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Initial Steps Towards Evaluating the Potential Disease Impacts of Propagated Marine Fish on Wild Stock: Examination of a New Herpes-like Virus
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Project Hypotheses
The project hypothesis is that with the development and application of the appropriate tools for pathogen detection, we can gain major initial insights into the potential impacts of diseases among propagated fish upon wild fish populations.

Project Goals and Objectives
The overall goal of this project is to establish the key criteria essential to evaluating the potential disease impacts of the use of hatchery-reared fish for the enhancement and or restoration of wild marine fish stocks. A corollary goal is to establish the key criteria essential to evaluating the potential disease impacts of free-ranging carriers on hatchery-reared fish. More specifically, we will develop the criteria for a newly discovered herpes-like virus among white seabass as a model for a range of potential microbial pathogens among a growing list of marine fish species earmarked for artificial propagation. The project will develop the needed diagnostic tools, evaluate modes of transmission in experimentally-infected fish, examine the pathogenesis of the agent among experimentally-infected fish, and finally compare the disease status of hatchery-reared to wild populations of fish.

Briefly describe project methodology
Herpes-like virus particles (WSB-HV) have been detected via electron microscopy in the intestinal tissues (lamina propria) of hatchery-reared white seabass. A series of studies will be conducted to: 1) isolate the virus, 2) develop key diagnostic reagents, 3) test potential routes of virus transmission, 4) characterize viral pathogenesis among experimentally-infected fish, and 5) evaluate virus prevalence in hatchery compared to wild populations of white seabass.

Methodology for each objective are as follows.
1) Isolate the virus
During the course of the project we have developed 3 different white seabass cell lines, the essential tools for virus isolation. The cell lines were initiated from kidney, whole larval, and skin tissues. The lines are stable and

have been subcultured numerous times and the conditions for growth and maintenance established. In addition, we have created several primary cell cultures from intestinal and kidney tissues.

2) Develop key diagnostic reagents
Two diagnostic tools for the white seabass herpesvirus (WSBHV) were proposed for development, an enzyme linked immunosorbent assay (ELISA) for detection of anti-virus antibodies in the serum of previously exposed fish and the polymerase chain reaction (PCR) test to detect viral DNA directly in fish tissues. Standard methods for the development of these tests was followed including production of a rabbit antibody to the white sea bass immunoglobulin (WSB Ig) and sequencing of a region of the WSBHV using degenerate primers to a region of the terminase gene, a gene unique to herpesviruses, including those from fish.

3) Test potential routes of virus transmission
Three experimental routes of transmission are to be tested including waterborne virus exposures of naïve fish using virus derived from cell cultures or ground, infected intestinal tissues from naturally infected white seabass, intraperitoneal injections of naïve fish with similar virus preparations and cohabitation of naïve fish with white seabass known to have been previously exposed to the herpesvirus.

4) Characterize viral pathogenesis among experimentally-infected fish.
Replicate groups of 100 experimentally-infected fish will be compared to replicate groups of 100 control fish by cumulative mortality over a 3 month period, sampling of selected tissues for viral presence and concentration, viral DNA by PCR, and microscopic pathological changes. Experimental route of infection will be either waterborne or cohabitation, guided by results of the transmission trials described above. Numbers of fish included here assume that mortality will not exceed 50% in the exposed groups but these can be adjusted according to outcomes of the transmission trials. From both one tank of experimentally-infected fish and one control tank, a minimum of 5 fish will be removed at each of the following time points: 1, 3, 7, 14, 21, and 30 days post-exposure, and all the surviving fish will be sampled at 90 days post-exposure. The development of microscopic lesions and the presence of viral DNA will be evaluated among sampled fish, fish that are freshly dead and survivors at 2 months post exposure. In addition, the presence of anti-WSBHV serum antibodies will be evaluated in survivors at 2 months post exposure.

5) Evaluate virus prevalence in hatchery compared to wild populations of white seabass.
Virus prevalence in HSWRI white seabass production lots for years 2005-2007 as well as in wild fish will be determined using the assays developed above. Serologic evidence of prior exposure to WSB-HV will be evaluated using the ELISA assay, while detection of herpesviral DNA in tissues will be assessed using the PCR test. Using these newly developed assays, we will be able to compare the disease status of hatchery-reared to wild populations of fish. We anticipate testing up to 100 hatchery origin fish, both in symptomatic and asymptomatic production lots, and in up to 100 wild juvenile and adult white seabass.

Describe progress and accomplishments toward meeting goals and objectives
Progress with respect to each of the objectives during Year 1 of the project are reported below.

1) Virus isolation
Several cell lines (n=3) and several primary cultures were established and then utilized for attempts at virus isolation. Despite numerous attempts (n>10), we were unable to isolate virus, even from fish tissues that we could confirm by electron microscopy harbored typical herpesvirus particles. This was of course frustrating but certainly not unknown among many of the fish herpesviruses. Approximately 50% of the fish herpesviruses recognized have failed to be isolated in cell culture. The reasons for this are not clear but may involve specific host cell requirements/factors not found in either primary or established cell lines.

