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Investigating Saxitoxin Resistance in Softshell Clams (*Mya arenaria*): Patterns of Inheritance and Improvements on Methodology for Tracking and Identification

Scott A. Hamilton

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**INVESTIGATING SAXITOXIN RESISTANCE IN SOFTSHELL CLAMS (*MYA
ARENARIA*): PATTERNS OF INHERITANCE AND IMPROVEMENTS ON
METHODOLOGY FOR TRACKING AND IDENTIFICATION**

By

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B.A. Goucher College, 2005

A THESIS

Submitted in Partial Fulfillment of the

Requirements for the Degree of

Master of Science

(in Marine Biology)

The Graduate School

The University of Maine

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Advisory Committee

Laurie Connell, Research Associate Professor, Advisor

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By Scott A. Hamilton

Thesis Advisor: Dr. Laurie Connell

An Abstract of the Thesis Presented in
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Attempts to characterize and study the population dynamics of the softshell clam *Mya arenaria* in relation to a mutation which confers resistance to paralytic shellfish toxins are complicated by a lack of non-lethal genotyping techniques, reliable tagging methods and an understanding of the inheritance patterns of the marker. Presented here, is a straightforward and non-lethal technique for clam genotyping, a new method for the long term tagging of clams, and the offspring genotype frequencies from a number of pair matings between clams of known genotype.

Hemolymph extracted from *M. arenaria* was used directly in a polymerase chain reaction (PCR) to successfully amplify a DNA fragment suitable for sequencing. Tested *M. arenaria* showed 100% (n=10) survival after a period of four weeks. In a separate experiment, passive integrated transponder (PIT) tags were inserted between the mantle and shells of 72 clams that were monitored for tag retention and survival. Among all PIT

tagged clams, there was 100% survival and 92 % tag retention. These methods provide a mechanism by which softshell clams can be genotyped and individually monitored, during field experiments.

Sixteen pair matings were conducted with adult *M. arenaria* of known genotype. Using polymerase chain reaction (PCR), the domain II (DII) pore region of the voltage-gated Na⁺ channel α -subunit, in which the mutation is found, was identified in a total of 344 larvae from these crosses. The data support the hypothesis that the mutant allele can be inherited from either sex and that there are no barriers to fertilization between gametes of different genotypes. Of the ten cross offspring genotype frequencies analyzed using goodness-of-fit tests, seven adhere to Mendelian expectations for inheritance, while three significantly deviated from expected ratios. These deviations are assumed not to be an actual representation of the sampled larval populations, but rather due to a combination of small larval sample size and a conservatively high significance level. This study demonstrates that the single nucleotide polymorphism in question is most likely inherited in a Mendelian fashion.

ACKNOWLEDGEMENTS

This research was supported by the Ecology of Harmful Algal Blooms project (ECO HAB #NA06NOS4780247) as well as a grant from the Mount Desert Island Biological Laboratory including both the NIEHS Center for Membrane Toxicity Studies (P30 ES003828-20) and the Maine INBRE (P20 RR-016463). I would like to thank the patience that my adviser and committee members had with my questions and sometimes frequent procrastinations. I could not have completed this work without their help. Additionally I would like to thank Dr. Bill Halteman for his assistance with statistical analysis. A large portion of this project would not have been possible without the huge support I received from Scott Feindel at the Darling Marine Center in setup and maintenance of clam broodstock, as well as the long hours he spent in helping me achieve the numerous pair matings. I am also grateful to all the student and faculty who aided me in and out of the field during my time here at University of Maine including Amber Bratcher, Anne Barrett, Janice Duy, Willis Beazley, Caleb Slemmons, Corinne Michaud, Viorel Popescue, Emily Knurek, Caroline Howes, Dr. Brian Beal, Scott MacQuarrie, and Dr. Monica Bricelj.

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CHAPTER 1. GENERAL INTRODUCTION

Species of toxic or harmful algae are a threat to human health and fisheries resources throughout the world (Anderson, Kaoru et al. 2000). The ingestion of shellfish which have been feeding on toxin algae, resulting in shellfish poisoning occurs worldwide and is one of the most serious human health issues associated with algal blooms. While there are several types of algal-borne toxins responsible for shellfish poisonings, paralytic shellfish poison (PSP) poses one of the highest threats to human health as it can cause full respiratory failure and lead to death (Gessner and Middaugh 1995). Bivalve mollusks, the primary vectors of paralytic shellfish toxins (PSTs), build up toxicity by filter-feeding on and ingesting toxigenic dinoflagellates (Prakash, Medcof et al. 1971). The primary vector of PSTs in North America, *Alexandrium* spp., is known to produce at least 20 paralytic shellfish toxin congeners, including the most potent derivative, saxitoxin (STX) (Maranda, Anderson et al. 1985; Cembella, Destombe et al. 1990). Saxitoxin and its derivatives are neurotoxins, that reversibly block sodium channels interfering with the Na^+ conductance responsible for generating the action potential in nerves (Narahashi, Haas et al. 1967).

Severe economic impacts are associated with PSP events due to both shellfish harvest closures and a halo effect of decrease in the purchase and consumption of shellfish. Economic losses associated with a single PSP event in 2005, which forced the entire coast of Maine and Massachusetts to cease shellfish harvesting, were estimated to be more than \$20M (Jin, Thunberg et al. 2008). Additionally, the costs associated with toxin monitoring are substantial, primarily due to the expense of the mouse bioassay, the method used to test for the presence of PSTs in frequently harvested populations of

shellfish. (Andersen 1996). Monitoring costs continue to rise as toxic bloom events have been increasing globally in both frequency and intensity (Hallegraeff 1993).

The softshell clam, *Mya arenaria*, is native to the Atlantic coast of North America. While its range includes the stretch of coast from the estuary of the Gulf of St. Lawrence, Canada through to Georgia, *M. arenaria* is primarily commercially important in New England, and Canada, where annual blooms of *Alexandrium* spp. are well documented (Anderson 1997). Bricelj and Shumway (1998) found that the range of toxin accumulation varies by up to 100-fold among bivalve species fed toxic algae. *Mya arenaria*, considered to have intermediate sensitivity to PSTs, is capable of accumulating high amounts of toxin and is often screened for PSTs during the months when toxic algae most frequently bloom. By subjecting isolated nerves to increasing concentrations of purified STX *in vitro*, Twarog and coworkers (1972) discovered that interspecific variation in toxin accumulation is attributed to differential nerve resistance to toxins. Additionally, Twarog (1974) proposed that species with resistant nerves would feed actively on toxic cells and attain high toxicity levels, whereas sensitive species would exhibit shell-closure and/or feeding inhibition and therefore accumulate relatively low toxin levels.

While the hypothesis of interspecific variation holds in general, MacQuarrie (2002) found evidence of variation among individuals in response to PSP toxin exposure within *M. arenaria* populations. Using a burrowing index, which measures the ability of *M. arenaria* to reburrow in sediments following exposure to toxic *Alexandrium* cells, Bricelj and coworkers (1996) demonstrated variation in toxin resistance among individual clams within a population. In addition, animals from different regions demonstrated

variability in feeding rates, survival, and *in vitro* nerve assay sensitivity when comparing populations with different histories of toxic bloom exposure (MacQuarrie and Bricelj 2000; MacQuarrie 2002). These results supported the hypothesis of Twarog (1974) that suggested populations of bivalves express genetic adaptations to toxins following repeated exposure to PSTs, and that resistant individuals, which could accumulate a higher level of toxin, may be found in areas with a higher frequency of toxin exposure.

Clams identified as toxin resistant using *in vitro* nerve assays were found to harbor a single amino acid change in the domain II (DII) pore region of the voltage-gated Na⁺ channel's α -subunit (Bricelj, Connell et al. 2005). This natural mutation, resulting in a change from glutamic acid (E) to an aspartic acid (D), causes a dramatic decrease (1,000-fold) in the binding of STX to the Na⁺ channel of resistant, but not sensitive (wild type) clams. Using this method of toxin resistance identification, a number of populations along the New England coast have been genetically characterized (Hamilton, Connell et al. 2009, Figure 1). These data also support the hypothesis that regions with histories of PSP blooms have a higher frequency of resistant genotypes. However, with the threat of future expansion of toxic *Alexandrium* blooms to previously unaffected waters in Southern New England, it is important to understand how more frequent toxic blooms could influence *M. arenaria* population dynamics in order to for communities to make more informed shellfish management decisions.

The overall goals of this study were to develop faster and more effective methods for genetic characterization, create more reliable methods of *in situ* field identification, and verify the Na⁺ channel mutation inheritance patterns of *M. arenaria*. This study was part of a larger project investigating the selective pressure imposed on *M. arenaria*

populations by natural PSP producing harmful algal blooms (HABs). Results from this project will help to understand how the potential spread of PST resistant clams into new areas may impact trophic transfer of these toxins and aid in the establishment of shellfish management strategies.

