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Effects of novel bacteriophage on red abalone (*Haliotis rufescens*) and white abalone (*H. sorenseni*) exposed to *Candidatus* Xenohaliotis californiensis – the causative agent of abalone withering syndrome.

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ABSTRACT

The *Rickettsiales*-like prokaryote - *Candidatus* Xenohaliotis californiensis (*Ca.* Xc) – has decimated black abalone populations along the Pacific coast of North America. White abalone, – *Haliotis sorenseni* – are also susceptible and nearly extinct in the wild due to overfishing in the 1970s. *Candidatus* Xenohaliotis californiensis proliferates in epithelial cells of the abalone gastrointestinal tract and causes clinical signs of starvation. In 2012, evidence of a putative phage associated with *Ca.* Xc in *Haliotis rufescens* was described. In this study, we investigated the virulence of *Ca.* Xc in red and white abalone at different environmental temperatures in the presence of phage. Using a comparative experimental design, we observed differences over time between the two abalone species in mortality, body condition, and bacterial load by quantitative real time PCR (qPCR). By day 251, all white abalone exposed to the *Ca.* Xc and its phage in the warm water (18.5ºC) treatment died, while red abalone exposed to the same conditions had a mortality rate of 10% despite a relatively heavy bacterial burden as determined by qPCR of posterior esophagus tissue and histological assessment at the termination of the experiment. These data support the phage as a protective and potentially therapeutic agent for withering syndrome in red abalone, while white abalone appear to remain susceptible to this disease even in phage presence. These findings have important implications for implementation of a white abalone recovery program, particularly with respect to the thermal regimes of locations where captively-reared individuals will be outplanted.

INTRODUCTION

Abalone are iconic benthic invertebrates that contribute to ecological health of the northern Pacific coast kelp forests and serve as a food source for endangered sea otters *Enhydra lutris*. California's wild abalone fishery flourished from the 1950s-1980s but was decommercialized in

response to population declines from overexploitation followed by disease. Farmed abalone is of increasing economic significance. In 2008, it was estimated that over 129,000 metric tons of farmed abalone was supplied to the world market (Cook 2016).

Withering syndrome (WS) was first reported in mid-1980's at the Channel Islands, California and decimated black abalone (*Haliotis cracherodii*) populations (Haaker et al. 1992). The causative agent of the disease, *Candidatus* Xenohaliotis californiensis (*Ca.* Xc) is a member of the Order *Rickettsiales* of the Alphaproteobacteria (Friedman et al. 2000). Analysis of five genes (16S rRNA, 23S rRNA, *ftsZ, vVirB11,* and *vVirD4*) suggested that *Ca.* Xc is most closely related to the *Neorickettsia* genus and is the most ancestral form of the *Anaplasmataceae* family studied to date (Cicala et al. 2017). Transmission of *Ca.* Xc appears to be fecal-oral (Moore et al. 2001). *Ca.* Xc infects the luminal epithelium of the posterior portion of the esophagus (PE) and digestive gland (Moore et al. 2001). The *Ca.* Xc bacterium forms large oblong inclusions in the digestive tract epithelium, which are easily identifiable in hematoxylin- and eosin-stained tissue sections (Friedman et al. 2000). The infection causes reduced feeding behavior and nutrient absorption; animals wither as they lose body mass through catabolism of the foot muscle (Gardner et al. 1995).

In red abalone, *H. rufescens*, exposure to warm water events exacerbates morbidity and mortality in two synergistic ways: it reduces nutritional content in feed, and is associated with increasing *Ca.* Xc burdens (Vilchis et al. 2005). Trends of increasing frequency and intensity of Pacific ocean warming El-Nino events correspond to dramatic reductions in giant kelp densities (Tegner et al. 1996), and nitrogen nutrient concentration in seawater is inversely related to temperature (Tegner et al. 2001). Furthermore, ocean warming trends facilitate *Ca.* Xc disease outbreaks (Harvell et al. 1999). The results of laboratory studies using juvenile farm-raised red abalone

showed that *Ca.* Xc has relatively little effect on the health of abalone held at temperatures of approximately 14°C, while animals held in water approximately 18°C suffer high mortality rates in association with higher *Ca.* Xc body burdens (Braid et al. 2005; Moore et al. 2000; Rosenblum et al. 2005; Vilchis et al. 2005). Braid et al. (2005) demonstrated that warm water stress alone is not responsible for clinical signs of withering syndrome.

Anecdotal observations from California red abalone farmers beginning in 2006 indicated that the incidence and severity of WS had diminished; this change correlated with the appearance of a novel bacteriophage hyperparasite associated with *Ca.* Xc (Crosson et al. 2014; Friedman & Crosson 2012). This bacteriophage was described in farmed red abalone from Cayucos, California examined in 2009 (Friedman & Crosson 2012). Its presence was visualized by histology as morphologically distinct inclusions and transmission electron microscopy confirmed the presence of phage particles (Friedman & Crosson 2012). Further characterization suggests that this phage is a member of the *Siphoviridae* family and employs a lytic life cycle (Cruz-Flores & Cáceres-Martínez 2016). Friedman et al. (2014) demonstrated significant improvement in black abalone survival when challenged with phage-infected *Ca.* Xc.

White abalone (*H. sorenseni)* were the first marine invertebrate species to be recognized as federally endangered. Outplanting animals bred and raised in a captive rearing program is considered the key restoration approach to increase densities quickly enough to reduce the probability of extinction (Rogers-Bennett et al. 2016; Stierhoff et al. 2014). White abalone are highly susceptible to WS and an initial captive breeding program in southern California suffered great losses due to the disease in 2002, prior to the presence of the phage (California Department of Fish and Wildlife unpublished observations, Friedman et al. 2007).

