Title

Characterizing the Role of Environmental Stressors in the Development of Withering Syndrome in Red Abalone

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R/A-117: Characterizing the Role of Environmental Stressors in the Development of Withering Syndrome in Red Abalone.

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Withering syndrome (WS) is a disease of wild and cultured abalone, caused by a Rickettsiales-like prokaryote (WS-RLP). While WS has decimated black abalone populations throughout most of California, both wild and cultured red abalone have shown resilience in certain environmental conditions. The changes in seawater temperature and food availability associated with El Niño events may, however, stimulate the pathogenesis of WS in WS-RLP-infected red abalone. This study sought to examine the relative contributions and synergistic effects of multiple stressors on development of WS, and establish sensitive and robust markers for characterizing the sequential pathological changes associated with disease progression. This information is critical for the proper management of WS by both private aquaculturists and state resource managers.

The core experiment was designed to characterize pathologic changes that occur during the progression of WS in red abalone. Animals were subjected to environmental stressors associated with El Niño conditions: thermal stress (18.3°C vs. 12.3°C) and food limitation (25% or less ration vs. full ration). Prior to the intitation of the experiment abalone were given a feed-based oxytetracycline therapeutic treatment to ensure WS-RLP-free status. Animals were subjected to a full factorial study investigating the impacts of WS-RLP exposure, temperature and food supply. The study included six replicate tanks per eight treatments, for a total of 48 tanks. Animals from each tank were periodically sampled over 447 days. Elevated temperature significantly affected WS-RLP transmission ($p < 0.05$): only 0.1 % of WS-RLP exposed abalone held at ambient temperature became infected, compared to 56 % of those held at elevated temperature. On sample day 382, RLP infection and digestive gland metaplasia was confined almost exclusively to the abalone of the infected/elevated temperature treatments Figure1 1.

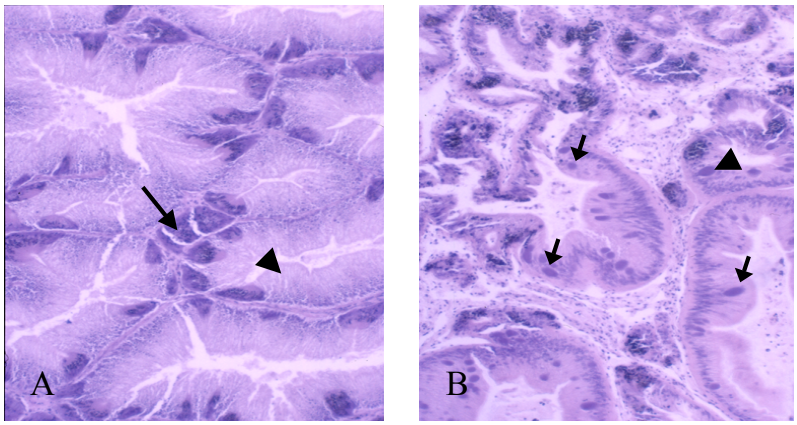

Figure 1. Abalone digestive gland (A) is composed of blind digestive tubules which are made up of crypt cells (arrow) and absorptive cells (arrowhead). The digestive tubules empty into transport ducts lined with ciliated columnar epithelium, not shown. (B) As the disease progresses. The digestive-enzyme secreting tubules are replaced by transport ducts and connective tissue. Note the RLP inclusions in the transport ducts (arrows) and metaplastic digestive tubules (arrowhead). (H&E)

While these effects were observed in both full-feed and reduced-feed, abalone of the infected/full-feed/elevated temperature group had the highest levels of metaplastic change and RLP infection intensity. The infected/reduced-feed/elevated temperature group had significantly more pedal degeneration than all other groups. Our results reaffirm previous findings and suggest that the presence of starvation, infection and elevated temperatures has an additive effect causing increased catabolism of foot muscle for energy. The percentage of withered animals also illustrates this point. At the termination of the experiment on Day 447, this group had the highest percentage of terminally withered animals: 32% compared to 22% for the uninfected/reduced-feed/elevated temperature group and 4% for the infected/full-feed/elevated temperature group. Thus, starvation and elevated temperature together seem to exacerbate the mortality associated with RLP infection (Figure 2).

