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## Assessing the Pathogenic Cause of Sea Star Wasting Disease in *Asterias Forbesi* Along the East Coast of the United States

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ASSESSING THE PATHOGENIC CAUSE OF SEA STAR WASTING DISEASE IN  
ASTERIAS FORBESI ALONG THE EAST COAST OF THE UNITED STATES

BY

CAITLIN JESSICA DELSESTO

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## ABSTRACT

As keystone predators, sea stars serve to maintain biodiversity and distribution through trophic level interactions in intertidal ecosystems. Sea Star Wasting Disease (SSWD) has caused widespread mass mortality of *Asterias forbesi* in locations along the Northeast Coast of the US in recent years. A similar disease has been described in several sea star species from the West Coast of the US. Recently, a densovirus has been associated with wasting disease in West Coast sea stars and a few limited samples of *A. forbesi*. The goal of this research is to describe the pathogenesis of SSWD in *A. forbesi* and other echinoderms in the Northeast Coast of the US and to determine if the densovirus isolated from West Coast sea stars (SSaDV) is associated with the SSWD of *A. forbesi* on the eastern US coast. Histological examination of *A. forbesi* tissues affected with SSWD showed cuticle loss, edema, and vacuolation of cells in the epidermis but little to no evidence of pathology caused by bacterial or parasitic agents. Inclusion bodies were noted in two of the stars sampled. Challenge experiments by cohabitation and immersion in infected water suggest that the cause of SSWD is viral in nature, since filtration (0.22  $\mu\text{m}$ ) of water from tanks with SSWD does not diminish the transmission and progression of the disease. Death of challenged sea stars occurred 7-10 days after exposure to infected water or sea stars. Of the 48 stars tested by qPCR, 29 (60 %) have tested positive for the SSaDV VP1 gene. These stars represent wild-collected sea stars from all geographical regions (South Carolina to Maine), as well as stars exposed to infected stars or water from affected tanks. However, a clear association SSaDV with SSWD in *A. forbesi* from the East Coast of

the US was not found in this study. Understanding the potential cause of this disease is a first step towards management and prevention of wide spread outbreaks.

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# CHAPTER 1

## INTRODUCTION

Sea stars act as keystone predators, maintaining ecosystem diversity and stable population densities of other marine invertebrates, including bivalve shellfish, through trophic and food web interactions (Dungan et al. 1982). They may compete directly for space and food with other intertidal invertebrates, or may indirectly affect the competition between organisms (Menge et al. 1994). Over the last four decades, there have been periodic reports of mass mortality events in sea star populations along the Pacific coast of the United States, though no responsible agent was identified (Dungan et al. 1982, Scheibling 2010). In the spring of 2012, anecdotal reports from fishers, aquaculturists, and sea star collectors for aquariums and educational institutions started to surface of health issues in aquarium and lab-held sea stars in the Northeast US (mainly in the Mystic and New England Aquariums), as well as significant drops in the numbers of wild populations in areas from Rhode Island, Buzzards Bay and Gulf of Maine (various individuals reported in acknowledgement section, personal communication). By June 2013, stars on both the Atlantic and Pacific coasts of the United States were reported as being affected by what was termed “Sea Star Wasting Disease” (SSWD) ([echinoblog.blogspot.com](http://echinoblog.blogspot.com)). Affected stars were flaccid and mucoid on the aboral surface, followed by dropping of the limbs and eventual disintegration of the central disc and body wall. This was often associated with the appearance of large multifocal lesions on the arms of stars, a sign of ulceration, sometimes followed by penetration through the test into the perivisceral coelom (Smolowitz, personal

communication). Wasting Disease appears to have caused chronic mortality in the sea star, *Asterias forbesi*, in locations from Maine to New Jersey (echinoblog.blogspot.com).

The rapid spread of SSWD, as well as the widespread geographical distribution and species affected, makes it of great concern to individuals and organizations interested in the stewardship of biodiversity and the conservation of ocean resources (Hewson et al. 2014). There have been reports of disease outbreaks in echinoderm species from over the world for decades. From 1983-1984, a mass mortality of the sea urchin *Diadema antillarum* occurred along their entire range in the Gulf of Mexico, leading to a loss of more than 93% of the existing population at each location (Lessios 1988). Sea Star wasting was first observed in the sunflower star, *Heliaster kubiniji* along the Gulf of California in June 1978. Stars exhibited white lesion on the aboral surface, while enlarged and led to fragmentation and death (Dungan et al. 1982). In the summer of 1997, more than ten species including *Asterina* (now *Pateria*) *miniata* and *Pisaster giganteus* were similarly affected at the Channel Islands. Stars exhibited similar signs, including loss of turgor, white lesions on the aboral surface, and finally fragmentation and death (Eckert et al. 1997). In many cases, episodes may be linked to increasing sea surface temperatures, though there is still no clear association (Menge et al. 1994; Scheibling and Lauzon-Guay 2010; Scheibling and Hennigar 1997). These echinoderm epizootics can have significant effects on ecosystem stability. For example, the sea urchin *Diadema antillarum* is an important grazer of coral reefs. When mass-mortalities of *Diadema* occurred in the Caribbean in the 1980's, the community faced an extreme shift in population dynamics. Without the sea

urchins to graze the reefs, microalgae began to overgrow the coral. Over time, dense mats of macroalgae smothered the reefs and completely changed the ecosystem (Lessios 1988). Though several pathogens have been suggested as cause for mortalities in echinoderms, none has been confirmed.

Forbes sea star, *Asterias forbesi*, is an intertidal (<30m deep) asteroid found along the Atlantic Coast of the United States from Maine to the Gulf of Mexico. These stars undergo external fertilization to create a free-swimming bipinnaria free-swimming larval stage, followed three weeks later by metamorphosis into the pentaradially symmetrical adult. Echinoderms have a complex innate immune system, and have been used for immunological research since the late 1900s (Sharlaimova et al. 2014). Located evolutionarily at the base of the Deuterostomes, echinoderms share many immune homologues to vertebrate groups, including Toll-like receptors, interleukins, complement systems, and cell adhesion regulation. Presence of adhesion activity is crucial in the coelomic cavity, because it allows for encapsulation of foreign bodies and wound repair (Pinsino et al. 2007). The first barrier of defense sea stars have against pathogens is the cuticle and epithelium. The rigid cuticle helps prevent bacteria and viruses from entering the body wall, and provides support for the epithelial cells just below the surface. The major humoral component of the echinoderm immune system are coelomocytes, which freely circulate in the coelomic cavity, as well as the water vascular system and connective tissues. These cells are responsible for recognition of foreign antigens, and the initiation of immune responses including phagocytosis and release of antimicrobial enzymes (Beck and Habicht 1986, Fuess et al. 2015).

Recently, a densovirus, named Sea Star Associated Densovirus (SSaDV), has been associated with SSWD in more than 20 species on the Pacific Coast of the United States, and in 9 of 14 samples of *A. forbesi* tested from the Atlantic Coast of the US. Densoviruses are genus of the *Parvoviridae* family, which includes single-stranded, non-enveloped, DNA viruses infecting invertebrates such as insects and crustaceans. The viruses replicate by attaching to host cell receptors, and are internalized into the cell through clathrin-mediated endocytosis, in which the plasma membrane buds inwards forming vesicles around the virus. If the virus can avoid detection by the immune system, it can replicate within the cell nucleus until it destroys the host cell, releasing viral copies into the area of other cells. Viral load and prevalence of SSaDV in Pacific Coast sea stars were reported as higher in moribund than in healthy-looking individuals, and stars with higher viral loads were more likely to show clinical signs of SSWD. Viral load also increased as disease signs progressed, suggesting a potential relationship between SSaDV and SSWD (Hewson et al. 2014). However, this research did not provide direct proof that SSaDV is the causative agent of SSWD. This is mainly due to the lack of invertebrate cell lines allowing for the isolation and culture of SSaDV, which would facilitate fulfilling Koch's Postulates by providing a source of pure virus to challenge healthy stars and reproduce the disease in controlled conditions (Falkow 1988).

Due to the small sample size of Atlantic coast sea stars tested in the Hewson et al. (2014) study, the association between SSWD and SSaDV on the Atlantic Coast of the US is still unclear. Moreover, the epidemiology and pathogenesis of SSWD in *Asterias* spp. has not been well characterized. The overall goal of the research is to



assess the pathogenesis of Sea Star Wasting Disease (SSWD) in affected echinoderm species from the Atlantic Coast of the US, mainly *A. forbesi*. The project has three objectives: 1) Determine if SSWD is an infectious disease and identify potential pathogenic agents; 2) Define and characterize the clinical signs of SSWD and the mode(s) of transmission; and 3) Assess the presence and relationship of SSaDV to clinical signs of SSWD in *A. forbesi*. This research will provide the tools necessary to study the disease in wild and captive populations of sea stars, elucidate potential links to environmental change, and develop potential management tools.

## CHAPTER 2

### METHODOLOGY

#### **Specimen Collection and animal husbandry**

Forbes sea stars (*Asterias forbesi*) were collected along the coast of Rhode Island between August 2013 and April 2015 from intertidal habitats in Narragansett Bay, including the pier at the University of Rhode Island Graduate School of Oceanography (GSO; GPS coordinates 41°26'56.0" N, 71°24'00.4"W) and Beavertail State Park (41°29'32.7", 71°25'11.1"W). Stars were classified as lacking or showing clinical signs based on the appearance of gross signs of Sea Star Wasting Disease (SSWD), including: a) loss of turgor pressure, b) presence of a mucus coat on the aboral surface, c) epidermal lesions of white foci along rays of body, and d) limb autotomy. Stars with clinical signs of disease (lesions) were used to characterize the pathogenesis of SSWD. Stars classified as lacking gross clinical signs (no lesions) were placed in a holding trough outside at the Graduate School of Oceanography, which received ambient unfiltered water from Narragansett Bay. Stars were monitored for 2-3 weeks for clinical signs of SSWD. After this initial acclimation phase, stars free of clinical signs were brought to the Pathology Laboratory and the Blount Aquaculture Research Building (GSO) and placed 3 to a tank, in 50 l glass aquaria containing filtered (down to 0.02  $\mu$ m) and UV-sterile sea water (FSSW). Stars were monitored for an additional two-3 weeks for signs of SSWD. Stars were kept at ambient seawater conditions for Narragansett Bay (20-23°C, 29-33ppt). If no clinical signs were observed, stars were used in experimental trials. One tank (3 stars) served

as a control group throughout trials, and was not exposed to any diseased individuals. Stars were fed every two weeks with snails or mussels collected from the GSO Pier. A complete list of all samples collected can be found in Appendix A.

### **Evaluation of disease range and timeline of epizootics**

A survey was designed to obtain information on location and extent of mortality events in the wild and the water conditions associated with the die-offs (Appendices B, C). The survey was distributed to 5 local dive groups (RI, MA) and aquarists at the New England Aquarium (Boston, MA), Mystic Aquarium (Mystic, CT), and the Maritime Aquarium (Norwalk, CT). Samples of sea stars were received from several sites along the reported range (Table 1) and handled as described in the section above, with the exception of sea stars from South Carolina, which were directly placed in tanks (n=5, n=6, respectively) of filtered artificial seawater (FASW) at ambient conditions (19-23°C, 29-32psu). Photographs, body condition scores, and swab samples were all taken upon arrival. The stars were observed daily for signs of disease onset (Table 2). These signs were documented through photography using an Olympus S2X10, with LG-PS2 illuminator scope and Olympus DP72 camera. Tissue samples were collected from moribund and dead stars and processed as described below for histological examination (all stars) and for microbiological analysis (moribund stars only). Categories of sea star health (turgor pressure, tube feet attachment strength, presence of absence of mucus, bloating or “pinched” look, and/or lesions) were established (Table 2), and each animal was evaluated and scored in each category.

Table 1: Geographic distribution of Asteroid samples collected

Site	# Collected	Sample Type	# Tested by qPCR
Beavertail, RI	17	Swab and Tissue	13
GSO Pier	37	Swab and Tissue	19
Maine State Aquarium	4	Swab and Tissue	3
Charleston, SC	11	Swab and Tissue	10

Table 2: Scoring table used to quantify extent of the gross clinical signs wasting in sea stars.

Score	0	1	2	3
<b>Condition</b>				
<b>Tube Feet attachment</b>	strong attachment	weak one to two rays	weak two to three rays	no attachment
<b>Turgor</b>	normal	Loss of pressure in 1-2 rays	Loss of pressure in 3-4 rays	no pressure, deflated
<b>Mucus</b>	None	Coat on aboral surface	-	-
<b>Bloating</b>	None	“pinched” look	-	-
<b>Spines</b>	normal	Some loss of orientation	spines disheveled	loss of spines
<b>Lesion size</b>	none	0-1cm	1-3 cm	3+ cm

### Sample Collection and Processing

*Sample collection:* For each star (both showing clinical signs and not), two 1.5mL microcentrifuge tubes were filled with 1 mL filtered artificial salt water (FASW, 28 psu), labeled appropriately (swab or tissue), and placed in ice. The animal to be sampled was placed in a sterile disposable Petri dish and rinsed three times with 10 mL FASW to remove surface debris. Photographs were taken as described above to document gross morphology of animal, as well as size, date, water quality from the holding tank, and body condition were recorded. Using a sterile swab, one 1cm<sup>2</sup> area

of rays was swabbed gently and the swab was placed into the corresponding microcentrifuge tube of 1 ml of FASW. If stars showed signs of disease, a swab each was taken from lesions and from an area with no visible lesions. Tissue clippings (2-3 mm<sup>3</sup>) were collected from the epithelium of diseased stars and placed into microcentrifuge tubes containing 1mL FASW and kept in ice. Autotomized limbs and whole bodies were preserved in 10% formalin for fixation for histological examination of tissue. Swabs (after plating for bacteriological isolation, see below) and tissue samples were centrifuged for 10 min at 12,000xg at room temperature. Once the supernatant was decanted, 1 mL of TRIzol fixative was added to each tube and the tube was stored in the -80°C for future analysis.

