Disease Management in Pacific Aquaculture, Year 11

General Information

Reporting Period

December 1, 2004–November 30, 2005 (Year 11 — M-Brock/OI Component, final report)

August 1, 2005–June 30, 2006 (Year 11 — HIMB Component, final report)

Funding Level

Year	Amount	Year	Amount
1	$41,638	7	$81,991
2	$63,725	8	$67,902
3	$45,956	9	$89,529
4	$44,030	10	$88,346
5	$66,451	**11**	**$100,000**
6	$51,934		
		TOTAL	$741,502

Participants

Teresa D. Lewis, Ph.D., Assistant Researcher
Hawaii Institute of Marine Biology (HIMB), University of Hawaii at Manoa

Ichiro Misumi, Ph.D., Student in Microbiology
Hawaii Institute of Marine Biology, University of Hawaii at Manoa

Dee Montgomery-Brock, Aquatic Health Specialist
Aquaculture Development Program, Hawaii Department of Agriculture

Objectives

1. Generate *Cryptocaryon irritans* expression libraries and initiate immunization/challenge trials in Pacific threadfin (moi). (The final objective in this project will be performed in Year 12: Identify and characterize antigens in the efficacious vaccine fractions, and characterize the antibody response in moi against *C. irritans*).
2. Identify the etiologic agent resulting in high mortality of juvenile Chinese catfish (*Clarias fuscus*).
3. Establish continuous cell lines for three fish species, longfin amberjack (*Seriola rivoliana*), flame angelfish (*Centropyge loriculus*), and the Chinese catfish (*Clarias fuscus*) for viral and rickettsial disease diagnostics in cultured and wild populations of fish.
4. Provide on-site workshops with individual aquaculture farmers in the area, examining water quality and general fish health.

Principal Accomplishments

Objective 1: Generate Cryptocaryon irritans *expression libraries and initiate immunization/challenge trials in Pacific threadfin (moi).*

It was determined that the free-swimming form of the parasite (theront) is the most immunogenic stage of the parasite and, therefore, the best one suited for development of expression libraries. This form of the parasite possesses cilia, and cilia facilitate establishment of infection on the surfaces of fish. Parasites move across fish surfaces actively feeding and eventually embed themselves into a fish's surface, forming a trophont. Using an immobilization assay originally developed for *Ichthyopthirius multifiliis*, we confirmed that serum (containing antibodies) from control fish do not immobilize *C. irritans* theronts, while serum from fish that were infected and then resolved the infection actually inhibit ciliary motion of the parasite (Figure 1). The fish immunized with theronts are still protected against re-infection with *C. irritans* 12 months later (periodic challenges are ongoing).

Of interest, immobilization assays using parasites hatched at different times in the laboratory reveal variation in the immobilization antibody response from vaccinated fish. Following what has been reported for *I. multifiliis*, this variation suggests that *C. irritans* possesses different serotypes, and we will need to study this further in the final year of the project. For initial experiments, tilapia were used (rather than moi) as they are easily spawned and reared in freshwater at HIMB

and can be acclimated to seawater immediately prior to use in *C. irritans* experiments. This method ensures the fish are naïve to prior exposure to the parasite. This aspect of our work is critical in the initial phases of our experiments, as prior exposure to the parasite may influence the results of immunization and challenge trials.

In the summer of 2005, two summer interns (sponsored by a UH-Manoa Marine Science Undergraduate Research Fellowship and a Howard Hughes Medical Institute Undergraduate Fellowship) assisted with the project, and the project was split along two separate tracks. The expression library construction was undertaken a third (and final) time by one student, and the other student was assigned to work with the graduate student on the project, Mr. Misumi, to identify any *C. irritans* proteins that were reactive with antibodies from immunized fish. The expression library construction was unsuccessful, and we believe this DNA approach may have failed due to the unique codon usage that occurs in ciliates. We have abandoned this approach but continue with vaccine development, as we were able to identify an immunogenic parasite protein using the more traditional method described below.

To identify possible immobilization antigens (i-antigens) from the parasite, *Cryptocaryon* theronts were sonicated in a buffer containing protease inhibitors and then assayed for total protein concentration. In other ciliates, both free living and parasitic, i-antigens have been identified ranging from 30-60 kilodalton (kD). We hoped to identify one or more i-antigens from *C. irritans* in this same range. The proteins were separated electrophoretically on a gel (by molecular weight) and then transferred to a PVDF membrane. Immunoblotting was performed to see which protein(s) fish antibodies recognized and whether this protein was found in samples from both immunized fish and control fish. A 30-kD molecular weight protein was observed to be immunoreactive with fish antibodies from the immunized fish and not recognized by fish antibodies in the control fish (Figure 3).