In this study, we aimed to elucidate the effects of phage on WS in red and white abalone species under thermal conditions that are known to either enhance or reduce expression of the disease.

MATERIALS AND METHODS

Abalone and Life Support. This experiment was conducted in the California Department of Fish and Wildlife's Pathogen Containment Facility at the UC Davis Bodega Marine Laboratory in Bodega Bay, California. Red abalone, approximately 2.2 cm in shell length, were purchased from an abalone farm in Goleta, California. White abalone, approximately 1.8 cm in shell length, were donated from the UC Davis Bodega Marine Laboratory's White Abalone Recovery Project. Prior to challenge, feces from all tanks were tested for 16S rRNA *Ca.* Xc genes by quantitative PCR (qPCR) following a validated protocol (Friedman et al. 2014b). Although fecal qPCR is treated only as a proxy for live pathogen, it has been shown to be the most sensitive assay for *Ca.* Xc detection (Friedman et al. 2014b). To support the absence of infection, five animals from both the red and white groups were sacrificed for histologic examination. Animals were supplied with a combination of wild-collected kelp (*Macrocystis pyrifera*) and cultured dulse (*Palmaria palmata)* two to three times per month throughout the experiment. Because *Ca.* Xc is known to be present in local abalone, feed was soaked in freshwater for at least 5 minutes prior to distributing to the tanks; our ongoing unpublished data have demonstrated that this is sufficient to inactivate residual *Ca.* Xc that may be present on algal feed (CDFW unpublished observations). All tanks received constant 20-µm filtered, aerated, UV-irradiated, flow-through seawater.

Experimental design. This study was constructed as a fully nested design with tanks nested within temperatures and *Ca.* Xc exposure challenge and abalone nested within tanks. One

hundred ninety-two red and 192 white abalone, ~2 cm in length, were randomly and evenly distributed into either of the two treatment groups (exposed), or the control group (**Figure 1**).

To avoid cross-contamination, we spatially organized the groups in lieu of random placing. Each treatment group was comprised of eight 3.8-L tanks, with eight animals housed in each tank. Two groups, one exposed to *Ca.* Xc and the other unexposed, received elevated temperature seawater (approximately 18.5°C); a second *Ca*. Xc exposed group received ambient water (approximately 13.6°C). Temperature was measured hourly by automated temperature recorder placed in one tank per treatment group.

Exposure to *Ca.* Xc. To initiate *Ca.* Xc exposure, inflowing seawater was routed through 11-L conical header tanks with farmed red abalone prior to supplying the experimental tanks. The two exposed groups of each species received effluent water from a header tank containing eight farmed red abalone, each approximately 123 gm in weight, from a population shown by histology to be infected with *Ca.* Xc and its phage. The control groups of red abalone and white abalone were headed by a tank holding 50 farmed red abalone, approximately 2.4 gm in weight; these animals' feces tested negative for *Ca.* Xc by PCR. The source water was directed through the headers through day 161 to ensure *Ca.* Xc exposure. Moore et al. (2001) showed that infection in red abalone was 100% after 111 days of *Ca.* Xc exposure to a header tank with infected red abalone at 18.5ºC (prior to appearance of the *Ca.* Xc phage).

Sampling schedule and processing. At selected time points (days 0, 62, 126, 161, 265, 343) all animals in the experiment were weighed and measured for shell length. A body mass Condition Index (CI) was calculated as: $\frac{Total\ Weight\ (gm)}{Shell\ Length\ (cm)^3}$. At day 161, header tanks were removed and two randomly selected animals per tank were sacrificed and tested for *Ca.* Xc infection by qPCR

from DNA extracted from post-esophagus (PE) tissue samples. Additionally, at day 161, six white abalone were randomly selected and sacrificed for histological confirmation of transmission of *Ca.* Xc and its phage. At day 343, all remaining experimental animals were also processed for analysis of infection in PE and digestive gland tissues by both qPCR and histology. For qPCR, PE tissue (~30mg) was excised from sacrificed animals and DNA extractions were performed using a DNeasy Blood and Tissue Kit (QIAGEN Germantown, MD) following the manufacturer's protocol for pathogen detection. Tissue samples were processed for histology as previously described (Moore et al. 2001). Davidson's-fixed (Shaw and Battle 1957), hematoxylin- and eosin-stained 5 μm paraffin tissue sections containing PE and digestive gland were prepared from sacrificed animals. After termination of the experiment, slides were blindly assessed for presence/absence of *Ca.* Xc inclusions and categorized as having morphologies indicating phage infection or lack of infection.

Fecal bacterial sampling regimen and processing. Feces were collected from each tank bi-monthly for qPCR analysis. Feces from four tanks per group were pooled for *Ca*. Xc 16S rRNA gene detection. Fecal samples were weighed and frozen at -20 °C upon collection until analysis. DNA from fecal samples (~250mg) was extracted and purified with a QIAamp DNA Stool Mini Kit (QIAGEN) according to the manufacturer's 'Isolation of DNA from Stool for Pathogen Detection' protocol. DNA obtained was eluted in 200 μl volumes and stored at -20 °C until analysis.

Quantitative PCR assays for *Ca.* Xc. We monitored *Ca.* Xc gene presence in post-esophagus and fecal samples using the methods developed and validated by Friedman et al (2014). Standard curves were constructed using PCR product of the WSN1 primers: WSN1 F (5'AGTTTACTGAAGGCAAGTAGCAGA3') and WSN1R (5'TCTAAC

TTGGACTCATTCAAAAGC3') and the P16RK3 plasmid (Friedman et al. 2014b). Plasmid concentration was quantified by Qubit fluorometer (ThermoFisher Scientific, Walthan, Massachusetts). Assayed tissue and fecal samples were considered positive if the mean copy number per ng of genomic DNA in triplicate samples was equal to or greater than one, and reactions prior to normalization calculations had at least three gene copies – as convention of Minimum Information for Publication of Quantitative Real-Time PCR Experiments guidelines describes (Bustin et al. 2009). For reporting purposes, and to meet assumption of residual normality for statistical analysis, reaction copy numbers were normalized by input DNA (ng) and log transformed.