*Histological examination:* Samples of sea stars were removed from the fixative and rinsed with FASW. Cross-sectional pieces (2-3cm in width) were cut through the ray and included the body wall, coelomic cavity, and tube feet, and placed into histology cassettes. Cassettes were decalcified in a 0.5M EDTA-OH solution (pH=8) for 48-96 hours (Howard et al. 2004). The decalcifying solution was changed every 24 hours. Once decalcified, cassettes containing sea star tissues were rinsed five times for five minutes each with ddH<sub>2</sub>O and placed in 70% ethanol to be processed by Mass Histology Services (Worcester, MA, USA). For each cassette, one 6-micron thick H&E stained section was received, photographed, and analyzed as described in Howard et al. (2004).

*Bacterial culture, DNA isolation, and species identification:* Swab samples were mixed using a Vortex (Service and Wardlaw 1985) before preparing serial 1/10 dilutions. An aliquot (20ul) from each of 4 dilutions was plated onto Seawater Tryptone (SWT, prepared with FASW at 30 psu), and Thiosulfate Citrus Bile Salt agar (TCBS) (Pfeffer and Oliver 2003) plates, incubated at room temperature (20 – 24°C), and monitored daily for bacterial colonies. The purpose of plating the samples was to identify culturable species present in lesions. Bacterial colonies in each of the media plates were classified based on morphology (color, shape, and type of growth) at 24 and 96 h after plating, and abundance of each colony type was recorded. Several colonies from bacteria that were present in the highest quantities in diseased animals, but in lower numbers or lacking in healthy individuals, were selected for storage and identification by sequencing of the 16S rDNA (Gauger and Gomez-Chiarri 2002). Selected colonies of bacteria were lifted using a sterile loop and suspended in 5mL SWT broth and grown overnight for two days at room temperature with shaking. From this culture, 1 mL was placed in a tube, glycerol was added (20% volume) and tubes were stored at -80°C. Another 1 mL was pipetted into a clean 1.5mL microcentrifuge tube and centrifuged at 1,792 RCF for five minutes. Bacterial pellets were washed two times with 500µL FASW, using a Vortex in between to resuspend samples. An aliquot of 539 µL Lysis Buffer was added to each tube containing bacterial pellet. To each tube, 11 µL of Proteinase K was added, and the tubes were incubated at 55°C until cells were lysed (about an hour). To bring the final salt concentration to 2M, 350 µL of 5M NaCl was added to each tube and mixed using the Vortex for fifteen seconds, then centrifuged at max speed for 30 min at room temperature. The aqueous portion

was then transferred into two clean 2 mL centrifuge tubes and 900  $\mu$ L 100% ethanol added before mixing well and storing in the  $-20^{\circ}\text{C}$  freezer overnight. To pellet the DNA, tubes were centrifuged at max speed for 30 minutes at room temperature. The ethanol was decanted, and the DNA pellet washed twice with 70% ethanol. The remaining liquid was decanted and the pellet allowed to air dry until all ethanol had evaporated. Pellets were suspended in 30 $\mu$ L ddH<sub>2</sub>O and stored in the  $-20^{\circ}\text{C}$  freezer until use. Bacterial genomic DNA was amplified using universal primers for the 16S rDNA (FWD: 5' - ACG AGC TGA CGA CAG CCA TG -3', REV: 5' CAG CAG CCG CGG TAA TAC -3', ~500bp) and the amplified product was sequenced at the Rhode Island Genomics and Sequencing Center. Sequences were compared to sequences in Ribosomal DNA Database (RDP Release 11) and the most significant (closest sequence identity) matches were used to determine species identification.

*DNA Isolation for quantitative real time PCR:* Sea star tissue and swab samples were removed from the  $-80^{\circ}\text{C}$  freezer and placed in an ice bath to thaw. Samples were then incubated at room temperature for 5 min to permit complete dissociation of the nucleoprotein complex by the TRIzol reagent. Tissue samples were homogenized in 1mL TRIzol reagent using sterile RNA free homogenizer. Chloroform (0.2  $\mu$ L) was then added to each sample, and the sample tube was shaken vigorously for 15 seconds by hand. After 2-3 minutes of incubation at room temperature, samples were centrifuged for 15 min at 12,000xg at  $4^{\circ}\text{C}$  to separate out the RNA, DNA, and protein phases. The aqueous layer containing RNA was decanted using filter tips (pipettes) and placed into a new, sterile microcentrifuge tube and stored in the  $-80^{\circ}\text{C}$  freezer.

After removing any remaining aqueous phase overlying the interphase, 0.3 mL 100% ethanol was added to the tube and inverted several times to mix. Samples were incubated for 2-3 min at room temperature and centrifuged at 448 RCF for 5 minutes at 4°C to pelletize the DNA. The phenol-ethanol supernatant was collected and placed in a new microcentrifuge tube and stored at -70°C for protein isolation. The DNA pellet was washed with 1 mL sodium citrate/ethanol solution (0.1M sodium citrate in 10% Ethanol, pH= 8.5) and incubated at room temperature for 30 minutes, mixing occasionally by inversion. The samples were centrifuged at 448 RCF for 5 min at 4°C, the supernatant was removed, and 1.5 mL of 75% ethanol were added to the supernatant and incubated at room temperature for 10-20 min. After centrifugation, the supernatant was discarded and the DNA pellet was left in the tube to air dry for 5-10 min. DNA from samples was then resuspended in 20 µL of 8mM NaOH, per manufacturer's protocol, and centrifuged for at 12,000 x g for 10 minutes at 4°C (Life Technologies). The supernatant containing the DNA was then transferred to a new microcentrifuge tube and stored at 20°C for analysis. Samples were moved to -80°C for long-term storage. To determine the concentration of DNA, the Thermo Scientific Nanodrop ND8000 version 2.2.1 system was used. Wells were blanked first with 5 µL of 8mM NaOH, and 2.2 µL of isolated DNA as used to determine concentration of DNA.

### **Bacterial challenge experiments**

One of the bacterial isolates identified in the bacterial sampling as a potential pathogen (based on abundance and predominance in disease sea stars and species



identity) was used in challenge experiments to try to fulfill Koch's postulates.

Postulates to be fulfilled include: 1) The candidate pathogen (bacterial isolate) must be abundant in samples taken from sick stars, and not from samples taken from healthy ones; 2) Exposure of sea stars to the bacterial isolate must lead to SSWD in previously healthy stars; and 3) The candidate pathogen must be reisolated from the experimentally exposed star showing signs of disease. If all of these conditions are met, then the candidate pathogen can be considered a causative agent (Falkow 1988).

Healthy-looking sea stars (5 - 200 g) were housed 2 to a tank in 10 tanks containing 19 L of filtered seawater (FSSW) from Narragansett Bay for 7-10 days prior to experimentation at ambient temperatures and salinity (12-25 °C, 28 – 32 psu).

Treatments (2 tanks per treatment) included: 1) Control (no pathogen exposure); 2) Animals immersed in seawater with  $10^6$  CFU/mL of bacteria; 3) Animals immersed in seawater with  $10^6$  CFU/mL of bacteria after cuticle abrasion (an area of  $1 \text{ mm}^2$  was eroded using sandpaper to induce cuticle loss to facilitate infection by breaking down the cuticle defenses; (Quinn et al. 2012); 4) Animals injected with 0.1 mL of a  $10^5$  CFU/mL solution of bacteria in seawater through the dorsal epithelium of one ray into the coelomic cavity to allow bacteria to immediately enter the coelomic fluid of the body; 5) Animals injected with 0.1mL FASW water (control for the effect of the injection). Stars were monitored twice daily for ten days, and signs of morbidity or mortality recorded.

## **Cohabitation challenge experiments**

The purpose of the cohabitation trials was to assess a timeline of disease progression, and to examine modes of transmission. Experiments predominantly assessed transmission between diseased and healthy *A. forbesi*. One trial involved sick echinoderms collected from the Maine State Aquarium. For all these trials, time to morbidity and mortality were recorded, as well as changes in behavior and physical appearance of stars. Swabs of lesioned areas were collected, as well as tissue clippings (2-3mm) for quantitative real time PCR analysis.

### *Cohabitation I*

The first trial involved stars that showed clinical signs while held in a trough at the Graduate School of Oceanography Aquarium Building (termed “*Source*”). Four moribund stars were placed into each of 4 tanks containing 38 L of filtered artificial seawater (FASW). Three *A. forbesi* that had previously passed both stages of acclimation (see animal husbandry section above) and did not develop gross signs of the disease (termed “*Challenged*”) were placed into each of the 4 tanks and allowed direct contact with moribund stars. Stars were then monitored for signs of wasting for 10 days. Once the *Source* stars died, their bodies were removed from the tank and a 20% water change was performed. Records of water quality, temperature, and body condition of cohabitation stars were taken daily for 10 days or until all *Challenged* stars were deceased.

### *Cohabitation II*

The second cohabitation trial involved moribund animals received from the Maine State Aquarium (one sea star *Asterias rubens*, one green sea urchin *Stronglyocentrotus droebachiensis*, and 2 sea cucumbers *Cucumaria frondosa*). Animals were received on April 1, 2014 and placed into each of 4 tanks containing 38 L of filtered sterilized sea water (FSSW) from Narragansett Bay (15-20°C, 29-31 psu). Plastic mesh dividers were placed down the middle of the tanks to prevent direct contact of *Source* animals with *Challenge* animals. Three stars that had passed the acclimation phase and were negative for gross SSWD lesions were placed into each of the 4 tanks. Stars were then monitored once daily for 3 weeks, or until all cohabitation stars were deceased.

### *Cohabitation III: Infected Water Accumulation*

The third cohabitation trial was named “Infected Water Accumulation”. The goal of this experiment was to generate infected water to use in filtration challenge experiments, while also collecting samples for analysis. This trial lasted for 40 days, with consistent turnover of diseased stars. Water was taken from a tank in which SSWD had caused mortality and placed into a clean, sterilized tank with two *A. forbesi*. Stars were sampled and monitored daily for signs of disease. When stars began to show signs, samples were taken for microbial analysis, and a new star was added to the tank. Each time a star was found moribund, the star was removed and a 20% water change was performed. Ammonia levels were recorded regularly to ensure proper water quality. A total of 15 stars were exposed in this way.

## **Filtration challenge experiments**

The purpose of the filtration experiments was to separate infected water in different size fractions, in order to determine the size of the pathogenic agent. Filtration through a 0.22-micron filter should remove any bacteria or larger particles. The process of UV sterilization should break down any microbes that may have passed through the filter, and thus serves as a negative control.

### *Filtration I*

Water from two tanks in which stars had experienced signs of SSWD was collected in 6-500mL bottles (named “*Infected water*”) immediately after stars started to show signs of disease. These bottles were frozen and stored at -80°C until challenge trials began. The rest of the water from the 2 infected tanks was separated into two treatment tanks: A) Whole, fresh untreated water from affected tank (infected water); B) 0.22µm filtered fresh infected water. Two *A. forbesi* that had gone through acclimation and showed no signs of disease were placed into each tank (n=2) and monitored daily. Trial continued for 10 days, or until all stars were dead.

### *Filtration II*

In order to determine if the pathogenesis of SSWD in *A. forbesi* had a viral component, groups of stars were challenged with previously frozen water collected from tanks containing diseased stars that had been filtered to eliminate particles > 0.22 microns (Omran and Eissa 2006, Ottesen 2011). Water was stored in 500 mL bottles in the -80°C freezer. The bottles were removed from the freezer and placed in an ice

bath to thaw (20-30 minutes) before being used in the experiment. As a negative control, a portion of this filtered water was then UV treated to inactivate potential viral particles remaining in the filtered water. A total of 1 L of filtered infected water was split into two 500 mL beakers (depth=15.2 centimeters) and placed inside a hood with UV light (TheSterilGARDHood, VBM400, the Baker Company, Inc.) for 4-5 hours. Twenty stars without clinical signs were allowed to acclimate for 2-3 weeks while being monitored for signs, before 3 stars were placed into each of 8 tanks containing 38 L of filtered artificial sea water (FASW) in a closed circulation system at ambient conditions (16 – 22°C, 28 – 32 psu). Stars were immersed in antibiotics (Enrofloxacin 2.5 mg/kg) for one hour prior to treatment. Infected water was filtered (down to 1 µm) and UV treated (EU25-U, Pentair-Emperor Aquatics) after each pass through the system (Figure 1). Four different treatments were established: 1) control, FASW, 2) 0.22-micron filtered infected water, 3) 0.22-micron filtered and UV treated infected water, and 4) whole, untreated infected water. Experimental stars were monitored for an additional 3 weeks for signs of SSWD. Swab samples were collected for processing before stars entered treatment, when they started to show clinical signs, and at death.

### *Filtration III*

A third trial was run in order to replicate results of *Filtration II*, but with some adjustments. Water for this trial was obtained from infected tanks and used immediately for exposure (fresh infected water). Stars in this trial did not receive any antibiotic treatment prior to exposure. The trial included the same treatment groups as *Filtration II*. Stars were monitored for 3 weeks for signs of SSWD. Samples were

collected for processing before stars entered treatment, when they started to show clinical signs, and at death.