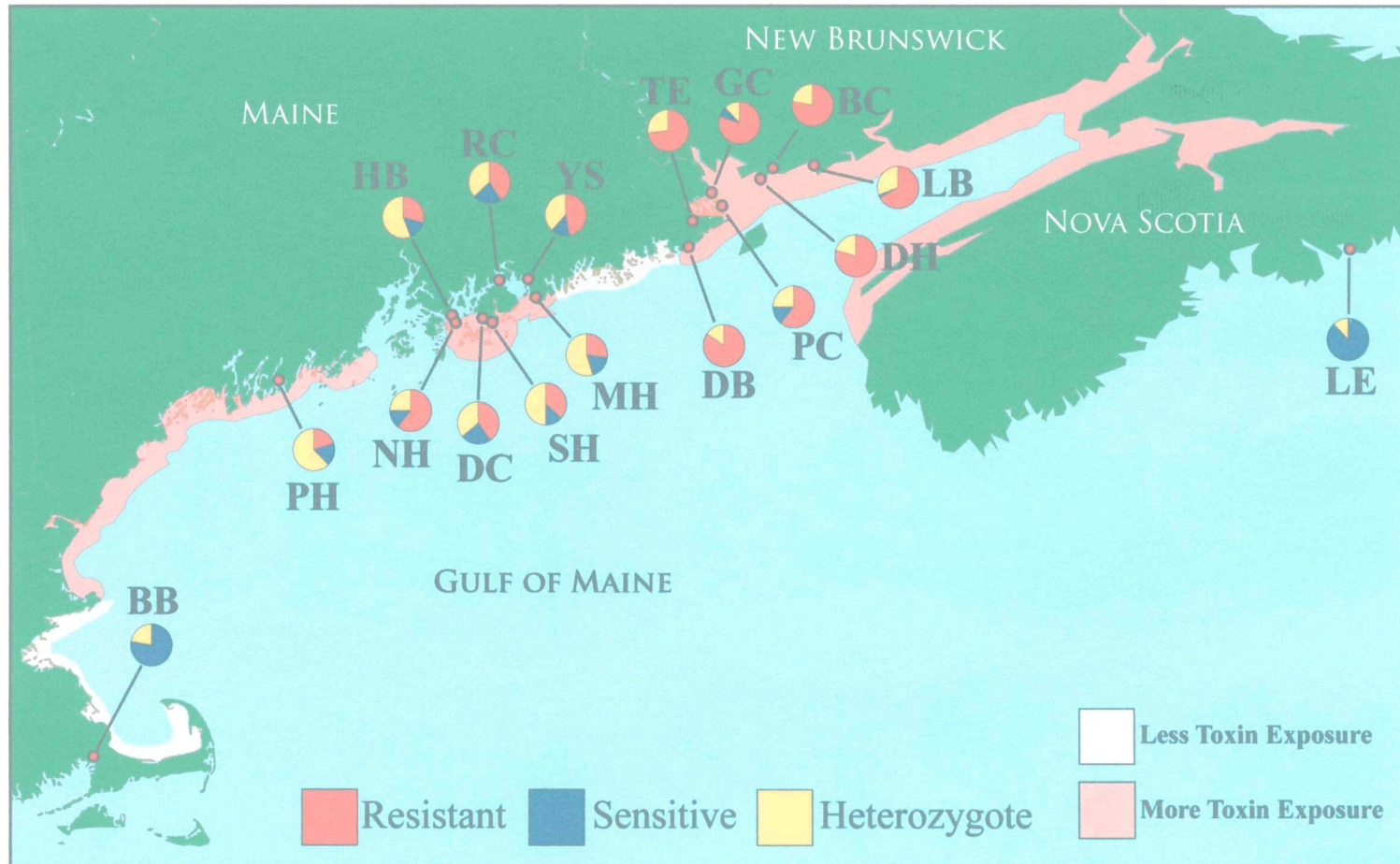


Figure 1. Na^+ channel mutation genotype frequencies of *Mya arenaria* populations around the New England and Canadian coasts. Location names are as follows; BB: Buttermilk Bay, BC: Beaver Cove, DB: Duck Brook, DC: Duck Cove, DH: Deadmans Harbour, GC: Gleason Cove, HB: Herrick Bay, LB: Lepreau Basin, LE: Lawrencetown Estuary, MH: Mosquito Harbor, NH: Naskeag Harbor, PC: Prince's Cove, PH: Poorhouse Cove, SH: Ship Harbor, TE: Tidemill Edmonds, RC: Raccoon Cove, YS: Young's Shore.

CHAPTER 2. IMPROVED METHODOLOGY TO TRACK AND GENETICALLY IDENTIFY THE SOFTSHELL CLAM *MYA ARENARIA*

2.1 INTRODUCTION

Analysis of population genetics in the softshell clam *Mya arenaria* (Linnaeus, 1758) in natural environments has been limited due to a number of technical considerations, particularly in studies attempting to evaluate the interplay between environmental factors and clam genotype. One specific problem involves the difficulties in obtaining genetic data from individual clams non-lethally. A second problem of equal concern is the challenge of prolonged tracking of each animal following their relocation at study sites. The power and scope of experimental methods based around *in situ* clam deployment could be improved with appropriate identification methods.

Passive integrated transponder (PIT) tags are radio-frequency devices that supply unique identification numbers when scanned (Prentice, Flag et al. 1990). These tags require no internal power source, as the minute electrical current required to run them is supplied by the incoming radio frequency signal emitted by the tag reader. Because of this, PIT tags have been implemented as a way to track individual organisms in their natural habitats (Whitfield Gibbons and Andrews 2004; Fuller, Henne et al. 2008). PIT tags offer advantages over other types of tagging or marking techniques, such as glue-on, anchor, painted or coded microwire tags (CW Tags), because they provide a nearly unlimited number of unique alphanumeric codes in relatively small and lightweight biocompatible glass capsules, that can be implanted into tissues or body cavities (Acolas, Roussel et al. 2007). Although fish tagging is one of the most widely used applications

for PIT tags (Achord, Matthews et al. 1996; Castro-Santos, Haro et al. 1996; Cucherousset, Paillisson et al. 2007; Meynecke, Poole et al. 2008), they are also being used to track and identify a wide variety of organisms with increasing regularity. For example, they are now routinely used as identification “chips” for pet dogs and cats. Recently, Kurth and coworkers (2009) successfully inserted PIT tags into the internal cavity of fresh water mussels as a means to evaluate the success of mussel translocations.

Because softshell clams spend the majority of their lives within the sediment, external tags can be easily dislodged due to abrasion, particularly when tagged shellfish are left in habitats of larger substrate size, such as gravel, for an extended period of time. In the past, CW tags, which can be inserted into organisms in a manner similar to PIT tags, have been used to bypass this issue in other invertebrate species (Joule 1983). However, these tags must be surgically removed before being deciphered. Additionally, as with many other tagging methods, data must be recorded and processed manually. Not only can these methods potentially increase the amount of error in a dataset, they can be especially cumbersome when recording data in the field, where time is often limited. Most PIT tag readers have the ability to directly read and store the data from each tag, in some cases without the need to remove the animal from the sediment. Using PIT tags, data can be transferred directly to a computer database at a later time, saving time in the field and appreciably reducing operator error during the data collection process. For these reasons, the efficacy of using internally placed PIT tags to monitor individual clams in field settings were explored.

While PIT tags can greatly aid in the monitoring and tracking of individuals, a faster, less expensive, and non-lethal tissue sampling method would be a boon to the

entire process of genetic experimentation with shellfish *in situ*. In previous studies that have isolated genetic material from *M. arenaria* for use in molecular based studies, animals were sacrificed in order to obtain a tissue sample (Olberding, Kelley et al. 2004; Bricelj, Connell et al. 2005). Not only are these methods time consuming when processing a large number of samples, but they also require the use of costly reagents for DNA extraction. Other workers have used a syringe to non-lethally extract hemocyte-containing hemolymph from the adductor muscle of *M. arenaria*, as well as other bivalves, such as mussels and oysters (Ashton-Alcox and Ford 1998; Riffeser and Hock 2002; Luengen, Friedman et al. 2004; Ford, Bricelj et al. 2008). Additionally, Schwab and coworkers (1998) demonstrated the feasibility of extracting RNA from a virus located in hemocytes harvested from oysters and hard shell clams. A similar method can be used to facilitate quick, non-lethal genotyping of *M. arenaria* without the need for tissue-based DNA extraction.

The goals of this study were (1) to develop a method for PIT tag insertion in *M. arenaria*, which would result in high survival and high tag retention, and (2) to assess the feasibility of non-lethal hemolymph extractions from *M. arenaria* as a means of genotyping each individual prior to experimental field deployment.

2.2 MATERIALS AND METHODS

2.2.1 EXPERIMENTAL ANIMALS

Softshell clams with shell lengths between 50mm and 75mm were obtained from a grocery store and transported on ice to the Darling Marine Center (DMC) in Walpole, Maine where they were allowed to recover in sand-filled baskets within a natural

seawater flow-through tank for two weeks prior to further handling. Both PIT tagging experiments and hemolymph sampling were carried out at the DMC.

2.2.2 HEMOLYMPH EXTRACTION AND PCR FRAGMENT AMPLIFICATION

Twenty *M. arenaria* were removed from the flow-through tank and allowed to dry for 15 minutes before their shells were labeled with permanent marker. Half of the animals (n=10) were labeled with permanent markers in preparation for hemolymph extraction. A 1ml, 26 gauge syringe was used to extract 200 μ l of hemolymph from the posterior adductor muscle, accessed from a spot halfway between the umbo and siphon. The needle was removed from the syringe to avoid hemocyte damage before the hemolymph was transferred to a pre-labeled 1.5ml microcentrifuge tube. Each hemolymph sample was centrifuged for 5 minutes at 8609xg. A cell pellet was not always easily visible in the tube following centrifugation. The supernatant was removed from each tube and the cell pellet was resuspended in 50 μ l of 10mM Tris-Cl buffer (pH 8.5) to stabilize DNA during storage. Cells were placed in a -20°C freezer to lyse the hemocytes and store until further processing. All animals were monitored for survival in natural seawater flow-through tanks over a period of one month.

As previously described by Connell and coworkers (2007), PCR was used to amplify a 172bp fragment containing the *M. arenaria* specific domain II α -subunit Na⁺ channel gene from DNA contained in the extracted hemolymph. PCR amplicons were purified using the Wizard SV gel and PCR clean-up system according to manufacturer's directions (Promega, Madison WI, USA). The PCR amplicon was sequenced by the University of Maine DNA Sequencing Facility (Orono, Maine, USA) and the resulting

chromatograms were analyzed using Vector NTI Advance 10 (Invitrogen, Carlsbad CA, USA) and Sequencher V 4.8 (GeneCodes, Ann Arbor MI, USA).