After the immunogenic protein was identified, additional samples of *C. irritans* proteins were separated 10% acrylamide gels and stained with Coomassie blue to identify the 30-kD protein. This band was excised (multiple copies) and sent for sequence analysis by LC MS/MS. This method varies from the more traditional N- or C-terminal protein sequencing method as 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 are then electronically compared with archived sequences from various databases to possibly identify the purified protein. Unfortunately, the 30-kD protein we sent for sequencing was determined to be unique. This may be interpreted in one of two ways: (1) The protein sequence obtained is truly unique from other proteins, or (2) more likely, there are not yet

enough archived sequences from ciliated organisms to have compiled a comprehensive database for comparative analyses.

At the writing of this final report, we are purifying more of the 30-kD protein for N-terminal sequencing and for a vaccine trial with moi. The HIMB wetlab facility is going to be renovated to a completely recirculating system, and the vaccine trial will be initiated once this renovation is complete.

*Objective 2: Identify the etiologic agent resulting in high mortality of juvenile Chinese catfish (*Clarias fuscus*).*

Numerous site visits were made to the facility where this disease was previously responsible for high mortality among catfish populations. However, we were not able to find fish displaying clinical signs of this disease. Some fish from the facility were sacrificed in the hopes of detecting the agent for this disease. However, all were negative by gross exam, histopathology, and bacteriology.

*Objective 3: Establish continuous cell lines for three fish species, longfin amberjack (*Seriola rivoliana*), flame angelfish (*Centropyge loriculus*), and the Chinese catfish (*Clarias fuscus*) for viral and rickettsial disease diagnostics in cultured and wild populations of fish.*

A number of primary cell lines were initiated from amberjack, flame angel, and Chinese catfish fibroblasts, but none of these lines transformed into continuous (immortalized) cell lines. This work will continue into the next funding cycle (with no additional funding requested for work with these species) and transfections will be performed to produce immortalized cells since natural *in vitro* selection of transformed cells did not occur during this year's tissue culture experiments.

Objective 4. Provide on-site workshops with individual aquaculture farmers in the area, examining water quality and general fish health.

Summary of CTSA/ site visits conducted in 2005

Number of aquaculture sitesvisited: 8

Number of water quality tests conducted: 272

Number of wet mount microscopic exams: 68

- Of the eight sites visited, four of them are on the island of Hawaii.
- Four of the sites visited were in the business of culturing ornamental fish.
- Three of the sites visited were in the business of raising fish for human consumption.
- One of the sites visited is in the business of raising crawfish for human consumption.

Impacts

Cryptocaryon irritans possesses very broad host specificity and has caused significant disease problems in many fish species in marine aquaculture and in private aquaria. Therefore, it is of concern to essentially all marine finfish aquaculture endeavors in the region as well as on a larger scale. Development of an effective vaccine will be beneficial to both food and ornamental finfish trades by limiting the losses that traditionally occur due to *Cryptocaryon* in cultured fish populations.

In terms of financial impact from the on-site stock/system assessments, about $8,000 was saved by a local producer during an outbreak of an ectoparasite disease detected while conducting clownfish health exams as part of a CTSA-funded site visit (Dollar figure supplied by owner and is based on the fact that there was a high mortality previous to treatment and zero mortality post-treatment).

Currently, the main diagnostic cell lines used for fish virus diagnostics are derived from carp and salmon. Viruses that thrive in other species often cannot be cultured in these cell lines. Therefore, development of cell lines for other locally important aquaculture species will greatly enhance our ability to diagnose viral pathogens.

Recommended Follow-Up Activities

We have concluded the second year of vaccine development, focusing on the objective outlined in the Disease Management in Pacific Aquaculture, Year 11, proposal. Although we were unsuccessful in generating an expression library for *Cryptocaryon*, using a different method we have identified an immunoreactive parasite protein from immunized fish that is a putative i-antigen. This protein is being purified for use in immunization/challenge trials. This protein was determined to be unique after sequencing using a LC MS/MS method, and we will follow up with additional sequence analysis using N-terminal sequencing in order to identify primers of utility for producing a recombinant protein in tandem with the immunization trials. If we see significant protection from the immunization trials, we plan to produce a recombinant protein suitable for use as a subunit vaccine and will determine the most effective delivery method at that time

Publications in Print, Manuscripts, and Papers Presented

Misumi, I. J. L. Keffer, and T. D. Lewis. 2006. AquaTips: Vaccine development against Cryptocaryon irritans — a progress report. Center for Tropical and Subtropical Aquaculture *Regional Notes* 16(4):4–5,10.