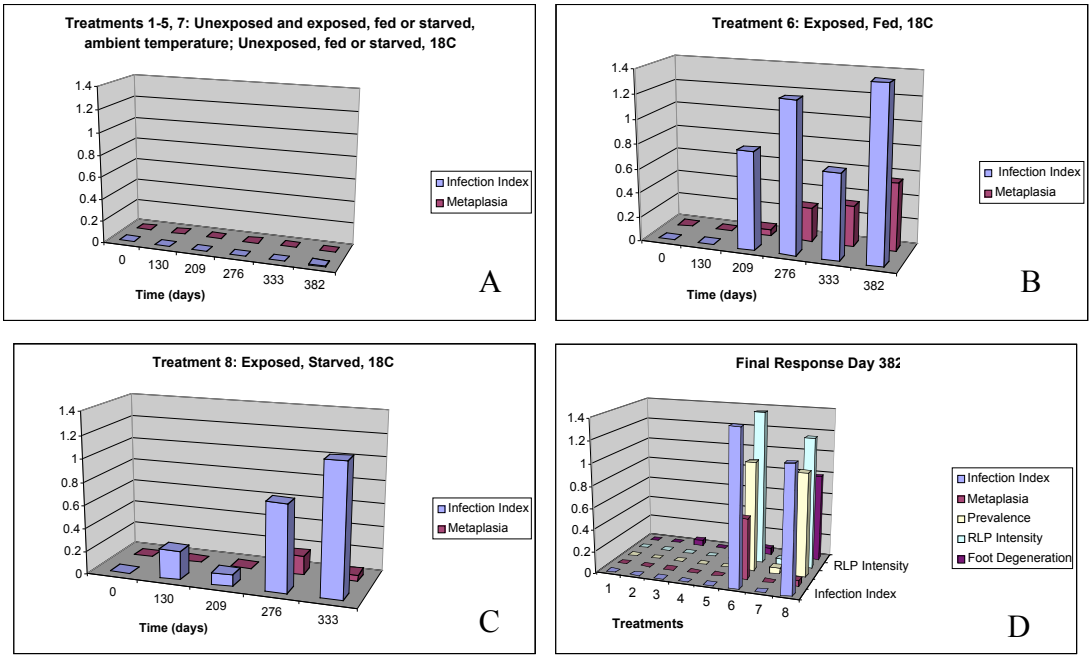

Figure 2. Response of abalone to RLP exposure. A. No RLP infection or metaplasia were observed in any unexposed abalone at ambient or elevated temperatures or exposed abalone at ambient temperatures through days 382 of the study. B. In contrast, exposed, fed abalone at 18C had moderate infection levels and increasing metaplasic infections with increasing duration of infection. C. While, exposed, starved abalone at 18C were also infected with the RLP, less metaplasia was observed in association with these infections. D. By 382 d of the study, only the exposed, 18C abalone in treatments 6 and 8 showed significant infections and signs of WS.

The gross symptoms of WS; wasting, weakness, lethargy - parallel those of starvation and it has been unclear as to whether the characteristic digestive gland changes in WS are a consequence of starvation or a physiologic response to WS infection. Comparisons between starved, uninfected abalone and the fed, infected abalone has shown that digestive gland metaplasia is specific for WS, not a result of starvation. These changes occurred only in infected abalone and were not associated with different patterns of cellular proliferation or apoptosis than those found in normal digestive gland.

Expression of HSP70 in the gill showed no obvious relation to treatment. Cessation of feeding resulted in precipitous decreases of foot and digestive gland glycogen levels, while WS-RLP infection was associated with gradual declines. An additional component of the study involved assessment of the digestive gland for drug residues. Oxytetracycline concentrations of up to 186 ppm were detected in the digestive gland at 105 days post-treatment. This quite remarkable drug residue retention may be related to the abundance of heavy metals found in this organ.

