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Evidence of selection for resistance to paralytic shellfish toxins during the early life history of soft-shell clam (*Mya arenaria*) populations

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Abstract

This study identifies early, postmetamorphic soft-shell clams, *Mya arenaria*, as the life-history stage most susceptible to effects of blooms of paralytic shellfish poisoning (PSP) toxin–producing *Alexandrium* spp. Laboratory experiments used progeny from predominantly susceptible (naïve) or resistant (annually exposed) NW Atlantic populations. Growth and survival of toxified veliger larvae did not differ from those fed nontoxic algae. In contrast, postlarvae (4–12-mm shell length) from both populations exposed to a highly toxic *Alexandrium tamarense* isolate ($\sim$ 100 cells mL^{-1}, 64–69 pg saxitoxin equivalents [STXeq] $cell^{-1}$) suffered burrowing incapacitation, toxin accumulation, and mortalities within 1 week of toxin exposure. These effects were greater and occurred sooner in the naïve population. Short-term toxification in the laboratory caused a significant shift in the genotypic composition of this population, determined with a molecular marker for sodium-channel resistance. Clams with the sensitive genotype were selectively eliminated relative to resistant or heterozygote clams. Ingestion of toxic cells (too large for larval capture) is thus required to elicit toxic effects. Exposure to mixed, toxic, and nontoxic algal suspensions demonstrated that adverse effects to fitness (survival and growth) were dose-dependent, occurring only at $\geq$ 50 cells mL^{-1} of the isolate used (PR18b). Paralysis and thus increased predatory risk occurred even at 10 cells mL^{-1}. Postlarvae $<$ 12 mm, which can co-occur with red tides throughout the Atlantic range of *M. arenaria*, were more susceptible to PSP than large ($>$ 30 mm) juveniles. Natural selection for resistance in Atlantic populations will thus vary latitudinally with the timing, duration, and intensity of toxic blooms.

Red tides caused by *Alexandrium* spp., producers of paralytic shellfish poisoning toxins (PSTs), play an important ecological role by influencing the distribution and abundance of marine organisms (Zimmer and Ferrer 2007) and the foraging behavior of keystone predators (Kvitek and Bretz 2004). These potent neurotoxins adversely affect susceptible marine invertebrates and vertebrates, including mammals, by blocking the Na^+ influx in excitable cells and thus inhibiting the nerve action potential, leading to paralysis and death.

Suspension-feeding bivalves are the main vectors of PSTs to humans and thus pose a public health hazard, incurring serious economic losses from shellfish harvesting closures and costly monitoring programs. The soft-shell clam, *Mya arenaria* L., is a native species with a wide latitudinal distribution along the Atlantic coast of North America, from Labrador, Newfoundland, to Georgia (Strasser 1999), which overlaps with areas affected by red tides. Throughout part of this range, Bay of Fundy to Chesapeake Bay, it can dominate the intertidal macrobenthos and supports valuable recreational and commercial fisheries (Congleton et al. 2006). A clear link between the prior history of exposure to paralytic shellfish poisoning (PSP) of *M. arenaria* populations and their toxin resistance has been demonstrated (Bricelj et al. 2004, 2005; MacQuarrie and Bricelj 2008). Thus, a population that has experienced recurrent annual toxic outbreaks and is predominantly composed of individuals with nerves resistant to PSTs from Lepreau Basin, Bay of Fundy, could accumulate toxins at significantly higher rates than a naïve population dominantly composed of sensitive clams from the Lawrencetown Estuary, southern Nova Scotia, when both were exposed to toxigenic *Alexandrium tamarense* in the laboratory.

Resistance in this species is caused by selection for a naturally occurring single-point mutation in domain II of the sodium-channel gene, which results in substitution of a single amino acid (glutamic in the wild type to aspartic in the mutant) that greatly reduces the binding affinity of PSTs (Bricelj et al. 2005). Sensitive genotypes (SS) are always homozygous at the identified locus, while resistant genotypes can be homozygous (RR) or heterozygous (RS) at this locus, and more than one nucleotide substitution (of adenine for either cytosine or thymidine) can yield a resistant genotype encoding for aspartic acid (Connell et al. 2007). These alleles are also known to follow simple Mendelian autosomal inheritance (Hamilton 2009). Differences in the resistance to PSTs in relation to prior history of PSP along the east coast of the U.S.A. have also been demonstrated for the copepod *Acartia hudsonica* (Colin and Dam 2002, 2004). The mechanism of adaptation in *A. hudsonica* is not fully understood, but it is not based on a Na^+ channel mutation that lowers toxin binding affinity (Chen 2010), as established in soft-shell clams. Therefore, the adaptation of populations to toxic algal blooms may be widespread across phyla of coastal marine organisms.

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While PSTs have the potential to act as strong natural selection agents, leading to the spread of toxin resistance in *M. arenaria* populations and increased risk of toxin accumulation, it is not clear how selection operates in nature in relation to the intensity and duration of toxic blooms, nor is the relative vulnerability of different *M. arenaria* life-history stages understood. The Atlantic coast of North America is characterized by wide latitudinal variation in the intensity and frequency of red tides (Franks and Anderson 1992; Anderson 1997), and the cell toxicity of *Alexandrium* isolates also varies extensively (Maranda et al. 1985; Anderson et al. 1994). Furthermore, there has been a well-documented historical southward dispersal of *Alexandrium* spp. and associated PSP along the coast, most recently, during the massive 2005 bloom, which extended the distribution of PSP in southern New England, thus posing a risk to previously unaffected regions (Anderson et al. 2005). Peak spawning and growth rates of *M. arenaria* also vary latitudinally, leading to potential exposure of all life-history stages to PSP. The vulnerability to PSP during ontogeny remains unknown because prior toxification studies only used large juveniles or pre-adults ($\sim$ 35–40 mm in shell length [SL]).

The specific objectives of this laboratory study were therefore: (1) to identify the *M. arenaria* early life-history stage most susceptible to toxic *A. tamarense* blooms by determining their effects on fitness of planktonic larvae and postmetamorphic clams (postlarvae or small juveniles) from a predominantly resistant and predominantly sensitive population, respectively, (2) to test whether natural selection for resistance to PSTs as evidenced by changes in genotypic composition can occur following exposure of clam postlarvae to a simulated bloom of toxic *A. tamarense*, and (3) to determine the concentration of a highly toxic *A. tamarense* isolate required to elicit adverse lethal and sublethal effects in sensitive postlarvae. To meet the first objective, naïve larvae and postlarvae bred from adults of the two source populations were exposed to unialgal suspensions of either nontoxic algae or *A. tamarense* at a cell density and toxicity comparable to that used for large juveniles in prior studies (Bricelj et al. 2005; MacQuarrie and Bricelj 2008) (experiment I). The third objective was met by exposing postlarvae from a predominantly sensitive population to varying proportions of toxic *A. tamarense* in a mixed diet with nontoxic algae, while maintaining a constant total algal cell volume to preclude confounding effects of this variable (experiment II). The potential for selection for resistance by PSTs (objective 3) was determined for postlarvae exposed to unialgal *A. tamarense* suspensions, representative of peak, high-intensity bloom conditions (worst-case scenario), in both experiments I and II. Taken together, results of this study will allow prediction of the evolutionary response of natural soft-shell clam populations to geographic variation in intensity and toxicity of algal blooms, and the relative response of different life-history stages.

Methods

Clam and algae production—Production of *M. arenaria* larvae and postlarvae (here defined as postmetamorphic clams ranging between $\sim$ 3- and 12-mm SL) and all experiments were conducted at the National Research Council, Institute of Marine Biosciences' (IMB) Marine Research Station, Ketch Harbour, Nova Scotia, Canada. *Mya arenaria* progeny were obtained by reproductive conditioning and mass spawning of adults of known genotype (determined from hemolymph extraction; Hamilton and Connell 2009) from each of two populations: Lawrencetown River Estuary (LE), Nova Scotia, a population with no history of PSP and dominated by clams sensitive to PSTs, and Lepreau Basin, Bay of Fundy (BF), New Brunswick, a site characterized by annual, summer PSP outbreaks and dominated by PST-resistant clams (Bricelj et al. 2005). Adults were conditioned on a mixed diet of algae obtained from the Center for the Culture of Marine Phytoplankton (CCMP), West Boothbay Harbor, Maine: *Pavlova pinguis* (CCMP 609), *Chateoceros muelleri* (CCMP 1316), and *Isochrysis galbana* (strain T-iso, CCMP 1324) at a total concentration of 6×10^4 cells mL^{-1}. Experiments with LE and BF clams (both larvae and postlarvae) were conducted sequentially. Larvae were reared in 400-liter conical tanks at 23°C, in 1-μm cartridge-filtered seawater, at 30 salinity, and were fed a mixed suspension of *P. pinguis* and strain Pav 459. Postlarvae were grown at 20°C in air-driven, 45-cm-diameter downwellers fitted with a 153-μm square Nitex mesh, in 1000-liter tanks, and fed a mixed diet of *P. pinguis* and *I. galbana* until they attained the appropriate experimental size.

