*Journal of Shellfish Research*, Vol. 26, No. 3, 877–885, 2007.

# OXYTETRACYCLINE AS A TOOL TO MANAGE AND PREVENT LOSSES OF THE ENDANGERED WHITE ABALONE, *HALIOTIS SORENSENI*, CAUSED BY WITHERING SYNDROME

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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. Captive rearing, whereas 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 days 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 (1,089 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 commercial and restoration aquaculture.

***KEY WORDS:*** white abalone, *Haliotis sorenseni*, oxytetracycline, withering syndrome, rickettsial, endangered

## INTRODUCTION

Due to a variety of causes such as over fishing, environmental conditions, and disease, many abalone populations are globally in decline including the white abalone, *Haliotis sorenseni*. In the United States, *H. sorenseni* is the first marine invertebrate protected by the Endangered Species Act of 1973. Because of concern over the health of white abalone populations, a captive rearing program has been successfully implemented in southern California. Fishing pressure is believed to be a key force contributing to the observed decrease in *H. sorenseni* population abundance (Hobday et al. 2000). Although disease has not been implicated in the demise of *H. sorenseni*, withering syndrome (WS), a disease that is now endemic in California, has been shown to be an impediment to recovery of this species (Moore et al. 2002, 2003). In fact, WS has caused catastrophic losses of black abalone, *H. cracherodii*, in California (Haaker et al. 1992, Altstatt et al. 1996) and has impacted populations of green, *H. fulgens*, and pink, *H. corrugata*, in Mexico (Tinajero et al. 2002). WS has also contributed to severe losses of cultured red abalone, *H. rufescens*, in California (Moore et al. 2000).

White abalone are highly susceptible to WS (McCormick et al. unpubl. data). However, no evidence of WS exists in the remnant *Haliotis sorenseni* populations that are found in the very deep waters off southern California (~180 ft) (Hobday & Tegner 2002, Moore et al. 2002, Butler unpubl. data). Given that all abalone culture facilities, including those rearing white abalone destined for population recovery efforts, are found within the WS endemic zone (San Francisco, CA to Mexico, Friedman & Finley 2003), methods to control this disease are needed.

Withering syndrome is caused by an intracytoplasmic rickettsiales-like prokaryote (RLP), "*Candidatus* Xenohaliotis californiensis" that infects abalone gastrointestinal epithelia (Friedman et al. 2000) and disrupts the function of this organ leading to catabolism of the pedal muscle and death (Kismohandaka et al. 1993, Braid et al. 2005). One of the few antibiotics approved for use in aquaculture, oxytetracycline (OTC, Schnick 1998), has recently been shown to effectively reduce RLP infection, WS development, and associated losses in red abalone *via* medicated feed and in black abalone *via* intramuscular injection (Friedman et al. 2000, 2003). In addition, the digestive gland (DG, the secondary target organ of RLP infection; Friedman et al. 2000) concentrates the OTC, requiring over six months to deplete below the 2 ppm federal tolerance level (Schnick 1998, Friedman et al. 2003, Friedman et al. unpubl. data, Rosenblum 2006). The prolonged OTC retention may provide protection to challenge with the RLP. However, as culture of the RLP has not been possible because of a lack of continuous cell lines for marine invertebrates, alternative means to determine the minimum inhibitory concentration are needed. We developed OTC protection challenge cohabitation experiments to assess the ability of varying levels of the therapeutant in the DG to protect abalone from RLP exposure. Whereas previous studies have focused on red abalone, we focus here on demonstration of the efficacy of OTC delivered *via* a medicated diet to treat and prevent RLP infections, as well as characterization of the pharmacokinetics of this drug, in the endangered white abalone.

## METHODS

### *General Methods*

#### DNA Extractions

Because the postesophagus (PE) is the primary target organ of infection by the WS bacterium (Friedman et al. 2000), dissections were conducted to ensure that excision of this tissue

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was optimial for RLP detection by PCR, the main diagnostic tool selected to assess the efficacy of OTC medication. Therefore PE tissue for PCR was removed just posterior to the right kidney-digestive gland (DG) junction, a site where RLP infections are most prevalent (Friedman & Moore, unpubl. data). DNA isolation from PE and DG (a secondary target tissue for the RLP, Friedman et al. 2002) tissues was performed with a QiaAmp DNA Stool Mini Kit (Qiagen Inc., Valencia, CA) following the manufacturers instructions with the following modifications. Tissues were homogenized in 1-mL lysis buffer before initial heating at 70°C and the DNA was eluted with 100 μl of Buffer AE. DNA isolation from the feces was performed in the same manner except that initial homogenization of these pellets was not necessary. All extractions were stored at –20°C until further analysis.

