CTSA Final Report

Project
Disease Management in Pacific Aquaculture, Year 12

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(Lewis and Laidley components)
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Executive Summary
The focus of the project Disease Management in Pacific Aquaculture is to address current disease and health management issues facing aquaculture operations. Resources in the project have been used to address applied research to health management issues important to the aquaculture community in Hawaii and throughout the Pacific. This support infuses necessary resources for the research and development of diagnostic and control services aimed at specific disease problems limiting aquaculture production. All project activities contribute to the long term goal of the project which is to assist aquaculture business operations in the region in developing solutions to emerging or long standing disease problems.
Cryptocaryon irritans is a ciliated ectoparasite known to cause heavy mortalities in many fish species in marine aquaculture and in private aquaria. This parasite causes mass mortality in hatchery conditions and monitoring fish for early signs of outbreaks is a significant component of disease surveillance in these systems. Despite the increasing occurrence of this epizootic, few methods are available to control it, especially in cultured food fishes. Currently the most effective control measure involves surface disinfection and the repeated transfer of fish from tank to tank, a practice that is labor-intensive as well as extremely stressful to the fish. Transfer in this fashion is meant to interrupt the life cycle of the parasite, which possesses host-associated (trophont) and non-host-associated (theront and tomont) stages. Two of this project’s Objectives investigate ways to manage C. irritans infections in fish.

Objective 1 of the project focuses on continued development of a vaccine against C. irritans. Funding for vaccine development has been ongoing since January 2004 (funded by CTSA in Years 16-17). Much progress has been made, including the identification of putative immobilization antigens of C. irritans in Hawaiian isolates and the identification of two distinct serotypes of the parasite. Protein sequencing of 28 and 29 KD proteins recognized by anti-Cryptocaryon antibodies from immunized fish reveals these proteins are distinct parasite proteins. Moi vaccine trials were conducted during the last phase of the project and anti-moi IgM is being generated under another grant to facilitate direct ELISA and immunoblot analysis. While moi also develop a protective response and immobilizing antibodies against C. irritans, fish remain more susceptible to re-infection after immunization when injected. More work is required to identify optimal vaccine dosage. Subunit vaccines identified in this study (28 and 29 KD parasite proteins) will continue to be studied since both proteins show promise for development of a multivalent recombinant vaccine against C. irritans.

Establishment of permissive cell lines is an important tool for the initial detection of pathogenic fish viruses. Viruses are an important class of microorganisms that contribute to high mortalities in aquacultured animals. Objective 2 of this project focused on development of cell lines from important Pacific aquaculture species. While a number of primary cell lines were established using methods developed in previous CTSA projects we were unable to transform cells to produce immortalized cell lines that could be cryopreserved and revived in culture.

The practice of importing and breeding freshwater ornamental fish is an increasingly lucrative aquaculture practice in Hawaii. Having completed a parasite screening for various industry members under the project’s Objective 3 there is now a data-set of the type of pathogens routinely imported with tropical ornamentals and prevalence data for parasite load from fish imported from specific countries. This information is readily available online in the March 2007 electronic version of the CTSA Regional Notes.

Control of ectoparasites remains a very challenging feature of warm water marine finfish aquaculture. Major culprits include microscopic protozoan parasites (e.g., Amyloodinium ocellatum and C. irritans), monogenes, and parasitic copepods. These ectoparasites, operating singly or in combination, cause major fish losses and are also problematic locally, especially in tank-based rearing systems with limited water exchange. The shortage of FDA-approved chemical treatments for parasite control compounds the difficulty of eradicating parasites.
Objective 4 tested the effectiveness of cleaner wrasses as a non-chemical approach to control of ectoparasites. Peroxide and hyposalinity treatments were also included in the study to determine the efficacy of these treatments alone or in conjunction with cleaner wrasse to manage ectoparasites. Although cleaner wrasse establish cleaning stations in captivity they are susceptible to the same types of parasites. This significantly reduced their effectiveness as a physical means of controlling ectoparasite infection.

In summary, positive results from this year’s Disease Management project include the identification of \( C. irritans \) proteins with utility as a subunit vaccine and documentation of the types of ectoparasites being brought into Hawaii with ornamental fish imports. Although primary cultures were obtained from a number of fishes there is still a need to identify a routinely successful strategy to immortalize cell cultures from fish species of regional interest for viral diagnostics. Finally, while cleaner wrasse establish cleaning stations in closed systems they are unable to successfully combat an ectoparasite outbreak like \( C. irritans \). As such they are not a sufficient means of ectoparasite control in tank-based rearing systems.

**Objectives**

**Objective 1.** Identification / characterization of antigens in the efficacious vaccine fractions and characterization of the antibody response in moi against \( C. irritans \).

