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TRANSMISSION OF WITHERING SYNDROME IN BLACK ABALONE, *HALIOTIS CRACHERODII* LEACH

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ABSTRACT Withering syndrome (WS) has been associated with catastrophic declines in black abalone populations in southern and central California. In an effort to identify the etiological agent of WS and to characterize the progression of this disease, we initiated a transmission study in which abalone from Ano Nuevo Island, a location free of WS, shared aquaria with animals from Vandenberg Airforce Base, a location where WS is epizootic. The mean incubation period of WS (time to develop overt signs of the disease) was 245 days with a mean time to death after development of clinical signs of 42 days. Median time to death was 41 wk in the experimentally exposed Ano Nuevo Island abalone and 16 wk in the positive control Vandenberg abalone. Cumulative mortality was significantly different between the negative control (unexposed) Ano Nuevo Island abalone (25% mortality) and both the exposed Ano Nuevo Island abalone (85% mortality; $P = 0.0001$) and the positive control Vandenberg abalone (100% mortality; $P = 0.0001$). In addition, significant differences in prevalences of a recently described Rickettsiales-like procaryote (RLP), "*Candidatus* Xenohaliotis californiensis," were observed between negative control animals (no RLPs) and those with WS (both the experimentally exposed Ano Nuevo Island and Vandenberg abalone were infected with RLPs; $P < 0.001$). All abalone infected with the RLP had signs of WS, including decreased condition indices, foot muscle atrophy, and digestive gland degeneration ($P < 0.05$). No correlation between intensity of RLP infection and degree of WS was observed ($P > 0.05$), suggesting a complex relationship between the RLP and clinical disease in black abalone. Despite this, these data in conjunction with a lack of observation of any other significant pathogens in the abalone provides evidence that the RLP infecting abalone ("*Candidatus* Xenohaliotis californiensis") is the etiological agent of WS.

KEY WORDS: withering syndrome, black abalone, *Haliotis*, rickettsiales, "*Candidatus* Xenohaliotis californiensis"

INTRODUCTION

Withering syndrome (WS) has been associated with catastrophic declines in black abalone populations in southern and central California (Haaker et al. 1992; Steinbeck et al. 1992, Friedman and Haaker unpublished data). Initial studies identified a previously undescribed coccidian parasite, *Margolisiella* (= *Pseudoklossia*) *haliotis* (Friedman 1991; Friedman et al. 1995, Desser & Bower 1997), in black abalone with WS that was subsequently determined to be nonpathogenic as evidenced by field and laboratory studies (Friedman et al. 1993, 1997). VanBlaricom et al. (1993) documented WS on San Nicolas Island in April of 1992. These researchers a observed Rickettsiales-like procaryote (RLP) that was recently described as a new taxon and has been given the provisional status of "*Candidatus* Xenohaliotis californiensis" (Friedman et al. 2000). Two of six abalone with clinical WS harbored RLPs, whereas apparently healthy animals were devoid of the RLP. The authors indicated that Rickettsiales-like bacteria were commonly observed in marine invertebrates and that the pathogenicity of these organisms was unknown. Gardner et al. (1995) also observed RLPs in association with WS in black abalone from San Nicolas and San Clemente Islands in southern California. Healthy abalone from Ano Nuevo Island in central California were not infected with RLPs, suggesting an association between the RLP and WS. Friedman et al. (1997) examined the association between the RLP, degeneration of the digestive gland, and mortality in a laboratory study. No clear associations between intensity of RLP infection and either condition of the digestive gland or time to mortality were observed. Recently, Moore et al. (2000) observed a significant relationship between the intensity of RLP infection and degree of WS in cultured red abalone. These conflicting data indicate further examination of the role of the RLP in WS is warranted. This study was designed to examine the transmissibility of WS and to determine the relationship between RLP infection and WS in black abalone.

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MATERIALS AND METHODS

Animals

Healthy black abalone were collected on March 28, 1995 from Ano Nuevo Island, where WS had never been observed. Black abalone with WS were collected from Vandenberg Airforce Base (Vandenberg) and Cayucos on April 24, 1995. Abalone were transported to the Pathology Quarantine Facility at the Bodega Marine Laboratory, where they were placed in an 88-L aquaria and received ambient (8–10°C), flow-through, full-strength seawater. *Macrocystis pyrifera* was collected from Bodega Bay and was surface sterilized by soaking in a tamed iodine solution (Prepodyne: Westagro, Kansas City, MO) for 15 min followed by a freshwater rinse. Animals were fed *M. pyrifera* twice per week. All abalone were tagged and the following data were collected: maximum length, foot length and total volume (TV), and total weight (TW). Animals were bled from the pallial sinus with a tuberculin syringe and a 26-gauge, 0.5-inch needle and the density, cell-type, and condition of circulating hemocytes was determined using a hemocytometer. Visual condition of the abalone was assessed according to the following scale: (3): healthy abalone with a foot and viscera that fills the entire shell volume; (2): visible mantle retraction and moderate atrophy of the foot muscle; and (1): severe atrophy of the foot muscle.

