Withering Syndrome Disease Dynamics in Wild and Cultured Northeastern Pacific Abalones

Lisa M. Crosson

A dissertation

submitted in partial fulfillment of the

requirements for the degree of:

Doctor of Philosophy

University of Washington

2020

Reading Committee:

Carolyn Friedman, Chair

Steven Roberts

Glenn VanBlaricom

Program Authorized to Offer Degree:

School of Aquatic & Fishery Sciences

©Copyright 2020

Lisa M. Crosson

University of Washington

Abstract

Withering Syndrome Disease Dynamics in Wild and Cultured Northeastern Pacific Abalones

Lisa M. Crosson

Chair of the Supervisory Committee:

Carolyn Friedman

School of Aquatic & Fishery Sciences

Withering syndrome (WS) is a chronic bacterial disease of abalones, *Haliotis* spp., caused by a *Rickettsia*-like organism (WS-RLO). The etiological agent, *Candidatus* Xenohaliotis californiensis, occurs along the eastern Pacific margin of North America in California, US and Baja California, Mexico. However, as infected abalones have been transported to Chile, China, Taiwan, Iceland, Ireland, Israel, Spain, Thailand, and Japan, the geographic range of the bacterium is likely broad especially where California red abalone (*Haliotis rufescens*) are cultured or in areas where native species have been exposed to red abalone. Disease susceptibility varies among abalones with up to 99% losses of black abalone (*H. cracherodii*) in lab and field studies in the US, to no losses among the small abalone (*H. diversicolor supertexta*) in Thailand. Some abalone

populations that have suffered severe WS mortality events have developed resistance to the disease. In addition, a newly identified phage hyperparasite of the WS-RLO may reduce pathogenicity and dampen associated losses. Proper diagnosis of WS requires the identification of infection with the pathogen (WS-RLO detected via *in situ* hybridization or histology coupled with PCR and sequence analysis) accompanied by morphological changes that characterize this disease (e.g. digestive gland metaplasia and pedal atrophy). A quantitative PCR (qPCR) assay was recently developed and validated for the detection of WS-RLO DNA in abalone tissues, feces, and seawater. While confirmation of infection cannot be done by PCR-based assays alone, they can be used as proxies for infection in areas where the WS-RLO is established and are recommended for inclusion in all abalone health examinations. Avoidance of WS is best accomplished by the establishment of a health history, good husbandry practices, and multiple health examinations prior to the movement of animals.

Population declines in wild and cultured abalones due to WS have been well documented along the northeastern Pacific Ocean. However, observed differences in species susceptibility to the disease are not well understood. The first objective of my dissertation was to examine the susceptibility of three temperate abalone species, the cool water (4-14°C) pinto or northern abalone (*H. kamtschatkana*), the intermediate water (8-18°C) red abalone, and the warm water (12-23°C) pink abalone (*H. corrugata*), to experimental WS infection at temperatures facilitating disease proliferation. Mortality data paired with histological and molecular detection of the WS pathogen confirmed that these abalone species exhibit different levels of susceptibility to infection and resistance to WS development ranging from high susceptibility and low resistance in pinto abalone

to moderate/low susceptibility and resistance in red and pink abalones. The temperature associated with WS induced mortalities also varied among species: pinto abalone died at the lowest experimental temperature (17.32 ± 0.09°C), while red abalone died at an intermediate temperature (17.96 ± 0.16°C), and pink abalone required the highest temperature (18.84 ± 0.16°C). When data from the current and previous studies were examined, susceptibility to WS was inversely related to phylogenetic distance from white abalone (*H. sorenseni*), which had the highest susceptibility and lowest resistance of all abalone species tested prior to the current study. These results provide further evidence that an abalone's thermal optima and phylogenetic relationship can determine its susceptibility to WS; species with cool water evolutionary histories are most susceptible to WS and the most susceptible species appear to be closely related. Differences among the thermal ranges of abalone species have broad implications for WS disease dynamics and highlight the importance of understanding the mechanisms governing the abalone-WS relationship in order to properly manage declining abalone populations.

My second dissertation objective was to elucidate important epidemiological information on the WS-RLO. The bacterium remains unculturable thereby limiting our understanding of WS disease dynamics. My goals were to: (1) determine the temporal stability of WS-RLO DNA outside of its abalone host in 14°C and 18°C seawater, (2) develop a standardized protocol for exposing abalones to known concentrations of WS-RLO DNA and (3) calculate the dose of WS-RLO DNA required to generate 50% infection prevalence (ID50) in the highly cultured red abalone. WS-RLO stability trials were conducted in October 2016, February 2017, and June 2017 during which qPCR analysis was used to quantify bacterial DNA for 7 days in seawater collected at an

abalone farm in southern California where the pathogen is endemic. For all trials and temperature treatments, WS-RLO DNA was not stable in seawater longer than 2 days. To determine an ID50, groups of uninfected juvenile red abalone were subjected to 3-hour bath exposures of WS-RLO at four concentrations: 0, 10^3, 10^4, and 10^5 DNA copies/mL. Abalone feces were monitored bi-weekly for the presence of WS-RLO DNA and abalone tissues were sampled 9 weeks after dosing for histology and qPCR examination. Results from the ID50 indicated that our protocol was successful in generating WS-RLO infections and a pathogen dose of 2.3×10^3 DNA copies/mL was required to generate 50% infection prevalence in the tissue of red abalone as assessed by qPCR.

The WS-RLO is considered an established bacterial pathogen in coastal CA seawaters and is of great concern to coastal managers and local abalone aquaculture facilities (AFs) conducting open or flow-through seawater culture methods. California AFs are at high risk for spillback (wild to farm) and spillover (farm to wild) disease transmission due to high abalone host densities and the use and release of coastal seawater that may contain the WS-RLO and its associated novel phage. To address these concerns, my third and final dissertation objective was to sample nearshore surface seawater from nine established wild black abalone sites and four red abalone AFs from Bodega Bay, Sonoma County, CA, US to Ventura County, CA, US including the Channel Islands over two consecutive summers to determine the presence and amount of WS-RLO and phage DNA via qPCR. In July 2010, WS-RLO DNA was detected as far north as Andrew Molera State Park, Big Sur, CA and as far south as San Nicolas Island (SNI). Phage DNA was detected from Monterey Bay, CA to SNI. In July 2011, WS-RLO DNA was detected as far north as Davenport, CA and as far south as SNI. The phage DNA

detection range remained the same as the 2010 survey. Phage DNA loads did not vary by year at AF or wild sites. However, WS-RLO DNA loads were greater in 2011 than 2010 at wild sites, while those at AFs did not vary by year. In October 2013, surface seawater surveys were conducted at the two southern-most AFs in Cayucos and Goleta, CA to assess fine-scale WS-RLO DNA dilution potential from a point-source discharge. In the 2013 samples, WS-RLO DNA loads in seawater directly adjacent to the AFs were less than the mean levels detected at all wild black abalone sites previously surveyed within 50 to 500 m of the AFs effluent outfalls. While these findings present management concerns for both wild and cultured California abalones, it is important to acknowledge that PCR-based assays do not indicate the presence of viable pathogen or active infection and serve as a proxy for WS exposure. In order to fully assess the potential for wild and cultured abalone disease interactions, additional experiments should be conducted to determine the longevity and infectivity of the WS-RLO and novel phage in seawater. Collectively, these findings are critical components of disease dynamics that will help assess WS transmission risk within and among abalone populations and facilitate appropriate management and restoration strategies for both wild and cultured abalone species in WS-endemic areas.

TABLE OF CONTENTS

Chapter 1. Introduction & literature review

1.1 Introduction……1

1.2 Literature review……4

1.2.1 Disease distribution……4

1.2.2 Role of temperature……6

1.2.3 Host susceptibility & disease resistance……8

1.2.4 Bacteriophage hyperparasite……13

1.2.5 Diagnostic methods……...16

1.2.6 Disease control……..17

1.3 Rationale & significance……..17

Chapter 2. Withering syndrome susceptibility of northeastern pacific abalones: a complex relationship with phylogeny & thermal experience

2.1 Introduction……..19

2.2 Methods……21

2.2.1 Animals……21

2.2.2 Experimental design……..22

2.2.3 Animal condition……..24

2.2.4 Histological determination of infection……25

2.2.5 DNA extractions……...25

2.2.6 Quantitative PCR……..26

2.2.7 Statistical analyses……26

2.3 Results……..29

2.3.1 Survival……29

2.3.2 Temperature……..31

2.3.3 RLO infections……..32

2.3.3.1 Posterior esophagus (PE)……...32

2.3.3.1.1 Initial PE infections…….32

2.3.3.1.2 Mortalities…….....33

2.3.3.1.3 Survivors versus mortalities……33

2.3.3.2 Digestive gland (DG)…….34

2.3.3.2.1 Initial DG infections……...34

2.3.3.2.2 Mortalities……...35

2.3.3.2.3 Survivors versus mortalities……35

2.3.3.2.4 All exposed abalones……..35

2.3.4 Host response……35

2.3.4.1 DG metaplasia……35

2.3.4.2 Pedal atrophy…….35

2.3.4.3 Body condition……...36

2.3.4.4 All control abalone…….36

2.3.5 Quantitative PCR……..37

2.3.6 Abalone phylogeny, thermal experience & WS relationship……38

2.4 Discussion……41

2.4.1 Pinto abalone have high susceptibility & low resistance to WS…...41

2.4.2 WS induction temperature varies among abalones……...43

2.4.3 Abalone immunity may vary among species & with temperature…44

2.4.4 Abalone phylogeny & thermal experience corresponds with WS resistance……45

2.4.5 Multiple RLOs & ST-RLO evolution……...50

2.4.6 Conclusions & conservation application……..50

Chapter 3. Abalone withering syndrome disease dynamics: infectious dose & temporal stability in seawater

3.1 Introduction……..52

3.2 Methods……55

3.2.1 WS-RLO temporal stability……..55

3.2.1.1 Experimental design……...55

3.2.1.2 Seawater sampling…….56

3.2.2 WS-RLO infectious dose……..56

3.2.2.1 Experimental design……...56

3.2.2.2 WS-RLO dosing…….58

3.2.2.3 Abalone tissue & fecal sampling……...58

3.2.3 DNA & RNA isolation……..59

3.2.4 Quantitative PCR……..60

3.2.5 Statistical analyses……61

3.3 Results……...61

3.3.1 WS-RLO temporal stability……..61

3.3.2 WS-RLO infectious dose……..64

3.4 Discussion……66

3.4.1 WS-RLO temporal stability……..66

3.4.2 WS-RLO infectious dose……..70

Chapter 4. A geographic survey of the *Rickettsia*-like organism and novel phage associated with abalone withering syndrome in California seawater using DNA-based qPCR assays

4.1 Introduction……..73

4.2 Methods……75

4.2.1 Seawater sampling……75

4.2.2 Quantitative PCR……..77

4.2.3 Statistical analyses……77

4.3 Results……..78

4.3.1 Pathogen DNA variation by year……..78

4.3.2 Pathogen DNA variation by zone…….78

4.3.3 Pathogen DNA variation by site type……...84

4.3.4 Pathogen DNA variation within site type…….86

4.3.5 Pathogen DNA variation by sub-site……87

4.3.6 Fine-scale seawater surveys……..88

4.3.7 WS-RLO DNA loads were less than the established infectious dose……89

4.4 Discussion……89

5. Acknowledgments……..94

6. References……..96

Chapter 1. Introduction & literature review

1.1 Introduction

Abalones are primitive marine vetigastropods of the genus *Haliotis* that inhabit the near shore intertidal and shallow subtidal zones. They are ecologically important in engineering habitat by grazing on micro and macroalgae, thereby maintaining open areas for recruitment of conspecifics and other benthic organisms (Geiger & Groves 1999, Roberts 2001), and also support economically valuable fisheries and aquaculture production throughout the world (Gordon & Cook 2001, 2004, Cook & Gordon 2010). Of the over 50 *Haliotis* species world-wide, eight inhabit the north-eastern Pacific (Haaker et al. 1986). They include subtidal species such as the commonly cultured red abalone (*H. rufescens*) and northern or pinto abalone (*H. kamtschatkana*) found in cool waters, the pink (*H. corrugata*) and green (*H. fulgens*) abalones of warmer waters, and the intertidal-shallow subtidal black abalone (*H. cracherodii*). Fishing pressure and disease threaten abalone populations globally (Hobday & Tegner 2000, Rothaus et al. 2008, Tan et al. 2008, Travers 2008). Currently, five California species experiencing population declines receive varying levels of federal protection ranging from "Species of Concern" (pinto, green and pink abalones) to "Endangered" (white and black abalones). These and other species tested to date are all susceptible to the primary established abalone disease in California, withering syndrome (WS; OIE 2012).

WS is a fatal bacterial disease characterized by a severely shrunken body and infection with a *Rickettsia*-like organism (RLO; Fig. 1.1). Friedman et al. (2000) identified and characterized a gastrointestinal RLO provisionally named "*Candidatus* Xenohaliotis californiensis" (WS-RLO) as the pathogen causing WS. The

WS-RLO is an obligate, intracellular bacterium that infects abalone digestive epithelia and causes severe morphological abnormalities within the digestive gland, resulting in physiological starvation followed by anorexia, absorption of pedal musculature, lethargy and death (Friedman et al. 2003, Braid et al. 2005). Transmission of the WS-RLO is likely fecal-oral (Friedman et al. 2002) and initial infections are in the posterior esophagus (Fig. 1.1D) and, to a lesser extent, the intestine of host abalone. Subsequently, metaplasia (the substitution of one mature tissue type for another, Fig. 1.1F) and infection occur in the digestive gland. These digestive gland changes are associated with depletion of glycogen reserves followed by pedal catabolism, atrophy (Fig. 1.1H) and, finally, death (Friedman et al. 2000, Braid et al. 2005). The severity of WS-RLO infection in juvenile red abalone has been directly correlated with negative physiological functions such as decreased feeding rates, metabolism, production of feces, and energy available for growth (Kismohandaka et al. 1993, González et al. 2012).

Two RLOs are known to infect California abalones: the WS-RLO and the stippled RLO (ST-RLO; Fig. 1.2, Table 1.1). Only "*Candidatus* Xenohaliotis californiensis" (WS-RLO) is known to cause WS (Friedman et al. 2000, 2003, Moore et al. 2001), while the ST-RLO appears, to date, to be non-pathogenic and is typically observed at low levels (Friedman et al. *in press*). The WS-RLO infects all members of the genus *Haliotis* examined to date including black abalone (Friedman et al. 1997, 2002), white abalone (Moore et al. 2002, Friedman et al. 2007), red abalone (Moore et al. 2000, 2001), pink abalone (Álvarez-Tinajero et al. 2002), green abalone (Álvarez-Tinajero et al. 2002), the small abalone (*H. diversicolor supertexta*; Wetchateng et al. 2010), Japanese black abalone (*H. discus discus*; Kiryu et al. 2013) and the European abalone (*H. tuberculata*,

Balseiro et al. 2006) in the wild or culture facilities, as well as flat abalone (*H. wallalensis*, Friedman *unpubl. obs.*) and Japanese abalone (*H. discus-hannai*, Friedman *unpubl. obs.*) in laboratory challenges. WS-RLO has not been identified in any non-haliotid hosts, including limpets and snails cohabiting with WS-RLO infected abalone (Moore et al. 2002, CDFW *unpubl. obs.*).

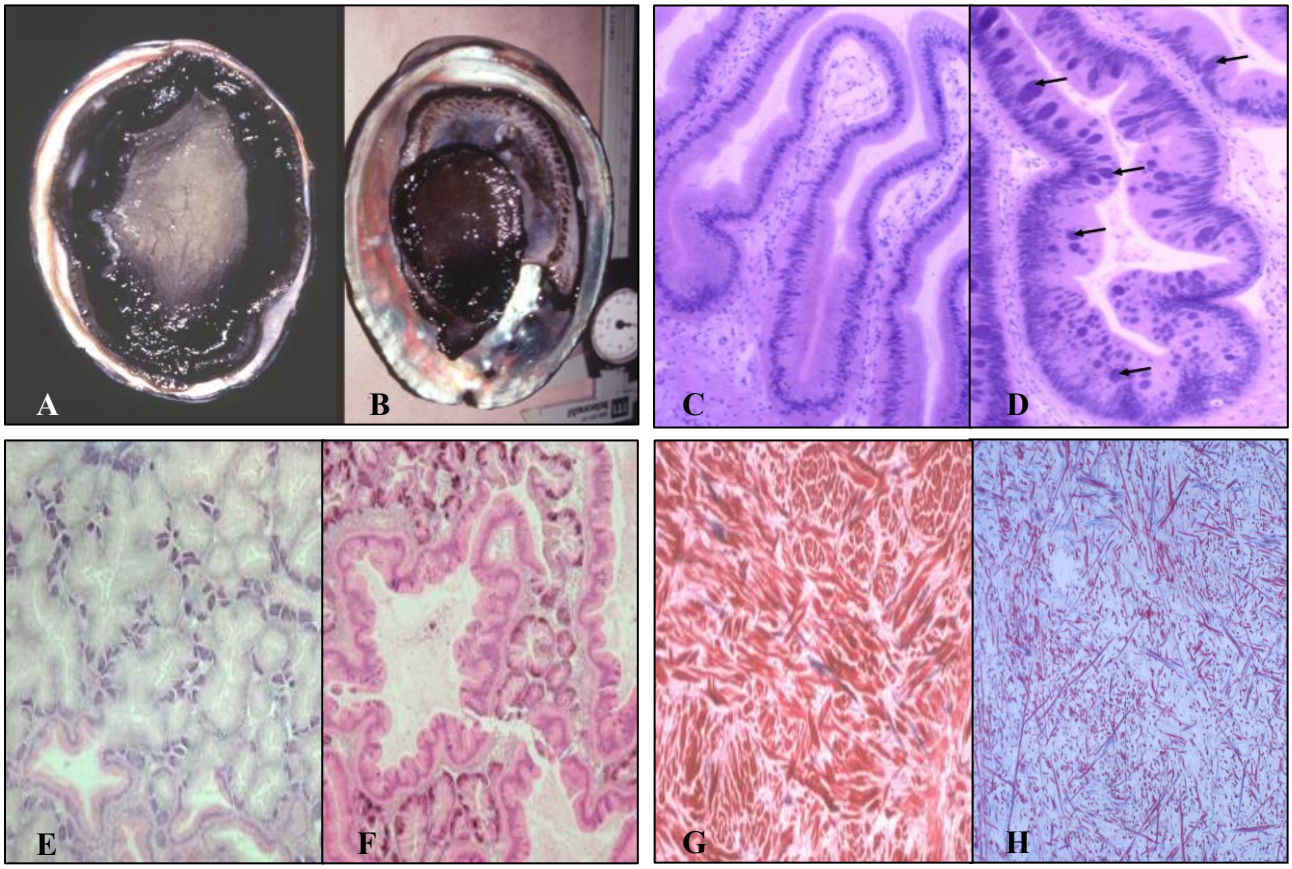

Figure 1.1. Effects of withering syndrome (WS) on abalones. A: Uninfected black abalone. B: Severely withered WS-RLO infected black abalone. C-H: Light micrographs of abalone tissues stained with hematoxylin and eosin (H&E). C: Normal post-esophagus. 200x magnification. D: WS-RLO infected post-esophagus with arrows indicating WS-RLO cytoplasmic inclusions (bacterial colonies). 200x magnification. E: Normal digestive gland. 100x magnification F: Metaplastic digestive gland. 100x magnification G: Normal pedal musculature. 400x magnification. H: Pedal atrophy. 400x magnification.

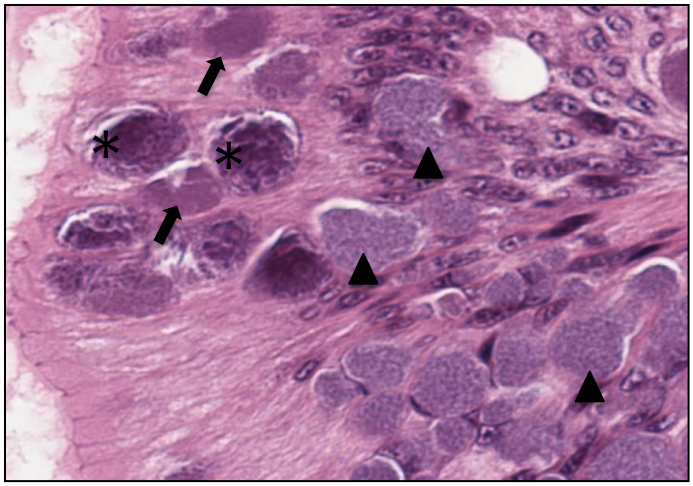

Figure 1.2. Light micrograph of WS-RLO (withering syndrome; arrows), ST-RLO (stippled; arrowhead), and RLOv (variant; asterisk) inclusions infecting the posterior-esophagus epithelium of California abalone. Individual rod to pleomorphic shaped RLOv (phage-infected WS-RLO) are visible by light microscopy while individual WS-RLO and ST-RLO are not. Note differential hematoxylin and eosin (H&E) staining properties of RLOs. 1000x magnification.

Table 1.1. Light microscopy: *Rickettsia* morphologies (mean ± s.d) and hematoxylin and eosin (H&E) staining properties (Friedman & Crosson 2012).

RLO	H&E Staining	Cellular Location	Histology Fixative	Inclusion Width[a]	Inclusion Length[a]	Bacterial Width	Bacterial Length
WS	Violet Violet	Apical Apical	Davidson's 1G4F	14.2±5.3 15.4±7.0	23.2±10.4 26.3±11.0	TSTM TSTM	TSTM TSTM
RLOv	Navy blue Navy blue	Apical Apical	Davidson's 1G4F	16.7±7.8 21.3±7.7	24.1±10.7 26.1±13.3	2.6±1.0 1.5±0.7	3.4±1.0 3.2±1.3
ST	Light blue Light blue	Basal Basal	Davidson's 1G4F	15.26±7.32 ND	19.34±5.49 ND	TSTM ND	TSTM ND

s.d standard deviation, *WS* withering syndrome, *TSTM* too small to measure, *ST* stippled, *ND* not determined

[a] Measured to the nearest 0.01 µm

1.2 Literature review

1.2.1 Disease distribution

WS was first observed in black abalone populations on the south shore of Santa Cruz Island, CA, US in 1985 shortly after the strong 1982-83 El Niño-Southern Oscillation (ENSO) event and subsequently spread to new locations and other abalone host species. From 1986-1989, black abalone population declines and WS were seen at

Anacapa Island followed by losses on Santa Cruz, Santa Rosa, Santa Barbara and San Miguel islands (Davis et al. 1992, Tissot 1995, 2007). WS associated declines in black abalone were first observed at San Nicolas Island (SNI) in 1992 and, as in other affected areas, resulted in markedly increased population declines as compared to pre-WS losses (VanBlaricom et al. 1993, Ruediger 1999). In 1988, WS was observed in Diablo Canyon (Steinbeck et al. 1992) but was not observed elsewhere along mainland California until its discovery north of Point Conception (Altstatt et al. 1996). By 1992, evidence of infected black abalone was reported at all southern California islands except Santa Catalina, especially during seasonal warm water events (Haaker et al. 1992, Tissot 1995, Raimondi et al. 2002).

The 1997-98 ENSO was associated with enhanced clinical signs of disease in wild abalones and also coincided with severe losses in cultured red abalone (Moore et al. 2000, Friedman et al. 2002). WS spread naturally and via anthropogenic movement of farmed red abalone (Friedman & Finley 2003, OIE 2012) throughout southern California, into the warmer waters of Baja California, Mexico (Casares-Martinez & Tinoco-Orta 2001, Álvarez-Tinajero et al. 2002, Garcia-Esquivel et al. 2007) and northward from the late 1980s to the present (Lafferty & Kuris 1993, Alstatt et al. 1996, Miner et al. 2006). Both clinical disease and the WS-RLO were observed as far north as Point San Pedro (San Francisco County, CA) by 1999 (Friedman & Finley 2003). Clinical WS continues to spread in a northward direction (Miner et al. 2006) and is strongly associated with declines in abundance co-occurring with increasing coastal warming and El Niño events (Tissot 1995, Alstatt et al. 1996, Raimondi et al. 2002). The WS-RLO is continuously distributed along the west coast of North America from Baja California, Mexico, to

southern Sonoma County, California, including the Channel and Farallon Islands (Álvarez-Tinajero et al. 2002, Friedman & Finley 2003, CDFW *unpubl. obs.*). During a 1999-2000 sampling event, WS-RLO was identified in two red abalone populations in northern California, Van Damme State Park and Crescent City (Friedman & Finley 2003), but has not been detected at those locations since, including histological examination of over 700 red abalone from Van Damme during 2001-2009 (CDFW *unpubl. obs.*). The anthropogenic introductions at these locations may have failed to become established because of low temperatures. As infected abalones have been transported to Chile, China (People's Rep. of), Taiwan, Iceland, Ireland, Israel, Spain, Thailand (Wetchateng et al. 2010), and most recently Japan (Kiryu et al. 2013), and possibly other countries, the geographical range of the etiological agent is suspected to be broad where California red abalones are cultured or in areas where native species have been exposed to this species.

1.2.2 Role of temperature

Climatic changes and short-term ocean temperature increases have the potential to significantly alter host-parasite dynamics in abalones infected with bacterial pathogens such as RLOs and make WS one of the most severe threats to abalone populations (Neuman et al. 2010). Temperature can modulate both the transmission and development of WS (Moore et al. 2000, Braid et al. 2005, Vilchis et al. 2005). Thermal induction and increased disease expression have been documented in both lab challenged and field RLO-infected animals including red (Vilchis et al. 2005, Moore et al. 2000, 2011) and black (Tissot 1995, Friedman et al. 1997, 2002) abalones. WS-RLO transmission and subsequent WS development in red abalone were nearly negated at 12.3^{o}C (only 1%

transmission and no clinical signs of disease), while up to 94% transmission and extreme clinical signs were observed at 18.7ºC (Braid et al. 2005). Although a relationship between food availability (fed or complete starvation) and WS-RLO transmission was observed (Braid et al. 2005), under more realistic feeding conditions (100%, 30% and 10% feeding rates) food availability and WS-RLO transmission were not correlated, further illustrating the importance of temperature in the ecology of this disease (Vilchis et al. 2005).

Temperature appears to have a significant influence on WS in the field. Since initial observation after the 1982-1983 ENSO, WS has been repeatedly associated with seasonal or decadal thermal events (Haaker et al. 1992). Steinbeck et al. (1992) investigated mortality of black abalone within, and adjacent to, the discharge plume of the Diablo Canyon Power Plant during 1988-1989 and found that animals with clinical signs of WS were located exclusively in the thermal discharge zone where water temperatures measured up to 11°C above ambient. Lafferty & Kuris (1993) also discovered a significant correlation between WS mortality rates and warmer locations. Tissot (1995) suggested high temperature was the most important factor limiting black abalone population recovery on Santa Cruz Island. Subsequently, during the severe 1997-1998 ENSO, when markedly elevated seawater temperatures occurred throughout southern and central California, up to 70% of black abalone at surveyed field sites showed clinical signs of WS (Raimondi et al. 2002, Friedman et al. 2003). High daily temperature variability may also increase the susceptibility of black abalone to WS infection, although disease expression was not seen in abalone until temperatures exceeded thresholds known to facilitate infection (Ben-Horin et al. 2013). Since the mid-

1970's decadal regime shift, thermal anomalies have been more common and of longer duration than during the previous 25 years (NOAA CoastWatch 1998). ENSO-neutral conditions were predicted through middle 2009. However, given that annual seasonal thermal maxima in southern California typically reach 17-19^{o}C, temperatures known to augment WS, understanding the role of both seasonal and anomalous ocean warming is crucial to understanding the ecology of marine diseases (NOAA CoastWatch 1998).