#### PCR Amplification of rDNA

Amplification of RLP DNA was used as a proxy for RLP infection. A 160 bp fragment of the 16S rDNA of the RLP was amplified in 20 μL reactions containing 2 μL template, 1.6 U of *Taq* DNA polymerase in 1X Storage Buffer A (Promega, Madison, WI), 1.5 mM $MgCl_2$, 200 μM dNTP's, 400 ng/mL BSA, and 0.5 μM of primers RA 3–6 and RA 5–1 (Andree et al. 2000 as modified by Friedman 2006). All amplifications were performed in a Mastercycler Gradient thermal cycler (Eppendorf, Westbury, NY) using an initial denaturation step of 95°C for 5 min followed by 40 cycles of 1 min at 95°C, 30 sec at 62°C, and 30 sec at 72°C, and a final extension of 72°C for 10 min.

#### Histology

Because of the small size of the abalone in this study, numerous analyses to be conducted on each tissue and lower sensitivity of histology in detecting evidence of RLP infection than PCR detection of pathogen DNA (Rosenblum 2006), histology was only used to confirm presence of RLP infection in the PE and DG in selected animals. A minimum of three abalone from every treatment group (negative control, experimental, and positive control) in each of the five temporal trials from the 30 and 60 day incubation periods was processed for histology. In addition, tissues from all PCR positive animals and all experimental animals from the 30-day incubation period of each of the five trials were also examined using the methods of Friedman et al. (1997) outlined later. Briefly, a standard 2–3 mm cross section was excised to include PE, DG, and foot muscle from all moribund abalone and sampled abalone, preserved in Invertebrate Davidson's solution (Shaw & Battle 1957) for 24 h and stored in 70% ethanol until processing for routine paraffin histology. Deparaffinized 5-μm sections were stained with hematoxylin and eosin (Luna 1968) and viewed by light microscopy. Given the small size of abalone, histological results are only reported for abalone in which the main target tissues, PE and DG (Friedman et al. 2000), were visualized on the stained tissue section.

To compare histology results with the PCR test described earlier, we extracted DNA from the PE and DG tissues of 40 abalone exposed to the RLP (22–35 mm in maximum dimension) (Table 1) that were scored according to the following (0)–(3) histology scale estimating the number of rickettsial colonies per ×20 field of view: (0) no infection, (1) 1–10, (2) 11–100, and (3) >100 (Friedman et al. 1997). For negative controls, we also extracted DNA from the DG of 10 wild adult red abalone (>90 mm) from a location in California that has never been exposed to the RLP (Friedman & Finley 2003). PCR was performed on all 50 samples in triplicate.

**TABLE 1.**

**Number of individuals from each species tested by PCR with known exposure history in each of the four histology categories.**

| | Histology Scale | | | |
|---|---|---|---|---|
| **Abalone species** | **0** | **1** | **2** | **3** |
| *Haliotis rufescens* | 12 | 7 | 0 | 1 |
| *Haliotis fulgens* | 0 | 3 | 0 | 8 |
| *Haliotis discus hannai* | 8 | 0 | 0 | 0 |
| *Haliotis sorenseni* | 0 | 0 | 10 | 1 |

#### Analysis of Oxytetracycline Residues in Abalone Tissues

Oxytetracycline (OTC) levels in the DG and foot muscle samples were quantified using a FDA-approved method for examining drug residues (AOAC 1990), as modified by Friedman et al. (2003; in review). Briefly, foot muscle and digestive gland tissues were collected during sampling and stored at –80°C until assayed. Tissues were homogenized and centrifuged at ×200*g* for 10 min at 4°C. The supernatant was then tested for inhibition of bacterial (*Bacillus cereus* ATCC 11,778) growth relative to known OTC standards. Mean OTC levels during each 3-wk exposure to infected red abalone (*Haliotis rufescens*) were calculated by averaging the OTC concentration at the start and end of the exposure period.