Histology

Selected tissues were placed in Invertebrate Davidson's solution (Shaw & Battle 1957) for 24 h and processed for routine paraffin histology. Deparaffinized 5-μm sections were stained with hematoxylin and eosin (Luna 1968) and viewed by light microscopy. The intensity of RLP infection was quantified using the following logarithmic scale at 200× magnification: (0): no bacterial foci; (1): 1–10 foci per field; (2): 11–100 foci per field; and (3): >100 foci per field (Friedman et al. 1997). Infection intensity was quantified in both the postesophagus (PE) and digestive gland (DG), and an overall infection intensity was calculated by summing the intensity in the PE and DG (range of 0–6 possible) (Moore et al. 2000). Intensities were examined according to tissue type to determine whether the location of infection was correlated with animal health. Unless otherwise specified, the term RLP infection refers to overall infection intensity. Condition of the digestive gland and foot muscle were assessed using the (1)–(3) scales of Friedman et al. (1997), in which normal was scored as (3), moderate (up to 30%) alteration from normal was scored as (2), and tissue that was severely (>30%) altered was scored as (1). Three specific morphologic changes that characterized observed alterations in digestive gland architecture were individually scored according to the following (1)–(3) scale: (1) normal architecture; (2) moderate (up to 25%) degeneration (characterized by an increase in connective tissue between digestive tubules, the primary tissues responsible for secretion of digestive enzymes and nutrient absorption in abalone) (Voltzow 1994), transport duct metaplasia, or inflammation; and (3) abundant (>25%) transport duct metaplasia, an increase in connective tissues between degenerating tubules, or inflammation.

Transmission Experiment

Groups of 12 abalone from Ano Nuevo Island were randomly placed in each of two negative control (NC) and two experimental aquaria (EA). Groups of 12 abalone with WS (EWS) were randomly added to each of the two experimental aquaria and to each of the two positive control aquaria (PC) (Fig. 1). Animals were maintained on ambient seawater for the first 3 mo of the study. During this time temperatures ranged between 8–10°C ($\overline{X}$ = 9.53°C) for the first 4 wk, 11–15°C ($\overline{X}$ = 12.89°C) for the following 4 wk and 10.5–15°C ($\overline{X}$ = 12.47°C) for the third month. After this time, the animals were acclimated over a 2-wk period to 18 ± 1°C, the temperature at which the abalone were maintained for the remaining 34 wk of the 46-wk study. Physical measurements and hemocyte counts were assessed approximately every 8 wk over the course of the experiment. All moribund abalone or mortalities were sampled as above, including shell weight (SW) and shell volume (SV), and selected tissues (foot, digestive gland, PE, and kidneys) were processed for histology. The intensity of RLP infection and condition of the digestive gland and foot muscle were quantified as described above. The condition of the abalone was also assessed upon death using the body weight condition index of Friedman et al. (1997) = [(TW – SW)/TW]. In addition, the percentage of live tissue volume relative to the entire volume of the animal was determined = [(TV – SV)/TV].

Statistical Analysis

The Fisher's exact test was used to test the independence of exposure to WS and mortality; abalone were grouped as exposed or unexposed and as alive or dead. Chi square contingency table analysis (X^2) was used to test independence between exposure to WS and measured health parameters. Abalone were grouped as exposed (laboratory or field exposed) and unexposed. The following health parameters or responses were grouped as normal (scores of 3 for animal condition and that of the digestive gland and foot and 0 for RLP presence), whereas those with signs of WS and RLP infection were grouped as abnormal. Observed versus expected frequencies in each category were compared using 2 × 2 contingency table analyses. The Fisher's Exact test was used when fewer than five observations were observed in any cells. These analyses