**Objective 2.** To establish a continuous cell line from grouper (\( Epinephelus lanceolatus \)) and barramundi (\( Lates calcarifer \)) for viral and rickettsial disease diagnostics.

**Objective 3.** To establish a pathogen screening program for companies that routinely import and breed freshwater ornamentals.

**Objective 4.** Evaluate the efficacy of cleaner fish as a non-chemical method to control ectoparasite levels in marine broodstock populations.

**Objective 5.** Technology transfer of project results.

**Anticipated Benefits**

This project looks at ectoparasite management strategies and cell line development for viral diagnostic support. Both are critical areas of research for Pacific aquaculture. The benefit of protective vaccination against \( C. irritans \) is obvious as much of the pathogen surveillance in hatchery systems in the region is to inhibit or control outbreaks of this insidious parasite. Knowledge regarding what specific parasites are being imported and whether specific freshwater tropicals are more susceptible to specific parasites allows local importers to make management decisions relating to quarantine and treatment for newly imported fish. While tissue culture remains the gold standard for viral diagnostics, especially for newly emerging pathogens, there are few cell lines established for local species of interest. Having cell lines available will allow for more rapid viral diagnostics when a viral etiology is suspected and when other diagnostics (i.e. PCR diagnostics) are not yet available. Finally, should a cohabiting fish species be
identified that successfully manages ectoparasite infection while remaining resistant to infection, workers may be able to reduce the amount of time spent performing routine parasite surveillance.

Work Progress and Principle Accomplishments

Objective 1. Identification / characterization of antigens in the efficacious vaccine fractions and characterization of the antibody response in moi against *C. irritans*.

Based on experiments in the previous year of this study a single parasite protein (30 KD) was identified as being immunogenic using immunoblot analysis. While purifying this protein from a sonicated protein suspension of *Cryptocaryon*, three additional proteins (40, 34, and 19 KD) were also identified as being recognized by serum antibodies from fish that had recovered from an experimental parasite infection. Thus, four purified proteins were selected for a preliminary vaccine trial and controls included injection with a sonicated, whole parasite protein suspension or PBS as the diluent (negative) control.

The injection/sampling schedule is provided below:

- **Week 0**  primary injection --- serum collection
- **Week 6**  tagging of individual fish --- serum collection
- **Week 9**  secondary injection (boost)
- **Week 14**  serum collection
- **Week 21**  parasite challenge
- **Week 24**  serum collection

ELISA results indicate fish developed an antibody response against *C. irritans* whole parasite homogenate and/or individual protein fractions. After the parasite challenge at week 21, it was noted that fish developed an infection but recovered naturally. In other words, these fish were NOT protected against subsequent infection although all immunized fish in previous experiments in this study had developed immunity against reinfection.

Serum was collected and the immobilization assays performed showed the parasite used for the challenge was serotypically different from the one we had been using at HIMB since initiating *Cryptocaryon* experiments. This was a surprising observation at this point of the study. It was originally anticipated there might be different serotypes of *Cryptocaryon* since this is known to be true for *I. multifiliis*, a related ectoparasite pathogen of freshwater fish. Three years were spent working with parasites from Kaneohe Bay before this second serotype was identified.

Immobilization assays using serum from immunized fish showed serum antibodies did not immobilize the parasite from the March 2007 challenge experiment but did immobilize the parasites from a 2006 culture that had been maintained *in vitro* in the laboratory (and was the source of the initial protein purification/injections). Instead of recognizing the 30 kDa protein using our standard immunoblotting method, a slightly smaller parasite protein (29? KD) was identified. The two parasite proteins, one being the same as previously described and the second being approximately one kDa smaller, were sent to Stanford for LC MS/MS sequence analysis.
This time the results were more promising than 30 KD sequencing results obtained from a different sequencing facility last year. LC MS/MS is different than N- or C-terminal protein sequencing methods in that the purified protein is digested into small fragments and the sequences of these smaller fragments are obtained using an LC MS/MS instrument that couples liquid phase chromatography with mass spectrometry. All fragment sequences from last year’s analysis were electronically compared with archived sequences from various databases and the 30 kD protein we sent was determined to be unique and therefore unidentifiable. This result had two possible interpretations. (1) The protein sequence obtained is truly unique from other proteins or, more likely, (2) there are not yet enough archived sequences from ciliated organisms to have compiled a comprehensive database for comparative analyses.