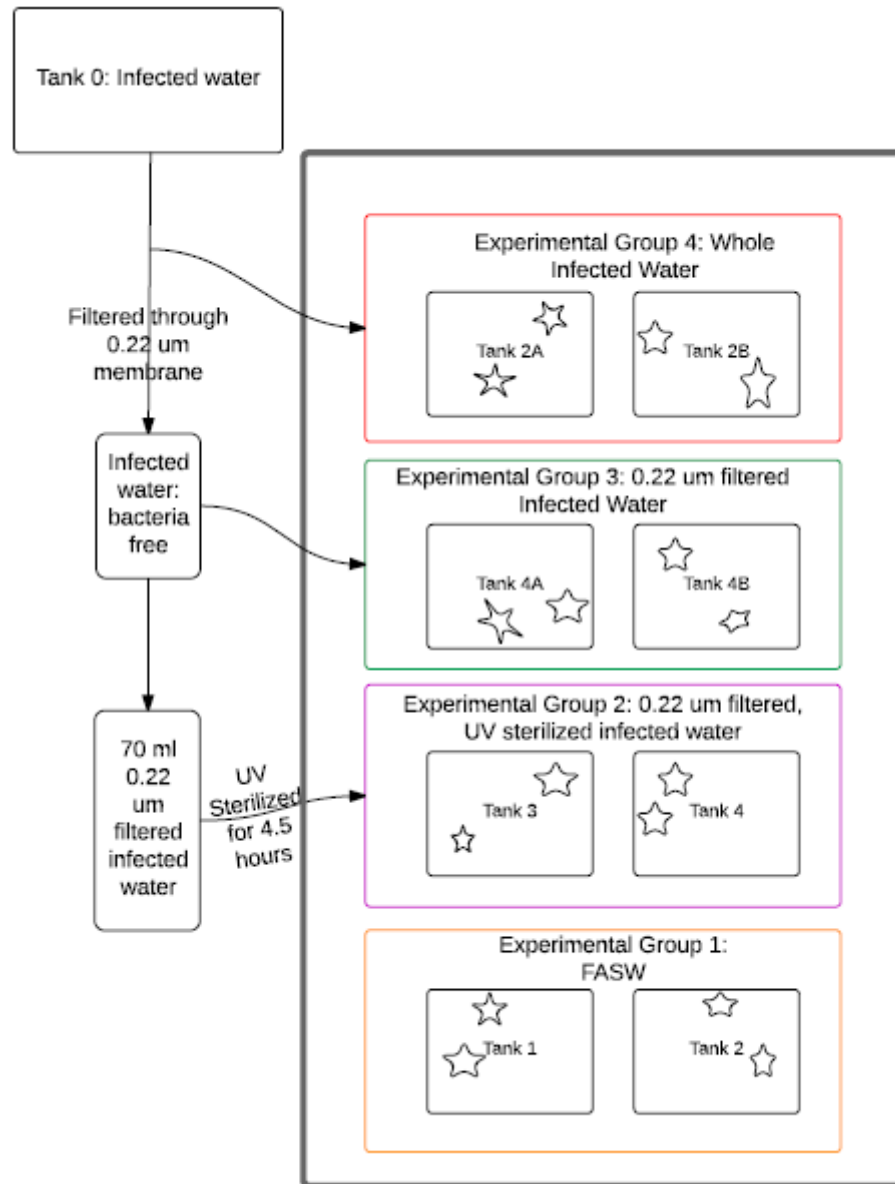


Figure 1: Experimental Tank Design for Filtration Protocol showing tank set-up, source of water, and treatment.

### **Amplification, cloning and sequencing of SSaDV DNA from *A. forbesi***

Polymerase Chain Reactions using transcript specific primers designed on the sequence of VP1 (accession no. PRJNA253121; FWD: 5'-ACGAAGATCCTGTGGTGAGTT-3'; REV: 5'-CATCGGTGTACAATATCCTGCTA-3') and VP 4 (FWD: 5'-GGAATCTTGCTGATGAAAC AGC-3'; REV: 5'-GAGCTGCTGATTTTGTTCAGG-3'), were carried out on isolated DNA from samples of *A. forbesi* with and without clinical signs of SSWD in order to assess the presence of SSaDV (VP1: VP4). A Platinum Taq Master Mix was prepared using 5µL 10x PCR buffer, 3µL 50µM magnesium chloride, 12µL 4x Q-solution, 1µL dNTPs, and 15.8µL Nuclease free H<sub>2</sub>O (Qiagen). Samples were tested in duplicate. For each sample, a volume of 37.3µL of Mastermix was added to each sample tube containing 2.5µL of DNA template, along with 5µL of the appropriate forward primer, and 5µL of the corresponding reverse primer. To this, 0.2µL Platinum Taq enzyme was added and the samples were mixed up and down with a pipette lightly to homogenize solutions. Samples were run through a Mastercycler<sup>®</sup> Nexus X2 (Eppendorf). The conditions of the PCR were 94 °C for 10 minutes, 94°C for 30 seconds, 50°C for 30 seconds, 72°C for 1 hour 30 minutes, 72°C for 5 minutes and kept at 4°C until removal. Amplicons were run on a 1% agarose gel in TAE buffer (40mM Tris, pH=7.6, 20mM acetic acid, 1mM EDTA) for 30 min at 100 Volts. The gel was stained in ethidium bromide for 15-20 min, then destained in ddH<sub>2</sub>O for 10 min, viewed through a UV transilluminator, and photographed using the BioRad Quantity 1 System. Bands corresponding to the predicted amplicon size for VP1 and

VP4 (285 and 942 bp, respectively) were cut using a sterile razor blade, and placed into individual 50 $\mu$ L microcentrifuge tubes to be cloned, sequenced, and compared to known sequences to determine identity to SSaDV VP1 and VP4 sequences.

Amplicon DNA was purified from the gel using the pGEM-T Easy Vector System 1 method (Promega) following the manufacturer's protocol. For the ligation of the purified DNA into a plasmid, 8 $\mu$ L of template was used, in addition to 1 $\mu$ L pGEM-T Easy Vector, 1 $\mu$ L 2x Rapid Ligation Buffer, and 1 $\mu$ L T4 DNA Ligase (Promega). The sample then incubated at room temperature for two hours. Ligation products were transformed into *E. coli* cells (Qiagen) and bacteria containing the plasmid/insert were selected using the Bacterial Cell Transformation System (Qiagen) following the manufacturer's protocol. Plasmid DNA was isolated using a QiaPrep Spin MiniPrep kit and DNA concentration ( $\mu$ g/ $\mu$ L) was quantified using the Thermo Scientific Nanodrop ND8000 version 2.2.1 system. Samples were prepared for sequencing following standard procedure for the RI Genomics and Sequencing Center at the University of Rhode Island. Plasmid DNA containing the VP1 insert was used to standardize target viral DNA results from quantitative real time PCR. Number of DNA copies per sample was calculated using the URI Genomics and Sequencing Center copy number calculator (<http://cels.uri.edu/gsc/cndna.html>).

### **Quantification of SSaDV in *A. forbesi* using quantitative real time PCR**

A Taq based assay for quantitative real time PCR for detection of the VP1 protein of SSaDV was used to quantify viral DNA following an adaptation of the methods of Hewson et al. (2014). Primers and probe for quantitative real time PCR



were developed by running a ClustalW alignment (SDSC Biology WorkBench) of 3 sequences obtained from *A. forbesi* samples using the methods described above and the sequence provided by the Hewson lab for VP1, and inputting the consensus sequence to the Integrated DNA Technologies Primer quest tool (FWD: 5'-GAC GTG CAA GAA GCT GAT AGA-3'; REV: 5'- TCATCGGTGTACAATATCCTGC -3'; PRB: 5'- GTC CAA TAT AAC CAG CAA TAG AAT -3'). Quantitative PCR was run in 20µL reactions containing 1X Probes Master (Roche), 200nM of each primer (4µL), 250 nM of probe (5µL), and 1µL of isolated DNA per single reaction. A Roche LightCycler480 Real-time PCR Instrument was used to perform thermal cycling. The program consisted of a 5 min denaturing step at 95°C, followed by 60 cycles of denaturing and annealing (95°C for 30 sec, 55.5°C for 30 sec, respectively). Dilutions (ten fold over six orders of magnitude) of a plasmid containing the VP1 target region were used as a standard to estimate VP1 concentration. A positive control (sample containing SSaDV DNA) was obtained from the lab of Ian Hewson (Cornell University).

## CHAPTER 3

### RESULTS

#### **Timeline and range of the SSWD epizootic in the Atlantic Coast of US**

An approximate timeline was established for the SSWD Atlantic Coast outbreak based on our direct observations and reports from the surveys (Figure 2). In March 2012, 10 *A. forbesi* were brought into a holding tank at the GSO Aquarium Building from the GSO Pier for an unrelated experiment. Within 5 days, all stars showed signs of wasting, including loss of turgor pressure, curled limbs, and lesions that lead to ulceration of internal tissue and death (not shown). All stars had perished within a week of placing them in the tank. During the summer and fall of 2012, similar episodes were reported by survey responders in aquaria held stars at the New England Aquarium, Boston, MA and the Mystic Aquarium, Mystic, CT. Reported cases at the New England Aquarium were limited to *A. forbesi* and *A. rubens* collected from Cape Cod and brought into holding tanks. At the Mystic Aquarium, both *A. forbesi* and the sunflower star, *Pycnopodia helianthoides*, showed signs of wasting. Samples from Mystic were sent to Cornell University (Hewson lab) for analysis (Alison Tuttle, personal communication). In March 2014, echinoderms collected from the wild and placed in the touch tank at the Maine State Aquarium started to show signs of lethargy, loss of turgor pressure, and lesion development shortly after introduction to the tanks. These echinoderms had experienced temperature stress during transport to the aquarium (Aimee Ayden-Rodrigues, personal communication). These clinical signs were also seen in echinoderms cohabitating with the diseased

specimens introduced in the tank. Specimens of cohabitating echinoderms showing signs of disease were sent to URI for analysis (Table 1). Another public aquariums reporting signs of disease in Atlantic and Pacific coast stars in display/touch tanks include the National Aquarium (2014, MD).

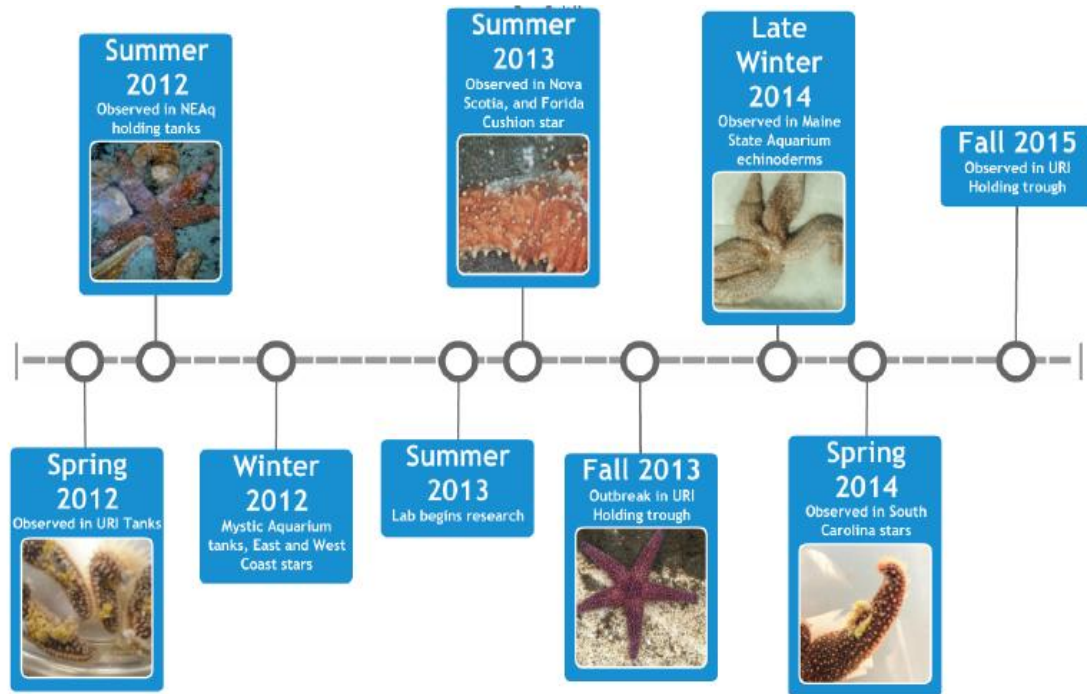


Figure 2: Timeline of Atlantic Coast SSWD reported episodes created from observations and collected citizen/aquarium reports. URI: University of Rhode Island. NEAq: New England Aquarium.

In 2014 - 2015, we received additional anecdotal reports of lesions in sea stars from the wild from Nova Scotia to Florida. The geographic extent of SSWD has been summarized in Figure 3. In the Northeast US and Canada, there have been reports of diseased *A. forbesi* and *A. rubens* in Marblehead, MA (B. Lebowitz, 2013, personal communication), Stonington, Northport, and Lincolnville, ME (R. Sprague, 2014 and G. McDonald, 2013, personal communication) and Dalhousie University and St.

Margaret’s Bay, Nova Scotia (R. Scheibling, 2015, personal communication). As for Rhode Island, mortalities have been seen at the Graduate School of Oceanography (URI Dive Group, 2014, personal communication), Fort Adams State Park (Newport, RI), Great Salt Pond (Narragansett, RI), and King’s Beach (Newport, RI; Kalipso Dive Group 2013-2014, personal communication). Wasting was also observed along the coast of Charleston (S. Boylan, 2015, personal communication) and Hilton Head, SC (K. Mahoney, 2014, personal communication). Morbidity (loss of turgor, lesions, disintegration) was reports in cushion stars, *Asteroidea oreaster reticulates*, along the coast of Florida (Lureen B., personal communication). A full list of reports can be found in Appendix D.



Figure 3: Reported Geographical range of disease episodes in sea stars along (A) New England coastal waters and (B) Atlantic Coast of United States from July 2013-March 2015.

## **Clinical Signs and Gross Morphology in wild and aquarium-collected echinoderms**

Two different pathologies were observed in *A. forbesi* collected from the Atlantic Coast of the US and those stars kept in our holding systems: an acute form (similar to that reported in Pacific coast epizootics; Hewson et al. 2014), leading to death within one week of the start of clinical signs, and one that was more chronic and slow progressing. Signs of the acute form included loss of body turgor, a bloated or puffy appearance, and/or the appearance of white lesions along the arms (Figure 4). Loss of turgor pressure was defined as a deflated appearance and lack of rigidity. Bloating was defined by a “pinching” of the body wall along or at the base of the rays. A mucus coat caused the spines to appear smooth and glossy. Stars from the wild, as well as those that were experimentally exposed to diseased stars, started to show signs and were dead within 7-10 days. The chronic form shows a much slower progression. Stars may exhibit lethargy and the development of small pinpoint (<3mm) lesions, but these signs may persist for weeks to months before any mortality is noted, if at all. A limited number of animal obtained (n=5) exhibited this form. These stars survived with minor lesions for  $115 \pm 74$  days.