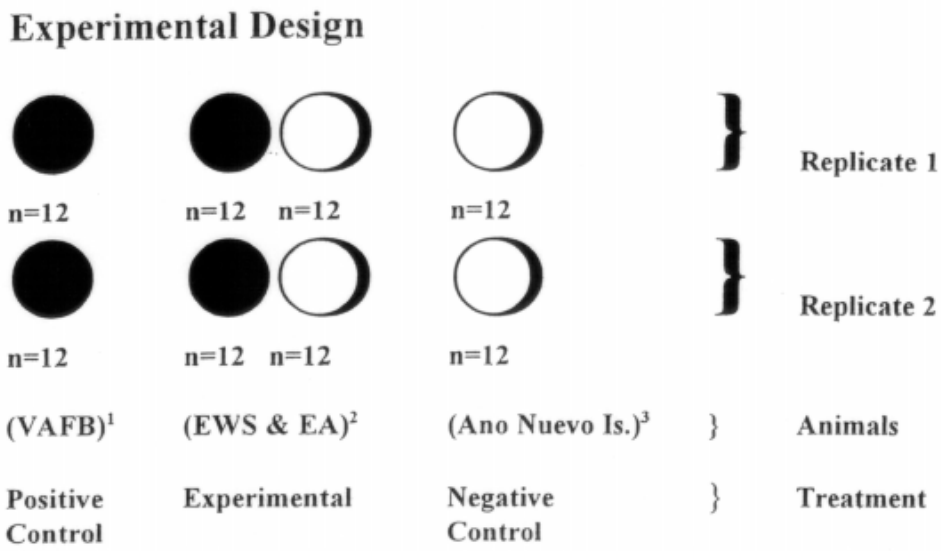

Figure 1. Experimental Design. The dark circles represent black abalone with WS collected from the field (Vandenberg Airforce Base or Cayucos (VBCS)). The open circles represent healthy, naive (no exposure to WS before study) black abalone from Ano Nuevo Island. [1]Animals with WS collected from Vandenberg Airforce Base and Cayucos in the positive control treatments. [2]Animals from VBCS (EWS) or Ano Nuevo Island (EA) with and without WS, respectively, in experimental treatments. [3]Animals in the negative control treatments without WS that were collected from Ano Nuevo Island.

were also used to test the independence of RLP infection and WS. Animals were grouped as infected and uninfected and as above for survival and health parameters. In a separate analysis to further assess the independence of specific lesions and RLP infection intensity animals were grouped as low overall infection levels (0–3) and high overall infection levels (4–6) and the response (e.g., metaplasia) was grouped as present or absent. Spearman rank correlation coefficients were calculated and tested for a (linear) relationship between intensity of RLP infection and condition of the digestive gland and foot, condition indices, density of circulating hemocytes, cumulative mortality, and time of exposure. Stepwise forward and backward regression models were used to predict the intensity of RLP infection in exposed abalone from the following variables: condition of the digestive gland and foot, weight condition index, visual condition assessment, and duration of exposure. Multiple logistic regression analysis was used to predict presence of RLP infection by using combinations of the five independent variables listed above.

RESULTS

All abalone from the PC treatment and all except three animals in the experimental treatment (EWS and EA) that died in this study had visible signs of WS, including weakness, weight loss, and visible atrophy of the foot muscle (Fig. 2). The two EA abalone in the experimental aquaria that died during the first week of the study lacked visual and histopathological signs of WS, RLP infections, or visible injuries. A third abalone from the experimental treatment that died during the 21st week of the study was too decomposed for gross or histologic examination. The six NC abalone that died during the experiment and 18 NC survivors sampled upon termination of the study did not have visible or microscopic signs of WS (Figs. 3 and 4). Both Vandenberg/Cayucos (PC and EWS) and Ano Nuevo Island (EA) abalone with clinical WS had histopathological and hematological signs of this disease, including degeneration and inflammation of and/or metaplastic changes in the digestive gland, depletion of muscle bundles in the foot, (Figs. 3–5), and the presence of necrotic cells, cellular debris, and small hemocytes (~4.5 μm) with a large nucleus to cytoplasmic ratio within the hemolymph. In addition, all PC and EWS animals and all except the two EA abalone that died during the first week of the study were infected with the RLP, whereas none of the NC animals were infected. Other than the nonpathogenic renal coccidian, *Margolisiella* (=*Pseudoklossia*) *haliotis*, no other parasites were observed in any of the abalone examined in this study. In this study, the incubation time for clinical WS is defined as the duration between initiation of the study and development of gross clinical signs such as mantle retraction or visible atrophy of the foot muscle. The mean incubation period for the EA abalone was 245 days ($n = 21$) with a range of 154–301 days. The duration between onset of visible signs of WS and mortality averaged 42 days ($n = 21$) with a range of 6–113 days. The two abalone that died during the initial week of the study from handling stress and the single animal that died at 21 wk did not show signs of WS or were too decomposed for assessment of WS, respectively, and were not included in these calculations. Cumulative mortality approached 100% in the PC aquaria, 85% of the EA animals in the experimental aquaria, and 25% in the NC aquaria (Fig. 6). A significantly higher proportion of abalone died upon exposure to WS (22/24) relative to unexposed animals (6/24; $P < 0.0001$, Fisher's exact test). Median time to mortality was significantly different between the exposed EA (41 wk) and PC (16 wk) abalone ($P < 0.0001$, Mann-Whitney test). As only a few NC abalone died during the study, median time to death was not calculated for this group.

A significantly higher proportion of abalone exposed to WS (EA and PC) had reduced condition indices, morphologic changes, and RLP infections than did unexposed animals (NC). Reduced condition indices were observed in 18/24 EA and 19/24 PC abalone, whereas only 3/24 NC animals lost condition ($P = 0.002$ and $P < 0.001$, respectively, X^2 test). Morphologic changes were observed in the digestive gland of 20/21 EA and 17/24 PC abalone, whereas only 1/24 NC abalone had an abnormal digestive gland architecture ($P < 0.001$, X^2 test). Of these, degeneration was observed in 14/21 EA and 6/10 PC animals, metaplastic changes in 9/21 EA and 4/10 PC abalone, and inflammation in 6/21 EA and 1/10 PC abalone, whereas 1/24 of the unexposed animals only had mild digestive gland degeneration. Pedal atrophy was observed in 15/21 EA, 17/24 PC, and only 1/24 NC abalone ($P = 0.001$ and $P < 0.001$, respectively, X^2 test). Infections with "*Candidatus* Xenohaliotis californiensis" were observed only in EA (22/21) and PC (24/24) treatments ($P < 0.001$, X^2 test and $P < 0.001$, Fisher's exact test). As above, significantly higher proportions of animals with RLP infections died and had clinical signs of WS than did unexposed abalone ($P < 0.001$, X^2 test).