Alexandrium tamarense (strain PR18b, a highly toxic isolate from the Gulf of St. Lawrence Estuary; mean equivalent spherical diameter [ESD] = 35 μm) was grown nonaxenically in L1 medium (Guillard and Hargraves 1993) with the addition of NH_4Cl at a final concentration of 5×10^{-5} mol L^{-1}, at 16°C, on a 14 : 10 h light : dark cycle in aerated 20-liter carboys. This strain was used in all experiments at a maximum exposure concentration of 100 cells mL^{-1}, which can occur in the natural environment, and which allows comparison with previous experiments using large juveniles of *M. arenaria* (Bricelj et al. 2005; MacQuarrie and Bricelj 2008). Nontoxic algae were grown under continuous light, at 20°C, in f/2 nutrient medium (Guillard and Ryther 1962). All algal species were harvested in late exponential growth phase.

Larval experiments—Larvae were produced by mass spawning of adults (15 males and 16 females from either the LE or BF population), and individuals were then reared to postlarval stages for experiment I. Prior to experiments, the temperature was gradually reduced to 18°C, and 1-week-old veligers were transferred to gently aerated 2-liter glass beakers filled with 0.22-μm cartridge-filtered seawater at a density of 4 larvae mL^{-1}. Veligers were exposed for 7 to 8 d, in triplicate, to the following diets: 3×10^4 cells mL^{-1} of *P. pinguis*, 3.5-μm ESD (nontoxic control), and the same concentration of *P. pinguis* spiked with 100 cells mL^{-1} of *A. tamarense* (strain PR18b). *A. tamarense* cells were added as a culture suspension ($\sim$ 25 mL), i.e., were not sieved to remove dissolved toxin. All larvae were retained on a 100-μm Nitex sieve and resuspended in a new algal suspension every 2 d. Larvae were sampled at 0, 2, 4, 6, and 8 d to

determine SL and percent mortality ($n = 50$ clams per beaker). Growth rates were obtained from the slopes of fitted linear regression equations.

Postlarval or juvenile experiment I (first exposure)—Temperature was reduced to 16°C for 2 weeks to acclimate clams prior to the start of the experiment. Clams from the two source populations were tested in sequential experiments following identical protocols. The initial mean size (± SE) of postlarvae was 3.5 ± 0.1 ($n = 20$) and 4.4 ± 0.1 mm SL ($n = 50$) for BF and LE progeny, respectively. Duplicate aquaria containing the postlarvae were exposed for 7 d to the following diets: a mixed suspension of 2.5×10^4 *P. pinguis* cells mL^{-1} and 1.1×10^4 *C. muelleri* cells mL^{-1} (nontoxic control), and a cell volume–equivalent suspension of 100 cells mL^{-1} of *A. tamarense* (PR18b) (toxified treatment). Algal cell concentrations were kept constant via delivery from concentrated stocks with peristaltic pumps, such that the delivery rate matched the clams' consumption. Algal concentrations in the tanks were monitored at least twice a day using a Multisizer 3 Beckman Coulter Counter. Clams were stocked at 4 clams cm^{-2} in 70-liter glass aquaria within a perforated polyvinyl chloride (PVC) tray (38 cm × 23 cm × 9 cm height) divided into six compartments (600 clams per compartment) filled to 5 cm with previously washed, natural sand from Crystal Crescent beach, Nova Scotia (*see* Barré [2007] for granulometric analysis). Aquaria were maintained at 16°C in a temperature-controlled, walk-in, environmental chamber. Each aquarium was fitted with an external, low-pressure, recirculating pump (Super King, Danner) to keep the water oxygenated and algal cells in suspension. Seawater was replaced in the tanks every 2–3 days. All clams were removed from one compartment from each aquarium tray at each sampling time (2 h, 4 h, 1 d, 3 d, 5 d, and 7 d) by sieving. A subsample ($n = 50$ clams) was placed on a graduated petri dish and scanned with a dissecting scope coupled to an analog camera (Pulnix TMC7-DSP), and images were recorded on a Sony DVCAM (DSR-V10) digital recorder for subsequent determination of SL. Triplicate samples of 50 pooled individuals each were frozen at −80°C for subsequent toxin analysis. Survival of toxified clams was determined after a 7 d-recovery period on the nontoxic diet, since dead animals and paralyzed clams were indistinguishable during toxification.

Postlarval or juvenile experiment I (second exposure)—Clams from the first trial were allowed to detoxify in downwellers for 60 d, a period more than sufficient to attain undetectable toxin levels. Naïve clams were held under the same conditions during this time period. Survivors of the first trial and naïve clams that had not been previously exposed to PST were exposed to the two experimental diets in duplicate systems as described already (control and toxified). The initial mean size ± SE of clams was 12.1 ± 0.2 ($n = 50$) and 11.1 ± 0.3 mm SL ($n = 30$) for BF and LE progeny, respectively. Clams with different histories of toxin exposure during the first trial (naïve, and survivors of initial 7-d toxin exposure) were held in separate compartments (250 per tray compartment); these groups were randomly assigned to a compartment in each of the two aquaria and one compartment from each aquarium was removed at each sampling date. Clam burrowing response was determined at intervals during the experiment (2 h, 4 h, 6 h, and 24 h of toxin exposure) by exposing all clams at the sediment surface and determining the percentage of the test population that burrowed at the end of 2 h (repeated measures). This burrowing assay was intended to determine the proportion of resistant (burrowers) and sensitive clams (nonburrowers), as used previously for larger (~ 35-mm SL) juveniles (MacQuarrie and Bricelj 2008). Burrowing behavior at these larger sizes was previously found to correlate well with in vitro nerve resistance (Bricelj et al. 2004), although it did not allow accurate discrimination between RR and RS genotypes. Burrowing incapacitation is expected to increase the clams' vulnerability to predators in nature and thus provides an additional measure of mortality risk under field conditions. The null hypothesis tested was that the proportion of nonburrowers would be greater in naïve progeny from parents of either source population than in those selected by pre-exposure to toxic *A. tamarense* in the laboratory.

Postlarval or juvenile experiment II (mixed suspensions)—Postlarvae (initial mean SL = 4.3 ± 0.1 mm SE) were produced by mass spawning of adults (pooled sperm from 20 males and 22 females) collected from the predominantly sensitive LE population. Postlarvae were exposed for 2 weeks at 16°C, in duplicate aquaria, to suspensions in which *A. tamarense* (strain Pr18b, cell volume ~ 22,450 μm^3) contributed varying proportions, 0%, 9%, 45%, and 100%, of the total cell volume concentration, and the remainder was made up with the nontoxic flagellate *Isochrysis galbana* (cell volume ~ 33.5 μm^3). This experimental design is useful to discriminate between toxic effects and feeding deterrence or nutritional deficiency in response to a harmful alga (Jónasdóttir et al. 1998; Colin and Dam 2002). Targeted concentrations in the end member, unialgal diets were 110 *A. tamarense* cells mL^{-1} and 7.4×10^4 *I. galbana* cells mL^{-1}. In the mixed diets, the concentration of *A. tamarense* was set at 10 and 50 cells mL^{-1} in the 9% and 45% diets, respectively. Algal concentrations in the mixed suspension were monitored as done previously, except for the 9% *A. tamarense* treatment, wherein the dinoflagellate cell density was too low to accurately determine with a Coulter Counter. In this case, the addition of *A. tamarense* was estimated based on the measured density of *I. galbana*, assuming that both algae were cleared from suspension in equal proportions. Concentrations were confirmed microscopically from Lugol-fixed samples.