2.2.3 PASSIVE INTEGRATED TRANSPONDER TAGGING

To test whether PIT tags could be inserted and retained in *M. arenaria*, two trials (A and B) were performed three months apart. For each trial, nine plastic baskets (33cm x 17.8cm x 10.8cm) were filled with 8cm of clean sand and placed in adjacent seawater tanks receiving a single source of water. In each trial, 108 animals were divided equally into three groups of three replicates (12 animals/replicate basket); untreated (control), tagged, and sham-tag control. Sham-tagged animals were subjected exclusively to the tag insertion process without insertion of an actual tag. To account for possible handling mortality, all animals were removed from the baskets, scanned with the tag reader, and replaced during each tag scanning process. Each animal was placed into a small hole excavated in the sand within each basket.

Two sizes of PIT tags (Destron Fearing, St. Paul, MN) were used during the experiment (trial A=12mm length and trial B=12.5mm length). Tag retention was monitored using an Avid Power Tracker VIII (East Sussex, UK) PIT tag scanner. To initiate the tagging process, each animal was first stimulated on the posterior end to induce an opening on the anterior end. A piece of pencil eraser was inserted between the two valves on the anterior side of the clam to prevent the animal from closing at the location intended for PIT tag insertion. A dissecting needle was then used to gently pry away a small amount of mantle from the valve before a PIT tag was pushed through the opening in the animal's extrapallial space (between the mantle and valve) using a plastic 20 μ l pipette tip. In the trial B, a more cautious approach was used during the insertion

step to angle the 20µl pipette tip so as not to damage the mantle while pushing in the tag. While the tag length in trial B was slightly larger (12.5mm), the insertion method remained the same.

All animals were monitored for PIT tag retention and survival for a period of five weeks (trial A) or nine weeks (trial B). If no tag was recognized by the reader, the animal was marked and measured, but not removed from the basket. Dead individuals (indicated by open shells and/or tissue decay) were removed immediately. Four to six months after each trial was finished, four clams that had retained their tags were dissected to examine the effects of tagging on the internal structures and shell nacre.

2.3 RESULTS

2.3.1 HEMOLYMPH EXTRACTION AND PCR FRAGMENT AMPLIFICATION

There was 100% survival among all animals subjected to the hemolymph sampling method and all individuals began feeding within 24 hrs following the hemolymph extraction procedure as determined by observation. Following PCR amplification, the 172bp fragment DNA products were sequenced to confirm that the fragment was the Na⁺ channel gene (Figure 2).

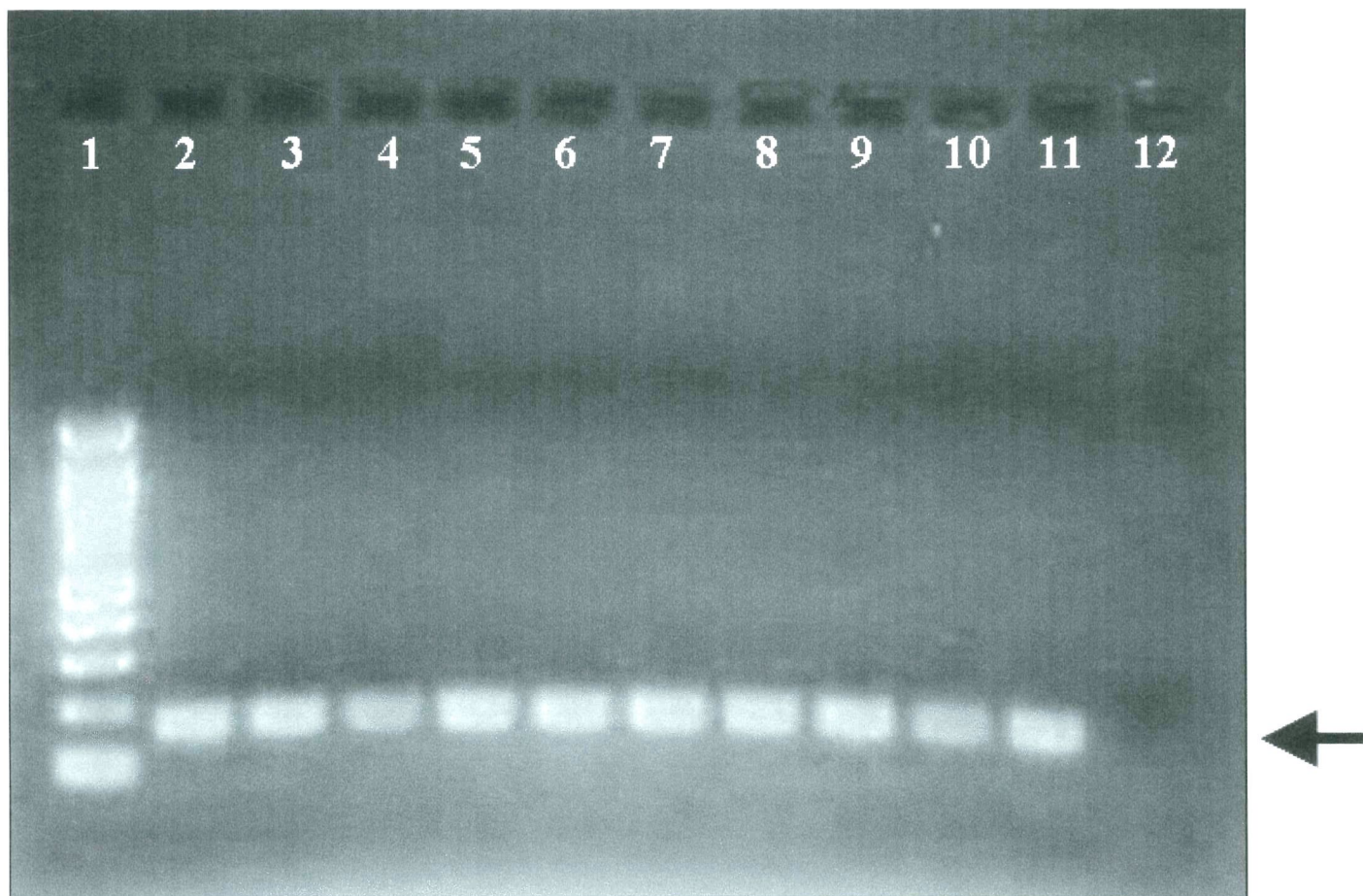


Figure 2. A 1.8% agarose PCR gel using primers oBTG99/oBTG100 on hemolymph extracted from ten individual *Mya arenaria*. Lane 1 is 100bp molecular marker. Lanes 2-11 represent each sampled individual. Lane 12 is the negative PCR (no DNA) control. The arrow indicates the 172bp amplicon.

2.3.2 PASSIVE INTEGRATED TRANSPONDER TAGGING

In trial A, 100% of control and sham-tagged animals survived, while 86.1% of tagged animals survived, only 47% of this group retained their tags. All experimental animals survived in trial B with 92% tag retention (Figure 3). Additionally, all cases of tag loss occurred in clams that survived the insertion, while tags were always found in deceased animals. As the tagging order of the three baskets in trial A increased, so did tag retention, suggesting a possible learning curve for successful tag insertion.

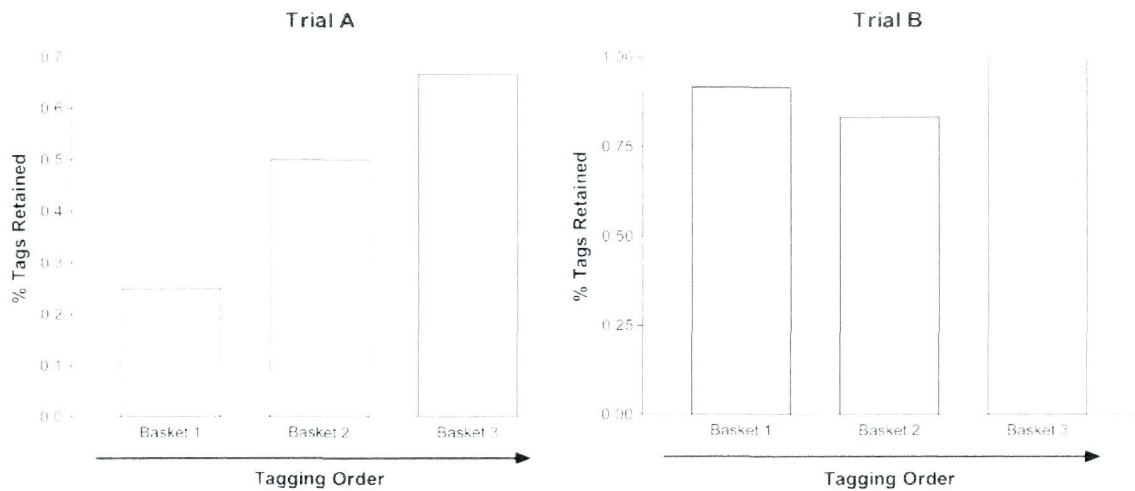


Figure 3. Percentage of *Mya arenaria* that retained the passive integrated transponder (PIT) tags. Trial A (n=12 per basket) is on the left and trial B (n=12 per basket) the right. The arrow indicates the order in which the animals in each experiment were processed by basket.

Four to six months after the conclusion of each trial, four tagged animals were dissected to inspect both the condition of the tag, and the clam itself. In each case, the animal had deposited a new layer of nacre over the tag, securely sealing it in place (Figure 4). At the time of inspection, there had been no additional mortality among the animals from each trial.