Spearman rank correlation coefficients for relationships between intensity of RLP infection of individuals in each WS-exposed group (EA, EWS, and PC) versus visual condition, condition indices, condition of the foot and digestive gland, and density of circulating hemocytes were low and ranged between –0.275 and 0.486 for the Ano Nuevo Island animals and –0.0175 and 0.0567 for Vandenberg animals. Except for metaplasia and overall

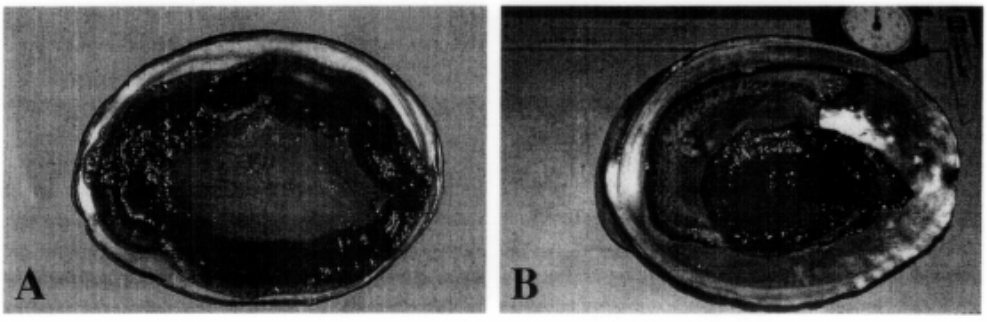

Figure 2. Black abalone with and without WS. A, Healthy animals from Ano Nuevo Island from a negative control treatment. B, An (EA) abalone from Ano Nuevo Island that contracted WS from infected black abalone in an experimental treatment.