1.2.3 Host susceptibility & disease resistance

While the WS-RLO infects all haliotids tested to date, susceptibility varies among species. Levels of WS range from little effect and no mortality (e.g. wild green and pink abalone: Álvarez-Tinajero et al. 2002, Moore et al. 2009) to moderate mortality (e.g. red abalone: Moore et al. 2000, 2001) to catastrophic impacts with up to 99% population mortality over a span of several years on large spatial scales (e.g. black abalone: Altstatt et al. 1996, Moore et al. 2002, Raimondi et al. 2002, Miner et al. 2006, Friedman et al. 2007), conferring significant alterations to marine nearshore biodiversity (Haaker et al. 1992, Tissot 1995, Friedman et al. 2000, Miner et al. 2006). Vilchis et al. (2005) conducted a long-term study in which development of clinical WS was observed in red but not green abalone at elevated temperatures. These results agreed with a similar study conducted by Moore et al. (2009) in which green abalone exposed to WS-RLO were relatively resistant to disease expression under ENSO conditions. However, thermal modulation remains a key factor as demonstrated by Garcia-Esquivez et al. (2007) who observed green abalone held at elevated laboratory temperatures of 25°C experienced more clinical signs of WS and higher mortality then those held at 20°C. A survey of wild abalones from Baja California showed 32% of the pink and 27% of green abalones had

clinical signs of WS. Little (<7%) to no abalones had advanced signs of WS and clinical signs of WS did not correlate with WS-RLO presence (Álvarez-Tinajero et al. 2002).In contrast, white and black abalones are highly susceptible to WS-RLO infection: up to 100% mortality (Fig. 1.3; Crosson et al. 2014) and 99% (Friedman et al. 2002, Raimondi et al. 2002), respectively. White abalone captive rearing programs have experienced substantial losses after ~2-3 years of culture when animals succumbed to WS-RLO infections and most died from the disease (Friedman et al. 2007). A laboratory study was conducted to compare the susceptibility of white and green abalones to WS when held at 18°C and few losses (<20%) were observed in green abalone over the course of 26 weeks, while 100% of white abalone died within 13 weeks (Fig. 1.3; Crosson et al. 2014). Initial losses of green abalone were attributed to handling stress, not WS, as no green abalone died after the first 10 weeks of study when few individuals were infected.

In addition to differences in disease susceptibility among species, it was recently observed that different populations of a single species appear to respond differently to the presence of WS. Black abalone from San Nicolas Island (SNI) have been under significant WS pressure for over 20 years (Fig. 1.4A; VanBlaricom et al. 1993). Between 1992-2001, a 99.2% decline in black abalone density (11.22 to 0.095/m^2, respectively, in permanent plots sited purposely in high-density patches of abalone) occurred on SNI due to WS. From 2002 to 2012, abalone densities on SNI increased over 200% from the minimum in 2001, via recruitment events and apparently improved survival rates despite presence of the WS-RLO (Fig. 1.4B; Crosson et al. 2014). Thus despite catastrophic losses, a small number of black abalone survived to reproduce. The observation of population increases on SNI suggested that the 1-2% of black abalone that survived WS

epidemics were more resistant to WS than populations not experiencing disease pressure (Friedman et al. 2014b). It was hypothesized that survivors were able to resist infection by mounting a sufficient immune response and/or resisting bacterial secretions thought to induce host metaplasia.

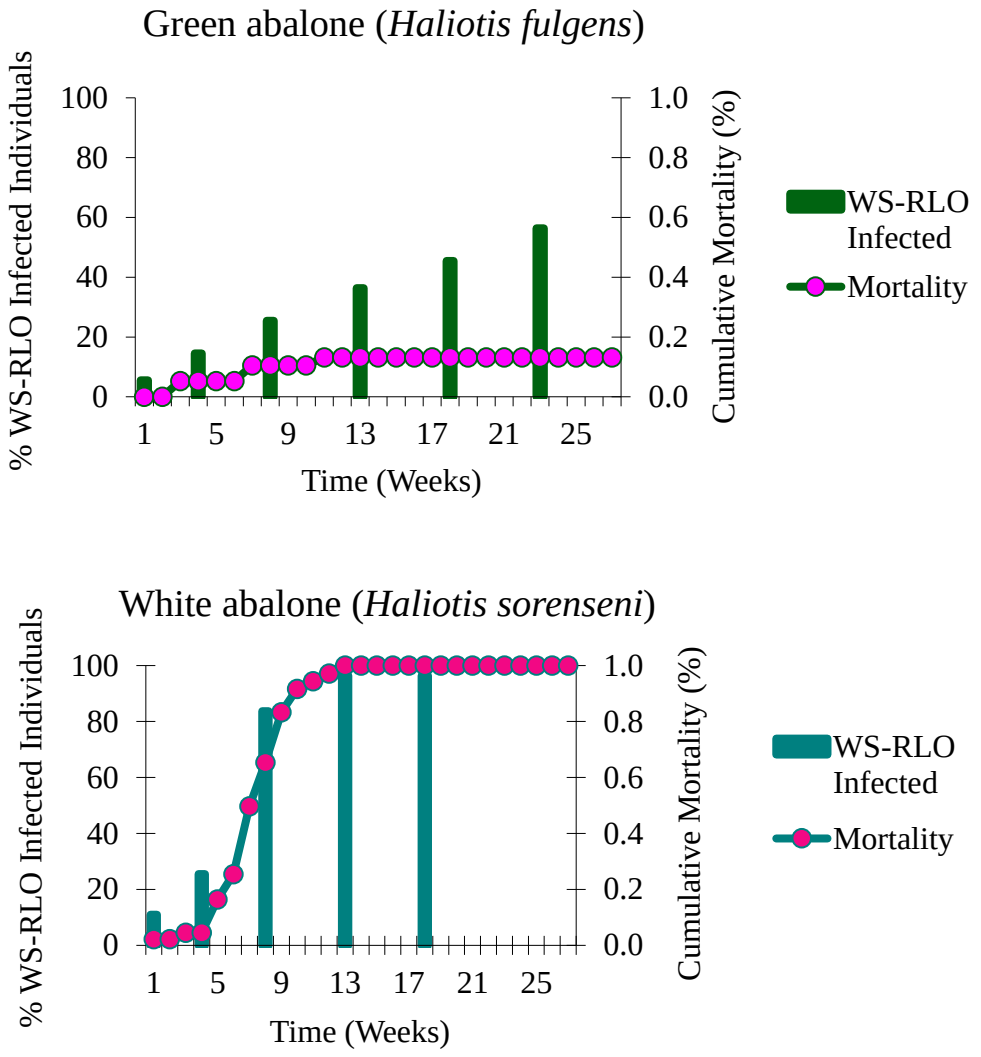

Figure 1.3. Differential susceptibility of green and white abalones to withering syndrome (WS). Bars represent the percent of WS-RLO infected individuals, while circles represent cumulative proportion mortality (Friedman, unpubl. data).

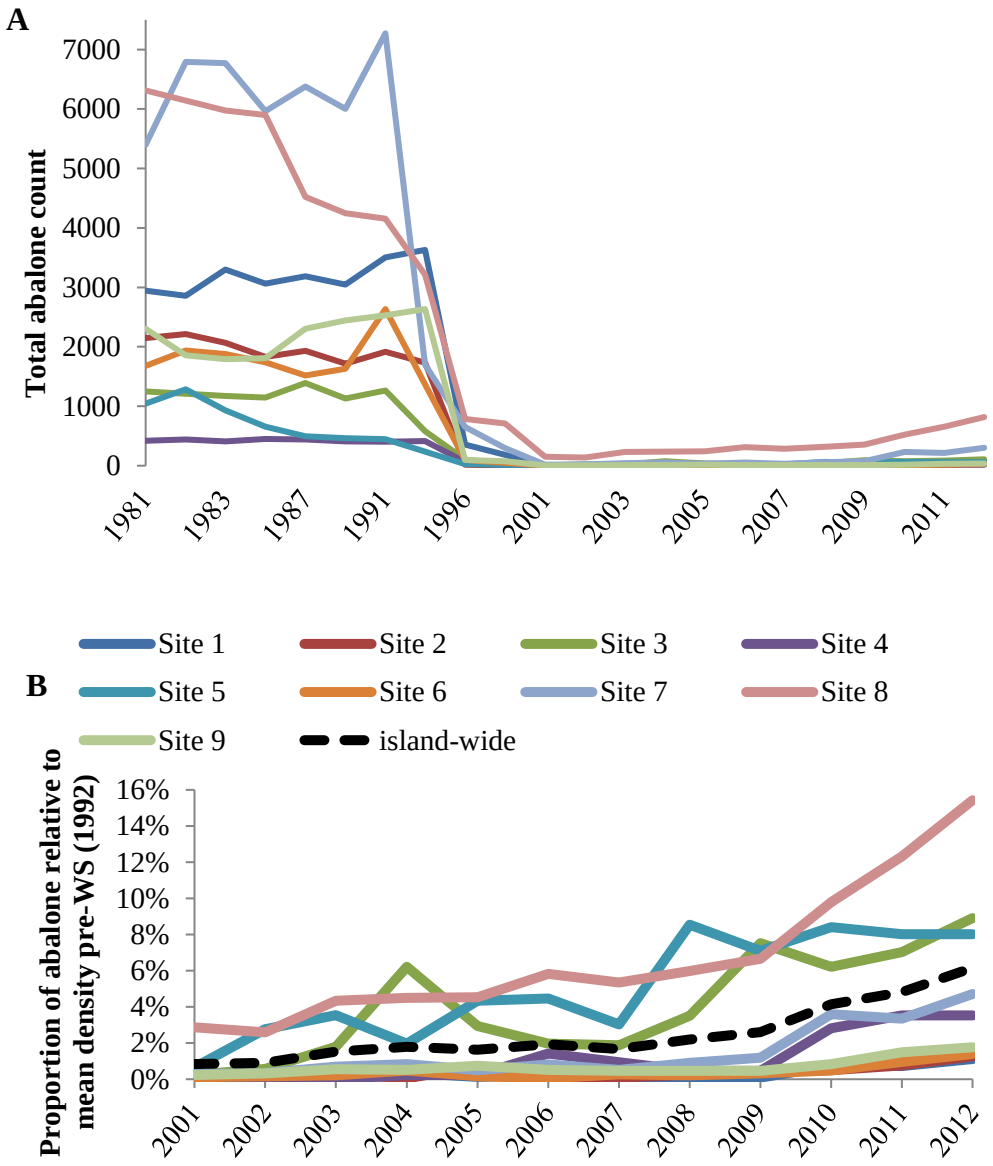

Figure 1.4. Black abalone population trends on San Nicolas Island (SNI), CA. A: Total abalone counts from 1981-2012. Note sharp population declines after withering syndrome (WS) was first observed in 1992. Declines at some sites prior to 1992 are likely due to overfishing. B: 2001-2012 only. Proportion of abalone relative to mean abalone density pre-WS epidemics (1992). Note strong recruitment event at Site 8, where abalone for disease resistance testing were collected (VanBlaricom 1993, unpubl. data).

The hypothesis that abalone populations under disease pressure selected for the development of disease resistance was tested in the laboratory using progeny of surviving abalone from SNI (Site 8 animals, Fig. 1.4B) and "naive" black abalone from Carmel, CA that had not been exposed to WS epidemics and thus were not under selection for improved tolerance (Friedman et al. 2014b). Upon RLO exposure at 19^{o}C, decreased mortalities were observed in SNI abalone compared to those from Carmel (Friedman et al. 2014b). Significant differences in survival were observed among treatments ($P < 0.001$); more RLO exposed abalone from Carmel died than did those from SNI ($P < 0.05$), while no differences in survival were observed between control groups ($P > 0.05$). All RLO exposed abalone that died had clinical signs of WS and microscopic examination suggested that resistance to WS might be more related to the host response to initial infection than to the ability to resist infection, as resistant abalone showed significantly less metaplasia and a corresponding lower RLO infection intensity in the digestive gland (Fig. 1.5; Friedman et al. 2014b). Analysis of WS-RLO DNA by quantitative PCR (qPCR) of feces from both populations showed that more WS-RLO DNA was excreted from Carmel abalone compared to those from SNI suggesting that abalone from SNI (survivors of high disease pressure) express a trait or have some characteristic that decreases the ability of RLOs to proliferate in the digestive gland (Fig. 1.5; Friedman et al. 2014b). Clearly a distinct difference in disease resistance exists among black abalone populations independent of temperature. Whether or not the observed differences are of genetic origin was explored in Crosson & Friedman (2018; Chapter 2).

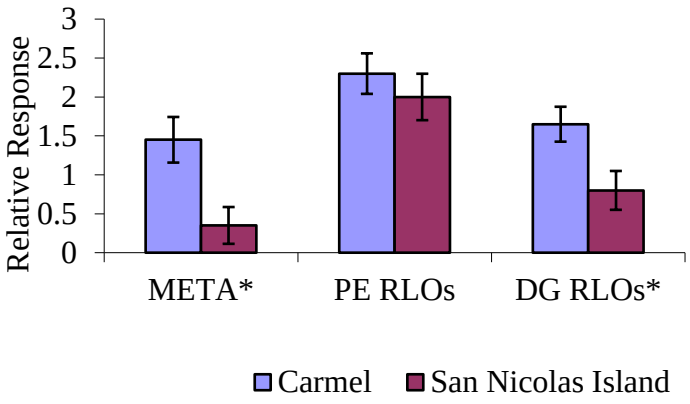

Figure 1.5. Microscopic observations of hematoxylin and eosin (H&E) stained black abalone tissues. Significant differences between San Nicolas Island (WS resistant) and Carmel (WS "naive") animals are noted (asterisk). Error bars represent standard error. Meta: Metaplastic response. PE RLOs: Combined RLO intensity in the posterior esophagus. DG RLOs: Combined RLO intensity in the digestive gland (Friedman et al. 2014b). See Friedman et al. 2002 for relative response scoring.

1.2.4 Bacteriophage hyperparasite

Friedman & Crosson (2012) recently observed a morphological variant of the WS-RLO infecting red abalone from central California and used a combination of light and electron microscopy, *in situ* hybridization and 16S rDNA sequence analysis to compare the WS-RLO and the RLO variant (RLOv). WS-RLO morphology has been consistent with its original taxonomic description (Friedman et al. 2000) and forms oblong inclusions within the abalone posterior esophagus (PE) and digestive gland (DG) tissues that contain small rod-shaped bacteria; individual bacteria within inclusions, which appear light purple upon hematoxylin and eosin (H&E) staining, cannot be discerned by light microscopy (Table 1.1, Fig. 1.2; Friedman & Crosson 2012). Like the WS-RLO, the RLOv forms oblong inclusions in the PE and DG but contain large, pleomorphic bacteria that stain dark navy blue with H&E (Table 1.1, Fig. 1.2; Friedman

& Crosson 2012). Transmission electron microscopy (TEM) examination revealed that the large pleomorphic bacteria within RLOv inclusions were infected with a spherical to icosahedral-shaped phage hyperparasite (Fig. 1.6; Friedman & Crosson 2012). Binding of the WS-RLO-specific *in situ* hybridization probe to the RLOv inclusions demonstrated sequence similarity between these RLOs. In addition, sequence analysis revealed 98.9-99.4 % similarity between 16S rDNA sequences of the WS-RLO and RLOv. Collectively, these data suggest that both RLOs infecting California abalone are "*Candidatus* Xenohaliotis californiensis," (WS-RLO) and that the novel variant is infected by a phage hyperparasite that induced morphological variation of its WS-RLO host.

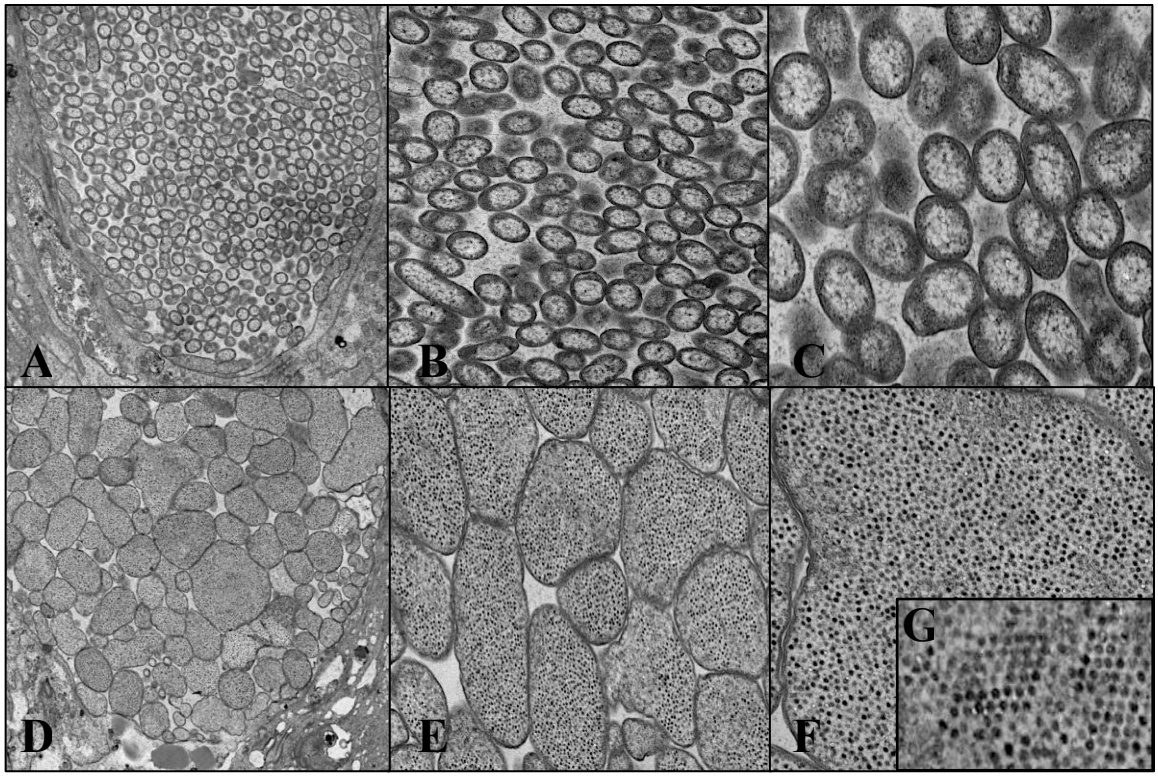

Figure 1.6. Transmission electron micrograph (TEM) of abalone rickettsial inclusions following preservation in Karnovsky's solution. WS-RLO at (A) 6800x, (B) 13000x, and (C) 30000x magnification. RLOv (phage-infected WS-RLO) at (D) 4800x, (E) 13000x, and (F) 30000x magnification. G: Inset illustrating phage morphology and virions in paracrystalline array (Friedman & Crosson 2012).

The presence of a phage hyperparasite exhibits interesting properties that appear to affect the host-pathogen relationship between the WS-RLO and abalone. For example, in a recent experiment with juvenile black abalone, both WS-RLO and phage-infected inclusions were statistically related to tissue pathology and mortality ($P < 0.05$; Friedman et al. 2014b). Like the WS-RLO, the phage-infected inclusions appeared to increase in prevalence and intensity with increasing temperature (Crosson & Friedman 2018). Curiously, mortalities of abalone infected with all RLO types appeared to be delayed and significantly reduced relative to previous studies with the WS-RLO alone (Friedman et al. 2014b). When black abalone were exposed to WS-RLO, ST-RLO, and RLOv in combination, the trial lasted 17 months during which 48% of the animals died. However, when abalone were exposed to the WS-RLO alone, they experienced 71% mortality in only 7 months. In addition, when the phage (RLOv) was absent, WS-RLO loads were higher and host metaplastic response was ~2x that observed when the phage was co-occurring (Friedman et al. 2014b). It is likely that the presence of the phage is attenuating WS disease development and consequences of infection will vary among host species and with temperature. Current studies are underway to discern if the phage infections alter physiological processes, such as virulence and pathogenicity, in its WS-RLO host. Also of interest, red abalone farms in California experiencing seasonal losses due to WS since ~ 1990 (e.g. Moore et al. 2000) and with confirmed RLO infected animals are currently reporting decreased losses in product-sized abalone relative to previous trends prior to the observation of the phage hyperparasite (R. Fields pers. comm.).

1.2.5 Diagnostic methods

Diagnosis of WS requires the identification of infection with the pathogen (WS-RLO via *in situ* hybridization or via histology coupled with PCR and sequence analysis) accompanied by morphological changes that characterize the disease (e.g. pedal and digestive gland atrophy, and digestive gland metaplasia). Definitive diagnosis of WS must be conducted according to World Organization of Animal Health (OIE) standards in the Manual of Diagnostic Tests for Aquatic Animals (OIE, 2012). *In situ* hybridization is the method of choice for WS-RLO confirmation because it allows visualization of a specific DNA probe hybridized to the target pathogen. The *in situ* hybridization technique for WS-RLO developed by Antonio et al. (2000) is extremely useful in visualizing initial stages of infection in sub-clinically infected abalone. Although this method was not formally validated, tests for specificity using several bivalve and fish RLOs suggested the test was specific for WS-RLO only (Antonio et al. 2000).

A conventional PCR assay that specifically amplifies a 160 bp segment of the WS-RLO 16S rDNA sequence available in GenBank (AF133090) was developed by Andree et al. (2000) and allows for greater sensitivity than histology alone. A quantitative PCR (qPCR) assay was also developed to specifically identify and enumerate bacterial loads of WS-RLO in abalone tissue, fecal and seawater samples based on 16S rDNA gene copy numbers (Friedman et al. 2014a). Both PCR assays designed to detect DNA of the WS-RLO were formally validated according to OIE (2012) standards (Friedman et al. 2014a). The conventional PCR assay limit of detection was 300 gene copies and 3 gene copies for qPCR. Thus, qPCR was over 100x more sensitive than conventional PCR in detecting target DNA (Friedman et al. 2014a). Also, the ability of qPCR to detect and

quantify very small amounts of WS-RLO gene copies in a variety of sample types will enable researchers to better understand WS transmission dynamics in both farmed and natural environments while providing a useful, non-lethal tool for WS monitoring. However, it is important to note that DNA-based PCR assays do not detect a viable agent or infection and serve only as a proxy for infection or exposure. Histological examinations remain the gold standard and show clear evidence of infection but may not enable one to discern the taxonomy of the agent (Burreson 2008, OIE 2012). Both conditionally independent tests should be used collectively for proper WS diagnosis.

1.2.6 Disease control

The most effective prevention of WS is avoidance of the pathogen. Avoidance is best accomplished by the establishment of a health history and multiple health examinations prior to movement of animals. Although histology or *in situ* hybridization are required to confirm infection, PCR is able to detect small amounts of pathogen DNA and is recommended for inclusion in health examinations. Good husbandry practices are essential for control of any bacterial disease and include reducing stocking densities, avoiding grading or mixing of disparate groups or families, and rinsing hands and equipment in fresh or iodinated water between groups/tanks. Holding abalones at cooler temperatures (<15 ^{o}C) may also reduce WS-RLO transmission (Braid et al. 2005). Infected groups should be isolated and culled or administered oral or bath treatments with oxytetracycline as per federal regulations (Friedman et al. 2003, 2007).

1.3 Rationale & significance

Much research on host-parasite relationships involves interactions between a single host and one parasite/pathogen. However, evidence from a wide variety of systems

suggests that mixed infections involving two or more parasite genotypes or species in a single host are becoming more common and, in some cases, may be the rule. Multiple pathogen infections have been examined in numerous host systems including a variety of invertebrates such as oysters (Stokes & Burreson, 2001), crustaceans (Tang et al. 2003) and abalones (Hine et al. 2002, Balseiro et al. 2006). The RLO-abalone system provides a unique opportunity to conduct multidisciplinary research examining the influence of single, dual and multiple parasites on closely related host species with varying susceptibilities to infection, disease development and subsequent RLO transmission. This system also provides insight not only for disease in abalones, but also in other marine hosts: intracellular bacterial pathogens are also problematic in marine shrimp (Loy et al. 1996), fishes (Fryer & Mauel 1997) and other species. In addition, new RLOs are being discovered with increased frequency in the marine environment (Delgado et al. 2007, Eddy et al. 2007, Chang et al. 2008, Friedman et al. 2008, Horwitz 2008). Understanding the role of abalone-RLO relationships under varying environmental conditions will be imperative to abalone resource management in the face of global climate change. To achieve protection and sustainable use of abalone resources, we must also understand interactions among wild and farmed animals and their potential impacts on disease transmission dynamics, especially in declining and endangered species.

Chapter 2. Withering syndrome susceptibility of northeastern pacific abalones: a complex relationship with phylogeny & thermal experience

2.1 Introduction

Abalones (*Haliotis* spp.) play important economic and ecological roles in the marine environment by supporting valuable fisheries and aquaculture production around the world (Cook & Gordon 2010) and acting as ecosystem engineers through their herbivory (Miner et al. 2006). Abalone graze macro and micro-algae, which maintains open habitat for benthic organisms (Roberts 2001). Of the over 50 abalone species worldwide, seven inhabit the northeastern Pacific Ocean (Haaker et al. 1986). Currently, five of these seven species are experiencing population declines and receive varying levels of federal protection ranging from "Species of Concern" [pinto (*Haliotis kamtschatkana*), green (*H. fulgens*) and pink (*H. corrugata*) abalones] to "Endangered" [white (*H. sorenseni*) and black (*H. cracherodii*) abalones; reviewed by Crosson et al. 2014]. One of the main drivers of black abalone population declines is a chronic bacterial disease, withering syndrome (WS), which has been responsible for moderate to catastrophic mortality events over the past three decades along the northeastern Pacific Ocean (Haaker et al. 1992, Friedman et al. 2000, Crosson et al. 2014).

Withering syndrome is a fatal disease of abalones characterized by a severely shrunken body and infection with a *Rickettsia*-like organism (RLO; Haaker et al. 1992, OIE 2014). The etiological agent was described and provisionally named "*Candidatus* Xenohaliotis californiensis" (WS-RLO; Friedman et al. 2000). The WS-RLO is an obligate, intracellular bacterium that infects abalone gastro-intestinal epithelia and causes severe morphological and functional abnormalities within the digestive gland (DG). The bacterium is transmitted horizontally via a fecal-oral route with initial

infections located in the posterior esophagus (PE) tissue and, to a lesser extent, the intestine of host abalone (Friedman et al. 2002). Metaplasia and subsequent RLO infection occur in the DG, which lead to physiological starvation, catabolism of the foot musculature, lethargy and eventually death (Gardner et al. 1995, Friedman et al. 2002, Braid et al. 2005).

Currently, three morphologically distinct RLOs infect California abalones: the WS-RLO, a phage infected WS-RLO variant (RLOv; Friedman & Crosson 2012) and an uncharacterized RLO with stippled morphology (ST-RLO; Crosson et al. 2014). The WS-RLO was described from California abalones with WS after its first observation in the mid-late 1980's (VanBlaricom et al. 1993, Gardner et al. 1995, Friedman et al. 1997, 2000). The ST-RLO was first observed in the mid-1990s (Friedman & Moore, pers. obs.) and appeared to be non- or lowly pathogenic (Friedman et al. 2014b, Crosson et al. 2014). The RLOv, first observed in the mid-2000s, is the most recently described RLO type and appears to modulate the effects of RLO infection (Friedman & Crosson 2012, Friedman et al. 2014b). Previous research demonstrated differences in WS susceptibility (the ability to become infected; Boots & Bowers 1999) and resistance (defense of pathogen once infected; Boots & Bowers 1999) among northeastern Pacific host species including the highly susceptible and low resistant white (Crosson et al. 2014) and black abalones (Altstatt et al. 1996, Friedman et al. 1997, 2002, 2014b, Miner et al. 2006, Tissot et al. 2007), the moderately susceptible and resistant red abalone (*H. rufescens*; Moore et al. 2000, Braid et al. 2005, Vilchis et al. 2005), and the near refractory or tolerant (no disease expression despite infection; Boots & Bowers 1999) green abalone (Álvarez-Tinajero et al. 2002, Vilchis et al. 2005, Moore et al. 2009, Crosson et al. 2014).

To characterize factors that contribute to susceptibility of RLO infection and resistance or tolerance to WS once infected, we exposed three northeastern Pacific abalone species to experimental infections with a combination of the three known RLO types from naturally co-infected animals and tracked disease development and abalone mortality at temperatures known to augment RLO proliferation. The abalone species tested included the warm water (12-23°C) pink abalone and the cool water (4-14°C) pinto or northern abalone for which little to no WS susceptibility data was previously available. We also tested red abalone, which inhabit intermediate water temperatures (8-18°C) overlapping the thermal ranges of the other two species tested and whose moderate susceptibility to WS has been extensively studied as a control (Dahlhoff & Somero 1993, Moore et al. 2000, Braid et al. 2005, Vilchis et al. 2005). We hypothesized that abalones adapted to cool water environments would be most susceptible to RLO infection and subsequent WS development, while warm water abalones would be most refractory. We also examined if WS-induced mortality was related to phylogenetic distance from the white abalone, which is the most susceptible host species tested to date. RLO susceptibility and WS resistance was hypothesized to be associated with abalone phylogeny; closely related species will have a similar host response to WS. Additionally, our experiment provides further characterization for evolving abalone-RLO relationships.