Data analysis. A Cox proportional hazard model was used for survival analysis of treatment groups. A Chi-squared test was used assess differences in survival of red abalone between this study and a historical study conducted prior to presence of the phage (Moore et al. 2000). Hazard ratio terminology refers to the likelihood of death associated with a variable. Variations in (1) condition index values and (2) qPCR data, were tested for significance by One-way ANOVA. Results of these models were assessed by *post hoc* Tukey comparisons. Residual errors from analysis were assessed for normality using the Wilk Shapiro test. Data from qPCR study was log-transformed. Both log-transformed qPCR data and condition index data was transformed by winsorization to meet the parametric test assumptions of normality. A test had a significant result if $p \leq 0.05$ (level of significance $\alpha = 0.05$). Statistical analysis was done with R version X R3.1.3 (R Core Team 2014).

RESULTS

Transmission of *Ca.* Xc. All *Ca.* Xc-exposed groups showed fecal shedding of *Ca.* Xc DNA after header tank removal from the system (day 161), indicating effective transmission to naïve

red abalone and white abalone under both temperature regimes. The unexposed tanks test negative by feces PCR (**Table 1**).

Pathogen transmission to all *Ca.* Xc-exposed tanks was confirmed by tissue qPCR from animals sacrificed at the end of the experiment with the exception of the *Ca*. Xc exposed, elevated temperature white abalone which all died prior to day 343. Abalone that died of natural causes during the experiment were frozen and PE was excised and screened by qPCR for *Ca*. Xc. Of these animals, 16% from the naïve groups produced low levels of *Ca*. Xc gene amplification (< three copies/ng input DNA. The source of the potential contamination is unknown. Other measurements from fecal qPCR, tissue qPCR from sacrificed animals, and histology assessments did not indicate transmission of *Ca.* Xc. to the naïve groups.

Analysis of survival. We examined survival in response to water temperature, abalone species, and *Ca.* Xc exposure (**Figure 2**).

Our analysis indicated that white abalone, exposed to *Ca.* Xc and elevated temperature were 10.9 times more likely to die than red abalone held in the same conditions (hazard ratio = 10.9; 95% CI = 5.97-19.87; P<0.001). By day 251, all *Ca.* Xc-exposed white abalone in the elevated temperature treatment died, while red abalone held under the same conditions maintained a survival rate of 91%. Elevated temperature increased mortality risk for both red and white abalone 3.3 times that of the ambient treatment groups (hazard ratio = 3.3; 95% CI = 1.1-10.2; P=0.039). Specifically, under elevated temperature, *Ca.* Xc-exposure increased the mortality risk of both species of abalone 12.5 times (hazard ratio = 12.5; 95% CI = 1.6-96.0; P=0.015). Generally, under all conditions, white abalone had a risk of mortality that was 41 times that of red abalone (hazard ratio = 41.0; 95% CI = 5.6-303.1; P<0.001).

The survival rate of red abalone in the current study, exposed to *Ca.* Xc with phage, was 36% higher than the previous experiment, in which animals were exposed to phage-free *Ca.* Xc (**Table 2**) (Moore et al. 2000).

We evaluated survival at day 220, immediately prior to termination day in the 2000 Moore et al. study (Moore et al. 2000). Between Days 1 and 220, in both experiments, the average temperature was 18.5°C (Moore et al. 2000). Both experiments used farm-origin juvenile red abalone. The 60 animals in the 2000 study averaged 8 cm in length and were selected from a farmed population with a known low-intensity *Ca*. Xc infection; however, this population did not express clinical symptoms of WS (Moore et al. 2000). Comparison of survival in the historical (phage-free) and current (phage-present) experimental data by chi squared test shows a significant difference ($p = 0.009$) favoring survival in the presence of phage.

Body condition indices. Assessment of animal health – as a function of weight, normalized by length – over time showed that red abalone remained heathy under all experimental conditions, unlike their white abalone counterparts (**Figure 3**).

While data were collected at additional time points, we focused on three – beginning, mid, and end – for visual clarity. At the outset of the experiment, the only group that showed a significant difference in CI values was the white, ambient group destined for *Ca.* Xc-exposure, with a greater condition index values than the other five groups. Mid-way through the experiment (Day 161) all three groups of white abalone overall had lower mean condition index values than their red counterpart groups. Statistical analysis showed significant differences between naïve, elevated temperature red and white groups (p-value = 0.007) with somewhat greater differences

between the exposed red and white groups in both elevated temperature (p-value < 0.001) and ambient (p-value < 0.001) seawater treatments. However, no differences were observed between elevated temperature and ambient white, *Ca.* Xc-exposed groups (p-value = 0.162) although more than half of the exposed elevated temperature white abalone had died prior to this time point. Based on visualization of longitudinal changes, white abalone fared worse under all treatments but nevertheless *Ca.* Xc exposure appeared to be associated with increased withering in white abalone while red abalone did not appear to decrease in condition index in response to *Ca.* Xc exposure. At the end of the experiment (day 343), white and red abalone showed significant differences in body condition under the same treatment of *Ca.* Xc-exposure in ambient seawater but we observe smaller differences between the naïve, elevated temperature groups. There was not a significant difference in body condition between the naïve, elevated temperature and *Ca.* Xc-exposed, ambient white groups (p-value = 0.896); additionally, there was no difference between red abalone in the naïve, elevated temperature and the exposed, elevated temperature groups (p-value = 0.99919).

