Abundance of Shell-Boring Polychaete Worms and Other Fouling Organisms in Aquacultured Oysters From Maine Used for Reef Restoration in Great Bay, NH

Haleigh Wright

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ABUNDANCE OF SHELL-BORING POLYCHAETE WORMS AND OTHER FOULING ORGANISMS IN AQUACULTURED OYSTERS FROM MAINE USED FOR REEF RESTORATION IN GREAT BAY, NH

by

Haleigh Wright

A Thesis Submitted in Partial Fulfillment of the Requirements for a Degree with Honors (Marine Science)

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ABSTRACT

Restoration projects on the oyster reefs in Great Bay, NH have been active since 2009 with the most recent involving the transfer of oysters from Maine oyster farms into the Bay. In an attempt to prevent the transfer of non-native species from oyster farms to the reefs, samples of oysters from each farm were inspected for shell-boring polychaete infestations. Polydora websteri, a common shell-boring species worldwide, was in high abundance in reference samples from oyster farms in Great Bay and in samples from the restoration grounds, themselves. A second shell-boring species, provisionally identified as P. onagawaensis, is present on oyster farms in Maine but has not been observed previously in Great Bay. I used microscopic analysis of morphological features and molecular analysis of the mitochondrial CO1 (mtCO1) gene to identify worms extracted from the oyster samples from Maine farms to species, when possible. When intact worms could not be extracted from the shells, I used the shape of their burrows, which is distinct for P. websteri and P. onagawaensis, to infer the presence of the latter. I found that the abundance of shell-boring polychaetes was variable along the coast of Maine, with farms in close geographical proximity having very different loads of burrows and worms. Both P. websteri and P. onagawaensis were identified by molecular analysis on some Maine farms while only P. websteri was found in the samples from New Hampshire farms and restoration sites.
ACKNOWLEDGEMENTS

I would like to thank Boze Hancock and The Nature Conservancy for allowing me to participate in the TNC SOAR restoration project. I am grateful to have played a role in an ongoing marine conservation effort and wish them the best of luck in continuing the restoration project in Great Bay. I would also like to thank my committee members who offered their time and guidance throughout the thesis process. Finally, a huge thank you to my advisor, Dr. Paul Rawson, who contributed an immense amount of time and effort to help me complete this project. He has inspired me to overcome hardships with hard work and to always persevere.
LIST OF FIGURES

Figure 1. Mud Blister burrow created by *Polydora websteri*..........................2
Figure 2. Distinctive u-shaped burrow created by *Polydora onagawaensis*.........8
Figure 3. Shell measurements........................................................................11
Figure 4. Worm extraction with peppermint oil..............................................12
Figure 5. Number of burrows observed at each farm........................................17
Figure 6. Number of worms observed at each farm..........................................17
Figure 7. Burrow from Farm 1 created by *Polydora onagawaensis*...............19
Figure 8. Penobscot East Region......................................................................20
Figure 9. Burrows and worms from the New Meadows Region.......................22
Figure 10. New Meadows Region......................................................................24
Figure 11. Burrows from the Casco Bay Region..............................................25
Figure 12. Spaghetti worms from Farm 12.........................................................27
Figure 13. Casco Bay Region ...........................................................................28

LIST OF TABLES

Table 1. Oyster farms and evidence for polychaete identification.....................18
# TABLE OF CONTENTS

BACKGROUND .......................................................................................................................... 1  
INTRODUCTION ........................................................................................................................ 4  
METHODS .................................................................................................................................. 10  
  Inspection of Oysters and Sampling of Shell-boring Polychaetes ........................................ 10  
  DNA Analysis to Confirm Species Identification ................................................................ 13  
RESULTS AND DISCUSSION .................................................................................................... 16  
  Penobscot East Region ........................................................................................................... 19  
  New Meadows Region ........................................................................................................... 21  
  Casco Bay Region .................................................................................................................. 25  
  New Hampshire ..................................................................................................................... 32  
CONCLUSIONS .......................................................................................................................... 35  
OPPORTUNITIES FOR FURTHER RESEARCH ........................................................................ 37  
BIBLIOGRAPHY ....................................................................................................................... 38  
AUTHOR’S BIOGRAPHY ........................................................................................................... 40
Oysters play an important role in the function of many coastal ecosystems. An adult oyster can filter up to 50 gallons of water a day, decreasing the turbidity of the water and taking up excess carbon and nitrogen (Konisky et al., 2014). However, natural populations of eastern oysters (*Crassostrea virginica*) have faced declining numbers as a result of disease and overharvesting. In response, resource managers have initiated restoration programs where oysters are planted and reintroduced into degraded habitats in the hope that they can thrive, reproduce, and help rebuild reefs, thereby restoring ecosystem services. While costly in terms of time and effort, many restoration projects have shown promising results and have helped to create healthier coastal ecosystems (Hernandez et al., 2018).

Oysters and other bivalves have been a prized food source since perhaps as early as 12,000 BC (Simon & Sato-Okoshi, 2015) and the purposeful culture of oysters extends back to Roman times (Günther, 1897). As oyster reefs have declined, worldwide, there has been increasing effort to culture oysters. Modern aquaculture grows oysters in relatively high densities in floating bags or other structures to maximize the use of aquaculture lease sites (Maine Sea Grant, n.d.)

Because bivalve farming often results in high numbers of animals in a relatively small area and there are limited ways to prevent or treat diseases, aquaculture farms can be high prevalence sites for disease (Bogwald & Dalmo, 2012). Several diseases are of concern in oysters including Roseovarius Oyster Disease (ROD), caused by the bacterium *Alliroseovarius crassostreae*, and Dermo Disease, SSO, and MSX (Multinucleated
Sphere Unknown) caused by the protists *Perkinsus marinus*, *Haplosporidium costale*, and *H. nelsoni*, respectively. They are of significant economic importance due to the high mortality at farms with disease outbreaks (Carnegie, 2009).

Cultured oysters can also be impacted by commensal and parasitic species that live on or in the shell valves (Watson *et al*., 2009). Of particular concern are shell-boring polychaetes, parasitic worms that can lead to declining health of both wild and aquaculture oysters. These worms can inhabit any crack or crevice in the surface a bivalve shell and with the use of a viscous, mucus-like fluid they can excavate a burrow into the shell (Zottoli & Carriker, 1974). In some cases, such as with the blister worm *P. websteri*, the worm packs the burrow with mud and detritus leading to the development of an unsightly blister (Fig. 1) that often decreases the market value of the oyster host due to the unattractive appearance of the blisters on the inside of the valves. All burrowing species, including *P. onagawaensis* and *P. websteri*, can increase shell fragility leading to decreased growth (Simon & Sato-Okoshi, 2015).