Clams were stocked in 70-liter glass aquaria within trays (600 clams per compartment) as described for experiment I. All clams were removed from one compartment from each aquarium tray at each sampling date (1 d, 3 d, 5 d, 7 d, and 14 d of exposure). The percentages of motile clams (as evidenced primarily by crawling of the foot but also by siphon movement), and those with intrapallial anoxic patches were determined using video imaging immediately following removal of two subsamples of 100 clams each,

per aquarium. Motility, which can be readily determined in the absence of sediment, was used as a proxy for burrowing capacity in this experiment. At the same intervals, three subsamples of 50 clams each per aquarium were frozen at −80°C for PSP toxin analysis. Initial and final samples only (n = 50 per aquarium) were frozen to determine shell lengths. At the end of the experiment, clams from all treatments were allowed to recover for 1 week on a nontoxic diet (5×10^4 *I. galbana* cells mL^{-1}) before determining mortalities from two subsamples of 100 clams each.

Toxin analysis—Toxin extracts of clam postlarvae and dinoflagellate cells were obtained following previously described methods (Doane 2007; MacQuarrie and Bricelj 2008). Frozen, pooled whole bodies (3 samples of 50 clams each) were lyophilized for 48 h, weighed to determine the total body dry weight (dry wt) per individual, pulverized, and extracted in 0.1 mol L^{-1} acetic acid (HOAc) with a Polytron homogenizer, and dinoflagellate cells (1×10^6 cells) were extracted in 3 mL of 0.03 mol L^{-1} HOAc to maintain the integrity of individual toxins using a Vibracell sonicator. Toxin analysis by high-performance liquid chromatography with fluorescence detection (HPLC-FD) was performed following methods of Oshima (1995) with minor modifications. Individual toxins were quantified using certified external standards provided by IMB's Certified Reference Materials Program (CRMP). Concentrations of individual toxins were converted to saxitoxin equivalents (STXeq) using toxin-specific conversion factors (mouse units [MU] μmol^{-1}) obtained using the standard mouse bioassay (Association of Official Analytical Chemists [AOAC] 1990) and a conversion factor of 0.23 μg STXeq MU^{-1} (Cembella et al. 1993). Individual toxicities were summed to yield total toxicity in μg STX eq 100 g^{-1} tissue wet weight (wet wt). A conversion factor between total body dry wt (soft tissues + shell) and wet wt of soft tissues for *M. arenaria* postlarvae was separately determined for postlarvae of comparable SL: soft tissue wet wt = (total body dry wt × 0.192) ÷ 0.235; n = 57. This enabled comparison of postlarval toxicity values in μg STXeq 100 g^{-1} soft tissue wet wt, the unit used by regulatory agencies, with published values for larger (~ 35 mm) juveniles. Pooled lyophilized dry wt was also used to calculate the instantaneous growth coefficient $k = [(\ln \text{dry wt}_f - \ln \text{dry wt}_o) \div t] \times 100$, where t = time interval, and dry wt_f and dry wt_o are final and initial values, respectively.

Genotypic analysis—All genotypic analyses were carried out at the University of Maine. The genotype at the *M. arenaria*–specific DII α-subunit Na^+ channel fragment locus was determined for individuals used in experiments I (both BF and LE clams) and II (only LE clams). A larger sample size in experiment II, obtained from duplicate aquaria, allowed improved characterization of genotypic changes and representation of all three genotypes. At the position homologous to E945 in the aligned rat amino-acid sequence, a substitution of aspartic acid (D) in resistant clams for glutamic acid (E) in sensitive clams causes a 1000-fold decrease in affinity at the STX binding site of the Na^+ channel pore (Bricelj et al. 2005). Heterozygote (ED) clams showed intermediate nerve resistance to STX (Connell et al. 2007; Bricelj et al. unpubl. results). Clams from experiment I (n = 25 to 30 per group) were stored frozen at −80°C, and those from experiment II were stored in RNA*later*® (Ambion, Austin, Texas) prior to deoxyribonucleic acid (DNA) extraction. Tissue was extracted from individual clams for sequence analysis by first removing the shell with sterile forceps and excising a small piece of tissue (< 100 mg), while avoiding the visceral mass. The tissue was placed into a sterile 1.5-mL microcentrifuge tube designed for use with a disposable pestle (Kimble-Kontes) and ground with a handheld motorized pellet grinder (Kimble-Kontes). DNA was extracted and the sequence determined following methods described by Connell et al. (2007). Genotype was determined for broodstock by directly applying polymerase chain reaction (PCR) and sequencing methods to extracted hemolymph as described by Hamilton and Connell (2009). DNA sequences were aligned and predicted amino acids were translated using Sequencher version 4.8 (Gene Codes Corp). Clams were scored as sensitive (SS) if their genotype at E945 was EE, resistant (RR) if their genotype was DD, and heterozygous (RS) if their genotype was DE.

Statistical analysis—Statistical analysis of postlarval experiments was conducted using Systat 10 (SPSS) software. Percentages (% mortality, % motility, % of clams with intrapallial anoxic patches, % burrowing) were arcsine transformed prior to analysis of variance (ANOVA). The burrowing responses (% nonburrowers) of naïve clams and those selected by pre-exposure to *A. tamarense* in experiment I were compared using a two-way repeated measures ANOVA (univariate test) of arcsine-transformed values, with time as the repeated, within-subjects measure. The assumption of sphericity was evaluated using Greenhouse-Geisser's (G-G) and Huynh-Feldt's (H-S) epsilon indices, and if violated, adjusted probabilities were used. Each repeated measures test was followed by post hoc F contrasts to compare the treatment effect (naïve vs. selected) at each time point. Separate analyses were conducted for BF and LE clams since trials with the two populations were run sequentially.

For experiment II, toxicity, % motility, % of anoxic clams, % mortality, and toxicity were tested by two-way ANOVA with time and treatment (diet) as factors. A one-way ANOVA followed by Tukey's unplanned multiple comparison was also conducted for all treatments at the end of the experiment (day 14) when mortalities had approached asymptotic levels and no further selection occurred. The slopes of the linear regressions fitted to the clam toxicity vs. time data were compared by analysis of covariance (ANCOVA). Shell growth rates were calculated as $(SL_f - SL_o) \div t$, assuming a linear increase over the short duration (time t) of the experiment, where SL_f and SL_o are final and initial SL values, respectively, and compared by one-way ANOVA followed by a Tukey's multiple comparison. Tissue dry wt values over time were best fitted by exponential equations; therefore, a semi-log transformation allowed calculation of the slopes of linear regressions (log

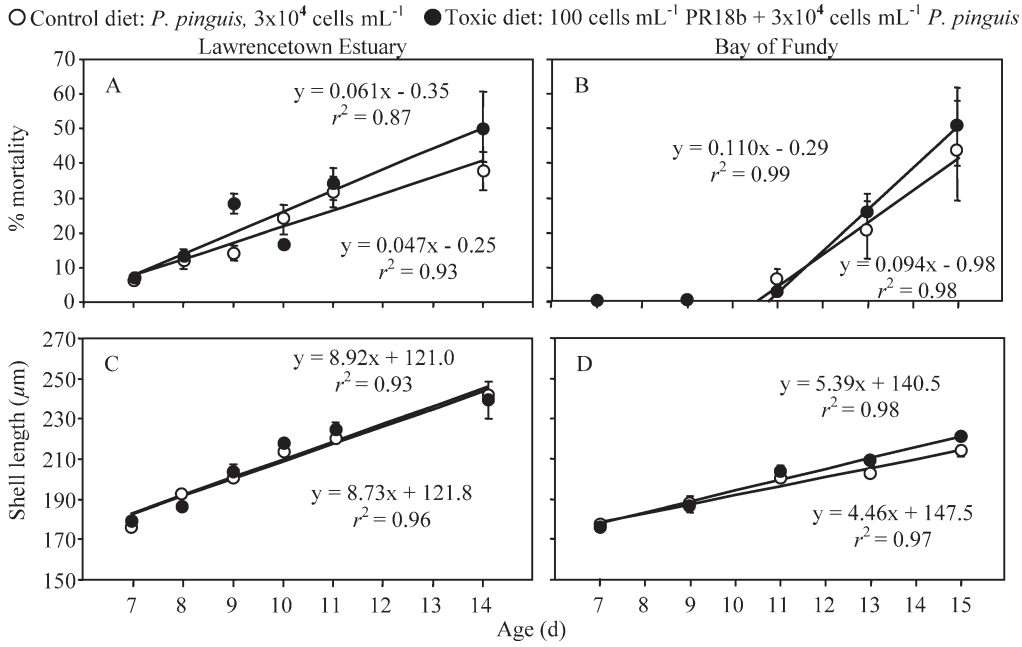

Fig. 1. (A, B) Percent cumulative mortality and (C, D) shell length of *M. arenaria* 1-week-old veliger larvae, progeny from adults of the two test populations, over 7-d exposure to a control, nontoxic, and toxic diet containing *A. tamarense* (strain PR18b). Values indicate means ± standard error (SE).

dry wt vs. time), followed by Tukey's multiple comparison of the slopes (ANCOVA).