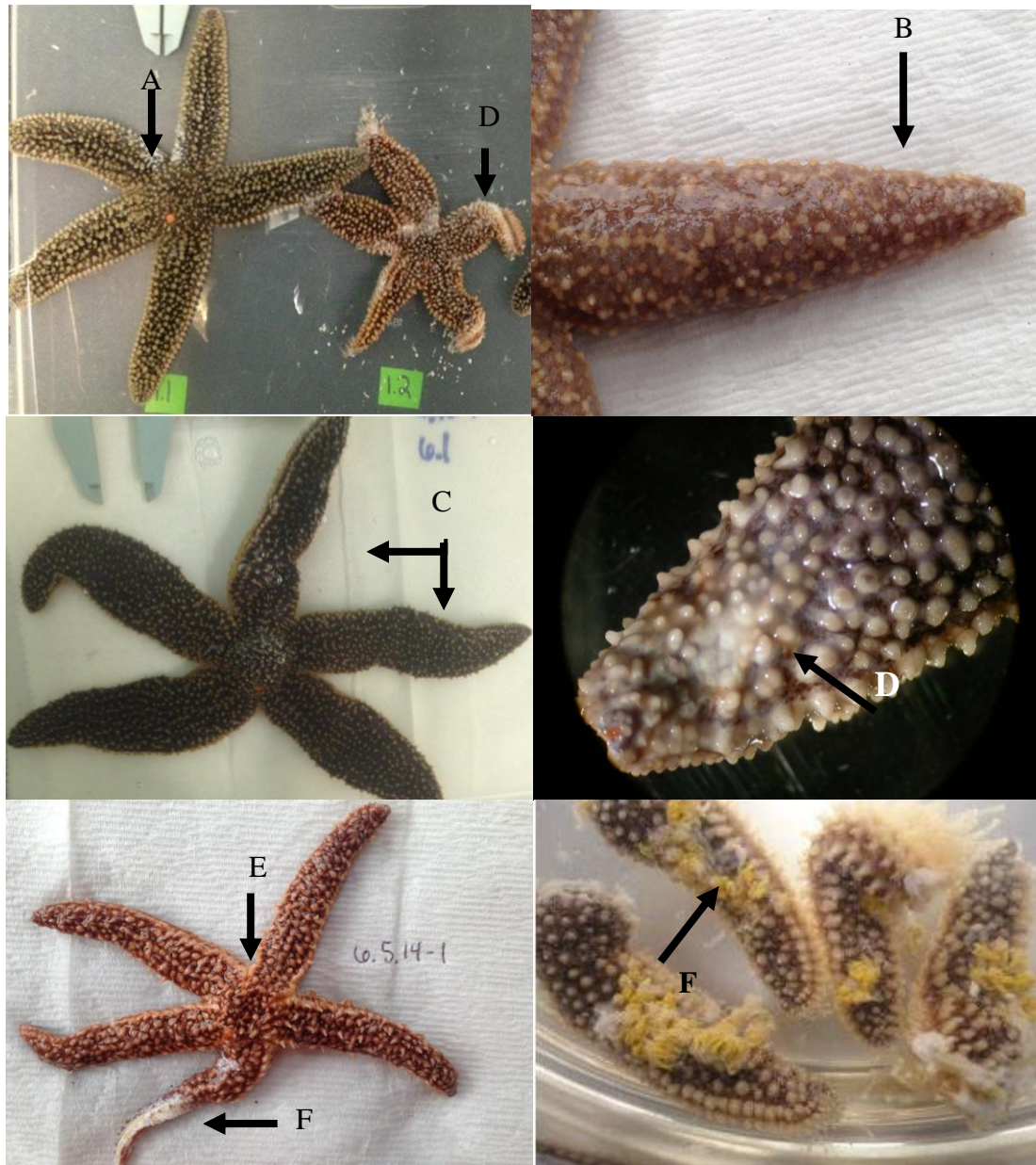


Figure 4: Photos depicting body gross signs of disease including: (A) limb curling, (B) mucus coat, (C) bloating, (D) lesion presence, (E) loss of spine orientation, (F) ulceration of tissue.

### **Histological Examination of wild and aquarium-collected *A. forbesi***

All stars examined for histology (n=22, Appendix A) showed gross morphological signs of disease, but with different levels of severity. Stars with lesions that could have been due to water quality issues in the tanks (lesions on tube feet,



accumulation of hemocytes and signs of tissue necrosis with bacterial proliferation in tube feet; not shown) were excluded from examination. Stars with gross signs of SSWD exhibited severe necrosis of epidermal and connective tissue with ulceration of the surface epithelium (Figure 5). Cuticle loss allowed for seawater to flow into epithelial tissues, causing disruption of normal cell structure and function. Edema (accumulation of excess coelomic fluid beneath the epidermis, leading to hemocytic infiltration) were common, accompanied by vacuolation and delamination of basal tissue in the epidermis. The presence of small numbers of ciliates and bacteria were noted in the dermis of some samples, but were not been consistently tied to lesion presence. Inclusion bodies (a potential sign of viral infection) in columnar epithelial cells were noted in some samples (n=2), but again were not seen consistently in diseased tissues.

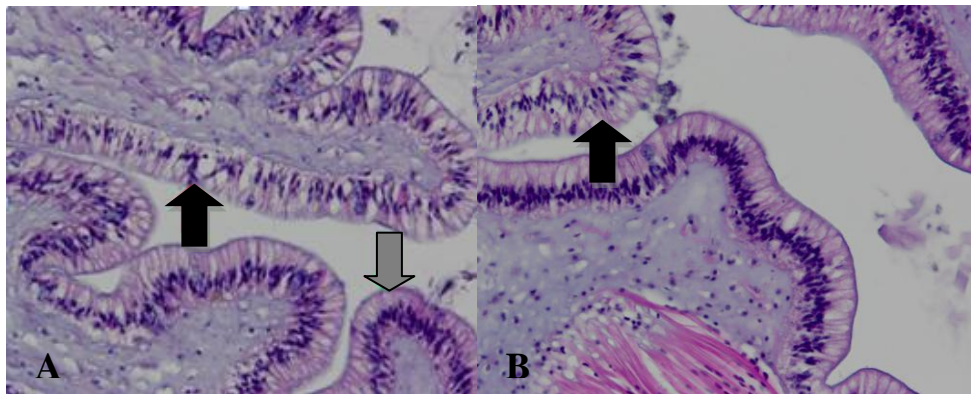


Figure 5: A) Gray arrow indicates area of normal epithelial cells; black arrow shows area of edema and vacuolation. B) Arrow indicated area of cuticle loss, edema, and breakdown of normal cellular structure.

### **Bacterial challenge experiments**

#### *Isolation and characterization of bacterial from SSWD *A. forbesi**

On average, stars that did not show lesions of SSWD had a lower amount of colony forming units (CFU) per mL of samples collected through swabbing, though it

was not statistically significant ( $p > 0.05$ ). The amount of CFU/mL was similar in areas with and without lesions in diseased stars (Figure 6), suggesting that the bacteria is not contained to visible lesions in infected individuals.

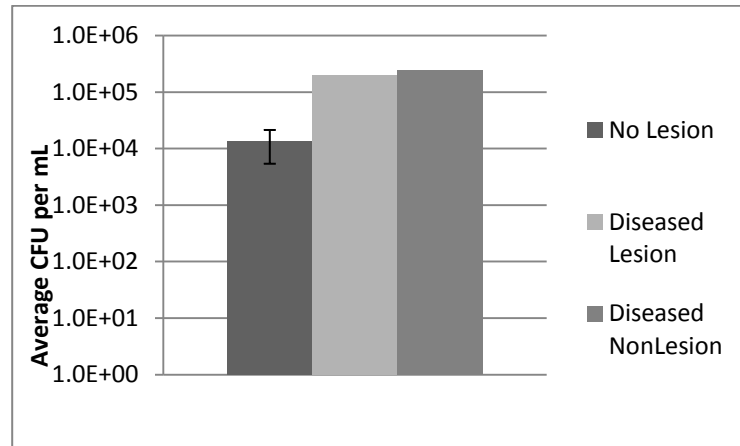


Figure 6: Culturable bacteria (average CFU/mL  $\pm$  SEM) from swabs obtained from a) stars with no visible lesions; b) a lesioned area of diseased stars; c) non-lesioned area of diseased star

The most abundant bacterial morphotype in stars with lesions and present in very low levels in stars without lesions (Figure 7) was collected from the plates and identified through sequencing of the 16S rDNA. These colonies were raised, and circular with an off-white/light yellow coloration. Margins of colonies were entire to undulating in shape. Sequences from this bacterial isolate showed the highest levels of sequence identity to sequences for a *Roseobacter* sp. (Accession no. J530587.1, 100% identity). The *Roseobacter* sp. was selected for the bacterial challenge experiment.

Stars (*A. forbesi*) exposed by immersion to the *Roseobacter* sp. isolated from lesions of SSWD stars exhibited lethargy, weak tube feet attachment to substrate, and slow righting response within 9 days of being exposed to the bacteria, while control stars not exposed to the bacteria did not show any signs of morbidity or mortality



(Figure 8). Mortality was also seen in 3 sea stars (25% cumulative percent mortality) injected with FASW (vehicle for bacterial injection), which may have been due to stress caused by handling or by at least one of these control stars being diseased prior to challenge. None of the animals used in this experiment exhibited the loss of turgor pressure, limb curling, or lesion development characteristic of SSWD. Microbiological examination procedures were performed when clinical signs started to develop, but were not able to reisolate the *Roseobacter* sp. from affected stars. These results indicate that this bacterium is not the causative agent of SSWD in sea stars, based on differences in lesions and time to morbidity and mortality.

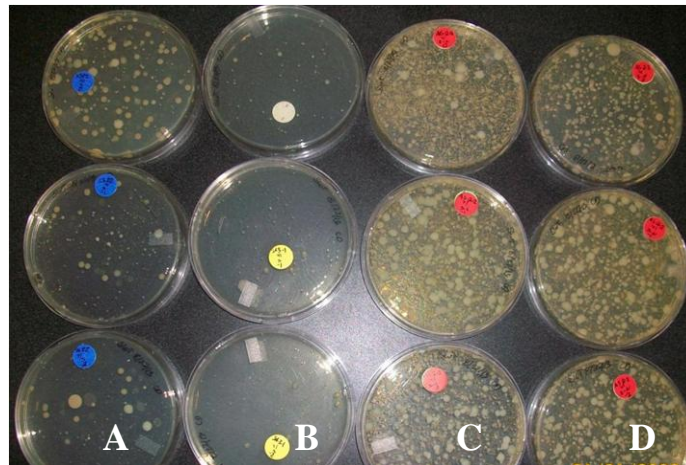


Figure 7: Plates showing bacteria cultured from swabs of aboral tissues collected from (A and B) sea stars without clinical signs of SSWD, (C) lesions from stars with signs of SSWD, and (D) areas with no visible lesions in diseased stars.

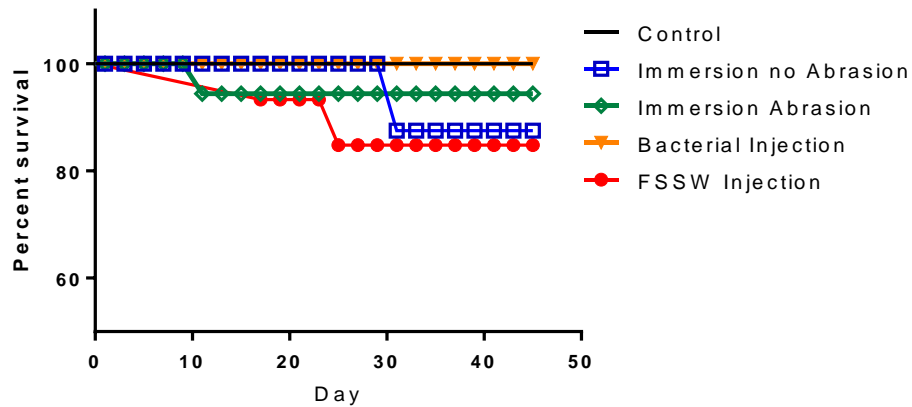


Figure 8: Effect of challenge with a *Roseobacter* sp. isolated from stars with signs of SSWD on the survival healthy-looking sea stars. Healthy-looking stars were exposed to four treatments (n = 6 per treatment): 1) Control; 2) Animals immersed seawater with  $10^6$  CFU/mL of bacteria for one hour with no other manipulation; 3) Animals immersed in seawater with  $10^6$  CFU/mL of bacteria after cuticle abrasion 4) Animals injected with 0.1mL of a  $10^5$  CFU/mL solution of bacteria in seawater through the dorsal epithelium of one ray into the coelomic cavity; 5) Animals injected with 0.1mL FASW water. Mortality was only observed in Immersion no Abrasion, FASW injection, and Immersion Abrasion.

### Cohabitation challenge experiments

Preliminary challenge experiments in which *Pateria miniata* affected by SSWD cohabitating with apparently healthy *A. forbesi* showed that transmission of the syndrome is independent of direct contact between hosts, and that exposure to water from a diseased animal's tank is enough to cause mortality in an otherwise healthy individual. Transmission also occurred between local species and a Pacific Coast species (*P. miniata*) in laboratory cohabitation trials, with progression to death occurring within 5 days of the start of the cohabitation (Wessel, personal communication). The first signs of infection included loss of turgor pressure, and an increase of mucus coat on the aboral surface. White lesions resulting from ulceration through the epidermis into the underlying calcified white plate eventually led to

ulceration of the gut and internal tissue. Transmission of the disease between *A. forbesi* collected from Narragansett Bay, RI, and a naïve Pacific Coast star in cohabitation experiments indicated that the pathogen is not species-specific.

### *Cohabitation I*

Within 3 days of placing a moribund *A. forbesi* into tanks (n = 4) each containing 3 sea stars without clinical signs, all *Source* stars with SSWD had died. Morbidity in cohabitating stars were seen 3 days post exposure, with 77.7% mortality occurring by day 5 post exposure. No mortality was observed in control stars (Figure 9). Clinical signs of wasting, including loss of turgor, lesion formation, and limb dissociation, were observed in cohabitation stars.