**Figure 1.** Mud blister burrow created by *P. websteri*. For this image the oyster host was sacrificed and the tissues were removed from each valve. The valves were then backlit to allow worm burrows to be directly observed in the intact valves.
Given the recent growth of oyster aquaculture and increasing conservation efforts to rebuild or sustain “wild” reefs there is concern over the interaction between the two (David, 2020). As shellfish aquaculture has grown in popularity, oysters have been transported around the world contributing to the dispersal and introduction of non-native fouling species that often inhabit their shells (Sato-Okoshi et al., 2017) and can potentially become established in nearby natural reefs. Similarly, if reef restoration programs are not properly designed they can result in the translocation of pests and non-native species to new locations that can potentially infest nearby shellfish farms.

The subject of my thesis lies at the intersection of oyster farming and oyster restoration. I worked with The Nature Conservancy (TNC) SOAR program which was established to buy up to 5 million eastern oysters from farms in multiple northeastern states and move them to nearby restoration grounds. The intention was to support oyster farmers who have had difficulty selling oysters during the COVID-19 pandemic. However, there are no restoration grounds in Maine to serve as “nearby” reefs. Instead, TNC managers sought to move oysters from Maine farms to conservation reefs in New Hampshire. This, in turn, necessitated inspections for disease and parasites. The Maine Department of Marine Resources coordinated disease inspections. Together with my advisor, Dr. Rawson, I inspected oysters for the presence of parasitic polychaetes and other fouling species.
INTRODUCTION

In recent years, Great Bay, New Hampshire has seen major decreases in oyster populations as a result of overharvesting, increased sedimentation, and disease (Konisky et al. 2014). The substantial decline in oyster abundance in the Bay has led to negative effects such as worsening water quality, high nitrogen levels, and loss of habitat and feeding grounds for many invertebrate and vertebrate species. To counteract these declines, the New Hampshire chapter of The Nature Conservancy established a program to rebuild reefs at multiple sites within Great Bay estuary. The ecosystem services of bivalve shellfish are often overlooked, but through their physical presence, feeding, and other activities, shellfish help to maintain and even restore some aspects of damaged ecosystems (Coen et al., 2007). Examples include greater abundances and diversity of species that utilize reef ecosystems for food or shelter, increased sea grass growth, decreased erosion of marshes and estuaries, and reducing turbidity of waters. While it can be difficult to quantify the effects of oyster restoration over a wide area, smaller studies have shown these positive results in ecosystems such as Chesapeake Bay and in the marshlands of southeastern states. Oyster restoration projects are costly, long-term commitments (Hernandez et al., 2018). Restoration efforts have been ongoing in the Chesapeake Bay since 1993 and along the coastlines of several southeastern states since 1992. However, they are generally worth the time and money due to the positive ecological effects that the oysters contribute. Protection of coastline and marshes means less need for subsequent reconstruction, while the increased size of finfish populations
helps sustain local fishing industries, and decreased turbidity allows for healthier ecosystems and more aesthetically pleasing tourist attractions.

In Great Bay, TNC has worked to buy and plant volunteer and hatchery-produced oysters in an attempt to restore the oyster population and reefs. This project has been in effect since 2009 and is continuing today (Konisky et al., 2014). Initially, barges full of surf-clam shells were ferried into the Bay and sprayed into the water with a fire hose to set the base for the constructed reefs. About 65,000 power washed oyster shells were loaded into wire cages to be used as substrate for bivalve larvae to settle and recruit. After about 2 months of growth the cages and juvenile oysters were hand-placed on the constructed reefs. In just five years the program planted about 3.14 million oysters over 13 acres in Great Bay.

With closures of restaurants and other pressures stemming from the COVID-19 pandemic, TNC proposed to buy aquaculture grown oysters to be used in the reef restoration project in Great Bay to lessen the economic stress on oyster farmers. Restaurant closures and the resulting decrease in consumption and sales left farmers with oysters that had grown past the ideal market size and were no longer desirable. The TNC offered to buy the oversized oysters to provide the farmers with monetary support without taking from their supply of oysters that could still be sold to dealers and restaurants. The opportunity was presented to oyster farmers in Maine and New Hampshire. The goal for this project was for TNC to buy upwards of 5 million oysters from aquaculture farms in the area that would then be planted in Great Bay (TNC SOAR Program, n.d.). In order for the oysters to be suitable for transfer to Great Bay, evaluation
of fouling species was important in order to limit the transfer of non-native species into the Bay.

Oysters and other bivalve species host a wide range of fouling organisms. The degree of fouling and composition of the fouling community vary tremendously (Watson et al., 2009). Variables that can impact fouling include whether a farm is located in intertidal or subtidal regions and the type of culture practiced on a farm. For example, farms that implement methods to periodically dry their oysters and cages tend to have lower loads of fouling species than those that do not implement such measures. Oyster farmers often struggle to contain biofouling and, although biofouling does not make the bivalves unsafe for consumption, some fouling species can alter the appearance of the shells which can turn customers away.

Among the fouling community, shell-boring polychaetes in the genera such as *Polydora*, *Boccardia*, and *Hydroides* have gained notoriety (Simon & Sato-Okoshi, 2015). Although species in these genera typically can burrow into a variety of calcareous substrates, they are particularly destructive when they infest the shells of bivalve shellfish. For example, they increase the fragility of the valves and can fill excavated burrows with pockets of mud and detritus on the inside of the shell that the host covers with nacre, resulting in a large and unsightly blister (Blake & Evans, 1970; Fig. 1). This diversion of energy to shell repair reduces the oyster’s growth rate and high infestations can lead to increased oyster mortality (Simon & Sato-Okoshi, 2015). Additionally, if worms burrow under the site of adductor muscle attachment they can cause uncontrolled gaping and perhaps make the oyster more susceptible to predation or dehydration during periods of exposure.
It is well known that shell-boring polychaete species and other fouling organisms are present in both Maine and New Hampshire waters. In order to reduce the potential of harmful and non-native fouling species being introduced to the restoration grounds in Great Bay, that could then make their way to the oyster farms of the region, it was decided that the oysters from each participating Maine farm would need to be inspected for fouling organisms prior to being accepted by the SOAR program.