Statistical analyses of genotype frequencies were run using the Fisher's exact test of the R open source statistical package from 2 × 2 and 2 × 3 contingency tables for experiments I and II, respectively. This test was used because of the low numbers obtained for some of the genotypes.

Results

Effects of PSTs on clam larvae—Growth and survival of 1-week-old veliger larvae, from either the BF or LE populations, when exposed to the highly toxic *A. tamarense* PR18b isolate were not adversely affected compared to nontoxic controls (Fig. 1). Mortalities were comparable in both toxic and control diets by the end of the experiment (Fig. 2A,B). Shell growth rates were linear (Fig. 1C,D) and equal to 8.7 and 8.9 μm d^{-1} in LE clams fed *A. tamarense* and *I. galbana*, respectively. They were lower, but again not different, between diet treatments in the experiment with BF larvae: 5.3 and 4.5 μm d^{-1}, respectively.

Effects of PSTs on postlarvae: Experiment I—The toxicity of *A. tamarense* cells was high in both toxification trials with resistant (BF) and sensitive (LE) progeny (experiment I, first exposure), averaging 64.4 (± SE = 2.6) and 68.9 (± SE = 13.7) pg STXeq cell^{-1}, respectively (Fig. 2). Postlarvae from both test populations experienced rapid mortality from exposure to a unialgal suspension of *A. tamarense* (100 cells mL^{-1}), whereas neither BF nor LE clams in the control nontoxic diet exhibited mortalities throughout the experimental period (not shown). However, LE clams exhibited higher mortalities and lower toxicities than BF clams, even though they were exposed to cells of comparable toxicity (Fig. 2). The latter attained 2.4× higher toxicities during the first 48 h of exposure than LE clams. Toxicities were only determined over the first 2–3 d,

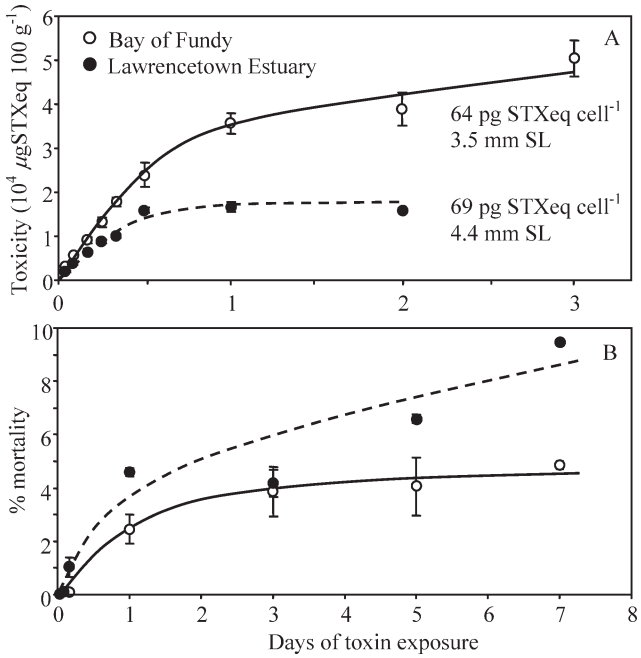

Fig. 2. (A) Toxicity (mean ± SE, n = 3 pooled samples, in μg STXeq g^{-1} wet weight of soft tissues) and (B) percent cumulative mortality (mean ± SE of two aquaria, 2 samples per aquarium) of *M. arenaria* postlarvae, progeny from adults of the two test populations, during experiment I. Postlarvae in the control, nontoxic diet experienced 0% mortalities (not plotted). Mean cell toxicities of *A. tamarense* (strain PR18b) and postlarval shell length are indicated.

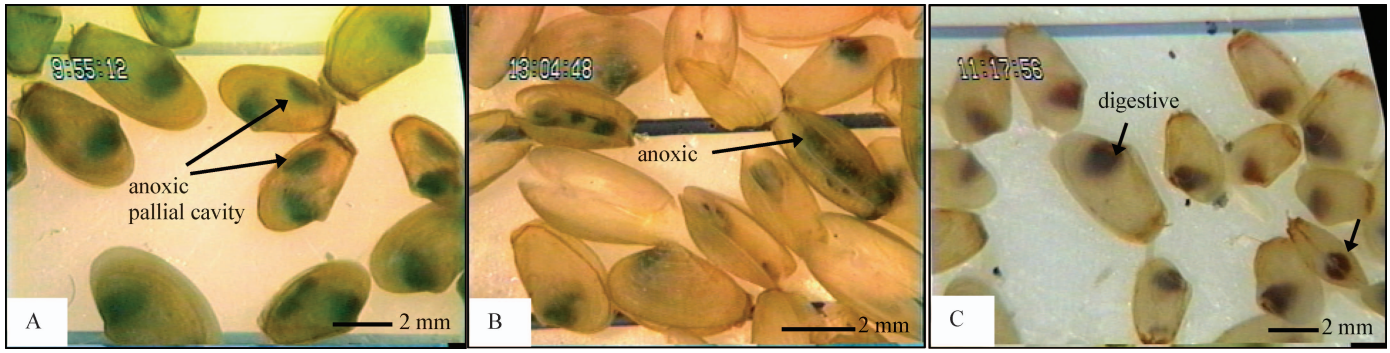

Fig. 3. Video images of *M. arenaria* postlarvae (~ 4 mm SL) following 5- to 7-d exposure to toxic *A. tamarense* cells (experiment I). Scale = 2 mm. (A, B) Progeny from LE parents after 5 d and 7 d of toxin exposure, respectively. Note the presence of numerous empty but still articulated shell valves in (B). (C) Progeny from BF parents after 7 d of toxin exposure; note the absence of anoxic patches in contrast to (A). Long arrows indicate the presence of anoxic patches within the pallial cavity, and short arrows point to the visceral mass, including the digestive gland.

to reduce the possibility of inclusion of dead clams. High mortalities of clams from the sensitive LE population occurred within 4 h of exposure to toxic cells, resulting in 95% cumulative losses following only 1 week of exposure (Fig. 2B). BF clams attained a lower cumulative mortality of 49% at 1 week. Since the two populations were tested sequentially, direct statistical comparison between populations is not possible, although the same protocols were used in both experiments, and postlarval sizes and cell toxicity and concentration were comparable. The LE progeny exhibited rapid paralysis of the foot and showed evidence of anoxic patches in the pallial cavity presumably due to reduced irrigation (Fig. 3).

Burrowing capacity as an indicator of sensitivity to PSTs was compared between naïve and selected clams that were re-exposed to PSTs following recovery from an initial, 7 d-simulated toxic bloom (Fig. 4). The toxicity of *A. tamarense* during this second exposure was 74.1 (± SE = 6.7) and 47.9 (± SE = 0.9) pg STXeq cell^{-1} for BF and LE clams, respectively. For both source populations the H-F epsilon index indicated acceptable symmetry of the variance-covariance matrix (H-F = 1), whereas the G-G epsilon index indicated substantial departure from homogeneity of variance (G-G = 0.4). Therefore, G-G adjusted probabilities are reported in Table 1, because they yield the more conservative outcome. The two-way repeated measures ANOVA showed that both treatment and time had a significant effect on burrowing in the BF populations (Table 1), and post hoc tests indicated that the differences were significant at all time points (caption to Fig. 4). The time × treatment interaction was only significant for BF clams. In the LE population, the difference between naïve and selected clams was only marginally significant (p = 0.05; Table 1), and there was a significant time effect; post hoc comparisons detected significant differences only at 4 and 24 h (caption to Fig. 4). In the BF population, the percentage of nonburrowers increased linearly over time, although the slope of the equation was greater in naïve than selected clams such that the two groups diverged increasingly over time (Fig. 4). In the LE population, the percent of nonburrowers over time was best fitted by a logarithmic equation, such that both selected and naïve clams approached asymptotic levels after 24 h of exposure.

