WITHERING SYNDROME SUSCEPTIBILITY OF NORTHEASTERN PACIFIC ABALONES: A COMPLEX RELATIONSHIP WITH PHYLOGENY AND THERMAL EXPERIENCE

Lisa M. Crosson [a], Carolyn S. Friedman [a,*]

[a] School of Aquatic and Fishery Sciences, University of Washington, Box 355020, Seattle, WA 98195 USA

*Corresponding author.

E-mail address: carolynf@u.washington.edu (C.S. Friedman).

Abstract

Population declines in wild and cultured abalones (*Haliotis* spp.) due to a bacterial disease called withering syndrome (WS) have been documented along the northeastern Pacific Ocean. However, observed differences in species susceptibility to the disease are not well understood. Here, we examined the susceptibility of three temperate abalone species, the cool water (4-14°C) pinto or northern abalone (*Haliotis kamtschatkana*), the intermediate water (8-18°C) red abalone (*H. rufescens*), 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.

Keywords: Abalone, Withering syndrome, *Rickettsiales*-like organism, Susceptibility, Phylogeny, Quantitative PCR

Abbreviations: WS, withering syndrome; RLO, *Rickettsiales*-like organism; WS-RLO, "*Candidatus* Xenohaliotis californiensis"; DG, digestive gland; PE, posterior esophagus; RLOv, phage infected WS-RLO variant; ST-RLO, uncharacterized RLO with stippled morphology; NOAA, National Oceanic and Atmospheric Administration; SD, standard deviation; OTC, oxytetracycline; UV, ultraviolet; TIC, tank independent control; qPCR, quantitative polymerase chain reaction; DNA, deoxyribonucleic acid; BSA, bovine serum albumin; rDNA, ribosomal DNA; ANOVA, analysis of variance; GLM, generalized linear model; LSR, least squares regression; LR, linear regression; ANCOVA, analysis of covariance; COI, cytochrome oxidase *c* subunit I

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 *Rickettsiales*-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 occurs 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. Materials and methods

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, USA. Red abalone (n = 84, mean size ± SD = 47.42 ± 5.53 mm) were received from The Abalone Farm, Inc. (Cayucos, CA, USA) 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, USA). 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 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. 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. 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.

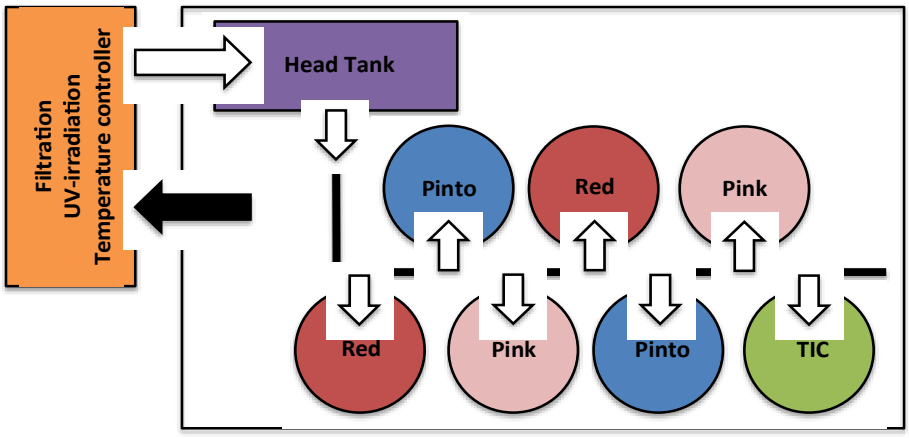


Figure 1. Recirculating seawater system used for RLO exposed and control abalones. Seawater was pumped through a series of filters, UV-irradiation, and temperature controllers prior to re-entering a head tank holding either infected (RLO exposed) or uninfected (control) red abalone. To ensure equivalent 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.

2.3 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 (cm^3) (Moore et al. 2000, Braid et al. 2005).

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).

2.5 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.) according to the manufacturer's "Isolation of DNA from Stool for Pathogen Detection" protocol and eluted in 100 µl. 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.

2.6 Quantitative PCR (qPCR)

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.), 320 nM of each primer, 200 nM hydrolysis probe (Applied Biosystems), 0.6 mg/µl BSA (New England BioLabs Inc.), 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.