2.2 Methods

2.2.1 Animals

Three species of abalones were used for this experiment conducted from January 2009 to June 2010: pinto, red, and pink abalones. All pinto abalone (n = 84, mean size ± SD = 32.36 ± 14.75 mm) were produced at the National Oceanic and Atmospheric

Administration's (NOAA) Mukilteo Biological Field Station using wild broodstock collected from Puget Sound, WA, US. Red abalone (n = 84, mean size ± SD = 47.42 ± 5.53 mm) were received from The Abalone Farm, Inc. (Cayucos, CA, US) and pink abalone (n = 84, mean size ± SD = 43.74 ± 5.69 mm) were supplied by the NOAA Southwest Fisheries Science Center (La Jolla, CA, US). In addition, we received the following abalone from The Abalone Farm, Inc.: 35 red abalone to serve as tank independent controls (TIC; mean size = ~45 mm) and 15 RLO infected red abalone (mean size = ~85 mm) to serve as donor animals for infection with all three RLO types. The RLOs remain unculturable and experiments involving single RLO types are currently not possible. All abalones were housed at the School of Aquatic and Fishery Sciences-University of Washington Pathogen Quarantine Facility and acclimated for 8 weeks at 14°C following methods outlined in Friedman at al. (2014b) prior to the start of the experiment. To remove any pre-existing RLO infections, all abalones, except RLO infected donor animals, were medicated with oxytetracycline (OTC) at a dose of ~90 mg/kg weight for three days (Friedman et al. 2007). OTC administration and depletion were conducted according to the methods of Friedman et al. (2003, 2007) and Rosenblum et al. (2008).

2.2.2 Experimental design

After OTC depletion, abalones were randomly distributed between two 400 L recirculating seawater systems. One system was designated for RLO exposed or treatment abalones and the other system for unexposed (control) abalones. Each system contained duplicate tanks of pinto, red and pink abalones (n = 6 tanks total) and each tank contained 21 abalones (n per species = 42 with a total n = 126; Fig. 2.1). Seawater was

circulated through a biological filter (canister with 50 μm polystyrene media), a mechanical/chemical filter (canister with 25 μm pleated filter and carbon media), UV-irradiation, and temperature controllers (±0.5°C) prior to re-entering each system via a head tank (Fig. 2.1). Weekly water chemistry and abalone feedings were conducted according to the methods of Friedman et al. (2014b). Fifteen RLO infected red abalone were added to the head tank of the experimental system to provide an equivalent dose of pathogen to each abalone tank; 15 uninfected red abalone were added to the control system head tank. At the start of the experiment all abalones were held at a seawater temperature of 18°C, a temperature known to facilitate RLO proliferation (Moore et al. 2000, Friedman et al. 2002, Braid et al. 2005). However, pinto abalone suffered mortalities due to thermal stress at 18°C (Paul & Paul 1981). Therefore, one-week post-RLO exposure the seawater temperature on all systems was lowered to 17°C. All infected and uninfected red abalone were removed from the head tanks after a six-week exposure period and an additional tank containing 10 uninfected red abalone (TIC) was added to each system to test the sterilization efficacy of the seawater recirculation systems. After 6.5 months at 17°C (and a 50% loss of pinto abalone but no losses of other species), seawater temperature was increased incrementally over the next 9.5 months in an effort to induce losses in red and pink abalones as follows: 18°C for 3 months to 18.5°C for 1 month to 19°C for 4 months and finally to 20°C for 1.5 months. Each system was monitored six days per week for temperature and the presence of moribund (lethargic and weakly attached) or dead abalones, which were immediately removed and sampled for animal condition, histological determination of infection, and qPCR analysis (methods below). Two abalone from each tank were also removed and sampled as above at 14, 30,

90, 150, 240, 360 and 420 days to examine temporal differences in disease progression. Abalone fecal samples were collected weekly from each tank and processed for assessment of WS-RLO DNA presence via qPCR (Friedman et al. 2014a). Upon termination of the experiment at 480 days, all remaining control and treatment abalones were sampled.

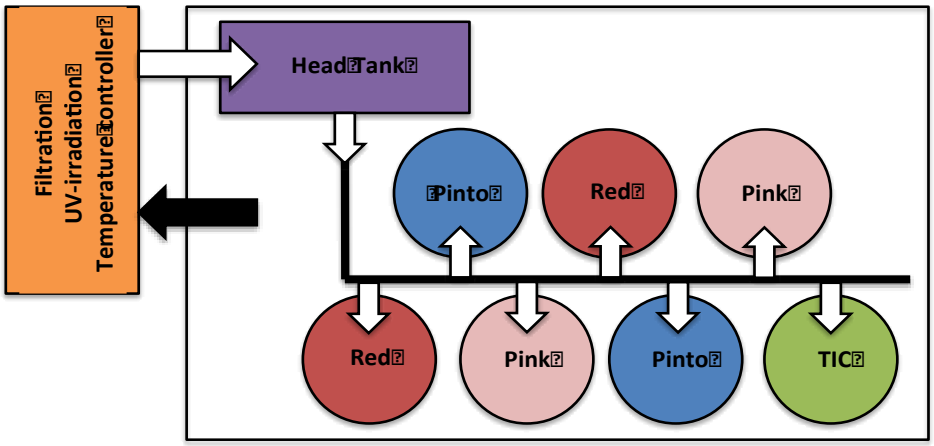

Figure 2.1. Recirculating seawater system used for RLO exposed and control abalones. Seawater was pumped through a series of filters, UV-irradiation, and temperature controllers prior to re-entering a head tank holding either infected (RLO exposed) or uninfected (control) red abalone. To ensure equivalent flow, head tank seawater was gravity fed to the duplicate pinto (*Haliotis kamtschatkana)*, red (*H. rufescens*) and pink (*H. corrugata*) abalone tanks. TIC represents a tank independent control of uninfected red abalone added six weeks post-exposure to test the sterilization efficacy of the recirculating seawater systems.

2.2.3 Animal condition

At the time of sampling, abalone were weighed (total weight, shell weight, body weight) and measured (maximum shell length). A body mass condition index was calculated from these data: total weight (g)/length (cm3) (Moore et al. 2000, Braid et al. 2005).

2.2.4 Histological determination of infection

A 2-3 mm tissue cross section including PE, DG, and pedal muscle was aseptically excised just posterior to the right kidney-DG junction from all moribund or dead abalone (referred to as mortalities) and those sampled at specific time points. Tissues were preserved in Davidson's fixative (Shaw & Battle 1957) for 24 h and stored in 70% ethanol until being processed by routine paraffin histology. Deparaffinized 5µm sections were stained with hematoxylin and eosin (Luna 1968) and examined by light microscopy. RLO infection intensities (each RLO type separately as well as total RLO intensity or co-infection with all three RLO types) in PE and DG tissues were scored on a (0) – (3) scale that estimated the number of RLO inclusions per 20x field of view: (0) no infection, (1) 1-10, (2) 11-100, and (3) >100 (Friedman et al. 2002). For each abalone examined, all PE and DG tissues were screened and the mean number of RLO inclusions per field of view was reported. Associated tissue changes (DG metaplasia and pedal atrophy) were also scored on a similar relative response scale where (0) represented normal tissue, (1) indicated ≤10% change, (2) indicated 11-25% change and (3) indicated >25% change (Friedman et al. 2002). Selected samples were examined by *in situ* hybridization to assess if the ST-RLO was a variant of the WS-RLO according to the methods of Antonio et al. (2000).

2.2.5 DNA extractions

A 150-200 mg sample of abalone PE tissue, which is the target tissue for RLO infections (Friedman et al. 2000), was aseptically excised directly adjacent to the histological section and preserved in 100% molecular grade ethanol. DNA was extracted using a QIAamp DNA Stool Mini Kit (Qiagen Inc., Hilden, Germany) according to the

manufacturer's "Isolation of DNA from Stool for Pathogen Detection" protocol and eluted in 100 µl of buffer. Abalone feces were collected, and DNA was immediately extracted as described above. All DNA extracted from tissue and fecal samples was stored at -20°C until qPCR analysis.

2.2.6 Quantitative PCR

Quantification of WS-RLO DNA was conducted using the validated qPCR assay of Friedman et al. (2014a). Briefly, qPCR reactions were conducted using 12.5 µl 2x GoTaq® Probe qPCR Master Mix (Promega Corp., Madison, WI, US), 320 nM of each primer, 200 nM hydrolysis probe (Applied Biosystems, Foster City, CA, US), 0.6 mg/µl BSA (New England Biolabs Inc., Ipswich, MA, US), 2 µl of DNA template, and sterile water to bring the final volume to 25 µl per reaction. Thermal cycling conditions were 95°C for 10 min, followed by 41 cycles of 95°C for 15 s and 60°C for 30 s. Each sample was run in duplicate along with a negative template control and plasmid-based standard curve of known WS-RLO copy numbers. The quantification cycle threshold was set to 580 relative fluorescence units for comparison of amplification among reactions. WS-RLO DNA copy numbers for each sample were determined using a standard curve. Tissue and fecal sample loads were calculated as copies per gram. All samples negative for WS-RLO DNA via qPCR were tested using 18S rDNA primers of Le Roux et al. (1999) to ensure the presence of amplifiable DNA.

2.2.7 Statistical analyses

All data were analyzed in SigmaPlot 11.0 (Systat Software Inc., Berkshire, UK) or JMP 12.0 (SAS Institute Inc., Cary, NC, US). Survival data were analyzed using a Kaplan-Meier log-rank survival analysis with pairwise multiple comparisons using the

Holm-Sidak method at a significance level = 0.05 (SigmaPlot 11.0). All pinto abalone mortalities prior to 43 days post-exposure (n = 12; 3 experimental and 9 control) were discarded from analyses because these animals died from thermal stress, not RLO infections (all RLO associated histological scores = 0). Five red abalone died on day 425 post-exposure but were too necrotic for histological assessment and were removed from all analyses except survivorship. Animal condition, temperature, and histological data were analyzed using least squares regression (LSR); qPCR data were analyzed by ANOVA with pairwise comparisons as above after square root transformation to normalize the data. qPCR data was compared among treatments per tank (for feces) and per abalone (for feces and tissue; JMP 12.0).

Temporal data from abalones sampled at discrete time points were analyzed by ANCOVA separately from mortality and survivor data. The mean temperature experienced among species and the relationship between time at temperatures between 16-20°C and mortality was examined using LSR. No exposed pinto abalone survived the experiment; thus, comparison of mortality to survivor animals was performed using data from pink and red abalones only.

A generalized linear model (GLM; Poisson distribution and log-link function) was used on square root transformed data to test for a relationship between WS and ST-RLOs in the PE of infected red abalone (JMP 12.0). GLMs were also used to test for relationships between phylogenetic distance of northeastern Pacific abalones, optimal growth temperature and the mean high temperature where each species is most abundant (range high) as reported in the literature (Table 2.1) versus two metrics: 1) mortality due to WS and 2) mean total RLO infection intensity and their interactions (JMP 12.0). For

black abalone, only optimal larval growth temperature was reported in the literature (Leighton 1974). Phylogenetic data included calculated mean pairwise distance from the mitochondrial DNA gene encoding cytochrome oxidase *c* subunit I (COI; Table 2.1, Straus 2010) of the following six northeastern Pacific abalone species: white, pinto, red, black, pink and green. Percent mortality and intensity of total RLO infection in the PE were averaged data from experiments conducted in our laboratory and with collaborators when all three RLOs were used in infection trials to ensure data consistency and quality (Table 2.1). In all but the current study and Friedman et al. (2014b) only total RLO infections (the RLOv had not yet been identified) were scored therefore we analyzed data using mean PE total RLO infection intensity.

Table 2.1. Phylogenetic distances of tested abalone species relative to white abalone (*Haliotis sorenseni*), mean WS-induced percent mortality, mean posterior esophagus (PE) total RLO infection intensity, range high temperature, optimum growth temperature, and associated references.

Species	COI Distance from *H. sorenseni*[1]	Mean % WS Mortality	Mean PE Total RLO Intensity	Range High Temp (°C)[2]	Optimum Growth Temp (°C)	References
White (*H. sorenseni*)	**0**	100	2.70	15	15	Crosson et al. 2014, McCormick et al. 2016
Pinto[3] (*H. kamtschatkana*)	0.004	100	2.10	10	14	Current study, Paul & Paul 1981
Red (*H. rufescens*)	0.023	29	1.60	16	18	Current study, Leighton 1974
Black (*H. cracherodii*)	0.081	49	2.15	22	15	Friedman et al. 2014b, Leighton 1974
Pink (*H. corrugata*)	0.123	20	1.78	20	22	Current study, Leighton 1974
Green (*H. fulgens*)	0.136	13.35	0.74	23	24	Crosson et al. 2014, Moore et al. 2009, Leighton 1974

[1]Data from Table 5 of Straus 2010. [2]Data from Table 1 of Dahlhoff & Somero 1993 for all species except white abalone, which was based on data from McCormick et al. 2016. [3]*H. kamtschatkana kamtschatkana*, the northern pinto abalone.

2.3 Results

2.3.1 Survival

Mortality rates for RLO exposed abalones were higher than those for control abalones (Log-rank statistic = 34.18, df = 1, $P < 0.001$; Fig. 2.2). Other than the nine pinto abalone that died from thermal stress and were discarded from all analyses, no control or unexposed pinto, red or pink abalones died during the experiment (Fig. 2.2). The rate and magnitude of mortality for exposed pinto abalone (100% by day 363) was higher than both exposed red and pink abalones (Log-rank statistic = 50.55, df = 2, $P < 0.001$), which experienced 29% and 20% mortality, respectively, upon experimental termination at 480 days (Table 2.2, Fig. 2.2). No red or pink abalones experienced mortality before 347 days post-exposure (Table 2.2), while 88% of pinto abalone experienced mortality by that time. Pairwise multiple comparisons revealed differences in survival for pinto and red abalones ($P < 0.05$), pinto and pink abalones ($P < 0.05$), but no difference in survival for red and pink abalones ($P > 0.05$). The mean survival time for RLO exposed pinto, red and pink abalones were 246 ± 19 days, 458 ± 7 days and 467 ± 7 days, respectively (Table 2.2).

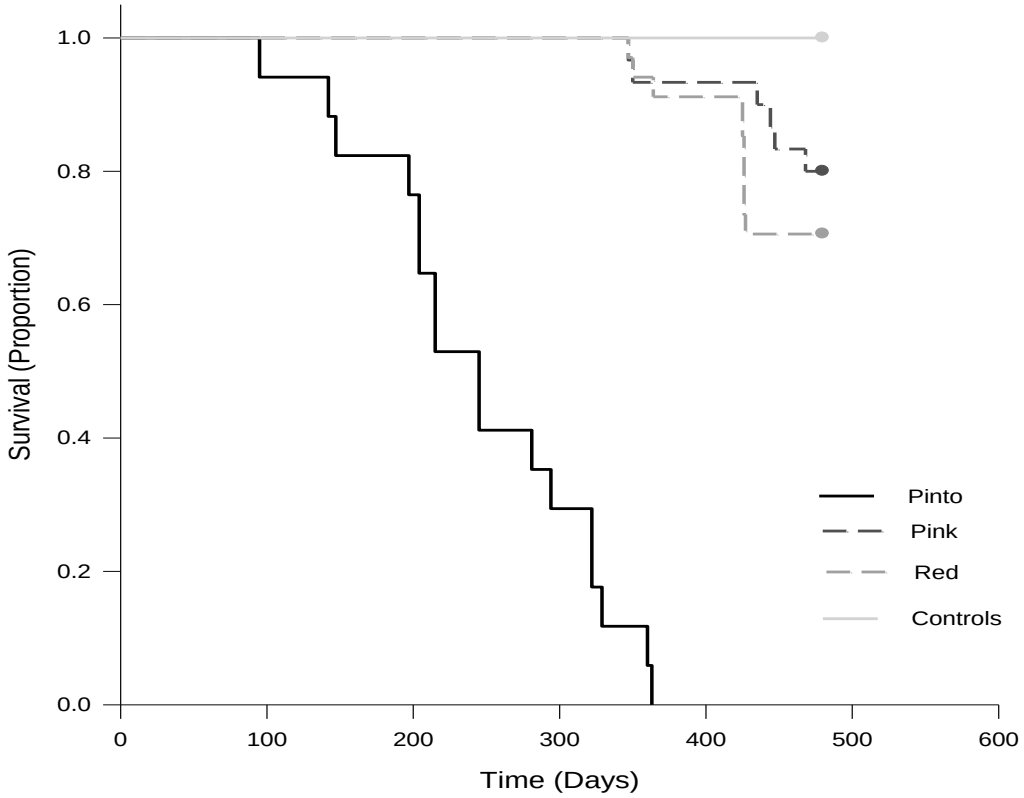

Figure 2.2. Kaplan-Meier survival curves for RLO exposed and unexposed (controls), pinto (*Haliotis kamtschatkana*), red (*H. rufescens*) and pink (*H. corrugata*) abalones.

Table 2.2. Withering syndrome dynamics in RLO exposed pinto (*Haliotis kamtschatkana*), red (*H. rufescens*) and pink (*H. corrugata*) abalones (includes mortality, survivor, and temporal data).

Time (days)	Pinto	Red	Pink
Initial PE infection[1]	90 (1.3)	240 (2.0)	240 (0.8)
Initial DG infection[1]	240 (1.8)	240 (0.5)	360 (0.1)
Initial WS-RLO DNA detection[2]	90 (7.68×10^7)	90 (7.36×10^3)	90 (5.84×10^3)
1st mortality	95	347	347
2nd mortality	142	350	350
50% mortality	245	ND	ND
Mean survival	246	458	467

[1]Total RLO infection intensity score.
[2]WS-RLO DNA copies per gram of tissue via qPCR analysis.

2.3.2 Temperature

The mean temperature experienced varied among species tested (LSR, F-ratio = 21.87, df = 2, $P < 0.0001$). Pinto abalone experienced the lowest temperature (17.32 ± 0.09°C), while red abalone experienced an intermediate temperature (17.96 ± 0.16°C), and pink abalone experienced the highest temperature (18.84 ± 0.16°C) before animals died upon RLO exposure ($P < 0.05$). In addition, the thermal exposure (time at temperatures between 16 and 20°C) and day of second mortality varied among species (LSR, F-ratio = 25.28, df = 5, $P < 0.0001$; Fig. 2.3). Time to second mortality varied with temperature (LSR, F-ratio = 77.85, df = 1, $P < 0.0001$) and species (pink = red > pinto; LSR, F-ratio = 15.00, df = 2, $P < 0.01$), and a temperature x species interaction was observed (LSR, F-ratio = 9.27, df = 2, $P < 0.01$).

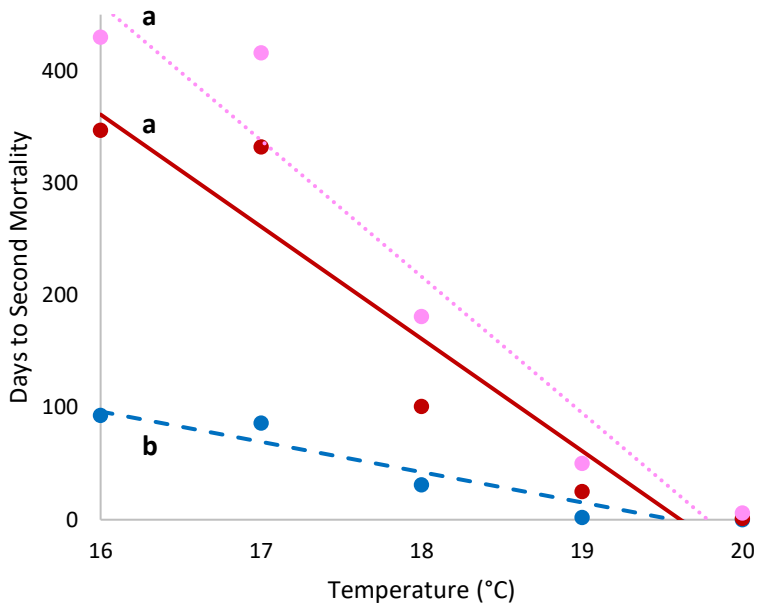

Figure 2.3. Relationship between temperature (°C) and the number of days at each temperature prior to the second RLO-induced mortality. Pinto abalone (*Haliotis kamtschatkana*) dashed line (blue), red abalone (*H. rufescens*) solid line (red) and pink abalone (*H. corrugata*) dotted line (pink). Letters indicate statistical differences ($P < 0.05$).

2.3.3 RLO infections

One to three morphologically distinct RLOs were observed in abalone histological sections (Fig. 2.4). We compared mortality samples for all three abalone species, but compared survivor versus mortality samples for only pink and red abalones as no pinto abalone survived the experiment. Overall, pinto abalone had the highest prevalence (percent of infected individuals) of total RLOs in the PE (76.9%), while red (53.3%) and pink (48.7%) abalones had lower but similar prevalence ($P < 0.0002$). Pinto abalone also had the highest prevalence of total RLOs in the DG (40%), while red (9.4%) and pink (5.1%) abalone had substantially lower prevalence ($P < 0.0001$).

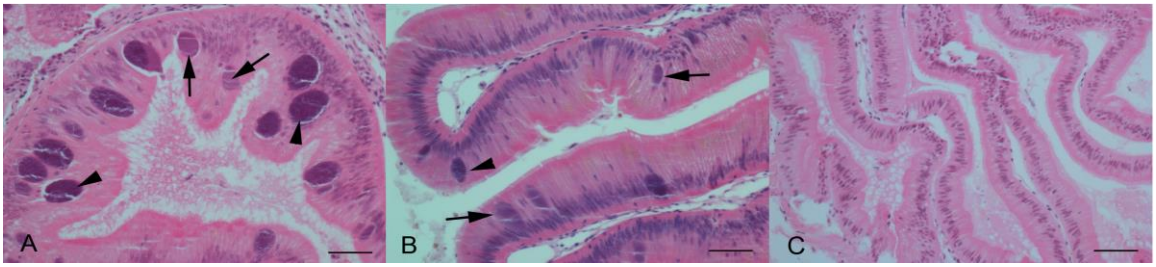

Figure 2.4. Light micrographs of hematoxylin and eosin stained posterior esophagus epithelium of pinto (*Haliotis kamtschatkana*; A), red (*H. rufescens*; B), and pink (*H. corrugata*; C) abalones at 240 days post-RLO exposure. Note high to moderate infections with WS-RLO (arrows) and RLOv (arrowheads) inclusions were observed in pinto and red abalones, respectively, while no inclusions were observed in pink abalone (bar = 50 µm).

2.3.3.1 Posterior esophagus (PE)

2.3.3.1.1 Initial PE infections

RLO infection in the PE of pinto abalone was first observed 90 days after initial RLO exposure, while RLOs in the PE of red and pink abalones were observed at day 240 (Table 2.2). Pinto and red abalones had similar total RLO infection intensities (histology scores of 1.3 and 2.0, respectively; $P > 0.05$) at first observation in the PE, while pink

abalone had fewer total RLOs (histology score of 0.8; LSR, F-ratio = 3.96, df = 2, $P < 0.05$; Table 2.2).

2.3.3.1.2 Mortalities

No differences in WS-RLO, RLOv or total RLOs were observed among host species that died (LSR, F-ratios = 0.25-0.49, df = 2, $P > 0.05$). However, the ST-RLO was found in higher abundance in moribund red abalone (histology score of 2.0) than in pink (histology score of 0.2) and pinto (histology score of 0.5) abalones (LSR, F-ratio = 8.20, df = 2, $P < 0.01$). No difference in ST-RLO abundance was detected between pinto and pink abalones ($P > 0.05$; Table 2.3).

Table 2.3. Mean histology scores of pinto (*Haliotis kamtschatkana*), red (*H. rufescens*) and pink (*H. corrugata*) abalone mortalities and survivors comparing the number of ST-RLO, WS-RLO and RLOv inclusions or infection intensity per 20x field of view: (0) no infection, (1) 1-10, (2) 11-100, and (3) >100 (Friedman et al. 2002). Bold number indicates significantly higher infection intensity.

Species	**Sample Type**	**Posterior Esophagus**				**Digestive Gland**			
		ST-RLO	**WS-RLO**	**RLOv**	**Total RLO**	**ST-RLO**	**WS-RLO**	**RLOv**	**Total RLO**
Pinto	Mortality	0.5	1.7	1.6	2.1	0.1	0.6	0.4	0.8
Red	Mortality	**2.0**	1.3	2.0	2.7	0.0	0.3	0.3	0.3
	Survivor	0.6	0.8	1.0	1.4	0.0	0.0	0.0	0.0
Pink	Mortality	0.2	1.4	1.6	2.1	0.0	0.0	0.2	0.2
	Survivor	0.0	1.3	1.1	1.7	0.0	0.0	0.0	0.0

2.3.3.1.3 Survivors versus mortalities

PE loads of WS-RLO, RLOv and total RLO loads did not vary between surviving and dead red and pink abalones (LSR, F-ratios = 1.00-1.42, df = 3, $P > 0.05$). However, more ST-RLO was observed in the PE of red mortalities (histology score of 2.0) than in all other sample types (histology score of 0.0-0.6; LSR, F-ratio = 9.61, df = 3, $P < 0.001$; Table 2.3). No correlation existed between the intensity of WS-RLO and the ST-RLO in

the PE (GLM, Chi-square = 1.05, df = 1, $P > 0.05$). In addition, the WS-RLO *in situ* hybridization probe bound to WS-RLO and not to ST-RLO inclusions (Fig. 2.5).

2.3.3.2 Digestive gland (DG)

2.3.3.2.1 Initial DG infections

RLO infections in the DG of pinto and red abalones were first observed 240 days after initial RLO exposure, while RLOs in the DG of pink abalone were first observed at 360 days (Table 2.2). Red and pink abalones had similar total RLO infection intensities (histology scores of 0.5 and 0.1, respectively) when first observed in the DG, while pinto abalone had higher total RLOs (histology score of 1.8; LSR, F-ratio = 12.72, df = 2, $P < 0.0001$; Table 2.2).

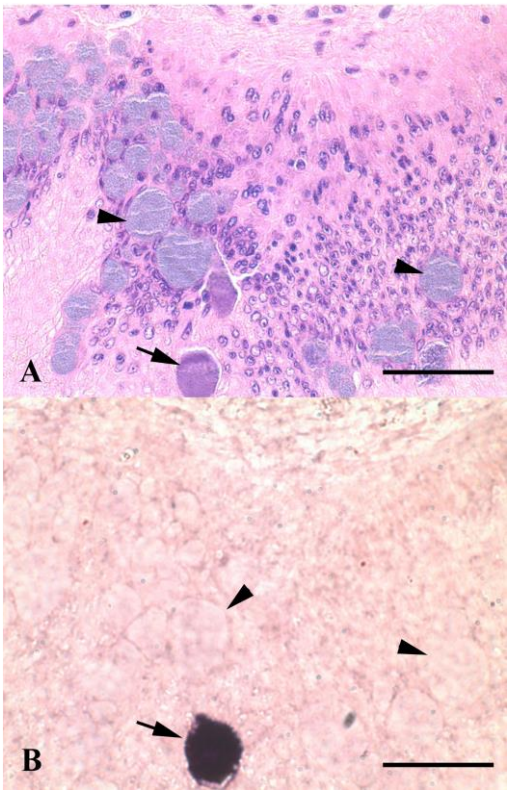

Figure 2.5. Histological section of a red abalone (*Haliotis rufescens*) containing both the ST-RLO (arrowhead) and WS-RLO (arrow) stained with hematoxylin and eosin (A) and probed by *in situ* hybridization for the WS-RLO. Note that only the WS-RLO binds with the probe (bar = 50 µm).

2.3.3.2.2 Mortalities

No differences in WS-RLO, RLOv, ST-RLO or total RLO abundance were detected among host species in dead abalones (LSR, F-ratios = 0.26-2.40, df = 2, $P > 0.05$). ST-RLOs were only observed in the DG of pinto abalone (Table 2.3).

2.3.3.2.3 Survivors versus mortalities

No differences in WS-RLO, RLOv or total RLO abundance were detected in the DG of surviving and dead red and pink abalones (LSR, F-ratios = 0.00-2.92, df = 3, $P > 0.05$). No ST-RLOs were observed in the DG of pink and red abalones (Table 2.3).

2.3.3.2.4 All exposed abalones

When all exposed abalone were examined, pinto abalone contained more RLOs (WS-RLO, RLOv and total RLOs) than did red and pink, which had similar loads (LSR, F-ratios = 15.21, 7.05, 12.72, resp., df = 2, $P < 0.0001$, $P < 0.01$, $P < 0.001$, resp.; Table 2.3). The ST-RLO loads were similar among all species (LSR, F-ratio = 2.89, df = 2, $P > 0.05$).

2.3.4 Host response

2.3.4.1 DG metaplasia

Metaplasia was similar in all abalones regardless of species (mean histology score of 1.3 for pintos, 0.3 for reds and 0.6 for pinks) or whether they were sampled discretely or as mortalities (LSR, F-ratio = 0.88, df = 3, $P > 0.05$).

2.3.4.2 Pedal atrophy

For both red and pink abalones, pedal atrophy was greater in moribund than surviving abalone (LSR, F-ratio = 52.64, df = 3, $P < 0.0001$). Red abalone that died had more pedal atrophy (mean histology score of 3) than those that survived (mean score of

0.3), and also had more advanced pedal atrophy than pink abalone that died (mean score of 0.9) or survived (mean score of 0.1; $P < 0.05$). Surviving pink abalone had less pedal atrophy than pink mortalities (mean scores of 0.1 and 0.9, respectively; $P < 0.05$). Among abalones that died, pedal atrophy of red abalone exceeded that of pink abalone ($P < 0.05$); pedal atrophy in pinto abalone (mean score of 2.0) mortalities was intermediate and was similar to that of both red and pink abalones ($P > 0.05$). Pedal atrophy correlated with PE ST-RLO infections in red abalone only (GLM, Chi-square = 53.47, df = 8, $P < 0.001$; $P < 0.05$ for ST-RLO in red abalone; $P > 0.05$ for all other RLOs in all species).