There are several potential ways in which fouling species like shell-boring polychaetes can spread. One way is through the production of planktonic larvae. Many shell-boring species produce larvae that are released into the water column post-hatch where they feed and complete development before settling. When competent and ready to metamorphose they seek out suitable substrates and can become established in habitats such as oyster reefs or farms. The longer the duration of time that such larvae spend in the water column increases the potential that they can disperse to new habitat.

Other species may produce less numerous adelphophagic or lecithotrophic larvae. Adelphagic larvae hatch within the maternal burrow and feed on brood eggs while lecithotrophic larvae are provided yolk to grow on and sustain them throughout a relatively short period of planktonic development. Adelphagic and lecithotrophic larvae have higher chances of survival and settlement due to the provisioning and little to no time spent in the plankton where predation is higher. They spend far less time in the plankton and thus have lower dispersal potential. Perhaps more problematic is that some species, such as *P. websteri*, can switch between the production of planktotrophic and adelphagotic larvae. While planktonic and lecithotrophic larvae may result in the dispersal of few larvae from reef to reef, reef to farm, or farm to farm, adelphagogy can
result in pest polychaetes quickly increasing in abundance in a suitable habitat, such as an aquaculture farm.

These natural processes can allow for dispersal and colonization of shell-boring species, however humans also play a role. It is believed that many polychaete species have been and continue to be transported as hitchhikers in the shells of bivalves (e.g., Rice et al., 2018). As a result of increasing interest in aquaculture worldwide, species such as oysters, and other bivalves, have been transported across the globe effectively transferring any shell-boring polychaetes that had colonized their shells (Sato-Okoshi et al., 2017).

One major concern in the TNC SOAR project was the potential transfer and introduction of *P. onagawaensis*, a non-native shell-boring polychaete, into Great Bay. This species, *P. onagawaensis*, has been recorded in oysters grown in several coastal estuaries in Maine (Silverbrand et al., 2021). Because it has not previously been observed

![Figure 2](image.png)

**Figure 2.** Distinctive, u-shaped burrow created by *P. onagawaensis*. This burrow is in the shell of *C. virginica* that is illuminated from behind. The burrow is unbranched, terminates before penetrating the inside of the oyster and has very little mud or detritus. The red coloration inside the burrow.
in New Hampshire waters, the SOAR program sought to have oysters from Maine farms inspected before they were used in this restoration project.

Another species of importance is *P. websteri*. As discussed above, this shell-boring polychaete species compromises valve strength with its large, mud-filled burrows. While the SOAR program sought to limit the transfer of *P. websteri* to Great Bay in order to preserve overall health of oysters in the region, this species was second in importance since it had previously been observed in cultured oysters from Great Bay and, therefore, the concern of a new introduction to the area was lessened. As stated above, *P. websteri* creates large u-shaped burrows that they tend to pack with mud creating the “mud blister” (Fig. 1). When compared to the u-shaped burrows of *P. onagawaensis* (Fig. 2), the burrows of *P. websteri* are generally more noticeable.

My thesis reports on the degree of infestation for both *P. websteri* and *P. onagawaensis* among oyster farms participating in the TNC-SOAR program. My observations also support efforts to understand the patterns of distribution of these two pest species in Maine and methods for controlling their abundance.
METHODS

*Inspection of Oysters and Sampling of Shell-boring Polychaetes*

Oysters were sent from oyster farms in Maine (n=12) and New Hampshire (n=4) to the University of Maine over the period of a month, from mid-October to mid-November, 2020. Each farm sent 30 oysters for inspection, with the exception of one farm in the New Meadows region of Maine which sent 60 oysters. In addition, oysters were sampled from 3 reef conservation regions, Adams Point (43.0913, -70.8654), Woodman’s Point (43.0715, -70.8609), and Squamscott (40.0618, -70.9080) in the Great Bay, NH estuary. Upon arrival, the oysters were stored in their shipping containers in a cold room until they could be processed. The oysters were typically processed the day after arrival with a few exceptions, such as those that arrived on Fridays or weekends. The oysters in each sample were inspected for the presence of shell-boring polychaetes (*P. websteri* and *P. onagawaensis*), and other fouling species such as shell-boring sponge (*Cliona sp.*). As a part of these inspections, the oysters were examined for surface fouling (e.g., barnacles, amphipods, tunicates) and the relative abundance of surface fouling species was noted for the sample as a whole. The shell heights and widths for each of the 30 oysters from each farm were measured (Figure 3) after which the valves were separated and the oyster meats removed and placed into individual 50 ml centrifuge tubes and frozen.
After the valves were separated and the meats removed, both valves of the shell were backlit using an LED microscope light source to illuminate and accentuate any burrows that were present. The burrows observed in the cupped and flat valves were counted and recorded separately. Where possible, photos of each burrow were taken to provide a permanent record using a Canon Rebel SLR outfitted with an EF50 compact-macro lens. Characteristics of the burrows, such as the general shape and heavy deposits of mud and detritus were recorded and compared to those shown in Figures 1 and 2 to make a preliminary species determination of the shell-boring polychaete species found at each farm.
If a worm was present in a burrow, usually indicated by a bright orange-red coloration in the burrow (Figure 2), we attempted to remove them for subsequent morphological examination and DNA testing. One approach used was to place a valve in a mixture of seawater and peppermint oil (as in Figure 4) and wait for the worms to leave the burrows on their own.

![Figure 4. A cupped valve placed in a seawater and peppermint oil mixture to extract worms from their burrows. The orange-redish worms can be seen exiting from burrow openings all along the shell.](image)

Because the majority of the worms were relatively inactive, however, I instead removed them from the burrow by cutting, breaking, and chiseling through layers of the host shell in order to access the burrow and gently remove the worm with dental tools. Many of the worms were not in good shape, perhaps due to seasonal affects or because the oyster hosts had been sampled and held out of water for an extended period during shipping. Due to this, the morphological characteristics of some could not be determined and many that I obtained were in multiple pieces. After being removed from the shell, each worm was placed in a petri dish with seawater and labeled with the oyster farm and
what number oyster it was removed from. Photos and observations (at 10 to 63x power) were taken using an Olympus SZ40-dissecting microscope and a Canon VIXIA HF-G20 still/video camera outfitted with a Martin Microscope MM99 through the lens adapter. Each worm was preliminarily identified as either *P. onagawaensis* or *P. websteri* based off of the characteristics of the burrow and the morphological characteristics of the worms themselves. In order to better view the worms, MgCl$_2$ was used as an anesthetic to slow worm movement while being viewed under the microscope. The worms were then preserved in 95% ethanol for subsequent DNA analysis.

