California Sea Grant College Program
Research Completion Reports
(University of California, San Diego)

Year 2007 *Paper Fisheries*07_02

Restoration of Endangered White Abalone, Haliotis sorenseni: Resource Assessment, Genetics, Disease and Culture of Captive Abalone

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Abstract

The white abalone, Haliotis sorenseni, supported valuable commercial fisheries only 30 years ago. In 2002, this species became the first marine invertebrate in the United States to achieve federal endangered species status. The overall goal of this project was to collect life history, genetic and disease susceptibility data that to support recovery.

California Sea Grant College
Project Number: R / F-196
Final Report

Project Title: **Restoration of Endangered White Abalone, *Haliotis sorenseni*: Resource Assessment, Genetics, Disease, and Culture of Captive Abalone.**

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RESEARCH OBJECTIVES:

The white abalone , *Haliotis sorenseni,* supported valuable commercial fisheries only 30 years ago. In 2002*, this species* became the first marine invertebrate in the United States to achieve federal endangered species status. The overall goal of this project was to collect life history, genetic and disease susceptibility data that to support recovery.

Specific objectives included:

1) field surveys to determine the abundance and distribution of white abalone in southern California.
2) collection of broodstock to enable captive rearing program.
3) development of captive rearing of white abalone for research and future outplanting
4) lab experiments to determine the effects of temperature and feeding regimes on t key life history characteristics of this species.
5) use of molecular genetic approaches to determine natural population structure, monitor the genetics of captive breed animals and develop tools for future monitoring of outplant success.
6) determination of the susceptibility of white abalone to withering syndrome and evaluation of treatment protocols for potential use in outplanting operations.

As detailed in the following component reports from each investigator, most of the objectives of the studies were met. One major problem was the collection of broodstock; delays in permitting and extremely low abundances of white abalone prevented any collection. All work was undertaken on animals that were collected prior to the ESA listing of the species and their captive-reared offspring. No samples were available for genetic analyses of population structure of white abalone.

COMPONENT 1: White Abalone Life Biology Investigations.
PI: Thomas B. McCormick

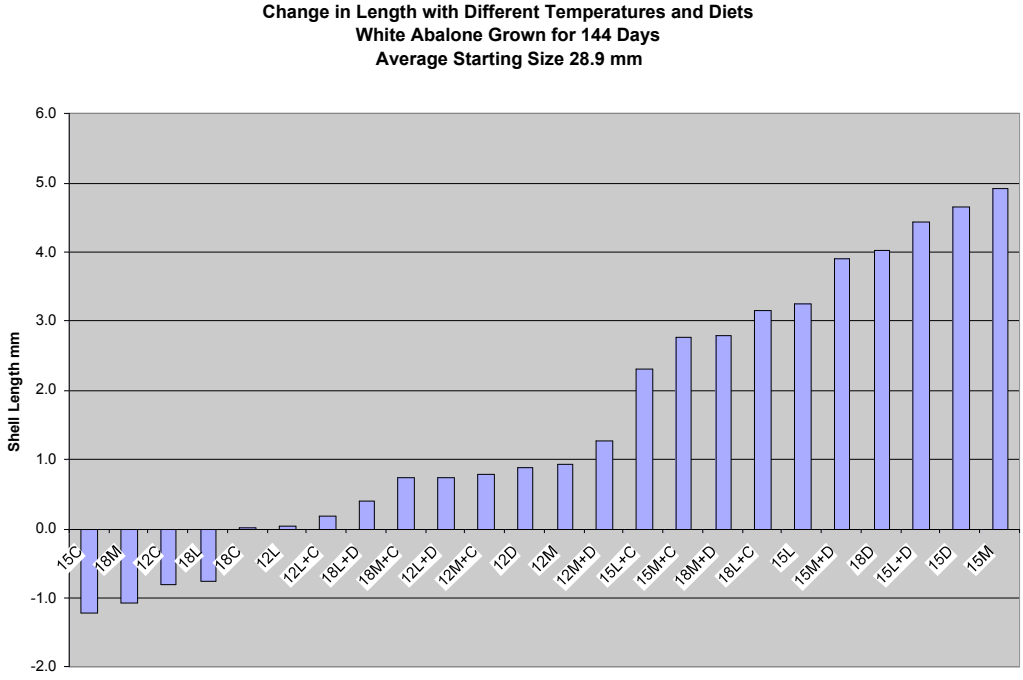
The goal of this component of the project was to use captive animals to gain as much information as possible about the biology of white abalone, a species that is too rare and inaccessible to study in the natural environment. Major studies and results are as follows:

Larval Development Rate – All abalone are ectotherms, that is, animals whose body temperature is the same as their environment. Seawater temperatures in the coastal water habitat of white abalone habitat off southern California and Baja California, Mexico vary as much as 10˚C over the course of the year. We examined the effect of a range of seawater temperatures (9˚, 12˚, 15˚, 18˚, and 21˚C) on the developmental rate and survival of white abalone eggs and larvae. Our findings indicate that the optimal temperature range for white abalone larvae was 12 - 18˚C. At 12˚C larvae were competent to settle in seven days. At 15˚C and 18˚C larvae were ready to settle in five days. These settlement times are significantly shorter than reported in previous work 35 years ago, but are more similar to rates for sympatric species such as red, green, and pink abalone in southern California.

Effect of Diet and Temperature – Juvenile and early adult white abalone were grown for 144 days at three seawater temperatures and fed a variety of macroalgae, both singly and in combination as follows:

Diet And Symbol	Temperature		
	12°C	15°	18°
Macrocystis (M)	Seven replicates of five abalone each	Seven replicates of five abalone each	Seven replicates of five abalone each
Laminaria (L)	"	"	"
Chondrocanthus (C)	"	"	"
Palmaria (Dulse -D*)*	"	"	"
Macrosystis & Chondrochathus (M + C)	"	"	"
Macrocystis & Palmaria (M + D)	"	"	"
Laminaria & Chondrocanthus (L + C)	"	"	"
Laminaria & Palmaria (L + D)	"	"	"

The following graph shows changes in white abalone shell length in µm / Day.