2.3.4.3 Body condition

Condition index (total body weight/length^3) of mortalities was similar among all three abalone species examined (pinto mean = 1.04×10^{-4}, red mean = 1.08×10^{-4}, pink mean = 1.23×10^{-4}; LSR, F-ratio = 1.44, df = 2, $P > 0.05$) but differed between survivors and those that died (LSR, F-ratio = 11.27, df = 3, $P < 0.0001$). Surviving pink and red abalones had similar condition indices (mean = 1.58×10^{-4} and 1.49×10^{-4}, respectively) that exceeded those of red and pink mortalities (mean = 1.23×10^{-4} and 1.08×10^{-4}, respectively).

2.3.4.4 All control abalone

For all above metrics, control abalones were devoid of RLO infections and had 100% survival. All control abalone lacked DG metaplasia and pedal atrophy. The mean body condition indices for control pinto, red and pink abalones were 1.29×10^{-4}, 1.44×10^{-4}, and 1.51×10^{-4}, respectively. The following host responses differed between control and exposed abalone: DG metaplasia (control mean = 0, exposed mean = 0.4; LSR, F-ratio = 21.63, df = 1, $P < 0.0001$) and pedal atrophy (control mean = 0, exposed mean =

0.6; LSR, F-ratio = 30.06, df = 1, $P < 0.0001$). Body condition was similar between control and exposed abalone (control mean = 1.43×10^{-4}, exposed mean = 1.44×10^{-4}; LSR, F-ratio = 0.0044, df = 1, $P = 0.9473$).

2.3.5 Quantitative PCR

No control abalone tissue, seawater or fecal samples contained WS-RLO DNA. No differences in WS-RLO DNA loads were detected in mortality and survivor tissues at the end of the experiment (ANOVA, F-ratios = 3.44 and 1.94, respectively; df = 1 and 3, respectively; $P > 0.05$). Mean WS-RLO DNA loads (± standard error) for exposed pinto, red, and pink abalone tissues were $2.48 \times 10^9 \pm 1.45 \times 10^9$, $4.11 \times 10^6 \pm 1.58 \times 10^9$, and $1.38 \times 10^6 \pm 1.45 \times 10^9$ copies/g, respectively. However, when WS-RLO DNA was first detected in abalone tissues via qPCR in temporal samples (90 days for all species), pinto abalone contained more WS-RLO DNA copies/g ($7.68 \times 10^7 \pm 5.49 \times 10^7$) than did red ($7.36 \times 10^3 \pm 4.33 \times 10^3$) or pink ($5.84 \times 10^3 \pm 5.84 \times 10^3$) abalones (ANOVA, F-ratio = 7.16, df = 2, $P = 0.0013$; Table 2.2). In addition, when all temporal abalone samples were examined, tissue loads varied among species (ANOVA, F-ratio = 7.16, df = 2; $P < 0.01$) with the highest loads of WS-RLO DNA in tissues from pinto abalone ($4.98 \times 10^9 \pm 1.05 \times 10^8$ copies/g) relative to those in red and pink abalone, which had similar loads ($2.22 \times 10^7 \pm 1.03 \times 10^8$ and $8.41 \times 10^6 \pm 1.08 \times 10^8$, respectively; $P > 0.05$; Fig. 2.6A).

Temporal fecal data from exposed pinto abalone tanks had more WS-RLO DNA copies than did exposed red and pink abalone tanks (ANOVA, F-ratio = 10.33, df = 2, $P = 0.0001$; Fig. 2.6B). Mean WS-RLO DNA loads in fecal samples were $2.48 \times 10^9 \pm 1.45 \times 10^9$ copies/g in pinto abalone tanks, $4.11 \times 10^6 \pm 1.58 \times 10^9$ copies/g in red abalone tanks and $1.38 \times 10^6 \pm 1.45 \times 10^9$ copies/g in pink abalone tanks. When fecal WS-RLO

copies were normalized per gram of feces per abalone, pinto abalone still excreted significantly more copies ($8.10 \times 10^8 \pm 2.39 \times 10^8$) than did red ($8.55 \times 10^6 \pm 2.14 \times 10^8$) and pink ($5.90 \times 10^6 \pm 2.14 \times 10^8$) abalones (ANOVA, F-ratio = 4.01, df = 2, $P < 0.05$). Presence of amplifiable DNA was demonstrated in all WS-RLO qPCR negative samples by amplification of 18S rDNA.

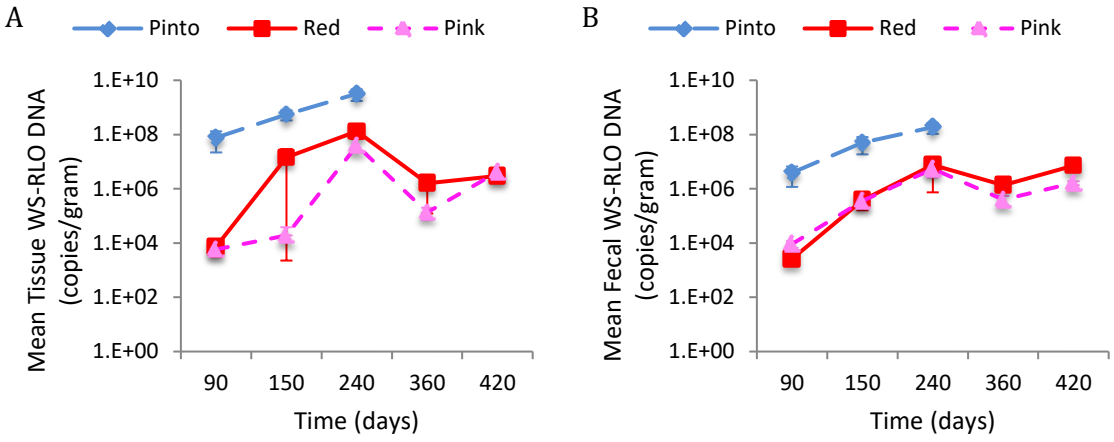

Figure 2.6. Mean WS-RLO DNA copy numbers per gram of pinto (*Haliotis kamtschatkana*), red (*H. rufescens*) and pink (*H. corrugata*) abalone post-esophageal tissues (A) and per gram of abalone feces (B) throughout the experiment. Error bars represent standard error.

2.3.6 Abalone phylogeny, thermal experience & WS relationship

One-way GLMs analyzed the relationship of the single factors (illustrated in Table 2.1) and percent mortality or mean PE total RLO infection intensity of NE Pacific abalones. Percent mortality due to WS was inversely related to the phylogenetic distance from white abalone based on pairwise distance of previously published COI gene sequences (Straus 2010; Fig. 2.7A; Table 2.4). Percent mortality and mean PE total RLO infection intensity of exposed abalone taxa were positively related (Fig. 2.7B), while percent mortality was inversely related to both range high and optimum growth temperature (Fig. 2.7C, 2.7D; Table 2.4). Interestingly, a phylogenetic distance by RLO

intensity interaction was not observed. In addition, no relationship was observed between mean PE total RLO infection intensity and range high or optimum growth temperature (Table 2.4).

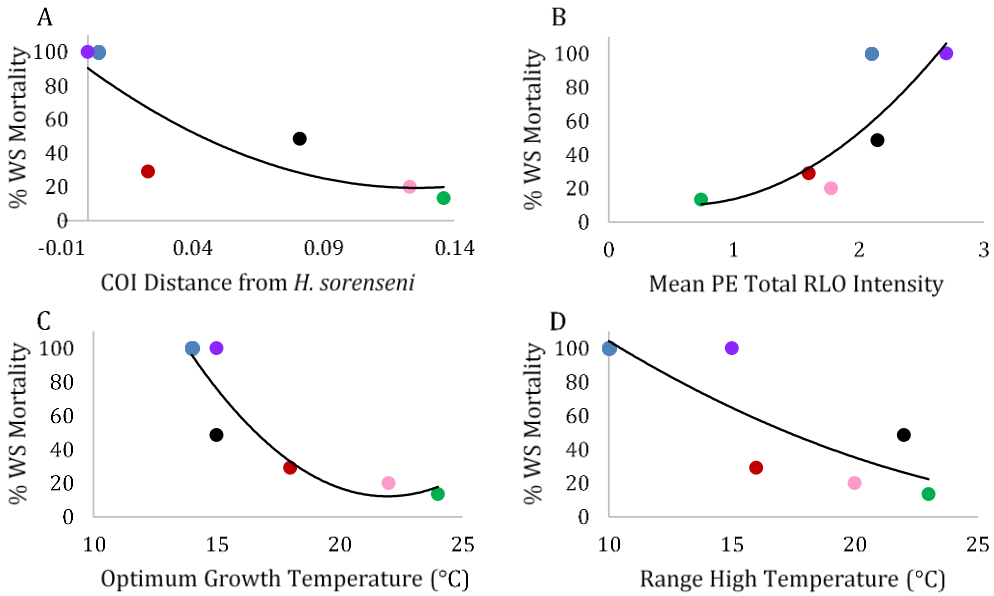

Figure 2.7. Regression plots (GLM) of six northeastern Pacific abalone species [white (*Haliotis sorenseni*; purple dot), pinto (*H. kamtschatkana*; blue dot), red (*H. rufescens*; red dot), black (*H. cracherodii*; black dot), pink (*H. corrugata*; pink dot) and green (*H. fulgens*; green dot)] demonstrating the relationship between mean percent WS-induced mortality and (A) COI distance from white abalone, (B) mean post-esophagus (PE) total RLO infection intensity, (C) range high temperature and (D) optimum growth temperature. See Tables 2.1 and 2.4 for associated data and references.

Two-way GLMs examined the influence of COI distance from white abalone and either mean PE total RLO infection intensity or range high or optimum juvenile (for black abalone, larval) growth temperature, as well as their interactions (Table 2.4). Although the whole models for all two-way analyses identified significant relationships, the factors that contributed to model significance varied (Table 2.4). When we modeled the relationship between percent mortality and both COI distance from white abalone and range high, both factors as well as their interaction were related to abalone survival. When COI distance from white abalone and optimum growth temperature were

examined, only optimum temperature and its interaction with COI were related to percent mortality. Both COI distance from white abalone and mean PE total RLO infection intensity (but not their interaction) influenced percent mortality (Table 2.4).

Table 2.4. Generalized linear model output to examine the relationship among phylogeny (COI distance), thermal experience (mean high temperature of range where taxa are most abundant and optimum growth temperature), mean post-esophagus (PE) total *Rickettsia*-like organism (RLO) infection intensity and percent mortality from withering syndrome. Significant factors are bolded.

Response	**Factor**	**df**	**Chi-square**	***P* value**
% Mortality	**COI distance**[1]	1	110.085	< **0.0001**
% Mortality	**PE RLO**[2]	1	106.019	< **0.0001**
% Mortality	**Range high temp.**[3]	1	86.558	< **0.0001**
% Mortality	**Optimum temp.**[4]	1	125.801	< **0.0001**
% Mortality	**Whole model**	3	129.580	< **0.0001**
	COI distance	1	23.376	< **0.0001**
	PE RLO	1	17.154	< **0.0001**
	COI distance x PE RLO	1	0.001	0.9797
% Mortality	**Whole model**	3	119.520	< **0.0001**
	COI distance	1	30.209	< **0.0001**
	Range high temp.	1	4.537	**0.0332**
	COI x Range high temp.	1	8.870	**0.0029**
% Mortality	**Whole model**	3	144.003	< **0.0001**
	COI distance	1	1.5239172	0.217
	Optimum temp.	1	32.142076	< **0.0001**
	COI x Optimum temp.	1	9.3263869	**0.0023**
PE RLO	COI distance	1	0.579	0.4468
PE RLO	Range high temp.	1	0.320	0.5716
PE RLO	Optimum temp.	1	0.876	0.3517

[1]COI distance from white abalone (*Haliotis sorenseni*). [2]Mean PE total RLO infection intensity. [3]Mean high temperature of range where taxa are most abundant. [4]Optimum growth temperature.

2.4 Discussion

2.4.1 Pinto abalone have high susceptibility & low resistance to WS

This is the first experimental infection study to demonstrate pinto abalone are highly susceptible to RLO infection and exhibit very low resistance to WS. Furthermore, we expand the number of relatively resistant northeastern Pacific abalone species to include red and pink abalone populations. Historically, black abalone were thought to have the highest susceptibility to WS of all northeastern Pacific abalone species due to catastrophic population declines seen in the field (upwards of 99%) and high mortality rates observed in laboratory trials (32-100%; VanBlaricom et al. 1993, Tissot et al. 1995, 2007, Altstatt et al.1996, Friedman et al. 1997, 2002, 2014b, Moore et al. 2000, Miner et al. 2006). Population scale losses of abalone species other than black abalone have not been documented but laboratory and aquaculture-related losses have been observed in white and red abalones (Friedman et al. 2000, 2007, Moore et al. 2002, Braid et al. 2005, Crosson et al. 2014). Laboratory-based RLO challenges demonstrated that white abalone appear to be more susceptible to WS than black abalone (100% vs. 74% mortality, respectively) when exposed at 18°C, a temperature known to promote WS development (Moore et al. 2000, Crosson et al. 2014, Friedman et al. 2014b). RLO infections were shown to cause moderate (28-33%) losses in red abalone and little to no mortality (< 14%) in green and pink abalones (Moore et al. 2000, 2009, Álvarez-Tinajero et al. 2002, Braid et al. 2005, Vilchis et al. 2005). In our study, pinto abalone were the only species to experience complete (100%) mortality when exposed to RLOs, while < 30% of the exposed red and pink abalones died, suggesting that pinto abalone parallel white abalone in their high susceptibility and low resistance to WS.

Virulence of the RLO was higher for pinto abalone than for red and pink abalone in our study as evidenced by the relative rates of transmission, development of clinical disease, and mortality among the three species experimentally evaluated (Steinhaus & Martignoni 1970). When WS-RLO DNA was first detected in tissue samples (90 days for all species), pinto abalone contained 10,000 times more copies/g than red and pink abalones. A similar trend was seen in fecal samples in which pinto abalone excreted 100 times more WS-RLO DNA copies/g than red and pink abalones. As compared to red and pink abalones, pinto abalone contained higher initial RLO loads, infections spread to the DG faster (130 days earlier), and DG infections were more advanced (intensity of 1.8 versus 0.5 and 0.1, respectively). Pinto abalone also became infected at a lower seawater temperature and exhibited rapid mortality with lower overall survivorship (no pinto abalone survived). Similar trends in RLO transmission and WS pathogenesis have been observed in white abalone (Crosson et al. 2014). Despite these trends, host response (DG metaplasia, pedal atrophy, and body condition) in pinto, red and pink abalones did not vary among species except for higher pedal atrophy in red abalone, which correlated with intensity of the ST-RLO and not the WS-RLO. Increased survivorship in red and pink abalones relative to pinto abalone was not attributed to reduced metaplasia and WS-RLO loads in the DG as previously suggested for black abalone populations with differing susceptibility and resistance to WS (Friedman et al. 2014b). However, our small sample size may have influenced the ability to detect differences in host response metrics. Together, these data demonstrate that WS disease dynamics differ among abalone species and that physiological factors related to disease susceptibility and resistance may vary among hosts, which warrants further study.

2.4.2 WS induction temperature varies among abalones

For all abalone species previously tested, increased water temperature was associated with the development of clinical disease, with the exception of green abalone for which clinical signs of WS were rare and not influenced by temperature (Vilchis et al. 2005, Moore et al. 2009, Crosson et al. 2014). In our study, pinto abalone were susceptible to RLO infection and WS at water temperatures much lower than those reported for all species examined to date (17.3°C), while red and pink abalones succumbed to WS at temperatures similar to those previously reported (≥ 18°C; Moore et al. 2000, Braid et al. 2005, Crosson et al. 2014). The observed increased susceptibility of pinto abalone at a lower water temperature but with similar RLO infection intensity and host response suggests that other aspects of disease physiology and/or thermal stress may contribute to this species' low resistance to WS. The absence of reported population losses of pinto abalone may be due to its historically low population levels (e.g. insufficient numbers existed to support a commercial fishery for this species) in the WS endemic zone (Karpov et al. 2000).

Temperature is an important factor in the disease physiology of ectothermic species, including abalones and their pathogens both of which may be locally adapted to specific thermal regimes (Huey & Stevenson 1979). Although the thermal maximum of the WS-RLO is not known, the bacterium is known to replicate faster and transmit more easily as water temperatures rise above 15°C to at least 21°C, the highest reported experimental temperature (Friedman et al. 1997, 2002, Moore et al. 2000, 2009, Braid et al. 2005, Ben-Horin et al. 2013). Abalone species that inhabit warmer waters, such as pink abalone, or can withstand significant thermal fluxes, like red abalone, may be better

adapted to respond to a secondary stressor such as disease during seasonal warm water events than the cool water pinto abalone (Dahlhoff & Somero 1993, Diaz et al. 2006). For example, intertidal black abalone exposed to daily temperature fluctuations are more susceptible to RLO infections, but disease expression occurs only at warm water temperatures (Ben-Horin et al. 2013). As water temperatures rise to levels that are physiologically stressful for the host but optimal for the RLO, the bacterium may outcompete host defenses and clinical disease can develop.

2.4.3 Abalone immunity may vary among species & with temperature

Host immune and stress responses may play key roles in resistance to WS upon RLO infection. Hemocytes are known to play key roles in response to tissue damage, transport of nutrients, and waste removal, and may impact immune health in digestive lumina (Fisher 1986). Although the intracellular nature of the RLOs may protect them from much of the host immune response, hemocytes may be involved in the host metaplastic response and pedal atrophy observed in the final stages of the disease (Friedman et al. 1997, 2000). The presence of increased numbers of fixed tissue phagocytes in the foot muscle and hemocytes in the digestive gland of black abalone with advanced signs of WS suggest they play a role in the catabolism and associated atrophy of the foot muscle and digestive gland (VanBlaricom et al. 1993, Friedman et al. 1997, 2000). However, hemocyte responses are not observed in the pathogenesis of WS in most abalone species (Friedman et al. 2000, Crosson et al. 2014).

Elevated water temperature can disrupt innate immune responses in abalones and influence their ability to combat pathogens. For example, abalone hemocyte numbers have been shown to increase rapidly in response to temperature stress but their

phagocytic ability was reduced (Cheng et al. 2004). In addition, humoral immune parameters may be delayed and antibacterial activity compromised after prolonged exposure to warm water (Dang et al. 2012). Adaptation to specific ranges in water temperature has been shown to influence the host stress response making warm water adapted species less stressed when exposed to water temperatures known to facilitate pathogen infection and disease development (Schade et al. 2014). In our study, abalones adapted to warm water temperatures were more resistant to WS than those adapted to cool water temperatures as shown by the inverse relationship of time from exposure to the second mortality. In addition, the subtropical Ezo abalone (*H. discus hannai*) and the tropical small abalone (*H. diversicolor supertexta*) were highly refractory to WS-RLO infection, development of clinical disease and mortality further demonstrating that warm water adapted abalones are less susceptible to RLO infection and disease (Wetchateng et al. 2010, Kiryu et al. 2013, Crosson et al. 2014). Thus, an increase in mortality at high temperatures after a bacterial challenge may be a function of both host immunomodulation/stress response and bacterial physiology.

2.4.4 Abalone phylogeny & thermal experience corresponds with WS resistance

Observed patterns in the distribution, susceptibility and thermal threshold for the onset of WS among northeastern Pacific abalone species tested suggests a link with host phylogeny, biogeography, thermal history and physiology. Species are known to vary in response to temperature changes both spatially and temporally using a suite of physiological, life history and behavioral strategies, which define their phenotype (Angilletta 2009). Although the geographic distribution of many northeastern Pacific abalones overlaps, a latitudinal gradient exists. Pinto abalone occupy the northernmost

distribution (from Alaska to Point Conception, California, US), followed by red abalone (distributed from Northern California, US to central Baja California, Mexico), black abalone (distributed from south Northern California to central Baja California, Mexico) and the southernmost pink and green abalones (distributed from central California/Point Conception, US to central Baja California, Mexico; Haaker et al. 1986, Geiger 2000). White abalone also occupy a southern distribution (from Point conception, California, US to central Baja California, Mexico; Haaker et al. 1986, Geiger 2000, Hobday & Tegner 2000). All northeastern Pacific abalones inhabit depths of 0-20 m except for white abalone, which inhabit depths of 20-60 m (Hobday & Tegner 2000). Depth and latitudinal distribution of northeastern Pacific abalones appear to be most related to temperature with an inverse relationship between thermal optima and latitude (Leighton 1974). The highest susceptibility to RLO infection and lowest resistance to WS in our study was observed in the northern latitude, cool water pinto abalone as compared to the warm water species tested with more southern distributions.

Measures of organismal performance (e.g. survival, disease resistance or reproduction) can vary with temperature and are maximized within a narrow thermal range (Angilletta 2008, Travers et al. 2008). Performance can also vary among genotypes and, if a species is isolated for a prolonged period, can lead to speciation or local adaptation via natural selection (Darwin 1859, Via 2009). Interestingly, pinto abalone are segregated into two subspecies: *H. kamtschatkana kamtschatkana* (the pinto or northern abalone, with a more northern range from Sitka, AK to central CA, US) and *H. k. assimilis* (referred to as the threaded abalone, with a more southern range from central CA, US into Baja California, Mexico; Cox 1962, Geiger 2000). Although recent genetic

data based on highly conserved nuclear genes (e.g. lysin) suggest that pinto and threaded abalones appear synonymous (Straus 2010), historical distributions may have influenced the observed thermal susceptibility of pinto abalone to RLO infection and WS development at a temperature lower than previously reported for other taxa (see review by Crosson et al. 2014). The pinto abalone used in our experiment were produced in WA State, located at the northern end of the species geographic range. While WA State pinto abalone can experience temperatures as high as 20°C for brief periods of time (Rothaus et al. 2008), the range high is only 10°C with an optimal juvenile growth temperature of 14°C (Paul & Paul 1981). The threaded abalone (pinto subspecies) that inhabit central California (US) south to Baja California (Mexico; Haaker et al. 1986) experience warmer waters with higher frequency and duration than their more northern conspecifics. The optimal growth temperature and susceptibility of the threaded abalone subspecies to RLO infection remains unknown. Additional studies on a broad range of abalone species, especially on the two possible subspecies of pinto abalone, throughout their ranges may shed light on the interplay of thermal history and other potential factors driving WS disease ecology.

The evolution of thermal range for a species may vary among taxa depending on the relative selective pressure of temperature on organismal survival or fecundity/reproduction (Angilletta 2009) and thus may vary among closely related taxa, such as among species within the genus *Haliotis*. White and black abalones are both highly susceptible to WS but are found in intermediate and warmer temperature waters, respectively (Leighton 1974, Geiger 2000, Crosson et al. 2014). Interestingly, both of these species occupy extremes in abalone habitats (white abalone occupy the deepest

depths, while black abalone are found in intertidal to shallow subtidal habitats throughout their ranges), which may play an important undiscovered role in their overall phenotype, including their susceptibility to RLO infection and resistance to WS (Haaker et al. 1986, Geiger 2000). Genetic distance of mitochondrial COI gene sequences of northeastern Pacific abalones relative to that of white abalone was inversely related to WS-induced mortality. The relatedness of these species to one another is supported by phylogenetic analysis based on sequence analysis of COI and a nuclear gene, lysin (Table 2.1; Metz et al. 1998, Estes et al. 2005) and, coupled with their relative ranges in depth, latitude, temperature and WS resistance, suggests a potential phylogenetic basis for RLO infectivity and WS pathogenesis. Fully annotated genomes for all northeastern Pacific abalone species are needed to identify genes responsible for observed differences in disease resistance and its relationship with thermal range.

The relationship among host genetics, thermal traits, and RLO infection is complex. A direct positive relationship was observed between percent mortality and RLO infection intensity. Resistance to the effects of RLO infection increased with greater phylogenetic distance from white abalone. White abalone are highly susceptible to RLO infection and have low resistance to the development of WS (Crosson et al. 2014). Closely related species, such as the pinto abalone, share these same traits with white abalone as evidenced by the current study. In addition, captive bred white abalone suffered high losses due to WS (McCormick, pers. obs.) and the disease is cited as a key impediment to the restoration of this species (see NMFS 2008). Additional traits that influence WS mortality may vary by host species and include the host species range high temperature and optimum growth temperature. We observed that both a species' range

high temperature and its optimum growth temperature were inversely related to WS-induced mortalities. Interestingly, when these factors were examined in relation to COI distance, the relative influence of each trait varied. Rapid acclimation of abalones to temperature (over one week) is known to influence their thermal tolerance. For example, 17% more black abalone acclimated to 16°C survived a thermal stress event relative to those acclimated to 11°C (Hines et al. 1980). However, the observed interaction of COI distance and range high temperature suggests that evolutionary history plays an important role in resistance to WS and that a careful examination of the interplay of temperature within physiological tolerable versus stressful ranges in response to WS resistance is needed.

All but two studies to date employed stable (versus variable) temperatures to examine the role of temperature on WS. Naturally infected (in the field) red abalone from San Miguel Island exposed to variable temperature experienced lower overall mortality due to WS and only the treatment with the highest mean temperature and prolonged periods over 18°C experienced significant losses (Moore et al. 2011). Black abalone exposed to water-borne WS-RLO and a range of thermal variability exhibited differences in both susceptibility to WS-RLO infection and WS development (Ben-Horin et al. 2013). Interestingly, increased thermal variation increased the risk of a black abalone becoming infected (higher prevalence) but this did not influence the development of WS clinical signs. However, more clinical signs of WS were observed in black abalone that experienced higher overall mean temperature, which is similar to observations of higher WS in red abalone exposed to variable thermal conditions (Moore et al. 2011, Ben-Horin et al. 2013). Population-wide losses of black abalone due to WS are linked to El Nino

Southern Oscillation (ENSO) events during which mean weekly average sea surface temperatures exceeded 18°C (Raimondi et al. 2002, Ben-Horin et al. 2013). Thus both temperature range and degree of variation appear to be linked to RLO susceptibility and WS development.

2.4.5 Multiple RLOs & ST-RLO evolution

Infection with multiple RLO types can affect abalone survival. Friedman et al. (2014b) found that the presence of RLOv substantially increased the survival of infected black abalone and that ST-RLO loads in black abalone DG were higher in moribund abalone from a naïve susceptible population than from those from a WS-resistant population. We observed no difference in WS-RLO and RLOv loads among pinto, red, and pink abalones. The ST-RLO was typically observed at low prevalence and intensity (Friedman and Moore, pers. obs.) until the observation of higher ST-RLO intensities in black abalone populations in infection trials (Friedman et al. 2014b). In the present study, high ST-RLO loads were observed in the PE of infected red abalone and correlated with increased pedal atrophy irrespective of WS-RLO presence, suggesting potential evolution and increased pathogenicity of the ST-RLO, especially for red abalone. However, a direct comparison of the pathogenicity of each RLO type remains unattainable due to the inability to culture these bacteria and conduct infection trials with a single bacterial type.

2.4.6 Conclusions & conservation application

Given that most northeastern Pacific abalone species are in decline and efforts to conserve and restore these species are underway, it is important to better understand threats to repopulation and protection efforts. Most abalone conservation and restoration efforts are currently being conducted within the endemic zone of the WS pathogen. In

addition, abalone aquaculture farms exist within the WS endemic zone in California, US (based primarily on red abalone) and Baja California, Mexico (based on green and pink abalones; Diaz et al. 2006). As organismal responses to stressors vary with both phenotype and genotype, to properly assess and manage declining abalone populations it is important to understand the mechanisms governing the abalone-RLO relationship. We demonstrated differential susceptibility to RLO infection and resistance to disease development among three host abalone species. Studies geared towards exploring adaptive differences among species under varying environmental conditions (e.g. increased sea surface temperature, decreased pH) will be crucial to achieve successful protection and restoration of abalone resources, especially for threatened and endangered species.

Chapter 3. Abalone withering syndrome disease dynamics: infectious dose & temporal stability in seawater

3.1 Introduction

Withering syndrome (WS) is an infectious marine wasting disease that affects numerous northeastern Pacific abalone *Haliotis* species. The causative agent is the *Rickettsia*-like organism "*Candidatus* Xenohaliotis californiensis" (WS-RLO; Friedman et al. 2000), which is an obligate intracellular bacterium that is transmitted horizontally via a fecal-oral route (Friedman et al. 2000, 2002). The WS-RLO creates bacterial inclusions in abalone post-esophageal (PE) epithelial cells and the digestive gland (DG) epithelia undergoes metaplasia, the transformation of one differentiated cell type to another mature cell type, which allows the bacterium to proliferate (Friedman et al. 2002). As WS progresses, abalones lose the ability to properly digest and absorb nutrients from their food. In response, abalones catabolize their pedal musculature for energy and begin to exhibit clinical signs of the disease including lethargy, mantle retraction and anorexia (Gardner et al. 1995, Friedman et al. 2002, Braid et al. 2005).