**DNA Analysis to Confirm Species Identification**

Often, burrow shape and the patterns of pigmentation and morphological features were not adequate for identifying the worms to species and molecular analysis was necessary. For larger worms, the heads and tails were fixed in formalin for reference and DNA was extracted from the middle segments, as per Silverbrand *et al.* (2021). DNA isolation employed a DNeasy Blood and Tissue Kit (Qiagen Inc.) For tissue lysis, 180 µL of buffer ATL, 20 µL of Proteinase K, and worm tissue samples were combined in 1.5 mL microcentrifuge tubes and allowed to incubate for 2 hours, with gentle mixing every 30 minutes. When the incubation period was complete the 1.5 mL microcentrifuge tubes containing the digested worm tissues were briefly centrifuged before 200 µL of Buffer AL was added to each sample, mixed, and incubated for another 10 minutes in a 70°C water bath. The tubes were centrifuged, briefly, and 200 µL of 96-100% ethanol was added to each sample, mixed by vortexing, and centrifuged briefly once again. The mixtures from each microcentrifuge tube were then transferred to QIA amp spin columns
and centrifuged for 1 minute at 8,000 RPM. The spin column was placed in a clean 2 ml collection tube and the filtrate was discarded and 500 µL of Buffer AW 2 was then added to the spin column containing the DNA and centrifuged at 13,200 RPMs for 3 minutes. Afterwards, the spin column was transferred to a new 2 ml collection tube, and the filtrate discarded. The samples were again centrifuged for 1 minute at full speed, 13,200 RPM.

The last step in preparation for using the DNA in a polymerase chain reaction (PCR) involved transferring the spin column to a 1.5 ml microcentrifuge tube, adding 50 µL of Buffer AE and incubating for 1 minute before centrifuging at 8,000 RPM for 1 minute. The spin column was then discarded and the purified DNA was stored in the 1.5 ml microcentrifuge recovery tube.

The isolated DNA was used in subsequent PCR reactions targeting the mitochondrial cytochrome c oxidase I (mtCO1) gene following the protocol described in Silverbrand et al. (2021) in which 24 µL of master mix comprised of 2.5 µL 10x PCR buffer, 0.75 µL 50 mM MgCl₂, 0.5 µL 10 mM deoxynucleotide triphosphates (dNTPs), 0.25 µL forward primer (Psp_CO1_331F: 5’ AGA GGA ATA TGA GTA GAA GA 3’), 0.25 µL reverse primer (Psp_CO1_966R: 5’ GTG GCC ATT CAG CTA AAT ACT TTA AT 3’), and 0.2 µL Taq DNA polymerase (Invitrogen Inc.) were combined in a 0.5 ml thin-walled PCR tube. With the final addition of 1 µL template DNA, the PCR reactions were run at a total volume of 25 µL. Reaction tubes were mixed and centrifuged briefly before being placed in an Eppendorf MasterCycler set for an initial 94°C denaturation soak for 2 minutes followed by 35 cycles of a 94°C denaturation step for 30 s, a 48°C primer annealing step for 30 s, and a 72°C extension step for 2 min. The PCR reactions were checked by electrophoresing them on a 1.5% agarose gel for 45 min at 75 v. Prior to
electrophoresis, the DNA was stained using EZ-Vision Two DNA dye and running buffer (Amresco, Inc.)

The PCR reaction produces a product that is 630 bp long in both *P. websteri* and *P. onagawaensis*. I employed restriction enzyme digests to detect species-specific sequence variation within the PCR products. The restriction digests were completed by mixing 5 µL of each PCR reaction with 1 µL of Cut Smart buffer (New England Biolabs, Inc.), 0.5 µL FokI (2 U), and 3.5 µL of distilled water. These digests were incubated at 37°C overnight. The resulting fragments were visualized on 1.5% agarose gel for 45 min at 75 v; the gels were photographed using a UV-transilluminator and Kodak DC120 digital camera outfitted with a SYBR green filter kit. The worm PCR and RFLP digest assays included positive control DNAs from a known New Hampshire *P. websteri* sample and a known Mount Desert Island *P. onagawaensis* sample, both described in a previous study by Silverbrand *et al.* (2021).

While the molecular analysis helped me to more reliably determine the species of the worms, it did not have an effect on the oysters that were planted in Great Bay since it occurred after the plantings had been completed. My observations of overall fouling, as well as burrow and worm counts with species identifications based on burrow and worm morphology were reported to the farms and TNC along with photographic evidence. The oysters from prospective farms were also subject to pathology testing for known oyster diseases. This latter work was coordinated by the Maine Department of Marine Resources and Kennebec River Biosciences (Richmond, ME). Oysters from farms that passed both the pathology testing and the shell-boring polychaete inspections were then used in rebuilding the reefs in Great Bay, NH
RESULTS AND DISCUSSION

The shell-boring worms in the oysters sent for evaluation were in variable condition. In some cases, I was able to easily extract whole worms or portions of worms that could be examined for morphological features indicative of the two species of interest. Patterns of pigmentation of each sampled worm were the main characteristic viewed since the timing of the inspections made it impossible for more detailed microscopical examination. I was unable to inspect the worms for diagnostic features such as the shape of the spines on the 5th chaetiger of specimens, as was done by Silverbrand et al. (2021). However, most of these worms were suitable for DNA-based analysis to identify them to species. For other oyster samples, degradation of the worm tissues within the burrows was apparent, likely due to oyster culture conditions, seasonal changes, delays in shipping, or a combination of factors. Morphological identification was impractical for these sites and identification of worms to species and estimation of the level of infestation were restricted to the comparison of burrow shape along with mud and detritus deposits.