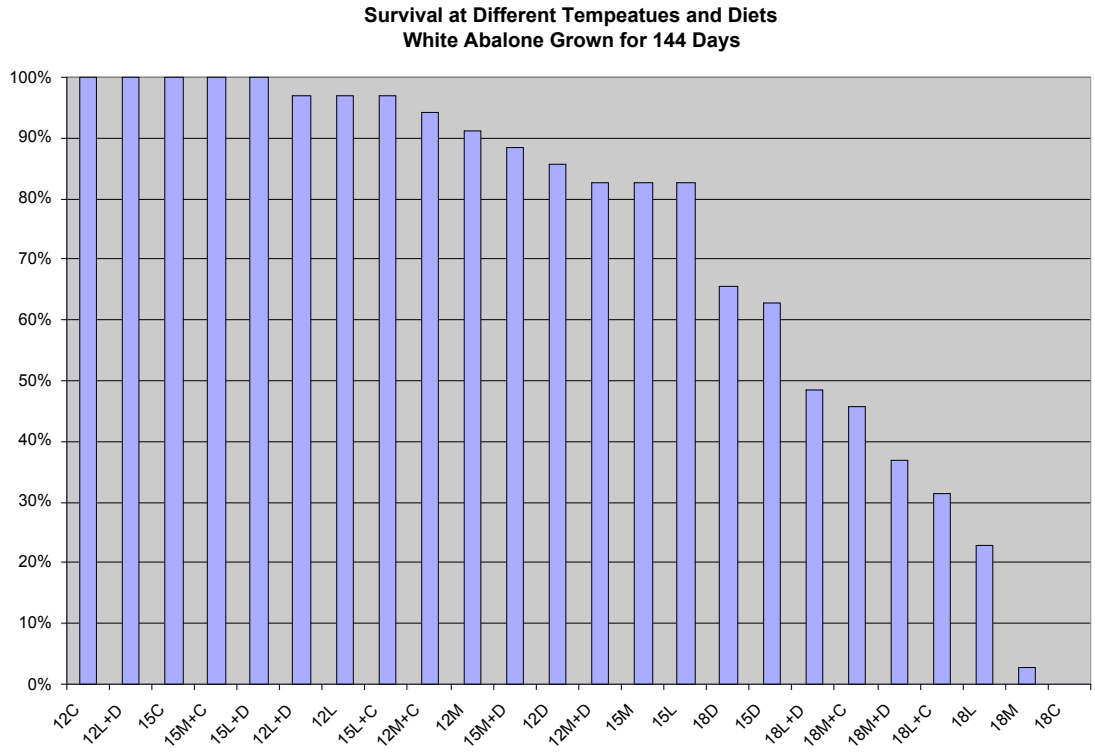


The X axis indicates the treatment temperature and diet: C = *Chondrocanthus* (Cow's tongue), M = *Macrocystis* (giant kelp), L = *Laminaria*, D = *Palmaria* (Dulse).

In general, growth is maximized at a temperature of 15ºC, although rapid growth was achieved at 18ºC on diets of Dulse*, Laminaria* + *Chondrocanthus,* and *Macrocystis* + Dulse. At 15ºC a diet of *Macrocystis* produced the fastest growth, followed by diets that included Dulse. *Chondrocanthus* is either not consumed or provides insufficient nutrients for growth. When fed by itself *Laminaria* provides the best abalone growth at 15 ºC. Better abalone growth is achieved when *Laminaria* is fed in combination with Dulse.

Abalone Survival

The combined effects of seawater temperature and diet on abalone survival are illustrated in the following graph:



Survival at 12ºC and 15ºC averaged more then 90% while that at 18 ºC averaged only 35%. Even though some diets at 18 ºC produced rapid growth, survival was low. Animals held at 18ºC exhibited signs of withering syndrome.

This work shows the interaction of seawater temperatures and diet on white abalone growth, survival, and susceptibility to WS. White abalone grow and survive best at temperatures of 15˚C or less on a mixed diet of giant kelp (*Macrocystis pyriferia*) and Pacific dulse (*Palmaria mollis*).

Expression of WS symptoms appears to be a function of water temperature and time: exposure to temperatures of 18 ºC or greater for a duration of over 3 months will reduce survival. Seawater temperatures in southern California vary annually, and white abalone are still found at sites where temperatures exceed 18 ºC for part of the year.

White abalone grown at low seawater temperature: Effect on the presence and expression of withering syndrome caused by rickettsia-like bacteria (WS –RLP). Our temperature diet experiment indicated that temperatures of 15˚C or less resulted in greater survival of abalone and little or no expression of withering syndrome (WS), a disease caused by a rickettsia-like procaryote (RLP) the bacterium "*Candidatus* Xenohaliotis californiensis". Withering syndrome is a chronic disease responsible for mass mortalities of wild and hatchery-raised abalone in southern and central California.

Working cooperatively with our co-PI, Dr. Carolyn Friedman at the University of Washington we undertook an investigation of how low seawater temperature affects the presence and expression of withering syndrome in white abalone. This information is necessary to develop a improved hatchery methods and recovery plans. Our research with Dr. Friedman was designed to determine if holding white abalone at a low temperature (10ºC) enables them to outgrow their RLP infection. Further, we asked how effective are low temperatures relative to OTC in reducing RLP burdens in infected abalone.

Untreated (no exposure to the antibiotic Oxytetracycline, OTC) hatchery-raised abalone were held in mini-raceways at the test temperature of 10ºC and at a control temperature of 15ºC for six months at the CIMRI hatchery in Oxnard, California. A second group of abalone drawn from the same pool were treated with OTC-medicated feed, then placed in separate mini-raceways at 10ºC and 15ºC. as shown below.

Temperatures			
10ºC		**15ºC Control**	
Untreated Abalone	OTC Treated Abs	Untreated Abalone	OTC Treated Abs
6 mini-raceways	6 mini-raceways	6 mini-raceways	6 mini-raceways
20 Abalone/raceway	20 Abalone/raceway	20 Abalone/raceway	20 Abalone/raceway

Animals were fed giant kelp (*Macrocystis pyriferia*) or Pacific Dulse (*Palmaria mollis*) on alternative weeks. The experiment was run for eight months. Both groups were sampled intermittently and sent to Dr. Friedman's laboratory for PCR detection of RLP, histology, and OTC residues. Results from Dr. Friedman's lab are forthcoming.

Treatment of WS with antibiotic Bath – Withering syndrome is a problem not only for wild populations but for farm-raised abalone as well. Formally antiobiotics would be administered during El Ni o periods via injections or medicated feeds. Injections are labor intensive and the feeds are not readily consumed and can result in poor water quality in rearing tanks.

In the course of our work on withering syndrome described above, we inadvertently found that abalone can absorb water soluable antibiotics directly from the water. With this in mind we teamed up with another of our Sea Grant co-PI's, Dr. Jim Moore of the Shellfish Health

Laboratory at the Bodega Marine Laboratory. For this project we secured additional funds from the National Marine Fisheries Service. Dr. Moore developed a protocol that entailed placing abalone in alternating antibiotic baths and flowing seawater over the course of several days. This protocol was initially tested in the Dr. Moore's laboratory, then on a larger scale on white and red abalone at CIMRI's hatchery. Dr. Moore will

Pathology and Histology – Dr. Jim Moore of the Shellfish Health Laboratory at the Bodega Marine Laboratory routinely conducted histological examinations of hatchery-raised white abalone. The information generated provides a picture of the etiology of withering syndrome (WS), resulting from a rickettsia-like procaryote bacteria (RLP) (see Final Year Narrative from Jim Moore). RLP reside in the post-esophagus and digestive gland of the abalone. In the digestive gland RLP is responsible for metaplasia, resulting in the loss of absortive tissue. In addition to the morphological changes associated with metaplasia, RLP infested animals show more clinical signs such as mantle retraction, lower weight gain, lower condition index associated with WS. Dr. Moore tracked these physiological changes over the course of our growth and treatment experiments.