The second round of LC MS/MS was more successful. The 30 kDa protein initially described is now known to be only 29 kD (mass spectrometry is more accurate for molecular weight determination known than sizing by electrophoresis and densitometry) and shares protein homology with an immobilization antigen protein sequence archived in a public access protein sequence database (10 amino acids: YMVTLAGTIK) which was sequenced by Hatanaka et al. (2007). The second protein is 28 kD and these two proteins are different based sequence and react differently to serum antibodies obtained from fish challenged with parasites from either the December 2006 culture or the March 2007 culture (Figure 1). This is a significant result and these proteins both require follow up vaccine trials. We have enough sequence data to use a molecular method (RACE; rapid amplification of cDNA ends) to obtain the full length cDNA (gene) sequence to produce recombinant proteins.

Limiting dilution experiments were conducted to optimize the number of parasites used to challenge fish in preparation for vaccine trials using purified parasite proteins. This work is the first to identify infectious dose 50 (ID_{50}) levels for *C. irritans*. This metric describes the dose of parasites required to infect 50% of fish in an experiment and allows for standardization of challenge experiments. Immersion challenge (direct exposure to theronts) was carried out on six groups of 15 fish (tilapia mean weight 30.1± 9.3 g /length12.3 ± 1.4 cm). *Cryptocaryon* theronts were suspended in 60 L of filtered, UV-treated seawater (28 ± 0.2 °C) at concentrations of 333, 3333, 10000, 20000, 30000, and 56667 theronts per fish (5000, 50000, 150000, 300000, 450000, and 850000 theronts per tank). The experiment was performed in duplicate a cumulative sample size of fish n=30 for each exposure dosage. Two days after the exposure, the number of infected parasites on the left pectoral fin was counted under the microscope (Figure 2, 3). Blood was collected and a number of serological assays performed. Immobilization assays were also conducted to better characterize what affect, if any, parasite dose has on antibody titers and immobilization. There was no significant difference between two replicates except the tanks exposed with 10K theronts per fish. All fish were infected by *C. irritans*.

The number of infected parasites on a pectoral fin is dose dependent and 50% of 0.3K infected fish did not show any trophont infection on fin. This experiment established an ID_{50} of 0.3K theronts for *C. irritans* infection and future vaccine trials will be conducted using this dose.
Summary of ID$_{50}$ experiment:

- The number of infected parasites as enumerated on the pectoral fin was dose dependent. This method can be used to monitor the effectiveness of future vaccine trials and is more standardized than performing skin swabs or scraping to enumerate level of infection.
- ID$_{50}$ was determined as 0.3 K theronts per fish.
- Although 50% of 0.3K exposed fish did not show any parasites on a pectoral fin, ELISA data showed all 0.3K exposed fish had an immune response against Cryptocaryon. We still need to find end points of ELISA for each sample. Then we can determine if antibody level is also dose dependent.
- Immobilization assay indicates that immobilization capacity is dose dependent. Antibody is involved in the immobilization response, so this result suggests that generation of protective antibody levels is dose dependent.
- The 50% lethal dose (LD$_{50}$) for Cryptocaryon infection was 85K theronts per fish. The 100% lethal dose (LD$_{100}$) for Cryptocaryon requires infection with over 100K theronts per fish.

After identifying what we considered a relevant infectious dose, a vaccine trial using moi was initiated. There are no commercial anti-moi IgM antibodies currently available to measure moi antibody responses via ELISA or immunoblot methods. We are working to develop anti-moi IgM using funding from another grant. Such an immunoreagent will have great utility studying moi antibody responses against various pathogens of interest in Hawaii. However, using immobilization as a metric we planned to determine whether moi respond immunologically in a way similar to tilapia and then use the two purified protein fractions (29KD and 28KD) identified in the tilapia trials. For this study, moi antibody responses were measured using the immobilization assay and serum was stored for ELISA and immunoblot analyses. These serological tests will be completed as soon the anti-moi IgM reagent is available.

Moi weighing 180 ± 20 g were used and the ID$_{50}$ dose of Cryptocaryon identified in the tilapia trails was used to infect fish (3300 theronts per fish). Three groups were included in the study:

Group 1: Fish were directly exposed to parasites by immersion (n=20). Theronts were suspended in 60 L of sea water (28 ± 0.2 °C) for one hour. After treatment to remove parasites, all fish were moved from 60 L tanks to one large tank (~500L). The same dose was used six weeks after primary exposure. The blood sample was collected nine weeks after the primary exposure and serum removed by centrifugation.

Group 2: Sonicated whole theronts (10 µg protein per fish) was injected i.p. into 20 fish. Fish received a secondary injection three weeks after primary injection with the protein emulsified in adjuvant (TiterMax Gold, Sigma Chemical). Blood was drawn six weeks after primary injection and serum removed by centrifugation for antibody analysis. One fish died before the challenge was conducted.

