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Biogeography of resistance to paralytic shellfish toxins in softshell clam, *Mya arenaria* (L.), populations along the Atlantic coast of North America

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ABSTRACT

Blooms of *Alexandrium* spp., the causative agent of paralytic shellfish poisoning (PSP), recur with varying frequency and intensity on the Northwest Atlantic coast of North America, from New York, USA, to northern Canadian waters. Along this latitudinal range blooms co-occur with abundant, intertidal populations of softshell clams, *Mya arenaria*. Prior work identified a naturally-occurring genetic mutation in Domain II α-subunit of the clams' voltage-gated sodium channels (Na_V), which significantly reduces the binding affinity of the paralytic shellfish toxin, saxitoxin (STX). This mutation provides clams with resistance to the deleterious effects of STX, allowing them to continue feeding during *Alexandrium* spp. blooms and attain very high tissue toxicities. This study used genetic sequencing of the Na_V mutation locus in clams from four coastal regions of the Bay of Fundy-Gulf of Maine and the mid-Atlantic to determine the percentage of clams in each region that possess the resistant Na_V mutation. The genotype composition was related to the occurrence and magnitude of PSP outbreaks based on shellfish toxicity, primarily that of mussels, *Mytilus edulis*, used as a proxy for the prevalence and severity of *Alexandrium* blooms in each region. As hypothesized, the proportion of clams bearing the resistant mutation generally matched up well with the historical incidence and intensity of *Alexandrium* spp. blooms. The highest percentage of homozygote resistant clams (RR = 70.0%), and the lowest percentage of sensitive clams (SS = 4.5%) were found in eastern Gulf of Maine populations. Exceptions at a few sites where anomalously high numbers of *M. arenaria* with the resistant mutation were found despite the absence of blooms, may be attributable to larval gene flow. There was no evidence that *Alexandrium* blooms occurring in Northport Harbor, Long Island, have resulted in a shift in genotypic composition of the local clam population, presumably due to their low cell toxicity. Seasonal mismatch of highly vulnerable *M. arenaria* postset with toxic blooms at this latitude may also partly explain this result. This study provides strong supporting evidence that *Alexandrium* blooms can select for resistance to PSP-toxins in *M. arenaria* populations and proposes a mechanism for the persistence of the sensitive allele throughout the region. Implications for clam aquaculture (seeding) efforts, as well as for shellfish toxicity monitoring are discussed.

1. Introduction

Harmful algal blooms (HABs) of dinoflagellate species, producers of paralytic shellfish toxins (PSTs), pose a threat to human health globally, primarily via consumption of bivalve mollusks that become contaminated via suspension-feeding. Such neurotoxins have played a critical role in the evolution of voltage-gated Na^+ channels (Na_V) essential for the function of excitable nerve and muscle cells across phyla (Anderson et al., 2005a; Catterall, 1992, 2000). The often annual recurrence of blooms of the *Alexandrium tamarense/fundyense* species complex and resulting paralytic shellfish poisoning (PSP) outbreaks have been well described along the Atlantic coast of North America (Anderson et al., 1994; Anderson, 1997; Bean et al., 2005; Thomas et al., 2010; Martin et al., 2014). Blooms vary in frequency and

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intensity (as measured by cell concentration and specific toxicity) along the coast; higher cell toxicities have been documented in northern *Alexandrium* isolates from Canada and Maine (ME), USA, waters than in those from southern New England and the mid-Atlantic (Maranda et al., 1985; Anderson et al., 1994; Anderson, 1997).

Toxicity in the main sentinel bivalve species, *Mytilus edulis*, provides a proxy for *Alexandrium* bloom occurrence and intensity, given that mussels accumulate PSTs very rapidly [achieve peak tissue toxicities within ~ 1 wk of peak *Alexandrium* densities (Bricelj and Shumway, 1998)]. The timing of peak PSP toxicity coincides for *M. edulis* and *Mya arenaria*, although the magnitude is ~ 2 to 4x greater in the former. Shellfish toxicity usually peaks in early summer (June) in the western Gulf of Maine (GoM) and southern New England, in late summer (late July/early August) in the eastern GoM and Bay of Fundy (BoF) (Anderson, 1997; Thomas et al., 2010), and in mid-late May on the north shore of Long Island, NY (Hattenrath et al., 2010). Populations of native Atlantic softshell clams, *Mya arenaria*, the target species in the present study, are a ubiquitous component of the intertidal benthos throughout the geographic range of *Alexandrium* blooms, from Québec, Canada, to New York, USA, coastal waters, and the timing of *Alexandrium* blooms overlaps with that of softshell clam postlarvae/juveniles, the life history stage most vulnerable to PSTs (Bricelj et al., 2010). This overlap provides a unique opportunity to understand the adaptive responses of marine bivalve populations to neurotoxic HABs and the evolution of resistance to neurotoxins in a tractable and relatively simple invertebrate model system.

A genetic/molecular basis for the resistance of *M. arenaria* to PSTs was previously established. A single base pair substitution in the pore region of Domain II (DII) of the Na_V gene in resistant individuals of this species (as measured by a nerve *in vitro* bioassay) results in a single amino acid substitution in the encoded protein, where glutamic acid (E) is replaced with aspartic acid (D) (Table 1), causing a 1000 fold decrease in the binding affinity for PSTs at this site (Bricelj et al., 2005; Connell et al., 2007). Six possible nucleotide combinations can occur at the Na_V mutation locus (Table 1): three that cause the E → D amino acid substitution and result in a STX-resistant genotype (RR), one wild type that results in a STX-sensitive genotype (SS), and two that produce a heterozygous genotype (RS). Both alleles were expressed in heterozygote individuals (Connell et al., 2007), which exhibited intermediate nerve resistance between that of RR and SS genotypes (Bricelj, MacQuarrie, and Connell, unpublished). These alleles are controlled at a single locus heritable through simple autosomal Mendelian inheritance patterns (Hamilton, 2009).