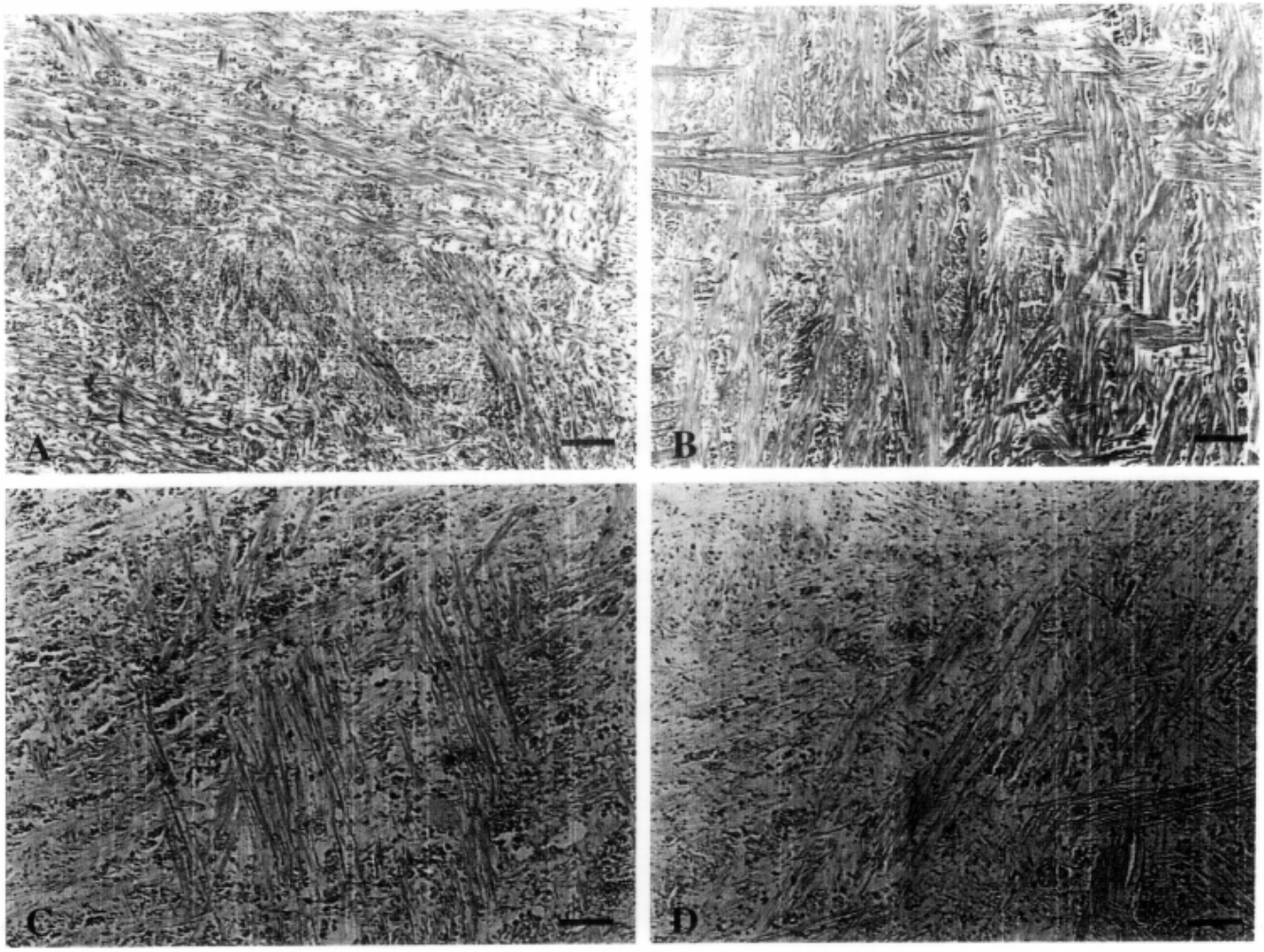

Figure 3. Microscopic anatomy of the foot muscle of black abalone with and without WS. The foot muscle of an uninfected animals are illustrated in A (Vandenberg abalone) and B (Ano Nuevo Island abalone). Note that dense bundles of muscle fibers comprise most the foot. The pedal muscle atrophy of an abalone that contracted WS in the field (C) is also observed in those that acquired WS in this laboratory study (D). Note the severe reduction in muscle fibers and increase in visible connective tissue in affected individuals. Hematoxylin and eosin, bar = 150 μm.

RLP burden in the EA abalone ($P < 0.05$), all coefficients were nonsignificant ($P > 0.05$). This relationship was also mirrored in X^2 analysis in which a higher proportion (8/9) of EA abalone with high overall RLP burdens (scores of 4–6) had metaplastic changes as the sole or partial response to RLP infections than did those with low (scores of 0–3) infections (1/7, $P < 0.01$). Several of the PC abalone were too necrotic to assess specific lesions in the digestive gland, a tissue that degrades more quickly than other tissues (Friedman, personal observation), and resulted in small samples sizes for this specific analysis. Intensity of RLP infection in laboratory EA abalone was predicted from the duration of exposure (time) with weight condition index, visual condition, condition of the foot and digestive gland, and time as independent variables in the model ($P = 0.0156$, Forward and Backward stepwise regressions). No prediction of presence of RLP infection could be made using Multiple logisitic regression analyses using all possible combinations of the five independent variables used in this study ($P > 0.500$). We did observe a significant correlation between hemocyte numbers and weight condition index of the EA abalone ($P = 0.0469$), PC abalone ($P = 0.0016$) and NC abalone ($P = 0.0015$). Correlation coefficients, however, were low to moderate and ranged between 0.2668–0.5612.

DISCUSSION

The present study describes the transmission of WS from black abalone with WS to previously healthy black abalone held in the same aquaria. The similarity in physical, histopathological, and hematological characteristics of WS between black abalone exposed to WS in the laboratory and field, combined with a lack of these signs in the negative control animals, confirmed that the experimental abalone contracted WS in this study (Haaker et al. 1992, VanBlaricom et al. 1993, Gardner et al. 1995, Shields et al. 1996, Friedman et al. 1997). These data also suggest that WS is directly transmissible between sympatric abalone by cohabitation.

WS is a chronic, slow-progressing malady in which clinical signs appear in the final stages of the disease. The presence of advanced microscopic morphologic changes throughout the pedal muscle and digestive gland of affected abalone supports this conclusion (Figs. 2–5). Our data also suggests a long incubation period for WS (~35 wk) followed rapidly by mortality (~42 days) under the conditions used in this study. As shown in Figure 6, once the animals developed clinical WS, the slopes of the mortality curves from the experimentally (EA) and field-exposed (PC) animals were very similar. However, median survival times between