2.7 Statistical analyses

All data were analyzed in SigmaPlot 11.0 (Systat Software Inc.) or JMP 12.0 (SAS). 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 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 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 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 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.

3. Results

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). 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). 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, Fig. 2). No red or pink abalones experienced mortality before 347 days post-exposure (Table 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).

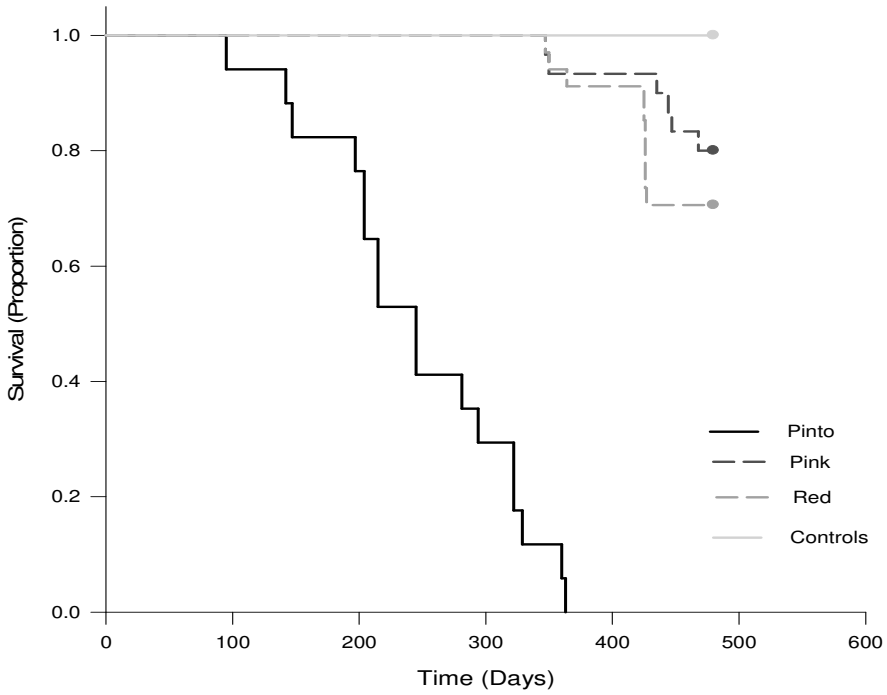


Figure 2. Kaplan-Meier survival curves for RLO exposed and unexposed (controls), pinto (*Haliotis kamtschatkana*), red (*H. rufescens*) and pink (*H. corrugata*) abalones.

Table 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.

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. 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, $P < 0.01$).