Behavioral Studies – The distribution of white abalone (*Halotis sorenseni*) extends throughout the southern California Bight and northern Baja Californa, a range of 900 km, despite a short five-day larval dispersal stage.

Observation of one to three-year old hatchery-raised white abalone revealed behaviors that may provide an alternative long-range dispersal mechanism. Juvenile and young adult white abalone assume a "standing" position, in response to the presence of a drifting substrate. Many then "climb" onto fragments of drifting kelp (*Macrocystis pyrifera,* giant kelp), other benthic macroalgae and drifting substrates in flumes. Such behavior has not previously been described for any abalone species.

To test the frequency and duration of the behavior, fragments of macroalgae typically found in white abalone habitat were passed down a flume stocked with juvenile and young adult white abalone. An average of more than 6% of the abalone "climbed" onto *Macrocystis* during the short 20-second transit time that the algae spent drifting down the flume. Significantly more abalone ($p<0.01$) "climbed" on *M. pyrifera* than any other test substrate including two other macroalgae and a rubber test substrate. Trials with red abalone resulted in no instances of "standing" or "climbing" behavior.

Duration of white abalone attachment on kelp suspended in the water column in the laboratory was prolonged (up to 51 days in tests), which could result in transport distance of hundreds of kilometers in the wild. The movement of the drift kelp may bring it and the rafting abalone to isolated rock outcrops that are adult habitat. Algal rafting could potentially transport benthic life stages or groups of small abalone far beyond the range of larval dispersal.

Wound Treatment – The hemolymph of abalone lacks clotting factors. Deep cuts inflicted during collection or handling may result in death. We are testing a series of theraputics that will either bond tissue together in the affected area or stop the bleeding. Testing is still underway.

Field Studies: BARTS - In December 2004 we deployed Baby Abalone Recruitment Trackers (BARTs) at three offshore sites that have potential for outplanting of white abalone for restoration. We returned to these sites in December 2005, May 2006, and October 2007. with SCUBA survey teams. As the teams surveyed the BARTs an underwater video camera provided live coverage to undergraduate students on the research vessel. Students were able to speak with the research diver, and helped document the invertebrate communities found on the BARTs. Water samples were taken as part of a cooperative effort with Carolyn Friedman, University of Washington, to test for the presence of rickettsiales-like-organisms (RLOs), the causative agent of withering syndrome.

Field Studies: Habitat Survey and Search for Wild Population Remnents
Submarine and diver surveys of white abalone habitat in the 1990s indicated that populations had dramatically declined and were at levels too low to sustain reproduction. We conducted a series of habitat surveys and searches for white abalone in areas that had previously been reported to be white abalone habitat. In brief, no live white (nor pink) abalone were found during our surveys. Only six red abalone were observed. Dives in the same areas in the 1970's and 1980's would have detected hundreds of abalone. With the loss of abalone populations habitat is often taken over by other invertebrates, especially the brittle star *Ophiopteris* which carpets the bottom, making it difficult for abalone and urchin communities to repopulate such areas.

Surveys were conducted by divers from the California Department of Fish and Game, Channel Islands National Park, and the Channel Islands National Marine Sanctuary using the RV Garibaldi and RV Shearwater.

Modifications: During the last year of this research we found that hatchery raised white abalone are susceptible to attack from a fungus-like infestation. A large percentage of abalone mortalities in the hatchery exhibited this fungus on the interior of the shell. Cooperative research to identify this disease was undertaken with Dr. Carolyn Friedman, University of Washington, and /Dr. Jim Moore, Bodega Marine Laboratory. Research is ongoing.

Benefits: This research dramatically expands our knowledge of the biology of the white abalone. The information that we are generating on the environmental requirements of this species is essential to resource managers who are working to recover this endangered species. Our work on abalone behavior is defining a new paradigm in thinking about distribution and defensive/ communication mechanisms for abalone and other marine invertebrates. In addition, the methods that we are employing to assess abalone and benthic invertebrate populations should prove of value to resource managers for other species in southern California**.**

International Implications: Abalone populations are spread throughout the world's oceans. The white abalone is the first species to make the endangered species list, however, there are other species that face similar perils. The results of our work to restore white abalone populations will provide a new perspective for resource managers in other countries.

Cooperating Organizations:

BHP Billiton, funding for research and hatchery operations.

California Department of Fish and Game, supply divers and research vessel for field work and collection of wild abalone.

Channel Islands National Marine Sanctuary , provide the research vessel, RV Shearwater, for field work.

Channel Islands National Park, provide Divers and support logistics for field work.

National Fish and Wildlife Foundation, provide funding for hatchery operations and research.

National Oceanic and Atmospheric Administration / NMFS, antibiotic treatment grant.

Reliant Energy, provides support for hatchery operations.

Conference presentation

McCormick, T. B., Research and recovery efforts for an endangered West Coast abalone (*Haliotis sorenseni*). American Fisheries Society Annual Meeting, September 10 – 14 Lake Placid, NY.

Manuscripts

McCormick, T. B., L.M. Buckley, J. Brogan, and M. Perry. Drift Macroalgae as a dispersal mechanism for the white abalone (*Haliotis sorenseni*), Bartsch 1940. 2007 Accepted – Marine Ecology Progressive Series.

McCormick, T. B., and G. Navas 200_, The effect of temperature and diet on the growth and survival of white abalone (*Haliotis sorenseni*). Manuscript in prep.

McCormick, T. B., and G. Navas 200_, The effect of density on the growth of white abalone (*Haliotis sorenseni*). Manuscript in prep.

Brogan J. and T. B. McCormick. 200_. The effect of temperature on larval development in white abalone (*Haliotis sorenseni*). Manuscript in prep.

COMPONENT 2: Genetic analyses.
PI: Ronald S. Burton

The goal of this component of the project was to provide genetic analyses of white abalone populations and broodstock structure in support of potential recovery actions such as translocation of adults, hatchery broodstock establishment, and outplanting of lab-reared abalone. As stated above, work was limited by the lack of population samples of white abalone. Using the pre-listing samples and families reared in captivity, progress was made on developing genetic markers that could be useful in tracking the success of future outplants. The work was completed by Year 1 Sea Grant trainee Kristen Gruenthal and has now been published. The citation and abstract are below:

Gruenthal, KM and RS Burton (2005) . Genetic diversity and species identification in the endangered white abalone (*Haliotis sorenseni*). Conservation Genetics 6:929-939.

Abstract: In 2001, the white abalone *Haliotis sorenseni* became the first marine invertebrate in United States waters to receive federal protection as an endangered species. Prior to the endangered species listing, twenty abalone were collected as potential broodstock for a captive rearing program. Using DNA from these animals, we have developed genetic markers, including five nuclear microsatellite loci and partial sequences of one nuclear (VERL) and two mitochondrial (COI and CytB) genes, to assess genetic variability in the species, aid in species identification, and potentially track the success of future outplanting of captive-reared animals. All five microsatellite loci were polymorphic and followed expectations of simple Mendelian inheritance in laboratory crosses. Each of the wild-caught adult abalone exhibited a unique composite microsatellite genotype suggesting that significant genetic variation remains in natural populations. A combination of nuclear and mitochondrial gene sequencing demonstrated that one of the original wild-caught animals was in fact not a white abalone, but *H. kamtschatkana* (possibly subspecies *assimilis*). Similarly, another animal of uncertain identity accidentally collected by dredging was also shown to be *H. kamtschatkana.* Inclusion of these two animals as broodstock could have resulted in unintentional hybridizations detrimental to the white abalone recovery program. Molecular genetic identifications will be useful both in preventing broodstock contamination and as markers for future restocking operations.

