of infection (MOI) of 1.0. Cells were infected with PDV USA 2006 for 1 h at 37 °C with 5% CO_2. Infected and non-infected cells underwent the same procedure with the equivalent volume of medium added and placed in a second incubator. Cells were then washed (5 min at 500 × *g*) to remove virus or control medium and gently mixed with 200 μl new medium. Cells were maintained in 200 μl pre-infection medium supplemented with 100 IU/ml IL-2hr. Medium used for virus infection contained 2% serum, double the concentration of penicillin/streptomycin (400 μg/ml) and an additional 0.5 μg/ml gentamicin. Cells continued to be maintained by adding 20 μl of this medium supplemented with IL-2 at days 3, 5, and 7.

2.6. RNA collection

Control and infected plates were sampled for RNA in separate biosafety cabinets to prevent viral contamination. Three fractions were collected from each well: tissue culture supernatant (S), lymphocytes (L) and monocytes (M). Tissue culture supernatant was carefully pipetted along with non-adherent cells, and lymphocytes were isolated from the supernatant following centrifugation for 30 s at 5220 × *g* (8000 rpm). Monocytes that adhered to the bottom of the well were collected from the plates using 200 μl of 0.25% (1×) trypsin (Hyclone). Plates were placed in the corresponding incubator at 37 °C with 5% CO_2 for 7–10 min to re-suspend adhered cells. Trypsin was subsequently removed by centrifugation for 30 s at 5220 × *g*. Samples for RNA were re-suspended in RLT buffer (Qiagen, Valencia, CA) and used in RNA extraction.

2.7. RNA extraction

Cryopreserved PBMCs each seal and samples from each fraction of the experiment were thawed in a 37 °C water bath, then extracted using an RNeasy plus mini kit (Qiagen) and further processed through a Qiashredder column (Qiagen). This kit includes a DNA eliminator column. RNA quantity (ng/μl) was determined using a Nanodrop 1000 Spectrophotometer (Thermo Scientific). Samples were kept at −80 °C until analysis.

2.8. Duplex RT-qPCR

The PDV and GAPDH primers and probes were used in a duplex reaction using Qiagen Quantifast Duplex RT-PCR +R in MicroAmp Optical 96-well reaction plates (Applied Biosystems) in the Applied Biosystems 7500 Real Time Cycler with copy numbers of PDV and GAPDH determined using 50 ng/μl of sample RNA in duplicate for each sample as previously described (Bogomolni et al., 2015a). GAPDH was measured for RNA quality control. The reaction initiated with a reverse transcription step at 50 °C for 20 min, followed by denaturation step at 95 °C for 5 min, and amplification for 40 cycles each of annealing at 95 °C for 15 s and an extension at 60 °C for 32 s. The baseline cycle threshold value was manually set at the exponential increase phase of the reaction for each of the two standards detected.

2.9. Assay sensitivity based on $TCID_{50}$ equivalents of standard PDV ds-DNA plasmid standard

The log10 $TCID_{50}$ of PDV USA 2006 was calculated using 10-fold dilutions of virus grown on transfected Vero cells expressing canine signaling lymphocyte activation molecules (SLAM) (Vero-DogSLAMtag cells) and results analyzed using the Spearmann–Karber Titre calculator as previously described (Bogomolni et al., 2015a). The PDV virus isolate was calculated to have a tissue culture infectious dose ($TCID_{50}$) of 5.52 (log10 $TCID_{50}$/ml), equivalent to 331,131 infectious particles/ml. The limit of detection and sensitivity of the assay was 12 infectious particles, equivalent to 0.25 copies of PDV standard, to 4.6×10^7 infectious particles with an equivalent of 524,807 copies PDV standard (Fig. 1).

2.10. Statistics

For lymphocyte proliferation, a mixed model one-way repeated measures analysis of variance was performed to compare exposed to unexposed cells using $p < 0.05$ for statistical significance. Dunnett's method was used for multiple pairwise comparisons *versus* control (0 ppb). For the infection study, outliers were determined using Minitab (Minitab Inc., State College, PA) for each

