CTSA Final Report

Project
Risk Assessment to Identify Potential Shrimp Virus Impacts in Hawaii and Development of Biosecurity Protocols

Principal Investigator
Teresa D. Lewis, Ph. D.

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Participants
Teresa D. Lewis, Ph. D., Assistant Researcher
Hawaii Institute of Marine Biology, University of Hawaii - Manoa.

Dee Montgomery-Brock, Research Technician
(previously HDOA Hawaii Aquaculture Development Program, then consultant at Oceanic Institute), now contract staff at the Hawaii Institute of Marine Biology, University of Hawaii at Manoa.

Amy Eggers, Research Technologist
Hawaii Institute of Marine Biology, University of Hawaii – Manoa.

Clyde Tamaru, Ph. D. Aquaculture Specialist
Sea Grant College Program, University of Hawaii – Manoa.

Don Lightner, Ph. D., Professor and Director
Aquaculture Pathology Laboratory, University of Arizona.

Feng-Jyu (Kathy) Tang-Nelson, Ph. D.Associate Research Professor
Aquaculture Pathology Laboratory, University of Arizona.

Rita Redman, Research Specialist
Aquaculture Pathology Laboratory, University of Arizona

Executive Summary
The focus of the project “Risk Assessment to Identify Potential Shrimp Virus Impacts in Hawaii and Development of Biosecurity Protocols” is a risk assessment involving potentially infectious commodity shrimp, farmed shrimp, and local decapod crustaceans. It is a well established fact that viral diseases have had a profound effect on commercial shrimp farming globally and that

biosecurity measures are needed to protect shrimp production facilities. In the past, Hawaii's shrimp industry has been affected by Taura Syndrome Virus (TSV) and Infectious Hypodermal and Hematopoietic Necrosis Virus (IHHNV), as well as an outbreak of White Spot Syndrome Virus (WSSV) on Kauai. More recently, an outbreak of TSV occurred in 2007 for which no source (i.e., introduction of new stock) could be identified. This highlights the need for a risk assessment in Hawaii to determine whether there is a baseline of virus already found in local species. Our efforts included a survey of local decapod crustacean species and commodity shrimp for "the Big Four" shrimp viruses reportable to the OIE (WSSV, IHHNV, TSV, and Yellowhead virus (YHV)) as well as transmission studies (bioassay) to determine the susceptibility of local crustacean species to these viruses.

Results from this study include more data about the risk posed by commodity shrimp purchased in local retail outlets, identification of virus susceptible species of decapod crustaceans in Hawaii, and information about the actual risk of transmission of virus from infected commodity shrimp to wild species to farmed shrimp (or from infected imported shrimp to farmed shrimp to wild populations of crustaceans). Results provide insight into the pathways through which introductions may occur and about spread potential. Based on these results, it is obvious the most serious risk to shrimp aquaculture in Hawaii is posed by the continued import of infectious shrimp virus into the State.

Wild-caught and commodity samples were collected from four islands (Oahu, Kaui, Molokai and the Big Island) and tested for viruses of concern (WSSV, IHHNV, TSV, and Yellowhead Virus (YHV)). No samples tested positive using PCR or RT-PCR methods described in the OIE Manual of Diagnostic Tests for Aquatic Animals (2006). Commodity shrimp testing conducted from samples purchased on these same islands identified virus-positive shrimp on all four islands. Bioassays using crabs from Kaneohe Bay showed WSSV that is first step PCR positive using an established two-step PCR (Lo et al. 1997) is transmissible *per os* and can cause mortality in these species.

A portion of our CTSA Year 20 project funds the continuation of this project, allowing testing of plankton species for shrimp viruses. Inclement weather affected some of the sampling success outer-island and since live plankton samples will also be used for bioassay experiments, especially for opae and small shrimp, some of the bioassay experiments proposed for the Year 19 scope of work will be conducted during the second year of the project. A complete summary of all bioassays will be submitted with the Year 20 report.

Objectives

1. Survey and sample wild decapod crustacean populations for the presence of WSSV, IHHNV, TSV, and YHV.
2. Sample local commodity shrimp, from grocery and bait shops, for WSSV, IHHNV, TSV, YHV, and for IMNV (Infectious Myonecrosis Virus) when the shrimp originates from Brazil.

3. Conduct bioassays feeding infected commodity shrimp to wild caught decapod crustaceans representing standing stocks of local populations to test transmissibility of viral pathogens.
4. Conduct bioassays feeding naturally or experimentally infected crustaceans to SPF shrimp to test for transmissibility and provide diagnostic support, pathogen testing, and disease surveillance services to local producers to establish baselines of pathogen prevalence in cultured shrimp populations.
5. Research results obtained from Objectives 1-4 will be used to identify where existing biosecurity measures are sufficient for individual farms and where refinements may improve biosecurity. An analysis of current best management practices/standard operating procedures within the industry will be performed in collaboration with local farmers.
6. Technology transfer.