2) Develop key diagnostic reagents
Polymerase Chain Reaction (PCR) Test: A PCR test for the detection of white seabass herpesviral (WSBHV)-specific DNA was developed. The diagnostic PCR primers 227F (5'- AGA GGC TGT ACT CTA CAT TC -3') and 228R (5'- GTT TCA ATG GTG TCA CAA TC -3') produce a 372 bp DNA fragment. The primers were designed manually based on the WSBHV gene which shows similarity to Ictalurid herpesvirus (IcHV)1 ORF44. The reaction cocktail (50 •l) is 2.5 mM MgCl2, 1• buffer, 200 •M dNTPs, 40 pmol primers and 0.5 U Platinum Taq polymerase (Invitrogen). The PCR cycling conditions are as follows: initial denaturation step of 95 °C for 5 min; then 40 cycles of 95 °C for 30 sec, 60 °C for 30 sec and 72 °C for 30 sec; followed by a final extension step at 72 °C for 5 min; then held at 4 °C. PCR products (10 •l) were electrophoresed on 2% agarose gels and the gels were stained with ethidium bromide for visualization of the banding pattern by UV transillumination. The assay can detect 0.01 fg of plasmid DNA carrying target sequence in the presence of 300 ng of fish genomic DNA. A discrimination test was performed using genomic DNA of 10 fish herpesviruses, including Salmonid herpesvirus (SalHV)1, SalHV2, Acipenserid herpesvirus (AciHV)1, AciHV2, CyHV1, CyHV2, CyHV3, Ictalurid herpesvirus (IcHV)1, IcHV2 and WSBHV, and DNA was only amplified from WSBHV.

Enzyme-Linked Immunosorbent Assay (ELISA): Initial attempts were made to prepare adequate WSBHV antigen that could be used in an ELISA test to detect serum antibodies to the virus. As the virus was not isolated in cell culture, it was not possible to purify virus directly from in vitro cultures. We instead made several attempts to isolate virus directly from infected intestinal tissues as provided by Dr. Okihiro. We were successful in purifying very small amounts of virus but amounts were inadequate for further pursuit of using directly as an antigen (coating ELISA plates) or as an antigen to make rabbit or mouse anti-WSBHV antibodies that could be used in an antigen capture ELISA protocol. We have pursued looking at amplification of the gene responsible for production of the major capsid protein, with the thought to make recombinant viral protein that might be used as an antigen.

3) Test potential routes of virus transmission
In the absence of in vitro cultures of the virus (see 1 above) or live infected fish, we attempted to transmit the white seabass herpesvirus (WSBHV) by exposure of healthy juvenile WSB to frozen tissues collected from WSB that had died during the 2005 epizootic. Presence of WSB herpesvirus-specific DNA in these tissues could be confirmed by PCR prior to exposure trials (but this is not confirmation of viability of the virus and thus viral infectivity). In two separate trials, replicate groups of 10 fish each were either fed or injected with homogenized tissues. Fish were sampled at 2 weeks, 1 month and 2 months post-exposure and tissues were collected for later PCR testing and histological

examination. No mortalities occurred during the experiments. No WSBHV-specific DNA was detected in any of the experimentally-exposed fish.

4) Characterize viral pathogenesis among experimentally-infected fish
Difficulties in reproducing the infection/disease by the inoculation of healthy juvenile white seabass with frozen tissues from suspected or known PCR positive fish was not successful (see above). Furthermore, there were no new reported outbreaks for the WSBHV from the hatchery. As a result of these two situations, we were unable to complete this important objective as proposed for year 2. An alternate strategy for completing related objectives has been submitted to and approved by Sea Grant for year 2.

5) Evaluate virus prevalence in hatchery compared to wild populations of white seabass.
Tissue samples (or in some cases whole fish) were collected by Dr. Okihiro from a population of hatchery-propagated white seabass following an apparent herpesviral epizootic in October 2005 and stored at -80C. Of these, 100 specimens were tested for the presence of herpesviral-specific DNA by PCR analysis. In 52% of the samples, herpesvirus-specific DNA was detected (52/100). A subset of these PCR products was purified and the DNA sequenced, confirming that these amplicons were indeed homologous to the white seabass herpesvirus. In contrast, no herpesviral-specific DNA was detected in a group of 62 hatchery-reared adult white seabass sampled in November 2006, shortly before release of this population. Also, no herpesviral-specific DNA has been detected to date in any wild juvenile or adult white seabass collected in the waters around or in various sites, including La Jolla, Santa Catalina Island, Carlsbad, Oceanside Harbor, Santa Rosa Island, Anacapa Island, Imperial Beach, Newport Bay, Pt. Loma, Oceanside, or Agua Hedionda Lagoon from April 2006-September 2007. We have analyzed tissues from over 83 wild fish (some were collected in 2-5 fish pools), with PCR testing of typically two tissues per fish (pyloric cecae and posterior intestine) or up to five tissues per fish (pyloric cecae, posterior intestine, skin, spleen, and head kidney).