***Candidatus* Xc prevalence and infection intensity.** *Candidatus* Xc 16S rRNA gene copy numbers obtained by qPCR from PE tissue can serve as a proxy for bacterial burden, and were assessed at days 161 and 343 (**Figure 4**).

The qPCR data from tissue samples taken on day 161 were too skewed to transform such that residuals met normality assumptions for appropriate parametric statistical. However, visualization of data trends suggests that at day 161, elevated temperature resulted in higher pathogen tissue burdens in white abalone (Figure 4); the mean *Ca.* Xc gene copy number per ng DNA from tissue samples of exposed white abalone in the elevated temperature regimen was 4,248; 326 times greater that of their red counterparts. Notably, white animals sacrificed at this

time point were survivors – 60% of their cohort already died. There was no *Ca.* Xc amplification in the red, ambient group at day 161. At day 343, red, exposed, ambient abalone had significantly lower pathogen gene numbers than both the exposed elevated temperature red group (p-value < 0.001) and exposed ambient white group (p-value < 0.001). No *Ca.* Xc DNA was amplified from PE tissue samples of sacrificed animals in the unexposed groups at either time point.

At day 161, six white animals from both ambient and elevated temperature conditions were sacrificed and used for histopathology to corroborate transmission data from fecal and tissue qPCR samples and determine whether any *Ca.* Xc inclusions present included those with morphology indicating phage infection. We morphologically identified the phage-infected inclusions in white abalone; this finding has not previously been documented (**Figure 5**).

Histological analysis at day 343 found *Ca.* Xc inclusions in tissue samples from the two groups: elevated temperature, red and ambient, white abalone (**Table 3**).

In concordance with the qPCR data, the exposed, elevated temperature, we observed nearly three times as many classical inclusions and more than five times as many phage-variant inclusions from the sample set of the red group compared to the samples taken from exposed, ambient white group. *Candidatus* Xc inclusions in digestive gland tissue were only observed in the *Ca.* Xc-exposed, elevated temperature, red abalone group.

DISCUSSION

White abalone, exposed to *Ca.* Xc and held in 18.5°C seawater, exhibited the highest mortality rate and most pronounced clinical signs of withering syndrome among the experimental groups. White abalone appear to be highly susceptible to WS infection and disease, even with current

268 phage-presence. While elevated temperature appeared to affect fitness of red abalone in terms of
269 condition index, *Ca.* Xc exposure with phage presence resulted in improved survival under
270 conditions that previously had been shown to exacerbate disease red abalone in absence of the
271 phage.

272 Transmission of *Ca.* Xc was slowest in the red, ambient group based on fecal and tissue qPCR
273 values. Variables that influenced fecal production and degradation, primarily feeding schedule
274 impaired normalization and thus interpretation accuracy of qPCR quantitative data, Table 1
275 presents only the presence/absence of *Ca*. Xc genes. Slow infection progression in cold water has
276 been show in previous studies (Braid et al. 2005; Moore et al. 2000). While the Moore et al. 2000
277 study identified *Ca.* Xc inclusions in 90% of experimental animals held at ambient temperature
278 (14.7°C) at day 220; in the current study no inclusions were found by histology in the red
279 abalone held at 13.6°C sacrificed on day 343. This finding from the 2000 study may be the result
280 of 'natural' farm-associated transmission at a time point prior to the start of the study.
281 Additionally, the small histology subsample taken at day 161 from white abalone, held under
282 ambient conditions did not have inclusions. We speculate that phage presence may also have
283 extended the disease incubation period in the ambient groups.

284 From weight and length data, we can infer that the white group destined for *Ca.* Xc-exposure and
285 elevated temperature treatment started with robust body condition, and thus were clearly not at a
286 disadvantage that would have predisposed them to withering and death. The similarity between
287 the elevated temperature, naïve and ambient exposed white groups' survival curves and
288 condition index values supports the general sensitivity of these animals to adverse conditions and
289 may also be a result of low genetic diversity (Gruenthal & Burton 2005). White abalone tend to

be a deeper dwelling species and experience less fluctuation in water temperature than other species that inhabit intertidal and shallower subtidal zones.

In order to examine the impact of the phage on *Ca.* Xc pathogenicity, it would of course be ideal to directly compare the phage-infected and uninfected pathogens. However, that was not possible because all abalone populations in California that are infected with *Ca.* Xc are now also infected with its phage (Moore, unpublished observations). Instead, we attempted to replicate a thermal induction study undertaken with red abalone prior to appearance of the phage (Moore et al. 2000). Comparing our results with those from that study strongly suggest that red abalone are better able to withstand withering syndrome now than in years previous to phage presence.

Furthermore, red abalone, exposed to *Ca.* Xc with phage showed no difference in body condition from their naïve counterparts, indicating that they were relatively unaffected by the pathogen in this experiment. In contrast to the white abalone, in which we observed trends in body condition and survival that appear to be directly related to *Ca.* Xc gene copy numbers detected by qPCR. At the mid-point of the experiment, coinciding with the highest mortality rate and lowest body condition index values, *Ca.* Xc-exposed white abalone in the elevated temperature group also showed the greatest bacterial burden by qPCR. Despite any measured evidence in body shrinkage in the red groups, at the end of the experiment, *Ca.* Xc-exposed red abalone in the elevated temperature group had the highest pathogen gene copy and after day 300, the *Ca.* Xc-exposed, elevated temperature red group's mortality rate appeared to increase; this might be indicative of disease expression after a nearly yearlong incubation period. The high level of detection of *Ca.* Xc DNA by tissue qPCR and histological changes at the end of the experiment could be associated with this apparent increase in mortality.