Montgomery-Brock, D., C. S. Tamaru. 2005. Weighing the risks of imported commodity shrimp to Hawaii's SPF shrimp industry. USMSFP *Industry Briefs* 11(2):3.

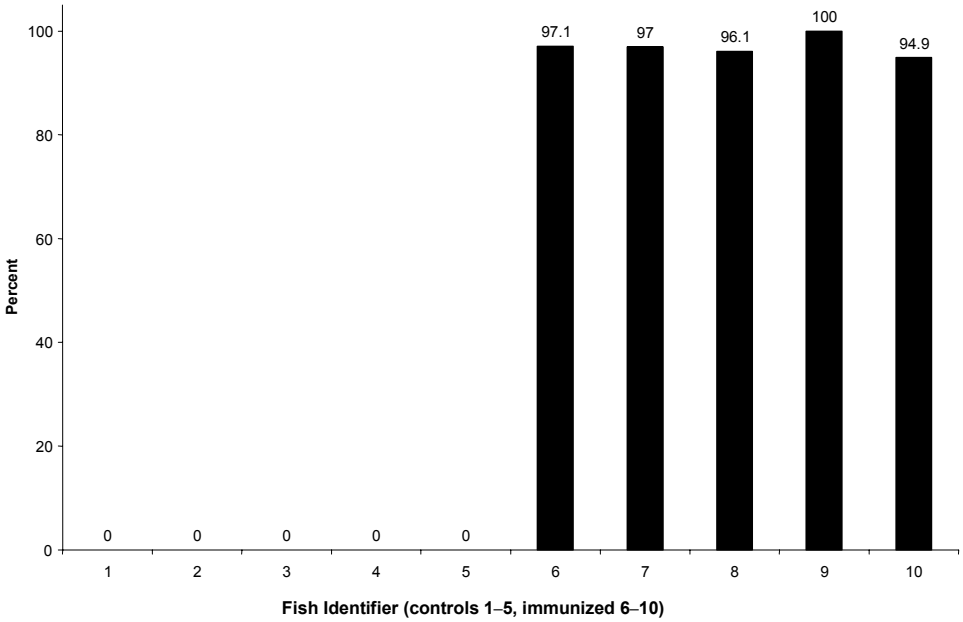
Keffer, J., I. Misumi, T. D. Lewis. 2006. Isolation and identification of the proteins of *Cryptocaryon irritans* responsible for soliciting the immune response in fish. Paper presented at the Ocean Sciences Meeting — a joint meeting of The American Society of Limnology and Oceanography (ASLO), Estuarine Research Federation (ERF), The Oceanography Society (TOS), and American Geophysical Union (AGU), February 20–24, in Honolulu.

Lewis, T. D. 2006. Vaccine development for use in Hawaiian aquaculture. Presentation made as an invited seminar held by the Department of Human Nutrition, Food, and Animal Sciences, of the college of Tropical Agriculture and Human Resources, University of Hawaii at Manoa, May 1, in Honolulu.

Misumi, I., T. D. Lewis, and J. C. Leong. 2006. Immune response of fish to saltwater ich, *Cryptocaryon irritans*, and identification of an antigenic protein. Presentation made at the meeting of the Hawaii chapter of the American Society of Microbiology, April 22, in Honolulu.

Appendix

FIGURE 1. In the five control samples, none of the fish were capable of immobilizing the parasite. For the five groups immunized against C. irritans*, an overall average of 97% of theronts was immobilized. If a parasite cannot move freely with ciliary action, then it cannot establish an infection on the skin surface or gills of fish.*