Group 3: PBS was injected into fish (n=20) and blood collected using the same schedule as group 2. Adjuvant was used at second injection but not in the primary injection.
Immobilization assays using established methods were done by adding 100 live theronts into serially diluted serum in microtiter plate wells and incubated for 30 minutes. The number of immobilized theronts was counted using an inverted microscope. Immobilization results are reported as the percentage of fish that generated antibodies capable of immobilizing ciliated theronts in the wells within 30 minutes.

<table>
<thead>
<tr>
<th>Group 1 (direct exposure):</th>
<th>40%, serum from 8 out of 20 fish immobilized theronts.</th>
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<tbody>
<tr>
<td>Group 2 (theronts injection):</td>
<td>10.5%, serum from 2 out of 19 fish immobilized theronts.</td>
</tr>
<tr>
<td>Group 3 (PBS injected):</td>
<td>0%, no immobilization of theronts.</td>
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The challenge experiment was limited due to the low availability of parasites available and tank restrictions. For each group, three fish were used for the challenge experiment and then the experiment was duplicated for a total of n=6. Fish were moved to a 60L tank and challenged with theronts. Percent mortality was the end point of this experiment.

<table>
<thead>
<tr>
<th>Group 1 (direct exposure):</th>
<th>0%, no fish died.</th>
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<tr>
<td>Group 2 (theronts injection):</td>
<td>50%, 3 out of 6 fish died.*</td>
</tr>
<tr>
<td>Group 3 (PBS):</td>
<td>83%, 5 out of 6 fish died.</td>
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*Serum was collected from the dead fish in Group 2 and these fish did not possess any immobilization antibodies.

The moi experiments reflect the species differences we see in susceptibility to Cryptocaryon. More work must be done to identify the optimal injection dose for moi to stimulate a protective antibody response and more parasites must be made available in order to increase the replicate size for challenge experiments. The final moi experiments will be conducted in the summer of 2008 and an amended final report submitted to CTSA. We are waiting for the availability of the anti-moi IgM so we can conduct ELISA and immunoblot analysis in tandem with the experiments using the 28 and 29 KD purified proteins. The fact every fish that was directly exposed to the parasite developed the ability to immobilize theronts and did not die upon subsequent challenge is quite promising. However, injection of sonicated parasites did not confer protection in moi to the same extent it does using tilapia. We need to repeat the ID$_{50}$ experiments using moi and repeat the experiment above using adjuvant with the primary injection as described for Group 2. When tilapia are injected with Cryptocaryon theronts (even without adjuvant), they develop strong immune responses. The amount of antigen injected may need to be increased to stimulate a protective response in moi.

The vaccine project was funded by CTSA for three years and a brief summary of findings is provided here. In the first year Cryptocaryon cultures were established using parasites that were naturally isolated from Kaneohe Bay. It was determined that the theront stage was the most immunogenic and ELISA and immobilization assays were validated in the lab. Tilapia was selected for the initial experiments since commercially available antibodies against tilapia IgM were available. The fact these tilapia are bred and reared in freshwater and only adapted to 5 µM filtered and UV treated seawater when they are used for Cryptocaryon experiments ensures the fish are immunologically naïve until immunized. This is a very important aspect of any vaccine study.
By the end of the first year and into the second the expression library immunization strategy initially proposed was pursued but had to be abandoned. Ciliates have a unique codon usage and we were unsuccessful in all attempts to create an expression library for *C. irritans*. It was decided to move forward using an immunologic approach to identifying antigenic proteins by taking serum from fish that were capable of immobilizing theronts *in vitro* and conducting immunoblot analysis. In this fashion, a 30 KD parasite protein was identified that serum from immunized fish but not naïve fish recognized. However, sequencing at this time by LC MS/MS was not very informative. Purification efforts to isolate the 30KD protein for vaccine trials resulted in the identification of 3 additional proteins (40, 34, and 19 KD) that were immunogenic based on immunoblot. A vaccine trial using these proteins (and the 30 KD protein) supported the hypothesis that the 30 KD protein was best suited for development as a subunit vaccine.

In the final phase of the project, we identified a second serotype of *C. irritans*. Although this result was not altogether unexpected (*I. multifiliis* possesses 5 characterized serotypes), it had not been an issue with previous studies. During follow up protein sequencing LC MS/MS analysis revealed the protein we thought was 30 KD was only 29 KD in length and the new serotype is a 28 KD protein. We have obtained enough protein sequence available to develop primers to obtain the full length cDNA sequence for these two proteins and work to produce recombinant proteins for use in vaccine trials. In the interim, moi were used in pilot studies to determine whether we had an optimum infectious dose based on the tilapia experiments. While immobilization and mortality metrics indicate moi can be protected against *Cryptocaryon* infection, additional vaccine trials must await the production of anti-moi IgM antibodies. Under a different research grant, rabbit anti-moi IgM antibodies are being produced at a commercial facility that will be used for ELISA and immunoblot analysis of vaccine trials scheduled for summer 2008.