Resistance to PSTs in softshell clams was associated with enhanced fitness in PSP-affected areas, as determined in laboratory studies in which clams were exposed to a simulated bloom of a highly toxic *Alexandrium* isolate [Pr18b, ~60 to 98 pg saxitoxin equivalents (STXeq $cell^{-1}$) (MacQuarrie and Bricelj, 2008)] originally collected from the estuary of the Gulf of St. Lawrence, Canada. Resistant juvenile clams exhibited increased burrowing capacity (and thus presumably reduced risk of predation, desiccation, and advection in nature), increased feeding (clearance rate) of toxic cells (and thus increased toxin accumulation), and ultimately higher survival than sensitive individuals (MacQuarrie and Bricelj, 2008). Laboratory studies demonstrated that post-metamorphic/juvenile clams represent the life history stage most susceptible to the disabling effects of PST exposure (Bricelj et al., 2010), and are also known to be the most vulnerable to predation in the field (Beal, 2006). Furthermore, when mixed populations of resistant and sensitive *M. arenaria* postlarvae (~4-12 mm in shell length, SL) were exposed under laboratory conditions to a single, one-week, simulated bloom of toxic *Alexandrium*, the latter acted as a strong selective agent favoring resistant (RR) clams via selective mortality of sensitive clams (Bricelj et al., 2010). Selection for PST resistance was also demonstrated when juveniles of known SS or RR genotype, generated by controlled breeding, were deployed together at several sites along the Atlantic coast varying in their history of PSP (Connell, Bricelj, and Martin, unpublished data).

Selective pressure imposed by naturally occurring neurotoxins can have significant effects on the genetic structure of populations of both terrestrial and aquatic organisms and affect their abundance and geographic distribution. Adaptation to tetrodotoxin (TTX), another potent Na_V blocker, is well characterized in the predator-prey system of garter snakes (*Thamnophis* spp.) and poisonous TTX-producing newts (*Taricha* spp.) that overlap in their distribution in the Pacific Northwest and California. Newts have evolved TTX resistance as a predator avoidance mechanism, and garter snakes have co-evolved adaptive resistance to TTX that allows them to prey on newts, an abundant and preferred food source (Brodie and Brodie, 1999; Geffeney et al., 2005; Feldman et al., 2009, 2010, 2012). This has resulted in considerable variation in TTX resistance among North American *Thamnophis* populations depending on their exposure to toxic newts (Brodie et al., 2002). Tetrodotoxin resistance in garter snakes is the result of several amino acid substitutions in DIII and DIV of the Na_V pore, which cause a variable, up to 40-fold decrease in binding affinity of the TTX molecule to the channel (Geffeney et al., 2005; Feldman et al., 2012). Differential resistance to PSTs in relation to historical toxin exposure was also demonstrated in the copepod, *Acartia hudsonica*, from the Atlantic USA coast, such that higher ingestion of toxic *Alexandrium* cells and higher fitness (egg production) were found in copepods from a PST-affected ME site than for naïve populations (Colin and Dam, 2002, 2004). The molecular basis for increased population resistance in this copepod species, however, remains uncertain. Although a mutation in the Na_V of *A. hudsonica* was identified, it differs greatly from that described in *Mya arenaria* and does not account for adaptation to PSTs in this copepod. It was not found at the toxin binding site (Chen et al., 2015), there was no evidence that it led to fitness advantages under toxic conditions or a cost under non-toxic conditions (Finiguerra et al., 2015), and the expression of the mutant Nav isoform was not related to exposure to toxic *Alexandrium* in the laboratory or to toxic blooms in the field (Finiguerra et al., 2014a, b).

Intraspecific differences in softshell clams' susceptibility to the effects of PSTs were first characterized using a burrowing incapacitation assay under simulated HAB conditions in the laboratory (Bricelj et al., 1996, Bricelj et al., 2002), as the genetic/molecular basis for the differential sensitivity to PSTs had not yet been described at the time. The main objective of the present study is to characterize the genotypic composition of toxin resistance of *Mya arenaria* populations in relation to their history of PSP along the northeastern and mid-Atlantic coasts of North America. Early characterization of resistance using the burrowing assay (conducted in 1996–2002) is compared to that determined by DNA sequencing of the Na_V mutation locus (2007–2013). It is hypothesized that *M. arenaria* populations in areas exposed to higher intensity HABs of toxic *Alexandrium* will be dominantly comprised of resistant individuals possessing the R allele (either RR or RS), whereas those where HABs are relatively rare or absent will be largely comprised of sensitive individuals with the SS genotype. Selection for resistance is further tested in a Long Island, NY, estuary where

Table 1
Six different nucleotide combinations observed at the Na^+ channel mutation locus, the encoded amino acid, and the resulting genotype exhibited in *M. arenaria:* SS, RR and RS indicate the STX-sensitive genotype, the STX-resistant genotype, and the heterozygous genotype, respectively. A = adenine, C = cytosine, T = thymidine; E = glutamic acid, D = aspartic acid.

Nucleotide	Amino Acid	Genotype
A/A	E/E	SS
A/C	E/D	RS
A/T	E/D	RS
C/C	D/D	RR
T/T	D/D	RR
C/T	D/D	RR