Principal Accomplishments

Objective 1. *Survey and sample wild decapod crustacean populations for the presence of WSSV, IHHNV, TSV, and YHV.*

Collection trips from four islands were conducted (Oahu, Kauai, Molokai, and the Big Island). All collections were covered by a scientific collection permit issued by the Hawaii Department of Land and Natural Resources Division of Aquatic Resources.

The first trip was made to the island of Molokai. Crustaceans were collected from two different environments. The first area was off the southwestern coast of Molokai where various species of crab and one native shrimp species were collected the from shoreline off a mangrove area. The second site was a fresh water stream located in southeastern Molokai, where opae were collected**.** Three sites were sampled in and around Hilo during the second trip. Once collection was done off the rocky shoreline of Hilo Bay. Numerous types of crabs were collected at this site. Crayfish were collected from a second Big Island site, the popular waterfall park called Boiling Pots. Shrimp and crabs were collected at the third site, Onekahaka Beach Park. The third survey was conducted in the area of Kailua-Kona on the Big Island of Hawaii. Several species of crabs were collected from beach parks and a small local shrimp were collected from an alkaline pond located adjacent to a beach area. The fourth sample set was collected from the island of Kauai. A number of crabs and local shrimp were collected from the beaches at Hanalei Bay and at Hanapepe. Opae and crabs were also sampled from Oahu, primarily from sites around Coconut Island and Heeia in Kaneohe Bay.

The samples from each individual site were pooled at three to five specimens (same species) per vial. A total of 489 of the specimens collected were sampled for PCR and RT-PCR assay. Tissue was collected from each individual specimen placed individually into separate vials or pooled into groups of one to five specimens per vial. Samples were extracted using a DNeasy Tissue kit for the isolation and purification of DNA (Qiagen, Valencia CA). RNA extraction of samples was done using a High Pure RNA Tissue extraction kit (Roche, Indianapolis IN). After nucleic

acid extraction, sample yield and quality of was measured spectrophotometrically. PCR for WSSV and IHHNV were done using methods outlined in the OIE Manual of Diagnostic Tests for Aquatic Animals (2006). This includes the Lo et al. (1997) two-step PCR diagnostic for WSSV and the Nunan et al. PCR test for IHHNV. For the analysis of TSV and YHV, cDNA was transcribed and RT-PCR diagnostics conducted using the GeneAmp EZ rTth RNA PCR kit (Applied Bioscience, Foster City CA). Primers for the amplification of TSV and YHV were those described in the OIE Manual of Diagnostic (2006) (Nunan et al. 1998, Wongteerasupaya et al. 1997)

Using agarose gel electrophoresis, the presence or absence of PCR amplicons was determined using a UV light box and gel documentation system. A molecular weight marker was run with each gel to provide a reference for any PCR positive reactions. Negative (no template DNA) and positive controls were included with each PCR. All PCR amplicons were sequenced at the EPSCoR core facility at HIMB.

None of the wild-caught crustaceans tested positive for WSSV, IHHNV, TSV, or YHV.

Objective 2. *Sample local commodity shrimp, from grocery and bait shops, for WSSV, IHHNV, TSV, YHV, and for IMNV (Infectious Myonecrosis Virus) when the shrimp originates from Brazil.*

A total of 632 commodity shrimp and crabs were sampled and tested for the presence of WSSV, IHHNV, TSV, and YHV. No shrimp from Brazil were identified in the supermarkets sampled so no testing for IMNV was done. Most samples were collected according to their visual appearance using any one or combination of the following criteria: (small white spots under the carapace, reddish coloration, or small size suggesting emergency harvest). Commodity shrimp samples were collected from 12 grocery stores over a six-month period. Seven of the grocery stores were located on Oahu. The shrimp from outer island stores were purchased from two stores on Molokai, two on the Big Island (Kailua-Kona town) and one store on Kauai. All samples were stored and transferred on ice to HIMB for PCR and RT-PCR assay. Upon arrival at HIMB, the samples were stored at -80 °C.

Commodity samples were pooled at three to five specimens per sample. The PCR and RT-PCR methods were the same as described in Objective 1. All PCR amplicons were submitted for sequence analysis and confirmed by sequencing to be virus-specific. A summary of these results and identification of the sample purchase site and country of origin is provided in Table 1 and 2. Shrimp tested from supermarkets on all islands surveyed were found to be positive for at least one of the “Big Four” shrimp viruses with all four represented in these commodity shrimp. Some commodity shrimp were co-infected with both WSSV and IHHNV.