**Objective 2.** To establish a continuous cell line from grouper (*Epinephelus lanceolatus*) and barramundi (*Lates calcarifer*) for viral and rickettsial disease diagnostics.

A number of primary cell lines were initiated from amberjack, flame angel, and Chinese catfish. We continued this from Year 17 but could not obtain grouper and barramundi for cell line development. A number of *in vitro* manipulations of primary cultures seemed promising but cells did not completely transform from primary to continuous cell lines. The ability to recover viable cells capable of re-establishing themselves in culture after cryopreservation was our first criteria for immortalization of cells. This was never accomplished. The methods used were adapted from cell line development protocols optimized by Yuanan Lu in previously funded CTSA research (Zhao et al. (2003) Meth Cell Sci 25:155-166; Zhao & Lu (2006) Dis Aquat Org 68:91-100).

In brief, tissue explants were collected from fish (liver, heart, spleen, and muscle) using sterile technique in a laminar flow hood. Tissues were minced and put into 25 cm² culture flasks (Corning) or processed through sterile mesh material to produce a single cell suspension and seeded into sterile 24 well tissue culture plates (Corning). The media used to maintain cultures was Leibovitz-15 (L-15) (Gibco/Invitrogen) containing 20% fetal bovine serum, 100 mM GlutaMax, epidermal and fibroblast growth factors (25 ng per ml each factor; BD Biosciences) and antibiotics (100 U streptomycin sulfate, 100 µg penicillin, 5 µg amphotericin B, and 50 µg
gentamicin sulfate per ml; all supplements from Invitrogen). Cultures were incubated in ambient temperature and no CO\textsuperscript{2} incubation was used since L-15 is formulated for growth in ambient air. Although cells would proliferate \textit{in vitro} (muscle fibroblasts only) most cultures dies out by 10 rounds of subculture. Cryopreservation at -80ºC in FBS containing 20% DMSO at a density of 2 million cells per 1 ml vial was sufficient to keep protect cells from ice crystal lysis but all cells were dead when thawed and put back into culture conditions using established fish cell line protocols.

\textbf{Objective 3.} To establish a pathogen screening program for companies that routinely import and breed freshwater ornamentals.

A total of 527 imported freshwater ornamental fish were examined for the presence of parasites. There were 19 different species represented in the study from 11 countries of origin. The work plan called for the microscopic examination of gill, skin, intestine, kidney, liver, spleen, gall bladder, muscle, and brain of each fish. Parasites were only observed in the skin, gill, and intestine samples. Ciliates and monogenean trematodes were observed microscopically on the skin of seven species of fish. There were a total of eight cases where ciliates, flagellates, digene and monogene trematodes were observed on the gills of fish. Flagellates and nematodes were observed in the intestine of two fish species of fish. The results are listed in Table 1.

\textbf{Objective 4.} Evaluate the efficacy of cleaner fish as a non-chemical method to control ectoparasite levels in marine broodstock populations.

Ectoparasite infestations continue to be the most frequently observed disease affecting the aquaculture of marine fishes in Hawaii. The problem is especially evident in broodstock holding operations where most stocks are wild-collected and must be maintained in a disease-free state for extended periods of time. Ectoparasites are also a significant concern for the offshore cage industry where wild fish populations attracted to cages present an uncontrollable vector for parasite transmission. A number of prophylactic techniques including lowered salinity and treatment with non-specific chemicals such as hydrogen peroxide and formalin represent the only approved treatments. However, in addition to creating considerable stress on valuable broodstock, these treatments offer only temporary relief in part due to the complex life cycles of many parasitic species. In the wild, many reef-associated fish develop synergistic relationships with “cleaner species” of shrimp and fishes to aid in parasite removal. This study explores the use of cleaner wrasses in comparison with a number of more traditional quarantine treatment protocols (hydrogen peroxide and hyposalinity) for quarantine of newly collected yellow tang.

Specimens of the Hawaiian cleaner wrasse (\textit{Labroides phthirophagus}) were obtained through a local aquarium supplier and adapted to culture tank conditions at OI. Fish were initially acclimated in 75L flow through tank systems provided with artificial cleaner stations made from 1 and 2” PVC pipe to familiarize them with the artificial structure. Fish were provided chopped fish, shrimp and commercial aquarium diets during the acclimation period.