Selective elimination of sensitive clams following toxin exposure was confirmed by genotypic analysis, which showed a significant increase in the prevalence of RS clams following 1 week of toxin exposure (from 10% to 47% of the total in LE clams) (p = 0.003; Fig. 5A). No RR clams were represented in this test population due to the relatively small sample size. Although 100% of the BF clams analyzed were resistant (RR or RS), the contribution of heterozygote clams was reduced from 42% to 17%. Although this difference was not significant (p = 0.11), it suggests that the increase in the prevalence of nonburrowers in the BF population over time of exposure (Fig. 5A) reflects the difference in susceptibility to PSTs between RR and RS clams.

Concentration-dependent effects on clam postlarvae from a sensitive population: Experiment II—The toxicity of *A. tamarense* cells over the course of this experiment averaged 35.7 pg STXeq cell^{-1} ± SE = 2.6 (n = 10 carboys). Clams in the nontoxic diet experiment and those fed the lowest *A. tamarense* cell density suffered negligible mortalities (0.2–0.7%, respectively), whereas clams exposed to diets with the two highest *A. tamarense* percentages (45% and 100%) attained significant cumulative mortalities of 15% and 50%, respectively, by day 14 (Fig. 6A). Mortalities began to level off by day 5. Clam toxicities increased with increasing contribution of *A. tamarense* to the diet, a pattern that was already apparent by day 1 of exposure. By day 14, mean toxicities were 1.5 × 10^3, 5.3 × 10^3, and 13.4 × 10^3 μg STXeq 100 g^{-1} in the 9%, 45%, and 100% *A. tamarense* diets, respectively, although at this time point, the difference was only statistically significant for the 100% *A. tamarense* diet (Fig. 6C). Toxin accumulation rates, given by the slopes of the fitted linear regressions (80, 154, and 247 μg STXeq 100 g^{-1} tissue wet wt), also increased significantly with increasing percentage of *A. tamarense* in the diet (ANCOVA, Fs = 312.8, df = 2, $p < 0.001$).

Sublethal effects, loss of motility, and the presence of anoxic patches are indicators of the paralyzing effects of

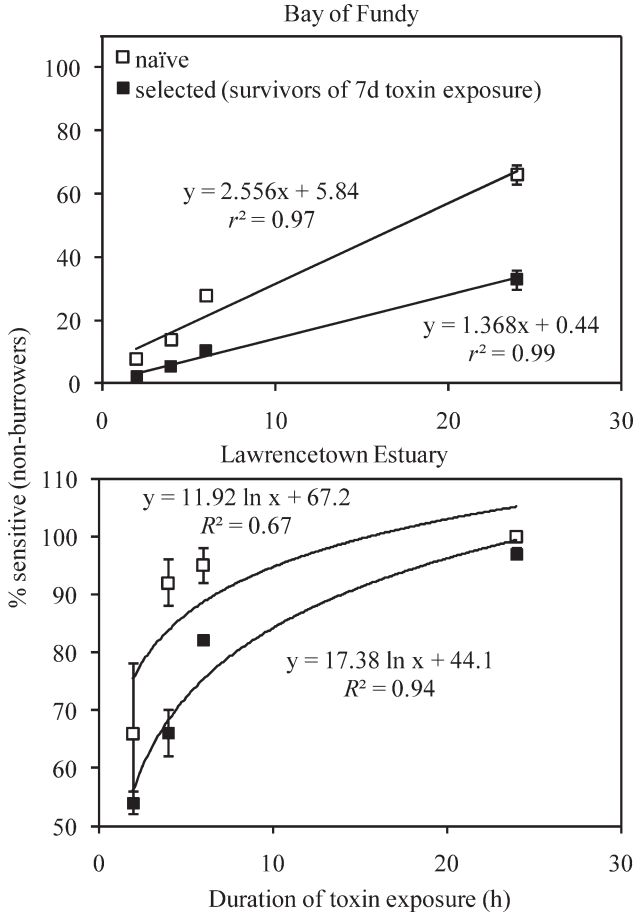

Fig. 4. Comparison of burrowing capacity of naïve (previously unexposed to PSTs) and selected (pre-exposed) *M. arenaria* postlarvae (11–12 mm mean SL) obtained as progeny from BF and LE brood stock. Selected clams are survivors of 7-d toxin pre-exposure, depurated on a nontoxic diet for ~ 2 months prior to re-exposure (experiment I). Relative sensitivity to PSTs was determined from the percentage of clams (mean ± SE) unable to burrow following varying periods of exposure to toxic *A. tamarense* (2, 4, 6, and 24 h) (second toxin exposure). Fitted equations are plotted, where x = hours of toxin exposure, and y = % nonburrowers. Differences between naïve and selected clams at each time point were tested by post hoc *F* contrasts following two-way, repeated measures ANOVA (Table 1): BF population, df = 1, *F*-ratio = 29.8, 759.4, 10,015, and 55.8 for analysis at 2 (*), 4 (**), 6 (***), and 24 h (*), respectively; LE population, df = 1, *F*-ratio = 0.24, 39.5, 9.5, and 33.3 at 2 ($p = 0.67$, ns), 4 (*), 6 ($p = 0.09$, ns), and 24 h (*), respectively. Asterisks indicate significant differences, * $0.01 < p \leq 0.05$; ** $0.001 < p \leq 0.01$; *** $p \leq 0.001$; ns = not significant.

PSTs. Percent motility and percent anoxia fluctuated over time, but, as expected, they varied in an inverse fashion (Fig. 6B,D). Marked differences in clam motility between diet treatments were detected very early on, within the first day of toxin exposure, and were greatest at this time point. Motility reduction at day 14 was greatest at the two highest *A. tamarense* concentrations (~ 41% and 67%, respectively) (Fig. 6B). Clams exposed to the lowest *A. tamarense* concentration showed only a small, yet significant reduction in motility (~ 10% at day 14) relative to controls.

Table 1. Results of two-way repeated measures ANOVA comparing naïve and selected clams (treatment, between-subjects factor) over time (within-subjects, repeated factor) in experiment I (data shown in Fig. 4). * $0.01 < p \leq 0.05$; ** $0.001 < p \leq 0.01$; ns = not significant; df = degrees of freedom; BF = Bay of Fundy; LE = Lawrencetown Estuary. G-G adjusted probabilities are given for time, and time × treatment effects.

	df	*F*-ratio	*p*
BF population			
Treatment	1	473.4	**
Time	3	323.9	**
Time×treatment	3	12.0	*
LE population			
Treatment	1	17.6	*
Time	3	26.8	*
Treatment×time	3	1.5	0.3 (ns)

Anoxic patches were only observed in clams exposed to diets that included toxic *A. tamarense* cells. Evidence of anoxia appeared as early as the first day of toxin exposure. The overall trend in these data was a decrease in the percent of clams with anoxic patches over the course of the experiment.

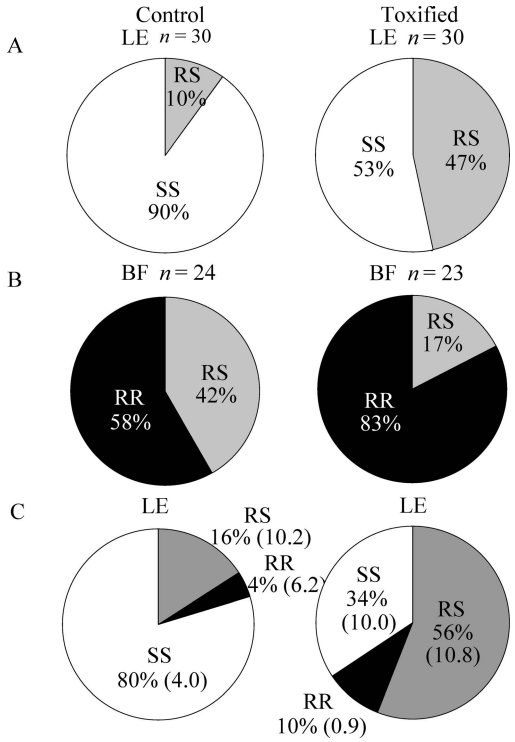

Fig. 5. Percentage of *M. arenaria* postlarvae of each genotype (resistant [RR], sensitive [SS], and heterozygous clams [RS]) in experimental populations after: experiment I (A) 7-d exposure to nontoxic algae (control) or (B) toxic *A. tamarense* (strain Pr18b, 100 cells mL^{-1}; 64–69 pg STXeq $cell^{-1}$), and (C) experiment II after 2-week exposure to nontoxic algae or strain Pr18b at 110 cells mL^{-1}, 36 pg STXeq $cell^{-1}$ (values represent the mean ± standard deviation of two aquaria; n = 22–29 clams per aquarium; n = 49 and 51 in control and toxified treatments, respectively).