Our molecular data was supported by histological analysis. At day 343, *Ca.* Xc inclusions were only found in tissue samples from the two groups with the highest qPCR *Ca.* Xc gene amplification. Red abalone had a greater ratio of phage-infected inclusions to classical (not phage infected) inclusions. However, it is difficult to assess and isolate the impact of temperature and species on our observations because we were only able to compare the red, elevated temperature group with the white, ambient group. This study is the first to document that white abalone could harbor the phage-infected variant of *Ca.* Xc. The granular morphology of some of the membrane-bound vacuoles seen in white abalone is consistent with the phage-infected variant of *Ca.* Xc as visualized by transmission electron microscopy in pink and green (Cruz-Flores & Cáceres-Martínez 2016) and red abalone (Friedman and Crosson 2012).

CONCLUSION

The results of this study have implications for restoration strategies to ultimately remove white abalone from the US Endangered Species list. The federal white abalone recovery plan concluded that outplanting of hatchery-produced animals must be the key restoration action for successful recovery of the species (Team TWAR 2008). White abalone in warm water appear highly susceptible to WS despite presence of the *Ca.* Xc phage, and outplanting efforts should take place in cooler water to minimize thermal enhancement of disease expression. The white abalone captive breeding program has successfully introduced new wild-origin to its broodstock pool, which may increase genetic variation and render the progeny to be less sensitive to natural environmental stressors and possibly more resistant to the effects of *Ca.* Xc. In the Southern California Bight, cooler water typically translates to deeper water but also certain geographic locations with strong upwelling such as San Miguel Island (Erlandson et al. 2008). Our findings with red abalone support the anecdotal reports from California abalone farmers that disease

caused by *Ca.* Xc has been much less frequent and severe since the phage became present. We conclude that phage-presence is associated with improved health and survival in red abalone under conditions that have previously exacerbated the disease. However, the stability of this development is unknown. Future efforts may be directed towards whole genome sequencing the *Ca.* Xc phage for further characterization to better understand the mechanisms associated with pathogenicity.

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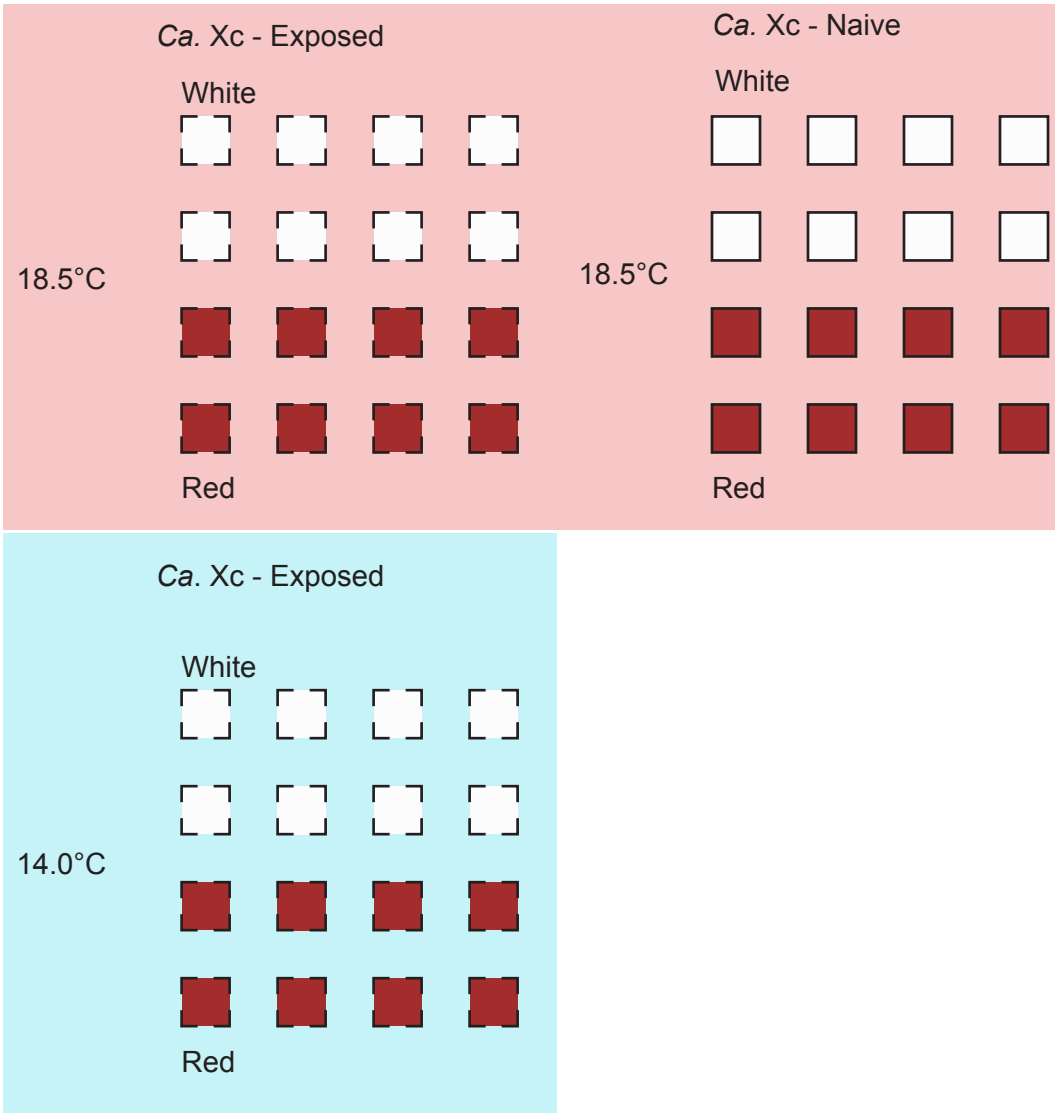


Figure 1. Experimental set up. Experimental units, tanks, are represented as boxes; red or white fill represents those stocked with red or white abalone respectively. Elevated (18.9° C) and ambient (14.5 °C) seawater flowed from header tanks holding infected or uninfected animals to for the first 161 days to transmit *Ca.* Xc. Experimental variables: species (red, white), water temperature (elevated, ambient) and *Candidatus* Xenohaliotis californiensis (*Ca*. Xc) exposed, Ca. Xc unexposed/naive.