In preliminary trials, cleaner wrasse (with their PVC cleaning stations) were introduced into a number of broodstock tanks systems for us to gain experience with the species, and test for cleaning behaviors. The Hawaiian cleaner wrasse readily adapted to captive holding conditions.
and soon initiated fish inspection and cleaning behaviors in tanks of yellow tang, Potters angelfish and kahala. Interestingly, the cleaner wrasses did not initiate cleaning behaviors with the Pacific threadfin (moi). Although successful in establishing cleaning behaviors, long-term maintenance of the Hawaiian cleaner wrasse in captivity proved quite challenging for several reasons. Firstly, wrasses maintained in larger tanks often disappeared within a couple of weeks of stocking, either lost down the drain or due to predation by larger fish. Secondly, captive wrasses lost condition in captivity (despite eating fish feed) and eventually died. This suggests a need to develop a more suitable feed to maintain these fish in captivity. In tanks with successful cleaning behaviors, the cleaner wrasses showed hovering and flashing behaviors to attract host fish and the host fish responded by allowing repeated cleaning throughout the day. At first, host fish appeared to seek wrasse cleaning, but after several days become less receptive to wrasse cleaning activities.

In collaboration with HIMB researchers, a full-scale yellow tang quarantine trial was run to test the effectiveness of cleaner wrasses in comparison with control (non-treated) recruits, fish receiving freshwater dips (2 min.) followed by repeated hydrogen peroxide (1h at 100ppm) treatments three times a week for four weeks, and fish undergoing a one-month hyposalinity (10ppt) treatment. Tanks (4m$^3$) were run in parallel with 15 tang and one cleaner fish per tank, with two replicates per treatment group. Artificial reefs (4 per tank) consisting of connected 6 inch PVC pipe pieces were placed into each tank to provide hiding places for the fish, and to help reduce the aggressive interactions among the fish. For the hyposalinity treatment, salinity was slowly lowered from 32 to 10 ppt over 4 days. Each tank received approximately four turnovers of water per day. All fish were fed three times daily, a variety of commercial marine ornamental fish feed (Marine Grow, Ocean Nutrition F2, Spirulina Flakes, Spectrum Diet) as well as sheets of nori. Tanks were cleaned as needed to reduce excess buildup of algal growth. In addition, a series of challenge tanks were setup at HIMB with Neobenedenia infested netting added to tanks containing yellow tang and cleaner wrasses to see if the cleaner wrasses specifically consumed this key monogenean parasite. Tanks were monitored for survival throughout the trial and sampled at the end of the trial to examine for residual parasite loads. The highest rates of mortality were seen in control and cleaner wrasse treatment groups which did not receive chemical or hyposalinity treatment (Figure 4). This was also true for the tanks set up at HIMB. These higher rates of mortality were mainly attributed to a series of Cryptocaryon outbreaks, which affect not only the yellow tang, but also the cleaner wrasses stocked with the yellow tang. It is unclear whether the cleaner wrasse were able to keep Cryptocaryon in check at OI, but it was noted that all the fish in one of the cleaner wrasse treatment tanks succumbed to Cryptocaryon soon after the loss of the cleaner wrasse and that all fish survived the tank containing a surviving wrasse. At HIMB, all fish were affected by Cryptocaryon early in the study prior to any Neobenedenia hatching from the netting material. The fish were housed in filtered, treated water but came in a low parasite burden. This was unexpected because HIMB staff thought the fish had been treated for parasites by the collector prior to transfer. While the cleaner wrasse were healthy, parasite burden on the tang was low but the wrasse rapidly developed Cryptocaryon infections as well and the experiment was halted. Both hyposalinity and hydrogen peroxide treatments proved effective in controlling Cryptocaryon through the quarantine period, although there was a noticeable loss of condition,
appearance of ulcerations, increased mucous production and some mortality in the fish toward
the end of the hyposalinity regimen. Further, these stocks were less robust and had a difficult
time recovering from anesthesia treatment, compared to the other treatments. However, the low
salinity treatment is maintained throughout the treatment period, in contrast to intermittent
peroxide treatments which may be less likely to fully eradicate Cryptocaryon and other parasites
with more complex life cycles.

Examination of fish (n=5 per tank) at the end of the four week quarantine period revealed the
presence of monogene tremadodes in 40% of the control (non-treated) fish but not under any of
the three quarantine treatments. The hydrogen peroxide and hyposalinity treatments also
appeared effective in getting rid of Cryptocaryon, although previous experience with hydrogen
peroxide treatments suggests that residual tomonts remain can later re-infect fish. However, both
treatments (peroxide and hyposalinity) did cause some damage to fish with epithelial
hypertrophy in one of the fish under peroxide treatment and excess gill and skin mucus
production in fish under the hyposalinity treatment. Similarly, the cleaner wrasse treatment
group, in addition to experiencing Cryptocaryon related mortality, also developed redness at the
base of pectoral fins, skin abrasion and early signs of head and lateral line erosion.