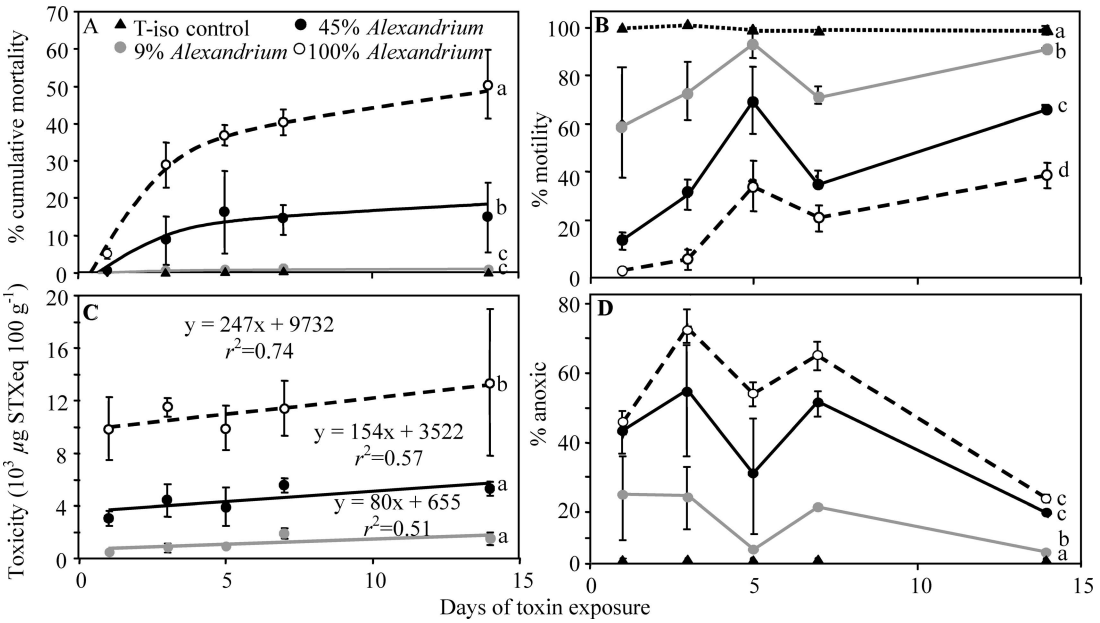

Fig. 6. (A) Percent cumulative mortality, (B) percent motility, (C) toxicity in μg STXeq 100 g^{-1} wet tissue weight with fitted linear regression equations, and (D) percent of test individuals with intrapallial anoxic patches, of postsettlement *M. arenaria* (progeny from LE brood stock) exposed to varying dietary proportions (0%, 9%, 45%, and 100%) of *A. tamarense* (strain PR18b) (experiment II). Values represent means $\pm$ SE (n = 2 aquaria). Different letters indicate significant differences in mean toxicity values at the end of the experiment (day 14) ($p \leq 0.05$) (ANOVA and Tukey's multiple comparison).

Overall, there was a highly significant effect of both treatment and time for all variables measured, including toxicities, mortalities, % motility, and % of clams with anoxic patches (two-way ANOVAs, $p < 0.001$; Table 2). The treatment $\times$ time interaction was also highly significant ($p < 0.001$), except for toxicities.

Clams in the control and lowest *A. tamarense* concentration did not differ significantly in shell growth rates, i.e., 141 and 148 μm d^{-1}, respectively (Fig. 7A), and showed visible signs of new growth at the edge of the shell. In contrast, clams exhibited a significant reduction in shell growth rate at the two highest *A. tamarense* concentrations ($\geq$ 50 cells mL^{-1}), i.e., both when exposed to the unialgal *A. tamarense* suspension and the mixed diet containing 45% *A. tamarense* (Fig. 7A). Thus, shell growth rate (y) declined exponentially with increasing contribution of *A. tamarense* to the diet (x). At the two highest concentrations of toxic algae (50 and 110 *A. tamarense* cells mL^{-1}, i.e., 45% and 100% contribution), final tissue dry wt on day 14 was significantly reduced (to $\sim$ 0.14 and 0.05 mg dry wt clam^{-1} d^{-1}, respectively) relative to the control and low *A. tamarense* treatment ($\sim$ 0.37 and 0.42 mg dry wt clam^{-1} d^{-1}, respectively) (Fig. 7B). Tissue growth rates, as indicated by the exponents of the fitted exponential equations (Fig. 7B), and instantaneous growth coefficients (k = 7.01%, 6.54%, 3.14%, and 1.32% d^{-1}) were inversely related to the proportion of toxic *A. tamarense* in the diet from 0% to 100%. At the two highest *A. tamarense* concentrations (50 and 110 cells mL^{-1}), tissue growth rates were significantly reduced ($p < 0.001$) compared to the control and low *A. tamarense* treatment, which did not differ from each other ($p = 0.75$) (ANCOVA and Tukey's multiple comparisons).

The LE test population exposed to a unialgal suspension of *A. tamarense* showed a 58% mean reduction in the percentage of the sensitive (SS) genotype relative to clams fed the control diet. The contribution of SS clams declined from 80% to 34% of the total, and the percentage of total resistant clams (RR + RS) increased from 20% to 66% following selective mortalities by PSTs (Fig. 5B). This shift

Table 2. Results of two-way analysis of variance comparing mortalities, percent motility, toxicities, and percent of anoxic patches over treatment (diet) and time in experiment II (progeny from the Lawrencetown Estuary). *** $p \leq 0.001$; ns = not significant; df = degrees of freedom.

Source	df	*F*-ratio	*p*
% mortality			
Diet	3	332.9	***
Time	4	37.7	***
Diet×time	12	10.3	***
% motility			
Diet	3	517.7	***
Time	4	0.4	***
Diet×time	12	8.4	***
Toxicity			
Diet	2	327.0	***
Time	4	6.4	***
Diet×time	8	1.0	0.4 (ns)
% anoxic patches			
Diet	3	314.4	***
Time	4	34.8	***
Diet×time	12	6.4	***

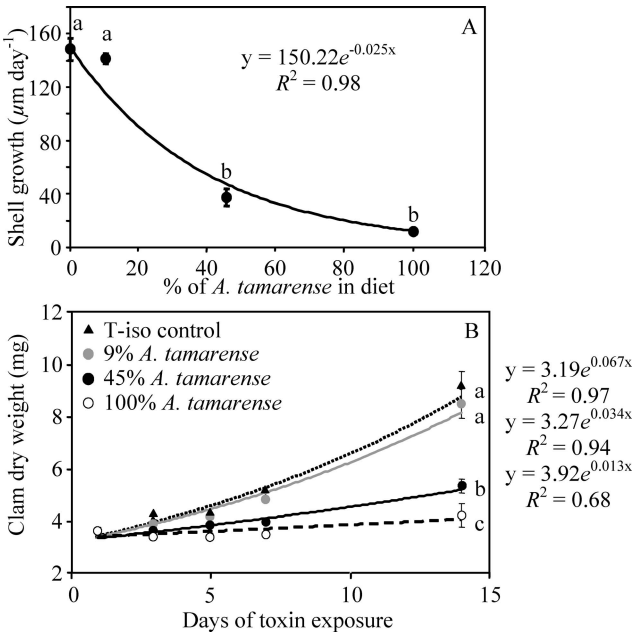

Fig. 7. (A) Shell growth rate (mean ± SE, $n = 2$ tanks) of postlarval *M. arenaria* (progeny from LE brood stock), calculated over 2 weeks, as a function of the volume contribution of *A. tamarense* to the diet, and (B) total body dry weight per individual ($n = 3$) over time of exposure to various diet treatments with fitted exponential equations (experiment II). Different letters indicate significant differences in final mean values (ANOVA and Tukey's multiple comparison, $p < 0.01$). The same results were obtained by comparing the slopes of the linearized regression equations, i.e., log dry wt vs. time (ANCOVA, *see* Methods).

in the genotypic frequencies was highly significant ($p < 0.001$). As a result of the larger sample size, a small proportion (4%) of RR clams was represented in the LE test population, and this increased to 10% following toxin exposure.