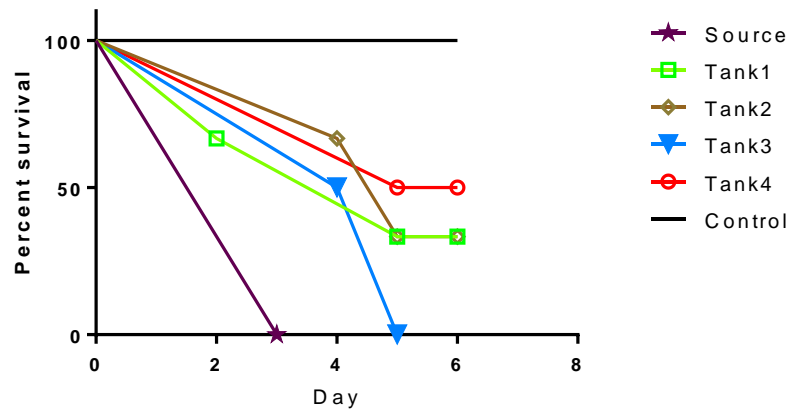


Figure 9: Effect of cohabitation of stars with clinical signs of SSWD on the survival of healthy-looking stars (Cohabitation I). *Source* diseased *A. forbesi* stars (n=4, purple line with star symbol) were placed into each of 4 tanks with *A. forbesi* (n=3 x 4; blue, green red, and brown lines). Control tanks n=3. Mortality was seen in all cohabitation tanks, but not in control tanks (black line).

## *Cohabitation II*

A sea star, sea urchin, and two sea cucumbers (*Asterias rubens*, *Stronglyocentrotus droebachiensis*, *Cucumaria frondosa*, respectively) showing signs of disease collected from the Maine State Aquarium (MSA) touch tank and transported to URI were introduced into a tank containing 3 acclimated *A. forbesi* not showing clinical signs. The sea star from the MSA showed signs as soon as it arrived, and was dead within 4 days of placement in the tank. By day ten, 100% mortality of *Challenged* stars was observed. The 3 cohabitation stars showed loss of turgor and limb curling, but no visible lesions on the skin. Histological analysis of one of these stars showed edema, inflammation, and vacuolation on one of the rays, signs consistent with SSWD (not shown). The green sea urchin from MSA began to lose spines 2-3 days after arrival to the lab, and within 6 days had died. Though none of the stars cohabitating with the sea urchin exhibited some of the lesions consistent with SSWD (ulcerations, limb curling), they did lose turgor pressure and developed a mucus coat, and had all died by 2 weeks post exposure (Figure 10). Similarity in clinical signs and time to morbidity and mortality in cohabitating stars indicate that the sea star and the sea urchin from MSA may have been suffering from SSWD.

Upon arrival from the MSA, one of the sea cucumbers (Cucumber 1) had a small, pinpoint (<1cm) lesion on the lateral surface. By the next day, the lesion measured over 5cm in length. The lesion continued to increase in size, by three weeks the lesion had ulcerated, and the internal tissue had been eviscerated. No change in physical condition was noted in the sea stars cohabitating with this sea cucumber (no loss of turgor, lesion formation), but 2 of the 3 were found dead 40 days post

exposure. The second sea cucumber (Cucumber2) never expressed signs of distress or disease, and neither did any of the stars in cohabitation. Cucumber 2 was found dead in tank at day 45. Differences in clinical signs in sea cucumbers from MSA, as well as differences in time to morbidity and mortality and the inability to transfer the disease to cohabitating sea stars suggest that the mortality seen in sea cucumbers at the Maine Aquarium was not due to SSWD.

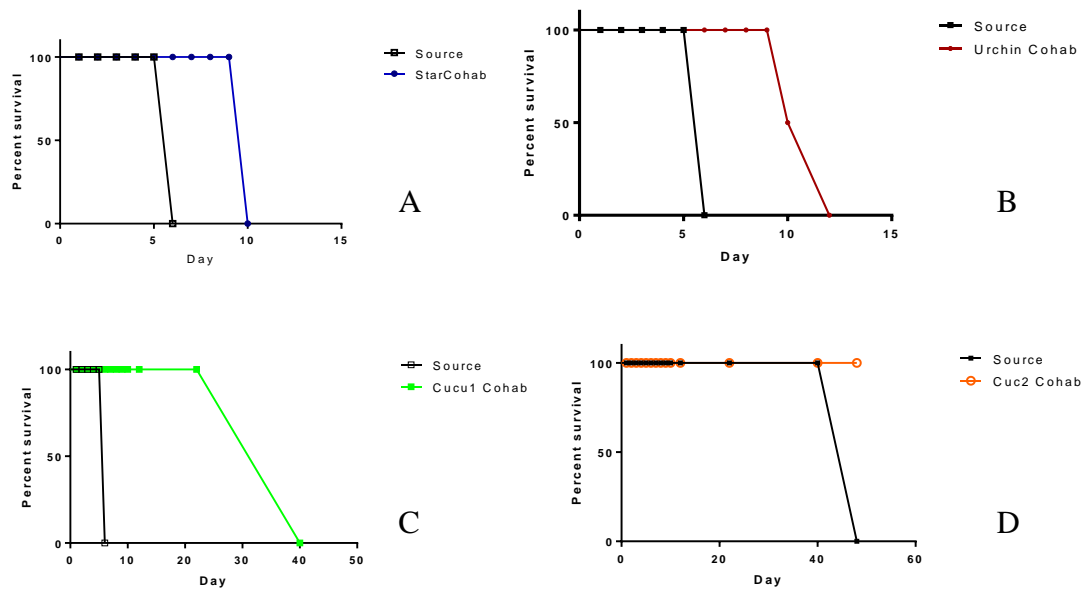


Figure 10: Effect of cohabitation of diseased echinoderms on the survival of healthy-looking stars (Cohabitation II). Acclimated *Asterias forbesi* (n=12) from Narragansett Bay not showing signs of disease were placed 3 to a tank, each with one animal received from the Maine State Aquarium (named *Source* in the graphs): (A) sea star, (B) sea urchin, (C) sea cucumber1, (D) sea cucumber2. Animals were monitored for signs of disease and mortality. Mortality was observed in all groups except cucumber 2.

### Cohabitation III: Infected Water Accumulation

Healthy-looking *A. forbesi* stars (n = 2) that were placed into the exposure tanks containing a diseased sea star began to show signs of SSWD (morbidity) at  $4.4 \pm 2.6$  days after exposure to a diseased sea star and mortality by  $5.3 \pm 2.6$  days post

exposure. By the end of the trial, 14/15 (93%) stars had presented signs of wasting and died within 5 days of being exposed to infected water and individuals (not shown). These results are consistent with those seen in *Cohabitation I*, which was also a species-specific trial.

### Challenge experiments using 0.22 $\mu\text{m}$ filtered water collected from SSWD stars

#### *Filtration I*

Stars in the Whole water treatment started to show signs of morbidity within 2 days, and 100% mortality had occurred in these groups by 4 days post exposure. Morbidity was noted in the Filtered treatments at 3-6 days, with 100% mortality by 15 days post exposure (Figure 11). No morbidity or mortality was noted in Control tanks during this time.

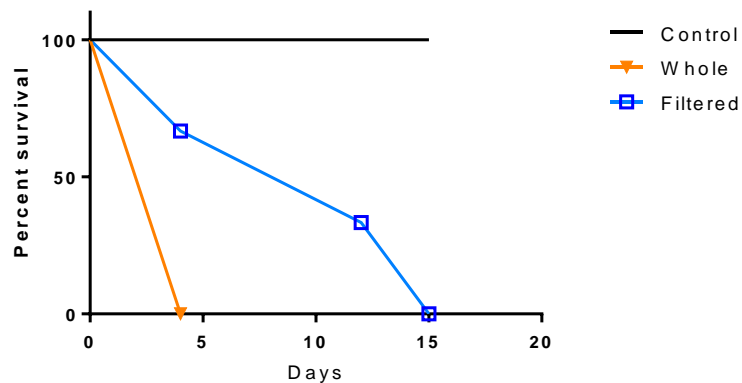


Figure 11: Effect of incubation in water collected from tanks with diseased sea stars on survival of healthy-looking stars (Filtration I). Healthy-looking acclimated stars from Narragansett Bay were exposed to: freshly collected infected water (*whole*), n=4, yellow line; 0.22  $\mu\text{m}$  filtered infected water (*filtered*), n=4, blue line; and no treatment (*control*), n=3., black line

### Filtration II

Stars in tanks receiving 0.22-micron filtered water were the only ones to express morbidity and mortality associated with SSWD in this trial. Clinical signs including loss of turgor, limb curling, and lesion formation were observed at 2-6 days post exposure, with 40% mortality occurring by day 10. Though no signs of disease were noted, one star in the *whole* water treatment was found dead by 32 days post exposure (Figure 12).

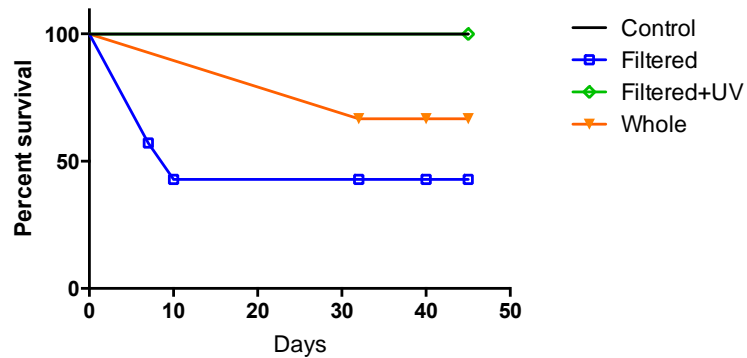


Figure 12: Effect of incubation in water collected from tanks with diseased sea stars on survival of healthy-looking stars (Filtration II). Healthy-looking acclimated stars from Narragansett Bay were treated with antibiotics and then exposed to: previously frozen infected water (*whole*, n= 6, orange line); 0.22  $\mu$ m filtered frozen infected water (*filtered*, n= 6, blue line); 0.22  $\mu$ m filtered and UV treated frozen infected water (*filtered + UV*, n= 6, green line); and no treatment (*control*, n=6, black line).

### Filtration III

In this trial, mortality was seen in all treatment groups. In control tanks, all stars were moribund by day 5, and 50% mortality occurred by day 13 post exposure. No other control stars expressed clinical signs associated with SSWD, but 83% mortality had occurred by day 35 post exposure. In tanks that received 0.22-micron filtered water, 33% mortality occurred within 26 days post exposure. In tanks with

0.22-micron filtered and UV treated water, 33% mortality occurs by day 31, though no clinical signs were expressed (Figure 13). No mortality was noted in Whole water treatment tanks during the trial period. Control tanks experienced 75% mortality, making this trial unsuitable for analysis.

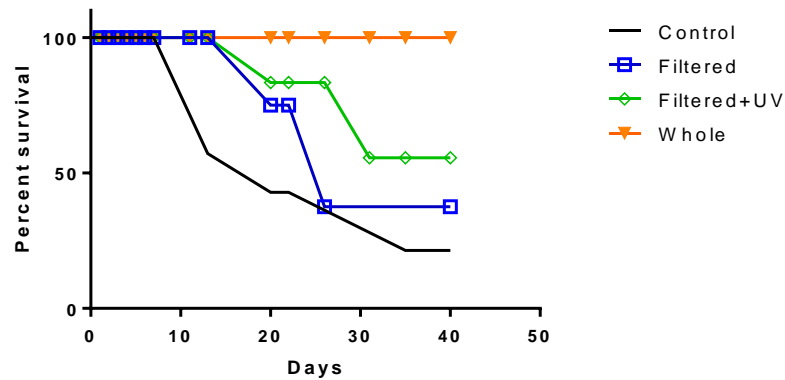


Figure 13: Effect of incubation in water collected from tanks with diseased sea stars on survival of healthy-looking stars (Filtration III). Healthy-looking acclimated stars from Narragansett Bay were treated with antibiotics and then exposed to: previously frozen infected water (*whole*, n= 6, orange line); 0.22  $\mu\text{m}$  filtered frozen infected water (*filtered*, n= 6, blue line); 0.22  $\mu\text{m}$  filtered and UV treated frozen infected water (*filtered + UV*, n= 6, green line); and no treatment (*control*, n=6, black line).

*Summary of challenge trials: comparison of time to morbidity and mortality between experiments*

Average time to morbidity and mortality varied between groups. Among the 9 groups analyzed, the means varied significantly for both morbidity and mortality (Figure 14). In the bacterial challenge experiment, stars showed signs of disease between  $8.7 \pm 5.2$  days, and mortality in  $24.7 \pm 7.5$  days. By contrast, the stars in Cohabitation I showed signs in  $3.5 \pm 0.5$  days, with mortality averaging  $4.4 \pm 0.5$  days. Time to clinical signs and mortality for sea stars used in the Cohabitation II trials



depended on the species of the source animal received from the Maine State Aquarium, ranging from 10 days for the stars exposed to a source sea star and sea urchin to no mortality seen for stars exposed to sea cucumbers. In the cohabitation trials named “Infected water accumulation,” morbidity consistently occurred within one week (7 days), and death within 2-8 days post exposure. Between the two most reliable filtration trials (in which no mortality was observed in control animals), morbidity occurred at  $3.1 \pm 1.5$  days (trial I) and  $9.6 \pm 10.4$  days, (trial II) and mortality at  $6.4 \pm 4.5$  (trial I) and  $12.6 \pm 10.9$  (trial II) days post exposure. Differences in time to morbidity and mortality between these 2 trials may have been due to the antibiotic treatment performed in trial II (but not on trial I), differences in environmental conditions or condition of acclimated animals, and/or differences in infective dose in the collected water.