With this approach, I observed a wide range of burrow abundance and intact worms among the sample sites that were submitted for inspection (Fig 5 and 6). This information was also used to infer the species of the shell-boring polychaetes present in each of the samples (Table 1). The following sections include the variation in worm presence and abundance among farms within the four regions from which oyster samples were submitted.
Figure 5. The total number of burrows observed in the oysters submitted by each of the 16 farms participating in the TNC-SOAR project during the fall of 2020. All farms submitted 30 oysters for inspection with the exception of farm 14 which submitted 60 oysters in total.

Figure 6. The total number of worms observed in the samples from the 16 farms that sent oysters for evaluation.
<table>
<thead>
<tr>
<th>Farm</th>
<th>P. onagawaensis</th>
<th>Evidence</th>
<th>P. websteri</th>
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<td>Burrow Shape</td>
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<td>Burrow Shape &amp; DNA</td>
</tr>
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</table>

**Table 1.** Evidence for the presence of shell-boring polychaetes at each of the oyster farms that participated in the program. The farms are categorized (1-16) from North to South based on the different regions they fall within along the coast of Maine and in New Hampshire. The table also shows which species of worms were observed at each farm and the evidence I used to identify them.
Penobscot East Region

Farm 1, the most northerly and easterly site in my study, was the only farm in this region to provide oysters for analysis. The oysters from Farm 1 were smaller than those we received from other farms. They were also clean with little mud and very little surface fouling, with the exception of few barnacles and superficial mud burrows created by *P. cornuta* and amphipods. I observed evidence of simple u-shaped burrows with little mud or detritus typical of the burrows created by *P. onagawaensis* (Fig. 7). However, I only detected such burrows in two of the oysters in the 30-oyster sample (Fig. 7).

![Figure 7](image)

**Figure 7.** An oyster valve from Farm 1 showing an empty burrow suspected to belong to *P. onagawaensis* due to the simple u-shape and lack of mud.

Each of the affected oysters had one clean, u-shaped burrow and no worms were present in the burrows so I could not conduct DNA analysis. No mud-blister burrows indicative of *P. websteri* were observed. My observations suggest this farm had a light load of shell-broing polychaetes and no on-going infestation. The identification of *P. onagawaensis* is consistent with the findings of Silverbrand *et al.* (2021) who, using morphological characteristics observed with scanning electron microscopy (SEM) as well
as molecular analysis of the 18s rRNA and mtCO1 genes, identified this species on other oyster farms near Mount Desert Island and Deer Isle area. On the other hand, Silverbrand et al. (2021) and Rice et al. (2018) found that shell-borers at farms to the north of Deer Isle, in the Brooksville Maine area, were *P. websteri*.

**Figure 8.** Enlarged view of the Penobscot East Region showing the area where Farm 1 was located. The blue wedge in the pie chart indicates the proportion of oysters out of the 30 sampled that contained polychaete burrows in at least one valve. The burrows in the farm 1 oysters were identified as belonging to *P. onagawaensis* (see Fig. 7).
**New Meadows Region**

Six farms located further west along the coast of Maine in the New Meadows region submitted samples for inspection (Farms 2-7, Table 1). There was no sign of shell-boring worm species at three of the farms (Farms 3, 6, and 7). The sample of oysters from Farm 5 was similar to those from Farm 1 in that the exterior of the oysters was clean, only having mud burrows created by *P. cornuta* and amphipods. However, thirteen out of sixty oysters from Farm 5 had *P. onagawaensis*-like burrows, nine of which contained live worms. Eight worms were sampled from the Farm 5 oysters, one of which showed dark pigmentation, a feature that occurs in *P. onagawaensis* but not *P. websteri*, while coelomic eggs were discovered within another specimen. There were no worms or burrows indicative of *P. websteri* in the sample from Farm 5 and, overall, there was an intermediate to high load of shell-boring worms. DNA analysis was conducted on eight worms that were provisionally identified by burrows shape to be *P. onagawaensis*. The results indicated that each worm was correctly identified as *P. onagawaensis*. These results were also consistent with Silverbrand *et al.* (2021) who described shell-boring *P. onagawaensis* at a sample site only about a mile from Farm 5 near Georgetown, Maine in the Sasanoa River.

The oysters from Farm 4 were relatively clean with some surface fouling by tunicates, byssal threads from mussels, bryozoans, and *P. cornuta* and amphipod mud burrows. Fewer of the oysters from Farm 4 had shell-boring worms than did those from Farm 5, with only three of 30 oysters having burrows that were indicative of *P.*
onagawaensis (Fig 9). I recovered three worm samples from the five burrows that contained live worms and preserved them for later DNA analysis. DNA analysis of all three worms confirmed they were *P. onagawaensis*.

**Figure 9:** Worms and burrows documented from the New Meadows region. The two images on the left are of a burrow appearing to contain a worm and a worm that was removed from one of the burrows. The middle picture shows an empty burrow in an oyster from Farm 4. The picture on the right shows two small burrows with worms present inside. The shape and lack of mud in these burrows is consistent with burrows formed by *P. onagawaensis*.

Most notably, Farm 2 had a high load of shell-boring polychaetes, most of which were provisionally identified as *P. onagawaensis* by burrow shape except for one oyster that had mud-blister burrows indicative of *P. websteri*. Of the 22 oysters from Farm 2 that had *P. onagawaensis*-like burrows, many contained live worms (Fig 5 and 6). Pigmentation patterns of the worms collected for morphological and DNA-based analysis were similar to the expected coloration of *P. onagawaensis* (Fig 9). Additionally, eight or more worms carried coelomic eggs and one burrow contained 25 egg cases with multiple eggs in each case. The oysters had very little surface fouling, the only noticeable sources being mud tubes from *P. cornuta* and amphipods. The worms in the burrows that were
morphologically indicative of *P. websteri* were not sampled and, therefore, the identification could not be confirmed with DNA-based analysis. However, twelve worms all initially suspected to be *P. onagawaensis* based off of morphology were sampled from Farm 2 oysters and used in DNA analysis where the species identification was confirmed.