Discussion

Results of this study combined with prior experimental results using larger juvenile *M. arenaria* (35–42 mm) (MacQuarrie and Bricelj 2008) identify postlarvae as the life-history stage most susceptible to natural selection by PSP. Blooms of toxic *Alexandrium* spp. in the NW Atlantic occur in the summer, typically peaking in June in southern New England, and in July–August in eastern Maine and Bay of Fundy (Anderson 1997). As illustrated for the Bay of Fundy, the seasonal occurrence of toxic blooms, reflected in the peak of *M. arenaria* toxicities, thus coincides with the main period of spawning and larval development of *M. arenaria* and also with the occurrence of small, second-year juveniles (Fig. 8). Blooms of *Alexandrium fundyense* in the Bay of Fundy are initiated in late May to early June and persist through the summer until mid- to late September, with peak cell densities up to 162 cells mL^{-1} occurring in mid-July (Martin et al. 1998, 2009). Soft-shell clam settlement peaks in the region typically occur from mid-August to mid-September (Emerson and Grant 1991; Bowen and Hunt 2009). The peak of shellfish

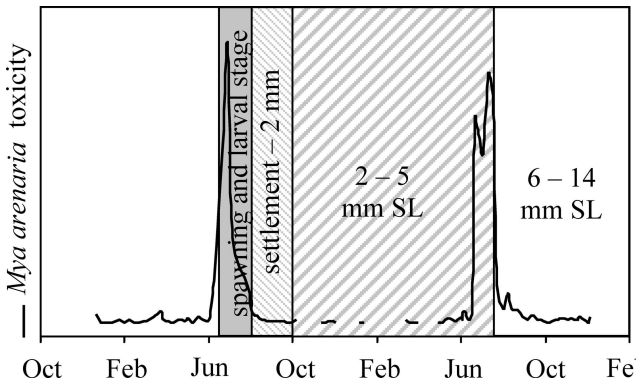

Fig. 8. Schematic showing the seasonality of PSP toxicity in *M. arenaria* adults (as determined by routine monitoring using the mouse bioassay) in relation to the occurrence of early life-history stages of soft-shell clams in the Bay of Fundy, Atlantic Canada. Sources: D. Richard and J. Martin (DFO, unpublished toxicity data), Newcombe (1935), Chandler et al. (2001), and Angus et al. (1985).

toxicities, and thus the timing of toxic blooms, occurs earlier in a southwestward direction along the coast from eastern Maine to Cape Cod, partly reflecting earlier coastal warming and stratification in the western region (Anderson 1997; McGillicuddy et al. 2005). Therefore, toxic *Alexandrium* blooms can co-occur throughout the Gulf of Maine, New England region, with the *M. arenaria* life-history stage most vulnerable to PSTs, i.e., juveniles less than ~ 20 mm SL. Selective pressure by PSP is expected, however, to vary latitudinally depending on *Alexandrium* spp. cell toxicity, and bloom intensity and duration.

Fitness effects of toxic A. tamarense *on* M. arenaria *veliger larvae*—Growth and survival of 1-week-old soft-shell clam larvae were not adversely affected by exposure to toxic *A. tamarense* cells for 1 week (Fig. 1). This is attributed to the fact that dinoflagellate cells were too large (mean = 35 μm ESD) to be ingested by clam larvae (size range ~ 180–240 μm SL in the present study), and that any potential release of dissolved PSTs by *A. tamarense* was insufficient to elicit harmful effects. Bivalve larvae have typically been shown to selectively retain ≤ 10-μm particles from natural seston irrespective of larval size, food quantity, and location of peak volume, but large oyster larvae (> 200 μm SL) can occasionally ingest large dinoflagellates (20–30 μm ESD) when very abundant (Baldwin and Newell 1995). These authors, however, never detected significant ingestion of > 30-μm-size particles. Use of cultured cells confirmed that larger, late veliger stages of *Mytilus galloprovincialis* approaching competence were able to ingest several large dinoflagellate species, including the nontoxic *Alexandrium affine* (24 μm ESD), and *Lingulodinium polyedrum* (compressed cells with 38 μm ESD), yet dinoflagellates were the less preferred food item when offered in a mixed suspension with *I. galbana* (Jeong et al. 2004).

Alexandrium tamarense in batch-culture has been shown to release PSTs into the surrounding medium, with maximum dissolved toxin concentrations occurring during

the late stationary growth stage (55 μg STXeq L^{-1}), a level comparable to that measured in the particulate phase (Hsieh et al. 2001). It has also been demonstrated that dissolved STX can impair the sensorimotor function (as measured by swimming avoidance and tactile response) of herring larvae within 1 h of exposure in a dose-dependent manner, and cause paralysis starting after 4 d, but the STX concentrations required to elicit these effects were $\geq$ 700 μg STXeq L^{-1}, depending on development stage (Lefebvre et al. 2005). Dissolved concentrations $\geq$ 229 μg STXeq L^{-1} were required to cause a similar reduction in sensorimotor function in larval zebrafish, which also experienced morphological abnormalities after 7 d of exposure to 481 μg STXeq L^{-1} (Lefebvre et al. 2004). The STX levels that elicited sublethal effects in these laboratory studies were, however, two orders of magnitude greater than the maximum dissolved STX level measured to date by enzyme-linked immunosorbent assay (ELISA) during natural red tides (K. Lefebvre, National Oceanic and Atmospheric Administration, National Marine Fisheries Service, Northwest Fisheries Science Center, pers. comm.). Mortalities of nonfeeding oyster trocophore larvae only occurred when they were exposed to $\geq 1 \times 10^7$ *A. tamarense* cells mL^{-1} (Matsuyama et al. 2001), a concentration unlikely to occur in nature. Similarly, a moderately toxic *A. tamarense* strain (11 pg STXeq $cell^{-1}$) had no negative effect on survival of scallop (*Argopecten irradians*) D-stage larvae that are too small to consume *A. tamarense* cells, even at extremely high cell densities (10×10^3 cells mL^{-1}) (Yan et al. 2003). This same strain caused reduction of egg-hatching success in *Clamys ferreri*, at lower concentrations ($\geq$ 100 cells mL^{-1}), but this was associated with a non-PST bioactive present on the algal cell surface (Yan et al. 2001).

Results to date on fish and scallop larvae and those of the present study using clam larvae thus suggest that the main pathway for PST contamination and induction of sublethal or lethal effects at ecologically relevant concentrations remains that of exposure to particulate food, i.e., toxic *Alexandrium* cells. This contrasts with lethal effects reported for bivalve larvae exposed to other harmful algal bloom (HAB) species such as *Karenia brevis*, which is prone to the release of dissolved brevetoxins via cell lysis (Leverone et al. 2007). The present study thus concludes that natural selection for resistance during PSP outbreaks is not expected to operate during *M. arenaria*'s planktotrophic period of larval development.

Effects of toxic A. tamarense *on* M. arenaria *postlarvae or juveniles (size-specific effects)*—In contrast to larvae, postmetamorphic clams experienced rapid mortalities following exposure to a simulated bloom of *A. tamarense* of comparable toxicity, especially the predominantly sensitive LE population, in which the onset of mortalities occurred at 2 h (Fig. 2B). This contrasts with previous findings for large juveniles collected from the same source population toxified under comparable laboratory conditions, in which mortalities were consistently delayed, i.e., only initiated after 8–10 d of toxin exposure, and reached much higher levels than in BF clams, although they varied considerably between experiments (26% to 72% in LE clams compared to 0% to 1% in BF clams in 2 weeks) (MacQuarrie 2002; MacQuarrie and Bricelj 2008). Thus, the effects of PSP are highly size specific. Postlarvae from the dominantly resistant population also experienced mortalities induced by exposure to toxic *A. tamarense*, although of a much lower magnitude than the LE progeny. In contrast, large juveniles experienced negligible or no mortalities under similar exposure conditions, indicating that postlarvae are the life-history stage most susceptible to the effects of PSTs. Peak toxicities attained by postlarvae (this study) were greater than those of large juveniles exposed to the same *A. tamarense* strain and concentration (MacQuarrie and Bricelj 2008; experiment II), i.e., 15×10^3 and 3.3×10^3 μg STXeq 100 g^{-1}, respectively, for the LE population, and 50.4×10^3 and 20×10^3, respectively, for the BF population.