**Objective 5.** Technology transfer of project results.

Dissemination of the results from each Objective is an important aspect of this project. Results
are informative to the industry and to researchers in the field of aquatic animal health and
without some means of technology transfer there is no benefit to performing the research.
Technology transfer is ongoing for each Objective. This is being accomplished through a series
of reports in the CTSA Regional Notes newsletter, pertinent peer-reviewed journal articles, video
footage, and conference presentations.

**Impacts**
The results from this project and the culmination of three years research into vaccine
development against *C. irritans* has yielded exciting information that illustrates the potential for
an effective vaccine is real. The Disease Management project has contributed much to a basic
understanding of disease issues in the region.

Establishing the effectiveness of a vaccine against *C. irritans* using purified parasite proteins is
the first step to commercial development of a vaccine with great benefit to regional aquaculture.
Thousands of dollars are spent annually in local hatcheries monitoring for parasite outbreaks and
disinfecting affected fish and systems if you calculate man-hours and supplies required for the
task. The research accomplished during this phase of the project has identified two parasite
proteins that will be a necessary component of an effective multivalent Cryptocaryon vaccine.
Vaccination will provide a significant economic impact to regional aquaculture. Characterization of the fish immune response against this parasite suggests an immersion vaccine
strategy will confer the best protection. If additional experiments support this observation the
impact will be quite positive for industry since immersion is the most economical approach in
terms of labor and supply costs.
In terms of financial impact from the onsite stock/system assessments performed in Objective 3, approximately $8,000 was saved by a local producer during an outbreak of an ectoparasite disease detected during clownfish health exams conducted as part of a CTSA-funded site visit (Dollar value supplied by owner and is based on the fact that there was a high mortality previous treatment and zero mortality post-treatment).

**Recommended Follow-Up Activities**

This is the final report for the Year 18 project and includes a comprehensive summary of the *C. irritans* vaccine trials funded for the past three years. Additional work arising from this study includes identifying as many parasite serotypes as possible and the production of recombinant parasite proteins (28 and 29 KD) for development of a multivalent recombinant vaccine. Anti-moi IgM will be available sometime in 2008 and ELISA and immunoblot assays will be done once this reagent is available. An update to this report will be submitted to CTSA once this work is completed. Ectoparasites will remain a health management issue until cost-effective treatments are developed. An effective *C. irritans* vaccine will be very beneficial to aquaculture hatcheries as well as the marine ornamental trade.

Fish cell line development for regional species of interest should continue although efforts this year were unsuccessful in creating new immortalized lines. Introduction of viral pathogens with imports or invasive fish introductions and emerging viral disease is a critical area of health management in the region. The ability to identify cytopathic effect in culture remains the gold standard for diagnosis of viral etiology, especially for a virus that hasn’t been previously documented in fish species cultured in the region.

**Publications, Manuscripts, or Papers Issued, Approved, or Presented**

Two manuscripts are in preparation reporting the results of the *C. irritans* vaccine research. Two reports were published in the CTSA Regional Notes describing work accomplished during this project (Objective 1 and 3) and Dr. Lewis will report on the *C. irritans* serotype results at the World Aquaculture Society meeting in Busan Korea in May 2008 (see Appendix). The graduate student, Ichiro Misumi, presented results at the 10th International Developmental and Comparative Immunology Congress in Charleston SC in July, 2006 and at an invited research seminar in Japan. Manuscripts will be forwarded to the CTSA Publications Office once accepted for publication.

**Publications in Print**


Manuscripts (in preparation)

Misumi, I. Development of vaccine against Cryptocaryon irritans for aquaculture fish. UH Manoa Microbiology Department. Ph. D. dissertation.


Papers Presented


Lewis TD, I Misumi, JC Leong. Vaccine research leads to identification of distinct serotypes of Cryptocaryon irritans. Abstract accepted for oral presentation at the 2008 World Aquaculture meeting in Busan, Korea. Meeting will be held May 19-23, 2008.
APPENDIX

Figure 1. Immunoblot illustrating (a) molecular weight markers or *C. irritans* from (b) December 2006 or (c) March 2007. In panel 1, the proteins are reacted with serum from fish exposed to parasites from the December culture prior to exposure (time 0). In panel 2, the proteins are reacted with serum from fish exposed to parasites from the March culture prior to exposure (time 0). In panel 3, the proteins are reacted with serum collected from fish approximately one month post-exposure to parasites from the December culture (note positive band in lane b and absence of band in lane c, indicating serum recognizes only one band and only from the December culture. In panel 4, the proteins are reacted with serum from fish approximately one month post-exposure to parasites from the March culture (note absence of band in lane b and presence of band in lane c, indicating serum recognizes only one band and only from the March culture.