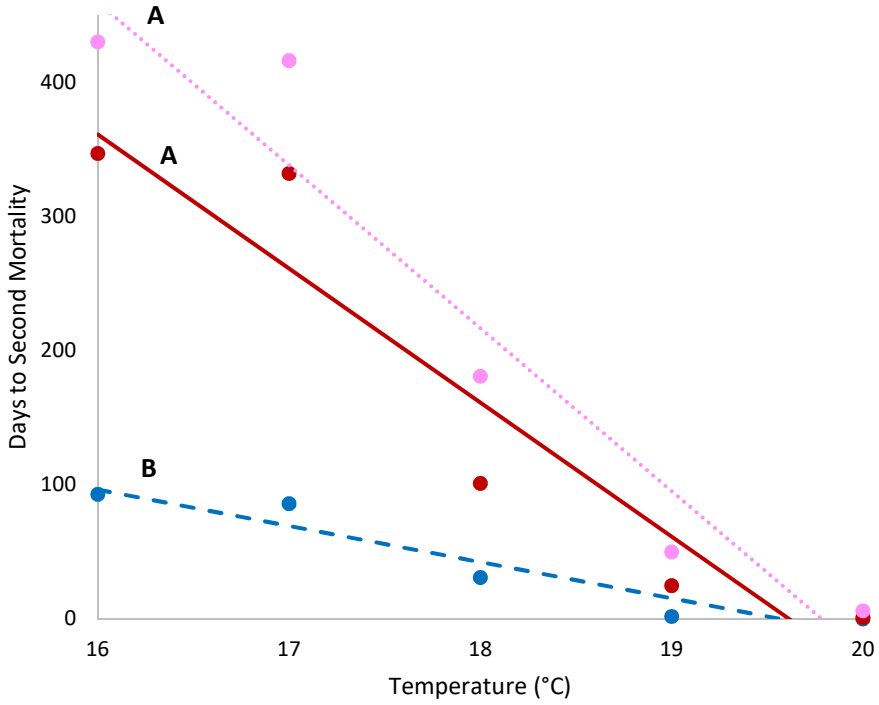


Figure 3. Relationship between temperature (°C) and the number of days at each temperature prior to the second RLO-induced mortality. Pinto abalone (*Haliotis kamtschatkana*) dashed line (blue), red abalone (*H. rufescens*) solid line (red) and pink abalone (*H. corrugata*) dotted line (pink). Letters indicate significance.

3.3 RLO infections

One to three morphologically distinct RLOs were observed in abalone histological sections (Fig. 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 prevalences ($P < 0.0002$). 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 prevalences ($P < 0.0001$).

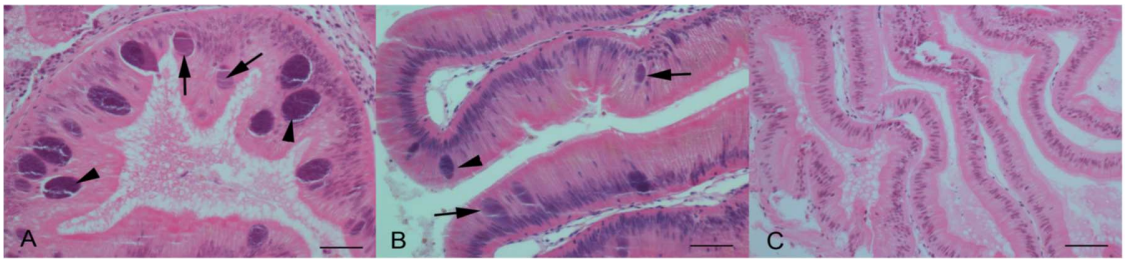


Figure 4. Light micrographs of hematoxylin and eosin stained posterior esophagus epithelium of pinto (*Haliotis kamtschatkana*; A), red (*H. rufescens*; B), and pink (*H. corrugata*; C) abalones at 240 days post-RLO exposure. Note high to moderate infections with WS-RLO (arrows) and RLOv (arrowheads) inclusions were observed in pinto and red abalones, respectively, while no inclusions were observed in pink abalone (bar = 50 µm).

3.3.1 Posterior esophagus (PE)

3.3.1.1 Initial PE infections

RLO infection in the PE of pinto abalone was first observed 90 days post initial RLO exposure, while RLOs in the PE of red and pink abalones were observed at day 240 (Table 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).

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 3).

Table 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

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 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. 5).

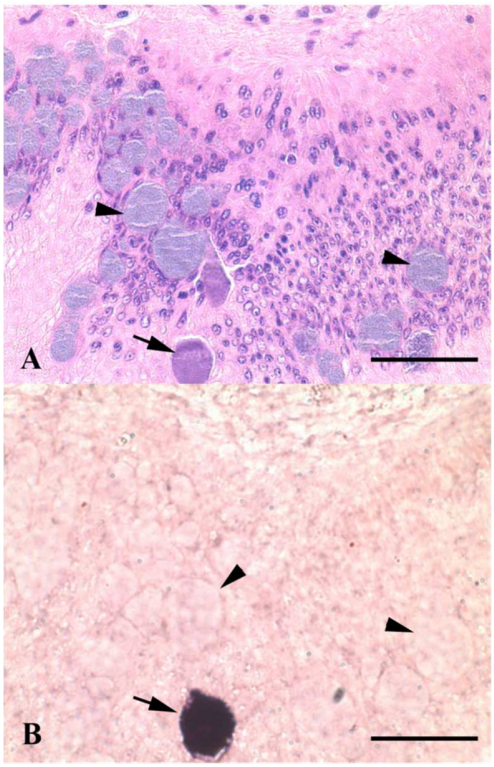


Figure 5. Histological section of a red abalone (*Haliotis rufescens*) containing both the ST-RLO (arrowhead) and WS-RLO (arrow) stained with hematoxylin and eosin (A) and probed by *in situ* hybridization for the WS-RLO. Note that only the WS-RLO binds with the probe (bar = 50 µm).