The observation of *P. onagawaensis* in the New Meadows region is consistent with Silverbrand *et al.* (2021) who described shell-boring *P. onagawaensis* at a sample site only about a mile from Farm 5 near Georgetown, Maine in the Sasanoa River. They also identified shell-boring worms as *P. onagawaensis* from a sample site near Brunswick, Maine, only about half a mile south of where Farm 2 is located in my study. I conducted a goodness of fit test to determine whether the variation in the proportion of oysters harboring *P. onagawaensis* at farms on the New Meadows River was statistically significant. Overall, among the farms from which I received samples in the New Meadows region there was very pronounced heterogeneity in the proportion of oysters that harbored shell-boring polychaetes (Fig 10) and the heterogeneity was statistically significant ($\chi^2 = 26.905$, d.f. = 2, $p = 1.44 \times 10^{-6}$). Even when only considering the two farms with relatively heavy worm loads there was significant heterogeneity in the proportion of oysters containing worms ($\chi^2 = 17.44$, d.f. = 1, $p = 3.0 \times 10^{-5}$). Perhaps most intriguing, however, was the sample from Farm 3 in which I did not observe a single burrow or shell-boring worm. This, despite the fact that Farm 3 is located only about a mile from Farm 2, the latter of which had the most pronounced infestation in the region, and slightly over half a mile from a site where Silverbrand *et al.* (2021) reported *P. onagawaensis*. Whether such small-scale heterogeneity is due to
differences in culture method, local habitat availability, movement of oyster hosts within the river, or other factors needs further investigation.

Figure 10: Map of the New Meadows Region and pie charts for the 6 participating farms in the region organized from North to South. Each pie chart presents the proportion of oysters affected with shell-boring polychaete burrows with blue for *P. onagawaensis* and purple for *P. websteri*. Each farm provided 30 oysters for analysis except for Farm 5 who provided 60 oysters.
Casco Bay Region

Five farms in the Casco Bay region submitted samples for inspection (Farms 8-12, Table 1). There was no evidence of *P. onagawaensis* or *P. websteri* at Farm 8. Oysters from Farm 9, about 5 miles south of Farm 8, had a total of 11 *P. onagawaensis*-like burrows among the sample of 30 oysters. Although I recovered two live worms from this farm, I was not able to complete DNA analysis, therefore, the identification of the worms was based off of burrow morphology alone (Table 1 and Fig 11). The oysters had minimal surface fouling including barnacles and few mud burrows created by *P. cornuta* and amphipods. There was no evidence of *P. websteri* or *P. websteri*-like burrows at Farm 9.

![Figure 11](image1.jpg)

**Figure 11:** Burrows found in oysters from Farms 9, 10, and 11, respectively, in the Casco Bay region. Given the shape and lack of mud in the burrows, we suspect that they were created by *P. onagawaensis*.

Oysters from Farm 10, located approximately 1.4 miles away to the south of Farm 9, had more burrows in total and more oysters had been impacted by shell-boring worms.
with 50% of the sample (15 oysters) having at least one burrow and four of the burrows contained live worms (Fig 5, 6, and 12). The worms at Farm 10 were first identified as *P. onagawaensis* based on morphology of the burrows and morphology of the worms, themselves (Fig 11). Two worms extracted from their burrows and used in subsequent DNA analysis were both found to be *P. onagawaensis*. Overall, Farm 10 had a light to intermediate infestation of *P. onagawaensis* with no signs *P. websteri* in the oysters. The exterior fouling was similar to Farm 10 with low numbers of barnacles and mud burrows from *P. cornuta* and amphipods.

Only three of the oysters in the sample from Farm 11 had evidence of worm burrows and only one of the burrows harbored a live worm. Although Farm 11 was only 0.3 miles from Farm 10, this farm has apparently been less impacted by shell-boring polychaetes (Fig. 12). The one live worm that was present was recovered but I did not conduct DNA analysis on that specimen. The worm was identified as *P. onagawaensis*, however, due to the prominent u-shape of the burrow and the lack of mud and detritus build-up within the burrow (Fig 11). The oysters from this farm also showed similar, minimal surface fouling in the form of barnacles and small amounts of fouling by *P. cornuta* and amphipods with no indication of *P. websteri* presence. Goodness of fit tests indicated there was no statistical difference in the number of oysters affected by worms or worm burrows for Farms 11 and 9 ($\chi^2 = 0.001919$, d.f. = 1, $p = 0.965$), but when Farm 10 was included the test was significant ($\chi^2 = 17.502$, d.f. = 2, $p = 1.6 \times 10^{-4}$). The latter indicates that a much higher proportion of the oysters in the sample from farm 10 had been impacted by shell-boring worms.
The last farm in the Casco Bay region showed the most severe signs of infestation, not only with *P. onagawaensis* and *P. websteri*, but many other species as well. Thirteen oysters from Farm 12 had *P. onagawaensis*-like burrows while sixteen oysters showed mud-blisters indicative of *P. websteri*. More than five live worms were found at this site (Fig 6) along with many additional polychaete species that are not known to burrow into calcareous substrates of any kind. I suspect that these worms had taken advantage of previously constructed and abandoned polydorid burrows. For example, multiple spaghetti worms (Fig 12) that are native to the area were found in the previously excavated burrows.

Three worms were sampled from Farm 12 and two underwent DNA-based analysis to determine the species. One of the worms was morphologically identified as *P. onagawaensis* and the other was indistinguishable. The worm that was thought to be *P. onagawaensis* and the other was indistinguishable. The worm that was thought to be *P. onagawaensis* and the other was indistinguishable. The worm that was thought to be *P. onagawaensis* and the other was indistinguishable. The worm that was thought to be *P. onagawaensis* and the other was indistinguishable. The worm that was thought to be *P. onagawaensis* and the other was indistinguishable. The worm that was thought to be *P. onagawaensis* and the other was indistinguishable. The worm that was thought to be *P. onagawaensis* and the other was indistinguishable. The worm that was thought to be *P. onagawaensis* and the other was indistinguishable. The worm that was thought to be *P. onagawaensis* and the other was indistinguishable.

![Figure 12: Examples of spaghetti worms from Farm 12. The top two pictures show the spaghetti worms residing in previously excavated polychaete burrows. The bottom pictures are of spaghetti worms after extraction from the burrows.](image-url)
*onagawaensis* failed twice in the PCR reaction and could not be identified, however, the worm that could not be identified by its morphology was found to be *P. onagawaensis*.