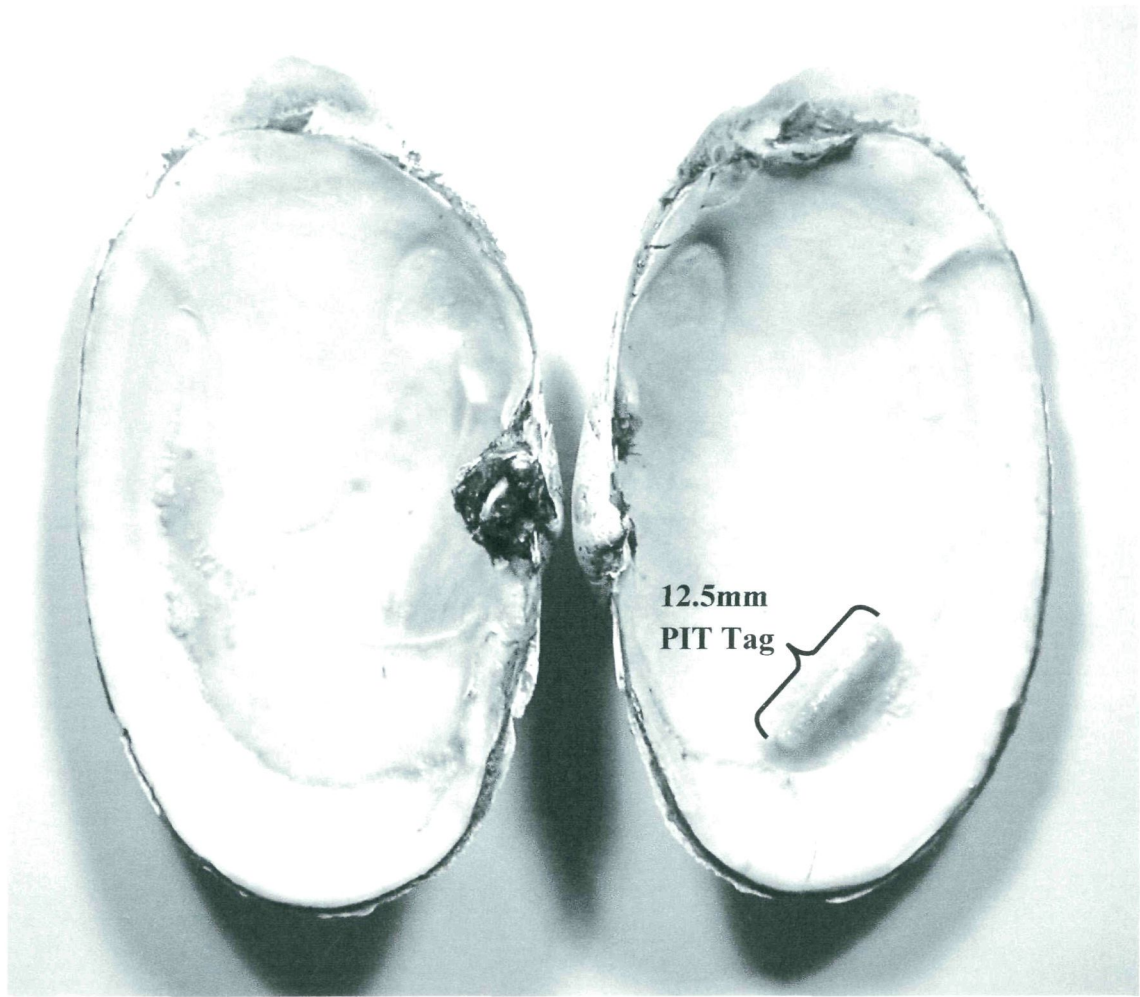


Figure 4. Shell of *Mya arenaria* dissected six months following the passive integrated transponder (PIT) tagging. The PIT tag has been sealed in place with a layer of nacre.

2.4 DISCUSSION

2.4.1 HEMOLYMPH EXTRACTION AND PCR FRAGMENT AMPLIFICATION

The primary purpose of the hemolymph project was to develop a non-lethal method to genetically identify individual *M. arenaria*. The current hemolymph collection method has successfully been used to genotype over 200 individual *M. arenaria* from populations along the NW Atlantic coast (Hamilton, Connell et al. 2009, Figure 1).

The storage of each individual cell pellet in the Tris-Cl buffer was an important part of the procedure to ensure a clean, repeatable amplification. Previous attempts at a

hemolymph collection method employed resuspension of the cell pellet in 50µl of hemolymph solute, obtained from the centrifuge process. While this method still resulted in successful PCR fragment amplification, hard-to-interpret sequences often resulted, possibly due to the deterioration of DNA quality. These problems were remedied with the use of the Tris-Cl buffer.

In the past, projects that required the sequencing of *M. arenaria* DNA, a tissue extraction process was necessary. In most cases, sampling of tissue was lethal. Non-lethal tissue sampling was also an option but still required an additional and expensive DNA isolation step prior to PCR amplification. While tissue extraction is still necessary for genetic characterization of juvenile *M. arenaria* due to their small size, the hemolymph extraction method is a practical, less expensive, and non-lethal option for adult *M. arenaria*.

2.4.2 PASSIVE INTEGRATED TRANSPONDER TAGGING

The goal of this study was to evaluate the efficacy of PIT tag insertion into *M. arenaria* as a means of tracking individual identity following field deployment. The differences in survival and tag retention between trials A and B are most likely due to a modification in technique between the two trials. As this was the first attempt to mark clams with PIT tags, multiple attempts at the tagging process were required to develop a working method.

A significant decrease in tag loss was observed when a more careful technique for tagging was applied in trial B. If a tag was placed within the pallial cavity of *M. arenaria*, it was typically ejected out of the opening through which the clam's foot is extended on the anterior end of the animal within 12 hrs of insertion. Additionally, following a

puncture in the mantle during the tag insertion process, the tag would pass into the clam's pallial cavity before quickly being expelled through the aforementioned "foot hole". To circumvent this problem, the technique was altered during trial B by positioning the pipette tip parallel with the lip of the valve as each PIT tag was wedged into the animal's extrapallial space, while ensuring that the mantle was not injured. In each trial, animals were sequentially tagged by basket number from lowest to highest. There was an increase in the rate of tag retention coincident with an increase in basket number (Figure. 2 A and B), indicating that the tagging success improved with practice. This resulted in more consistent tag retention during the second trial (Figure. 2 B). Higher survival in trial B was also likely a result of decreased damage to the mantle. The results from this study are similar to those obtained with the fresh water mussel *Lampsilis radiata radiata* by Kurth and coworkers (2007) who reported high retention rates (75-100%) and tag rejection only during the first three weeks post-tagging. This method appears to be feasible for long-term deployment of PIT tagged clams. However, because the retention of the tag must be verified prior to field deployment, a four-week lag between the tagging process and the use of the tagged animal is recommended.

CHAPTER 3. INHERITANCE OF A DNA POLYMORPHISM LEADING TO SAXITOXIN RESISTANCE IN *MYA ARENARIA*

3.1 INTRODUCTION

Paralytic shellfish poisoning (PSP) poses a severe human health risk and causes closures of shellfisheries worldwide. From May to July of 2005, an exceptional bloom of *Alexandrium fundyense* occurred along the coast of southern New England and high toxin levels were measured farther south than ever before. In fact, toxicity was above quarantine levels in locations where this had previously never been the case (Anderson, Keafer et al. 2005). It is hypothesized that cysts left behind by toxic dinoflagellates to overwinter can ignite blooms in the right conditions (Anderson and Wall 1978; Fast, Cembella et al. 2006). Therefore, in years following the 2005 bloom, it is possible that the remaining cysts could lead to heavier and more frequent bloom in these previously unaffected areas. In turn, this increase in bloom density and frequency would have an effect on shellfish populations in this area.

When comparing several species of east coast bivalves, Twarog and coworkers (1972) ranked *M. arenaria* (collected from Woods Hole, Massachusetts) as having an intermediate sensitivity to saxitoxin (STX), a major component of PSTs. Based on this work, Macquarrie (2002) found evidence of intraspecific variation in toxin sensitivity in *M. arenaria* populations in Nova Scotia, Canada. By examining the genetic data collected from these animals, Bricelj and coworkers (2005) discovered a sodium channel mutation in softshell clams that results in intraspecific variation in STX resistance when the animals are exposed to paralytic shellfish toxins (PSTs). Clam populations in areas with frequent toxic algal blooms have a high occurrence of the Na⁺ channel mutation and thus

are predominantly resistant to STX, while populations infrequently exposed to PSTs are primarily wildtype and sensitive to the effects of STX (Hamilton, Connell et al. 2009, Figure 1). Because *M. arenaria* carrying the resistance mutation are capable of accumulating more PSTs than those without (Bricelj and Shumway 1998), a shift in the genetic structure of a predominantly sensitive population towards resistant genotypes may require important changes within the toxin monitoring efforts of local communities. For this reason, it is becoming increasingly important to understand what effect toxic blooms have on the Na⁺ channel gene allele frequencies of natural *M. arenaria* populations.

Before it is possible to understand the effect this mutation will have on the overall toxicity levels of a *M. arenaria* population, the inheritance pattern of this gene from parents to offspring must be determined. There are a number of factors that can complicate the inheritance of genetic variations. For example, close linkage of a locus to a sex determining gene could cause sex-biased inheritance of alleles which could be incorrectly interpreted as a deviation from the standard of Mendelian expectations (Côte-Real, Holland et al. 1994). Two other types of deviations often reported in bivalve studies are a deficiency or an excess of heterozygotes (Gaffney 1994; Launey and Hedgecock 2001; Hedgecock, Li et al. 2004). Hypotheses accounting for one or the other of these deviations invoke typing artifacts, null alleles, partial aneuploidy, genomic imprinting, deleterious genes, population substructuring, or partial inbreeding (Bierne, Launey et al. 1998). These deviations highlight the importance of investigating the inheritance patterns of a particular gene prior to larger studies of bivalve population genetics.