3.3.2 Digestive gland (DG)

3.3.2.1 Initial DG infections

RLO infections in the DG of pinto and red abalones were first observed 240 days post initial RLO exposure, while RLOs in the DG of pink abalone were first observed at 360 days (Table 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).

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 3).

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 3).

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 3). The ST-RLO loads were similar among all species (LSR, F-ratio = 2.89, df = 2, $P > 0.05$).

3.4 Host response

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, $P > 0.05$).

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).

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 x 10^{-4}, red mean = 1.08 x 10^{-4}, pink mean = 1.23 x 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 x 10^{-4} and 1.49 x 10^{-4}, respectively) that exceeded those of red and pink mortalities (mean = 1.23 x 10^{-4} and 1.08 x 10^{-4}, respectively).

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 x 10^{-4}, 1.44 x 10^{-4}, and 1.51 x 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 x 10^{-4}, exposed mean = 1.44 x 10^{-4}; LSR, F-ratio = 0.0044, df = 1, $P = 0.9473$).

3.5 Quantitative PCR (qPCR)

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 x 10^9 ± 1.45 x 10^9, 4.11 x 10^6 ± 1.58 x 10^9, and 1.38 x 10^6 ± 1.45 x 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 x 10^7 ± 5.49 x 10^7) than did red (7.36 x 10^3 ± 4.33 x 10^3) or pink (5.84 x 10^3 ± 5.84 x 10^3) abalones (ANOVA, F-ratio = 7.16, df = 2, $P = 0.0013$; Table 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. 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. 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.

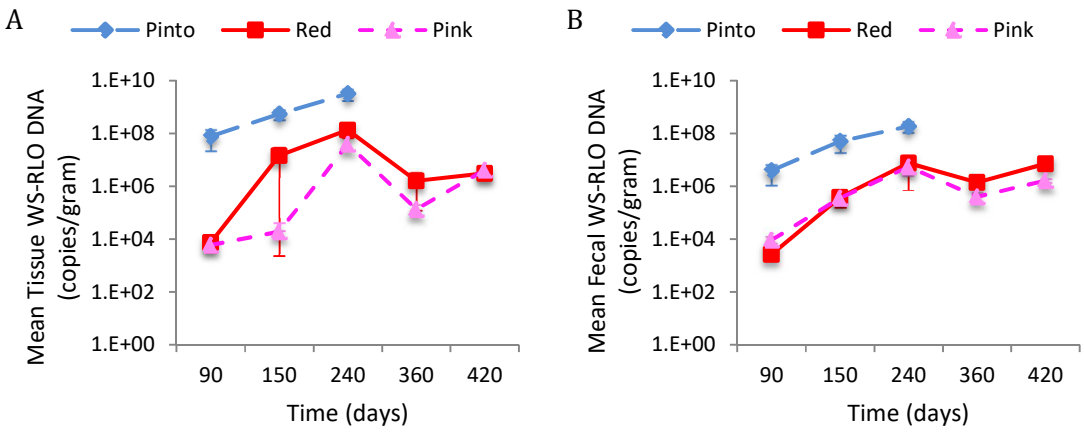


Figure 6. Mean WS-RLO DNA copy numbers per gram of pinto (*Haliotis kamtschatkana*), red (*H. rufescens*) and pink (*H. corrugata*) abalone post-esophageal tissues (A) and per gram of abalone feces (B) throughout the experiment. Error bars represent standard error.

3.6 Abalone Phylogeny, Thermal Experience and WS Relationship

One-way GLMs analyzed the relationship of the single factors (illustrated in Table 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. 7A; Table 4). Percent mortality and mean PE total RLO infection intensity of exposed abalone taxa were positively related (Fig. 7B), while percent mortality was inversely related to both range high and optimum growth temperature (Fig 7C, 7D; Table 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 4).

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 4). Although the whole models for all two-way analyses identified significant relationships, the factors that contributed to model significance varied (Table 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 4).