**Figure 13**: View of the Casco Bay Region with pie charts depicting the number of oysters affected with shell-boring polychate burrows at each farm where blue indicates suspected *P. onagawaensis* and purple indicates suspected *P. websteri*.
Comparisons of the levels of infestations on farms participating in the TNC-SOAR project indicates a high degree of heterogeneity in the impact of worms on oyster aquaculture. This observation is consistent with the description of the distribution of *P. websteri* and *P. onagawaensis* in some of the same regions that was described by Silverbrand *et al.* (2021). It is intriguing that farms with no signs of burrowing worm (e.g., farms 3, 6, 7 and 8) are often in close proximity to farms that have evidence of past or current infestations. Farm 3 in the New Meadows region was located within a mile of two other sites for which there was evidence of infestations by *P. onagawaensis*. In the Casco Bay Region (Figure 13), Farm 8 was also in close proximity to three other farms (Farms 9, 10, and 11), all of which had mild to intermediate signs of infestation by *P. onagawaensis*.

It is not clear whether and how often dispersal of larvae results in connectivity for worm species between farms. In addition, many shell-boring polychaetes burrow into the shells of species that are not part of aquaculture, such as periwinkles and slipper snails. As yet, there is little information on whether such “wild” populations may contribute to infestations on nearby shellfish farms. However, in some of the samples that I examined there was clear evidence that *P. websteri* and *P. onagawaensis* have both found suitable habitat within shellfish farms with enough resources to complete their life cycle and release enough offspring to perhaps sustain an infestation. Worms extracted from oysters sampled at Farm 2 supported this idea as eight of the worms were reproductive and contained coelomic eggs while 25 egg cases with multiple eggs in each case were discovered in the burrow of one worm from this farm. These eggs suggest that the polychaetes at this farm are reproducing and if the larvae they produce are adelphophagic
they are most likely to end up recruiting to that farm or a neighboring farm.

Adelphophagic larvae are those that feed upon brood eggs while lecithotrophic larvae are provided yolk for nourishment until they are able to feed freely (Simon & Sato-Okoshi, 2015). The larvae usually do not spend time in the water column like planktotrophic larvae, therefore, they are not often carried by currents to other locations (Simon & Sato-Okoshi, 2015). It is much more likely that these larvae will instead inhabit the burrow they were born in, a burrow near them, or an adjacent oyster to create a new burrow and repeat the life cycle.

Growth rates of host bivalve species can also play a role in the impact of infestation by shell-boring polychaetes. Slower growing host species allow the polychaetes more time to become established and reproductive which, in turn, creates a more severe, lasting infestation (Simon & Sato-Okoshi, 2015). This may also be true for bivalve host species grown near slow growing species since larvae may disperse from the slower growing host species to the faster growing host species (Simon & Sato-Okoshi, 2015). Eastern oysters (*C. virginica*) can grow to a harvestable size within 1-3 years, depending on environmental conditions (Wallace, 2001). Simon and Sato-Okoshi (2015) have suggested that infestations by worm species with adelphophagic and lecithotrophic larvae are less likely when the host culture time is less than 2 years. In contrast, at higher latitudes it can take upwards of 2-3 years for oysters to grow to market size which suggests in such regions infestation is more likely for *C. virginica*. As a result of reduced feeding or no feeding during winter periods, oysters lose organ mass and growth rates are slowed in areas with colder water (Mayrand *et al.* 2017). Therefore, it is likely that oyster growth rates due to cold waters of the Gulf of Maine may require at least two and perhaps
even closer to three years for oysters to reach market size, allowing sufficient time for shell-boring species to create sustained infestations.

Many of the oyster samples in my study contained small burrows, empty burrows, and burrows with degraded worm tissue. For example, the oysters sampled from Farms 9 and 10 had intermediate numbers of small burrows present, but very few were inhabited by worms. Since the oysters were sampled in October and November one explanation for small worm numbers may be seasonal mortality. These results are consistent with those from Zajac (1991) who found that the abundance of polychaete species in the waters around Connecticut began to decrease in September and the trend continued through November. These die offs are thought to be food source or temperature related along with other disturbances.

The presence of eggs and egg cases in the burrows of worms in some of the samples I examined provides another potential type of evidence for species identification. All of the worms that were sampled and for which I completed DNA-based analysis were found to be *P. onagawaensis*. In all cases I observed specimens to be carrying coelomic eggs, as well as the one worm that was brooding egg cases were identified by DNA typing as *P. onagawaensis*. Typically, *P. websteri* brood their larvae in the spring and summer months (Blake, 2012) while *P. onagawaensis* has been noted to brood their eggs anytime from October to June when the seawater is below 15°C (Teramoto et al., 2013). This suggests that the worms were in fact *P. onagawaensis* since *P. websteri* would be less likely to have been brooding eggs according to previous research documenting the seasonal patterns in reproduction for this species. In addition, water temperatures were
below 15°C throughout October (NOAA Gulf of Maine Satellite Data), creating suitable conditions for *P. onagawaensis* to reproduce (Teramoto et al., 2013).

**New Hampshire**

Four farms (Farms 13-16) in Great Bay, New Hampshire sent oysters for analysis. Farms 13-16 also showed a wide range of infestation levels. The oysters from Farm 13 were large in size and had extensive surface fouling by algae, barnacles, jingle shells, limpets, and mussels, as well as mud burrows from colonization of *P. cornuta* and amphipods. We assumed there was interior fouling by both species of shell-boring polychaetes, *P. onagawaensis* and *P. websteri*, due to burrow morphologies. Large, overlapping mud-filled burrows created by *P. websteri* were likely greatly underestimated in number. We also observed small, mud-less, u-shaped burrows typical of *P. onagawaensis* worms. In total, over 100 burrows were detected in oysters from Farm 13 (Fig 5) and twelve live worms were sampled from Farm 13, eight of which were subjected to DNA analysis. Of the eight worms, two were provisionally identified *P. websteri* and six were identified as *P. onagawaensis* based off of the morphologies of the burrows they were sampled from. The DNA-based analysis showed that all eight worms were *P. websteri*.

Farm 14 submitted oysters whose condition was similar to those from farm 13. They were heavily loaded with burrows (> 88 total burrows; Fig. 5) that had the appearance of those from *P. websteri* but also contained burrows morphologically similar to those made by *P. onagawaensis*. Five live worms were sampled from the oysters supplied by Farm 14, three of which I was unable to identify by morphology alone. DNA
analysis indicated that one of the worms removed from a burrow with a shape like that of *P. onagawaensis* was actually *P. websteri*.