Additional research focused on the identification of energetic and metabolic indicators of stress and infection. HPLC (High Pressure Liquid Chromatography) analysis of perchloric acid extracts of both the foot muscle and digestive gland tissue was investigated to identify the potentially synergistic roles of infection, elevated seawater temperature and food availability on the energetic (metabolic) status. We evaluated two established metabolic indices (adenylate energy charge (AEC) and the ATP/ADP ratio) on both foot muscle and digestive gland tissue derived from the core experiment. Of the two tissue types investigated we found foot muscle tissue to be the most responsive to the effects of the stressors investigated. Within the foot muscle we observed significant decreases occurring in both indices due to food reduction and RLP infection. Food limitation along with elevated temperatures resulted in the greatest reductions in both AEC and ATP/ADP ratios. Temperature, food limitation, and RLP infection had a less severe affect on abalone digestive gland and in many cases the observed changes occurred in the opposite direction to those found in foot muscle. In the digestive gland of stressed animals we observed, significantly higher AEC values than the values that were measured in control abalone. This trend suggests the foot muscle serves as storage organ and that transport occurs from the foot muscle to the hepatopancrease in the California red abalone.

In a concurrent study we sought to identify biochemical markers that could track disease expression and help explain the pathologic changes that occur. The goal of this study was to develop new methods for assessing the chronic effects of chemical, physical and biological stressors on organisms in the environment. We first investigated WS in farmed red abalone using a metabolomic approach that combines the metabolic profiling capabilities of nuclear magnetic resonance spectroscopy (NMR) with pattern recognition methods. Foot muscle, digestive gland, and hemolymph samples were collected from abalone that were healthy, showing early WS signs and Advanced WS signs. Following spectral pre-processing, principal components analyses of the metabolite profiles were conducted. Our results confirmed that NMR-based metabolomics could successfully distinguish the biochemical profiles of the three groups of animals, in every type of tissue or biofluid studied. This discovery-based approach successfully identified novel metabolic biomarker profiles associated with withering syndrome and is the first application of this cutting edge approach in an aquatic organism. It allowed us to observe disease related changes occurring in valine, alanine, carnitine, glycine, tyrosine, phenylalanine, tryptophan, glutamine, adenylates, glucose, glycine-betaine, hypotaurine, glycogen, formate and homarine.

We finally used this novel ^{1}H NMR-based metabolomics approach described above to examine foot muscle and digestive gland samples that were harvested throughout the core experiment. High-resolution nuclear magnetic resonance (NMR) spectroscopy was particularly appropriate for investigating metabolic status, since potentially hundreds of endogenous metabolites could be quantified rapidly in both the foot muscle and digestive gland tissues with minimal sample preparation. Following spectral pre-processing of the NMR data, principal component analyses of the metabolite profiles were conducted. Food limitation caused dramatic reductions in all classes of foot muscle metabolites while at the same time metabolite levels

within the digestive gland were preserved or increased. We also found that food limitations along with the additional stress of elevated seawater led to greater metabolic perturbations in both tissue types than those observed under food limitation alone. WS-RLP infection and food reduction resulted in many of the same metabolic changes within the tissues studied although the effects of infection were more modest. We observed increased levels of homarine in the digestive gland of both food limited and infected animals yet only observed increased homarine levels in the foot muscle of WS-RLP positive abalone. This allowed the recently established 'glucose:homarine' ratio to successfully differentiate foot muscle tissue of infected animals from tissue of both healthy and starved abalone. We found the NMR metabolomics data correlated well with classic histology, supporting the use of unique methodologies in characterizing both normal and pathological events in marine species, particularly to characterize environmental stress.

Publication:
Viant MR, Rosenblum ES, Tjeerdema RS. 2003. NMR-based metabolomics: A powerful approach for characterizing the effects of environmental stressors on organism health. *Environmental Science and Technology* 37:4982-4989.

Publications submitted:

Braid B.A., Moore J.D., Robbins T.T., Hedrick R.P., Tjeerdema R.S., Friedman C.S. Health and survival of red abalone, Haliotis rufescens, under varying temperature, food supply and exposure to the agent of withering syndrome. Journal of Invertebrate Pathology.

Rosenblum, E.S., Viant, M.R., Braid, B.M., Moore, J.D., Friedman, C.S., and Tjeerdema, R.S. Investigating the effects of pathogen, elevated temperature and starvation on the metabolic profiles of California red abalone, *Haliotis rufescens. Metabolomics.*