This study investigates the inheritance of a single nucleotide polymorphism (snp) found in domain II of the coding sequence of the Na⁺ channel α -subunit protein in the softshell clam *M. arenaria*. This mutation involves the substitution of cytosine (C) or thymine (T) for adenine (A) and results in an amino acid substitution from the wildtype glutamic acid (E) to aspartic acid (D). There are six possible nucleotide combinations that produce three phenotypes in relation to STX resistance. Wild type animals (AA) are homozygous for the glutamic acid containing alleles and show full sensitivity to STX. Animals with an A to C or A to T mutation in both alleles (CC, CT or TT) are homozygous for an aspartic acid allele and show up to a 1000 fold increase in resistance to STX during nerve assays (Bricelj, Connell et al. 2005). Individuals with either an A to C or A to T mutation in just one allele (AC or AT) are heterozygous and show an intermediate phenotype of resistance to STX (Connell, MacQuarrie et al. 2007).

The goal of this study was to investigate the inheritance of the snp found in domain II of the Na⁺ channel α -subunit gene in softshell clams. Inheritance was assessed using PCR and DNA sequencing to determine the individual genotypes of 344 larvae resulting from 16 controlled laboratory crosses between parental clams whose genotypes were determined prior to spawning. The following questions were addressed with the data obtained: (a) Is there evidence of sex-biased inheritance? (b) Were any unexpected genotypes observed in any pair matings and what factors may have contributed to this? (c) Are the ratios of genotypes obtained from each cross compatible with strict Mendelian patterns of inheritance and, if not, what mechanisms may be to blame for any observed genotypic ratio distortion?

3.2 MATERIALS AND METHODS

3.2.1 ANIMAL COLLECTION AND TREATMENT

Broodstock animals were obtained from a number of locations along the New England and Canadian coasts including Lawrencetown Estuary, Nova Scotia and Lepreau Basin New Brunswick, Canada; Gleason Cove, Prince's Cove, Frenchman's Bay, Naskeag Harbor, and Poorhouse Cove along the coast of Maine, and Buttermilk Bay in Massachusetts (Figure 1). Additionally, a number of F1 offspring from the Lawrencetown Estuary and Lepreau Basin broodstock were themselves also conditioned and used for spawning. Immediately following field collection, 200 μ l of hemolymph was extracted from each clam to determine genotype using methods described in chapter 2. All broodstock were brought to the Darling Marine Center (DMC) in Walpole, Maine and held in suspended baskets in 400 L quarantined filtered seawater tanks.

In order to stimulate gonadal ripening, water temperature was maintained in a recirculating system at 13°C using an Aquatic Ecosystems (Apopka, FL, USA) Cyclone Chiller. Twice each day, the broodstock were drip-fed a mixed diet consisting of *Isochrysis* (T-iso), *Pavlova lutheri*, *Chaetoceros meulleri*, *Tetraselmis* sp., and *Rhodomonas* sp. A number of genetically unidentified clams (sentinels) were conditioned alongside the broodstock and sacrificed on a weekly basis to gauge gonadal ripeness. Once the clams were conditioned properly, the spawning process (either thermal shock or strip spawning) was initiated to acquire gametes for fertilization.

3.2.2 THERMAL SHOCK SPAWNING

Pair matings were produced using a thermal shock spawning protocol (Widman, Choromanski et al. 2001). To prevent larval contamination between separate pair

matings, animals were individually cleaned using a brush and rinsed to remove any residue on their outer shell. Following this, all broodstock were placed in a 4'x3' spawning tray in filtered seawater at 13°C. Once animals had recovered from handling and were actively filtering, indicated by slight extension of the siphon, the water temperature was raised to 23°C. Water temperature was subsequently allowed to cool to 20°C and the animals were observed for any sign of spawning. The further cycling of water temperature between 12°C and 23°C, as well as the addition of algae to the spawning tank, was often necessary to induce spawning.

Once an animal began releasing gametes, it was immediately removed from the tank, thoroughly rinsed with filtered seawater, and placed in an individual pre-labeled plastic cup of filtered seawater at 23°C where the release of gametes continued. Following the release of spawn, each clam was removed from its cup. As a final preparation for fertilization, the gametes were filtered through a 50µm sieve to remove contaminants such as pseudofeces.

3.2.3 STRIP SPAWNING

Some of the *M. arenaria* broodstock were found to be unresponsive to thermal shock stress, therefore a number of pair matings were created using a strip spawning method. Once each animals shell was cleaned as described above, clams were opened to expose their gonad. A sterile scalpel was then used to obtain a gonadal biopsy, which was examined under an Olympus CH30 microscope at 10X magnification to determine the sex of the individual. Females were stripped of their ova by cross-cutting small incisions into the gonadal tissue and gently rinsing gametes through a 50µm sieve into a pre-labeled plastic cup using 0.5µm filtered seawater filled squirt bottle. In order to

preserve sperm viability, males were labeled and set aside while females were stripped of their ova. Males were then stripped of sperm immediately prior to the fertilization step, and rinsed using the method described above.

To initiate germinal vesicle breakdown and maturation of the ova, 2ml of a 0.1 M NH_4OH solution was introduced to each 250ml cup of eggs (Masahiro 1999). The germinal vesicle was allowed to dissolve in this solution for a period of 20 minutes before the eggs were rinsed and collected onto a 20 μm sieve and re-suspended in 0.5 μm filtered seawater, at which point each egg cohort was ready for fertilization.

3.2.4 EGG FERTILIZATION AND GROWTH

To achieve the requisite pair matings, eggs from each female were distributed evenly into 1 l cups. Each cup containing ova was then brought to a total volume of 1 l using 0.5 μm filtered seawater. To fertilize the eggs, enough sperm (roughly 10 sperm to each egg) from each specified male was added to each cup according to the predetermined set of desired crosses. All cups were held at 20°C for a period of 18-24 hours until larvae reached D-stage. Larvae were then collected on a 35 μm sieve to remove any remaining sperm or unfertilized eggs, and either immediately isolated for PCR analysis or preserved in 5ml of 70% ethyl alcohol for separation at a later time. Random samples were observed under a microscope using a Sedgewick Rafter cell counting slide to quantify fertilization success as number of developing embryos.

3.2.5 COLLECTION AND TREATMENT OF LARVAE

Preserved samples of larvae (10 μ l) from each cross were placed on a Sedgewick Rafter cell counting slide and diluted to 1ml with de-ionized (DI) water. Using a heated and stretched glass pipette tube, individual larvae were extracted from the Sedgewick Rafter slide under 10X magnification and placed into 0.2ml PCR inhibitor-free thin-wall polypropylene tubes containing 2 μ l PCR grade water. Larvae were frozen (-20°C), heated (95°C) for five minutes, and then stored at -20°C until processing. Between 15 and 30 larvae were processed for each pair mating.

3.2.6 GENETIC IDENTIFICATION

A 172bp portion of the domain II α -subunit Na⁺ channel gene was amplified from each larvae directly from the larval preps. PCR amplification relied on previously published *M. arenaria* specific primers and thermalcycling parameters (Connell, MacQuarrie et al. 2007). The PCR reactions were carried out in volumes of 25 μ l within each 0.2 μ l polypropylene tube using illustraTM PuRe TaqTM Ready-To-GoTM PCR Beads (GE Healthcare) in a PTC-100 thermalcycler (MJ Research). A No-DNA control reaction was run with every set of PCR samples to test for procedural contamination. Amplification products were visualized by electrophoresis on 1.8% I.D.NA agarose gels (Lonza Group, Switzerland) containing SYBR Safe DNA gel stain (Invitrogen, Carlsbad, CA, USA).

The remaining PCR product for each larva was purified using the Wizard SV gel and PCR clean-up system (Promega, Madison WI, USA). The purified products were sequenced either by the High Throughput Genomics Unit (Seattle WA, USA) or the University of Maine DNA Sequencing Facility (Orono Maine, USA) using BigDye

Terminator Cycle Sequencing. Chromatograms were proofread and aligned using Vector NTI Advance 10 (Invitrogen, Carlsbad CA, USA) or Sequencher V 4.8 (GeneCodes, Ann Arbor MI, USA) and resulting sequences were compared to previously published results to determine snp alleles (Connell, MacQuarrie et al. 2007).

3.2.7 STATISTICAL ANALYSIS

A binomial exact test for goodness-of-fit (exact test) was used to compare the observed larval genotype frequencies from pair matings with one heterozygous parent. Because crosses with two heterozygous parents are trinomial, a chi-square test (χ^2 test) for goodness-of-fit was used instead. Both of these tests are commonly used to statistically test for deviations from expected values. Analysis of pair matings involving two homozygous parents had zero degrees of freedom, while those in which one or both parents were heterozygous had one and two degrees of freedom, respectively. An alpha level of 0.1 was chosen for this analysis. This higher significance threshold helped avoid type II error (false positives). This choice simultaneously incurred a greater chance of type I error. However, in the case of this analysis, it is more advantageous to avoid false positives than false negatives. Therefore, a significance level of 0.1 is a more conservative test for each pair mating and will ultimately allow for a more confident prediction of the inheritance patterns of this snp. Additionally, this alpha level allowed for a slightly higher power level when considering the limited sample sizes collected for this analysis.