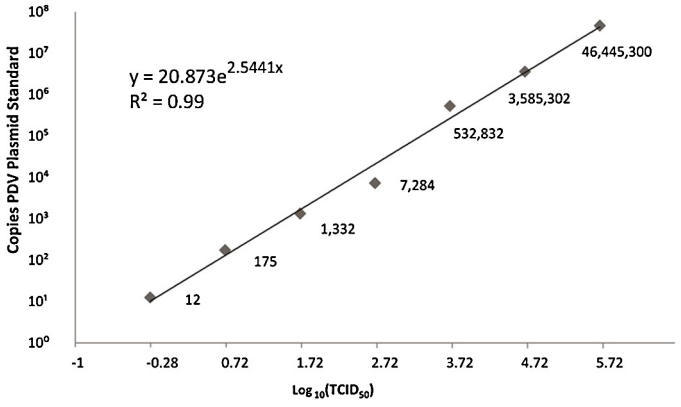

Fig. 1. *Assay Sensitivity based on $TCID_{50}$ Equivalents of standard PDV ds-DNA plasmid standard.* The PDV USA 2006 virus isolate was calculated to have a $TCID_{50}$ of 5.72 (log10 $TCID_{50}$/ml), equivalent to 331,131 infectious particles per ml. The limit of detection and sensitivity of the assay was 0.5 infectious particles, equivalent to 12 ± 8 copies of PDV standard, to 524,807 infectious particles with an equivalent of 46,445,300 ± 231,364,674) copies PDV standard.

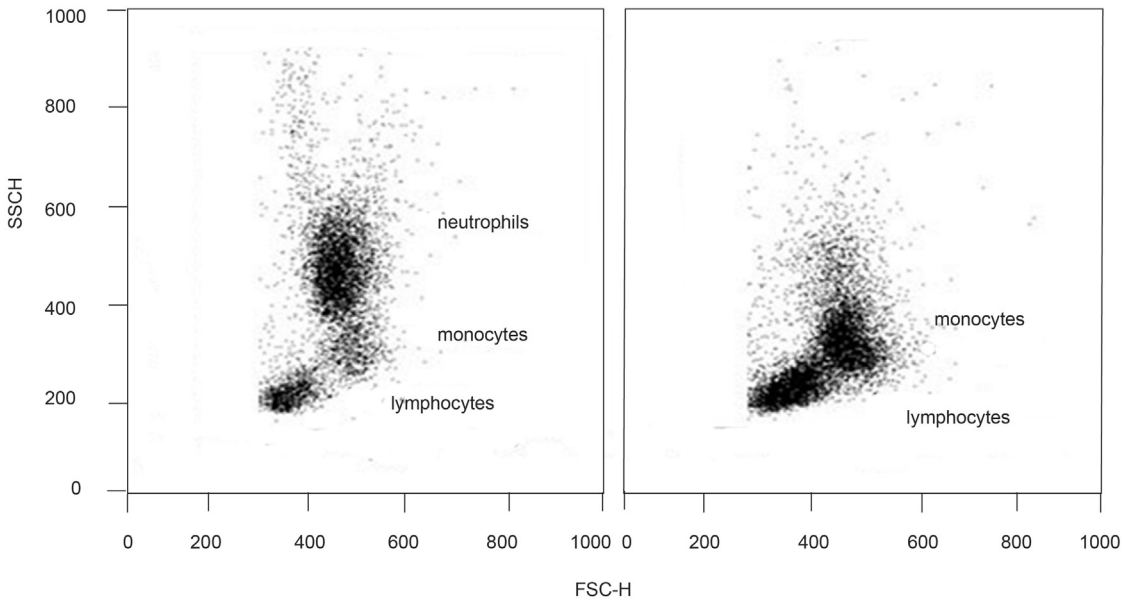

Fig. 2. *PBMC isolation.* Representative scatterplots of the flow cytometric profile of harbor seal peripheral blood leukocytes before (A) and after ficoll gradient centrifugation (B) demonstrates the selective loss of granulocytes and enrichment in mononuclear cells (lymphocytes and monocytes) in harbor seal cells isolation.

separate run. A generalized linear mixed model two-way repeated measures analysis of variance was performed for each fraction (S, L, M) to compare the effects of treatment (exposed or unexposed to STX) and time (Days 3, 5, 7 and 9 post-infection). Statistical analyses were performed using SigmaPlot 12.5 (Systat Software, San Jose, CA). Datasets that lacked normality and equal variance were transformed using a Johnson transformation using Minitab. The Johnson transformation determines an optimal transformation from three different distribution families (Kotz and Johnson, 1993). All error bars represent mean±SD.

3. Results

3.1. Animal samples

RT-qPCR of PBMC samples indicated that no animals used in the study had detectable PDV RNA, *i.e.* there was no evidence of current infection. GAPDH copies in PBMCs were within range of detection of the standards indicating suitable quality RNA for subsequent amplification tests.