Table 4. Generalized linear model output to examine the relationship among phylogeny, thermal experience (mean high temperature of range where taxa are most abundant and optimum growth temperature), mean PE total RLO infection intensity and percent mortality from WS. 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.

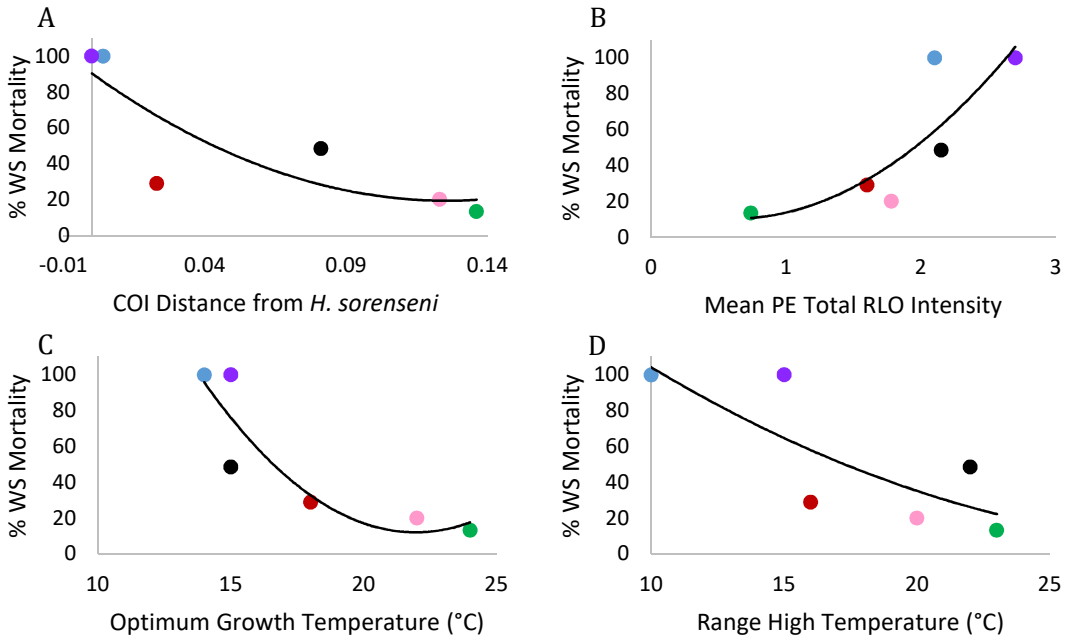


Figure 7. GLM regression plots of six northeastern Pacific abalone species [white (*Haliotis sorenseni*; purple dot), pinto (*H. kamtschatkana*; blue dot), red (*H. rufescens*; red dot), black (*H. cracherodii*; black dot), pink (*H. corrugata*; pink dot) and green (*H. fulgens*; green dot)] demonstrating the relationship between mean percent WS-induced mortality and (A) COI distance from white abalone, (B) mean PE total RLO infection intensity, (C) range high temperature and (D) optimum growth temperature. See Tables 1 and 4 for associated data and references.

4. Discussion

4.1 Pinto abalone have high susceptibility and 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.

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.

4.3 Abalone immunity may vary among species and 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.

4.4 Abalone phylogeny and 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, USA), followed by red abalone (distributed from Northern California, USA 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, USA to central Baja California, Mexico; Haaker et al. 1986, Geiger 2000). White abalone also occupy a southern distribution (from Point conception, California, USA 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, USA) and *H. k. assimilis* (referred to as the threaded abalone, with a more southern range from central CA, USA 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 (USA) 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 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.

4.5 Multiple RLOs and 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.

4.6 Conclusions and 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, USA (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.

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 California Department of Fish and Game, and the School of Aquatic and Fishery Sciences at the University of Washington. We thank the abalone aquaculture industry for participating in our research, especially Ray Fields at The Abalone Farm, Inc., Josh Bouma at Puget Sound Restoration Fund, and John Butler at NOAA Southwest Fisheries Science Center. We also thank Nate Wight, Samantha Adams, and Sean Bennett for assistance with animal care and sample processing, Robert Egbert for abalone illustrations, and Dr. Colleen Burge, Dr. Stanley Langevin, Daniel Gillon, and other anonymous reviewers for their editorial comments. The views expressed herein are those of the authors and do not necessarily reflect the views of NOAA or any of its sub-agencies. The U.S. government is authorized to reproduce and distribute this paper for governmental purposes.