WS was initially observed in wild black abalone *Haliotis cracherodii* populations on the south shore of Santa Cruz Island, California, US in 1985 (Tissot 1995). Since then, WS has spread to numerous abalone species and is considered continuously distributed from Baja California, Mexico to Sonoma County, California, US including the Channel and Farallon Islands (reviewed by Crosson et al. 2014). Research investigating potential environmental drivers of the WS epidemics revealed that as seawater temperatures increased, the prevalence and severity of the disease also increased (Steinbeck et al. 1992, Tissot 1995, Moore et al. 2000, Braid et al. 2005, Vilchis et al. 2005). The onset of warmer than normal seawater conditions associated with the large El Niño Southern

Oscillation (ENSO) events of 1982-83 and 1997-98, as well as the anthropogenic transport of WS-RLO infected abalone to previously uninfected areas (Friedman & Finley 2003) likely facilitated the spread of the disease (Tissot 1995, Crosson et al. 2014).

The threat of WS to abalones has substantial ecological implications. Abalones are considered ecosystem engineers that, via herbivory, maintain open space on rocky substrate for utilization by other benthic organisms and conspecifics (Miner et al. 2006). Currently, five out of seven northeastern Pacific abalone species are experiencing population declines and receive varying levels of federal protection, ranging from "Species of Concern" (pinto abalone *H. kamtschatkana*, green abalone *H. fulgens*, and pink abalone *H. corrugata*) to "Endangered" (white abalone *H. sorenseni*, and black abalone; reviewed by Crosson et al. 2014). Many of these abalone species overlap in geographic range and demonstrate differential susceptibility and resistance to WS (Crosson & Friedman 2018). Therefore, the need to understand potential WS transmission risk is critical to facilitate more informed management decisions for the successful protection and restoration of threatened and endangered abalone species.

WS can also have profound economic effects on the commercial abalone industry. Abalones are one of the world's most expensive seafood products and current production has shifted from wild to farmed abalones with 95% of the global market supplied by aquaculture (FAO, 2017). Over the last 10 years, global abalone aquaculture production has increased a dramatic 500% (24,400 metric tons in 2006 to over 150,000 metric tons in 2015; FAO, 2017) and production in the United States (California and Hawaii) for 2015 was an estimated 362 metric tons (Cook 2016) valued at over $13.1 million dollars

(Ray Fields, The Abalone Farm, Inc., pers. comm.). Given the economic importance and explosive growth of abalone aquaculture combined with declining wild abalone numbers, concern has risen regarding potential WS transmission between wild and cultured abalone populations, including both pathogen spillback (wild to cultured abalones) and spillover (cultured to wild abalones; Daszak et al. 2000). A previous study implicated shore-based abalone aquaculture in southern California as a potential threat to critical habitat for endangered wild abalones (Lafferty & Ben-Horin 2013). However, without fundamental information regarding WS transmission mechanisms and associated pathogen dynamics, the potential threat is largely unknown.

The paucity of baseline information on WS disease dynamics can be primarily attributed to the inability of the WS-RLO bacterium to grow *in vitro* and the lack of established marine invertebrate cell lines making disease transmission experiments a challenge (Rinkevich 2005). Many studies attempting to explore WS transmission mechanisms have relied on cohabitation of healthy abalone with WS-RLO infected abalone (e.g. Friedman et al. 1997, 2002, Braid et al. 2005). While cohabitation experiments more closely simulate natural WS infections, the amount of pathogen or dose needed to establish infection remains unknown. Development of tools and protocols that facilitate both *in vitro* and *in vivo* studies with the WS-RLO are needed. Friedman et al. (2014a) recently developed and validated a quantitative PCR (qPCR) assay for the detection of WS-RLO DNA in abalone tissue, feces and seawater. While the presence and amount of WS-RLO nucleic acid does not confirm the pathogen's viability or infectivity, it can serve as a proxy for pathogen detection and potential transmission risk. Other studies comparing bacterial quantification via PCR-based assays to traditional

culture-based methods found that PCR-based assays are a viable alternative, although relative quantities of DNA copies detected and limits of detection relative to traditional methods can vary by taxa being examined (Wright et al. 2007, Ricchi et al. 2017). These findings are especially valuable for the detection and quantification of unculturable bacteria such as the WS-RLO.

To better understand important components of WS disease dynamics, our study aimed to: (1) determine the temporal stability of WS-RLO DNA outside of its abalone host in 14°C and 18°C seawater, (2) develop a standardized protocol for exposing abalones to known concentrations of WS-RLO DNA and (3) calculate the dose of WS-RLO DNA required to generate 50% infection prevalence (ID50) in the highly cultured red abalone *H. rufescens*. These findings will provide critical information for modeling potential WS transmission risk within and among abalone populations and will aid management decisions for the restoration and culture of abalone species in WS-endemic areas.

3.2 Methods

3.2.1 WS-RLO temporal stability

3.2.1.1 Experimental design

Approximately 19 L of effluent seawater from a commercial abalone farm located in Goleta, CA, US, where the WS-RLO pathogen is established in local waters, was shipped overnight on ice to the School of Aquatic and Fishery Sciences-University of Washington-Pathogen Quarantine Facility [SAFS-UW-PQF (Seattle, WA, US)]. Upon arrival, effluent seawater was thoroughly mixed, immediately aliquoted into glass beakers (500 mL each), covered with parafilm, and placed in dark incubators at their respective

temperature treatments, 14°C and 18°C. Each temperature treatment consisted of 16 replicate effluent seawater beakers and 4 sterile seawater (SSW) beakers used as negative controls, all with independent aeration. Two effluent seawater beakers per treatment were randomly sampled daily for 8 days and two SSW control beakers were sampled per treatment on days 2 and 8 to ensure no pathogen cross contamination at the beginning or end of the experiment. Three experimental trials were conducted in October 2016, February 2017 and June 2017 to assess seasonal variability of WS-DNA levels in the farm effluent seawater.

3.2.1.2 Seawater sampling

Sampling consisted of aseptic filtration of seawater replicates through a 0.2 µm Supor® membrane disc filter (Pall Corp., Port Washington, NY, US) along with 5 mL of 1X TE buffer [10 mM Tris-HCl (pH 8.0), 1 mM EDTA] for nucleic acid preservation. All filters were aseptically halved; one half was stored at -80°C for subsequent DNA isolation and the remaining half was stored in 1 mL of TRI Reagent® (Sigma-Aldrich Corp., St. Louis, MO, US) at -80°C for subsequent RNA isolation (methods below).

3.2.2 WS-RLO infectious dose

3.2.2.1 Experimental design

Independent shipments of WS-RLO-free juvenile red abalone (n = 120), to serve as experimental animals, and WS-RLO infected adult red abalone (n = 26), to serve as a source of infectious material, were received from a commercial abalone farm located in Cayucos, CA, US and housed at the SAFS-UW-PQF. WS-RLO infected abalone were placed in a 270 L recirculating seawater system and healthy abalone were equally distributed among four 175 L recirculating seawater systems containing triplicate 11.4 L

tanks with 10 abalone per tank and system-independent biological/mechanical filtration, UV-irradiation, and temperature control (Fig. 3.1). Abalone were acclimated for approximately two weeks prior to WS-RLO dosing (method below) and maintained at 18°C, a temperature known to augment pathogen proliferation (Moore et al. 2000). Feces were tested by qPCR to ensure abalone were free of detectable levels of WS-RLO DNA prior to experimentation. Abalone were fed 8-10 pellets of S-A diet (Cosmo Business Support, Japan) per tank daily for the duration of the experiment. Seawater pH, dissolved oxygen and ammonia were assessed on alternate days, while temperature and abalone mortality were assessed daily. Lethargic or dead abalone were immediately removed and sampled for histology and quantitative PCR (qPCR) analyses (methods below). Abalone tank feces were monitored bi-weekly for the presence of WS-RLO DNA via qPCR and abalone post-esophageal tissues were sampled at 9 weeks post WS-RLO dosing for histology and qPCR examination.

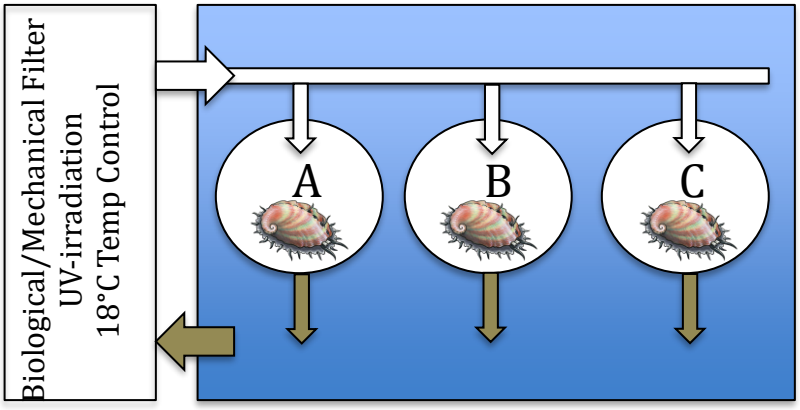

Figure 3.1. Schematic of recirculating seawater systems used for the WS-RLO infectious dose experiment at four concentrations: 0 (sterile seawater control), 10^3, 10^4, and 10^5 WS-RLO DNA copies/mL. Each 175 L system contained triplicate 11.4 L tanks (A, B, C) of 10 juvenile red abalone (*Haliotis rufescens*). Post-dosing, effluent seawater (gray arrows) was pumped through a series of biological/mechanical filtration, UV-irradiation, and an 18°C temperature controller prior to reentry to individual abalone tanks (white arrows).

3.2.2.2 WS-RLO dosing

Post-esophageal (PE) tissue, the target tissue type for WS-RLO infections (Friedman et al. 2000), was excised from all infected adult red abalone. PE was pooled, suspended in 100 mL of SSW and homogenized on ice with a 7 mL Tenbroek homogenizer (Fisher Scientific, Pittsburg, PA, US). Approximately 19 L of effluent seawater was received from the same commercial abalone farm from where the infected adult red abalone originated and filtered on to 0.2 µm Supor® membrane disc filters (n = 5; Pall Life Sciences, Port Washington, NY, US). All filter retentate (primarily WS-RLO infected abalone feces) was added to the infected tissue homogenate to ensure a maximum yield of WS-RLO source material. To generate an appropriate volume for dosing, 3.3 L of SSW was added to the WS-RLO homogenate and two 1:10 serial dilutions in SSW were generated. The homogenate stock (10^0) and dilutions (10^{-1}, 10^{-2}) were sampled via the seawater sampling method (described above) except the entire filter retentate was immediately processed for DNA isolation and quantified via qPCR (methods below) to determine WS-RLO dosing concentrations in DNA copies per mL. Abalone treatment tanks received 1 L of the appropriate WS-RLO dose, while control tanks received 1 L of SSW. All abalone remained submerged for 3 h with aeration after which the dosing medium was decanted and replaced with flow-through SSW.

3.2.2.3 Abalone tissue & fecal sampling

For histological examination, a 2-3 mm tissue cross-section of PE was aseptically excised posterior to the right kidney-DG junction. Tissues were preserved in Davidson's fixative (Shaw & Battle 1957) for 24 h and stored in 70% ethanol until processing by routine paraffin histology. Deparaffinized 5µm sections were stained with hematoxylin

and eosin (Luna 1968) and examined by light microscopy. WS-RLO infection intensities in PE tissues were scored on a (0) – (3) scale that estimated the number of bacterial inclusions per 20x field of view: (0) no infection, (1) 1-10, (2) 11-100, and (3) > 100 (Friedman et al. 2002). For each abalone examined, all tissues were screened and the mean score per field of view was reported. A 150-200 mg sample of PE was also excised directly adjacent to the histology cross-section and preserved in 100% molecular grade ethanol until subsequent DNA isolation and qPCR analysis described below. Abalone fecal samples (~ 200 mg) were collected bi-weekly from each tank (or pooled by dosing treatment when tank fecal quantities were low) and immediately processed for DNA isolation and qPCR analysis for continuous monitoring of WS-RLO DNA levels.

3.2.3 DNA & RNA isolation

DNA was isolated from seawater filters using a DNeasy Blood & Tissue Kit (Qiagen Inc., Hilden, Germany) according to the manufacturer's "Gram-positive bacteria" protocol with the following modifications: volume of lysing buffer was doubled to ensure complete filter coverage and DNA was eluted in 100 µL. DNA was isolated from abalone fecal and tissue samples using a QIAamp DNA Stool Mini Kit (Qiagen Inc.) according to the manufacturer's "Isolation of DNA from Stool for Pathogen Detection" protocol with the following modifications: DNA was eluted in 100 µL. All DNA samples were stored at -20°C until qPCR analysis. RNA was isolated from seawater filters using TRI Reagent® (Sigma-Aldrich, St. Louis, MO, US) according to the manufacturer's protocol and suspended in 50 µL of 0.1% DEPC-treated water. To ensure complete removal of genomic DNA (gDNA), all RNA was treated with DNase using the Turbo DNA-free Kit (Invitrogen Corp., Carlsbad, CA, US) according to the

manufacturer's protocol and verified gDNA-free via qPCR. RNA was then reverse transcribed to complimentary DNA (cDNA) using M-MLV reverse transcriptase, 10 mM dNTPs and oligo Dt primer (Promega Corp., Madison, WI, US) according to the manufacturer's protocol. All cDNA samples were stored at -20°C until qPCR analysis as described below.

3.2.4 Quantitative PCR

Quantification of WS-RLO nucleic acids was conducted using the validated qPCR assay of Friedman et al. (2014a), which amplifies the single copy per bacterium of the16S ribosomal gene as determined by the annotation of the WS-RLO partial genome (Langevin et al., *in manuscript*). Thus, qPCR copy numbers quantified may serve as a proxy for bacterial cell numbers present, but do not signify bacterial viability (Friedman et al. 2014a). Briefly, qPCR reactions were conducted using 0.5 µL 2x GoTaq, 12.5 µm qPCR Master Mix (Promega Corp.), 0.8 mg/µL BSA (New England Biolabs Inc., Ipswich, MA, US), 2uL of template, and sterile water for a total volume of 25 µL per reaction. Thermal cycling conditions were 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 s and 60°C for 30 s. Each sample was run in duplicate along with a negative template control and a plasmid-based standard curve of known WS-RLO copy numbers with a limit of detection of 3 gene copies for both genomic and cDNA (Friedman et al. 2014a). WS-RLO copy number was determined via regression analysis of the standard curve. Seawater filter quantities were calculated as copies per mL, while tissue and fecal quantities were calculated as copies per gram. All samples negative for WS-RLO via qPCR were evaluated using a Qubit™ 3.0 fluorometer (Invitrogen Corp.) to ensure the presence of nucleic acid.

3.2.5 Statistical analyses

All data were analyzed in JMP 12.0 (SAS Institute Inc., Cary, NC, US). Histological data were analyzed using least squares regression (LSR) with dose and time as fixed factors and tank as a random factor. qPCR data were natural log transformed for normalization and analyzed by ANOVA with pairwise multiple comparisons using the Holm-Sidak method at a significance level = 0.05 to test for relationships between temperature, replicates, and time. Infection prevalence per tank (fecal shedding) and per individual abalone (PE tissue) were estimated using qPCR data (WS-RLO DNA copies). Differences in fecal shedding were tested using LSR with dose and day nested within dose as factors. Differences among factors were post-hoc tested using the Tukey-Kramer multiple comparison method. Infectious dose values were calculated according to the methods of Reed & Muench (1938) with the percentage of infected abalone per replicate as the unit of measurement due to the fact that infection by cross-contamination during a relatively short incubation period was unlikely to occur as demonstrated by previously published cohabitation infection experiments with red abalone (Crosson & Friedman, 2018).

3.3 Results

3.3.1 WS-RLO temporal stability

Initial mean ± SE WS-RLO DNA levels were 18.09 ± 0.61 copies/mL, 2.99 ± 0.39 copies/mL, and 93.60 ± 27.20 copies/mL in water received from the commercial abalone farm for the October 2016, February 2017, and June 2017 trials, respectively (Fig. 3.2). The highest mean ± SE WS-RLO DNA levels were detected for the first 24 h post-arrival for all trials (October = 32.60 ± 5.20 copies/mL, February = 3.61 ± 0.73

copies/mL, June = 95.50 ± 1.50 copies/mL; Fig. 3.2). WS-RLO DNA copies declined rapidly between 24-72 h for the October trial, and between 24-48 h for the February and June trials ($P < 0.05$; Fig. 3.2). WS-RLO DNA levels declined below the qPCR assay limit of detection (3 copies/mL) between 72-168 h of the October trial (Fig. 3.2A), by 48 h of the February trial (Fig. 3.2B), and by 120-144 h of the June trial (Fig. 3.2C). No significant temperature or replicate effects on WS-RLO DNA stability were observed (Table 3.1). WS-RLO RNA was below qPCR detection at all time points for all trials (data not shown).

Table 3.1. ANOVA output examining the relationship among temperature, replicates, and time for the WS-RLO stability trials. Significant factors are bolded.

Trial	Factor	df	SS	MS	F Ratio	*P* Value
October 2016	**Whole model**	4	29.35	7.34	26.31	**0.0113**
	Replicate	1	0.29	-	1.04	0.3837
	Time	3	29.07	-	34.74	**0.0079**
February 2017	**Whole model**	10	22.15	2.22	25.67	< **0.0001**
	Temperature	1	0.22	-	2.54	0.1247
	Replicate	1	0.03	-	0.31	0.5851
	Time	7	19.99	-	33.09	< **0.0001**
June 2017	**Whole model**	10	384.51	38.45	63.62	< **0.0001**
	Temperature	1	0.11	-	0.18	0.6714
	Replicate	1	0.001	-	0.002	0.9650
	Time	7	327.02	-	77.30	< **0.0001**

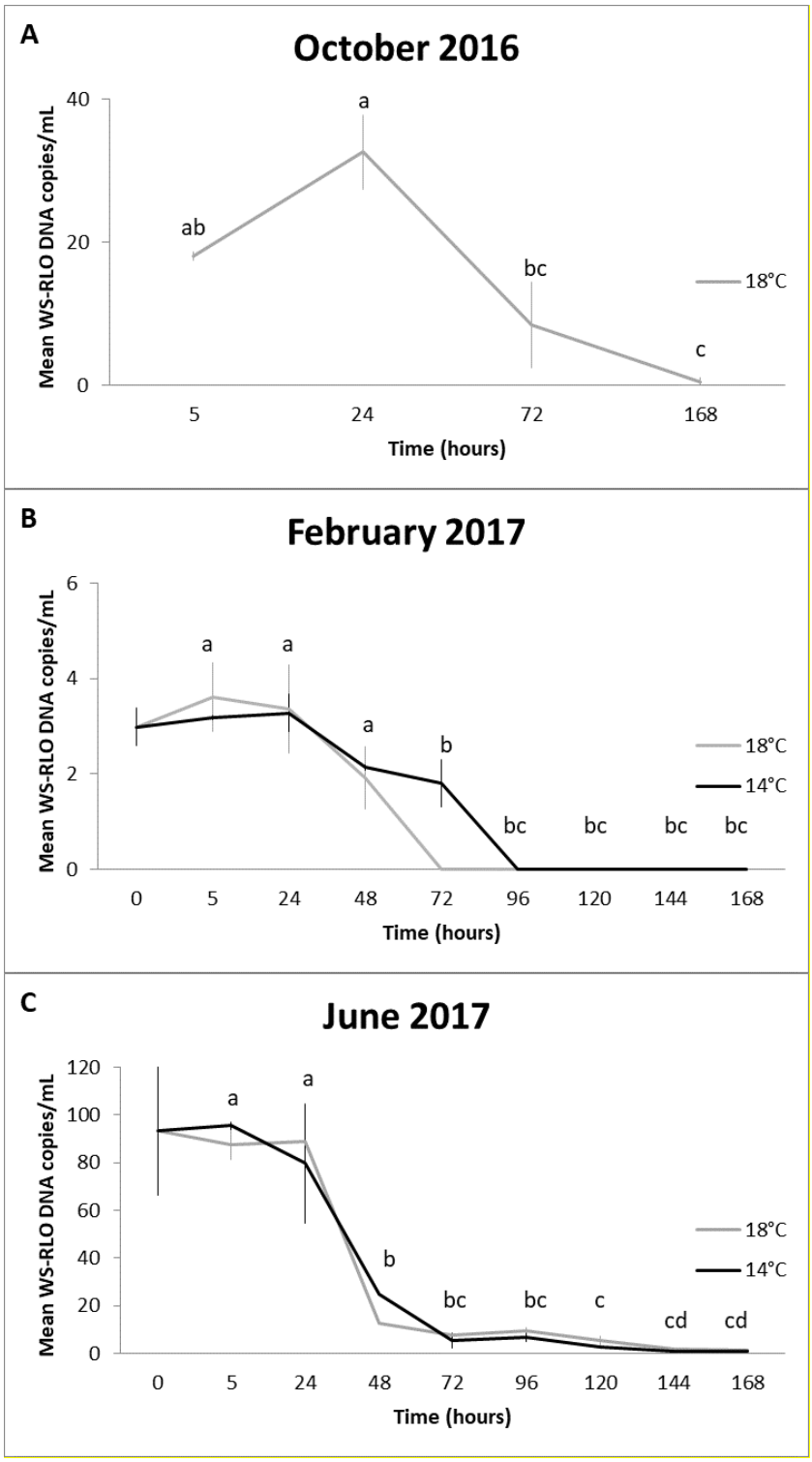

Figure 3.2. Mean WS-RLO DNA copies/mL in seawater effluent from a commercial abalone farm located in Goleta, CA, US and collected in October 2016 (A), February 2017 (B) and June 2017 (C). Bars represent standard error and letters indicate statistical differences ($P < 0.05$).

3.3.2 WS-RLO infectious dose

Initial WS-RLO homogenate dosing concentrations were 4.08 x 10^5 ("10^0 stock"), 6.49 x 10^4 ("10^{-1} dilution") and 5.07 x 10^3 ("10^{-2} dilution") WS-RLO DNA copies/mL, which were used to calculate infectious dose curves. The infectious dose required to generate 50% infection prevalence based on qPCR of abalone PE tissues and shed feces were similar: 2.26 x 10^3 and 3.16 x 10^3 WS-RLO DNA copies/mL, respectively. An ID50 could not be calculated using visual observation of bacterial inclusions within abalone PE tissues via histology. However, the infectious dose required to generate 25% infection prevalence (ID25) based on histology was 6.98 x 10^4 WS-RLO DNA copies/mL (Table 3.2, Fig. 3.3).

Table 3.2. WS-RLO dose (DNA copies/mL) required to generate 50% or 25% infection prevalence in red abalone (*Haliotis rufescens*). na = not available.

Infectious Dose	Feces qPCR	Tissue qPCR	Histology
50%	3.16 x 10^3	2.26 x 10^3	na
25%	na	na	6.98 x 10^4

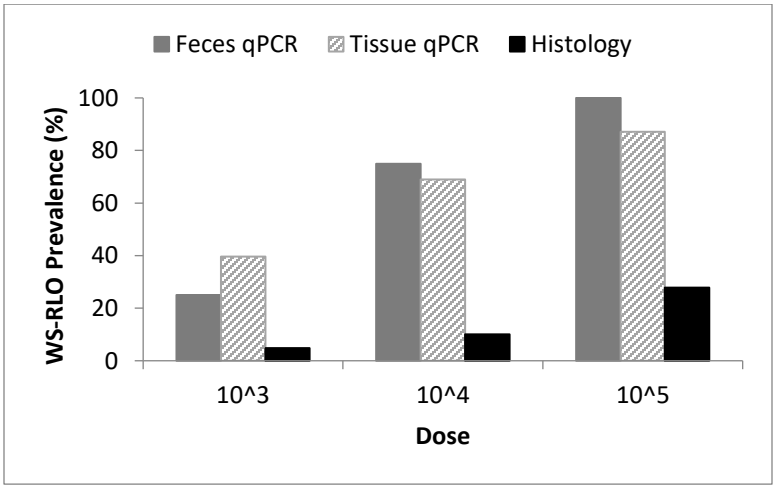

Figure 3.3. WS-RLO prevalence in red abalone (*Haliotis rufescens*) assessed by quantitative PCR (qPCR) of feces and post-esophageal (PE) tissues and by histological examination of PE tissues upon termination of the experiment on day 61.

Only abalone from the highest dose (10^5) shed significantly more WS-RLO DNA than did the other doses ($P < 0.05$; Fig. 3.4). The infection prevalence of abalone tanks positive for WS-RLO DNA as determined by qPCR of fecal samples was 100% for the 10^5 dose, 75% for the 10^4 dose and 25% for the 10^3 dose (Fig. 3.3). Abalone feces from SSW control tanks contained no WS-RLO DNA throughout the duration of the experiment. No WS-RLO DNA in abalone feces was detected in any treatment 6 days post-dosing (Fig. 3.4). The amount of WS-RLO DNA in abalone feces increased with time ($P = 0.0068$) and by dose ($P = 0.0042$).

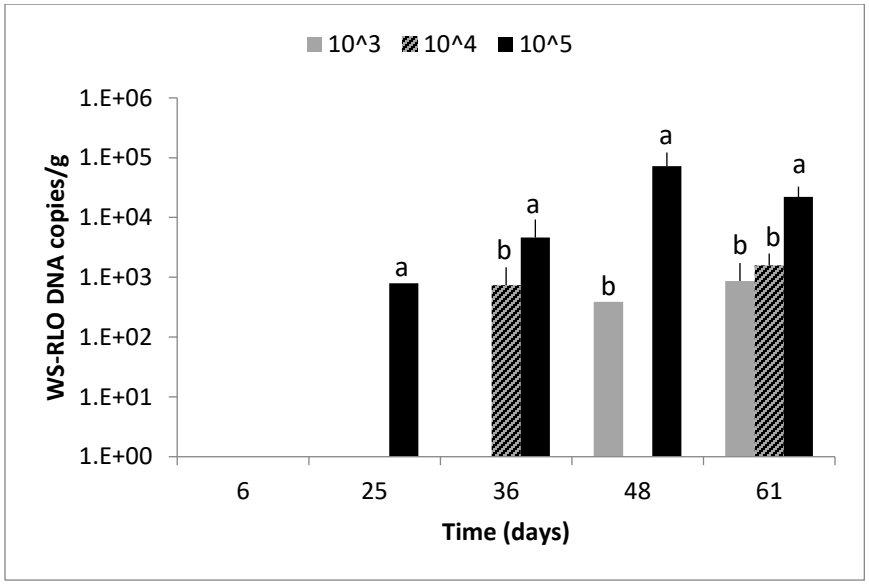

Figure 3.4. Mean WS-RLO DNA copies/g of red abalone (*Haliotis rufescens*) feces for the duration of the WS-RLO infectious dose experiment. Bars represent standard error and letters indicate statistical differences ($P < 0.05$).

Mean ± SE WS-RLO DNA copies/g of abalone PE tissue for the 10^5, 10^4, and 10^3 doses upon termination of the experiment on day 61 were $8.87 \times 10^5 \pm 3.75 \times 10^5$, $4.16 \times 10^5 \pm 1.49 \times 10^5$, and $6.03 \times 10^4 \pm 2.45 \times 10^4$, respectively. Abalone PE tissues from SSW control tanks contained no WS-RLO DNA. The infection prevalence of abalone positive

for WS-RLO DNA as determined by qPCR of PE tissues was 87% for the 10^5 dose, 69% for the 10^4 dose, and 40% for the 10^3 dose (Fig. 3.3). Mean ± SE WS-RLO infection intensity and prevalence in PE tissues scored by histology were 0.10 ± 0.05 and 28% for the 10^5 dose, 0.02 ± 0.02 and 10% for the 10^4 dose, and 0.07 ± 0.04 and 5% for the 10^3 dose (Fig. 3.3)

3.4 Discussion

3.4.1 WS-RLO temporal stability

This was the first study attempting to assess WS-RLO temporal stability outside of its abalone host at seawater temperatures spanning the seasonal range of southern CA waters (https://www.nodc.noaa.gov). Despite differences in initial pathogen levels during the three seasonal trials, WS-RLO DNA was not stable in seawater longer than 48 h. WS-RLO DNA levels fell below the qPCR assay's limit of detection (3 copies/mL) by 48-168 h, while WS-RLO RNA levels were below detection at all time points of all trials. Cellular and extracellular DNA is often very persistent in the environment (Taberlet at al. 2012). However, bacterial DNA persistence in seawater can vary considerably, as short as 6.5 h to an estimated 2 months (see review by Neilsen et al. 2007). Key environmental factors influencing DNA persistence in aquatic ecosystems are temperature and sunlight as prolonged exposure to high temperatures and/or UV radiation result in single stranded and fragmented DNA molecules (Paul et al. 1987, Huver et al. 2015). All of our stability trials were conducted in the dark to eliminate the risk of UV-induced DNA degradation and allow us to examine the influence of seawater temperature (14°C and 18°C) and time outside of an abalone host (up to 168 hours or 7 days) on WS-RLO DNA persistence. Time proved to be the only significant factor tested that influenced WS-RLO DNA

stability. Although we observed no influence of temperature on WS-RLO DNA stability, the strong correlation between pathogen abundance and seawater temperature remains an important consideration when conducting disease transmission experiments and managing both wild and cultured abalone populations. Seawater temperatures of 18°C are known to facilitate WS-RLO proliferation (Moore et al. 2000, Friedman et al. 2002, Braid et al. 2005, Crosson & Friedman 2018) and likely explains the higher observed levels of pathogen DNA detected in our June trial when mean ambient coastal seawater temperatures are typically highest in CA (https://www.nodc.noaa.gov). Although elevated seawater temperature is the key environmental factor driving WS expression, thermal variation has been shown to influence WS-RLO transmission in black abalone (Ben-Horin et al. 2013). Coastal seawater temperature records for southern CA consistently show high variance over time and often include intervals best described as chaotic; future studies should consider the influence of thermal variation on WS-RLO stability.