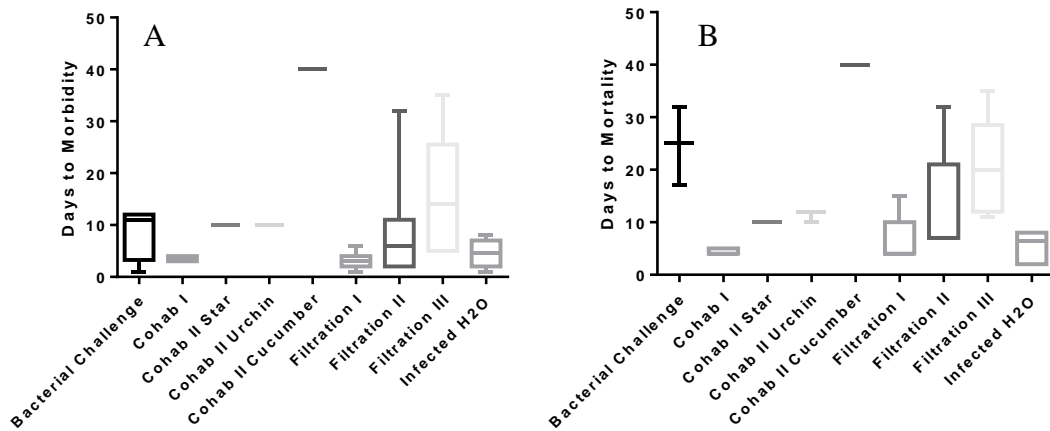


Figure 14: Average time to morbidity (A) and mortality (B) in different experimental groups (n=9). Bacterial challenge n=5, Cohab I n= 9, Cohab II Star n=4, Cohab II Urchin n=4, Cohab II Cucumber n=4, Filtration I n=4, Filtration II n=7, Filtration III n= 9, Infected water (H<sub>2</sub>O) accumulation n=14. Box and whisker plot represents minimum and maximum values, quartiles, and mean of days to morbidity or mortality after exposure.

### Detection and cloning of SSaDV VP1 in *A. forbesi*

Samples selected for end-point PCR testing showed amplification using primers designed from the VP1 and VP4 sequences of SSaDV. Of the samples tested, 11/14 (78.6%) elicited DNA bands in the region associated with VP1 and 8/14 (57%) bands in the region associated with VP4 (Figure 15). Bands matching the expected range of VP1 and VP4 (285 and 492 bp, respectively) were selected for cloning and sequencing. Sequencing of these bands yielded 3 positive identifications of the VP1 gene with 100% identity to the sequence in GenBank (consensus sequence shown in Figure 16). However, no sequences were recovered with any percent identity to the VP4 sequence in GenBank (not shown). Using the sequencing results for VP1 amplified from *A. forbesi* samples, a ClustalW analysis was used to determine conserved VP1 gene sequences to be used in the design of SSaDV qPCR primers and probe (see highlighted area in Figure 16: FWD and REV=yellow, PRB=blue).

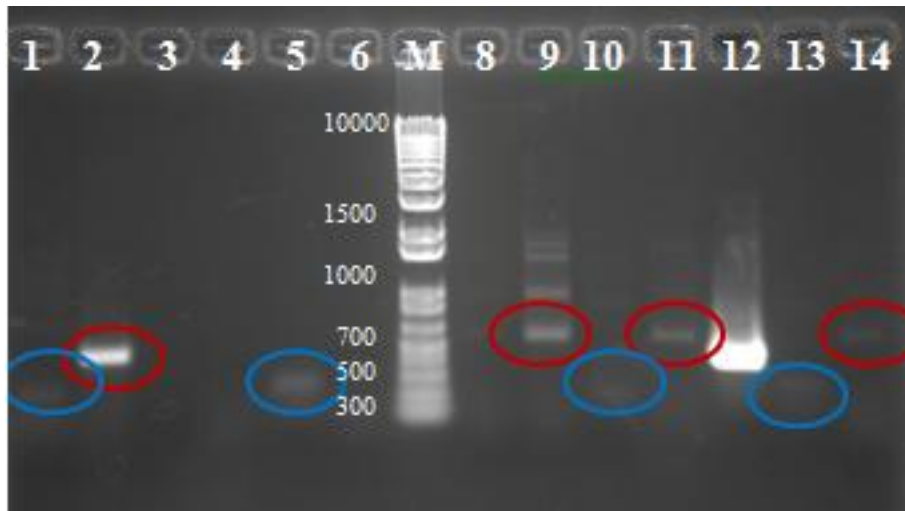


Figure 15: Gel electrophoresis of products from the amplification of individual samples of *A. forbesi* (lanes 1 & 2: star 1; lanes 3 & 4: star 2; and so on) to detect presence of SSaDV VP1 (blue, odd lanes) and VP4 (red, even lanes) DNA. M: molecular markers.

Hewson_VP1	-----ACGAAGATCCTGTGGTGAGTTTACCTGATCATAGATATATTGGTCCTGGT
VZ23_2.5.15	GAATTCGATTACGAAGATCCTGTGGTGAGTTTACCTGATCATAGATATATTGGTCCTGGT
VZ06_2.5.15	ACTAGTGATTACGAAGATCC-GTGGTGAGTTTACCTGATCATAGATATATTGGTCCTGGT
VZ08_2.5.15	ACTAGTGATTACGAAGATCC-GTGGTGAGTTTACCTGATCATAGATATATTGGTCCTGGT
Hewson_VP1	AATACTATTGACGATACTGAACCTGTTGATCTTGATGACGATATAGCAAGAGAGCAGAT
VZ23_2.5.15	AATACTATTGACGATACTGAACCTGTTGATCTTGATGACGATATAGCAAGAGAGCAGAT
VZ06_2.5.15	AATACTATTGACGATACTGAACCTGTTGATCTTGATGACGATATAGCAAGAGAGCAGAT
VZ08_2.5.15	AATACTATTGACGATACTGAACCTGTTGATCTTGATGACGATATAGCAAGAGAGCAGAT
Hewson_VP1	ATTAATTACGAAAAGCTAAAACCTCAAGAAAGACGTGCAAGAAGCTGATAGAGAAGGGGCT
VZ23_2.5.15	ATTAATTACGAAAAGCTAAAACCTCAAGAAAGCTGCAAGAAGCTGATAGAGAAGGGGCT
VZ06_2.5.15	ATTAATTACGAAAAGCTAAAACCTCAAGAAAGCTGCAAGAAGCTGATAGAGAAGGGGCT
VZ08_2.5.15	ATTAATTACGAAAAGCTAAAACCTCAAGAAAGCTGCAAGAAGCTGATAGAGAAGGGGCT
Hewson_VP1	GGTGAATTTCTTACTGACGTTATTTCATAATTCAAATCCTCATTCTATTGCTGGTTATATT
VZ23_2.5.15	GGTGAATTTCTTACTGACGTTATTTCATAATTCAAATCCTCATTCTATTGCTGGTTATATT
VZ06_2.5.15	GGTGAATTTCTTACTGACGTTATTTCATAATTCAAATCCTCATTCTATTGCTGGTTATATT
VZ08_2.5.15	GGTGAATTTCTTACTGACGTTATTTCATAATTCAAATCCTCATTCTATTGCTGGTTATATT
Hewson_VP1	GGACTTAAAGCCAAGGAAAAAGTTGAAAGTGTGATCGGTGTACAATATCCTGCTA-----
VZ23_2.5.15	GGACTTAAAGCCAAGGAAAAAGTTGAAAGTGTGATCGGTGTACAATATCCTGCTAATAATCA
VZ06_2.5.15	GGACTTAAAGCCAAGGAAATAGTTGAAAGGTGTCATCGGTGTACAATATCCTGCTAATC-
VZ08_2.5.15	GGACTTAAAGCCAAGGAAATAGTTGAAAGGTGTCATCGGTGTACAATATCCTGCTAATAATCA

Figure 16: ClustalW analysis used to develop VP1 from sequences recovered from n=3 *Asterias forbesi* samples (VZ06, VZ08, VZ23) and the sequence provided by Ian Hewson for SSaDV VP1 (Cornell).

### Concentration of SSaDV VP1 gene in sea star samples

#### *Comparison of VP1 detection between swab and tissue samples*

Two types of samples were collected from stars: a skin swab (DNA resuspended in 1000  $\mu$ L) and a tissue sample (approximate weight = 0.2 mg). Samples obtained from a skin swab averaged  $8.92 \times 10^{19} \pm 3.90 \times 10^{19}$  copies/ $\mu$ L, while tissue samples averaged  $1.25 \times 10^{20} \pm 9.79 \times 10^{19}$  copies/ $\mu$ L (Figure 17). Of the 33 swab samples tested, 22 (33.3%) were positive for the target VP1 sequences. Of the 15 tissue samples tested, 7 (46.6%) were positive for VP1. Unfortunately, we were not able to run a comparison of both sample types collected from the same star. Although the values for tissue samples were slightly higher and showed smaller variation, differences in concentrations between sample types did not differ significantly

( $p=0.7388$ ). Since no significant difference between swabs and tissue samples were obtained, data from either tissue was included in further analysis.

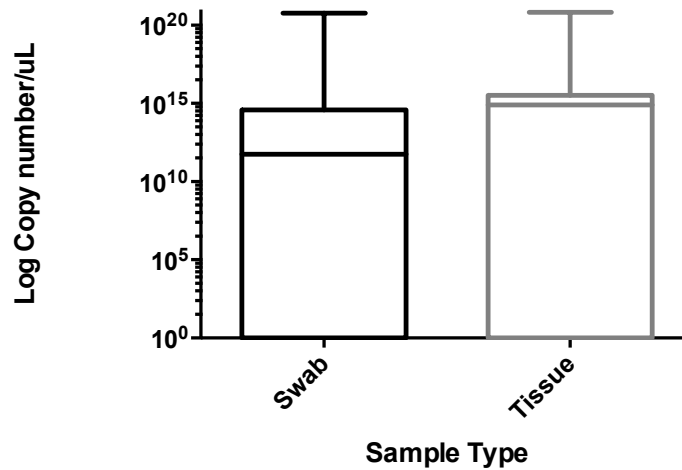


Figure 17: Concentration of SSaDV VP1 (copy number/ $\mu$ L) in swab and tissue samples from challenged stars that were positive for VP1 (Swab samples  $n=22$ , Tissue samples,  $n=7$ ). Box and whisker plot represents minimum and maximum values, quartiles, and mean.

#### *Quantification of SSaDV VP1 in all sea star samples*

Of the 48 stars tested, 29 (60 %) tested positive for the VP1 gene through qPCR. These stars represent all geographical regions (South Carolina to Maine), and include stars collected from the wild ( $n = 6$ ) and stars used in challenge experiments ( $n = 9$ ) (Appendix A). Of these 29 SSaDV-positive stars, 15 (51%) exhibited gross morphological lesions consistent with SSWD. Atlantic US coast stars positive for SSaDV (both wild and experimentally exposed) showed comparable or higher levels of SSaDV (average  $C_p$  of  $28.06 \pm 9.8$ , corresponding to a concentration of  $3.1 \times 10^{10} \pm 6.2 \times 10^{10}$  copies/ $\mu$ L based on a standard curve using the cloned VP1 target) as the positive control from the Pacific US coast provided by Ian Hewson ( $C_p$  of  $33.61 \pm 3.79$ ; estimated concentration of  $6.85 \times 10^5 \pm 9.69 \times 10^5$  copies/ $\mu$ L based on the same

standard curve). Concentrations of SSaDV in *A. forbesi* positive for SSaDV ranged from 1.2 copies/ $\mu\text{L}$  to  $2.2 \times 10^{11}$  copies/ $\mu\text{L}$ , with samples from Beavertail, RI showing the highest concentrations of SSaDV (Figure 18).

#### *Quantification of SSaDV VP1 in wild-collected samples*

Of the 34 wild-collected samples tested, 14 were positive for VP1 (41.2%), with a concentration (mean  $\pm$  SD) of  $9.8 \times 10^{19} \pm 1.9 \times 10^{20}$  copies/ $\mu\text{L}$ . Concentrations of VP1 in wild-collected samples ranged from  $3.91 \times 10^{10}$  to  $6.08 \times 10^{20}$  copies/ $\mu\text{L}$ , with the highest viral levels seen in selected samples from Beavertail and the GSO pier in Rhode Island. According to location, 10/13 (76.9%) of the samples collected from Beavertail, RI, tested positive for VP1, with 9/15 (60%) positive samples from the GSO pier (RI), and 6/16 (37.5%) positive samples from South Carolina (Figure 18). Only one out of 3 echinoderms (the sea star) collected from the Maine State Aquarium tested positive for VP1, with a concentration of  $9.7 \times 10^{10}$  copies/ $\mu\text{L}$ .

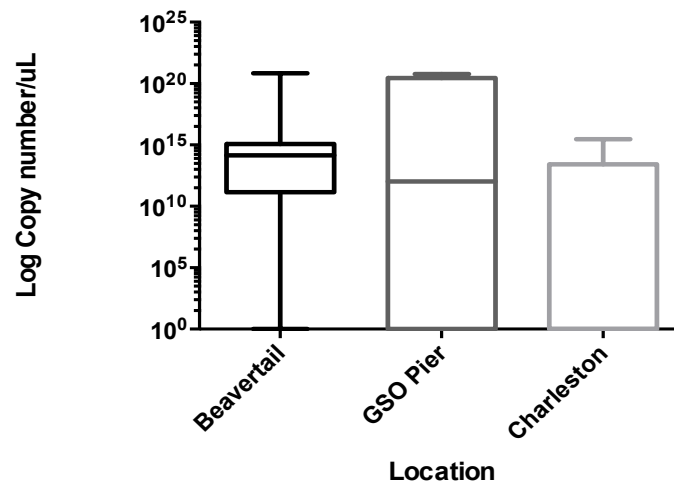


Figure 18: Concentration of SSaDV VP1 (copy number/ $\mu$ L) in all sea stars collected in different locations from the Atlantic coast of the US between 2013 and 2015. Locations include: Beavertail (RI) n=13, GSO Pier (RI) n=15, Charleston (SC, 2014) n=16. Box and whisker plot represents minimum and maximum values, quartiles, and mean.

*Quantification of SSaDV VP1 in sea stars from challenge experiments*

Of the 14 sea stars exposed to the disease through experimental challenges, 9 were positive for VP1 (64.3%), with an average concentration of  $9.7 \times 10^{19} \pm 2.3 \times 10^{20}$  copies/ $\mu$ L (Figure 19). VP1 concentration was not statistically significant ( $p=0.462$ ) between wild-collected and experimentally challenged samples that are positive for VP1 (negative samples excluded from this analysis). The 9 (out of 14) experimentally exposed sea stars positive for VP1 included 3 (33.3%) that showed no gross signs of wasting, and 6 (66.6%) that expressed limb curling, bloating, or other SSWD lesions. Within 10 days of exposure, 14/15 (93.3%) stars had died, with the last one dead by day 15, with gross signs of wasting.