#### Pharmacokinetic Data Analysis

Prior to analysis we eliminated sample points below the limit of detection (0.1 ppm) from foot muscle and DG tissue residue data. Raw data were analyzed as a function of time with one and two compartment models using the naïve-pooled data approach (Ette & Williams 2004) and WinNonlin 5.0 pharmacokinetic data fitting software (Pharsight Corp., Mountain View, CA). The equations for the one and two compartment models used are: (1) one-compartment model: $C_{(t)} = C_{zero}e^{-\beta t}$; (2) two-compartment model: $C_{(t)} = Ae^{-\alpha t} + Be^{-\beta t}$
where C is the OTC concentration in the tissue at time ($t$), $C_{zero}$ is the intercept of the one-compartment model elimination rate constant, $A$ is the $t_0$ intercept of the distribution phase, α is the distribution rate constant, $B$ is the $t_0$ intercept of the elimination phase and β is the terminal elimination rate constant. Selection of the best-fit model was based on the Akiake information criteria (AIC; Akaike 1974), and visual inspection of the residual plots and curve fit to the data. The elimination half-life ($t_{1/2}$) of OTC from the foot muscle and digestive gland was calculated as $t_{1/2} = 0.693/\beta$.

### *Experimental Methods*

#### Experiment I: Efficacy of Oxytetracycline Treatments *via* Medicated Feed in Eliminating the RLP

To assess the ability of efficacy of oxytetracycline (OTC) delivered *via* medicated feed to treat RLP infections, changes in RLP burdens and associated histopathology in infected white

abalones were assayed after OTC treatment according to Friedman et al. (2003) with the following modifications. White abalone (32.8 ± 8.66 mm) naturally exposed to the RLP at ambient temperatures (14.1°C ± 1.3°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% to 46%) of the abalone sampled from the source tank at the Channel Islands Marine Research Institute (CIMRI, Port Hueneme, CA) where the captive rearing program for endangered white abalone is located. The population ($N = 6{,}000$) 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°C). Animals in half of the tanks were fed the proprietary medicated diet (The Abalone Farm, Inc.) with 9% Terramycin (TM-100, Pfizer Inc.) (1.625% OTC) at a rate of 90.82 mg OTC/kg abalone body weight daily for 20 days, whereas control abalone were fed fresh kelp (*Macrocystis pyrifera*) and dulse (*Palmaria mollis*) to satiation. After the 20 days medication, all animals were fed the live macroalgae. Given the short duration of the study, our desire to mimic on-farm practices and low seawater temperature of ~14°C used in this study, a temperature shown to not facilitate RLP transmission or replication (Braid et al. 2005, Moore et al. 2000), using kelp to feed control abalone was deemed acceptable. 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, WA, 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 days post medication) an additional 22 abalone per tank were shipped to the UW for OTC protection challenges described in Experiment II later. 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 ± 8.66 mm were exposed to the RLP and medicated with OTC as described earlier. Because unexposed white abalone in the same size class as the infected group were unavailable, a second group of abalone that measured 15.1 ± 3.50 mm and had never been exposed to the RLP or OTC were used as a positive control group in this experiment. Because abalone as small as 3 mm can be infected by the RLP (Altstatt et al. 1996, Moore et al. 2002), use of smaller abalone as positive controls for the presence of infectious RLP in this experiment was deemed acceptable. Abalone were held in 12 × 13.5 in (D × H) plastic storage aquaria (Consolidated Plastics, Twinsburg, OH) and received aerated seawater that was collected from Puget Sound, WA. Water quality was maintained *via* biweekly 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; moribund (lethargic and weakly attached) animals were promptly removed and selected tissues (postesophagus, digestive gland, and foot muscle) were excised for PCR and histological analyses.

*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. On completion of the medication on withdrawal days 24, 40, 67, 146, and 171 days post medication animals were acclimated to 19°C ± 1°C over a 24 h 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 weeks of cohabitation with RLP-infected red abalone (75–100 mm), a known method of RLP transmission (Moore et al. 2001, Friedman et al. 2002, Braid et al. 2005). 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), whereas 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 (Table 2). 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 days

**TABLE 2.**

**Tank design for Experiment II: oxytetracycline protection challenges.**

| Tanks (3 each) | White Abalone | | Red Abalone |
|---|---|---|---|
| | Medicated | Non-medicated | RLP Infected |
| Experimental | $n = 11$ | $n = 0$ | $n = 3$ |
| Positive Control | $n = 0$ | $n = 11$ | $n = 3$ |
| Negative Control | $n = 11$ | $n = 11$ | $n = 0$ |

and the remaining half at 60 days after the 3 wk exposure period. Because of the small size of the abalone and difficulty in obtaining adequate amounts of each desired digestive tissue (PE and DG) for all three tests (PCR, OTC, and histology), 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 earlier.

To ensure RLP exposure, fecal samples were collected prior to the removal of the red abalone by pipetting debris off the bottom of the tanks and centrifuging into a pellet. The DNA was then extracted and tested for RLP as described previously.