Mortalities of sensitive postlarvae are likely attributable to anoxia of the pallial cavity resulting from toxin-induced muscular paralysis and reduced irrigation of the pallial cavity rather than direct effects of PSTs. Morphological abnormalities induced by exposure to dissolved STX in zebrafish larvae have also been attributed to secondary effects of edema and osmotic disruption associated with paralysis, rather than a direct effect of STX on heart tissue (Lefebvre et al. 2004). Burrowing of an increasing proportion of BF clams became compromised after $>$ 4 h of toxin exposure (Fig. 4), whereas that of larger BF juveniles ($\sim$ 35 mm) was not affected even after 24 h of toxin exposure (MacQuarrie and Bricelj 2008), again demonstrating that the effects of PSTs are highly size specific.

Concentration-dependent effects of A. tamarense *on* M. arenaria *postlarvae*—Although *A. fundyense* and *A. tamarense* densities in the Gulf of Maine typically attain maxima of only up to 3–5 cells mL^{-1} (Townsend et al. 2005*a,b*), higher abundances occur in some years (e.g., 20 cells mL^{-1} in Cape Cod Bay and offshore waters in 2005; Anderson et al. 2005) and in nearshore waters (e.g., 200 cells mL^{-1} in Cape Cod ponds in 2008; B. Keafer, Woods Hole Oceanographic Institution, pers. comm.). Higher *A. tamarense* densities commonly occur in the Gulf of St. Lawrence Estuary, with maxima of 50 to 500 cells mL^{-1} recorded over 6 yr along the south shore of this estuary (Blasco et al. 2003), and in the Bay of Fundy, where between 1987 and 2004 annual peak concentrations of *A. fundyense* exceeded 90 cells mL^{-1} in 5 yr (Martin et al. 2009). Thus, the unialgal concentration of *A. tamarense* used in our study is representative of high, naturally occurring densities in the region. Few studies, however, have determined the relative abundance of *Alexandrium* spp. in naturally occurring mixed phytoplankton assemblages. *Alexandrium* spp. can be dominant during very severe blooms in the Bay of Fundy (J. Martin, Department of Fisheries and Oceans [DFO], St. Andrews, pers. comm.), but contributed $\leq$ 28% of total phytoplankton carbon during 2 yr of low *Alexandrium* densities ($\leq$ 2 cells mL^{-1}) at an inshore location in the western Gulf of Maine where diatoms were dominant (Teegarden et al. 2001).

In the present study, a cell density of *A. tamarense* ≥ 10 cells mL^{-1} (≥ 9% volume contribution to the diet) was required to induce deleterious effects in postlarvae generated from broodstock from a predominantly sensitive population, and this is dependent on the dinoflagellates' cellular toxicity. For this highly toxic isolate, which attained on average 36 pg STXeq $cell^{-1}$ in experiment II, concentrations ≥ 50 cells mL^{-1} were required to induce significant mortalities and shell growth inhibition of *M. arenaria* juveniles (Figs. 6A, 7A, respectively). Although a density of only 10 *A. tamarense* cells mL^{-1} did not elicit significant mortalities or reduced shell or tissue growth, this low level was sufficient to significantly reduce clam motility (Fig. 6B). This transient paralysis could therefore lead to exposure at the sediment surface, greater susceptibility to predators, desiccation, and/or advection or bed-load transport to unsuitable habitat by tidal action under field conditions. Juvenile soft-shell clams live just below the sediment surface and are therefore especially vulnerable to these processes (Emerson and Grant 1991). Since, as is general for most bivalves, predatory risk is an inverse function of body size, toxin-induced burrowing incapacitation will be most deleterious for postsettlement stages, and toxin-induced reduction in growth rates will only serve to prolong the period of vulnerability to predators.

All postlarval parameters measured yielded intermediate values at the intermediate *A. tamarense* cell volumes tested (100% > 45% > 9%) (Fig. 6). As indicated earlier, there is very limited information from field studies throughout the region on the contribution of toxic cells of *Alexandrium* spp. to the total phytoplankton assemblage in terms of cell volume or biomass, or on the relationship between this parameter and shellfish toxicities, because often only *Alexandrium* cell densities are reported.

Cumulative mortalities of clams by day 7 in relation to *A. tamarense* cell toxicity can be compared between experiments I (LE initial exposure) and II, since they used a comparable size of postlarvae and *A. tamarense* cell density, and the LE populations had a similar initial percentage of sensitive (S) clams (90% and 80%, respectively). Thus, the mean mortality at this time was approximately twice as high in experiment I (95%), during which clams were exposed to the isolate of higher toxicity (69 pg STXeq $cell^{-1}$ vs. 36 pg STXeq $cell^{-1}$ in experiment II), indicating that mortality (and selective pressure) was a direct function of cell toxicity over this range.

Genotypic effects on M. arenaria *postlarvae*—Results of this study demonstrate strong selection for toxin resistance in *M. arenaria* postlarvae or juveniles, leading to a marked shift in population gene frequencies that can occur within ≤ 1–2 weeks of exposure to a high-intensity *A. tamarense* bloom (Fig. 5). Exposure to a highly toxic strain of *A. tamarense* led to a marked increase in the ratio of homozygous (RR) to heterozygote (RS) resistant clams in the BF population from 1.4 to 4.9 within a week of exposure (experiment I), indicating that RS clams show intermediate resistance, and led to a 41% to 57% reduction in sensitive clams in the LE population (experiments I and II, respectively).

Selective mortalities in both large (~ 35 mm; MacQuarrie and Bricelj 2008) and small clams (4 to 12 mm juveniles; this study), however, only occurred under conditions of severe toxin exposure (≥ 50 cells mL^{-1} of a highly toxic *Alexandrium* strain [36–98 pg STXeq $cell^{-1}$]). These conditions are atypical of southern New England coastal waters and many parts of the Gulf of Maine. *Alexandrium* isolates from this region can exhibit variable toxicity profiles and cell toxicities as a function of physiological status and source location (Anderson 1990; Anderson et al. 1994; Etheridge and Roesler 2005). Superimposed on this variability, there is a strong north-to-south decreasing trend in the cell toxicity of *Alexandrium* isolates along the U.S. coast, ranging from ~ 66 pg STXeq $cell^{-1}$ to undetectable (Maranda et al. 1985). Higher cell toxicities, ranging from 30 to 130 pg STXeq $cell^{-1}$, were obtained for isolates from the Gulf of St. Lawrence Estuary (Cembella et al. 1988). As indicated earlier, cell densities of *Alexandrium* spp. that elicited postlarval mortalities in this laboratory study primarily occur in the northern portion of this algal species' range on the NW Atlantic coast. Thus, natural selection for resistance may only occur in years and regions characterized by particularly severe toxic blooms and when there is overlap between a severe red tide outbreak and the presence of small recruits. Brief exposure to more moderate blooms will lead only to transient paralysis and growth inhibition rather than lethal effects.

Preliminary results indicate that there is a latitudinal pattern in the gene frequencies of NW Atlantic *M. arenaria* populations (S. A. Hamilton, L. B. Connell, and A. R. Bratcher unpubl.). Northern populations from the Bay of Fundy and eastern Maine have a lower proportion of SS clams and a lower ratio of RS relative to total resistant clams (RR + RS) than populations in western Maine and Bay of Fundy. The observed degree of genetic differentiation among these populations is likely influenced not only by the concentration, duration, and toxicity of *Alexandrium* blooms along the coast, but also by larval transport and gene flow resulting from physical circulation patterns. The predominant direction of the net alongshore flow occurs in a southwestward direction via the Maine Coastal Current (Anderson 1997). Finally, superimposed on these natural processes, there has been fairly extensive movement of hatchery-produced *M. arenaria* seed for intertidal planting along the U.S. coast (B. F. Beal, University of Maine, pers. comm.). This anthropogenic activity may potentially accelerate natural evolutionary change via the introduction of the RR genotype from north to south along the coast. In contrast, selection for resistance may be slowed down if resistance is associated with fitness costs, thus favoring the sensitive genotype in areas characterized by reduced severity or absence of red tides (Avery and Dam 2007).

Verification of rapid natural selection for resistance in the natural environment, as demonstrated in the present study for *M. arenaria* under laboratory conditions, is of special significance in coastal areas that have experienced a geographic spread in PSP over the past decades, as documented on the Atlantic coast of North America, and northward along the Pacific coast of South America (Guzmán et al. 2002). *Mya arenaria* populations, which

are sedentary and widely distributed along a gradient of PSP intensity and frequency, thus serve as an ideal model to track evolutionary change over relatively short timescales.

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