Although WS-RLO DNA proved to be relatively unstable *in vitro*, transmission of WS continues to occur in the natural environment (VanBlaricom et al. 1993, Altstatt et al. 1996, Miner et al. 2006). Host behavior and pathogen reservoirs or vectors may influence the potential for WS transmission. The WS-RLO is transmitted horizontally via a fecal-oral route (Friedman et al. 2002) and abalones are gregarious by nature with some species, such as the intertidal black abalone, even displaying a stacking behavior (Douros 1987, VanBlaricom et al. 1993). This behavioral dynamic can readily facilitate the spread of disease within a population. Unknown reservoirs or vectors of the WS-RLO bacterium can also serve as potential disease transmission mechanisms. A marine bacterium in the

family *Rickettsiaceae* was discovered living in the cytoplasm of the protozoan ciliate, *Diophrys appendiculata* (Vannini et al. 2005). While the novel bacterium does not appear to cause disease in the ciliates, this symbiotic relationship reveals that members of the *Rickettsiaceae* are more diverse and widespread than once thought. More recently, an intertidal snail belonging to the *Turbinidae* family was discovered harboring WS-RLO inclusions in Japan (Iku Kiryu, pers. comm.), demonstrating that the WS-RLO bacterium has the ability to infect other marine vetigastropods. Expansion of the WS-RLO host range coupled with episodic ENSO or strong seasonal warm water events could readily facilitate spread of the pathogen among abalone populations. The potential geographic spread of pathogenic RLOs in the natural environment by non-target organisms and variable oceanic conditions has significant ecological implications and warrants further research.

The role of virus-induced bacterial mortality in seawater should also be considered when assessing WS transmission risk. Bacteriophages constitute a significant controlling factor for bacterial abundance and activity with estimates of bacteriophage infection being responsible for ~10-50% of bacterial mortality in both seawater and sediment (Fuhrman & Noble 1995, Glud & Middelboe 2004). Friedman & Crosson (2012) recently discovered a bacteriophage infecting the WS-RLO and, while it remains unclear as to whether viral replication occurs via a lytic or lysogenic cycle, co-infection was shown to attenuate WS disease progression in black abalone (Friedman et al. 2014b). The potential for the bacteriophage to modulate WS disease dynamics has significant ecological and economic impacts with regards to abalone survival and disease

management. Future studies further characterizing the role of the bacteriophage on WS-RLO survival are needed.

Our study confirms that southern CA abalone farm effluent seawater contains WS-RLO DNA and that RLOs could spillover to nearby wild abalone populations as suggested by Lafferty & Ben-Horin (2013). However, no established method exists to determine the source of the pathogen and results from our WS-RLO stability trials indicate pathogen DNA persistence is limited to ~2 days making transmission over broad geographic ranges unlikely. These observations combined with the low concentration of WS-RLO DNA in the farm effluent (a maximum of 94 copies/mL) and the large dilution factor once the RLO reaches coastal waters bring question to the assertion of Lafferty & Ben-Horin (2013) that an abalone farm was a likely source of WS-RLO DNA over 20 km from the farm outfall. The potential for species other than abalone, such as kelps or other macroalgae to which the WS-RLO may adhere, could serve as vectors or a link from sources of the WS-RLO in the natural environment to off-shore abalone populations (Fuller, 2017). It is important to note: (1) the WS-RLO was first detected in wild abalones and has since spread to farmed populations, (2) the WS-RLO is now considered established in southern and central CA waters and thus farm influent seawater may contain the pathogen creating spillback concerns, and (3) due to the rapid decline (degradation) of detectable WS-RLO nucleic acid in seawater, the relative risk of an abalone farm acting as a significant source of pathogen spillover depends on its proximity to wild abalone populations and local seawater circulation patterns. Future research should examine oceanographic patterns surrounding CA abalone farms and determine their proximity to wild abalone populations in order to accurately assess WS-RLO

transmission risk. A recent sentinel study conducted in southern CA estimated WS-RLO transmission risk *in situ* by deploying modules containing uninfected abalone at two sites, one near an onshore commercial abalone farm and one in proximity to wild aggregations of abalone (Fuller, 2017). WS-RLO DNA was detected in seawater via qPCR at the site where modules were deployed near wild abalones but not at those near farm site modules even though WS-RLO DNA was detected in the farm's effluent seawater (Fuller, 2017). Infection prevalence and intensity of the sentinel abalone were also very low and similar at both wild and farm site modules. These *in situ* WS-RLO transmission data support our findings of minimal temporal stability of the WS-RLO and highlight the importance of thoroughly assessing all aspects of WS disease dynamics for the proper management of both wild and cultured abalones.

3.4.2 WS-RLO infectious dose

This was the first study to successfully infect abalone with known concentrations of WS-RLO and calculate an infectious dose. We demonstrated that 2.26×10^3 and 6.98×10^4 WS-RLO DNA copies/mL of seawater are needed to infect 50% (using qPCR as a proxy for infection) or 25% (using histology to determine infection), respectively, of juvenile red abalone with a 3 h exposure period. The difference between quantification methods is likely due to the increased sensitivity of qPCR relative to histology. In both white (Friedman et al. 2007) and black abalones (Friedman et al. 2014b) exposed to WS-RLO, 23-30% more individuals were positive for WS-RLO DNA using qPCR as a proxy relative to histology. In fact, visual observation of bacterial inclusions correlated with qPCR copy numbers of 10^6 copies/mg of tissue (Friedman et al. 2007, 2014b). The WS-RLO DNA levels from our infectious dose experiment were 100-1,000 times higher than

those quantified from abalone farm effluent seawater during our stability trials (~3 to 94 WS-RLO DNA copies/mL). The ID50 as assessed by qPCR was similar when calculated using PE tissues from individual abalone (2.26×10^3 copies/mL) or feces shed per tank of abalone (3.16×10^3 copies/mL). These data suggest that determining WS-RLO DNA levels via abalone feces is a reliable alternative to lethal PE tissue sampling. Quantification of WS-RLO DNA from PE tissues was performed on individual abalone upon experimental termination, while fecal sampling was pooled by treatment tank allowing us to track WS-RLO DNA levels over time. Future dosing experiments should include replicates of individual abalone to avoid concern of possible WS-RLO cross-transmission.

Efficient disease transmission relies primarily on initial infectious dose (including pathogen concentration and host exposure time), pathogen virulence, host immunity and important environmental drivers such as temperature and salinity (Grassly & Fraser 2008). Dose effect must be considered as a function of the bacterial strain and the life stage and species of the host. Our study demonstrated a relatively high WS-RLO dose was needed for a 3 h exposure period at an optimal pathogen temperature (18°C) for the moderately susceptible red abalone (Crosson & Friedman 2018). The composition of at-risk abalone populations will be an important factor given the substantial differences in susceptibility and resistance to WS that have been observed among abalone species (Crosson & Friedman, 2018). Fuller's (2017) sentinel study illustrated that red abalone developed low level infections after 6 weeks of exposure in CA waters near an abalone farm where no WS-RLO DNA was detected in the seawater and near wild abalone populations where WS-RLO DNA was detected in the seawater. These data suggest a

disease vector, such as macroalgae to which the WS-RLO could adhere and concentrate, may be responsible for transmission and warrants further examination. Collectively, these studies highlight the need to characterize the relationship among pathogen dose, exposure time, host species and seawater temperature in order to better understand WS-RLO transmission dynamics. Our novel dosing method can be used to facilitate these types of studies and will help address remaining questions associated with WS disease dynamics such as bacterial reproduction, shedding rates (pathogen spread potential) and critical thresholds (host density needed for pathogen invasion).

In order to generate predictive models and accurately assess transmission risk associated with WS, more studies addressing the fundamentals of disease dynamics need to be conducted. Host, pathogen, and potential vector diversity/species composition should be explored in relation to ecological processes at community, ecosystem and landscape levels. Collectively, our findings are essential components of understanding WS disease dynamics and will help to assess transmission risk within and among abalone populations for better informed management decisions to successfully protect and restore abalone species in WS-endemic areas.

Chapter 4. A geographic survey of the *Rickettsia*-like organism and novel phage associated with abalone withering syndrome in California seawater using DNA-based qPCR assays

4.1 Introduction

Pathogens play critical roles in the marine environment, from recycling of nutrients to shaping local biodiversity. However, most pathogens are underappreciated components of marine ecosystems until a disease outbreak occurs. One of the most well-documented marine disease outbreaks and associated mass mortality events occurred in California black abalone (*Haliotis cracherodii*) beginning in the mid-1980s, shortly after the strong 1982-83 El Niño-Southern Oscillation (ENSO) event. The rapid and extensive die-offs, up to 99% losses in some areas, were the result of an infectious disease called withering syndrome (WS; Tissot 1991, 1995; Haaker et al. 1992; VanBlaricom et al. 1993, 2008; Altstatt et al. 1996; Friedman et al. 2000; Neuman et al. 2010). Withering syndrome is a fatal abalone disease characterized by a severely shrunken foot muscle and gastrointestinal infection with a *Rickettsia*-like organism (RLO), provisionally named "*Candidatus* Xenohaliotis californiensis" (Friedman et al. 2000), herein referred to as "WS-RLO". The WS-RLO is an intracellular bacterium that infects abalone digestive epithelia and causes digestive gland metaplasia resulting in physiological starvation followed by anorexia, catabolism of the foot musculature, and eventually death (Friedman et al. 2003, Braid et al. 2005). The distribution of the WS-RLO subsequently spread naturally and via anthropogenic movement in all directions from its first observation on the Channel Islands, CA and is now considered an endemic pathogen in California waters (Moore et al. 2002, Friedman & Finley 2003, Crosson et al. 2014).

The WS-RLO can infect all California abalone species including the subtidal and highly cultured red abalone (*H. rufescens*), the cool water northern or pinto abalone (*H. kamtschatkana*; Crosson & Friedman 2018), warm water pink (*H. corrugata*) and green (*H. fulgens*) abalones (Vilchis et al. 2005, Alvarez-Tinajero et al. 2002, Crosson et al. 2014), endangered, deep-water white abalone (*H. sorenseni*; Friedman et al. 2007) and the intertidal-shallow subtidal black abalone (*H. cracherodii*; Altstatt et al. 1996, Friedman et al. 2000, 2002, Miner et al. 2006). Withering syndrome-associated mortality events have been attributed to high, and prolonged, increases in sea surface temperature and disease induction by high temperature has been well documented in laboratory studies (Moore et al. 2000, Braid et al. 2005, Vilchis et al. 2005). In the mid-2000s, a morphologically distinct RLO was observed in abalone tissues and discovered to be the WS-RLO infected with a phage (Friedman & Crosson 2012, Vater et al. 2018). Presence of the phage substantially increased the survival of infected black abalone (Friedman et al. 2014b) and red abalone but not the highly susceptible white abalone (Crosson et al. 2014, Vater et al. 2018). Characterizing the distribution of the WS-RLO and novel phage has large implications for the management and restoration of both wild and cultured abalones.

The goals of this study were to: (1) quantify WS-RLO and phage DNA levels in seawater along the California coast from Bodega Bay, Sonoma County to Ventura County including the Channel Islands over two consecutive summers (July 2010 and 2011), (2) compare pathogen DNA levels at sites with established wild black abalone populations to those at red abalone aquaculture facilities (AFs), and (3) examine WS-

RLO DNA dilution potential in seawater directly adjacent to a point source discharge at the two southern-most AFs in Cayucos and Goleta, CA.

4.2 Methods

4.2.1 Seawater sampling

Given that WS can be horizontally transmitted via seawater (Friedman et al. 2014b), nearshore surface seawater samples were collected to quantify WS-RLO and phage DNA at four red abalone AFs and nine sites with established wild black abalone populations along the California coast and Channel Islands in July of 2010 and 2011 (Fig. 4.1). Wild black abalone sites included: (from north to south) Bodega Bay, Carmel Point, Andrew Molera State Park, Point Sierra Nevada, Rancho Marino, San Miguel Island (Hare Rock and Cuyler Harbor), Santa Cruz Island (Painted Cave and Prisoners Harbor), Santa Rosa Island (Carrington Point and Talcott) and San Nicolas Island (permanent intertidal sites 3 and 8; see VanBlaricom et al. 1993), and AF sites included: (from north to south) Davenport (AF1), Monterey (AF2), Cayucos (AF3), and Goleta (AF4). Duplicate 2 L seawater samples from each wild site were collected at 5 stations within 1000 m of established black abalone populations, except for Andrew Molera State Park and Rancho Marino where only one station each was accessible. Duplicate 2 L seawater samples were also collected from each AF directly adjacent to the farm effluent outfall (0 m) and 100 m and 500 m north and south of the outfall, except for AF4 where samples were east and west relative to the outfall and AF2, which utilizes an ocean-based in-water basket culture method and therefore had no direct outfall. In October 2013, fine-scale seawater surveys were conducted at the two southern most AFs in Cayucos and Goleta, CA (AF3 and AF4, respectively) where nearshore surface seawater was collected from 0

to 100 m in 25 m increments, 300 m, and 500 m. Only AF3 was sampled over three consecutive days to assess daily variation in WS-RLO DNA levels.

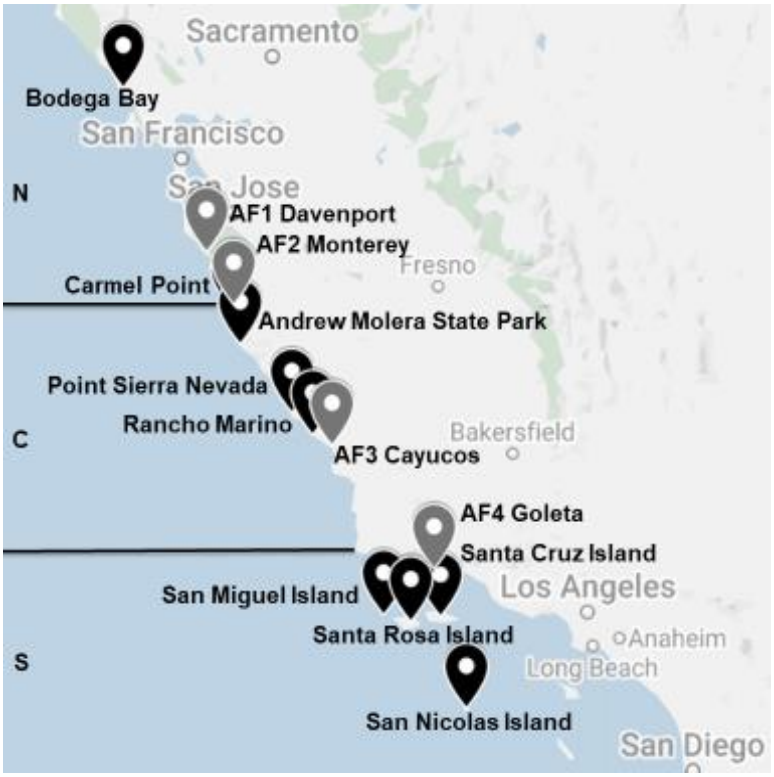

Figure 4.1. Map (Google, n.d.) of WS-RLO and phage DNA surface seawater surveys conducted along the California coast, US. Black pins represent wild sites with established black abalone populations (n = 9) and grey pins represent red abalone aquaculture facilities (AFs; n = 4). Horizontal lines demarcate zones: N = northern zone, C = central zone, and S = southern zone.

After collection, individual seawater samples were aseptically filtered through a 0.2 µm Supor® membrane disc filter (Pall Life Sciences, Port Washington, NY, US) followed by 5 ml of low TE buffer (10 mM Tris pH 8.0, 100 mM EDTA, 0.5 M NaCl) for nucleic acid preservation and stored in liquid nitrogen (-80°C). DNA from the filters was extracted using a DNeasy Blood & Tissue Kit (Qiagen Inc., Hilden, Germany) according to the manufacturer's "Gram-positive bacteria" protocol with double the volume of reagents. Filters were incubated overnight at 56°C in Buffer AL and

Proteinase K to facilitate bacterial cell lysis. DNA was eluted in 100 µl of Elution Buffer and stored at -20°C until subsequent qPCR analysis.

4.2.2 Quantitative PCR

Quantification of WS-RLO and phage DNA was accomplished using the qPCR assays of Friedman et al. (2014a) and Langevin et al. (*in manuscript*), respectively. Briefly, qPCR reactions were conducted using 12.5 µl 2x GoTaq qPCR Master Mix (Promega Corp., Madison, WI, US), 320 nM of each primer, 200 nM Black Hole Quencher 5' FAM/3' probe (LGC, Biosearch Technologies, Novato, CA, US), 0.6 mg/µl BSA (New England Biolabs Inc., Ipswich, MA, US), 2 µl of DNA template, and sterile water to bring the final volume to 25 µl per reaction. Thermal cycling conditions were 95°C for 10 min, followed by 41 cycles of 95°C for 15 s and 60°C for 30 s. Each sample was run in duplicate along with a negative template control and plasmid-based standard curve of known copy numbers. Copy number for each sample was determined via regression analysis of the standard curve and calculated as copies per liter of seawater. All samples negative for WS-RLO and phage DNA via qPCR were evaluated using a Qubit™ 3.0 fluorometer (Invitrogen Corp., Carlsbad, CA, US) to ensure the presence of nucleic acid.

4.2.3 Statistical analyses

All seawater qPCR data (DNA copies/L) were log transformed for normalization and analyzed in JMP Pro 15 (SAS Institute Inc., Cary, NC, US) using least squares regression (LSR) and a Tukey's Honest Significant Difference (HSD) test or a Student's t-test. Comparisons were conducted by year, zone (northern, central, and southern), site type (wild versus AF), sites within site type, and sub-sites within AF sites.

4.3 Results

Tables 4.1, 4.2 and 4.3 provide mean metrics for WS-RLO and phage DNA copies/L of seawater ± standard deviation. Values with no reported standard deviation had no biological replicate. Statistical analyses with LSR outcomes and effect magnitude are provided in Table 4.4.

4.3.1 Pathogen DNA variation by year

In 2010, 22% of seawater samples collected from wild black abalone sites contained WS-RLO DNA and 56% contained phage DNA (Table 4.1), while 50% of seawater samples collected directly adjacent to AFs contained WS-RLO DNA and 75% contained phage DNA (Table 4.2). In 2011, 56% of wild samples contained both WS-RLO DNA and phage DNA (Table 4.1), while 100% of AF samples contained WS-RLO DNA and 75% contained phage DNA (Table 4.2). WS-RLO DNA levels at AFs were not significantly different by year, while loads at wild sites were greater in 2011 than 2010 (Table 4.4, Rows 2-3). Phage DNA levels at both wild and AF sites did not vary by year (Table 4.4, Rows 4-5).

4.3.2 Pathogen DNA variation by zone

When data from all sites were compared, central CA had the highest levels of both WS-RLO and phage DNA. Phage DNA levels were similar in northern and southern CA, while WS-RLO DNA loads were higher in southern CA than northern CA (Fig. 4.2; Table 4.4, Rows 6-7). Wild black abalone sites in southern CA had higher WS-RLO DNA levels than northern CA, while central CA wild sites had higher phage DNA loads than both northern and southern CA wild sites, which were similar (Table 4.4, Rows 8-9). AF3, located in central CA, had higher WS-RLO DNA levels than both northern (AF1

and AF2) and southern (AF4) facilities, which had similar loads, while AF3 and AF4 had higher phage DNA levels than northern AFs (Table 4.4, Rows 10-11).

Table 4.1. Seawater WS-RLO and phage qPCR data for wild black abalone sites (from north to south) surveyed along the California coast, US in July 2010 and 2011. WS-RLO and phage units are expressed as mean DNA copies/L (± standard deviation). Black abalone count data provided by the Partnership for Interdisciplinary Studies of Coastal Oceans (PISCO) and the Multi-Agency Rocky Intertidal Network (MARINe). nd = not determined.

Site	Lat., Long.	2010			2011		
		Count	WS-RLO	Phage	Count	WS-RLO	Phage
Bodega Bay	38.33325, -123.04805	nd	0	0	nd	0	0
Carmel Point	36.54307, -121.92882	231	0	$2.91x10^4$	227	$2.28x10^2$ ($\pm 5.10x10^2$)	$8.52x10^4$
Andrew Molera State Park	36.28648, -121.84309	425	$1.11x10^2$	$1.66x10^3$	321	0	$1.35x10^4$
Point Sierra Nevada	35.71385, -121.31548	164	0	$5.05x10^3$	155	$6.48x10^1$ ($\pm 9.75x10^1$)	$1.31x10^4$
Rancho Marino	35.54007, -121.08841	27	0	$2.52x10^3$	26	$8.48x10^2$	$2.25x10^4$
San Miguel Island[2]	34.0651151, -120.3580118 Hare Rock	0[1]	0	0	0[1]	0	0
	34.0519621, -120.3580701 Cuyler Harbor		0	0		0	0
Santa Cruz Island[2]	34.0705009, -119.9265448 Painted Cave	0[1]	0	0	0[1]	0	0
	34.022279, -119.6890585 Prisoners Harbor		0	0		0	0
Santa Rosa Island[2]	34.0375686, -120.0866869 Carrington Point	0[1]	0	0	0[1]	$5.55x10^3$	0
	34.006047, -120.196582 Talcott		0	0		0	0
San Nicolas Island[2]	33.232136, -119.537351 Site 8	521	$2.40x10^1$ ($\pm 5.37x10^1$)	0	656	$2.08x10^2$ ($\pm 3.57x10^2$)	$6.74x10^3$
	33.260256, -119.465804 Site 3	76	$7.38x10^2$ ($\pm 1.37x10^3$)	$1.28x10^4$	86	$6.27x10^2$ ($\pm 9.76x10^2$)	$4.88x10^4$
Wild Prevalence			22%	56%		56%	56%

[1]Counts provided for entire island; no black abalone observed.
[2]Channel Islands site.

Table 4.2. Seawater WS-RLO and phage qPCR data for red abalone aquaculture facilities (AFs; from north to south) surveyed along the California (CA) coast, US in July 2010 and 2011. Units are expressed as mean DNA copies/L (± standard deviation). Sub-sites are expressed as distance in meters north/west (+) and south/east (-) from the AF effluent seawater outfall (0 m). AF1 = Davenport, CA. AF2 = Monterey, CA. AF3 = Cayucos, CA. AF4 = Goleta, CA. nd = not determined.

Site	Lat., Long.	Sub-site (m)	2010		2011	
			WS-RLO	Phage	WS-RLO	Phage
AF1	37.01352, -122.19665	500+	0	nd	nd	nd
		100+	0	nd	$1.02x10^{2}$	nd
		0	0	0	0	0
		100-	0	nd	0	nd
		500-	0	nd	nd	nd
		Mean	0	0	$3.40x10^{1}$ ($\pm 5.89x10^{1}$)	0
AF2	36.60023, -121.89467	500+	0	nd	0	nd
		100+	0	nd	0	nd
		0	0	$6.21x10^{2}$	0	$9.63x10^{3}$
		100-	0	nd	0	nd
		500-	0	nd	$8.00x10^{3}$	nd
		Mean	0	$6.21x10^{2}$	$1.60x10^{3}$ ($\pm 3.58x10^{3}$)	$9.63x10^{3}$
AF3	35.44275, -120.89212	500+	0	nd	0	nd
		100+	$1.21x10^{3}$	nd	0	nd
		0	$1.65x10^{3}$	$1.44x10^{5}$	$2.71x10^{5}$	$3.81x10^{7}$
		100-	$1.26x10^{3}$	nd	$4.26x10^{2}$	nd
		500-	$2.79x10^{2}$	nd	$2.86x10^{2}$	nd
		Mean	$8.80x10^{2}$ ($\pm 7.04x10^{2}$)	$1.44x10^{5}$	$5.43x10^{4}$ ($\pm 1.21x10^{5}$)	$3.81x10^{7}$
AF4	34.41683, -119.8322	500+	0	nd	0	nd
		100+	$1.49x10^{3}$	nd	0	nd
		0	$7.84x10^{3}$	$1.51x10^{5}$	$2.61x10^{2}$	$4.77x10^{5}$
		100-	$1.85x10^{2}$	nd	0	nd
		500-	$2.44x10^{2}$	nd	0	nd
		Mean	$1.95x10^{3}$ ($\pm 3.34x10^{3}$)	$1.51x10^{5}$	$5.22x10^{1}$ ($\pm 1.17x10^{2}$)	$4.77x10^{5}$
AF Prevalence			50%	75%	100%	75%

Table 4.3. Seawater WS-RLO qPCR data for fine-scale surveys conducted in October 2013 at the two southern-most red abalone aquaculture facilities (AFs) in Cayucos and Goleta, CA, US (AF3 and AF4, respectively). Only AF3 was sampled over three consecutive days to assess daily variation in DNA levels. Units are expressed as mean WS-RLO DNA copies/L (± standard deviation). Sub-sites are expressed as distance in meters north/west (+) and south/east (-) from the AF effluent seawater outfall (0 m). nd = not determined.