Farm 16 also had a large infestation of shell-borers with about 38 burrows counted. However, that is likely to be an underestimate as many burrows overlapped. We were not able to use morphological characteristics for identification; therefore, DNA analysis was necessary for these worms as well. Out of five worms sampled from Farm 16, two were morphologically similar to *P. onagawaensis* and three were similar to *P. websteri*. DNA-based analysis for the two worms thought to be *P. onagawaensis* determined that they were also *P. websteri*. In addition, the oysters from Farm 16 had a high degree of surface fouling including club tunicates, jingle shells, boring sponge, barnacles, and some exterior burrows formed by *P. cornuta* and amphipods.

Farms 13, 14, and 16 were located nearer to the southern end of the Great Bay ecosystem. In contrast, at a more northern site (Farm 15) the oysters were cleaner with less surface fouling from barnacles, jingle shells, and club tunicates. There were no burrows or worms present indicating that there was no infestation of either type of shell-boring polychaete at this farm.

Oysters from three sample sites located on the restoration reefs in Great Bay were also evaluated in order to understand the condition of the oysters at the restoration sites, themselves. The restoration sites that were sampled were near the middle and southern end of Great Bay. All three restoration sites were heavily infested with burrows typical of *P. websteri* as well as many other different fouling organisms. The muddy burrows were stacked on top of each other to create galleries and it was nearly impossible to determine an accurate number of shell-boring species that were present in the valves. At one site
(Squamscott), only sixteen of the thirty oysters provided were inspected due to the fragility of the shells. The shells were heavily loaded with burrows typical of *P. websteri*, however some smaller, cleaner *P. onagawaensis*-like burrows were also present, but in lower numbers.

The other two restoration sites contained oysters with such thick shells that burrows could not be viewed by backlighting. As a result, only five of thirty oysters from these two sites were analyzed by breaking through the shells. Upon inspection with this technique, it was discovered that the valves were loaded with burrows and polychaetes. The heavy fouling and large amount of mud indicated presence of the shell-boring *P. websteri*, however, it was impossible to determine their true identification through morphological means.

As described above, the worm burrows were initially identified to be associated with *P. websteri* or *P. onagawaensis* based on the morphology of the burrows. *Polydora websteri* burrows are known to contain more mud and create dark colored, larger blisters while *P. onagawaensis* generally forms smaller, cleaner, “pimple”-like burrows with a very defined u-shape (Figures 1 and 2). However, it is possible that some of the smaller, simpler burrows that I observed were actually early-stage *P. websteri* burrows that had not yet been packed with mud. This was demonstrated in the worm identifications from New Hampshire where multiple worms from different farms were originally identified *P. onagawaensis* based off of burrow morphology, but were proven to be *P. websteri* after DNA analysis. However, many were also correctly identified as *P. onagawaensis* in the regions in Maine with justification from subsequent DNA-analysis.
CONCLUSIONS

Analysis of worm samples from the aquaculture farms and restoration sites showed a wide range of burrow shapes and worm abundance. Four farms in Maine (Farms 3, 6-8) and one in New Hampshire (Farm 15) showed no indications that any shell-boring polychaete species had inhabited the oysters. Five farms in Maine (Farm 1, 4, 9-11) showed low levels of shell-boring polychaete abundance and many had evidence of burrows with very few worms occupying them. All of the worms sampled from these farms and identified through DNA-based analysis were found to be *P. onagawaensis*. The empty burrows are thought to be the result of seasonal mortality due to changes in food sources and temperatures or other disturbances heading into the winter months, when the study took place. Other factors that may have contributed to the empty burrows include delays in the transport of oysters to UMaine or perhaps culture methodology. Separating these possibilities will require further study.

Farms 2, 12-14, and 16 were all found to have high loads of shell-boring polychaete burrows and worms, with Farm 5 having an intermediate load. Molecular analysis showed that *P. onagawaensis* was present at Farms 2, 5, and 12 and there was a *P. websteri* infestation at Farms 13, 14, and 16. Our results and identifications of *P. onagawaensis* are consistent with a previous study by Siverbrand et al. (2021) who described *P. onagawaensis* at sample sites close to the sample sites in this study. Farm 2 was also found to have *P. websteri*, however, the worms were identified based on their morphology and the morphology of the burrows they were found in. The farms with high loads of worms were occasionally in close proximity to those that had low or no infestation at all. For example, Farm 2 was only about half a mile from Farm 3 and was
also close to Farm 8, neither of which had any signs of shell-boring polychaetes. From this observation I predict that the polychaetes at these sites are mainly adelphophagic and that the worm offspring produced on those farms stay on those farms. Observations of egg cases as well as gravid females within burrows from Farm 2 indicates that this is likely the scenario contributing to high loads of worms at select farms with little transfer of larvae to neighboring farms.
OPPORTUNITIES FOR FURTHER RESEARCH

Further research may include completing further molecular analysis on the worms sampled from the farmed oysters to understand the species infection rates of the farms or continue to sample from farms periodically to understand how the infestations are progressing. It may also be worthwhile to study the causes of the empty burrows found at many of the farms and if these findings may be a result of declining infestations.

Repeating this project would be helpful to the aquaculture farmers in times of need and could play an integral part in continuing the restoration project in Great Bay, NH. It would also be beneficial to study the health of the oysters as well as the general ecology of Great Bay to determine if the restoration efforts have been effective. This may include tracking turbidity and nitrogen levels to understand how the oyster plantings have affected water quality as well as researching species population to determine if the oyster reefs have increased dependent fish populations by replenishing feeding grounds and habitat. Periodically sampling oysters from the restoration sites and evaluating the infestations of shell-boring worm species may also be important in tracking the health of the oysters and determining the effect the worms may have on the success of the restoration project.


AUTHOR’S BIOGRAPHY

Haleigh Wright was born in Middlebury, VT on June 22nd, 1999. She was raised in Ticonderoga, NY where she attended and was a graduate of the class of 2017 from Ticonderoga Senior High School. She pursued a degree in Marine Science with a concentration in Marine Biology at the University of Maine. Haleigh was awarded the Flagship Scholarship throughout her time at the University of Maine, as well as the John H. and Bethel B. Dearborn Scholarship. She played on the club softball team and was the president and captain during her junior and senior years. Haleigh will be attending Louisiana State University’s School of Veterinary Medicine in the fall of 2021. She has received the Dean’s Circle Scholarship, the most prestigious scholarship offered. She plans to focus on small animal medicine before continuing training to become a veterinarian for marine species.