3.3 RESULTS

The genotypes of 344 larvae resulting from 16 controlled laboratory crosses between parental clams of known genotype were determined (Table 1). Four of the 16 crosses were completed during a single experiment using thermal shock stress spawning, while the remaining twelve matings were completed during two separate strip spawning experiments. Due to the difficult nature of obtaining spawn from *M. arenaria*, the majority of crosses were carried out using a strip spawning technique slightly altered from one used with pearl oysters (Masahiro 1999). During the first strip spawning experiment, 14 pair matings were attempted. Eight of these crosses successfully produced viable offspring. A second strip spawning experiment was undertaken to produce the six additional matings, of which, only four were successful. Due to time constraints, the remaining two replicate crosses were not completed. Fertilization success rate in each cross was typically observed between 30 and 40% in each successful strip spawning.

The genotype frequencies of the offspring produced from the majority of crosses were consistent with those expected under Mendelian inheritance (Table 1). For pair matings in which only one possible genotype was expected, a lower number (~15) of larvae were genotyped. More larvae (~30) were genotyped from crosses where multiple genotypes were expected. In crosses 14 (♀CAx♂AA) and 4 (♀AAx♂AA), the target number of offspring were not available for analysis due to a combination of unproductive matings and unsuccessful PCR amplification. Unexpected genotypes were found in two of the 16 pair matings. In cross 11 (♀CAx♂CC), four larvae with unexpected genotypes out of 34 (11.8%) were found, while in cross 1 (♀CCx♂CC), one unexpected larval genotype out of 15 (6.6%) was found. Due to the small number of aberrant larvae, these

unexpected genotypes were assumed to be the result of contamination. Because neither binomial exact goodness-of-fit tests nor chi-square goodness-of-fit tests are capable of handling unanticipated categories, cross 11 was not analyzed.

Out of the 10 crosses analyzed for goodness-of-fit, only the offspring frequencies of three crosses [7 (♀CCx♂CT), 9 (♀AAx♂CA), and 12 (♀CAx♂CC)] varied significantly from expected Mendelian ratios with p-values of 0.059, 0.084 and 0.068 respectively. The test statistics of the remaining seven crosses all had p-values greater than 0.1 suggesting no significant deviance from expected ratios (Table 1).

Table 1. Genotypes at the domain II pore region of the α -subunit of the voltage gated Na^+ channel locus for offspring from laboratory crosses of *Mya arenaria*. Statistical significance of deviance of observed ratios from expected were determined using both binomial exact and chi-square goodness-of-fit analyses excluding genotypes incompatible with parental genotypes.

Cross Type/Cross No.	Parental Genotypes	Spawn Method	Offspring Scored	Offspring Genotypes						Goodness-of-fit (p)	
				CA	TA	CT	CC	TT	AA	Binomial Exact	χ^2
No Heterozygous Parents											
1	♀CCx♂CC	Strip	15	1	0	0	14	0	0	n/a	n/a
2	♀CCx♂AA	Strip	14	14	0	0	0	0	0	n/a	n/a
3	♀AAx♂AA	Strip	11	0	0	0	0	0	11	n/a	n/a
4	♀AAx♂AA	Strip	7	0	0	0	0	0	7	n/a	n/a
5	♀AAx♂CC	Thermal Shock	16	16	0	0	0	0	0	n/a	n/a
One Heterozygous Parent											
6	♀CCx♂TA	Strip	33	14	0	19	0	0	0	0.243	n/a
7	♀CCx♂CT	Strip	15	0	0	11	4	0	0	* 0.059	n/a
8	♀AAx♂CA	Thermal Shock	33	16	0	0	0	0	17	0.500	n/a
9	♀AAx♂CA	Strip	26	9	0	0	0	0	17	* 0.084	n/a
10	♀AAx♂CT	Strip	13	9	4	0	0	0	0	0.133	n/a
11	♀CAx♂CC	Thermal Shock	34	11	0	0	19	0	4	n/a	n/a
12	♀CAx♂CC	Strip	29	10	0	0	19	0	0	* 0.068	n/a
13	♀CAx♂AA	Strip	23	8	0	0	0	0	15	0.105	n/a
14	♀CAx♂AA	Strip	11	7	0	0	0	0	4	0.274	n/a
Two Heterozygous Parents											
15	♀CAx♂CA	Thermal Shock	30	18	0	0	7	0	5	n/a	0.480
16	♀CAx♂CA	Strip	34	19	0	0	10	0	5	n/a	0.379
Total:			344								

3.4 DISCUSSION

3.4.1 SPAWNING

Two different spawning techniques were used to collect gametes from genetically identified broodstock of *M. arenaria*. Initially, thermal shock spawning techniques were used as the sole method of obtaining gametes. While obtaining sperm and eggs in this manner has its advantages, it also carries a number of difficulties. Using thermal stress spawning with *M. arenaria*, the proportion of adults from which gametes were obtained was ~5%. Furthermore, there was no way of controlling which genotypes spawned, nor was there any sex specific marker available to predict the sex of individual animals prior to a spawning experiment.

Following a number of unsuccessful attempts using the thermal shock protocol to induce natural spawning in a suitable number of broodstock, the decision was made to focus on strip spawning techniques. The use of strip spawning solved both of the aforementioned difficulties by simplifying the process of obtaining gametes from parents with target genotypes. Strip spawning also helped avoid the unintentional mixing of eggs and sperm between individual pair matings, which can be a problem when thermally inducing animals. The downside of the stripping method is that the clams were sacrificed. Because labeling and genetically identifying each animal incurs a significant cost, it is desirable to use them in more than one spawning event. Additionally, there is no way to know the gonad index of each animal prior to stripping, therefore the concentration and quality of stripped gametes vary and the gametes may not always be viable.

The other primary difficulty with strip spawning *M. arenaria* is the issue of germinal vesicle breakdown following the collection of eggs. The oocytes of *M. arenaria*

are surrounded by a germinal vesicle during development, as are the majority of invertebrates. In some bivalves, such as *Crassostrea virginica*, the germinal vesicle breaks down during fertilization or upon contact with sea water (Fong, Kyozyuka et al. 1994). However, in a number of species, including the clams *Mercenaria mercenaria* and *M. arenaria*, the germinal vesicle is broken down naturally before being released. When manually stripped, *M. arenaria* eggs retain their germinal vesicle, which can block fertilization. In previous work, Loosanoff and Davis (1963) and Masahiro (1999) successfully strip spawned *M. mercenaria* and *Pinctada margaritifera*, respectively, by exposing the stripped ova to low concentrations of ammonium hydroxide (NH_4OH) which dissolved and broke down the germinal vesicle, allowing fertilization to occur. To successfully strip spawn and mate specific pairs of *M. arenaria*, NH_4OH was used to stimulate germinal vesicle breakdown. The first three pair matings attempted had a success rate of ~40% egg fertilization. In subsequent attempts, 35-40% fertilization rates were observed following the treatment with 0.1M NH_4OH . These results closely matched those of Loosanoff and Davis (1963) who obtained a 32% fertilization rate with *M. mercenaria*. During the second strip spawning event, 16 additional pair matings were attempted, many of which produced a very small number of larvae or were completely unsuccessful. As a result of the lengthy washing process following the ammonium hydroxide soak, the eggs from a number of the crosses were left in the solution for far longer than the planned 20 minutes, which may have rendered them non-viable. Less successful fertilization outcomes may be due to the length of time the eggs were exposed to the NH_4OH solution. This hypothesis is supported by the fact that a higher rate of fertilization was obtained in the third strip spawning experiment (signified by a higher

concentration of larvae) when the soaking time for each cup of oocytes was limited to exactly 20 minutes.

3.4.2 LARVAE ISOLATION AND GENETIC IDENTIFICATION

The goal was to genetically identify 10-15 larvae for each pair mating between homozygous individuals and 25-30 larvae for each mating involving one or two heterozygote parents. Fewer larvae were genotyped for the homozygous matings as there was only one genotypic outcome possible. Assuming no unexpected genotypes were observed in a specific cross, any significant deviation from the expected outcome could be recognized without the need for a large sample size. However, matings involving one or two heterozygous parents were expected to result in two or three separate genotypes, respectively. An examination of larval genotype frequencies from crosses where more than one genotype was expected was used to determine if there were deviations from Mendelian expectations. While this goal of 25-30 larvae was achieved with the majority of the matings, there were a few cases in which identification of the desired number of larvae was not achieved (<25 Larvae). This was due not only to the spawning difficulties mentioned above, but also to problems associated with the sampling and genotyping process.

To identify the genotype frequencies of the offspring from each pair mating, a DNA fragment containing the snp was amplified and sequenced from individual larvae. Because the larvae were only ~70 μ m in size, the method by which each animal was isolated and processed in individual tubes was problematic. PCR reactions were run in the 0.2ml PCR polypropylene larvae collection tubes. Therefore, if the PCR was unsuccessful, the DNA of that particular larva was lost and a second PCR could not be

attempted. Furthermore, if procedural contamination of any type was found in the control of any set of PCR reactions, all samples were discarded and lost to further analysis. These problems, coupled with the occasional low number of larvae available from a number of pair matings, made it difficult to consistently reach the target number for each cross.

3.4.3 GENOTYPE FREQUENCY ANALYSIS

One of the goals was to rule out the possibility of any blocks to the inheritance of the snp conferring toxin resistance. The successful transmission of both the individual A and C snp alleles from males and females was confirmed by the abundance of larvae with the heterozygous C/A genotype in crosses with ♀AAx♂CC (Cross 5) and ♀CCx♂AA (Cross 2) parents. The offspring from ♀CCx♂TA (Cross 6), ♀CCx♂CT (Cross 7) and ♀AAx♂CT (Cross 10) confirm the transmission of allele T from males. This nucleotide was not present in any of the female parents, and therefore transmission from the female was not confirmed. There appear to be no blocks to transmission.