3.2. Leukocyte flow cytometry profile

Flow cytometry was used to determine the proportions of sub-populations of leukocytes following purification of PBMCs using Ficoll gradient centrifugation. Sub-populations can be distinguished by relative size (forward scatter) and complexity (side scatter). Granulocytes were large and complex (granular), lymphocytes were smaller and less complex and monocytes were slightly larger than lymphocytes and less complex than granulocytes (Fig. 2). Ficoll gradient centrifugation is designed to allow selective loss of granulocytes and enrichment in mononuclear cells (lymphocytes and monocytes) from whole blood samples. The purification resulted in 71–95% mononuclear cells following Ficoll gradient centrifugation.

3.3. Lymphocyte proliferation

PBMCs from seven wild captured harbor seals were used to assess the effects of STX on harbor seal lymphocyte proliferation. Exposure of PBMCs to 10 ppb STX significantly increased ConA-induced lymphocyte proliferation compared to control ($p = 0.029$) representing a 78% increase over control (Fig. 3).

3.4. Quantity of PDV during in-vitro infection

Experimental *in-vitro* infections were performed to assess the potential influence of STX exposure on viral infection in harbor seal PBMCs. One outlier was identified in the monocyte fraction during the infection experiment and removed from the dataset. The STX-exposed lymphocyte fraction had significantly higher PDV loads at day 5 ($p = 0.041$), compared to infected cells without STX (Fig. 4A) representing an eight-fold difference in viral quantity. There were no significant differences in viral loads between time points in lymphocytes exposed to STX (PDV+, STX+). However, in cells unexposed to STX (PDV+, STX−), there was significantly more virus in the lymphocyte fraction between day 9 and 3 ($p < 0.001$), day 9 and 5 ($p = 0.001$) and day 9 and 7 ($p = 0.019$).

Exposure to STX did not significantly affect the viral loads in the monocyte fraction following infection with PDV (Fig. 4B). There were no significant differences in viral loads between time points in monocytes exposed to STX. However, in cells unexposed to STX,

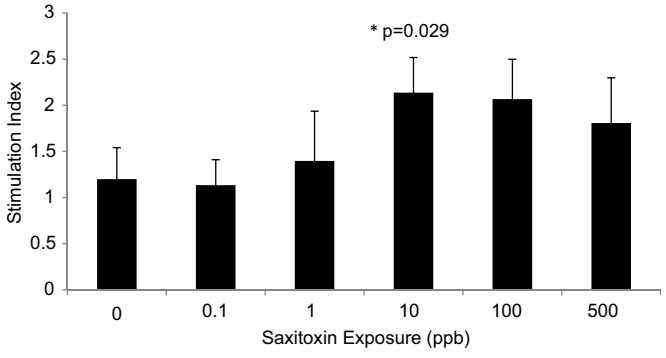

Fig. 3. *Lymphocyte Proliferation.* Exposure of harbor seal PBMCs to 10 ppb saxitoxin (STX) significantly increased spontaneous Con-A induced lymphocyte proliferation of stimulated *versus* unstimulated PBMCs (mean, ±SD, RM ANOVA, * $p < 0.05$).