Figure 2. The average number of *Cryptocaryon* trophonts on the left pectoral fin of tilapia two days post primary exposure (n=30). These results are based on combined data of two replicates.
Figure 3. Images of tilapia pectoral fin used to enumerate the level of infection. Top two images were visualized using a dissecting stereomicroscope and the bottom two images visualized using a standard compound microscope.
Table 1. Parasites identified from a survey of imported freshwater ornamental fish and percent prevalence for each parasite identified. N.P. = no parasites found.

<table>
<thead>
<tr>
<th>Family Name</th>
<th>Common Name</th>
<th>Country of Origin (n=11)</th>
<th>Skin</th>
<th>Gill</th>
<th>Intestine</th>
</tr>
</thead>
<tbody>
<tr>
<td>Belontiidae</td>
<td>Sparkling gourami</td>
<td>Indonesia</td>
<td>N.P.</td>
<td>Trichodina 5%</td>
<td>nematodes 100%</td>
</tr>
<tr>
<td>Characcidae</td>
<td>Neon Tetra</td>
<td>Hong Kong</td>
<td>Ichthyobodo 5%</td>
<td>Ichthyobodo 5%</td>
<td>Hexamita 5%</td>
</tr>
<tr>
<td>Cichlidae</td>
<td>Oscar</td>
<td>Thailand</td>
<td>Trichodina 5%</td>
<td>Dactylogyrus 70% digenes 100%</td>
<td>N.P.</td>
</tr>
<tr>
<td>Cichlidae</td>
<td>Electric yellow cichlid</td>
<td>Taiwan</td>
<td>Chilodonella 100%</td>
<td>Trichodina 100%</td>
<td>N.P.</td>
</tr>
<tr>
<td>Cobitidae</td>
<td>Clown loach</td>
<td>Indonesia</td>
<td>Gyrodactylus 15%</td>
<td>N.P.</td>
<td>N.P.</td>
</tr>
<tr>
<td>Cobitidae</td>
<td>Clown loach</td>
<td>Malaysia</td>
<td>Trichodina 100%</td>
<td>Ichthyobodo 100%</td>
<td>N.P.</td>
</tr>
<tr>
<td>Cyprinidae</td>
<td>Goldfish</td>
<td>China</td>
<td>Gyrodactylus 100%</td>
<td>N.P.</td>
<td>N.P.</td>
</tr>
<tr>
<td>Cyprinidae</td>
<td>Hi-fin shark</td>
<td>Singapore</td>
<td>Trichodina 2.5%</td>
<td>Digenes 100%</td>
<td>N.P.</td>
</tr>
<tr>
<td>Cyprinidae</td>
<td>Shubukin</td>
<td>USA</td>
<td>Trichodina 2.5%</td>
<td>N.P.</td>
<td>N.P.</td>
</tr>
<tr>
<td>Cyprinidae</td>
<td>Koi</td>
<td>USA/Malaysia</td>
<td>N.P.</td>
<td>Dactylogyrus 10%</td>
<td>N.P.</td>
</tr>
<tr>
<td>Cyprinidae</td>
<td>Rainbow albino shark</td>
<td>Thailand</td>
<td>N.P.</td>
<td>Ichthyobodo 20% digenes 40%</td>
<td>N.P.</td>
</tr>
<tr>
<td>Cyprinidae</td>
<td>Rainbow albino shark</td>
<td>Taiwan</td>
<td>N.P.</td>
<td>Digenes 100%</td>
<td>N.P.</td>
</tr>
<tr>
<td>Gobidae</td>
<td>Dragon goby</td>
<td>Ecuador</td>
<td>Chilodonella 100%</td>
<td>Chilodonella 100%</td>
<td>N.P.</td>
</tr>
<tr>
<td>Poeciliidae</td>
<td>Sailfin molly</td>
<td>Singapore</td>
<td>Trichodina 5%</td>
<td>Gyrodactylus 5%</td>
<td>N.P.</td>
</tr>
<tr>
<td>Poeciliidae</td>
<td>Lyretail swords</td>
<td>Malaysia</td>
<td>N.P.</td>
<td>Digenes 10%</td>
<td>N.P.</td>
</tr>
<tr>
<td>Poeciliidae</td>
<td>Hi-fin swords</td>
<td>Singapore</td>
<td>Gyrodactylus 15%</td>
<td>N.P.</td>
<td>N.P.</td>
</tr>
<tr>
<td>Tetraodotidae</td>
<td>Green puffer</td>
<td>Vietnam</td>
<td>N.P.</td>
<td>Ichthyobodo 100%</td>
<td>N.P.</td>
</tr>
</tbody>
</table>
Figure 4. Survival of yellow tang (*Zebrasoma flavescens*) exposed to various treatments to help control parasites at the Oceanic Institute.