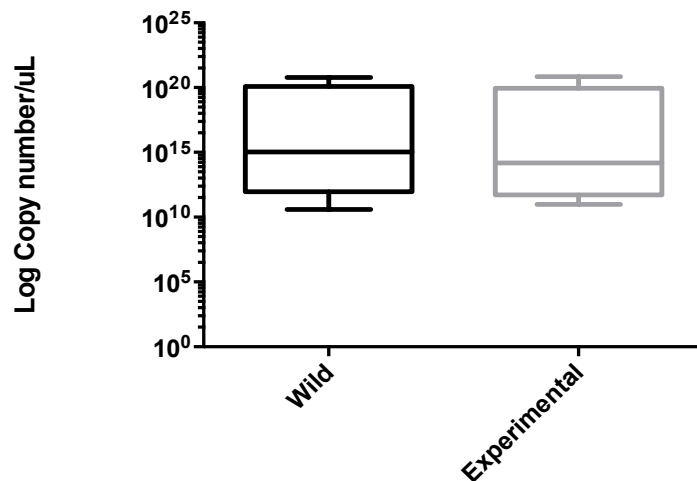


Figure 19: Concentration of SSaDV VP1 (copy number/ $\mu$ L) in wild-collected and experimentally exposed sea stars that tested positive for VP1 (Wild samples n=20, Experimental samples, n=9; negative samples were excluded from this analysis). Box and whisker plot represents minimum and maximum values, quartiles, and mean.

*Relationship between concentration of VP1 and signs of SSWD*

There was no significant difference in VP1 load between VP1-positive stars showing signs of disease and those not showing signs of disease ( $p=0.478$ ; Figure 20). Additionally, stars used in experimental challenges that exhibited lesions did not show significantly higher levels of VP1 than those sampled from wild stars ( $p= 0.405$ , Figure 20).

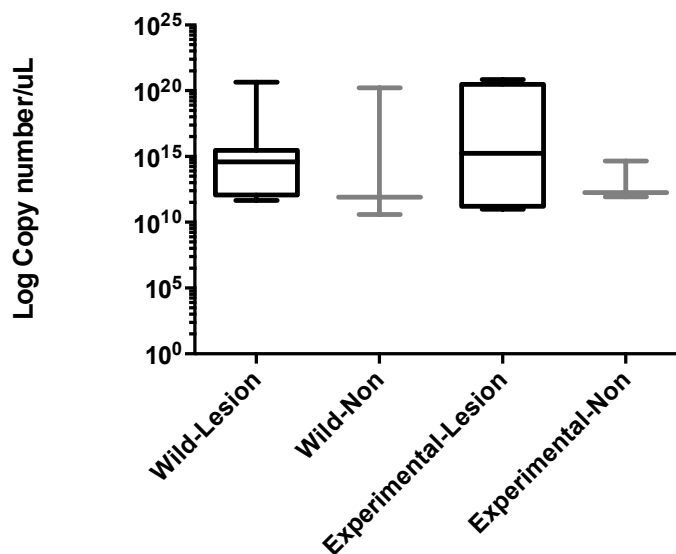


Figure 20: Concentration of SSaDV VP1 (copy number/ $\mu$ L) in swabs collected from wild and experimental stars showing signs of SSWD (*Lesion*) and not showing signs of disease (*Non*). Box and whisker plot represents minimum and maximum values, quartiles, and mean.

Of all the samples analyzed in this study (wild and experimental), 20/48 (42%) of samples tested positive for the VP1 gene also showed clinical signs of disease (Table 4). Additionally, 5/48 (10%) of the samples testing negative for the VP1 gene were also negative for clinical signs. A total of 23/48 (48%) of the samples either tested positive for the VP1 region, yet showed no clinical signs, or tested negative and

showed clinical signs. Contingency analysis using Fisher’s exact test yields a p-value of 1.0, suggesting that there is no statically significant relationship between presence of clinical signs of SSWD and VP1, from the samples analyzed. Concentration of VP1 DNA in VP1-positive stars with no lesions ranged from  $4.75 \times 10^2$  to  $1.24 \times 10^{11}$ . Additionally, of the ten samples with the highest VP1 concentration (copy number/ $\mu\text{L}$ , range= $2.97 \times 10^{15}$ - $6.92 \times 10^{20}$ ), only sixty percent showed clinical signs of disease.

Table 3: Contingency Table expressing relationship between presence or absence gross morphological (signs clinical signs) and results of qPCR analysis for VP1 region of SSaDV. No relationship was observed between presence of VP1 and clinical signs of SSWD (Fisher’s exact test:  $p=1.0$ )

	+ Gross	- Gross	<b>Totals</b>
+ qPCR	20 (42%)	9 (19%)	<b>29</b>
- qPCR	14 (29%)	5 (10%)	<b>19</b>
<b>Totals</b>	<b>34</b>	<b>14</b>	<b>48</b>

*Changes in VP1 concentration in sea stars*

We also sought to determine if viral load would increase in disease stars as the disease progressed. For stars received from Charleston, South Carolina, we were able to collect samples from the same stars at different stages of the disease for quantification of VP1. On the day of arrival from Charleston, VP1 concentrations in these stars averaged  $6.5 \times 10^1 \pm 10.0 \times 10^1$  copies/ $\mu\text{L}$  (Figure 21). At this stage, stars did not show any signs of SSWD. By the third day after placement of stars in the tanks, these stars experienced 100% mortality showing signs of SSWD. Concentrations had increased to  $2.9 \times 10^6 \pm 3.8 \times 10^6$ . Although not statistically significant at a 0.05 alpha level ( $p=0.0907$ ) due to variability, all stars showed lethargy, weak tube feet attachment, limb curling, and lesion formation. Comparative



analysis of 6 of the stars sampled at both time points (at arrival from SC on day 1 and at time in which signs of disease were shown at day 3) showed an increase in VP1 levels in 3/6 (50%). The mean increase in VP1 load was  $6.42 \pm 10^{15}$  (Figure 21).

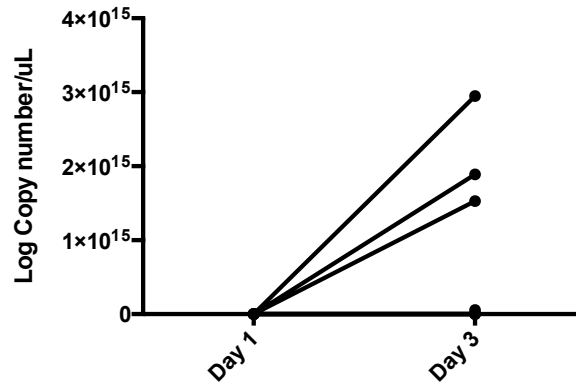


Figure 21: Change in viral VP1 DNA concentration (copies/μL) in South Carolina stars at two time points. Stars did not show signs of disease at day 1, while showing morbidity and mortality by day 3. Data for n=6 stars sampled at both time points. Box and whisker plot represents minimum and maximum values, quartiles, and mean.

## CHAPTER 4

### DISCUSSION

In this study, we sought to characterize SSWD in sea stars (*A. forbesi*, *A. rubens*) collected from the Atlantic US, determine if a virus could be causing this disease through experimental challenges, and assess the presence and relationship of SSaDV to clinical signs of SSWD in *A. forbesi*. Our results show that: 1) SSWD has affected populations of sea stars from Florida to Canada in the period from 2012 – 2015; 2) Clinical signs of SSWD in sea stars from the Atlantic coast of the US are similar to those seen in sea stars in the Pacific coast of the US; 3) A viral pathogen is the most likely cause of SSWD in *A. forbesi*, and *A. rubens*; and 4) A clear association between the presence of SSaDV in sea stars and SSWD could not be proven with the limited amount of samples available in this study.

The first objective of our research was to assess the geographical extent of SSWD on the East Coast, which was achieved by distributing surveys to local aquaria and dive groups. Based on the citizen survey data, the geographic range of wasting along the Atlantic Coast has been identified as Nova Scotia to Florida, with reports as early as Spring of 2012. Signs of SSWD were still evident in some populations as late as Fall 2015. The disease appears to have had a significant impact on sea star population levels. Anecdotal reports from Rhode Island show that, while populations of sea stars were highly abundant prior to 2012 (causing numerous complaints from local oyster growers that experienced high predation pressure), populations of sea stars were severely depleted in Narragansett Bay and the Rhode Island coastal ponds during

2012 – 2015, with local divers reporting some signs of recovery in the late summer and Fall of 2015 (personal communication). However, the survey results exhibited a strong response bias, with only a limited number of reports obtained (Appendix D). A more comprehensive survey should be conducted in order to assess the true range of the disease, as well as which species are affected, and the extent of population decline in these species. Currently there is no long-term comprehensive intertidal monitoring system in place in the Atlantic coast of the US similar to the one in the Pacific Coast (Pacific Rocky Intertidal Monitoring, UC Santa Cruz), but such a system would be extremely beneficial in determining the extent and impact of wasting in sea stars, as well as potential impacts on the ecosystem. It would also help to establish disease timelines and shed light on how the disease spread, as well as determine the potential role of changing environmental conditions on triggering disease epizootics. Along the Pacific Coast, historical reports of sea star wasting outbreaks have been loosely tied to increased water temperatures (Eckert et al. 1997, Scheibling and Lauzon-Guay 2010; Scheibling and Hennigar 1997). Interestingly, we observed two major die-off events in our holding tanks, which occurred in both October of 2014 and 2015. This is the time when seawater temperatures decrease rapidly in Narragansett Bay (~0.3 °C per day) suggesting that this changing temperature conditions may be a trigger for disease epizootics. A more thorough analysis of the timeline of the Atlantic coast of the US outbreak is needed to examine the relationship of mortality events to changes in environmental conditions such as temperature, salinity, and pH, as well as potential relationships with changes in food availability.

Clinical signs and a timeline of disease progression have been defined for *A. forbesi* based on gross observations and histological examination. These signs include lethargy, limb-curling, loss of tube feet attachment to substrate, and lesion formation leading to ulceration of internal tissue and death, and are similar to those described for sea stars affected by SSWD in the Pacific coast of the US (Hewson et al. 2014). Until now, very little was known about sea star wasting on the Atlantic coast of the US. This research provides some of the first histological examination *A. forbesi* tissue affected by SSWD. This data has allowed us to visualize the impact the disease is having on sea star tissue, and define both gross and microscopic clinical signs associated with wasting in this species. A more comprehensive analysis of SSWD infected tissue from both coasts is important to defining the morphological and immunological changes associated with the wasting phenotype. Among the *Asterias* spp. stars studied in this research, we observed two different manifestations of the disease: an acute and a chronic form. The chronic manifestation (observed in only a handful of stars) may present itself with clinical signs early (3-5 days post exposure), but then linger for weeks to months before progressing rapidly to deterioration of sea star tissue. The two manifestations may be a reflection of different modes of progression of the same disease due, for example, to differences in host susceptibility, environmental conditions, or the impact of secondary and possibly tertiary invaders. Alternatively, this may reflect two different diseases or pathogens. On the Pacific Coast only the acute form has been reported; stars live 1-2 weeks after exposure before succumbing to disease (Hewson et al. 2014). Future work should seek to assess the relationship of

the two manifestations of SSWD in *Asterias* spp. to determine if they have the same cause, or just similar physical expression.

Some of the clinical signs observed in stars affected with SSWD may be outward expressions of the star attempting to fight off an infection. The limb curling behavior was observed in Pacific coast stars, *Pycnopodia helianthoides*, with SSWD could be related to an upregulation in expression of genes related to cell adhesion, nervous system, and connective tissue management (Fuess et al. 2015). These three systems are crucial to maintaining structure and shape in sea stars, so an upregulation of gene expression may reflect the gross morphological appearance of infected individuals. Fuess et al. (2015) also report the upregulation of genes associated with immune cell production (coelomocytes, macrophages) in response to injection of viral sized particles from diseased animals. In our study, examination of diseased *A. forbesi* tissue consistently shows an influx of hemocytes and immune cells, supporting the idea that Atlantic coast sea stars may mount a similar immune response to SSWD exposure.

Results from our cohabitation suggest that the disease is highly transmissible in *A. forbesi* and *A. rubens*, leading to rapid and severe morbidity and mortality within 10 days of exposure of a healthy-looking star to a diseased star. Furthermore, filtration trials involving water collected from tanks with sea stars experiencing mortality to SSWD indicate that a viral pathogen is the most likely cause of SSWD in *Asterias* spp. in the Atlantic coast of the US. Although several bacterial species have been found to be pathogenic to echinoderms (Becker et al. 2008) and some morbidity and mortality of *A. forbesi* was observed in stars exposed to bacteria isolated from SSWD stars, the

time to morbidity and mortality (significantly longer for stars exposed to bacterial challenge) and the gross and histological signs of disease observed in stars from the bacterial challenge are not consistent with SSWD as observed in both wild and experimentally exposed stars. A viral pathology is also consistent with the conclusions by Hewson et al. (2014) for the SSWD outbreaks in the Pacific coast of the US. Interestingly, in our experiments, high levels of morbidity and mortality were observed in *A. forbesi* after exposure by immersion of healthy-looking sea stars to fresh or frozen water from infected tanks, while injection challenges with viral sized particles (which should lead to enrichment in the pathogen) were used in experimental challenges of Pacific Coast sea stars (Hewson et al. 2014).

Studies from the SSWD outbreaks in the Pacific coast showed that several species of sea stars are susceptible to SSWD (Hewson et al. 2014). In the Atlantic coast of the US, signs of SSWD have been observed in *A. forbesi*, *A. rubens*, and one sea urchin *Stronglyocentrotus droebachiensis*. Moreover, we have seen transmission of SSWD between a sea urchin collected from the Maine State Aquarium and healthy looking *A. forbesi*, as well as between diseased *P. miniata* from the Pacific Coast and *A. forbesi*. Our results also indicate that sea cucumbers, *Cucumaria frondosa*, may not be susceptible to SSWD. These results suggest that the causative agent of SSWD in the Atlantic Coast of the US may also have a broad host range including several sea star species and at least one sea urchin species. Further field and experimental research, however, is needed to determine which echinoderm hosts present in the Atlantic coast of the US are susceptible to SSWD, since our experiments were extremely limited in sample size.