COMPONENT 3: Withering Syndrome: Susceptibility and Treatment
PI: James Moore

INTRODUCTION

Withering syndrome (WS) is a chronic, progressive wasting disease of abalone (*Haliotis* spp.) in the coastal waters of California, USA and Mexico (Moore *et al.* 2002). It was first noted in the Channel Islands of Southern California in the mid-1980s, and resulted in black abalone (*Haliotis cracherodii*) population declines of up to 99% (Haaker *et al.*, 1992). WS is caused by "*Candidatus* Xenohaliotis californiensis", an intracellular Rickettsiales-like prokaryote (WS-RLP) that infects epithelial cells of the gastrointestinal tract (Friedman *et al.*, 2000; Moore *et al.*, 2001). WS-RLP infections have been observed in pink (*H. corrugata*), flat (*H. walallensis*), red (*H. rufescens*; Moore *et al.*, 2000; Caceres-Martinez and Tinoco-Orta, 2001), black (*H. cracherodii*; Friedman *et al.*, 1997) and green (*H. fulgens*;

Caceres-Martinez *et al.*, 2000) abalone. In 2002, it was discovered that white abalone are susceptible to WS-RLP infection and subsequent expression of clinical signs of the disease (Moore et al. 2003).

The gross signs of WS parallel those of starvation: weakness, lethargy, body mass shrinkage, and death. Microscopically, the myofibers of the foot muscle atrophy and are replaced by connective tissue, presumably due to starvation-induced catabolism. WS-affected abalone consume less food than healthy abalone (Moore *et al.*, 2000, 2001). The area occupied by functional digestive/absorptive cells in the digestive gland diminishes while that occupied by transport duct tissue increases (Gardner *et al.*, 1995, Moore *et al.* 2001). These changes, described by Gardner *et al.* (1995) as metaplastic, are presumed to impair digestive gland function, contributing to further physiological decline.

Elevated water temperature has been shown to hasten the progression of WS and to increase mortality (Lafferty and Kuris, 1993; Friedman *et al.*, 1997; Moore *et al.*, 2000; Raimondi *et al.*, 2002, Braid et al. 2005, Vilchis *et al.*, 2005). Cultured red abalone infected with WS-RLP and maintained at 14.7°C for 220 days experienced no clinical signs of WS while those held at 18.5°C showed WS signs and 33% mortality (Moore et al. 2001). The first observation of WS in white abalone occurred in hatchery stocks (2001 spawn) during a period of elevated temperature. Subsequent experience has indicated a temperature sensitivity of WS expression similar to that of red abalone.

The 1997-1998 El Niño condition resulted in mass mortalities at California red abalone farms due to WS induced by elevated temperatures. This led to investigation of potential therapeutic treatments, and a feed-based administration of oxytetracycline (OTC) was found to be highly effective at reducing signs of the disease (Friedman et al. 2003). When WS was observed in cultured white abalone, the population was administered OTC treatment and it appeared to have significantly reduced losses to this disease. In both red abalone and white abalone OTC treatment did not completely eliminate the pathogen. This is not unexpected, as the medicated feed did not appear to be highly palatable, and abalone would have varying access to it during the treatment period.
The following studies were conducted to further investigate the effects of WS on white abalone and whether a therapeutic treatment regimen could be developed that completely eliminates WS-RLP from infected captive populations. Investigations of broodstock mortalities that occurred during the study period are also included.

GENERAL MATERIALS AND METHODS

Histological and visual assessment of WS-RLP burdens and WS-associated changes: WS-RLP infection intensities in the postesophagus and digestive gland were scored using the following scale: 0 = absent, 1 = 1 - 10 inclusions per 200x field of view, 2 = 11 – 100 inclusions, 3 = > 100 inclusions. Foot muscle condition was assessed using the following scale: 0 = Muscle fibers comprise > 90 % of tissue present, 1 = 76 – 90 %, 2 = 50 –75 %, 3 = < 50 % of tissue present. Digestive gland metaplastic changes, in which functional acini are replaced with transport duct, were assessed using the following scale: 0 = transport ducts comprise < 5 % of tissue, 1 = 5 – 10 %, 2 = 11 – 25 %, 3 = > 25 % of tissue. Body shrinkage, including reduction in foot size and recession of the mantle, was visually scored

with 0 = no shrinkage and 1, 2 and 3 indicating mild, moderate and severe body shrinkage respectively.

PCR detection of WS-RLP in tissues and feces: The presence of the WS-RLP in fecal samples was determined by using a modification of the PCR test of Andree et al., (2000). Briefly, DNA was purified from abalone feces collected from a tank or pooled postesophagus and digestive gland from individual abalone using a Qiagen Mini Stool Kit. Negative control feces or tissue and positive control feces or tissue were purified and amplified with every PCR run, along with one blank (water). A 160 bp segment of the 16s rDNA gene was amplified from WS-RLP infected tissue or feces using the primers of Andree et al. (2000). All amplifications were performed in 50µL reactions containing 100ng of template DNA, 2U Sigma JumpStarTAq, 1x JumpStarTAq buffer, 3mM MgCl2, 400ng/ul BSA, 200uM dNTPs, and 0.5uM of each primer. The rickettsial DNA was amplified with an initial denaturation step of 95ºC for 3 minutes; followed by 40 cycles of 95ºC for 1 min, 62ºC for 30 seconds, 72ºC for 30 seconds; and a final extension of 72ºC for 10min using an Eppendorf Master Cycler Gradient thermal cycler. After amplification, DNA was separated and visualized on 2% agarose gels containing ethidium bromide.

SPECIFIC METHODS, RESULTS AND DISCUSSION

OTC Administration Regimen to Completely Eliminate WS-RLP Infections: Three hundred juvenile white abalone (2001 year class "O" tanks; 41.6±1.4mm shell length, 12±0.62g total weight, mean±std. error) were shipped from the Channel Islands Marine Research Institute (CIMRI) under NOAA Scientific Research Permit # 1346-01 to the CDFG Pathogen Containment Facility at the U.C. Bodega Marine Laboratory on February 3, 2005. A health exam on thirty individuals was conducted on October 29, 2004 prior to receiving the abalone (Table 1). Almost all had clinical signs of WS (body shrinkage score 1.6±0.2). Histology revealed light WS-RLP infections (0.5±0.1 and 0.6±0.1 for the postesophagus and digestive gland respectively) yet they had remarkably high levels of digestive gland metaplasia (2.3±0.2) and reduced foot muscle condition (0.4±0.2). These animals were still showing advanced signs of WS although pathogen infection intensity had been greatly reduced by previous OTC treatment. Upon arrival at BML, the animals were stocked in two Vexar mesh baskets suspended in 176 gallon polypropylene tanks on flow-through ambient (13.3^{o}C) seawater. Ten abalone were sampled for histology. No WS-RLP inclusions were detected in this sample but moderate levels of body shrinkage, (0.4±0.1), digestive gland metaplasia (0.5±0.2) and foot atrophy (0.6±0.2) were present.