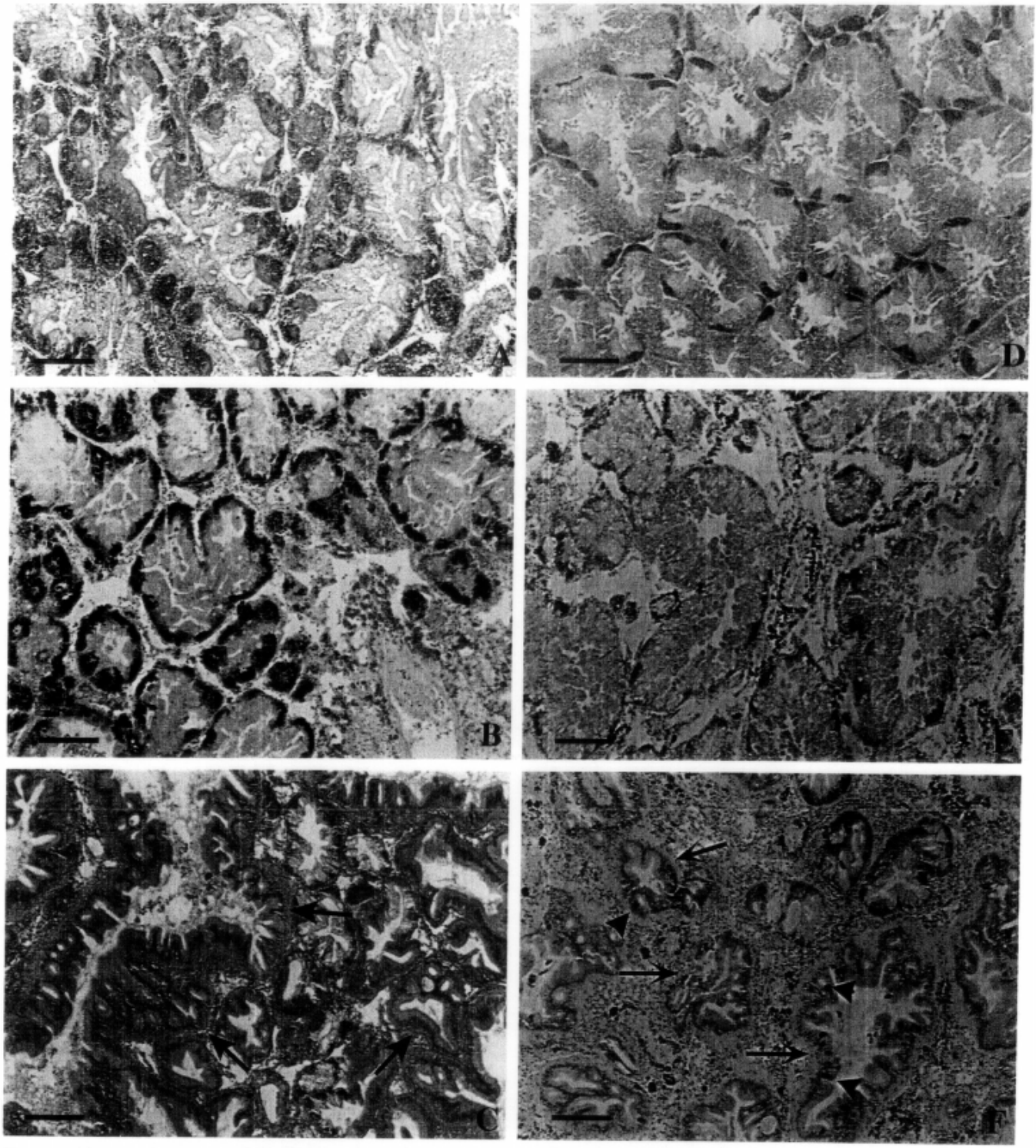

Figure 4. Microscopic anatomy of the digestive gland of black abalone with and without WS. The digestive gland of unexposed abalone from both Vandenberg (A) and Ano Nuevo Island (D) is composed primarily of terminal tubules and little transport/absorptive duct epithelia, whereas those of abalone that contracted WS in the field (B, C) and laboratory (E, F) show a reduction in numbers of terminal tubules and an increase in connective tissue and transport/absorptive duct epithelia. The digestive gland of some abalone with WS is characterized by an atrophy and loss of terminal tubules (B, E), while other individuals respond to RLP infection (arrow heads) with a transport/absorptive duct metaplasia (arrows; C, F). Hematoxylin and eosin, bar = 150 μm.

these two groups were quite different (41 wk for Ano Nuevo and 16 wk for Vandenberg/Cayucos animals) and may be due to a variety of factors. The Ano Nuevo Island EA abalone was uninfected before initiation of the study, whereas the Vandenberg and Cayucos PC abalone were in varying stages of WS. In addition, differences in susceptibility may exist between abalone from these geographically distant locations. In an earlier study in which asymptomatic but previously exposed black abalone were col-