In each of the 16 crosses analyzed, the expected genotypes were found, suggesting no major barriers to fertilization between animals with either sensitive, resistant, or heterozygote genotypes. However, in two of the 16 crosses (crosses 1 and 11) a minority of larvae with genotypes incompatible with Mendelian inheritance were found (one and four larvae, respectively). Deviations from Mendelian inheritance have been found in similar studies with other bivalves (Foltz 1986; Côte-Real, Holland et al. 1994). While the frequency of unexpected genotypes was small among all of the laboratory crosses in this study, it is still important to consider both the technical and biological mechanisms that may be responsible for these deviations.

The simplest explanations invoke experimental contamination. For example, any of the following could lead to larvae with unexpected genotypes: (1) presence of extraneous gametes or larvae in natural seawater supplies, (2) contamination of analyzed larvae with adhered sperm, (3) contamination of PCR template or (4) transfer of gametes or larvae between experiments. The first possibility, in which *M. arenaria* gametes were present in the natural seawater prior to clam stripping or spawning has been discussed by Mallet and coworkers (1985) and Foltz (1986). However, this explanation is unlikely because there were no *M. arenaria* spawning in local waters during the time of the experiment. Additionally, the seawater used was filtered through a 0.5µm filter that would have eliminated eggs or sperm from local waters. The second hypothesis, in which adhered sperm contributed to deviations from Mendelian inheritance in our samples, seems unlikely for two of reasons. Côte-Real (1994) found that successful amplification of sperm DNA from the bivalve *Mytilus edulis* requires a very high concentration of sperm under PCR conditions very similar to those used in this study. More importantly, during their collection for PCR, larvae from each cross were collected on a 35µm mesh. At this step, any remaining sperm should have been rinsed through the screen, leaving the larvae behind for collection. However, it is possible that some individual sperm remained, clinging to some larvae prior to collection, in which case they could have been accidentally included in the collection tubes. But, because the genotypes in both cases of contamination were not from either allele of either father (e.g. unexpected larvae with only AA allele from ♀CAx♂CC cross), sperm contamination remains unlikely. The third hypothesis for the unexpected genotypes is related to possible inclusion of foreign DNA in the larval collection process and/or the high sensitivity of PCR and is a common worry

when using this technique. During the larval collection process, a number of larval samples were removed from their sample tubes and placed on a clean slide to confirm the absence of any extraneous materials or larvae. In addition, extreme care was taken to avoid contamination during the preparation of all PCR reactions, however these sources of contamination cannot be completely ruled out.

The fourth explanation for contamination, in which there was a transfer of larvae between experimental crosses is the most likely of the four mentioned above. It is unlikely that gamete transfer occurred during the safer strip spawning method (used for ♀CCx♂CC (Cross 1), in which one CA larvae was found) because extra care was taken to clean and sterilize equipment between matings. On the other hand, ♀CAx♂CC (Cross 11), in which four AA larvae were found, was created using the thermal shock spawning methods, which may have allowed for the mixing and fertilization of gametes in the spawning bin prior to gamete collection. Eggs fertilized by an AA male may have carried over into the larval growth containers that would have resulted in the unexpected allele in that particular cross. More likely however, is the possibility that insufficient care was taken when rinsing the 35µm screen between the filtering of each 1l cup of larvae prior to microscopic separation. Because the same screen was used to filter all larvae, larvae from one cross may have been left behind and contaminated may the next group of larvae collected on the screen. This step in the experimental design is the most reasonable explanation for the unexpected larvae observed.

In addition to the possible technical mistakes mentioned above, there are a few biological explanations that may also account for the presence of unexpected genotypes in our crosses. Cells, individuals, or populations of animals in which the chromosome

complement is typified by the presence, or absence, of one or more whole chromosomes compared with the normal haploid or diploid condition are referred to as aneuploid. While aneuploidy is typically lethal at the embryonic stage, a small number of individuals can often survive to maturity. Dixon (1982) reported that ~8% of embryos were aneuploid in laboratory spawning of the mussel *Mytilus edulis* from a site in Whitsand Bay, England. This frequency of aneuploidy is similar to 'spontaneous' levels reported for other animal groups at a corresponding stage in development. Therefore, it is possible that aneuploidy may have resulted in the incorrect scoring of heterozygous larvae as homozygotes for one allele or the other. For example, in cross 11 (♀CAx♂CC), chromosomal loss from heterozygotes could account for the unexpected genotypes scored as AA (genotypically A0). For this to be possible, it would be necessary to assume that each chromosome in *M. arenaria* is equally prone to aneuploidy, an assumption for which there is no evidence for, or against. However, even if this were the case, only a very small percentage of chromosome loss should be associated with the chromosome on which the Na^+ channel gene is located. Additionally, because aneuploidy could not explain the unexpected CA genotype found in cross 1 (♀CCx♂CC), it is doubtful that aneuploidy played any role in the observed anomalous offspring in cross 1.

Hu and Foltz (1996) found that priming site polymorphisms may be responsible for heterozygote deficiencies in population studies of oysters and other animals. This may be a secondary biological explanation for the unexpected larvae found in cross 11 (♀CAx♂CC) as it could have lead to the improper scoring of CA offspring as AA (genotypically A0). All the same, this cannot explain the inconsistencies found in cross 1 (♀CCx♂CC), nor were there comparable issues observed in any of the other fourteen

crosses. Thus, there is little evidence to support priming site polymorphisms as an explanation for the anomalous offspring genotypes detected. While a number of these explanations provide reasoning for the unforeseen genotypes, the most likely cause for both cases of contamination remains the repeated use of a single 35 μ m sieve for the collection of larvae. In this scenario, if the sieve was not properly rinsed between each collection, larvae from one pair mating could have remained on the screen and contaminated another.

The primary goal this experiment was to determine whether the variation at the Na⁺ channel snp is inherited in a Mendelian fashion. The question was address by examining the frequency of offspring genotypes relative to Mendelian expectations in crosses 6-16 (with the exception of cross 11). Genotype frequencies were not statistically different from Mendelian expectation in seven of the ten crosses (Table 1). In crosses with only one heterozygote parent, a 1:1 ratio of heterozygotes to homozygotes is predicted among offspring, while a 1:2:1 genotype ratio is expected for double heterozygote matings. In the latter crossed offspring, genotype frequencies conformed closely to those expected under Mendelian inheritance (p=0.480 and p=0.379).

Among the matings between one homozygous and one heterozygous parent, the majority had larval genotype frequencies that did not differ from Mendelian expectations based on the significance threshold of 0.1. However, the larvae genotype frequencies of the three remaining matings, crosses 7, 9 and 12 deviated significantly from Mendelian expectations with p-values of 0.059, 0.084 and 0.068 respectively (Table 1). While these values are above the 0.05 significance level that is usually used in such analyses, they did not exceed the more conservative significance level (0.1) chosen for this study.

Therefore, the outcomes of these pair matings do not conform with Mendelian inheritance. Because many (crosses, 7, 10, 13, and 14) of these pair matings had sample sizes below adequate (a power level of 80% would have required $n=76$), small differences in specific genotype frequencies can make large differences in significance values for both χ^2 and binomial goodness-of-fit tests. While some of these pair matings deviate from expected Mendelian inheritance patterns, the majority of them do not. Therefore, an increase in sample size would most likely result in all crosses showing no significant deviations. Thus, there are no significant factors blocking the transfer of this marker from either sex and the gene is inherited in a Mendelian fashion.

In conclusion, this study sought to understand the inheritance pattern of this single nucleotide polymorphism conferring STX resistance to natural populations of *M. arenaria*. This understanding is vital to future studies measuring population shifts in resistance/susceptibility to PSTs in *M. arenaria*. For example, data obtained from measuring how one bloom season effects the survival of clam larvae or juveniles could be extrapolated to understand the structure of these populations many bloom cycles into the future. Combined with an understanding of differences in toxin depuration between the three phenotypes, this information could help local and statewide shellfish management agencies in deciding which genetic stock of *M. arenaria* to plant in shellfish beds. Because the majority of these crosses conformed to expected patterns of Mendelian inheritance, the deviations observed were assumed to be the result of small sample size. However, despite the issues with sample size, there remains strong evidence that this trait displays a Mendelian inheritance pattern and therefore, this work can be applied to future projects studying the effects of PSTs on the population dynamics of *M. arenaria*.

REFERENCES

- Achord, S., G. M. Matthews, et al. (1996). "Use of passive integrated transponder (PIT) tags to monitor migration timing of snake river chinook salmon smolts." North American Journal of Fisheries Management **16**(2): 302-313.
- Acolas, M. L., J. M. Roussel, et al. (2007). "Laboratory experiment on survival, growth and tag retention following PIT injection into the body cavity of juvenile brown trout (*Salmo trutta*)." Fisheries Research **86**(2-3): 280-284.
- Andersen, P. (1996). Design and implementation of some harmful algal monitoring systems. Intergovernmental Oceanographic Commission technical series. UNESCO: pp116.
- Anderson, D. M. (1997). "Bloom dynamics of toxic *Alexandrium* species in the northeastern U.S." Limnology and Oceanography **42**(5): 1009-1022.
- Anderson, D. M., Y. Kaoru, et al. (2000). Estimated annual economic impacts from harmful algal blooms (HABs) in the United States. Woods Hole, MA, Woods Hole Oceanographic Institution: pp97.
- Anderson, D. M., B. A. Keafer, et al. (2005). "Initial observations of the 2005 *Alexandrium fundyense* bloom in southern New England: General patterns and mechanisms." Deep-Sea Research Part II-Topical Studies in Oceanography **52**(19-21): 2856-2876.
- Anderson, D. M. and D. Wall (1978). "Potential importance of benthic cysts of *Gonyaulax tamarensis* and *G. excavata* in initiating toxic dinoflagellate blooms." Journal of Phycology **14**: 224-234.
- Ashton-Alcox, K. A. and S. E. Ford (1998). "Variability in molluscan hemocytes: a flow cytometric study." Tissue and Cell **30**(2): 195-204.
- Bierne, N., S. Launey, et al. (1998). "Early effect of inbreeding as revealed by microsatellite analyses on *Ostrea edulis* larvae." Genetics **148**(4): 1893-1906.
- Bricelj, V. M., L. Connell, et al. (2005). "Sodium channel mutation leading to saxitoxin resistance in clams increases risk of PSP." Nature **434**(7034): 763-767.
- Bricelj, V. M., C. A. D., et al. (1996). Comparative physiological and behavioral responses to PSP toxins in two bivalve molluscs, the softshell clam, *Mya arenaria*, and the surfclam, *Spisula solidissima*. Harmful and Toxic Algal Blooms. T. Yasumoto, Y. Oshima and Y. Fukuyo, Intergovernmental Oceanographic Commission of UNESCO: 405-408.