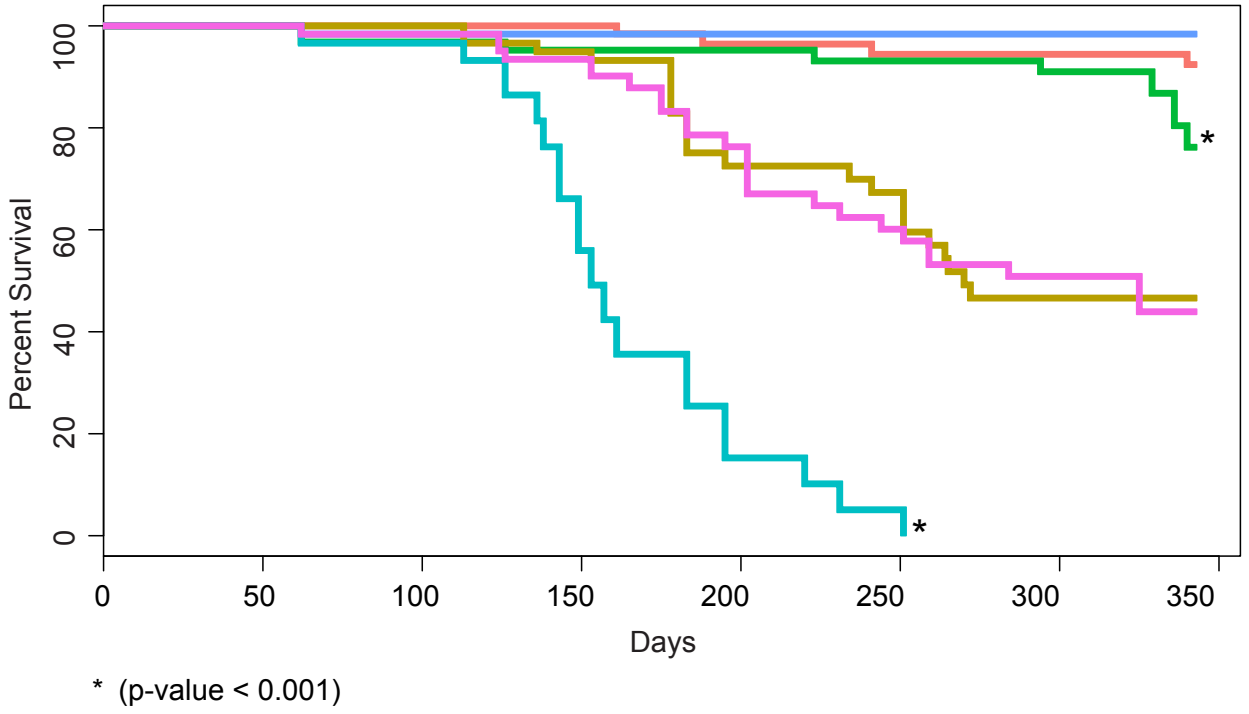
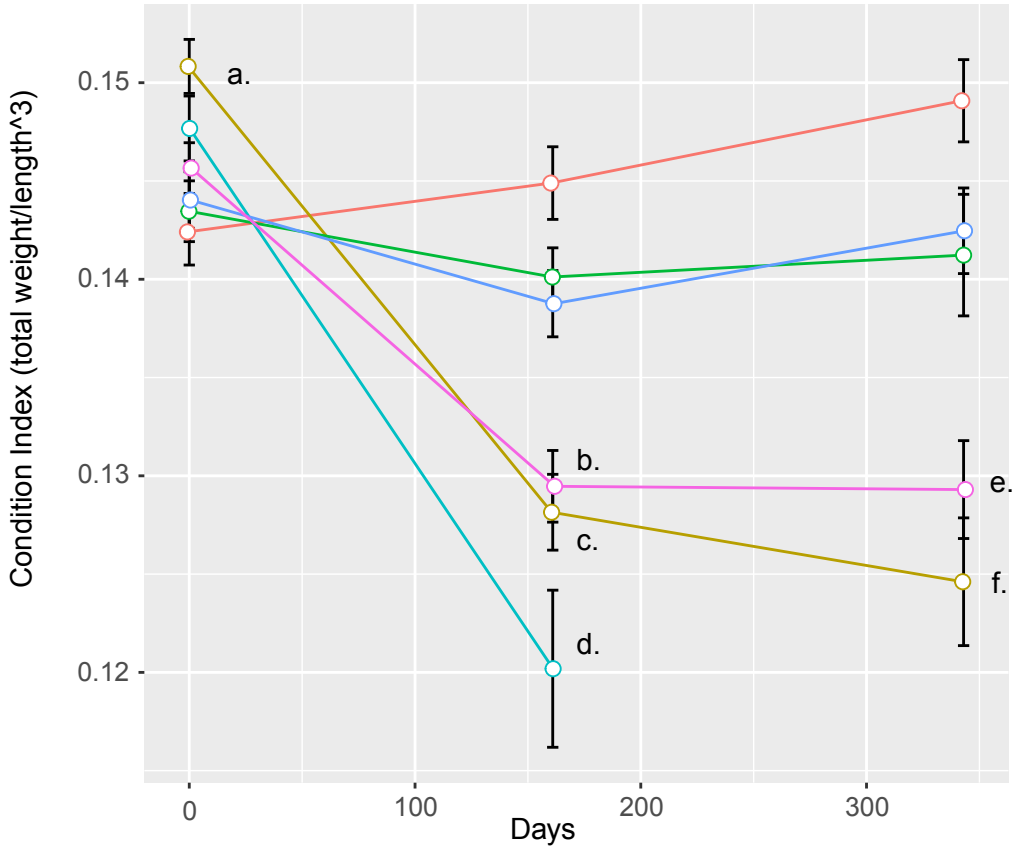