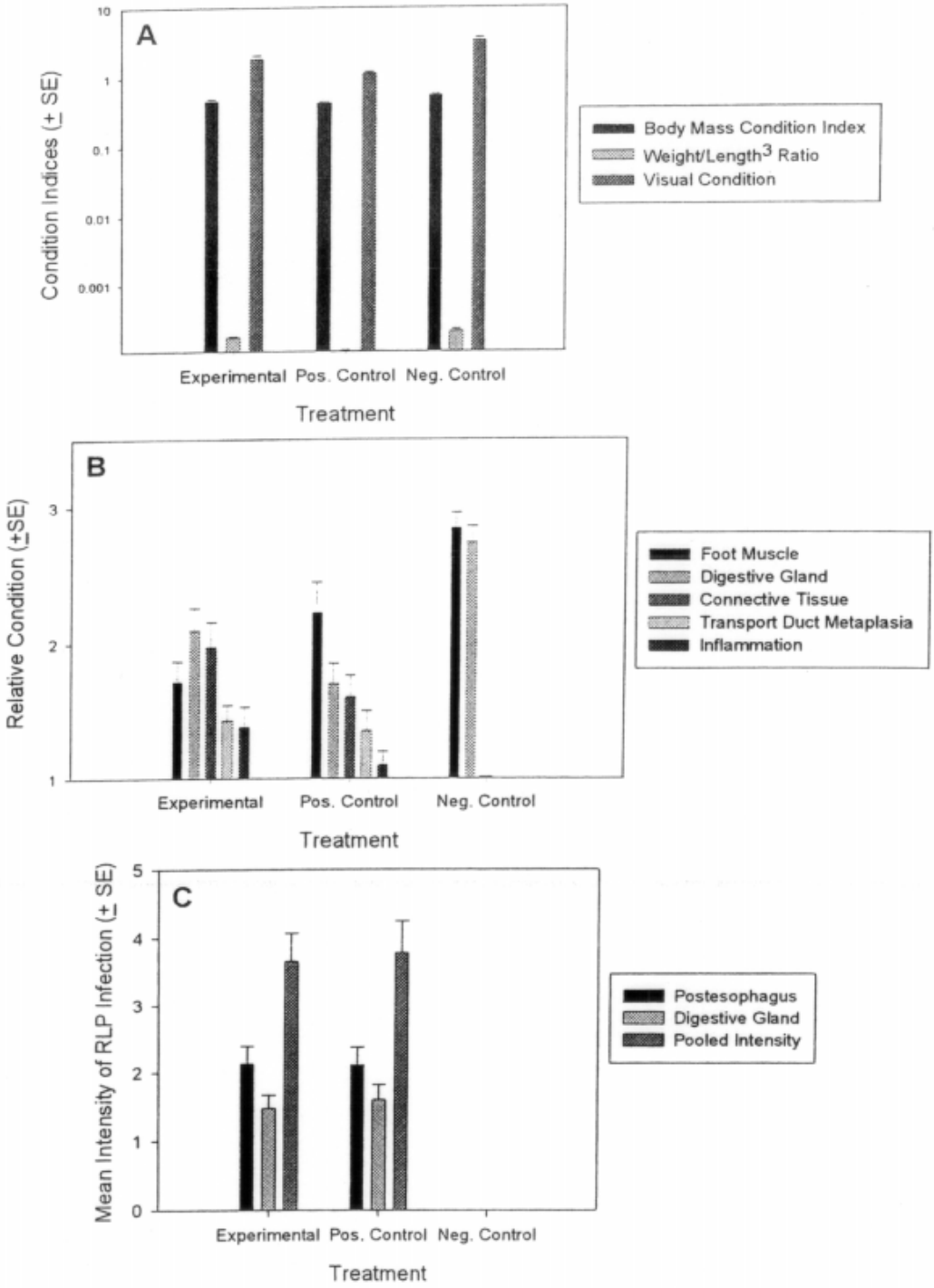

Figure 5. Microscopic morphologic changes of black abalone that acquired WS in the field (Pos. Control) or in the laboratory (Experimental) relative to unexposed (Neg. Control) animals. A, Animal condition; B, relative condition of the foot muscle and digestive gland, C, RLP intensity of infection. Each bar represents the mean of 12 abalone in each of two replicate treatments ± standard error (SE).

lected from Vandenberg and held at 18°C, the initiation of mortality at 15 wk was similar to that observed in the PC and EWS abalone in the current study and also supports a long incubation period for WS (Friedman & Fan 1998).

The observation of RLPs in the EA Ano Nuevo Island animals and not in the NC Ano Nuevo Island animals (Fig. 5) suggests that this bacterium, like WS, is horizontally transmitted by cohabitation and is the etiological agent of this disease. This is further supported by a lack of observation of any pathogens besides "*Candidatus* Xenohaliotis californiensis" in any abalone examined in this study. Transmission of this RLP is thought to be via a water-borne/fecal-oral route because of the presence of bacterial foci in the digestive epithelium and the observation of both intact and lysed RLP foci in lumina of the digestive tract (unpublished observations). Mortalities of the European Saint-Jacques scallop, *Pecten maximus*, have been associated with a branchial RLP infection (Le Gall et al.

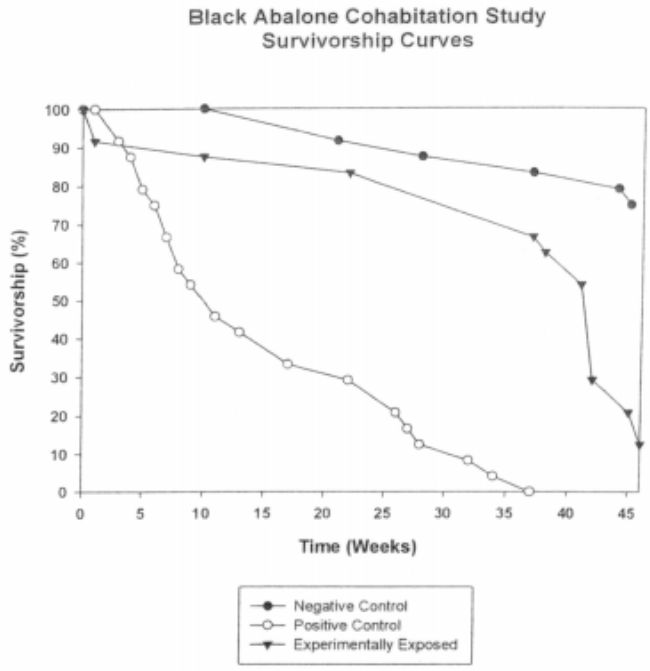

Figure 6. Survivorship curve of abalone in the cohabitation study. The closed circles represent the negative control animals, the open circles represent positive control animals, and the closed triangles represent the (EA) Ano Nuevo Island animals in the experimental treatment.