The abalone were held without feed for one week and then were fed a medicated feed at a rate of 103mg oxytetracycline /kg abalone (Friedman et al. 2003; feed donated by The Abalone Farm Inc., Cayucos, CA). Feeding began on February 11, 2005 and lasted for thirty days (Table 1). The medicated feed was added fresh daily and particulates were removed from the basket once weekly. This population experienced steady mortalities over the following three months and on March 2nd, 2005 a second shipment of 300 juvenile white abalone were obtained from the same holding tanks at CIMRI to augment the initial population. These animals were placed in a tank identical to the one already present and fed the same medicated diet for thirty days. PCR of feces samples taken in May 2005 showed the presence of WS-RLP DNA in Tank 1. Steady mortalities were occurring in both tanks, with a loss of approximately 8% per month; based on similar experience with the source

population at CIMRI along with the absence of detectable inclusions in the ten animal pre-sample, we believe that these mortalities occurred due to pathological changes as a result of WS in the source population prior to treatment at CIMRI.

On June 2nd, 2005 all abalone from the two tanks were moved to smaller containers and treated again for a second round of OTC-medicated feed (Table 1). These smaller containers were used to increase access of abalone to the feed and also because it was believed that OTC leached from the feed may be responsible for some portion of that taken up by abalone. The abalone in the original tank were separated into two tanks (7.5L Rubbermaid containers) designated D & E with approximately 97 animals/tank, while the abalone in the second tank were divided between two tanks designated G and K with approximately 113 animals/tank. They were fed the medicated feed for 30 days ending on July 3rd, 2005.

Feces collected on July 19th, 2005 indicated that all tanks were positive for WS-RLP DNA (Table 1). On August 9th, 2005, shrunken animals were culled from each tank, between 6 and 8 animals from each of the four tanks for a total of 29 animals. Histological examination revealed no WS-RLP inclusions in the postesophagus or in the digestive gland, but severe digestive gland metaplastic changes (mean score: 2.0±0.1) were still present in this select group.

A third treatment with OTC-medicated feed was initiated on September 2, 2005, this time with a double dosage to 0.206g OTC/g of abalone, for 23-days (Table 1). PCR tests were conducted on feces collected on a weekly basis starting on October 18th, 2005, approximately one month after the third round of feeding ended, until January 2nd, 2006. During this period mortality rates began to slow. These results indicated that WS-RLP DNA was still present in each of the four tanks. A fourth round of feeding was initiated on January 3, 2006, again with the higher dose of 0.206g OTC/kg of abalone, for 20 days. Feces were collected starting on February 21st and every 1-2 weeks after this date until April 18, 2006 and less frequently after that. The 2/28/06 sampling of Tank E showed a positive signal for the presence of WS-RLP, but nine subsequent samplings revealed negative results. After the final sampling it was concluded that animals were WS-RLP-free.

Mortalities over this period slowed dramatically and at the end less than 1% were visibly shrunken. These abalone were subsequently used in an ongoing unrelated experiment. Results collected during that study provide additional evidence that the populations were WS-RLP-free; on 8/16-17, 2006 36 individuals (approximately half the combined population) randomly selected from the four tanks were distributed into 12 containers with three animals each. PCR of feces from these containers collected four months later (December 2006) indicated that all were WS-RLP-free, even though the temperature was raised from ambient (~13.3°C) to 18.5°C after two months. The animals have been held at this temperature through May 2007 with no signs of WS.

These results indicate that a very prolonged OTC treatment regimen was required to completely eliminate WS-RLP from captive white abalone populations held at a relatively cool temperature (~13.3°C). While a single OTC treatment has been shown to be highly effective in both red abalone and white abalone at reducing signs of WS and attendant mortality, complete elimination (in white abalone) appears much more difficult to achieve,

yet was still possible. Completely eliminating WS-RLP from captive abalone will be particularly important if WS-RLP-positive broodstock are collected from the wild in the future. The patchy appearance of positive WS-RLP DNA signals from the four tanks suggests that low-level WS-RLP infections persisted in a small proportion of the animals present. Clearly, detection of low-level infections in such abalone in cold water presents a challenge, even using the highly sensitive PCR method. The simplest method of detecting such low-level infections is to elevate water temperature to approximately 18°C, a condition much more permissive to rapid proliferation of WS-RLP and expression of WS signs. The observation of no WS signs after seven months at 18.5°C strongly corroborates our conclusion that the abalone are now WS-RLP-free.

Untreated source animals at CIMRI: While the white abalone held at the Bodega Marine Lab were being treated with OTC, the original source animals in "O" tanks at CIMRI were also examined. The first exam was done prior to the shipment of white abalone while the next was a pre-sample of the white abalone received at the lab (described above). The subsequent four samples were taken from July 2005 to June 2006. Data from all samples is shown in Table 2.

Table 1. Treatment of WS-RLP-infected white abalone with a oxytetracycline medicated feed and PCR results (positive/negative) for the presence of WS-RLP DNA in feces samples from holding containers.

Date				
10/29/2004	Presample at CIMRI: Light WS-RLP infections			
2/3/2005	Arrival of **Tank 1** animals			
2/11-3/12/2005	Tank 1 OTC treatment (30d, 0.103g OTC/kg abalone)			
3/2/2005			Arrival of **Tank 2** animals	
3/7-4/6/2005			Tank 2 OTC treatment (30d, 0.103g/kg)	
5/5/2005	Tank 1 feces PCR **positive**		Tank 2 feces PCR negative	
6/2/2005	Tank 1 split to Tanks D & E		Tank 2 split to Tanks G & K	
	Tank D	**Tank E**	**Tank G**	**Tank K**
6/2-7/3/2005	Second OTC treatment (30d, 0.103g/kg)			
7/19/2005	**positive**	**positive**	**positive**	**positive**
9/2-9/24/2005	Third OTC treatment (23d, 0.206g/kg)			
10/28/2005	negative	negative	negative	negative
11/4/2005	negative	positive	negative	**positive**
11/9/2005	negative	**positive**	negative	negative
11/15/2005	negative	negative	negative	negative
11/22/2005	negative	negative	**positive**	negative
11/28/2005	negative	negative	negative	negative
12/5/2005	negative	negative	negative	**positive**
12/12/2005	negative	**positive**	negative	negative
12/19/2005	negative	negative	negative	negative
12/27/2005	negative	positive	negative	negative
1/2/2006	**positive**	negative	negative	negative
1/3-1/22/06	Fourth OTC treatment (20d, 0.206g/kg)			
2/21/2006	negative	negative	negative	negative