Figure 2. Percent survival of experimental red and white abalone held in ambient water (13.6 °C) or at elevated temperature (18.5°C) with and without *Candidatus* Xenohaliotis californiensis (*Ca.* Xc) exposure for 343 d; N = 64 for each group at day 0. Each curve represents one of the six treatment groups, with variables: *Ca.* Xc exposure, seawater temperature, and species. We observe a significant difference between survival curves of red and white abalone held under elevated seawater temperature and Ca. Xc exposure conditions.



Experimental Treatment Groups

- Red, *Ca.* Xc−exposed, ambient
- White, *Ca.* Xc−exposed, ambient
- Red, *Ca.* Xc−exposed, elevated
- White, *Ca.* Xc − exposed, elevated
- Red, Naive, elevated
- White, Naive, elevated

a. Initial CI values of white abalone, destined for *Ca.* Xc exposure were significantly greater than the other groups. (p-values < 0.05)

b. CI values of white, naive abalone were significantly lower than red, naive abalone (p-value = 0.007)

c. CI values of white, *Ca*. Xc-exposed, ambient abalone were significantly lower than red, *Ca.* Xc-exposed, ambient abalone (p-value < 0.001)

d. CI values of white, *Ca*. Xc-exposed, elevated abalone were significantly lower than red, *Ca.* Xc-exposed, elevated abalone (p-value < 0.001)

e. CI values of white, naive abalone were significantly lower than red, naive abalone (p-value = 0.007)

f. CI values of white, *Ca*. Xc-exposed, ambient abalone were significantly lower than red, *Ca*. Xc-exposed, ambient abalone (p-value < 0.001)

Figure 3. Longitudinal plot of mean (+/- standard error) values in condition index (calculated by total abalone weight divided by shell length cubed) over time of experimental groups. Error bars represent standard error of the mean.