*Statistical analyses*

Analysis of deviance with binomial errors (logit link) was used to test differences in infection prevalence among treatments in the rechallenge experiments, with OTC treatment, withdrawal day, incubation period (30 or 60 days), 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

### *Histology*

Negative control wild adult red abalone from California showed no histological or PCR evidence of RLP infection (Fig. 1). Of those samples exposed to the RLP but scored as (0) or noninfected according to histology, 30% were PCR positive for the RLP. All samples that scored *via* histology as (1) lightly infected; (2) moderately infected; or (3) heavily infected were PCR positive for RLP (Fig. 1).

### 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 (Fig. 2). Elimination curves for digestive gland and foot muscle were best described with a one-compartment model (Table 3). Individual animal data points and the predicted concentrations of OTC using a one-compartment model for foot muscle and digestive gland after oral medication with 90.82 mg OTC/kg body weight daily for 20 days are illustrated in Figure 3. Although OTC levels peaked at 24 days post medication at 992 ± 303 ppm in the DG, foot muscle drug residues peaked at only 23.6 ± 2.5 ppm three days after cessation of medication. Significantly less OTC accumulated in foot muscle relative to digestive gland tissue (ANCOVA, $P < 0.0001$; Fig. 3, Table 3). The respective rates of elimination were also significantly different (ANCOVA, $P < 0.0005$); no tank effects were observed ($P > 0.05$). The rate constants of elimination, the corresponding half-life of elimination and the predicted concentration at time = 0 ($C_{zero}$) are shown in Table 3 for both foot muscle and digestive gland. 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, whereas the digestive gland values, predicted or observed, did not fall below this limit over the entire 198 days of the study (Fig. 3).

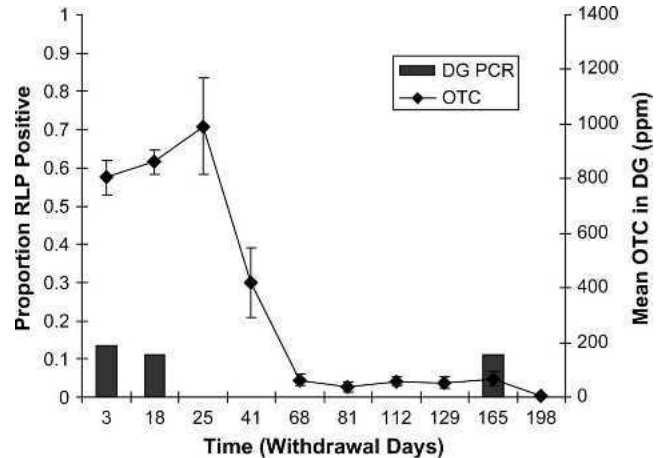


**Figure 2. Analysis of oxytetracycline concentration in the digestive gland and proportion of rickettsia-infected animals from on-farm white abalone sampled during the withdrawal period. Values represent mean ± SE. Note groups of abalone from these tanks were challenged on days 24, 40, 67, 146, and 171 of the withdrawal period.**

### Experiment II: Prevention of RLP Infections Using *Per Os* Oxytetracycline Treatments

Evidence of RLP exposure was provided by PCR examination of feces during the 3 wk exposure period. Positive PCR reactions were observed by examination of triplicate fecal

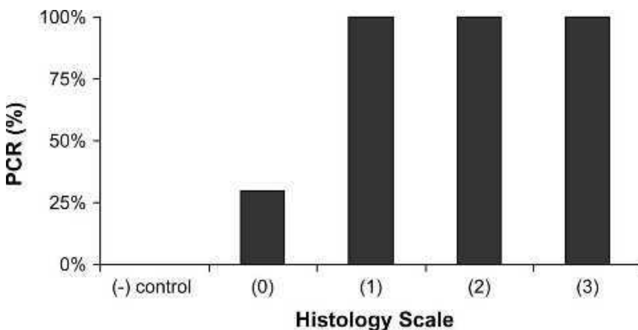


**Figure 1. Percent of individuals positive for the withering syndrome rickettsia based on the 0–3 histology scale of Friedman et al. (1997).**

**TABLE 3.**

**Pharmacokinetic parameters for foot muscle and digestive gland as determined by a one-compartment model.**

| Tissue | $C_{zero}$ (ppm) | Equation $C_{(t)}$ = | β | $t_{1/2}$ (days) | $r^2$ |
|---|---|---|---|---|---|
| DG | 1089 | $1089e^{-0.021t}$ | 0.021 | 32.3 | 0.88 |
| Foot | 29.4 | $10.4e^{-0.069t}$ | 0.069 | 10.1 | 0.96 |