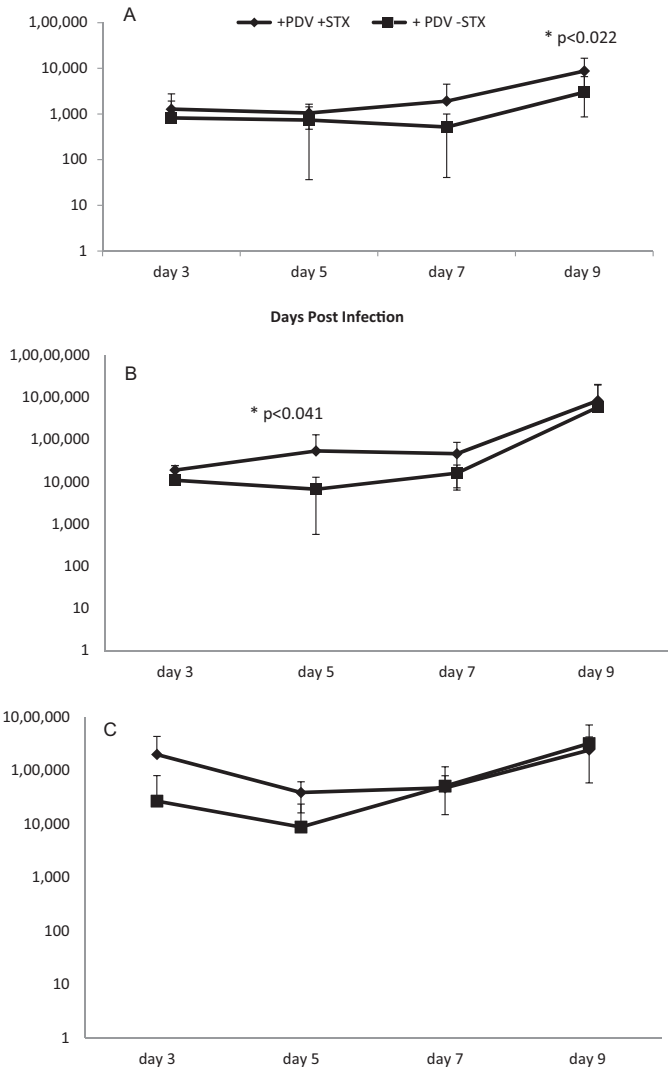

Fig. 4. *Quantity of PDV during in-vitro infection.* PDV quantity measured by RT-qPCR in (A) lymphocyte, (B) monocytes and (C) supernatant fractions from harbor seal cells exposed to 10 ppb STX (PDV+ and STX+) and unexposed control cells (PDV+ and STX−). Samples taken at day 3,5,7 and 9 post infection. Results are presented as mean, ±SD ($n = 5$), * $p < 0.05$.

there was significantly more virus in the monocyte fraction at day 9 than at days 3 ($p = 0.049$) and 5 ($p = 0.014$).

STX exposure significantly ($p = 0.022$) increased the viral loads in the supernatant fraction on day 9 post-infection (Fig. 4C), compared to the supernatant fraction without STX exposure, representing a 2.5-fold increase in virus. However, time did not significantly affect the viral loads in the supernatant in either STX-exposed (PDV+, STX+) or unexposed control samples (PDV+, STX−).

GAPDH levels were reduced in both control and infected samples in all fractions at day 9.

4. Discussion

In this study, exposure to 10 ppb STX increased mitogen-induced T cell proliferation. The effects were observed at the lower to middle range of concentrations tested, which were based on those measured in seal tissues/fluids in the Northeast US and were, therefore, environmentally relevant. As suggested by the National Toxicology Program, any chemically-induced disturbance of immune functions (up- or down-regulation) has the potential to be deleterious and increase susceptibility to infectious diseases (Luster et al., 1992, 1993). Specifically, an increase in the stimulation of T cells has the potential to result in anergy, a state of unresponsiveness, autoimmune disorders or cancer (Ichihara et al., 2003; Levin et al., 2007). Therefore, it is possible that *in-vivo* exposure to STX may increase the susceptibility of seals to morbillivirus infections, through immune dependent mechanisms.

Further, exposure to STX increased the ability of PDV to replicate in harbor seal cells *in-vitro*. Although the effects on lymphocytes occurred early (day 5) and appeared transient, these effects in lymphocytes resulted in a significant increase in free virus in the supernatant on day 9, which suggests the potential for increased systemic virus dissemination upon *in-vivo* infection.

Blooms of the dinoflagellate *Alexandrium fundyense* are common along the New England coastline between April and September, with shellfish closures commonly occurring later in the summer months (Anderson, 1997; Martin et al., 2014). The cysts produced by the dinoflagellate and the quantity of STX accumulated in shellfish has been monitored extensively due to the significant risk to human health (Anderson et al., 2014; Kleindinst et al., 2014; Martin et al., 2014). The highest level of STX recorded in New England was found in Maine shellfish (80,000 ng/g, or ppb) in 2009 (Fire et al., 2012a,b). This is 100 times higher than the level accepted by the US federal government for human consumption (Martin et al., 2014). While these levels not only exceeded human safety limits, mortality in endangered sturgeon was attributed to the high levels of STX (37–2300 ng/g) in the gut contents of these dead animals (Fire et al., 2012a,b). Despite these findings, little work has been done to assess the consequences of STX exposure in seals in the same region.