Bricelj, V. M. and S. E. Shumway (1998). "Paralytic shellfish toxins in bivalve molluscs: occurrence, transfer kinetics, and biotransformation." Reviews in Fisheries Science 6(4): pp69.

Bricelj, V. M. and S. E. Shumway (1998). "Paralytic Shellfish Toxins in Bivalve Molluscs: Occurrence, Transfer Kinetics, and Biotransformation." Reviews in Fisheries Science 6(4): 69.

Castro-Santos, T., A. Haro, et al. (1996). "A passive integrated transponder (PIT) tag system for monitoring fishways." Fisheries Research 28(3): 253-261.

Cembella, A. D., C. Destombe, et al., (Eds.) (1990). Toxin composition of alternative life history stages of *Alexandrium excavatum*, as determined by high-performance liquid chromatography. Toxic Marine Phytoplankton. New York, Elsevier.333-338

Connell, L. B., S. P. MacQuarrie, et al. (2007). "Population differences in nerve resistance to paralytic shellfish toxins in softshell clam, *Mya arenaria*, associated with sodium channel mutations." Marine Biology 150(6): 1227-1236.

Côrte-Real, H. B. S. M., P. W. H. Holland, et al. (1994). "Inheritance of a nuclear DNA polymorphism assayed in single bivalve larvae." Marine Biology 120(3): 415-420.

Cucherousset, J., J.-M. Paillisson, et al. (2007). "Using PIT technology to study the fate of hatchery-reared YOY northern pike released into shallow vegetated areas." Fisheries Research 85(1-2): 159-164.

Dixon, D. R. (1982). "Aneuploidy in mussel Embryos (*Mytilus edulis* L.) originating from a polluted dock." Marine Biology Letters 3: 155-161.

Fast, M. D., A. D. Cembella, et al. (2006). "In vitro transformation of paralytic shellfish toxins in the clams *Mya arenaria* and *Protothaca staminea*." Harmful Algae 5(1): 79-90.

Foltz, D. W. (1986). "Segregation and Linkage Studies of Allozyme Loci in Pair Crosses of the Oyster *Crossostrea virginica*." Biochemical Genetics 24: 941-956.

Fong, P. P., K. Kyojuka, et al. (1994). "*In vivo* and *In vitro* induction of germinal vesicle breakdown in a freshwater bivalve, the zebra mussel *Dreissena polymorpha* (Pallas)." The Journal of Experimental Zoology 269(5): 467-474.

Ford, S. E., M. Bricelj, et al. (2008). "Deleterious effects of a nonPST bioactive compound(s) from *Alexandrium tamarense* on bivalve hemocytes." Marine Biology 154: 241-253.

Fuller, S. A., J. P. Henne, et al. (2008). "Performance of commercially available passive integrated transponder (PIT) tag systems used for fish identification and

interjurisdictional fisheries management." North American Journal of Fisheries Management **28**(2): 386-393.

Gaffney, P. M. (1994). Heterosis and heterozygote deficiencies in marine bivalves: more light? Genetics and evolution of aquatic organisms. A. R. Beaumont. London, Springer: 146-153.

Gessner, B. D. and J. P. Middaugh (1995). "Paralytic shellfish poisoning in Alaska: a 20-year retrospective analysis." Am. J. Epidemiol. **141**(8): 766-770.

Hallegraeff, G. M. (1993). "A review of harmful algal blooms and their apparent global increase." Phycologia **32**(22): 79-99.

Hamilton, S. A., L. Connell, et al. (2009). The effect of red tide bloom history on the gene frequency of paralytic shellfish poisoning (PSP) resistance in populations of the softshell clam, *Mya arenaria*. National Shellfish Association 101st Annual Meeting. Savannah, GA.

Hedgecock, D., G. Li, et al. (2004). "Widespread null alleles and poor cross-species amplification of microsatellite DNA loci cloned from the Pacific oyster, *Crassostrea gigas*." Journal of Shellfish Research **23**: 379-385.

Hu, Y. P. and D. W. Foltz (1996). "Genetics of scnDNA polymorphisms in juvenile oysters (*Crassostrea virginica*) Part I: Characterizing the inheritance of polymorphisms in controlled crosses." Molecular Marine Biology and Biotechnology **5**(2): 123-129.

Jin, D., E. Thunberg, et al. (2008). "Economic impact of the 2005 red tide event on commercial shellfish fisheries in New England." Ocean & Coastal Management **51**(5): 420-429.

Joule, B. J. (1983). "An effective method for tagging marine polychaetes." Canadian Journal of Fisheries & Aquatic Sciences **40**(4): 540-541.

Kurth, J., C. Loftin, et al. (2007). "PIT tags increase effectiveness of freshwater mussel recaptures." **26**(2): 253-260.

Kurth, J., C. Loftin, et al. (2009). "PIT tags increase effectiveness of freshwater mussel recaptures." Journal of the North American Benthological Society **26**(2): 253-260.

Launey, S. and D. Hedgecock (2001). "High Genetic Load in the Pacific Oyster *Crassostrea gigas*." Genetics **159**(1): 255-265.

Loosanoff, V. L. and H. C. Davis (1963). Rearing of Bivalve Mollusks. London, Academic. pp136.

- Loosanoff, V. L. and H. C. Davis (1963). Rearing of Bivalve Mollusks. London, Academic.
- Luengen, A. C., C. S. Friedman, et al. (2004). "Evaluation of mussel immune responses as indicators of contamination in San Francisco Bay." Marine Environmental Research **57**(3): 197-212.
- MacQuarrie, S. P. (2002). Inter- and intra-population variability in behavioral and physiological responses of the softshell clam, *Mya arenaria*, to the PSP toxin-producing dinoflagellate, *Alexandrium tamarense*. Halifax, NS, Dalhousie University: 141pp.
- MacQuarrie, S. P. and V. M. Bricelj (2000). "Does the history of toxin exposure influence bivalve population responses to PSP toxins in *Mya arenaria*?: II) feeding, survival and toxin accumulation." Journal of Shellfish Research **19**(1): 636 (abstract).
- Mallet, A. L., E. Zouros, et al. (1985). "Larval viability and heterozygote deficiency in populations of marine bivalves: evidence from pair matings of mussels." Marine Biology **87**(2): 165-172.
- Maranda, L., D. M. Anderson, et al. (1985). "Comparison of toxicity between populations of *Gonyaulax tamarense* of eastern North American waters." Estuarine, Coastal and Shelf Science **21**: 401-410.
- Masahiro, I. (1999). Technical guidance on pearl hatchery development in the Kingdom of Tonga, Food and Agriculture Organization of the United Nations: 52.
- Meynecke, J.-O., G. C. Poole, et al. (2008). "Use of PIT tag and underwater video recording in assessing estuarine fish movement in a high intertidal mangrove and salt marsh creek." Estuarine, Coastal and Shelf Science **79**(1): 168-178.
- Narahashi, T., H. G. Haas, et al. (1967). "Saxitoxin and tetrodotoxin: comparison of nerve blocking mechanism." Science **157**(3795): 1441-1442.
- Olberding, K. E., M. L. Kelley, et al. (2004). "A HECT E3 ubiquitin-protein ligase with sequence similarity to E6AP does not target p53 for degradation in the softshell clam (*Mya arenaria*)." Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis **552**(1-2): 61-71.
- Prakash, A., J. C. Medcof, et al. (1971). Paralytic Shellfish Poisoning in Eastern Canada. Ottawa, Fisheries Research Board of Canada. pp87.
- Prentice, E. F., T. A. Flag, et al. (1990). "Feasibility of using implantable passive integrated transponder (PIT) tags in salmonids " American Fisheries Society Symposium **7**: 317-322.

Riffeser, M. and B. Hock (2002). "Vitellogenin levels in mussel hemolymph--a suitable biomarker for the exposure to estrogens?" Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology **132**(1): 75-84.

Schwab, K. J., F. H. Neill, et al. (1998). "Distribution of norwalk virus within shellfish following bioaccumulation and subsequent depuration by detection using RT-PCR." Journal of Food Protection **61**: 1674-1680.

Twarog, B. M. (1974). "Immunity" to paralytic shellfish toxin in bivalve molluscs. Proceedings from the Second International Coral Reef Symposium. Brisbane, Australia. **1**: 505-512.

Twarog, B. M., T. Hidaka, et al. (1972). "Resistance to tetrodotoxin and saxitoxin in nerves of bivalve molluscs." Toxicon **10**: 273-278.

Whitfield Gibbons, J. and K. M. Andrews (2004). "PIT tagging: simple technology at its best." BioScience **54**: 447-454.

Widman, J. C., J. Choromanski, et al. (2001). Manual for Hatchery Culture of the Bay Scallop, *Argopecten irradians irradians*. Milford, CT, Connecticut Sea Grant College Program and NOAA National Marine Fisheries Service. pp50.

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