1988, 1991). Transmission of this scallop-pathogenic RLP via horizontal, water-borne transmission has also been documented (Le Gall et al. 1991). Field and laboratory studies suggested that transmission of the scallop RLP occurred between ~5–28 wk of exposure (Le Gall et al. 1991). Additional field studies reported heavy RLP infections in scallops during the winter months followed by mortalities in the spring (Le Gall et al. 1991), suggesting a relatively long incubation period for the scallop rickettsial disease as we have observed for the RLP-induced WS in this study.

The relationship between the RLP and WS in black abalone is complex as evidenced by higher proportions of mortality and clinical WS in groups of abalone either exposed to WS-affected animals or infected with the RLP. With one exception, a lack of significant correlation existed between intensity of RLP infection and WS in both the experimental and PC animals coupled with a lack of ability to predict intensity of RLP based on gross or histologic signs that characterize WS (regression models). The single significant positive correlation between overall intensity of RLP infection and degree of metaplasia in the EA abalone that responded, in part, with this morphologic change suggests that sustained high RLP burdens may lead to metaplasia in black abalone as has been observed in red abalone (Moore et al. 2000). This relationship was not observed in the small number (10) of PC abalone in which the presence or absence of metaplasia was quantified; the small sample size may account for an inability to detect a relationship. However, when the overall DG condition (alteration from normal, including all three specific morphologic changes) was assessed in the EA and PC, these relationships were not observed. This lack of correlation between RLP infection and DG overall condition in black abalone may relate to the host response to RLP infection (primarily degeneration of digestive tubules) (Figs. 4 and 5) combined with the high turnover rate of the target tissue (digestive epithelia) infected by the WS-bacterium relative to the bacterium's growth rate. Significant correlations between intensity of RLP infection and degree of WS have recently been observed in both field and laboratory studies using wild and cultured red abalone (Moore et al. 2000, Friedman, unpublished observation). The authors also suggested that differences in correlations between intensity of RLP infection and disease in red and black abalone might relate to species differences in host response to infections. Red abalone respond to the RLP infections predominantly by a metaplastic change in which digestive gland tubules are replaced by transport duct epithelium (Moore et al. 2000). Black abalone respond to RLP infection by a combination of digestive tubule degeneration and, to a lesser extent, transport duct metaplasia (Gardner et al. 1995, Friedman et al. 1997) (Figs. 4 and 5). Both of these tissue changes result in a loss of key functional tissue in the digestive gland, the terminal tubules (Voltzow 1994), which may lead to starvation and account for the utilization of foot muscle as an energy source followed by death as observed in abalone with WS (Friedman unpublished data.). As the RLP infects transport duct epithelia and not terminal digestive tubules, this may result in an increase in RLP intensity of infection in red and not in black abalone as the infections progress and clinical disease develops. Figure 5, however, does illustrate alterations in condition indices and changes in the condition of the foot and digestive gland only in abalone with RLP infections. This provides further evidence that the RLP is the etiological agent of WS. RLPs have been associated with atrophy and degenerative changes in other invertebrate species (Min & Benzer 1997).

Infections with RLPs have been reported in a variety of molluscs and crustaceans, including the sea scallop, *Placopecten magellanicus* Gmelin (Gulka & Chang 1984a), the blue mussel, *Mytilus edulis* Linné (Gulka & Chang 1984b), the manila clam, *Tapes japonica* Adams and Reeve and the Japanese scallop, *Patinopecten yessoensis* (Elston 1986), the European flat oyster, *Ostrea edulis* Linné (Friedman et al. 1989), the black abalone, *Haliotis cracherodii* Leach (VanBlaricom et al. 1993), and the penaeid shrimp, *Penaeus marginatus* Randall (Brock et al. 1986). These infections varied greatly in tissue specificity (nonspecific to highly specific), length of incubation period, and pathogenicity, ranging from no apparent harmful effects to lethal effects in the host (Gulka & Chang 1984a, Brock et al. 1986, Frelier et al. 1993, Gardner et al. 1995, Bower et al. 1996). In addition, the pathogenicity of a specific RLP has been shown to vary between host species (Brock et al. 1986). As in these studies, which document that RLPs are pathogenic for marine invertebrates, our data provides evidence that "*Candidatus* Xenohaliotis californiensis," the recently identified RLP observed in abalone in California, is the etiological agent of WS. Future studies that examine the interaction between host gastrointestinal cells and the RLP may provide insight into the cellular physiology of the host and the physiology and disease mechanisms of the bacterium.

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