2/28/2006	negative	**positive**	negative	negative
3/6/2006	negative	negative	negative	negative
3/14/2006	negative	negative	negative	negative
3/21/2006	negative	negative	negative	negative
3/28/2006	negative	negative	negative	negative
4/5/2006	negative	negative	negative	negative
4/10/2006	negative	negative	negative	negative
4/18/2006	negative	negative	negative	negative
6/2/2006	negative	negative	negative	negative
6/9/2006	negative	negative	negative	negative
8/8/2006	negative	negative	negative	negative
8/16-17/2005	Subset from each tank distributed to 12 tanks with 3 abalone each			
10/10-10/15/2007	Temperature in subset increased to 18.5°C			
12/6/2006	All 12 subset tanks negative			
5/31/2007	Abalone in 12 subset tanks and tanks D, E, G, K remain healthy with no signs of WS			

Table 2. Clinical signs and morphological changes in white abalone from the source population at CIMRI. Data = mean±std. error

Date (n)	Body Shrinkage	PE WS-RLP	DG WS-RLP	DG Metaplasia	Foot Atrophy
10/29/2004 (30)	1.6±0.2	0.5±0.1	0.6±0.1	2.3±0.2	0.4±0.2
2/3/2005 (10)	0.5±0.2	0	0	2.6±0.2	0.6±0.2
7/1/2005 (10)	1.7±0.3	0	0.1±0.1	2.3±0.3	0.5±0.2
10/4/2005 (10)	2.3±0.2	1.2±0.3	0.7±0.2	2.1±0.4	0.7±0.2
4/12/2006 (10)	0.8±0.3	2.5±0.2	1.5±0.2	2.1±0.4	0.5±0.3
6/23/2006 (22)	n.d.	2.2±0.2	1.4±0.2	2.8±0.2	n.d.

The severity of metaplastic changes in the digestive gland seen at the beginning of this series was unprecedented. Over time, WS-RLP infection intensities decreased (following OTC treatment) and then gradually increased, presumably as the medication diminished. During the period in which WS-RLP infection intensities were reduced there was not a recovery from body shrinkage, digestive gland metaplastic changes and foot atrophy, which we had anticipated. Thus, OTC-mediated recovery from WS in white abalone appears to be a relatively slow process.

Broodstock Mortalities: Three white abalone broodstock mortalities occurred at CIMRI over the study period. A brief description of their examination is provided here.

Broodstock #1118, was observed to be weak and shrunken on 5/24/05 and was sent overnight to the CDFG Shellfish Health Laboratory. Upon arrival the abalone was observed to be severely shrunken (score = 3) and slightly necrotic, with an extensive shell-boring polychaete infestation *(Polydora* spp.*)*. Histologically, there was massive metaplasia of the digestive gland (score = 3) with an almost complete loss of functional tissue. No definitive WS-RLP inclusions were observed in the postesophagus or digestive gland although alterations in tissue architecture were suggestive of resolving lesions, and a postesophagus

tissue sample was PCR-positive for WS-RLP DNA. No kidney coccidian were present and the sex was indeterminate. It appeared likely that the changes in the digestive gland could result in dramatically reduced ability to process and absorb nutrients, substantially contributing to the death.

Broodstock #4017 was found dead on June 1, 2005 and portions of various tissues were placed in fixative by CIMRI and shipped to the CDFG Shellfish Health Laboratory. This abalone had no visible body shrinkage upon death and had a fully developed female gonad. CIMRI reported that there was a large number of boring organisms in the shell and an unusual large protrusion into the interior of the shell, which was sent to Dr. Carolyn Friedman for further examination. Following the death of Broodstock #1118, all remaining broodstock had been treated with OTC by injection (21mg/kg on May 14, 16, 18). By histology, moderate metaplastic changes and no WS-RLP inclusions were observed. It is likely that stress associated with OTC injection handling played a role in the death.

Broodstock #04-3 was discovered dead on September 26, 2005 and had been reported to be moderately shrunken for several months. This animal was collected from the wild in December 2004 and had been treated with the OTC injection in May 2005 as described in the paragraph above. Mild (score=1) WS-RLP infections were observed in the postesophagus and digestive gland while no metaplastic digestive gland changes were present.

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COMPONENT 4: Oxytetracycline Treatment and Withering Syndrome Protection
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Abstract

Research and captive rearing programs targeted to restore the endangered white abalone, *Haliotis sorenseni*, are being conducted in California and Washington state, USA. Captive rearing, while successful, has demonstrated that this species is highly susceptible to withering syndrome (WS), a rickettsial disease of abalone; WS has not been demonstrated in remnant wild white abalone populations. Thus, WS may limit white abalone production and supplementation of captive abalone must include measures to preclude the introduction of WS into wild populations. Oxytetracycline (OTC) is approved for use in aquaculture and

has been demonstrated to effectively reduce rickettsial loads, WS development and associated losses. White abalone were medicated at 90.82 mg/kg of OTC daily for 20 d and the efficacy, elimination and potential to protect against exposure to the WS rickettsia were examined. This study illustrated that OTC effectively eliminates rickettsial infections. High concentrations of OTC (1089 ppm) were observed in the digestive gland after medication; depletion occurred over a prolonged period providing protection to rickettsial challenge in abalone with a mean of over 72 ppm in this tissue. These data highlight the need for further optimization of this drug for use in both commercial and restoration aquaculture.

Experimental Methods

Experiment I: Efficacy of oxytetracycline (OTC) treatments via medicated feed in eliminating the RLP: In order to assess the ability of OTC delivered via medicated feed to treat RLP infections, changes in RLP burdens and associated histopathology in infected white abalones were assayed following OTC treatment according to Friedman et al. (2003). White abalone (mean size of 32.8 mm) naturally exposed to the RLP at ambient temperatures (14.1 $\pm$ 1.3^{o}C) were examined by histology to quantify the proportion of infected animals. Prior to treatment, moderate intensity RLP infections were observed in 25% (range = 5 - 46%) of the abalone sampled from the source tank at the Channel Islands Marine Research Institute (CIMRI, Port Hueneme, California, USA) where the captive rearing program for endangered white abalone is located. The population of experimental abalone at CIMRI (on-farm) was equally divided into six replicate tanks (n=1,000 each) and received flow through seawater at ambient temperatures of Southern California (mean = 14.1^{o}C). Animals in half of the tanks were fed the proprietary medicated diet (The Abalone Farm, Inc.) at a rate of 90.82 mg OTC/kg abalone body weight daily for 20d, while control abalone were fed fresh kelp (*Macrocystis pyrifera*) and dulse (*Palmaria mollis*) to satiation. After the 20d medication, all animals were fed the live macroalgae. At selected time points (withdrawal days 3, 18, 24, 40, 67, 80, 110, 129, 165, and 198) after medication, animals (n=3 per tank) were shipped to the University of Washington (UW, (Seattle, Washington, USA)) for PCR, histological and OTC analyses. At each sampling animals were weighed, and measured prior to analysis. At five of the time points (withdrawal days 24, 40, 67, 146

and 171 d post medication) an additional 22 abalone per tank were shipped to the UW for OTC protection challenges described in Experiment II below. The last day of medication was considered withdrawal day 0.