References

Altstatt, J.M., Ambrose, R.F., Engle, J.M., Haaker, P.L., Lafferty, K.D., Raimondi, P.T., 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.C., Caceres-Martinez, J., Aviles, J.G.G., 2002. Histopathological evaluation of the yellow abalone *Haliotis corrugata* and the blue abalone *Haliotis fulgens* from Baja California, Mexico. J. Shellfish Res. 21(2), 825-830.

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

Antonio, D.B., Andree, K.B., Moore, J.D., Friedman, C.S., Hedrick, R.P., 2000. Detection of *Rickettsiales*-like prokaryotes (RLPs) by *in situ* hybridization in black abalone *Haliotis cracherodii* with Withering Syndrome. J Invert. Path. 75, 180-182.

Ben-Horin, T., Lenihan, H.S., Lafferty, K.D., 2013. Variable intertidal temperature explains why disease endangers black abalone. Ecology 94(1), 161-168.

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

Braid, B.A., Moore, J.D., Robbins, T.T., Hedrick, R.P., Tjeerdema, R.S., Friedman C.S., 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.

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, P.A., Gordon, H.R., 2010. World abalone supply, markets, and pricing. J. Shellfish Res. 29(3), 569-571.

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

Crosson, L.M., Wight, N., VanBlaricom, G.R., Kiryu, I., Moore, J.D., Friedman, C.S., 2014. Abalone withering syndrome: distribution, impacts, current diagnostic methods and new findings. Dis. Aquat. Org. 108(3), 261-270.

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

Dang, V.T., 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).

Díaz, F., Re, A.D., 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.

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

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

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

Friedman, C.S., Thomson, M., Chun, C., Haaker, P.L., Hedrick, R.P., 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, C.S., Andree, K.B., Beauchamp, K., Moore, J.D., Robbins, T.T., Shields, J.D., 2000. "*Candidatus* Xenohaliotis californiensis", a newly described pathogen of abalone, *Haliotis* spp., along the west coast of North America. Int. J. Syst. Evol. Microbiol. 50, 847–855.

Friedman, C.S., Biggs, W., Shields, J.D., Hedrick, R.P., 2002. Transmission of Withering Syndrome in black abalone, *Haliotis cracherodii* Leach. J. Shellfish Res. 21(2), 817-824.

Friedman, C.S., Trevelyan, G., Mulder, E.P., 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, C.S., Scott, B.B., Strenge, R.E., McCormick, T.B., 2007. Oxytetracycline as a tool to manage and prevent losses of the endangered white abalone, *Haliotis sorenseni*, to withering syndrome. J. Shellfish Res. 26(3), 887-885.

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

Friedman, C.S., Wight, N., Crosson, L.M., White, S.J., Strenge, R.M., 2014a. Validation of a quantitative PCR assay for detection and quantification of '*Candidatus* Xenohaliotis californiensis'. Dis. Aquat. Org. 108, 251-259.

Friedman, C.S., Wight, N., Crosson, L.M., VanBlaricom, G.R., Lafferty, K.D., 2014b. Reduced disease in black abalone following mass mortality: phage therapy and natural selection. Front. Microbiol. 5(78), 1-10.

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

Gardner, G.R., Harshbarger, J.C., Lake, J.L., Sawyer, T.K., Price, K.L., Stephenson, M.D., Haaker, P.L., Togstad, H.A., 1995. Association of prokaryotes with symptomatic appearance of withering syndrome in black abalone *Haliotis cracherodii*. J. Invert. Path. 66, 111–120.

Geiger, D.L., 2000. Distribution and biogeography of the recent Haliotidae (Gastropoda: Vetigastropoda) worldwide. Boll. Malacol. 35, 57–120.

Haaker, P.L., Henderson, K.C., Parker, D.O., 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, P.L., Richards, D.V., Friedman, C.S., Davis, G., Parker, D.O., Togstad, H., 1992. Abalone withering syndrome and mass mortality of black abalone, *Haliotis cracherodii* in California. In: Shephard, S.A., Tegner, M., Guzman del Proo, S. (Eds.), Abalone of the World. Blackwell Scientific, Oxford, pp 214-224.