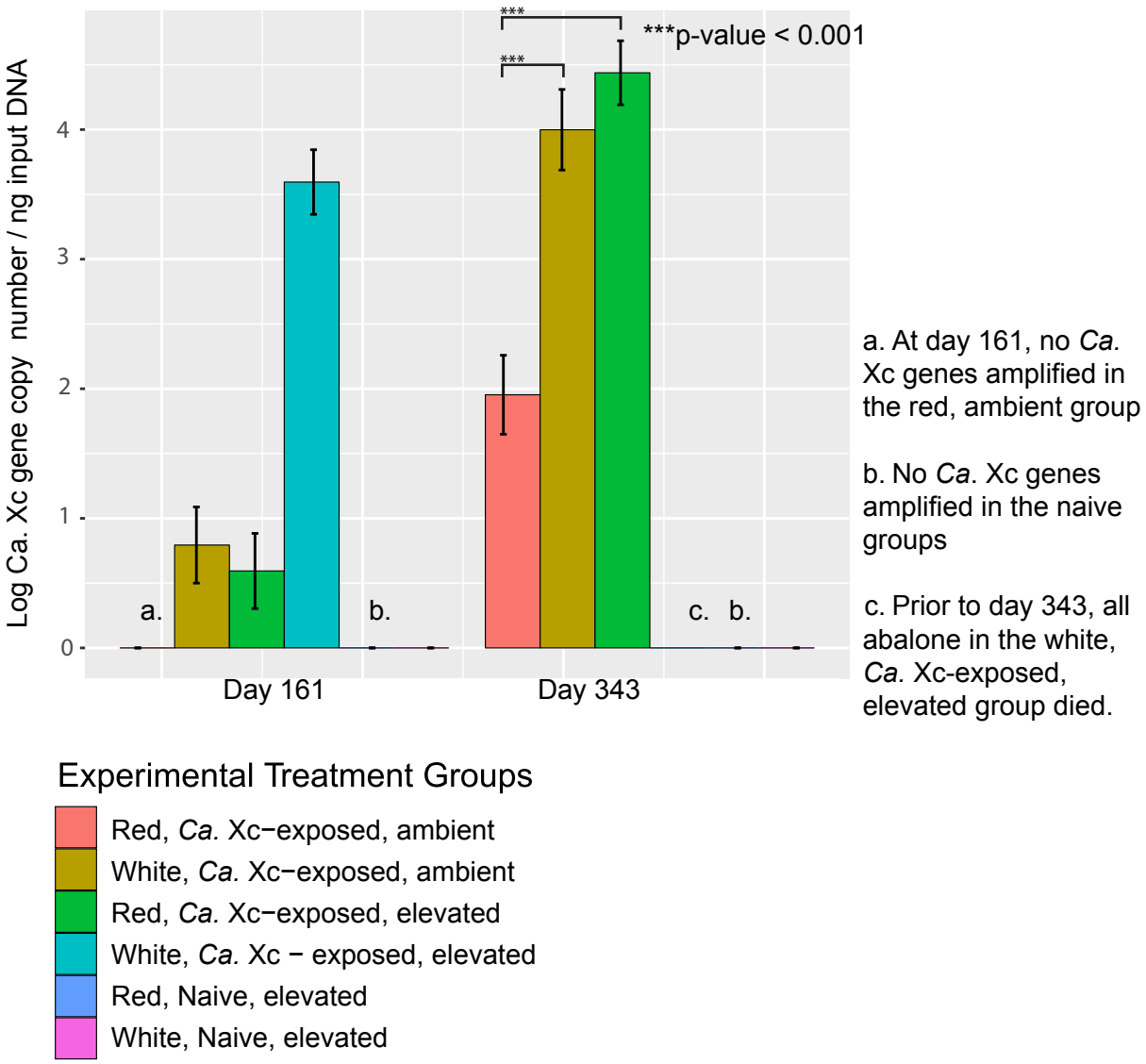


Figure 4. Log transformed qPCR-derived *Candidatus* Xenohaliotis californiensis gene copy numbers from PE tissue at days 161 and 343.

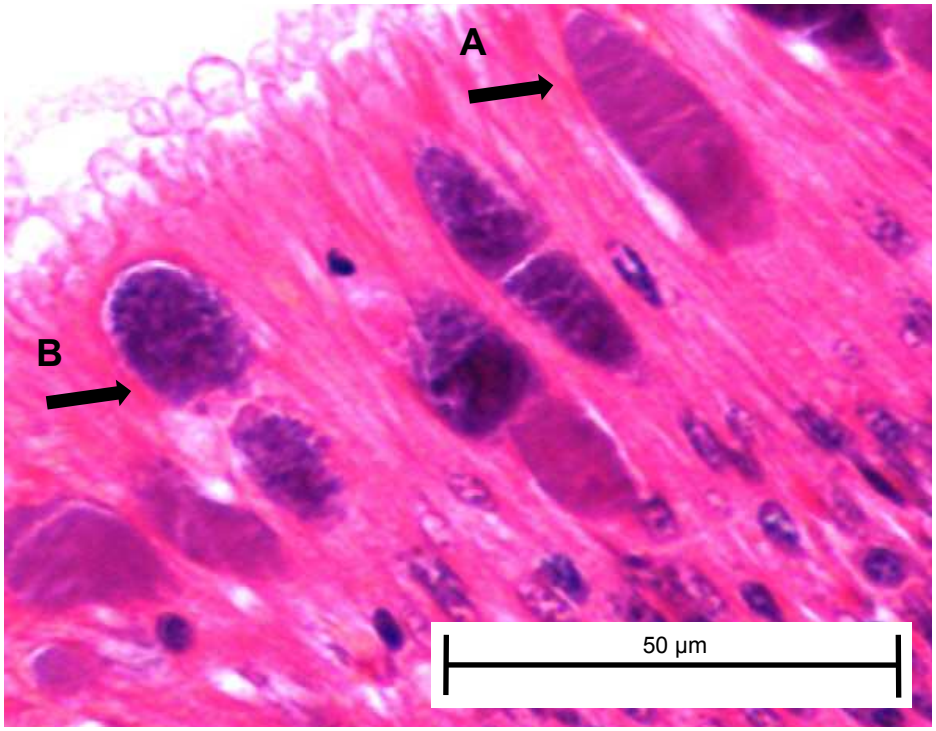


Figure 5. *Candidatus* Xenohaliotis californiensis inclusions within posterior esophagus epithelia from white abalone held at 18.5°C and *Ca.* Cx exposed at 161 days. Arrows indicate (A) classical inclusions, (B) Phage-infected variant inclusions. Haematoxylin and eosin. Bars, 50 µm and 100 µm.

Table 1. Presence or absence of *Candidatus* Xenohaliotis californiensis (*Ca.* Xc) DNA in feces from experimental groups between day 0 and 342 by qPCR. DNA, extracted from tank feces PCR was pooled by group and time period for PCR. Presence is defined as mean *Ca.* Xc gene copy number greater than or equal to three. * NA, no animals remain at this time point.

Group: species, treatment, temperature	Days		
	0-113	126-236	251-342
Red, *Ca*. Xc-exposed, ambient	-	+	+
White, *Ca*. Xc-exposed, ambient	+	+	+
Red, *Ca*. Xc-exposed, elevated	+	+	+
White, *Ca.* Xc-exposed, elevated	+	+	NA
Red, naive, elevated	-	-	-
White, naive, elevated	-	-	-

+ greater or equal to 3 gene copies - less than 3 gene copies

Table 2. Comparison of cumulative mortality rates between 2000 and current study of thermal induction of Candidatus Xenohaliotis californiensis in red abalone.

	Average Seawater Temperature	N	Cumulative mortality events at day = 220	Percent survival
Current study	18.5	46	4	91%
Moore et al. 2000	18.5	30	10	67%

Table 3. Histological analysis of *Candidatus* Xenohaliotis californiensis - inclusion type prevalence in post esophagus and digestive gland tissue samples by group at day 343.

Species, treatment, temperature condition	Post-esophagus			Digestive gland		
		Inclusion type*			Inclusion type*	
	N	Classical	Phage-variant	N	Classical	Phage-variant
Red, *Ca*. Xc-exposed, ambient	34	0%	0%	54	0%	0%
White, *Ca*. Xc-exposed, ambient	14	29%	14%	19	0%	0%
Red, *Ca*. Xc-exposed, elevated	35	89%	77%	46	20%	15%
White, *Ca*. Xc-exposed, elevated	0	NA	NA	0	NA	NA
Red, naïve, elevated	27	0%	0%	62	0%	0%
Red, naïve, elevated	8	0%	0%	22	0%	0%

* % of tissue samples with inclusion type