Site	Sub-site (m)	Day 1	Day 2	Day 3
AF3	500+	0	0	0
	300+	$2.38x10^3$	0	0
	100+	$2.54x10^2$	0	0
	75+	$2.23x10^2$	0	0
	50+	0	0	0
	25+	$2.97x10^3$	$1.74x10^5$	$2.85x10^4$
	0	$1.44x10^5$	$1.94x10^5$	$2.80x10^4$
	25-	$3.98x10^4$	$3.88x10^4$	$8.45x10^3$
	50-	$1.48x10^4$	$4.18x10^4$	$2.03x10^3$
	75-	$5.12x10^3$	$8.04x10^3$	$4.95x10^2$
	100-	$4.08x10^3$	$1.46x10^3$	$9.07x10^2$
	300-	$2.11x10^2$	$1.07x10^3$	$1.20x10^3$
	500-	$7.20x10^2$	$3.89x10^2$	0
	Mean	$1.65x10^4$ ($\pm 3.98x10^4$)	$3.54x10^4$ ($\pm 6.77x10^4$)	$5.35x10^3$ ($\pm 1.04x10^4$)
AF4	500+	0	nd	nd
	300+	0	nd	nd
	100+	0	nd	nd
	75+	0	nd	nd
	50+	0	nd	nd
	25+	$4.02x10^3$	nd	nd
	0	$1.58x10^3$	nd	nd
	25-	$1.36x10^3$	nd	nd
	50-	0	nd	nd
	75-	0	nd	nd
	100-	0	nd	nd
	300-	0	nd	nd
	500-	0	nd	nd
	Mean	$5.35x10^2$ ($\pm 1.18x10^3$)	nd	nd

Table 4.4. Least squares regression (LSR) table. Outcomes determined by Tukey's HSD test or Student's t-test. DF = degrees of freedom. AF = aquaculture facility. W = wild black abalone site. N = northern zone. C = central zone. S = southern zone. Bold *P*-values indicate a significant difference. ns = non-significant

Row	Metric	Year	Factor	DF	Sum of Squares	Mean Square	F-Ratio	*P*	Effect Magnitude	Outcome
2	AF WS-RLO	2010 & 2011	Year	1	14.39	14.39	0.67	0.4162	0.01	ns
3	W WS-RLO	2010 & 2011	Year	1	61.49	61.49	5.70	**0.0184**	0.04	2011>2010
4	AF Phage	2010 & 2011	Year	1	23.33	23.33	0.47	0.5056	0.03	ns
5	W Phage	2010 & 2011	Year	1	7.54	7.54	0.22	0.6376	0.00	ns
6	WS-RLO	2010 & 2011	Zone	2	1132.04	566.02	28.55	**<0.0001**	0.16	C>S>N
7	Phage	2010 & 2011	Zone	2	1213.17	606.59	20.09	**<0.0001**	0.37	C>N=S
8	W WS-RLO	2010 & 2011	Zone	2	85.60	42.80	4.01	**0.0205**	0.06	S>N
9	W Phage	2010 & 2011	Zone	2	521.63	260.81	10.87	**<0.001**	0.35	C>N=S
10	AF WS-RLO	2010 & 2011	Zone	2	978.40	489.20	20.83	**<0.0001**	0.19	C>N=S
11	AF Phage	2010 & 2011	Zone	2	652.86	326.43	25.11	**<0.0001**	0.71	C=S>N
12	WS-RLO	2010	Site Type	1	171.06	171.06	14.30	**0.0003**	0.12	AF>W
13	Phage	2010	Site Type	1	23.75	23.75	0.72	0.4049	0.03	ns
14	WS-RLO	2011	Site Type	1	3.30	3.30	0.19	0.6665	0.00	ns
15	Phage	2011	Site Type	1	279.58	279.59	6.62	**0.0148**	0.17	AF>W
16	WS-RLO	2010 & 2011	Site Type	1	111.67	111.67	7.45	**0.0069**	0.04	AF>W
17	Phage	2010 & 2011	Site Type	1	317.71	317.71	8.38	**0.0052**	0.12	AF>W
18	W vs AF WS-RLO	2010	Whole model	5	671.11	134.22	18.26	**<0.0001**	0.48	
19			Residual	98	720.31	7.35				
20			Site Type	1	343.95		46.79	**<0.0001**	0.25	AF>W
21			Zone	2	342.89		23.33	**<0.0001**	0.25	S=C>N
22			Zone x Site Type	2	196.51		13.37	**<0.0001**	0.14	S-AF=C-AF>N-AF,N-W,C-W,S-W
23		2011	Whole model	5	192.30	38.46	2.34	**0.0472**	0.11	
24			Residual	94	1541.96	16.40				
25			Site Type	1	11.13		0.68	0.4121	0.01	ns
26			Zone	2	100.60		3.07	0.0513	0.06	ns trend: C>N
27			Zone x Site Type	2	98.76		3.01	0.0541	0.06	ns trend: C-AF>N-W
28	W vs AF phage	2010	Whole model	5	439.08	87.82	3.64	**0.0142**	0.44	
29			Residual	23	554.59	24.11				

30			Site Type	1	120.27		4.99	**0.0355**	0.12	AF>W
31			Zone	2	204.36		4.24	**0.0271**	0.21	C>N
32			Zone x Site Type	2	146.60		3.04	0.0674	0.15	
33		2011	Whole model	5	958.00	191.60	7.76	**<0.0001**	0.57	
34			Residual	29	716.10	24.69				
35			Site Type	1	321.82		13.03	**0.0011**	0.19	AF>W
36			Zone	2	483.65		9.79	**0.0006**	0.29	C>N
37			Zone x Site Type	2	87.75		1.78	0.1871	0.05	
38	W WS-RLO	2010 & 2011	Site	12	328.14	27.35	2.88	**0.0017**	0.23	SNI3>PSN=CAR=BB
39	W Phage	2010 & 2011	Site	12	1492.14	124.35	31.95	**<0.0001**	0.92	CP=SNI3=PSN=RM=AMSP>all other W
40	W WS-RLO	2010	Site	12	124.91	10.41	1.86	0.0634	0.30	ns
41	W WS-RLO	2011	Site	12	326.66	27.22	2.24	**0.0233**	0.34	
42	W Phage	2010	Site	12	848.52	70.71	527.69	**<0.0001**	1.00	
43	W Phage	2011	Site	12	961.32	80.11	602.28	**<0.0001**	1.00	
44	AF WS-RLO	2010 & 2011	Site	3	403.03	134.34	8.03	**0.0001**	0.25	AF3>AF2=AF1
45	AF Phage	2010 & 2011	Site	3	681.55	227.18	65.87	**<0.0001**	0.94	AF4=AF3>AF2>AF1
46	AF WS-RLO	2010	Site	3	477.13	159.05	17.23	**<0.0001**	0.59	AF4=AF3>AF2=AF1
47	AF WS-RLO	2011	Site	3	121.27	40.42	1.95	0.1409	0.15	ns
48	AF Phage	2010	Site	3	267.58	89.19	520.25	**<0.0001**	1.00	AF4=AF3>AF2>AF1
49	AF Phage	2011	Site	3	431.29	143.76	13494.37	**<0.0001**	1.00	AF3>AF4>AF2>AF1
50	AF WS-RLO	2010 & 2011	Sub-site	4	220.10	55.03	2.82	**0.0314**	0.14	0m>all other sub-sites
51	WS-RLO	2013	Site	1	355.41	355.41	12.38	**0.0007**	0.11	AF3>AF4
52	Phage	2013	Site	1	65.57	65.57	94.46	**<0.0001**	0.94	AF3>AF4
53	AF4 WS-RLO	2013	Sub-site	12	426.37	35.53	104.46	**<0.0001**	0.99	0=25+=25->all other sub-sites
54	AF3 WS-RLO	2013	Whole model	25	2135.29	85.41	13.04	**<0.0001**	0.86	
55			Residual	51	334.17	6.55				
56			Sub-site	12	1826.03		23.22	**<0.0001**	0.74	
57			Day	1	110.47		16.86	**0.0001**	0.04	
58			Day x Sub-site	12	222.59		2.83	**0.0048**	0.09	

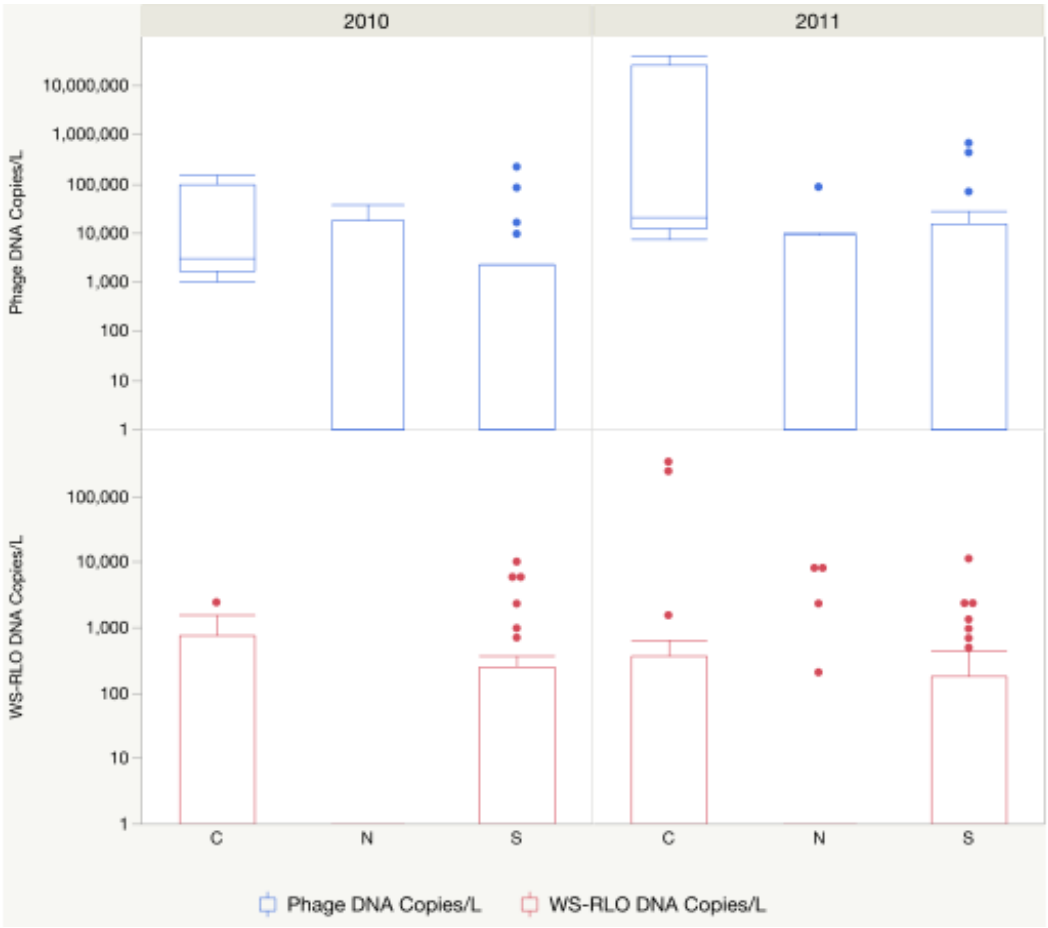

Figure 4.2. Box plot of seawater WS-RLO and phage qPCR data by year (July 2010 and 2011) and geographic zone along the California coast, US. Units are expressed as DNA copies/L of seawater and dots represent outliers. N = northern zone, C = central zone, and S = southern zone.

4.3.3 Pathogen DNA variation by site type

In 2010, AFs had higher WS-RLO DNA levels than wild black abalone sites and similar phage DNA loads. However, in 2011, AFs had higher phage DNA levels than wild sites, while their WS-RLO DNA loads were similar (Fig. 4.3; Table 4.4, Rows 12-15). Overall, when both years' data were combined, AFs had higher WS-RLO and phage DNA levels than wild sites (Table 4.4, Rows 16-17). To address differences among years, we analyzed pathogen DNA levels by site type, zone and their interaction. In 2010, all factors influenced WS-RLO DNA with site type, zone, and their interaction accounting

for 64% of the whole model and southern and central AFs having higher loads than northern AFs and all wild sites (Table 4.4, Rows 18-22). These factors were not significant in 2011, although there was a trend of the central AF (AF3) having higher WS-RLO DNA levels than northern wild sites (Table 4.4, Rows 23-27). Site type and zone influenced phage DNA levels in both 2010 and 2011 with AFs having higher loads than wild sites and central CA having higher loads than northern CA. No interaction between site and zone was observed for phage DNA levels during either year (Table 4.4, Rows 28-37).

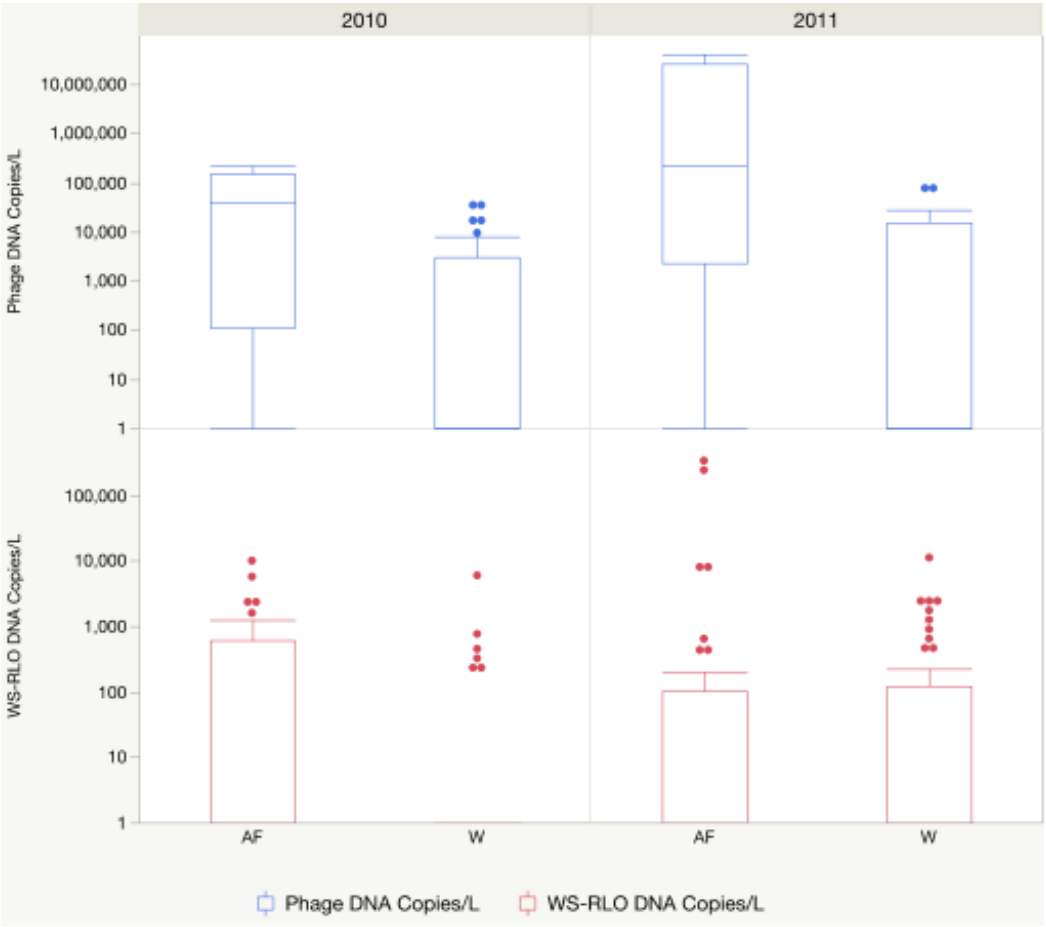

Figure 4.3. Box plot of seawater WS-RLO and phage qPCR data by year (July 2010 and 2011) and site type along the California coast, US. Units are expressed as DNA copies/L of seawater and dots represent outliers. AF = red abalone aquaculture facilities. W = wild sites with established black abalone populations.

4.3.4 Pathogen DNA variation within site type

When all data were examined among wild black abalone sites, SNI Site 3 had greater WS-RLO DNA levels than those at Point Sierra Nevada, Carmel Point, and Bodega Bay, while loads at all other wild sites were similar (Table 4.1; Table 4.4, Row 38). Phage DNA levels varied among wild sites with seawater from Carmel Point, SNI Site 3, Point Sierra Nevada, Rancho Marino, and Andrew Molera State Park containing higher loads than all other wild sites (Table 4.1; Table 4.4, Row 39). When WS-RLO DNA levels were compared among wild sites by year, they were similar in 2010 and varied in 2011, while phage DNA levels varied both years with Carmel Point having almost twice the amount of phage DNA than all other wild sites (Fig. 4.4; Table 4.4, Row 40-43). Bodega Bay, San Miguel Island, Santa Cruz Island, and Santa Rosa Island lacked phage DNA detection in both years (Table 4.1).

Among AFs, WS-RLO DNA levels at AF3 were significantly higher than all other AFs, while phage DNA loads were similar and highest at AF3 and AF4 (Table 4.2; Table 4.4, Row 44-45). When WS-RLO DNA levels were compared among AFs by year, they varied in 2010 with AF3 and AF4 having similar loads, and AF2 and AF1 lacking WS-RLO DNA detection. However, in 2011 all AFs had similarly low WS-RLO DNA loads (Fig. 4.5; Table 4.2; Table 4.4, Row 46-47). When phage DNA levels were compared among AFs by year, they varied in 2010 with AF3 and AF4 having similarly high levels, AF2 having lower levels, and AF1 lacking phage DNA detection. In 2011, phage DNA loads were highest at AF3, followed by AF4, AF2, and no detection at AF1 (Fig. 4.5; Table 4.2; Table 4.4, Row 48).

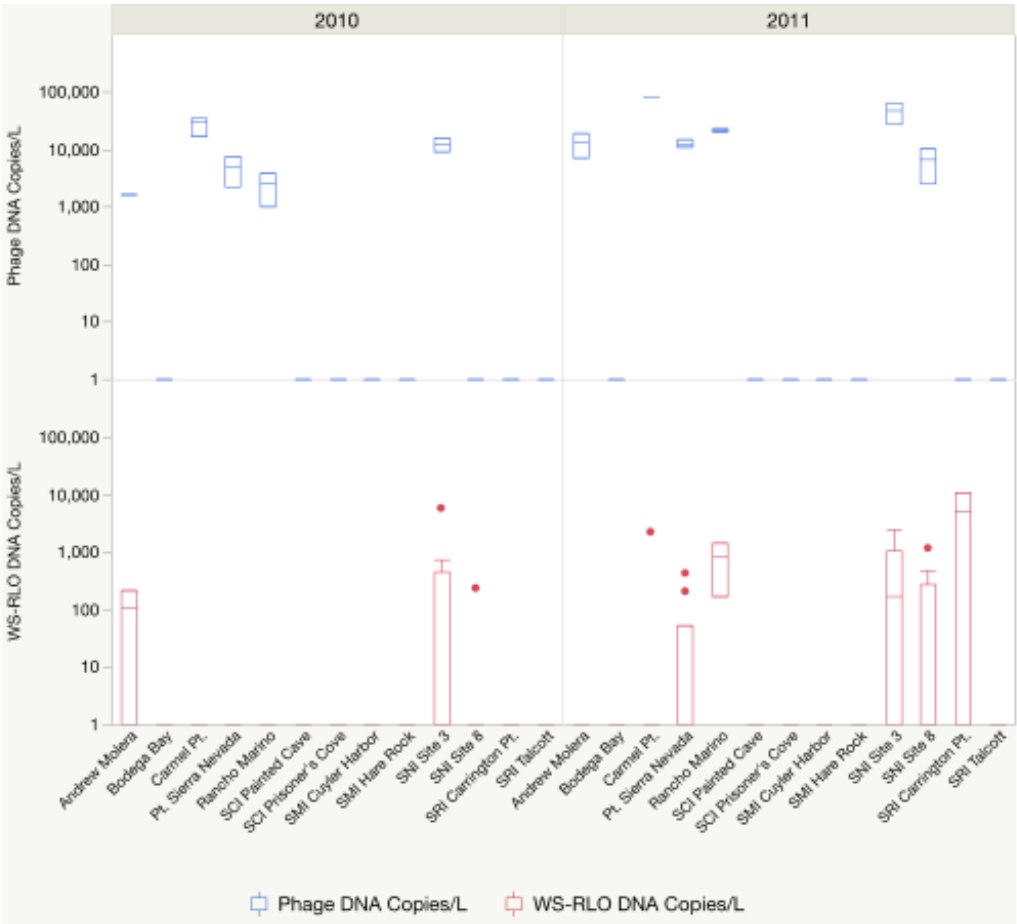

Figure 4.4. Box plot of seawater WS-RLO and phage qPCR data by year (July 2010 and 2011) among wild sites with established black abalone populations along the California coast, US. Units are expressed as DNA copies/L of seawater and dots represent outliers. SCI = Santa Cruz Island. SMI = San Miguel Island. SRI = Santa Rosa Island. SNI = San Nicolas Island.

4.3.5 Pathogen DNA variation by sub-site

When 2010 and 2011 data were combined, WS-RLO DNA levels at AFs were nearly 2x higher directly adjacent to effluent outfalls (0 m) than all other sub-sites (Table 4.2; Table 4.4, Row 50). Analysis of phage DNA levels by sub-site was not performed since data were collected only from 0 m.

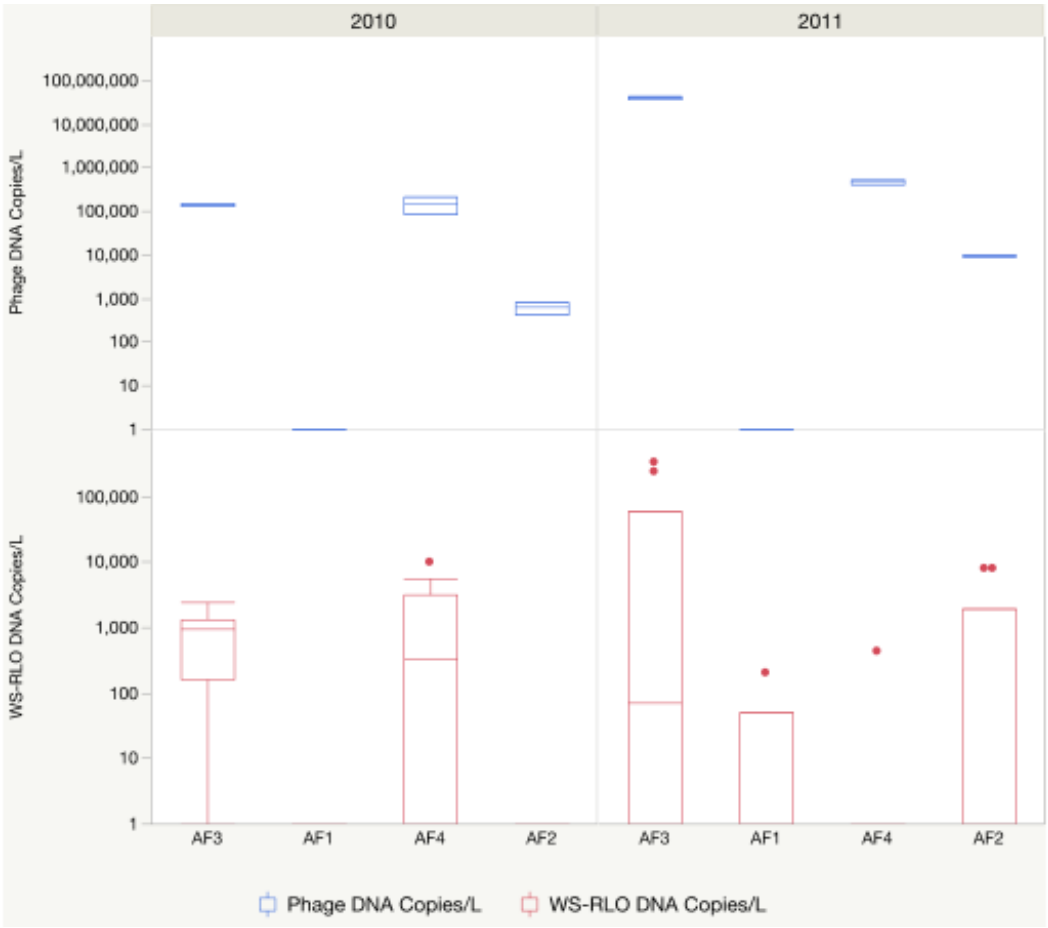

Figure 4.5. Box plot of seawater WS-RLO and phage qPCR data by year (July 2010 and 2011) among red abalone aquaculture facilities (AFs) along the California (CA) coast, US. Units are expressed as DNA copies/L of seawater and dots represent outliers. AF1 = Davenport, CA. AF2 = Monterey, CA. AF3 = Cayucos, CA. AF4 = Goleta, CA.

4.3.6 Fine-scale seawater surveys

For the fine-scale seawater surveys conducted at the two southern most AFs in October 2013, AF3 had more WS-RLO and phage DNA than AF4 (Table 4.3; Table 4.4, Row 51-52). At AF4, which was sampled on a single day, WS-RLO DNA levels were similar and highest at the 0, +25 and -25 m sub-sites, while all other sub-sites lacked WS-RLO DNA detection (Table 4.3; Table 4.4, Row 53). At AF3, which was sampled over three consecutive days, WS-RLO DNA levels were largely influenced by sub-site, while day and a day x sub-site interaction had a lesser effect (Table 4.4, Row 54-58).

4.3.7 WS-RLO DNA loads were less than the established infectious dose

The highest observed WS-RLO DNA load at a wild site was $5.55x10^3$ copies/L at Carrington Point on SRI in 2011 (Table 4.1), while the highest at an AF was $2.71x10^5$ copies/L at AF3 in 2011 directly adjacent to the effluent outfall (Table 4.2), both of which are less than the established dose to infect 50% of abalone after a 3-hour exposure to the WS-RLO ($2.26x10^6$ copies/L; Crosson et al. 2020).

4.4 Discussion

This was the first large-scale, multi-year survey to assess the geographic distribution of the RLO and novel phage associated with abalone withering syndrome in California seawater using DNA-based qPCR assays. We employed the highly sensitive and specific qPCR assays of Friedman et al. (2014a) and Langevin et al. (*in manuscript*) to detect and quantify WS-RLO and phage DNA, respectively. During our surveys, WS-RLO DNA was detected as far north as Davenport, CA and as far south as San Nicolas Island (SNI), while phage DNA was detected from Monterey Bay, CA to SNI. Previous surveys have shown the WS-RLO to be continuously distributed along the west coast of North America from southern Sonoma County, California, including the Channel and Farallon Islands, to Baja California, Mexico (Álvarez-Tinajero et al. 2002, Friedman & Finley 2003, CDFW unpubl. obs.). Friedman & Finely (2003) identified the WS-RLO in two red abalone populations in northern CA, Van Damme State Park and Crescent City, during a 1999−2000 survey. However, the WS-RLO has not been detected at those locations since and may have failed to become established due to cool seawater temperatures (CDFW unpubl. obs.).

This was the first study to compare withering syndrome pathogen DNA levels in seawater between wild and cultured abalone sites and evaluate WS-RLO DNA dilution potential in seawater directly adjacent to a point source discharge. Our findings suggest WS-RLO DNA dilution potential in surface seawater directly adjacent to AFs is high. WS-RLO DNA loads were less than the mean levels detected at all wild black abalone sites previously surveyed within 50 to 500 m of the AFs effluent outfalls. High dilution potential, coupled with the rapid degradation of WS-RLO DNA in seawater (stability limited to ~2 days; Crosson et al. 2020), make the relative risk of an AF acting as a significant source of pathogen spillover dependent on its proximity to wild abalone populations and local seawater circulation patterns. Future studies to examine fine-scale oceanographic patterns surrounding CA AFs and to determine AF proximity to wild abalone populations are essential in order to accurately assess WS-RLO transmission risk.

Interestingly, no WS-RLO DNA levels detected in our surveys exceeded the established dose to infect 50% (ID50) of red abalone after a 3-hour exposure to the WS-RLO in 18°C seawater ($2.26x10^6$ copies/L; Crosson et al. 2020). However, exposure time, seawater temperature, and abalone species susceptibility are all important factors to consider and further ID50 challenges are needed in order to accurately assess WS-RLO transmission occurring in the natural environment. A recent sentinel study conducted in southern CA estimated WS-RLO transmission risk *in situ* by deploying modules containing uninfected red abalone at two sites, one ~500 m off-shore to AF4 near Goleta, CA and one ~400 m off-shore and in proximity to a wild abalone population near Corona Del Mar, CA, a site not included in the present study (Fuller, 2017). WS-RLO DNA was

detected in seawater at the wild module site, but not at the AF module site even though WS-RLO DNA was detected in the AF's effluent seawater (Fuller, 2017). After six weeks of exposure, sentinel abalone at both sites developed low level WS-RLO infections. These *in situ* transmission data suggest a WS-RLO vector other than abalone, such as kelps or other macroalgae to which the pathogen could adhere and concentrate, may be a link from sources of WS-RLO in the natural environment to off-shore abalone populations and warrant further examination (Fuller, 2017).

Our findings suggest there is a high degree of spatial and temporal variability in WS-RLO and phage DNA levels in CA seawater. For example, when all survey data were combined and analyzed by site type there was more WS-RLO and phage DNA detected at AFs than sites with establish wild black abalone populations. However, when analyzing combined data by year, there was no significant difference between AF and wild site phage DNA loads or AF WS-RLO DNA loads, while wild site WS-RLO DNA loads were greater in 2011 than 2010. When analyzing combined data by zone, WS-RLO and phage DNA loads were highest in central CA and WS-RLO DNA loads were higher in southern CA than northern CA, while their phage DNA loads were similar.

Two key environmental factors likely driving pathogen DNA spatial and temporal variation are seawater temperatures and abalone host densities. Seawater temperatures of 18°C are known to facilitate WS-RLO proliferation (Moore et al. 2000, Friedman et al. 2002, Braid et al. 2005, Crosson & Friedman 2018) and likely explains higher observed levels of pathogen DNA detected in southern CA where mean ambient coastal seawater temperatures are typically highest (https://www.nodc.noaa.gov). The highest observed levels of pathogen DNA were in central CA, which was largely driven by AF3 in

Cayucos, CA. This facility was the largest of all the CA AFs, producing over 100 tons of red abalone per year and accounting for over 50% of all cultured abalone in the US. In late winter of 2020, AF3 closed production (R. Fields, The Abalone Farm, Inc., pers. comm.) and it will be interesting to see if and how WS-RLO disease dynamics in central CA may change in response to the closure.

We also observed high levels of pathogen DNA at SNI, which has abundant black abalone populations whose numbers are steadily increasing relative to other wild sites. The availability of relatively large numbers of highly susceptible hosts and warm seawater temperatures make southern CA a prime location for WS transmission to occur as evidenced by large WS-induced die-offs in black abalone populations in this area (Altstatt et al. 1996, Miner et al. 2006, Neuman et al. 2010, Crosson et al. 2014, Friedman et al. 2014b). In addition, sites in the northern and north-central zones of CA with high abalone densities and lower seawater temperatures have not yet suffered losses due to WS, even at sites with relatively high WS-RLO DNA loads quantified in seawater by this study, further highlighting the influence of temperature as a key driver of WS transmission and disease development (Moore et al. 2000, Ben-Horin et al. 2013).