Objective 3. *Conduct bioassays feeding infected commodity shrimp to wild caught decapod crustaceans representing standing stocks of local populations to test transmissibility of viral pathogens.*

Bioassays for WSSV were conducted at HIMB in strict quarantine conditions. Quarantine facility and protocols were covered under a Biological Safety Protocol reviewed and approved by the UH Institutional Biosafety Committee. A pilot bioassay was conducted using SPF shrimp obtained from the Oceanic Institute to assess the *per os* protocol planned for feeding WSSV-infected commodity shrimp to decapod crustaceans. First step positive shrimp tissue identified using the nested PCR protocol developed by Lo et al. (1997) was fed to shrimp in individual beakers to ensure each shrimp ingested an equivalent amount. The shrimp were returned to 30 gal aquarium (static conditions) and observed 3 times daily. After 24 h, all shrimp were active, apparently healthy, and no mortalities were observed. Within 48 h shrimp began to die and within 5 d the mortality rate was =95% in all tanks. All shrimp were confirmed WSSV-positive by PCR and sequencing at the end of the study. Control shrimp remained healthy throughout the duration of the trial and the quarantine conditions were well suited for small-scale experiments at HIMB.

Three species of crabs (*Thalamita crenata, Portunus pubescens, Pilodius areolatus*) from Kaneohe Bay were used in a larger scale bioassay (n=32) to test transmissibility of WSSV. The feeding protocol was similar to that for the SPF shrimp pilot study but the results were very different, confirming observations by others that crabs are more resistant to mortalities caused by some strains of this virus. Of this group, only 2 crabs that died during the study were PCR positive for WSSV and only second step positive (Lo et al. 1997). Cannibalism of a small portion of these dead crabs did not transmit sufficient virus to be detected in additional samples.

During the bioassay, samples were collected for PCR diagnostics at HIMB as well as preserved for histological analysis at the University of Arizona Shrimp Pathology Laboratory. Once all bioassays are complete, paired preserved samples will be submitted for histological analyses.

Objective 4. *Conduct bioassays feeding naturally or experimentally infected crustaceans to SPF shrimp to test for transmissibility and provide diagnostic support, pathogen testing, and disease surveillance services to local producers to establish baselines of pathogen prevalence in cultured shrimp populations.*

Experiments to meet this Objective are contingent upon the completion of Objective 3. Since bioassays using plankton samples will be conducted under the Year 20 scope of work it was decided it would be more economical to finish all bioassays when plankton samples were available for the opae and shrimp bioassays. Diagnostic support to local producers is being provided through the ADP-DP Hawaii Shrimp Surveillance and Certification Program (HSSCP).

Objective 5. *Research results obtained from Objectives 1-4 will be used to identify where existing biosecurity measures are sufficient for individual farms and where refinements may improve biosecurity. An analysis of current best management practices/standard operating procedures within the industry will be performed in collaboration with local farmers.*

The results from Objective 1-2 clearly indicate the greatest biosecurity risk to individual farms is from commodity shrimp. Once the plankton surveys and bioassays from Year 20 are completed, results will be analyzed and a review of how local producers can benefit from the results of this study. These results will be shared with the HDOA ADP-DP so information can be relayed to industry involved in the HSSCP and also disseminated via the Hawaii Aquaculture Association membership.

Objective 6. *Technology transfer.*

Dissemination of results is an important aspect of this project. The results of the work completed suggests Hawaii's natural environment currently poses less risk of as a reservoir for harmful shrimp viruses than other regions involved in shrimp aquaculture. Results of the survey of wild and commodity shrimp will be published in the CTSA Regional Notes. At least one manuscript will be prepared for submission to a peer-reviewed journal once the plankton surveys and all bioassay results have been analyzed.

Impacts

The results of the work completed suggests Hawaii's natural environment currently poses less risk of harboring harmful shrimp viruses than other regions involved in shrimp aquaculture. Having this established baseline is extremely important for Hawaii's aquaculture industry. Should Hawaii be able to establish clear SPF zones, it will be important to minimize possible introduction of shrimp viruses from commodity shrimp that is being brought into the state and distributed freely throughout the main Hawaiian Islands.

Recommended Follow-Up Activities

A number of observations were made in this project that will be followed up during the Year 20 plankton survey and bioassay component. Expanded sampling, including some overnight sampling outer-island, must be completed in order to obtain a representative number of species. Funds were set aside for this purpose from the Year 19 budget since the requested Year 20 budget was revised from the original. The remaining bioassays will be done with virus-positive commodity shrimp as well as live collected plankton samples. This is the most economical way to complete the proposed work. Finally, development of public education materials to make the public aware of the potential harm using commodity shrimp for bait is recommended.