Statistical analyses. Analysis of covariance was used to test differences in OTC levels and depletion rates between DG and foot tissues with withdrawal day as the covariate. Model simplification was conducted using the Akaike information criterion as implemented in S-Plus (Insightful Corporation).

Experiment II: Prevention of RLP infections using oral oxytetracycline treatments

Given the apparent long-term protection of the OTC oral treatments in red abalone (Friedman et al. 2003, Braid et al. 2005), the lack of observation of RLP-infected wild white abalone, and the culture of this endangered species within the endemic zone of the RLP and WS, it was important to assess whether OTC pretreatment may reduce losses of white abalone destined for enhancement.

Animals: Two groups of white abalone were collected from CIMRI and transported to the Pathogen Quarantine Facility at the UW for use in this study. Abalone that measured a mean of 32.8 mm were exposed to the RLP via cohabitation and medicated with OTC as described above. Since unexposed white abalone in the same size class as the infected group were unavailable, a second group of abalone that measured 15.1 $\pm$ 3.50 mm and had never been exposed to the RLP or OTC were used as a positive control group in this experiment. Abalone were held in 12 x 13.5 in (D x H) plastic aquaria nd received aerated seawater that was collected from Puget Sound, Washington, USA. Water quality was maintained via bi-weekly water changes and continuous filtration with standard aquarium filters. Water quality tests were performed three times per week and additional water changes were performed as needed if excess ammonia or nitrites were detected. All abalone were fed *Palmaria mollis ad libitum*. Containers were checked daily for the presence of moribund (lethargic and weakly attached) animals, which were promptly removed and selected tissues (post-esophagus, digestive gland and foot muscle) were excised for PCR and histological analyses (Friedman et al. 1997, Friedman 2006).

Challenge methods. To test the hypothesis that OTC levels in the DG can protect animals from infection, we initiated the following study using abalone from Experiment I. A control group of animals fed only *Palmaria mollis* was also being used in this study. After medication on withdrawal days 24, 40, 67, 146 and 171 d post medication animals were acclimated to 19 $\pm$ 1^{o}C over a 24 hr period, a temperature known to promote RLP transmission and WS development (Friedman et al. 1997, Moore et al. 2000, Braid et al. 2005). After the one day acclimation period, animals were challenged with the RLP via 3 wk of cohabitation with RLP-infected red abalone. Of the 22 abalone received for experimentation from each medicated tank, 11 animals were placed in a tank and commingled with three RLP-infected red abalone (experimental treatment), while the remaining 11 animals were held separately without exposure to infected red abalone as negative controls. Groups of naïve white abalone (n=11) were also held with groups of medicated but unexposed white abalone (n=11) as negative controls. Groups of naïve white abalone (n=11) that did not receive OTC treatments were also exposed to infected red abalone via cohabitation as a positive control for RLP transmission. After each 3 wk exposure, red abalone were removed and white abalone were maintained for up to 60 additional days to allow development of RLP infections. Half of the surviving white abalone were sampled by PCR and histology at 30 d and the remaining half at 60 d after the 3 wk exposure period. Only preserved tissues from selected abalone (e.g. all PCR positive and at least three PCR negative samples per treatment) were processed for routine paraffin histology as a confirmation of RLP infections as described above.

Statistical analyses. Analysis of deviance with binomial errors (logit link) was used to test differences in infection prevalence among treatments in the re-challenge experiments, with OTC treatment, withdrawal day, incubation period (30 or 60d) and analysis (PCR or histology) as factors (S-Plus 7.0, Insightful). The Sidak T-test (Games 1977) was used to identify differences among factor levels. In a separate analysis, RLP prevalence within each treatment was weighted by relative percent infection (RPI) as some previously medicated negative control abalone were still RLP infected during the first three trials. The Pearson

Moment Correlation was used to examine if a relationship existed between mean OTC levels during exposure and RPI.

Results

Experiment I: Efficacy of per os *oxytetracycline (OTC) treatments in eliminating the RLP*

The 20 day OTC medication eliminated evidence of RLP infection beginning on sample day 24 until day 165 of the withdrawal period when PCR evidence of infection was observed (Figure 1). Although OTC levels peaked at 24 d post medication at 992 $\pm$ 303 ppm in the DG, foot muscle drug residues peaked at only 23.6 $\pm$ 2.5 ppm three days after cessation of medication. Significantly less OTC accumulated in foot muscle relative to digestive gland tissue (ANCOVA, $p<0.0001$; Figure 2). The respective rates of elimination were also significantly different (ANCOVA, $p<0.0005$); no tank effects were observed ($p>0.05$). The DG of animals medicated for 20 days fell below the FDA tolerance level for OTC of 2 ppm after a predicted 39 days (41 days measured) in the foot muscle, while the digestive gland values, predicted or observed, did not fall below this limit over the entire 198 days of the study (Figure 2).

Experiment II: Prevention of RLP infections using per os *oxytetracycline treatments*

Low levels of infection were observed in the experimental (medicated) abalone during the first two trials at withdrawal days 24 and 40 when OTC levels in the DG exceeded 400 ppm (Figures 2, 3). Significantly higher levels of infection were observed in experimentally challenged animals beginning on withdrawal day 146 ($p<0.05$) when mean DG OTC levels during RLP exposure fell to 37ppm (day 146 trial) and 23ppm (day 171 trial). No evidence of RLP infection was observed in re-challenged experimental abalone examined from the trial conducted on day 67 of the withdrawal period (30 d incubation period) when mean OTC residues of 51 ppm were observed in abalone during the 3 wk RLP exposure via cohabitation (Figures 2, 3). Although OTC medication and withdrawal period significantly influenced the level of infection ($p<0.0001$ for both), incubation period (30 or 60 d) was not a significant factor ($p>0.05$). A significant inverse correlation was observed between mean OTC level during the 3 wk cohabitation period and RPI of experimentally medicated

abalone (C= -0.544 & -0.897, $p<0.05$ and $p<0.001$, respectively, for post exposure analyses at 30d and 60d).

PCR evidence of RLP infection (presence of amplifiable RLP DNA) was confirmed in experimental, negative and positive control abalone using histology; however, fewer RLP infected abalone were observed upon microscopic examination than using PCR ($p<0.002$).

Conclusions

Collectively these data, combined with our evidence of protection from reinfection in abalone with relatively low amounts of OTC in the DG, suggest that further examination of lower OTC doses in abalone are needed to benefit both commercial and restoration abalone aquaculture. These data also suggest that administration of OTC as a protection against RLP infection may be a useful management tool for restoration of this endangered species. All California abalone farms, including CIMRI which is conducting captive rearing of white abalone, are within the WS endemic zone (Friedman and Finley 2003). The lack of RLP detection in the deep water remnant population tested to date (Moore et al. unpubl. data) combined with the high susceptibility of white abalone to WS (Moore et al. 2002, 2003) call for a mechanism to ensure that restoration efforts do not introduce the RLP into presumably uninfected wild populations.

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Figures

Figure 1. Analysis of oxytetracycline concentration in the digestive gland and proportion of infected animals from on-farm white abalone sampled during the withdrawal period. Values represent mean ± s.e. Note groups of abalone from these tanks were challenged on days 24, 40, 67, 146 and 171 of the withdrawal period.