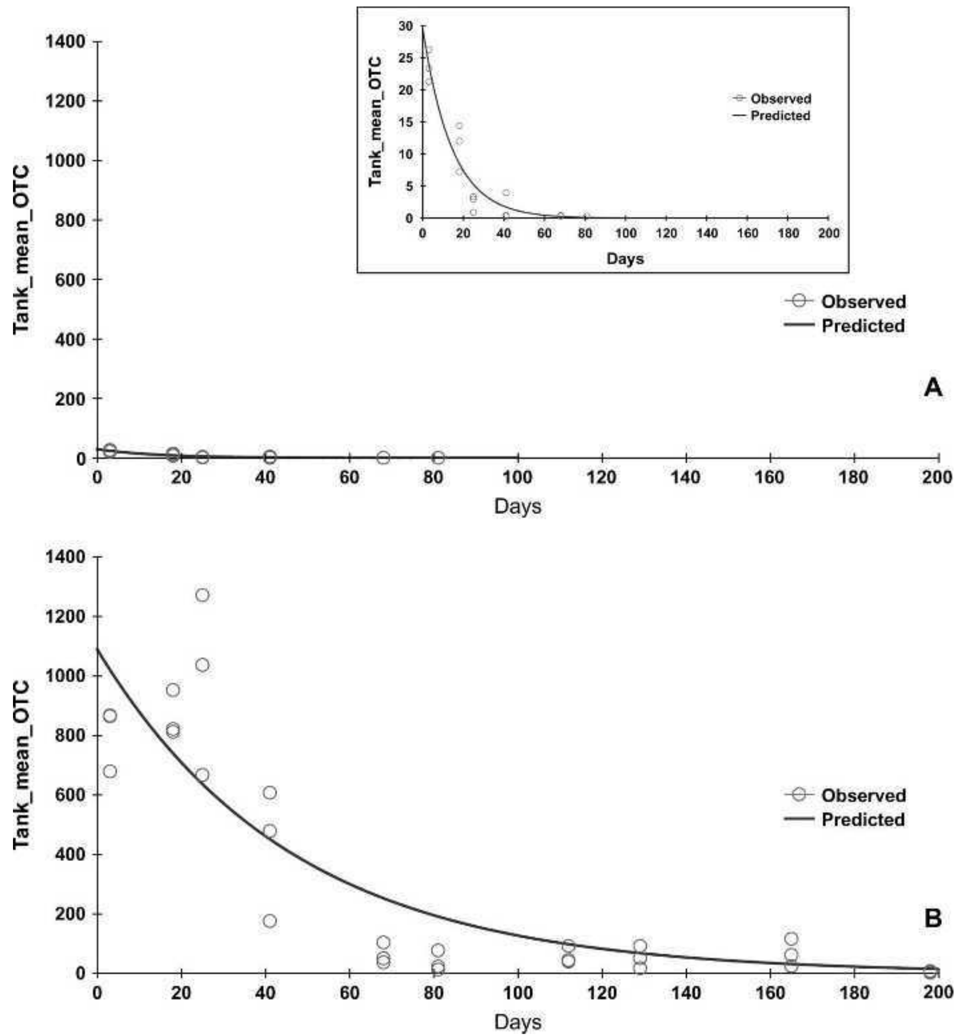


**Figure 3. Observed and predicted log oxytetracycline levels in white abalone foot muscle (A) and digestive gland (B) during the withdrawal period. The inset illustrates the depletion kinetics of the foot using smaller axis scales.**

samples (one per tank amplified in duplicate) during exposure to RLP-infected red abalone in all experimental and positive control tanks. No evidence of RLP exposure was observed in the negative control tanks, with the exception of one PCR positive amplification from a single tank during trials 1 and 2 on days 24 and 40 of the withdrawal period.

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 (Figs. 2–5). Significantly elevated 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 37 ppm (day 146 trial) and 23 ppm (day 171 trial) (Fig. 4). No evidence of RLP infection was observed in rechallenged experimental abalone examined from the trial conducted on day 67 of the withdrawal period (30 days incubation period) when mean OTC residues of 51 ppm were observed in abalone during the 3 wk RLP exposure *via* cohabitation (Fig. 4). At the start of the day 67 trial OTC levels in the DG ranged from 37–104 ppm with a mean of 64 ppm; at the end of the 3-wk challenge period DG OTC levels ranged from 13–77 ppm and a mean of 38 ppm (Fig. 3b). DG tissues of abalone examined 60 days after exposure in the third trial on day 67 became contaminated and were excluded from analysis. Although OTC medication and withdrawal period significantly influenced the level of infection ($P < 0.0001$ for both), incubation period (30 or 60 days) 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 30 days and 60 days).