Hewson et al. (2014) identified a densovirus that has been associated with SSWD in species along the Pacific coast. The research team identified three gene sequences (VP1, VP4, NS1) that code for parts of the Sea Star associated Densovirus (SSaDV) genome. The VP4 and NS1 sequences were used primarily for analysis in Pacific coast samples, but we were not able to recover these sequences in our samples, probably due to differences in sequences between the viruses from different locations. We therefore relied on the sequenced obtained for the VP1 gene to quantify viral DNA in *Asterias* spp. A majority of the samples tested (61%) have tested positive for the SSaDV VP1 gene, showing that East coast stars do in fact carry the VP1 sequence for SSaDV, though at varying concentrations. However, although the few stars (n = 3) in which we were able to quantify viral DNA at different stages of the disease show a very clear increase in viral DNA copy number, our results do not provide conclusive evidence that presence of SSaDV is associated with SSWD lesions in *A. forbesi*. All the stars analyzed in this study, however, came from areas experiencing SSWD and most experienced mortality during the performance of this research, so even stars not showing clinical signs of the disease may have been infected at the time of sampling. Only a portion (48/196, 24%) of the samples collected in this project has been analyzed by qPCR so far. Future research should seek to analyze all samples collected, as well as to establish a collection schedule for more stars to assess viral levels, including stars from areas not experiencing SSWD. Future research should also track changes in viral DNA (indicating presence of the virus) and RNA (indicating active replication of the virus) copy number in healthy-looking stars at different time points before and after exposure to the disease through experimental challenges.

DNA isolated from sea star samples were not processed in the same way as those in Hewson et al. (2014), which prevents us from performing direct comparisons on viral loads between studies. In our study, viral DNA was not isolated from samples, meaning that they reflect microbial as well as sea star DNA, and may result in a dilution of target DNA values. Furthermore, results from the Pacific coast are reported as copies/mg<sup>-1</sup> tissue. Samples testing positive for SSaDV from ten Pacific sea star species ranged in concentration from 1.0 x 10<sup>3</sup> to 1.0 x 10<sup>9</sup> copies/mg<sup>-1</sup> for the VP4 sequence. From the fifteen tissue samples analyzed from Atlantic coast stars in Hewson et al. 2014, the concentration of VP1 ranged from 2.68 x 10<sup>11</sup> to 3.46 x 10<sup>18</sup> copies/mg<sup>-1</sup>, with an average of 3.89 x 10<sup>10</sup> ± 8.09 x 10<sup>10</sup> copies/mg<sup>-1</sup>. These values fall above the reported range of viral load for *Mediaster aequalis*, *Pisaster giganteus*, *Pisaster brevispinus*, and *Patiria miniata* (Hewson et al. 2014). Many of the stars tested in our study showed similar or higher viral loads than the positive control obtained from the Hewson laboratory. Future research should focus on analyzing more tissue samples from the east coast in order to provide substantial comparisons to results from the west coast, and to assess the efficacy of tissue as opposed to swab samples. Our research has led to the development of a tool that could be used to screen samples for presence or absence of SSaDV in sea stars in the Atlantic coast of the US. Our results obtained through quantification of VP1 should also be confirmed through analysis of additional targets in the sequence of SSaDV. Sequencing and characterization of SSaDV from *Asterias* spp. would allow for the development of other screening tools for SSaDV.



More research is also needed to confirm a viral etiology for this disease and determine if SSaDV is the causative agent of SSWD. Results from our filtration challenge experiments were not always consistent. Factors leading to differences in the outcome of these 3 filtration experiments include whether stars were treated with an antibiotic (Enrofloxacin by injection) prior to challenge, whether water from infected tanks was frozen or not prior to the challenges, and/or differences in viral load between samples of water used in the filtration experiments. Furthermore, there are several major pitfalls for identifying the causative agent of SSWD through challenge experiments, including: 1) the lack of a reliable source of sea stars from an area in the Atlantic coast of the US potentially free of the disease; 2) difficulties in confirming that stars used for the experiments are not already infected with the pathogen causing SSWD due to a lack of a screening tool for the pathogen (qPCR for VP1 was not developed until the final stages of this research); 3) lack of knowledge on the environmental conditions triggering SSWD outbreaks; and 4) the lack of marine invertebrate cell cultures that could be used to isolate and culture candidate viral pathogens.

It should also be noted that there is the potential that some of the stars have developed a resistance to SSWD. Recent reports (Fall 2015) have stated that some populations of *A. forbesi* around Rhode Island seem to be rebounding. It is unclear whether these stars are the result of spawning post-outbreak, or if some managed to survive the outbreak altogether. Future work should seek to assess any populations that may be untouched by wasting disease. Finding such populations could be

beneficial for future work by providing better controls, lending support to any conclusions.

In summary, our research shows that outbreaks of SSWD similar to the ones affecting several species of sea stars in the Pacific Coast of the US have also affected *Asterias* spp. in the Atlantic Coast of the US. Challenge experiments confirm a viral etiology for the disease. In addition, SSaDV has been detected in *Asterias* spp. from the Atlantic coast of the US, although a clear association of SSaDV with SSWD has been found in these stars. More research is necessary to characterize the epizootiology of the disease and identify the causative agent.

APPENDICES

**Appendix A: Sample Collection Data**

Experiment type: 1: wild, 2: challenged/experimental

Presence of lesions: 0: none, 1: lesions present

Samples collected: 1: swab, 2: tissue

qPCR: Results of qPCR analysis for VP1: 0: negative, 1: positive, -: not tested

Histo: Results of Histological examination: 0: negative, 1: positive, -: not tested

Sample Name	Sample Collected	Location	Experiment Type	Presence of Lesions	Samples collected	qPCR	Histo
2A.1	6/9/2014	Beavertail, RI	2	1	2	1	1
2B.1	6/8/2014	Beavertail, RI	2	1	1	1	1
4A.1	6/9/2014	Beavertail, RI	2	1	2	1	1
4B.1	6/9/2014	Beavertail, RI	2	1	1	1	-
4B.2	6/9/2014	Beavertail, RI	2	1	1	0	-
6.2.14-2	6/2/2014	Beavertail, RI	1	1	1	1	1
6.4.14-1	6/5/2014	Beavertail, RI	1	1	1	1	1
6.5.14-2	6/5/2014	Beavertail, RI	1	1	1	1	1
6.5.14-4	6/6/2014	Beavertail, RI	1	1	1	1	-
CohabStar 1	4/14/2014	Beavertail, RI	2	0	1	1	0
CohabStar 2	4/14/2014	Beavertail, RI	2	0	1	1	-
CohabStar 3	4/14/2014	Beavertail, RI	2	0	1	1	-
Cucumber 2	4/19/2015	Maine	1	1	2	-	-
H2O TransStar	4/23/2014	Beavertail, RI	2	1	2	0	1
MSA Star	4/13/2014	Maine	2	1	1	1	1
SCA-C1	4/25/2015	Charleston, SC	1	1	2	1	1
SCA-C2	4/25/2015	Charleston, SC	1	1	2	0	-
SCA-C3	4/23/2015	Charleston, SC	1	1	1	1	-
SCA-C4	4/25/2015	Charleston, SC	1	1	2	-	-
SCA-C5	4/23/2015	Charleston, SC	1	1	1	1	1
SCA-C6	4/25/2015	Charleston, SC	1	1	2	-	-
SCA-F1	4/25/2015	Charleston, SC	1	1	2	0	-
SCA-F2	4/25/2015	Charleston, SC	1	1	2	0	-
SCA-F3	4/23/2015	Charleston, SC	1	1	1	0	1
SCA-F4	4/23/2015	Charleston, SC	1	1	1	0	1

Sample Name	Sample Collected	Location	Experiment Type	Presence of Lesions	Samples collected	qPCR	Histo
SCA-F4	4/25/2015	Charleston, SC	1	1	2	1	-
SCA-F5	4/23/2015	Charleston, SC	1	1	1	1	-
SickTank# 1	10/7/2014	GSO Pier	2	1	2	-	-
SickTank# 11	11/11/2014	GSO Pier	2	1	2	-	-
SickTank# 12	11/11/2014	GSO Pier	2	1	2	0	1
SickTank# 13	11/11/2014	GSO Pier	2	1	2	1	1
SickTank# 15	11/20/2014	GSO Pier	2	1	2	-	-
SickTank# 3	10/15/2014	GSO Pier	2	1	2	-	-
SickTank# 4	10/15/2014	GSO Pier	2	1	2	-	-
SickTank# 6	10/23/2014	GSO Pier	2	1	2	0	-
SickTank# 8	10/29/2014	GSO Pier	2	1	2	-	-
T3S1	3/16/2015	GSO Pier	1	0	1	1	-
T3S10	4/8/2015	GSO Pier	1	0	1	-	-
T3S11	4/8/2015	GSO Pier	1	0	1	1	-
T3S12	4/9/2015	GSO Pier	1	1	1	0	-
T3S13	4/8/2015	GSO Pier	1	0	1	1	-
T3S14	4/9/2015	GSO Pier	1	0	1	-	-
T3S15	4/9/2015	GSO Pier	1	0	1	-	-
T3S2	3/16/2015	GSO Pier	1	0	1	-	-
T3S3	3/16/2015	GSO Pier	1	0	1	1	-
T3S4	3/16/2015	GSO Pier	1	0	1	-	-
T3S5	3/16/2015	GSO Pier	1	0	1	0	-
T3S6	4/8/2015	GSO Pier	1	0	1	-	-
T3S7	4/8/2015	GSO Pier	1	0	1	1	-
T3S8	4/8/2015	GSO Pier	1	0	1	-	-
T3S9	4/8/2015	GSO Pier	1	0	1	1	-
Trial 1 5.1	7/21/2014	Beavertail, RI	2	0	2	-	1
Trial 1 5.3	7/22/2014	Beavertail, RI	2	1	2	-	-
Trial 1 5.4	8/2/2014	Beavertail, RI	2	1	2	-	-
Trial 1 7.2	7/7/2014	Beavertail, RI	2	0	2	0	1
Trial 1 9.2	7/30/2014	Beavertail, RI	2	1	2	-	-
Trial 2	9/1/2014	GSO Pier	2	1	1	-	1
Trial 2 1.1	8/23/2014	GSO Pier	2	1	2	-	1
Trial 2 1.3	8/25/2014	GSO Pier	2	1	2	-	-

<b>Sample Name</b>	<b>Sample Collected</b>	<b>Location</b>	<b>Experiment Type</b>	<b>Presence of Lesions</b>	<b>Samples collected</b>	<b>qPCR</b>	<b>Histo</b>
Trial 2 1.4	9/1/2014	GSO Pier	2	1	2	-	-
Trial 2 2.3	9/16/2014	GSO Pier	2	1	2	-	-
Trial 2 3.2	9/22/2014	GSO Pier	2	1	2	-	-
Trial 2 4.1	9/1/2014	GSO Pier	2	1	1	-	-
Trial 2 6.1	9/7/2014	GSO Pier	2	1	1	-	-
Trial 2 6.3	9/1/2014	GSO Pier	2	1	1	-	-
TS5	3/12/2015	GSO Pier	1	1	1	0	-
TS6	3/12/2015	GSO Pier	1	1	1	1	-
TS7	2/20/2015	GSO Pier	1	0	1	0	-
TS8	2/20/2015	GSO Pier	1	0	1	1	-
TS9	3/12/2015	GSO Pier	1	1	1	-	-
Urchin	4/19/2015	Maine	1	0	2	0	-



### **Appendix C: Survey distributed to local Aquaria**

- 1) Which species of Sea star are in holding?
- 2) Are they in a touch tank, display, or holding tank?
- 3) How dense are their numbers in the tanks?
- 4) What type of filtration is being used on the system (ie. Carbon, paper, UV sterilizer, etc.)?
- 5) Is the system isolated or on flow-through?
- 6) What are the water conditions (pH, temperature, salinity)?
- 7) How frequently are tanks cleaned/maintained?
- 8) How long after acquisition are the first lesions seen?
- 9) Where are the lesions located?
- 10) How long does it take for an animal to die from the issue?
- 11) Do you notice any fluctuations in mortality from the issue (seasonal, spawning)?
- 12) Any additional information/observations?

#### **For Collection Trips:**

Location, date, depth, approximate water temperature

What are their relative densities in the wild?

What is the average size of animals seen in the wild?

Do you notice higher densities in shallow or deeper waters?

What are the conditions of transport/treatment before being placed into holding tank?

Any specific locations where you notice a difference in population numbers?

Would you be interested in sending us some sample specimens (alive or dead/preserved)?

**Appendix D: Table of Citizen Survey Reports for Presence/Absence of SSWD**

<b>Reporter</b>	<b>Date (Month/Year)</b>	<b>Location</b>	<b>Presence/Absence</b>
Lawrence	7/13	Fort Adams, RI	Absent
L. B.	7/13	Florida	Present- cushion star
N. Jakobowski	7/13	Riverside, RI	Present
O. Pisano	7/13	Dalhousie, Nova Scotia, Canada	Present
P. Voskamp	8/13	Block Island, RI	Present
T. Joslin	8/13	Cohasset, RI	Absent
B. Lebowitz	8/13	Marblehead, MA	Present
F. Monteforte	8/13-10/13	Narragansett, RI	Present
M. Hall	9/13	Quonset, RI	Present
G. McDonald	11/13	Stonington, ME	Present
C. Wells	2/14	Maine State Aquarium, ME	Present
T. Newlands	8/14-10/14	Narragansett, Jamestown, Newport, RI	Present
K. Mahoney	8/14	Hilton Head, SC	Present
R. Sprague	8/14	Northport and Lincolnville, ME	Present
R. Scheibling	3/14	St. Margaret's Bay, Nova Scotia, Canada	Present
S. Boylan	3/15	Charleston, SC	Present



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