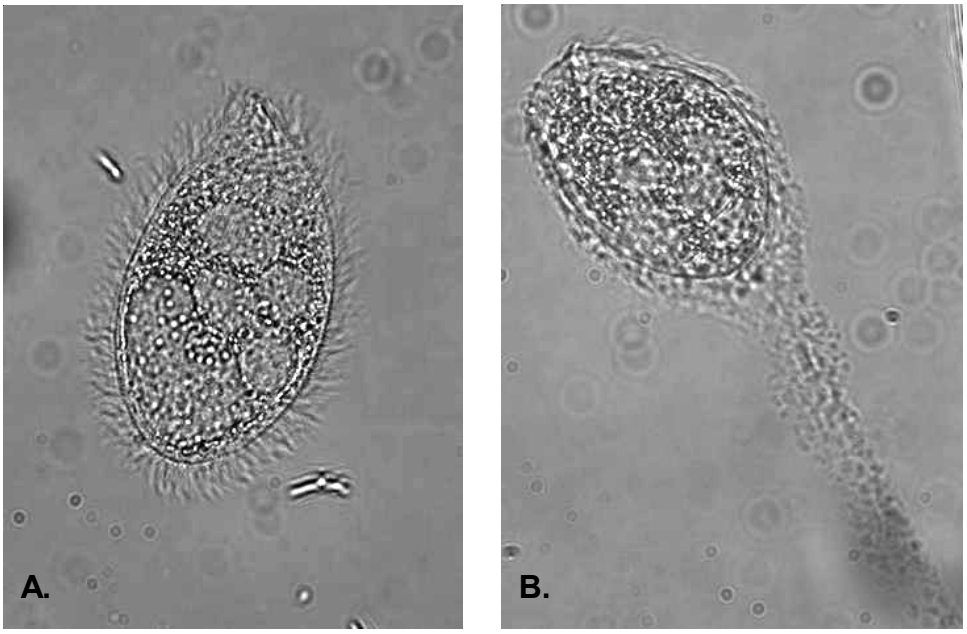


FIGURE 2. Note the differences in cilia between the (A) free-swimming theront and the (B) immobilized theront. These C. irritans *theronts were co-incubated with tilapia serum from (A) control fish that have never been exposed to* C. irritans *and from (B) fish who have recovered from a previous* C. irritans *infection, resulting in clumping of the cilia and immobilization of parasite.*

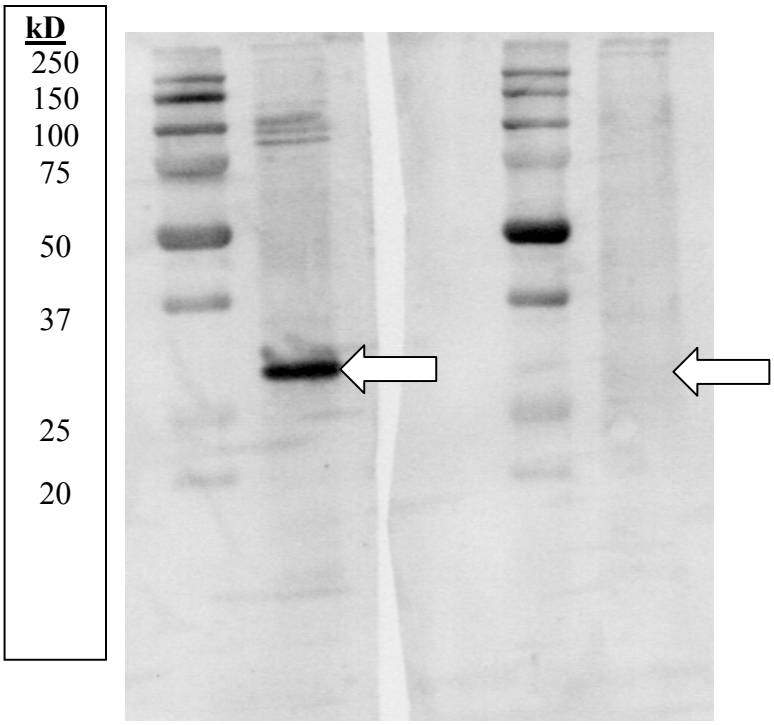


FIGURE 3. Immunoblot (A) of C. irritans *proteins when incubated with fish serum from an immunized fish. The left lane of the membrane contains a dual-color, pre-stained, broad-range protein standard. Approximate sizes (kD) of the protein bands are labeled at left. The lane adjacent to the molecular weight standard contains the non-reduced proteins of* C. irritans *with a prominent band reactive at about 30 kD (arrow). Immunoblot (B) of* C. irritans *proteins when incubated with fish serum from a freshwater (control) fish. The 30-kD band seen in A is not recognized by antibodies in B, the sample from a control fish.*