The lack of detection of herpesviral-specific DNA in wild fish tested to date highlights two potential limitations of our testing: (1) the virus may be present in latent form in these fish but in levels below detection limits of the assay or in tissues not tested, and (2) our sample size is inadequate to detect the virus if found in low prevalence. In the absence of access to populations of fish known to have been exposed to the herpesvirus (either wild or experimentally-exposed fish), we cannot determine prevalence rates and thus adjust our wild fish sampling strategy to ensure a statistically adequate sample size.

Project modifications

A significant redirection of efforts is proposed and has been approved by CASG for year 2. Due to a sudden disappearance of the white seabass herpesvirus among propagated populations of white seabass there is no longer adequate materials for pursuing year 2 objectives. Instead, the same basic hypothesis will be tested using a new sporozoan infection recently detected in hatchery stocks of white seabass.

Project outcomes
Principal outcomes of this project are the development of a sensitive and specific diagnostic test for detection of the white seabass herpesvirus by PCR.

The test works quite well and has been utilized in two laboratories to date. Testing of over 100 wild white seabass captured as part of the CDFG program indicated no evidence for the presence of the white seabass herpesvirus. This suggests that currently conservative approaches to the release of virus-infected white seabass from hatchery propagated programs are warranted. Testing of the 2008 year class of hatchery propagated white seabass indicated no evidence for presence of herpesvirus and these fish were released. In contrast, testing of a production cycle of juvenile white seabass in the hatchery in 2009 demonstrated that an active mortality episode was due to the herpesvirus and these fish were subsequently destroyed and the hatchery disinfected. This management approach, while severe, continues to be a good approach until there is some demonstration the virus is present in wild fish populations. As part of the modification for Year 2 objectives, which were planned when there was no 2008 herpesvirus outbreak, we characterized a sporozoan parasite found in the kidney and intestine of white seabass. This sporozoan is viewed as an equally important problem for the hatchery production of white seabass. We were able to partially characterize the agent describing developmental stages as well as ultrastructural features by electron microscopy. We are still pursuing the genetic characterization of the agent that will lead to new diagnostic tools as we were able to do with the white seabass herpesvirus. The new PCR test as well as the new knowledge of the herpesvirus and sporozoan parasite have been shared with personnel at the HSWRI as well as with the California Department of Fish and Game, the two most critical entities involved in the propagation and release of white seabass.

Impacts of project
The development of the new diagnostic tool has allowed the first insights into the presence of key pathogens in wild populations of white seabass. These initial investigations suggest that neither the herpesvirus or the sporozoan are likely found in high prevalences in wild fish and that conservative approaches to release of potentially infected hatchery propagated fish is warranted. In contrast to the herpesvirus and sporozoan, our earlier investigations revealed that a nodavirus agent (VNNV) is likely present in wild white seabass as indicated by the presence of serum antibodies in these fish to the virus. Thus, we have at least two different situations that suggest two different management approaches to the release of hatchery propagated fish that may harbor important pathogens, one where release is not warranted and a second where the impacts of release are apt to be much less or negligible.

Benefits, commercialization and application of project results
Development of a sensitive and specific white seabass herpesvirus polymerase chain reaction (PCR) capable of detecting low levels of infection in fish. Can be used as a management tool for the evaluation of infections among propagated and wild white seabass populations. Additionally, initial characterization of a new sporozoan parasite provides knowledge that can be pursued for the development and utilization of new diagnostic methods.

Economic benefits generated by discovery
The prudent use of the new diagnostic methods are apt to help considerably in controlling losses of fish in the hatchery and the potential release of infected fish that might impact wild fish resources. These both would have significant economic impacts.

Issue-based forecast capabilities

The development and initial use of the new diagnostic tools for pathogens in marine fish are providing the first data and insights into the potential role of pathogen impacts on wild fish populations. The data collected to date indicate that among 3 of the most important pathogens found in hatchery propagated white seabass that evidence for the presence of only 1 pathogen is found in wild fish. While this does not prove that the wild fish are free of the other 2 pathogens, the initial data is suggesting a low prevalence or pathogen levels at such low concentrations that our sensitive diagnostic methods are not detecting it. It would certainly be prudent to follow management approaches that preserve this type of host-pathogen relationship which is more apt to reduce pathogen impacts on such fish populations. This initial data on pathogen prevalence in wild fish also provides a baseline for future results that might be associated with ecosystem changes.

Tools, technologies and information services developed

Development of a sensitive and specific white seabass herpesvirus polymerase chain reaction (PCR) capable of detecting low levels of infection in fish. Can be used as a management tool for the evaluation of infections among propagated and wild white seabass populations.

Publications

Journal publications that describe the initial development and utilization of the white seabass herpesvirus diagnostic PCR are under preparation. This report will also provide the first results of the use of the PCR in both hatchery and wild fish populations.

Cooperating organizations

Regional

Hubbs SeaWorld Research Institute, Carlsbad, CA

Local and state

California Department of Fish and Game

International implications

No

Awards

None

Keywords

White seabass, herpesvirus, fish sporozoan