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 during microscopic examination than using PCR ($P < 0.002$; Figure 5). When examining tissues from animals that were analyzed by PCR and histology (with both PE and DG present in the sample), 97% of samples that were histology positive for the RLP were also PCR positive ($n = 39$), whereas histological confirmation of PCR results was observed in only 30% of the samples analyzed by both methods ($n = 148$). The single animal that was considered RLP positive by histology but PCR negative had a light, focal infection

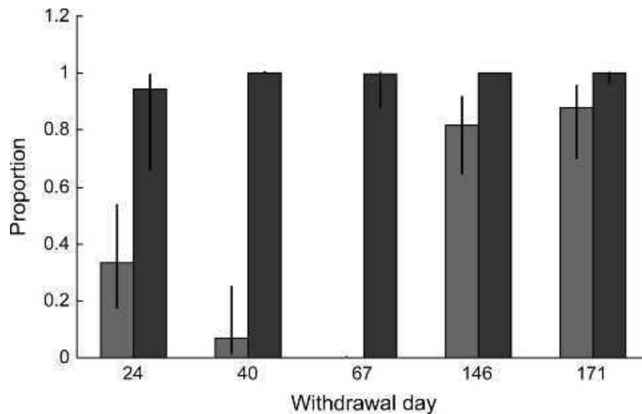


**Figure 4. Proportion infected of medicated (light bars) and control (dark bars) abalone examined by PCR (30 and 60 days exposures pooled) after a 3-wk exposure to the rickettsial pathogen *via* cohabitation. Bars represent treatment 95% confidence intervals.**

(histology score of 1). In addition, histology data from 22 abalone (14 positive control, 2 negative control and 6 experimental animals) were not included in the analysis because PE and DG were not observed on the stained tissue section.

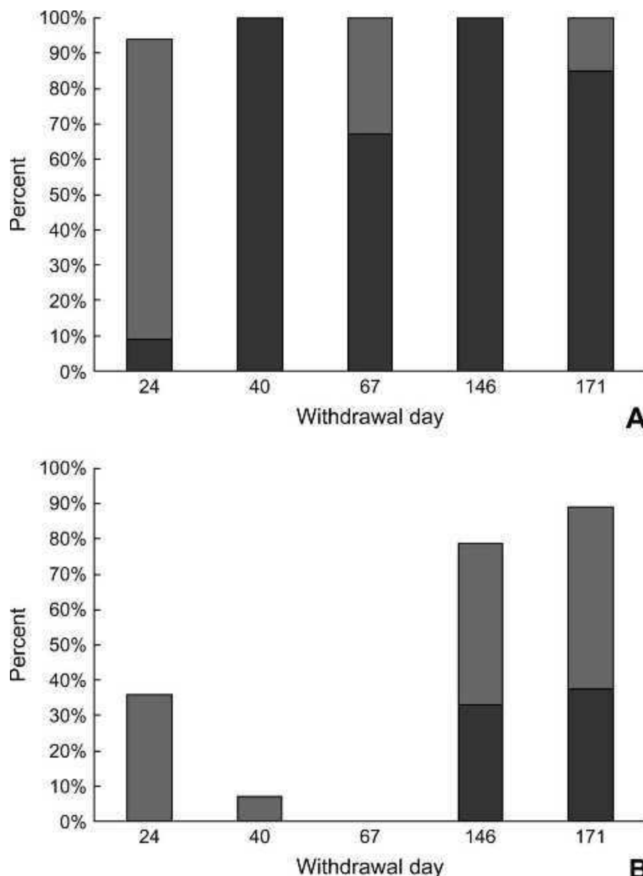


**Figure 5. Percent detection of infection *via* PCR and histology of positive control (A) and medicated (B) abalone after a 3-wk exposure to the rickettsial pathogen *via* cohabitation. Total bar height represents PCR data; detection *via* histology is represented by the dark portion of each bar. When PCR and histology results were identical, bars are dark.**