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, A.J., Tegner, M.J., 2000. Status review of white abalone (*Haliotis sorenseni*) throughout its range in California and Mexico. US Department of Commerce, National Oceanic and Atmospheric Administration, National Marine Fisheries Service, Southwest Region Office.

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

Karpov, K.A., Haaker, P.L., 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., Kamaishi, T., Yatabe, T., 2013. First Detection of *Candidatus* Xenohaliotis californiensis, the Causative Agent of Withering Syndrome, in Japanese Black Abalone *Haliotis discus discus* in Japan. 魚病研究 48(2), 35-41.

Leighton, D. L., 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, L.G., 1968. Manual of Histological Staining Methods of the Armed Forces Institute of Pathology 3rd Edn. McGraw-Hill Book Co., New York pp. 258.

Metz, E.C., Robles-Sikisaka, R., Vacquier, V.D., 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, C.M., Altstatt, J.M., Raimondi, P.T., Minchinton, T.E., 2006. Recruitment failure and shifts in community structure following mass mortality limit recovery prospects of black abalone. Mar. Ecol. Prog. Ser. 327, 107-117.

Moore, J.D., Robbins, T.T., Friedman, C.S., 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, J.D., Finley, C.A., Robbins, T.T., Friedman, C.S., 2002. Withering syndrome and restoration of southern California abalone populations. Reports of California Cooperative Oceanic Fisheries Investigations 43, 112-119.

Moore, J.D., Juhasz, C.I., Robbins, T.T., Vilchis, L.I., 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.

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

OIE: World Organisation for Animal Health, 2012. Principles and methods of validation of diagnostic assays for infectious diseases. In: Manual of diagnostic tests for aquatic animals sixth ed., France.

Paul, A.J., Paul, J.M., 1981. Temperature and growth of maturing *Haliotis kamtschatkana* (Jonas). Veliger 23, 321-324.

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

Rosenblum, E.S., Robbins, T.T., Scott, B.B., 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, D.P., Vadopalas, B., Friedman, C.S., 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(12), 2703-2711.

Schade, F.M., Shama, L.N., Wegner, K.M., 2014. Impact of thermal stress on evolutionary trajectories of pathogen resistance in three-spined stickleback *(Gasterosteus aculeatus).* BMC Evol. Bio. 14(1), 164.

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

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

Straus, K.M. 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.

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

Tissot, B.N., 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., pp. 43.