Several factors may explain the increase in virus in harbor seal lymphocytes exposed to STX. It is possible that the cell activation associated with increased lymphocyte proliferation resulted in differences in the dynamics of expression of cell receptors such as IL-2R or CD150, facilitating the entry of PDV into host cells (Farina et al., 2004; Yanagi et al., 2009). It is also possible that the increased proliferation of T cells detected 66 h following exposure to STX would have increased the number of host cells available for replication, as detected 5 days following exposure. However, it is unlikely that the replication process of PDV would be associated with changes in antigen-specific immune response, which requires complex cellular interactions in specific environments (such as lymph nodes). Such interactions would be difficult in our *in-vitro* system, and the very low likelihood that antigen-specific cells (with the appropriate T cell receptor) would be found consistently in our *in-vitro* system, given the low number of cells used (5×10^4 cells per well) and considering the diversity of T cell receptors.

GAPDH levels were low to undetected at the end of the experiment, indicating that host nucleic acid was minimal whereas viral quantity increased. The duplex RT-qPCR reaction may favor the more abundant target and be unable to detect the remaining low levels of the host nucleic acid. Previous studies indicate that in controlled exposure of harbor seal PBMCs (without stressor, without virus), cellular GAPDH decreased at the end of the experiment, which was attributed to the loss of cells from the primary culture (Bogomolni et al., 2015b; Bogomolni et al. unpublished data). Low levels of GAPDH were reported in a similar experiment in harbor seal PBMCs exposed to Aroclor 1260 (Bogomolni et al., 2015b). It was therefore more likely that in our *in-vitro* system, viable host cells were the limiting factor to virus replication, with cytopathic effects resulting in cell death following viral infection and replication in the infected fractions. If this was the case, the transient nature of the increase in virus replication in lymphocytes may be the result of early use of viable cells by the virus. This finding would underrepresent the potential effects of STX on viral replication upon *in-vivo* exposure where the pool of available target cells would not be exhausted as quickly.

Several studies reported that early morbillivirus infection of lymphocytes resulted in a lethal infection. Ferrets, mink and dogs experimentally infected with CDV showed increased risk of death with a lowered host immune response during early onset of infection (Gröne et al., 1998; Von Messling et al., 2006; Svitek and von Messling, 2007; Nielsen et al., 2009). Early immune suppression by morbillivirus allows for the infection of receptive cells expressing the signaling lymphocyte activation molecule (SLAM) receptor, and destruction of these cells that are needed to fight the viral infection (Minagawa et al., 2001; Tatsuo and Yanagi, 2002; Von Messling et al., 2006). STX appeared to enhance the infection of cells *in-vitro* and could contribute to earlier infection *in-vivo* whereby the host could be more susceptible to a more lethal, early infection. In light of those studies, it is reasonable to expect that in seals exposed to STX and infected with PDV, the immune alterations suggested by the observed effects on T cell proliferation upon *in-vitro* STX exposure would combine with the immune-independent effects on host cells resulting in an increased viral replication upon *in-vitro* STX exposure, and likely exacerbate the infection and ensuing disease.

5. Conclusions

The complex relationship between large-scale mortality events where morbillivirus co-occurs with HAB toxins has led to much interest on the ultimate cause of mortality and the impact of one or the other on the survival of the exposed populations. During the 1997 die-off of the highly endangered Mediterranean monk seal, both STX and a morbillivirus were isolated from seals. Without baseline information on the prevalence of virus antibodies or levels of algal toxins in seals and prey before the event, however, it was impossible to make a definitive diagnosis as to the specific causative agent (Hernandez et al., 1998). PSP symptoms in humans that have ingested STX also vary greatly due to differences in susceptibility among individuals (Krogh, 1983; Bricelj et al., 2010). Despite the difficulty associated with understanding the relationships between exposure to biotoxins and outcomes of viral infections at the population level in seals, these results support potentially relevant effects and pathways that may be a factor in current population declines. This may be especially relevant given similar findings in regions of the Northeast Atlantic, specifically the Orkney and Shetland Islands, Scotland, where HAB toxins in harbor seals have been found to correlate to immunomodulation in regions experiencing population decline (Jensen et al., 2015). Current estimates of the harbor seal populations in the northwest Atlantic, where the seals in this study are derived, also indicate a decline of nearly 30% (Waring et al., 2014).

This study represents the first *in-vitro* exposure of seal immune cells to STX and the first to determine the effects of exposure and resulting changes in morbillivirus replication. The effects of STX on harbor seal lymphocytes include an increase in proliferation as well as allowed for an increase in PDV. These results suggest that it is critical to monitor environmental levels of STX within the context of marine mammal health and as a potential facilitating factor in the development of PDV-related disease and mortality events.

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