## DISCUSSION

### *Pharmacokinetics*

Pharmacokinetic trends of OTC in white abalone followed patterns established for red abalone at ambient temperatures (Friedman et al. 2003, Rosenblum 2006). In both species, digestive gland concentrations of OTC exceeded the United States federal tolerance level of 2 ppm (FDA 1998) for five months or more, whereas foot muscle OTC concentrations depleted to below 2 ppm within about 6 wk (the present study, Friedman et al. 2003 and unpubl. data, Rosenblum 2006). Like many aquatic species studied to date, OTC elimination was best described by a one-compartment model (Chen et al. 2004, Wang et al. 2004) and digestive gland (or liver) OTC levels exceeded those in muscle at all sampling periods (e.g., Rigos et al. 2002, Chen et al. 2004, Wang et al. 2004, Friedman et al. unpubl. data, Rosenblum 2006). Foot muscle OTC uptake and elimination rates were similar to all previous studies using red abalone; typically mean foot $C_{zero}$ peaked at 10–25 ppm (Friedman et al. 2003, Rosenblum 2006), similar to that observed in our study. Interestingly, when red abalone were medicated at a rate of 103.4 mg/kg for 20 days, peak concentrations ($C_{zero}$) of OTC in the DG varied between studies and ranged from a low of 928 ppm (Rosenblum 2006) to a high of 2,710 ppm (Friedman et al. unpubl. data). Despite this variation in OTC accumulation, a long $t_{1/2}$ was observed in white abalone (32.3 days) that is similar to that calculated for red abalone (22.4–27.5 days, Rosenblum 2006, Friedman et al. in review) and sea urchins (24.6 days, Campbell et al. 2001). The red abalone experiments by Friedman et al. (2003 and unpubl. data) were conducted at 15.7°C and 13.4°C, respectively, similar to the 14.1°C temperature used on-farm in the current study. Our observation of $C_{zero}$ at 1,089 ppm is more similar to that observed by Rosenblum (2006) whose study was conducted at 17.2°C. Whereas the influence of temperature on OTC accumulation in fish species varies (Haug & Hals 2000, Rigos et al. 2002, Chen et al. 2004) and its depeletion kinetics are typically directly temperature dependent, our current study and previous trials with abalone have shown no consistent effect of temperature on OTC accumulation. Terminal elimination rates for white abalone tissues (Table 3) are similar to those reported previously for red abalone; foot muscle (0.069) exceeded those of the DG (0.021) reflecting a more rapid elimination of OTC from pedal muscle than DG (Rosenblum 2006, Friedman et al. unpubl. data). The elevated DG retention has been attributed to the presence of di-valent cations, lipids, and, possibly, an acidic environment in this organ relative to foot muscle (Friedman et al. 2003, unpubl. data, Rosenblum 2006).

### *Efficacy of Oxytetracycline to Reduce RLP Infections*

Our efficacy study illustrates that short-term administration of OTC *via* medicated feed is an effective treatment for the RLP in white abalone. Medicated abalone remained free of the RLP for a prolonged period at the farm (days 24–129), whereas control abalone remained RLP-infected. As in previous studies using red abalone, approximately one month after cessation of medication, the RLP was effectively eliminated from host tissues *via* oral OTC administration (Friedman et al. 2003, Friedman et al. unpubl. data, Rosenblum 2006). In these studies

using red abalone, efficacy of the treatment lasted up to 1 y after medication (Friedman et al. 2003, Friedman et al. unpubl. data, Rosenblum 2006).

Red and white abalones medicated with the bacteriostatic antibiotic OTC appear to need approximately 1 mo to purge themselves of RLP infection. In the present study, evidence of RLP infection was observed in the first two sampling periods (days 3 and 18 of the withdrawal period) when animals contained ≥800 ppm in the DG. By the third sampling period (day 24) when abalone contained >900 ppm OTC in the DG, no RLP evidence of infection was observed; however, some previously medicated negative control animals remained infected as evidenced by PCR analysis of fecal samples and the presence of low level infections in some negative control animals in Experiment II. In a study with red abalone, reductions in RLP prevalence were observed as early as 5 days after a 14 day medication of 143.6 mg/kg ($P < 0.01$; 33% in medicated *versus* 87% in control abalone), yet even fewer previously medicated abalone were infected with the RLP than nonmedicated controls 329 days after medication ($P < 0.001$; 0.03% *versus* 63%, respectively, Friedman et al. 2003). Collectively, these studies suggest that the efficacy of OTC cannot be measured until 3–4 wk after medication to allow the host to purge RLP infections from gastrointestinal epithelia.