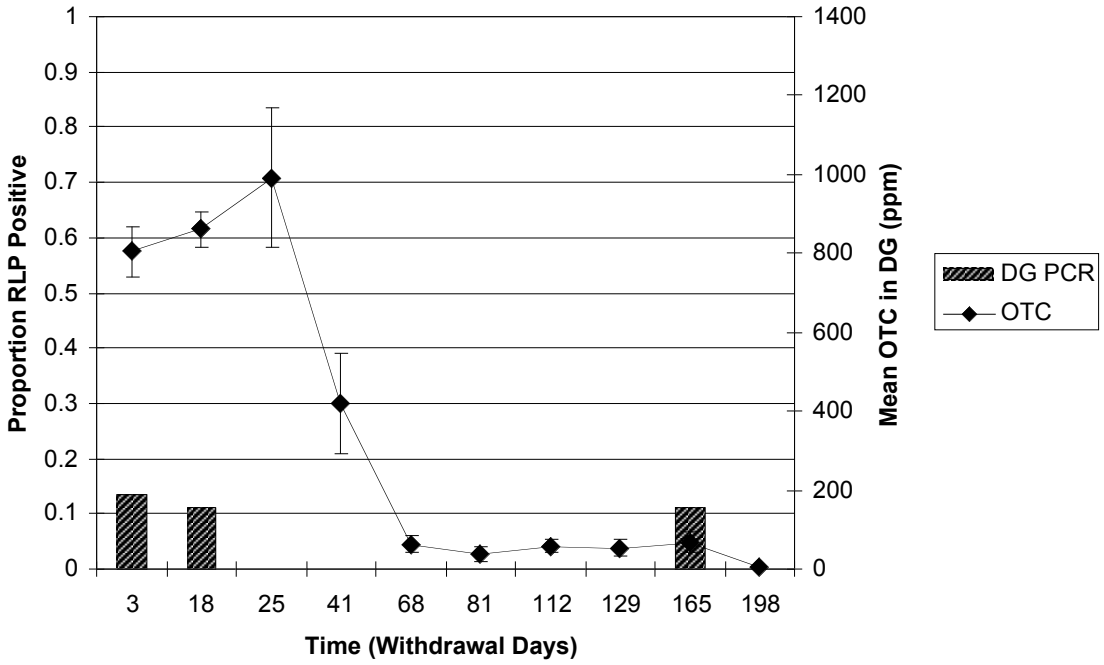
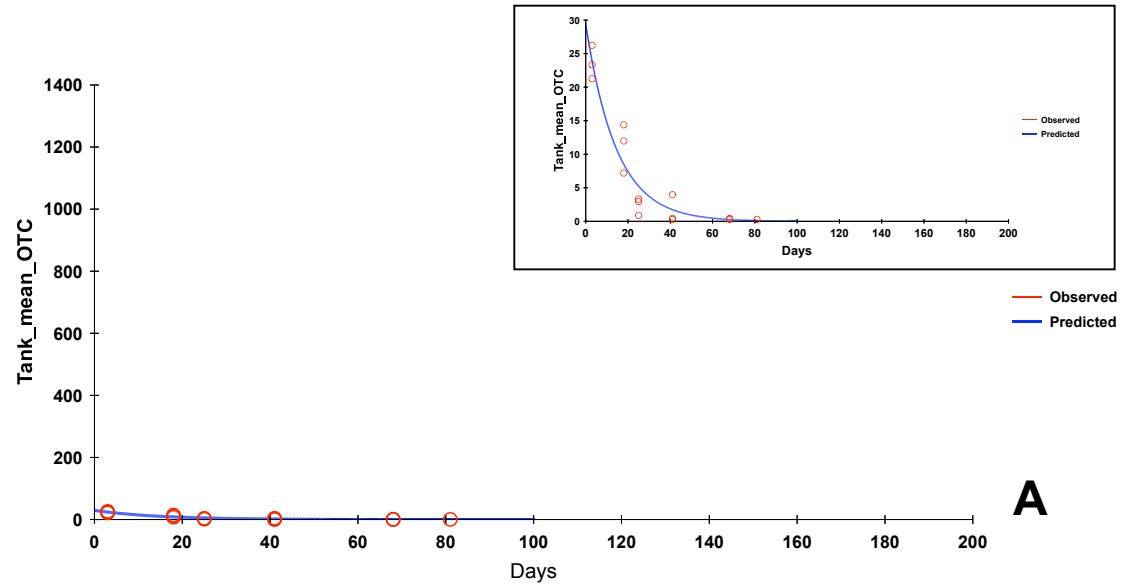


Figure 2. Observed and predicted log oxytetracycline levels in white abalone foot muscle (A) and digestive gland (B) during the withdrawal period. The inset illustrates the depletion kinetics of the foot using smaller axis scales.



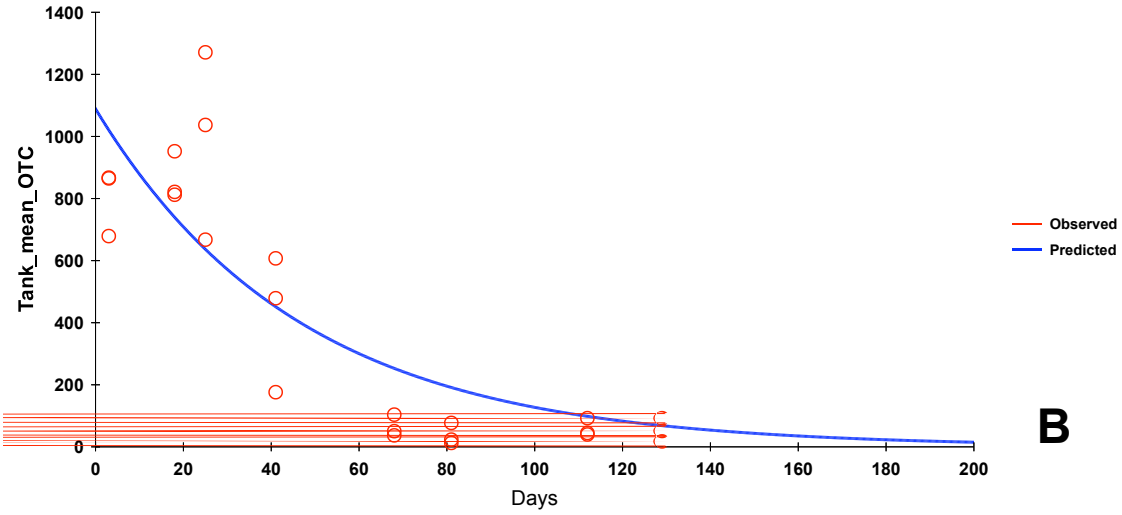


Figure 3. Proportion infected of medicated (light bars) and control (dark bars) abalone examined by PCR (30 and 60d exposures pooled) after a 3 wk exposure to the rickettsial pathogen via cohabitation. Bars represent treatment 95% confidence intervals.