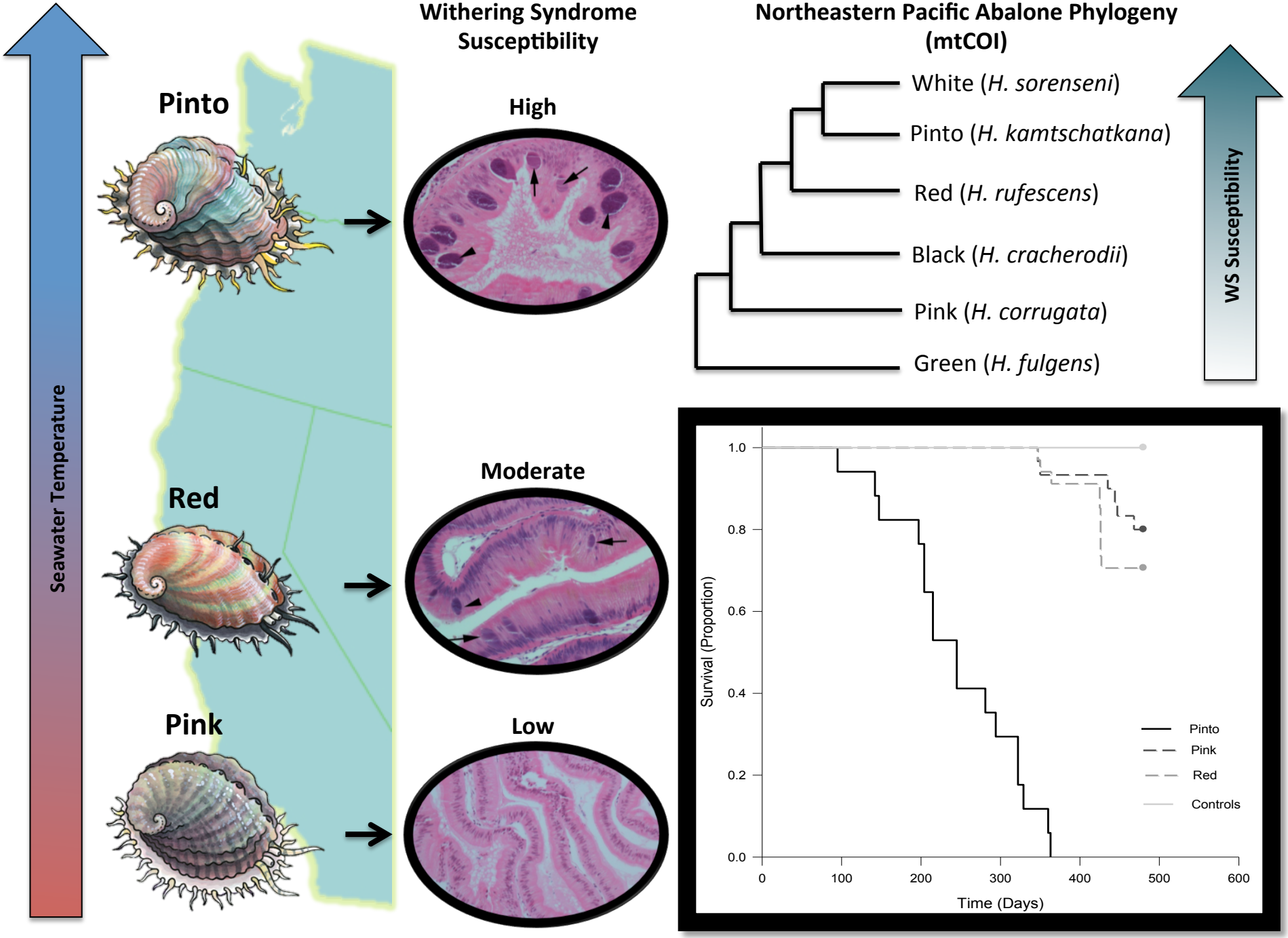
Travers MA, Le Goïc N, Huchette Skoken M, Paillard C., 2008. Summer immune depression associated with increased susceptibility of the European abalone *Haliotis tuberculata* to *Vibrio harveyi* infection. Fish Shellfish Immunol. 25, 800-808. DOI:10.1016/j.fsi.2008.08.003.

VanBlaricom, G.R., Ruediger, J.L., Friedman, C.S., Woodard, D.D., Hedrick, R.P., 1993. Discovery of withering syndrome among black abalone *Haliotis cracherodii* Leach, 1814, populations at San Nicolas Island, California. J. Shellfish Res. 12, 185-188.

Via, S., 2009. Natural selection in action during speciation. Proc. Nat. Acad. Sci. 106 (Suppl. 1), 9939-9946. DOI:10.1073/pnas.0901397106.

Vilchis, L.I., Tegner, M.J., Moore, J.D., Friedman, C.S., Riser, K.L., Robbins, T.T., Dayton, P.K., 2005. Ocean warming effects on growth, reproduction, and survivorship of southern California abalone. Ecol. App. 15, 469-480.

Wetchateng, T., Friedman, C.S., Wight, N.A., Lee, P.Y., Teng, P.H., Sriurairattana, S., Withyachumnarnkul, B., 2010. Withering syndrome in the abalone *Haliotis diversicolor supertexta*. Dis. Aquat. Org. 90(1), 69-76.


Seawater Temperature
Withering Syndrome Susceptibility
Northeastern Pacific Abalone Phylogeny (mtCOI)
WS Susceptibility
Pinto
High
Red
Moderate
Pink
Low
White (H. sorenseni)
Pinto (H. kamtschatkana)
Red (H. rufescens)
Black (H. cracherodii)
Pink (H. corrugata)
Green (H. fulgens)
Survival (Proportion)
Time (Days)
Pinto
Pink
Red
Controls