In the surveys of wild crustaceans completed, no virus-positive samples were identified. This is a contrast to reports of the prevalence of WSSV and other shrimp viruses in natural crustacean populations in other regions. It is evident that commodity shrimp pose a real risk as all "Big Four" viruses were found in locally purchased shrimp. This highlights the need inform the public of the risk commodity shrimp may pose to wild and farmed populations of decapod crustaceans in Hawaii.

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

Results of the surveys from wild decapod crustaceans and commodity shrimp will be published in the CTSA Regional Notes. At least one manuscript will be prepared for submission to a peer-reviewed journal when the plankton surveys and all bioassay results have been analyzed. Project results were not available prior to the November 2007 submission deadline for the next World Aquaculture Society (WAS) meeting. However, a late poster abstract will be submitted to the WAS meeting organizers and will hopefully be accepted for presentation in Busan, Korea in May 2008. Dr. Lewis will also present project results at the 2008 American Association for Advancement of Science Pacific Division (AAAS-PD) 89th Annual Meeting in Waimea HI, June 15-20, 2008.

Presentation

TD Lewis, D Montgomery-Brock, AR Eggers, JC Leong. Don't use that shrimp for bait! 89th Annual Meeting of the American Association for Advancement of Science-Pacific Division (AAAS-PD) in Waimea HI, June 15-20, 2008.

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APPENDIX

Table 1. DNA virus test results of commodity shrimp and crabs (PCR and sequencing).

Case# /Number/Source	Country of Origin	Samples (n=)	IHHNV (% positive)	WSSV (% 2nd step positive)
07-01/Safeway/Oahu	Thailand	12	**45%**	**100%**
07-02/Safeway/Oahu	Thailand	12	**50%**	**30%**
07-04/Costco/Oahu	Indonesia	50	0%	0%
07-05/Sam's Club*/Oahu	Ecuador Nicaragua Thailand India Hawaii	50 35 50 7 8	0% 6% 0% 0% 0%	0% 0% 0% 0% 0%
07-06/Foodland/Oahu	Taiwan	10	**50%**	**50%**
07-07/Foodland/Oahu	Thailand	45	**55%**	**65%**
07-11/Friendly Market/Molokai	Vietnam	50	0 %	**40%**
07-12/Misaki's/Molokai	Thailand	50	**60 %**	**70%**
07-16/Safeway/Oahu	Thailand	50	0 %	0 %
07-17/Don Quijote/Oahu	Not identified	20	0 %	0 %
07-18/Times Supermarket/Oahu	Thailand	35	0 %	**25 %**
07-19/Times Supermarket/Oahu	Thailand	10	0 %	0 %
07-23/Safeway/Big Island	China	20	0 %	0%
07-24/KTA/Big Island	Thailand	50	0 %	0 %
07-25/KTA/Big Island	Indonesia	20	0 %	0 %
07-28/Safeway/Kauai	Thailand	40	0 %	0 %
07-29/Safeway/Kauai	India	17	0 %	0 %

*Samples from Ecuador, Nicaragua, and Thailand are shrimp. Samples from India and Hawaii are crabs.

Table 2. RNA virus test results of commodity shrimp (RT-PCR and sequencing).

Case# /Number/Source	Country of Origin	Samples (n=)	TSV (% positive)	YHV (%positive)
07-01/Safeway/Oahu	Thailand	4	0 %	**100 %**
07-02/Safeway/Oahu	Thailand	12	0 %	0 %
07-04/Costco/Oahu	Indonesia	50	0 %	0 %
07-05/Sam's Club*/Oahu	Ecuador Nicaragua Thailand	50 35 50	0 % 0 % 0 %	0 % 0 % 0 %
07-06/Foodland/Oahu	Taiwan	10	0 %	0 %
07-07/Foodland/Oahu	Thailand	44	0 %	0 %
07-11/Friendly Market/Molokai	Vietnam	10	0 %	0 %
07-12/Misaki's/Molokai	Thailand	10	0 %	0 %
07-16/Safeway/Oahu	Thailand	12	0 %	0 %
07-17/Don Quijote/Oahu	Not identified	8	0 %	0 %
07-18/Times Supermarket/Oahu	Thailand	35	0 %	**94 %**
07-19/Times Supermarket/Oahu	Thailand	10	0 %	0 %
07-23/Safeway/Big Island	China	20	**10%**	0 %
07-24/KTA/Big Island	Thailand	50	0 %	0 %
07-25/KTA/Big Island	Indonesia	20	0 %	0 %
07-28/Safeway/Kauai	Thailand	40	0 %	0 %
07-29/Safeway/Kauai	India	15	0 %	0 %