#### *Minimum Effective Dose*

The RLP challenge trials (Experiment II) provide the first data illustrating the minimum effective concentration of OTC needed in the DG during a RLP challenge is >50 ppm. Treatment of Gram negative bacteria such as *Vibrio* and *Pasturella* have a minimum inhibitory concentration (MIC) of 0.10–12.5 ppm and up to 32 ppm in shrimp and finfish, respectively (Takahashi et al. 1985, Catry et al. 2006) and thereby require OTC doses far lower than observed in the present study. However, other researchers have suggested using four times the MIC for treating bacterial infections in shrimp (Bermudez-Almada et al. 1999), a dose similar to that observed in our study. It is important to note that MIC values for culturable bacteria may not be directly comparable to values calculated *in vivo* as in the present study. In addition, rickettsial bacteria, whereas susceptible to OTC, often require higher therapeutic doses of OTC than other antirickettsial compounds (Coetzee et al. 2005).

OTC in the DG protected abalone from reinfection with the RLP. Only when OTC levels in abalone DG fell below 100 ppm at the start of an exposure trial and had a mean <51 ppm OTC during the entire exposure period, did white abalone become susceptible to infection by the RLP. Rosenblum (2006) found reduced susceptibility of red abalone to RLP infection for up to 88 days after OTC medication when digestive gland OTC residues contained 9–60 ppm OTC; however, no experimental abalone containing >60 ppm were included in his study. As in the current study, Rosenblum (2006) identified more abalone as RLP-positive by PCR than by histology, a common observation when comparing these two methods (Carnegie et al. 2003). In fact, when Rosenblum (2006) examined OTC treatment in red abalone, significant differences in RLP prevalence between medicated and control abalone were only found using PCR data. Thus, current practices of medicating abalone for 10–20 days exceed the minimum effective dose. If the target accumulation of OTC in the DG is only 50–100 ppm for effective treatment, shorter duration treatments and lower OTC doses need to be investigated. A shorter duration, effective treatment would result in the accumulation of a lower amount of the therapeutant within abalone tissues and would reduce the currently prolonged depuration period needed to allow the DG to deplete to below the federal tolerance level of 2 ppm (FDA 1998, Schnick 1998). A shorter withdrawal period would be a distinct advantage for farmers who could more rapidly obtain harvest authorization after treatment. In addition, many countries (e.g., in the European Union) have a stricter tolerance level for OTC in milk and animal flesh of 0.1 ppm (EMEA 1990), which further necessitates faster depletion of this drug from tissues, should a farm want to export to countries with tolerance levels equal to or less than those imposed by the United States. In addition, judicious administration of OTC is also imperative to avoid the development of resistance to this drug in target and other bacteria (Chopra & Roberts 2001).

#### *Histology Versus Polymerase Chain Reaction*

Results from the studies previously provided evidence that PCR is a more sensitive tool in revealing evidence of RLP presence in abalone. Of all histology-PCR assessments for RLP presence, only tissue from one very lightly infected animal (histology score of 1) was scored as histology positive and PCR negative and the focal nature of early RLP infections likely accounts for this observation. In both groups of animals tested in the present study, more animals contained evidence of RLP presence based on PCR than histology. Carnegie et al. (2003) also found that PCR was more sensitive than histology in detecting the protistan parasite, *Mikrocytos mackini*. In addition, Rosenblum (2006) using the same PCR test used in the present study, also found significantly more animals were PCR than histology positive for the RLP. Because PCR detects presence of amplifiable DNA and not whether an animal is truly infected, the potential that rickettsial DNA and viable or nonviable bacteria were within or on the animals tested must be considered; such presence could suggest that an animal was exposed or yield false evidence of an active infection. Characterization of the sensitivity and accuracy of these two diagnostic tests is needed. Whereas the PCR test is clearly a viable diagnostic tool because of increased sensitivity, lower expense, and greater speed, histology tests provide valuable and complementary confirmation of PCR positive specimens.

## CONCLUSION

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 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 & 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. 2003)

call for a mechanism to ensure that restoration efforts do not introduce the RLP into presumably uninfected wild populations.

## ACKNOWLEDGMENTS

The authors thank Pharsight Corp. who donated Win NonLin 5.0 for pharmacokinetic analyses, Dr Graham Young, Nathan Wight, and two anonymous reviewers for their editorial comments. This research was funded, in part, by a grant from the National Sea Grant College Program, National Oceanic and Atmospheric Administration, United States Department of Commerce, under grant number NA04OAR4170038, project numbers R/F196D and R/A-122B, through the California Sea Grant College, the School of Aquatic and Fishery Sciences at the University of Washington and the California Department of Fish and Game. 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 United States government is authorized to reproduce and distribute this paper for governmental purposes.

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