It is important to acknowledge potential issues surrounding the use of PCR-based methods for monitoring pathogen DNA in aquatic ecosystems. Foremost, the presence and amount of pathogen DNA do not confirm pathogen viability or infectivity, instead serving as proxies for pathogen detection and potential exposure. PCR-based assays are especially valuable for the detection and quantification of unculturable pathogens such as the WS-RLO and its associated phage. Other studies comparing bacterial quantification via PCR-based assays to traditional culture-based methods found PCR to be a viable

alternative, although DNA quantities and limits of detection relative to traditional methods can vary by the taxon being examined (Wright et al. 2007, Ricchi et al. 2017). Numerous environmental factors can influence DNA dispersal and persistence in aquatic ecosystems. Bacterial DNA persistence in seawater can vary considerably, as short as 6.5 h to an estimated 2 months (see review by Neilsen et al. 2007). Abiotic factors, such as prolonged exposure to high seawater temperatures and UV radiation from sunlight, can result in single stranded and fragmented DNA molecules affecting PCR results (Paul et al. 1987, Huver et al. 2015), as well as biotic factors, such as host metabolism and DNA excretion rates (Thomsen et al. 2012).

Collectively, our findings help elucidate WS disease dynamics along the CA coast and have large implications for the management and restoration of all CA abalone species. In order to fully assess the potential for wild and cultured abalone disease interactions, additional experiments should be conducted to: (1) determine the longevity and infectivity of the WS-RLO and novel phage in seawater, (2) survey local oceanographic patterns surrounding both wild and cultured abalone populations, and (3) examine non-target organisms potentially serving as pathogen reservoirs or vectors. These experiments will assess disease transmission risk within and among abalone populations for better informed management decisions to successfully protect and restore abalone species in WS-endemic areas.

5. Acknowledgements

This research was funded, in part, by a grant from the National Sea Grant College Program, National Oceanic and Atmospheric Administration, U.S. Department of Commerce, under project number R/FISH-208 through the California Sea Grant Program, the School of Aquatic and Fishery Sciences at the University of Washington, and the California Department of Fish and Wildlife. I would like to express my sincere gratitude to the California abalone aquaculture industry for participating in my research, especially Ray Fields, Brad Buckley, Doug Bush, Tom Ebert, Art Seavey and Trevor Fay. Without their collaborative support much of this research would not have been possible. This research utilized data collected by the Partnership for Interdisciplinary Studies of Coastal Oceans (PISCO) and the Multi-Agency Rocky Intertidal Network (MARINe): a long-term ecological consortium funded by many groups, including the Bureau of Ocean Energy Management (BOEM), PISCO, the National Park Service (NPS) and the California Ocean Protection Council (OPC). For more information, please visit pacificrockyintertidal.org. I thank my PISCO collaborators Peter Raimondi, Melissa Miner and Christy Bell as well as David Kushner and Stephen Whitaker from the NPS. I thank Jennifer Dugan and Mark Paige from UC Santa Barbara, Don Canestro from UC Santa Barbara Kenneth S. Norris Rancho Marino Reserve, John Butler at NOAA Southwest Fisheries Science Center and Jim Moore, Blythe Marshman and Jim Snyder from the California Department of Fish and Wildlife. To my colleagues at the University of Washington who helped with animal care, sample processing, project logistics and editorial comments, thank you - especially to Dr. Brent Vadopalas, Samuel White, Nate Wight, Samantha Adams, Dr. Colleen Burge, Dr. Kristina Straus, Josh Bouma, Bethany

Stevick, Vanessa Lowe, Elene Dorfmeier, Dan Gillon, Sean Bennett, Bryanda Wipple, Nina Lottsfeldt, Mariah Weavil-Abueg and Dr. Stanley Langevin. To my committee, Dr. Carolyn Friedman, Dr. Glenn VanBlaricom, Dr. Steven Roberts, Dr. James Winton and Dr. Jeffrey Riffell, I am extremely grateful for your understanding, wisdom, patience, enthusiasm, and encouragement throughout my project. Most of all, thank you to all my friends and family for a decade-worth of emotional support and never letting me give up.

The views expressed herein are those of the author and do not necessarily reflect the views of NOAA or any of its sub-agencies. Any use of trade product or firm name herein is for descriptive purposes only and does not imply endorsement by the US Government. The US government is authorized to reproduce and distribute this paper for governmental purposes.

6. References

Altstatt JM, Ambrose RF, Engle JM, Haaker PL, Lafferty KD, Raimondi PT. 1996. Recent declines of black abalone *Haliotis cracherodii* on the mainland coast of central California. Mar Ecol Prog Ser 142:185-192.

Álvarez-Tinajero M del C, Caceres-Martinez J, Aviles JGG. 2002. Histopathological evaluation of the yellow abalone *Haliotis corrugata* and the blue abalone *Haliotis fulgens* from Baja California, Mexico. J Shellfish Res 21:825−830.

Andree KB, Friedman CS, Moore JD, Hedrick RP. 2000. A polymerase chain reaction for detection of genomic DNA of a *Rickettsiales*-like prokaryote associated with Withering Syndrome in black abalone (*Haliotis cracherodii*). J Shellfish Res 19:213-218.

Angilletta MJ. 2009. Thermal adaptation: a theoretical and empirical synthesis. Oxford University Press. DOI:10.1093/acprof:oso/9780198570875.003.0001

Antonio DB, Andree KB, Friedman CS, Hedrick RP. 2000. Detection of *Rickettsiales*-like prokaryotes (RLPs) by *in situ* hybridization in black abalone *Haliotis cracherodii* with Withering Syndrome. J Invert Path 75(2):180-182.

Balseiro P, Aranguren R, Gestal C, Novoa B, Figueras A. 2006. *Candidatus* Xenohaliotis californiensis and *Haplosporidium montforti* associated with mortalities of abalone *Haliotis tuberculata* cultured in Europe. Aquaculture 258:63-72.

Ben-Horin T, Lenihan HS, Lafferty KD. 2013. Variable intertidal temperature explains why disease endangers black abalone. Ecology 94(1):161-168.

Boots M, Bowers RG. 1999. Three mechanisms of host resistance to microparasites-avoidance, recovery and tolerance-show different evolutionary dynamics. J Theoretical Bio 201(1):13-23.

Braid BA, Moore JD, Robbins TT, Hedrick RP, Tjeerdema RS, Friedman CS. 2005. Health and survival of red abalone, *Haliotis rufescens*, under varying temperature, food supply, and exposure to the agent of withering syndrome. J Invert Path 89(3):219-231.

Burreson EM. 2008. Misuse of PCR assay for diagnosis of mollusc protistan infections. Dis Aquat Org 80:81-83.

Casares-Martinez J, Tinoco-Orta GD. 2001. Symbionts of cultured red abalone *Haliotis rufescens* from Baja California, Mexico. J Shellfish Res 29(2):875-881.

Chang PH, Yang MC, Kuo ST, Chen MH, Cheng CH. 2008. Occurrence of a *Rickettsia*-like prokaryote in the small abalone, *Haliotis diversicolor supertexta*, cultured in Taiwan. Bull Euro Assoc Fish Path 28:52-57.

Cheng W, Hsiao I, Hsu C, Chen J. 2004. Change in water temperature on the immune response of Taiwan abalone *Haliotis diversicolor supertexta* and its susceptibility to Vibrio parahaemolyticus. Fish & Shellfish Immun 17(3):235-243.

Cook PA, Gordon HR. 2010. World abalone supply, markets, and pricing. J Shellfish Res 29(3):569-571.

Cook PA. 2016. Recent trends in worldwide abalone production. J Shellfish Res 35(3):581-583.

Cox KW. 1962. California abalone, family *Haliotidae*. The Resources Agency of California, Department of Fish and Game, Fish Bull 118:130.

Crosson LM, Wight N, VanBlaricom GR, Kiryu I, Moore JD, Friedman CS. 2014. Abalone withering syndrome: distribution, impacts, current diagnostic methods and new findings. Dis Aquat Org 108:261-270.

Crosson LM, Friedman CS. 2018. Withering syndrome susceptibility of northeastern Pacific abalones: A complex relationship with phylogeny and thermal experience. J Invert Path 151:91-101.

Crosson LM, Lottsfeldt NS, Weavil-Abueg ME, Friedman CS. 2020. Abalone withering syndrome disease dynamics: infectious dose and temporal stability in seawater. J Aquat Animal Health.

Dahlhoff EA, Somero GN. 1993. Effects of temperature on mitochondria from abalone (genus *Haliotis*): adaptive plasticity and its limits. J Exper Bio 185(1):151-168.

Dang VT, Speck P, Benkendorff K. 2012. Influence of elevated temperatures on the immune response of abalone, *Haliotis rubra*, Fish & Shellfish Immun 32(5):732-740.

Darwin C. 1859. On the Origin of Species by Means of Natural Selection or the Preservation of Favored Races in the Struggle for Life (J. Murray, London).

Davis GE, Richards DV, Haaker PL, Parker DO. 1992. Abalone population declines and fishery management in southern California. In: Shepard SA, Tegner MJ, Guzman del Proo SA (eds) Abalone of the World: Biology, Fisheries and Culture. Proceedings of the 1st International Symposium on Abalone, La Paz, Mexico.

Daszak P, Cunningham AA, Hyatt AD. 2000. Emerging infectious diseases of wildlife-threats to biodiversity and human health. Science 287(5452):443-449.

Díaz F, Re AD, Medina Z, Re G, Valdez G, Valenzuela F. 2006. Thermal preference and tolerance of green abalone *Haliotis fulgens* (Philippi, 1845) and pink abalone *Haliotis corrugata* (Gray, 1828). Aquaculture Research 37(9):877-884.

Douros WJ. 1987. Stacking behavior of an intertidal abalone: an adaptive response or a consequence of space limitation? J Exper Mar Bio & Ecol 108(1):1-14.

Estes JA, Lindberg DR, Wray C. 2005. Evolution of large body size in abalones (*Haliotis*): patterns and implications. Paleobiology 31(4):591-606.

FAO, Food & Agriculture Organization of the United Nations. 2017. GLOBEFISH: analysis & information on world fish trade.
http://www.fao.org/in-action/globefish/market-reports/resource-detail/en/c/902597/

Fisher WS. 1986. Structure and functions of oyster hemocytes. Immunity in invertebrates. Springer Berlin Heidelberg, 25-35.

Fisher WS, Auffret M, Ballouet G. 1987. Response of European flat oyster (*Ostrea edulis*) hemocyte to acute salinity and temperature changes. Aquaculture 67:179-190.

Friedman CS, Thomson M, Chun C, Haaker PL, Hedrick RP. 1997. Withering syndrome of the black abalone, *Haliotis cracherodii* (Leach): water temperature, food availability, and parasites as possible causes. J Shellfish Res 16(2):403-411.

Friedman CS, Andree KB, Beauchamp K, Moore JD, Robbins TT, Shields JD, Hedrick RP. 2000. "Candidatus Xenohaliotis californiensis", a newly described pathogen of abalone, Haliotis spp., along the west coast of North America. Inter J Syst Evol Microbiol 50:847-855.

Friedman CS, Biggs W, Shields JD, Hedrick RP. 2002. Transmission of Withering Syndrome in black abalone, *Haliotis cracherodii* Leach. J Shellfish Res 21(2):817-824.

Friedman CS, Trevelyan G, Mulder EP, Fields R. 2003. Development of an oral administration of oxytetracycline to control losses due to withering syndrome in cultured red abalone *Haliotis rufescens*. Aquaculture 224(1-4):1-23.

Friedman CS, Finley CA. 2003. Evidence for an anthropogenic introduction of "*Candidatus* Xenohaliotis californiensis", the etiological agent of withering syndrome, into northern California abalone populations via conservation efforts. Can J Fish Aquat Sci 60:1424-1431.

Friedman CS, Scott BB, Strenge RE, Vadopalas B, McCormick TB. 2007. Oxytetracycline as a tool to manage and prevent losses of the endangered white abalone, *Haliotis sorenseni*, caused by withering syndrome. J Shellfish Res 26(3):877-885.

Friedman CS, Crosson LM. 2012. Putative phage hyperparasite in the rickettsial pathogen of abalone, "*Candidatus* Xenohaliotis californiensis". Micro Ecol 64(4):1064-1072.

Friedman CS, Wight N, Crosson LM, White SJ, Strenge RM. 2014a. Validation of a quantitative PCR assay for detection and quantification of '*Candidatus* Xenohaliotis californiensis'. Dis Aquat Org 108:251-259.

Friedman CS, Wight N, Crosson LM, VanBlaricom GR, Lafferty KD. 2014b. Reduced disease in black abalone following mass mortality: phage therapy and natural selection. Front Microbiol 5(78):1-10.

Fuhrman JA, Noble RT. 1995. Viruses and protists cause similar bacterial mortality in coastal seawater. Limnol & Ocean 40:1236-1242.

Fuller AM 2017. Transmission dynamics of the withering syndrome *Rickettsia*-like organism to abalone in California. Master's Thesis. University of Washington. Seattle, WA. http://hdl.handle.net/1773/40574

Gao LY, Kwaik YA. 2000. The modulation of host cell apoptosis by intracellular bacterial pathogens. Trends in Micro 8(7):306-313.

Garcia-Esquivel Z, Montes-Magallon S, Gonzales-Gomez MA. 2007. Effect of temperature and photoperiod on the growth, feed consumption, and biochemical content of juvenile green abalone, *Haliotis fulgens*, fed on a balanced diet. Aquaculture 262(1):129-141.

Gardner GR, Harshbarger JC, Lake JL, Sawyer TK, Price KL, Stephenson MD, Haaker PL, Togstad HA. 1995. Association of prokaryotes with symptomatic appearance of withering syndrome in black abalone *Haliotis cracherodii*. J Invert Path 66:111–120.

Geiger DL, Groves LT. 1999. Review of Fossil Abalone (Gastropoda: Vetigastropoda: Haliotidae) with Comparison to Recent Species. J Paleo 73(5):872-885.

Geiger DL. 2000. Distribution and biogeography of the recent Haliotidae (Gastropoda: Vetigastropoda) worldwide. Bull Malacol 35:57–120.

González RC, Brokordt K, Lohrmann KB. 2012. Physiological performance of juvenile *Haliotis rufescens* and *Haliotis discus hannai* abalone exposed to the withering syndrome agent. J Invert Path 111(1):20-26.

Gordon HR, Cook PA. 2001. World abalone supply, markets and pricing: historical, current and future. J Shellfish Res 20(2):567-570.

Gordon HR, Cook PA. 2004. World abalone fisheries and aquaculture update: supply and market dynamics. J Shellfish Res 23(1):935-939.

Grassly NC, Fraser C. 2008. Mathematical models of infectious disease transmission. Nature Rev Microbiol 6(6):477.

Guld RN, Middelboe M. 2004. Virus & bacteria dynamics of a coastal sediment: Implications for benthic carbon cycling. Limnol & Ocean 49:2073-2081.

Haaker PL, Henderson KC, Parker DO. 1986. California Abalone. Marine Resources Leaflet No. 11. State of California, The Resources Agency, Department of Fish and Game, Marine Resources Division, Long Beach, CA.

Haaker PL, Richards DV, Friedman CS, Davis G, Parker DO, Togstad H. 1992. Abalone withering syndrome and mass mortality of black abalone, *Haliotis cracherodii* in California. In: Shephard SA, Tegner M, Guzman del Proo S (eds) Abalone of the World. Blackwell Scientific, Oxford, p214-224.

Hackstadt T. 1998. The diverse habitats of obligate intracellular parasites. Current Opinion Microbiol 1:82-87.

Hine PM, Wakefield S, Diggles BK, Webb VL, Maas EW. 2002. Ultrastructure of a haplosporidian containing *Rickettsiae*, associated with mortalities among cultured paua *Haliotis iris*. Dis Aquat Org 49(3):207-219.

Hines A, Anderson S, Brisbin M. 1980. Heat tolerance in the black abalone, *Haliotis cracherodii* (Leach, 1814): effects of temperature fluctuation and acclimation. Veliger 23:2.

Hobday AJ, Tegner MJ. 2000. Status review of white abalone (*Haliotis sorenseni*) throughout its range in California and Mexico. NOAA Technical Memorandum NOAA-TM-SWR-035, U.S. Department of Commerce, National Oceanic and Atmospheric Administration. National Marine Fisheries Service, Long Beach, CA.

Huey RB, Stevenson RD. 1979. Integrating thermal physiology and ecology of ectotherms: a discussion of approaches. American Zoologist 357-366.

Huver JR, Koprivnikar J, Johnson PTJ, Whyard S. 2015. Development and application of an eDNA method to detect and quantify a pathogenic parasite in aquatic ecosystems. Ecological Applications 25(4):991-1002.

Karpov KA, Haaker PL, Taniguchi I, RogersBennett L. 2000. Serial depletion and the collapse of the California abalone fishery. Canadian Special Publication on Fisheries and Aquatic Sciences 130:11–24.

Kiryu I, Kurita J, Yuasa K, Nishioka T, Shimahara Y, Kamaishil T, Ototake M, Oseko N, Tange N, Inoue M, Yatabe T, Friedman CS. 2013. First detection of *Candidatus* Xenohaliotis californiensis, the causative agent of withering syndrome, in Japanese black abalone *Haliotis discus discus* in Japan. Fish Path 48(2):35-41.

Kismohandaka G, Friedman CS, Roberts W, Hedrick RP. 1993. Investigation of physiological parameters of black abalone with withering syndrome. J Shellfish Res 12:131-132.

Lafferty KD, Kuris AM. 1993. Mass mortality of abalone *Haliotis cracherodii* on the California channel-islands - tests of epidemiologic hypotheses. Mar Ecol Prog Ser 96:239-248.

Lafferty KD, Ben-Horin T. 2013. Abalone farm discharges the withering syndrome pathogen into the wild. Frontiers Microbiol 4:373.

Langevin SA, Closek CJ, Burge CA, White SJ, Crosson LM, Wippel BJ, Solberg OD, Friedman CS. *In manuscript*. Molecular characterization of a bacteriophage and its pathogenic *Rickettsia* host, *Candidatus* Xenohaliotis californiensis, reveals a segmented phage genome that exhibits novel biological mechanisms to ensure the survival of this dual endosymbiont relationship.

Leighton DL. 1974. The influence of temperature on larval and juvenile growth in three species of southern California abalones. Fish Bull 72(4):1137-1145.

Le Roux F, Audemard C, Barnaud A, Berthe F. 1999. DNA probes as potential tools for the detection of *Marteilia refringes*. Mar Biotechnol 1:588-597.

Luna LG. 1968. Manual of histological staining methods of the Armed Forces Institute of Pathology, 3rd ed. Am Reg Pathol, MaGraw-Hill Book Co., New York, 258 pp.

Metz EC, Robles-Sikisaka R, Vacquier VD. 1998. Nonsynonymous substitution in abalone sperm fertilization genes exceeds substitution in introns and mitochondrial DNA. Proceedings of the National Academy of Sciences 95(18):10676-10681.

Miner CM, Altstatt JM, Raimondi PT, Minchinton TE. 2006. Recruitment failure and shifts in community structure following mass mortality limit recovery prospects of black abalone. Marine Ecology Progress Series 327:107-117.

Moore JD, Robbins TT, Friedman CS. 2000. Withering syndrome in farmed red abalone, *Haliotis rufescens*: Thermal induction and association with a gastrointestinal *Rickettsiales*-like prokaryote. J Aquat Animal Health 12:26-34.

Moore JD, Robbins TT, Hedrick RP, Friedman CS. 2001. Transmission of the *Rickettsiales*-like prokaryote "*Candidatus* Xenohaliotis californiensis" and its role in withering syndrome of California abalone *Haliotis* spp. J Shellfish Res 20:867-874.

Moore JD, Finley CA, Robbins TT, Friedman CS. 2002. Withering syndrome and restoration of southern California abalone populations. *Reports of California Cooperative Oceanic Fisheries Investigations*, *43*, pp.112-119.

Moore JD, Juhasz CI, Robbins TT, Vilchis LI. 2009. Green abalone, *Haliotis fulgens* infected with the agent of withering syndrome do not express disease signs under a temperature regime permissive for red abalone, *Haliotis rufescens*. Mar Bio 156(11):2325-2330.

Moore JD, Marshman BC, Chun CSY. 2011. Health and survival of red abalone *Haliotis rufescens* from San Miguel Island, California, USA, in a laboratory simulation of La Niña and El Niño conditions. J Aquat Animal Health 23:78-84.

Neuman M, Tissot B, VanBlaricom GR. 2010. Overall status and threat assessment of black abalone (*Haliotis cracherodii* Leach, 1814) populations in California. J Shellfish Res 29(3):577-586.

Nielsen KM, Johnsen PJ, Bensasson D, Daffonchio D. 2007. Release and persistence of extracellular DNA in the environment. Environmental Biosafety Research 6:37-53.

NMFS, National Marine Fisheries Service, 2008. White Abalone Recovery Plan (*Haliotis sorenseni*). National Marine Fisheries Service, Long Beach, CA.

NOAA, National Oceanic and Atmospheric Administration, CoastWatch. 1998. http://coastwatch.pfel.noaa.gov (accessed June 2013).

OIE, World Organization for Animal Health. 2012. Principles and methods of validation of diagnostic assays for infectious diseases. In: Manual of diagnostic tests for aquatic animals, 6th edition. Paris, France.

Paul AJ, Paul JM. 1981. Temperature and growth of maturing *Haliotis kamtschatkana* (Jonas). Veliger 23:321-324.

Paul JH, Jeffrey WH, DeFlaun MF. 1987. Dynamics of extracellular DNA in the marine environment. Applied Environmental Microbiology 53:170-179.

Raimondi PT, Wilson CM, Ambrose RF, Engle JM, Minchinton TE. 2002. Continued declines of black abalone along the coast of California: are mass mortalities related to El Niño events? Mar Ecol Prog Ser 242:143-152.

Reed LJ, Muench H. 1938. A simple method of estimating fifty percent endpoints. American J Epidemiol 27(3):493-497.

Ricchi M, Bertasio C, Boniotti MB, Vicari N, Russo S, Tilola M, Bertasi B. 2017. Comparison among the quantification of bacterial pathogens by qPCR, dPCR, and cultural methods. Front Microbiol 8:1174.

Rinkevich B. 2005. Marine invertebrate cell cultures: new millennium trends. Mar Biotech 7(5):429-439.

Roberts R. 2001. A review of settlement cues for larval abalone (*Haliotis* spp.). J Shellfish Res 20:571-586.

Rosenblum ES, Robbins TT, Scott BB, Nelson S, Juhasz C, Craigmill A. 2008. Efficacy, tissue distribution, and residue depletion of oxytetracycline in WS-RLP infected California red abalone *Haliotis rufescens*. Aquaculture 277:138-148.

Rothaus DP, Vadopalas B, Friedman CS. 2008. Precipitous declines in pinto abalone (*Haliotis kamtschatkana kamtschatkana*) abundance in the San Juan Archipelago, Washington, USA, despite statewide fishery closure. Can J Fish Aquat Sci 65:2703-2709.

Ruediger J. 1999. Ecological characteristics of abalone withering syndrome in the California Channel Islands: discovery, effects, and persistence. MS thesis, University of California, Santa Cruz, CA.

Schade FM, Shama LN, Wegner KM. 2014. Impact of thermal stress on evolutionary trajectories of pathogen resistance in three-spined stickleback *(Gasterosteus aculeatus).* BMC Evol Bio 14(1):164.

Shaw BL, Battle HI. 1957. The gross and microscopic anatomy of the digestive tract of the oyster, *Crassostrea virginica* (Gmelin). Can J Zool 35:325-347.

Steinbeck JR, Groff JM, Friedman CS, McDowell T, Hedrick RP. 1992. Investigations into a mortality among populations of the California black abalone, *Haliotis cracherodii*, on the central coast of California. In: Shepard SA, Tegner MJ, Guzman del Proo SA (eds) Abalone of the World: Biology, Fisheries and Culture. Proceedings of the 1st International Symposium on Abalone, La Paz, Mexico.

Steinhaus EA, Martignoni ME. 1970. An Abridged Glossary of Terms Used in Invertebrate Pathology (2nd edition). USDA Forest Service, PNW Forest and Range Experiment Station.

Stokes NA, Burreson EM. 2001. Differential diagnosis of mixed *Haplosporidium costale* and *Haplosporidium nelsoni* infections in the eastern oyster, *Crassostrea virginica*, using DNA probes. J Shellfish Res 20:207-213.

Straus KM. 2010. Shellfish aquaculture and conservation of two Puget Sound molluscs: The pinto abalone (*Haliotis kamtschatkana kamtschatkana*) and the Pacific geoduck (*Panopea generosa*). PhD Dissertation, University of Washington School of Aquatic and Fishery Sciences, Seattle, Washington. 240 pp.

Taberlet P, Coissac E, Hajibabaei M, Rieseberg LH. 2012. Environmental DNA. Molecular Ecology 21(8):1789-1793.

Tan J, Lancaster M, Hyatt A, VanDriel R, Wong F, Warner S. 2008. Purification of a herpes-like virus from abalone (*Haliotis* spp.) with ganglioneuritis and detection by transmission electron microscopy. J Virol Meth 149(2):338-341.

Tang KF, Durand SV, White BL, Redman RM, Mohney LL, Lightner DV. 2003. Induced resistance to white spot syndrome virus infection in *Penaeus stylirostris* through pre-infection with infectious hypodermal and hematopoietic necrosis virus-a preliminary study. Aquaculture 216(1-4):19-29.

Thomsen PF, Kielgast J, Iversen LL, Møller PR, Rasmussen M, Willerslev E. 2012. Detection of a diverse marine fish fauna using environmental DNA from seawater samples. PLoS one 7(8).

Tissot BN. 1991. Geographic variation and mass mortality in the black abalone: the roles of development and ecology. PhD dissertation, Oregon State University, Corvallis, Oregon.

Tissot BN. 1995. Recruitment, growth, and survivorship of black abalone on Santa Cruz Island following mass mortality. Bull So Cal Acad Sci 94:179-189.

Tissot BN. 2007. Long-term population trends in the black abalone, *Haliotis cracherodii*, along the eastern Pacific coast. Unpublished report for the Office of Protected Resources, Southwest Region, National Marine Fisheries Service, Long Beach, CA. p43.

Travers MA. 2008. Interaction of the bacterium *Vibrio harveyi* with its host the abalone *Haliotis tuberculata*: physiological, cellular and molecular approaches. PhD Dissertation, Universitaire de Bretagne Occidentale-Centre Nationale de Recherche Scientifique, Brest, France.

Travers MA, Le Goïc N, Huchette Skoken M, Paillard C. 2008. Summer immune depression associated with increased susceptibility of the European abalone *Haliotis tuberculata* to *Vibrio harveyi* infection. Fish Shellfish Immunol 25:800-808.

VanBlaricom GR, Ruediger JL, Friedman CS, Woodard DD, Hedrick RP. 1993. Discovery of withering syndrome among black abalone *Haliotis cracherodii* Leach, 1814, populations at San Nicolas Island, California. J Shellfish Res 12:185-188.

VanBlaricom GR, Butler J, DeVogelaere A, Gustafson R, Mobley C, Neuman M, Richards D, Rumsey S, Taylor B. 2008. Status review report for black abalone (*Haliotis cracherodii*). U.S. Department of Commerce, National Oceanic and Atmospheric Administration, Long Beach, California: National Marine Fisheries Service, pp. 1-135.

Vannini C, Petroni G, Verni F, Rosati G. 2005. A bacterium belonging to the *Rickettsiaceae* family inhabits the cytoplasm of the marine ciliate *Diophrys appendiculata* (Ciliophora, Hypotrichia). Microbial Ecology 49(3):434-442.

Vater A, Byrne BA, Marshman BC, Ashlock LW, Moore JD. 2018. Differing responses of red abalone (*Haliotis rufescens*) and white abalone (*H. sorenseni*) to infection with phage-associated *Candidatus* Xenohaliotis californiensis. PeerJ 6:5104.

Via S. 2009. Natural selection in action during speciation. Proc Nat Acad Sci 106(1):9939-9946.

Vilchis LI, Tegner MJ, Moore JD, Friedman CS, Riser KL, Robbins TT, Dayton PK. 2005. Ocean warming effects on growth, reproduction, and survivorship of southern California abalone. Ecol Appl 15: 469−480.

Wetchateng T, Friedman CS, White NA, Lee PY, Teng PH, Sriurairattana S, Wongprasert K, Withyachumparnkul B. 2010. Withering syndrome in the abalone *Haliotis diversicolor supertexta*. Dis Aquat Org 90(1):69-76.

Wright AC, Garrido V, Debuex G, Farrell-Evans M, Mudbidri AA, Otwell WS. 2007. Evaluation of postharvest-processed oysters by using PCR-based most-probable-number enumeration of *Vibrio vulnificus* bacteria. Applied & Environmental Microbiology 73(22):7477-7481.