Proposal Title: **Post Harvest Salinity Processing as a Means of Reducing *Vibrio Vulnificus* in Oysters Harvested from Apalachicola Bay**

Principal Investigator: John Teem, Ph.D., Florida Department of Agriculture and Consumer Services, Division of Aquaculture

EXECUTIVE SUMMARY

Oysters harvested from Apalachicola Bay in the months April-November (warm water-harvested oysters) present an increased risk of *Vibrio vulnificus* (Vv) infection to at-risk individuals as compared to oysters harvested in the months of December-March (cool water-harvested oysters). The Interstate Shellfish Sanitation Conference (ISSC) in conjunction with the FDA have set a goal to reduce the incidence of *Vibrio* illness by 60% for years 2007 and 2008 (average) from the average illness rate for the years 1995 - 1999 of 0.303/million, but this goal has not yet been achieved. In order to allow oyster harvesting in summer months and meet the illness reduction goal, new oyster harvest practices are required.

This proposal is seeks to define a procedure to reduce Vv and Vp in harvested oysters by the transfer of oysters to a high-salinity environment for a 1-2 week period. On a biweekly basis, oysters will be collected at Cat Point in Apalachicola Bay where the salinity is low and then transferred to Alligator Harbor where the salinity is high. The Vv and Vp associated with the oyster meat will be determined before and after the transfer to the high-salinity environment. A loop mediated isothermal amplification (LAMP) assay will be used in making determinations of Vv and Vp. By using the relatively inexpensive LAMP assay to quantify *Vibrio* levels, a cost-effective in-house capacity for Vv and Vp measurement will be developed that will allow the effectiveness of the high-salinity *Vibrio* reduction treatment to be determined.

PROJECT MANAGEMENT APPROACH

Post Harvest Salinity Processing (PHSP) of oysters may provide a means of reducing Vv numbers in warm water-harvested oysters from Apalachicola Bay, and reduce the incidence of *Vibrio* illness. Oysters harvested from lower salinity water have higher levels of *Vibrio vulnificus* than oysters harvested from higher salinity water (Motes et al., 1998, Kaspar and Tamplin, 1993, Randa et al., 2004, Johnson et al., 2010, Audemard, 2013, Larsen, 2013). It may thus be possible to reduce the Vv levels in warm water-harvested oysters from Apalachicola by transferring them to high salinity water for a period of time sufficient to significantly reduce Vv to levels that approximate the levels found in cool water-harvested oysters (Lewis et al., 2010, Supan and Cake, 1982, Larsen et al. 2013). Once Vv levels are reduced, oysters taken from the site could be rapidly cooled and processed, providing a warm water-harvested product with a Vv risk that is comparable to a cool water-harvested product.

A preliminary study was conducted to test the feasibility PHSP as a means of reducing Vv and Vp from oysters harvested from Apalachicola Bay. Oysters were removed from Apalchichola Bay (salinity 27.7 ppt) and transferred to Alligator Harbor (salinity 34 ppt) for two weeks, followed by Vv and Vv assessment within the oysters. As shown in

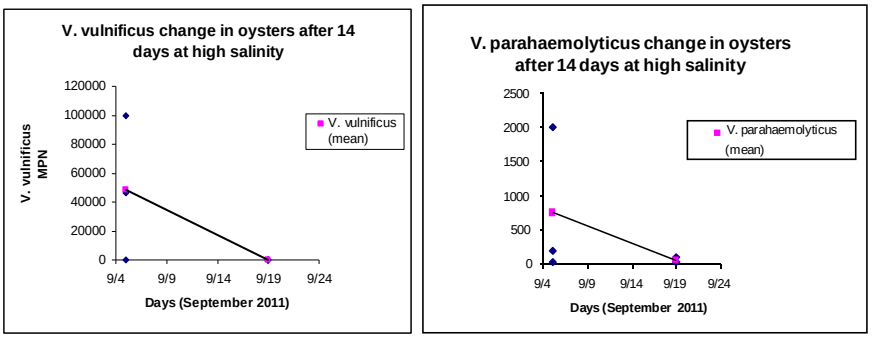

Figure 1, both Vv and Vp levels were reduced in the oysters as a result of PHSP treatment. *Figure 1. Vv and Vp decrease resulting from post harvest salinity processing (PHSP) of oysters from Apalachicola Bay. Vibrio levels were determined by staff in the laboratory of Steve Otwell of the University of Florida, using a standard real-time PCR assay.*

This data suggests that a PHSP treatment could be an effective means reducing Vv and Vp, allowing Florida to meet the mandated Vv reduction goal. One of the goals of this proposal is to further investigate the efficacy of PHSP and develop a pilot program to test the practical application of PHSP treatment by oyster processors.

SCOPE, APPROACH, AND METHODOLOGY

Approximately 150 oysters will be harvested on a bimonthly basis from the Cat Point oyster harvest area of Apalachicola Bay during the months of August-November 2014 and April-July 2015. Oysters will be collected by Division personnel and taken divided among three plastic mesh bags (50/bag) and transported by truck to Alligator Harbor. Two of the bags will be transferred to the lease area and secured to the bottom using PVC anchors at each bag corner. The other bag will be used for a sample collection representing the Vv levels at the time of introduction into high salinity waters (Day 0, or D_0). Each of the two bags introduced into Alligator Harbor will represent a timepoint for Vv sampling at 7 and 14 days post-transfer to high salinity water (D_7, D_{14}). At the time a sample time point is collected, the bag will be removed from the water and 36 oysters removed for processing. The oysters will be taken to the Apalachicola lab for shucking and processing.

Oysters will be processed using the SOPs for Vv enumeration using an APW media enrichment protocol followed by DNA extraction of positive tubes and PCR confirmation of Vv. Briefly, three tissue homogenate samples (12 oysters per sample) will be prepared from 36 oysters collected from each timepoint. A dilution series for each sample will be prepared (10^1-10^4) from each homogenate, and three tubes of APW growth media inoculated from each dilution. Tubes will be incubated 12-18 hours. Growth-positive tubes in each dilution will be enumerated, and DNA extracted from each assayed by loop mediated isothermal amplification (LAMP) analysis using LAMP primers specific for Vv (Han et al.) and Vp (Chen et al.)

Vibrio LAMP assays will be used to identify Vv and Vp positive tubes in the MPN assay instead of real-time PCR. LAMP assays can be performed with a 65^{o}C water bath, removing the necessity for an expensive real-time PCR machine. Additionally, the reagent components for detecting Vv and Vp by real-time PCR are prohibitively expensive. The LAMP assays cost less than $1 per sample whereas the real time PCR

assays cost about $9 per sample. The LAMP assay is thus a cost-effective means for state agencies without the capacity for real-time PCR to collect *Vibrio* data for regulatory purposes. All oyster samples and MPN enrichment tubes derived from samples will be frozen at -20^{o}C to provide a means to validate Vv and Vp determinations using the traditional real-time PCR method at a later time should it be deemed necessary.

This proposal will:
1.) provide baseline data for Vv and Vp in oysters from Apalachicola Bay, data which FDA has requested that states generate in order to regulate the shellfish harvest.
2.) determine the effect of a 7-day and 14 day high salinity treatment of oysters taken from a low salinity environment, responsive to FDA's request for states to reduce Vibrio illness by 60% (and program priorities I.B, 1 and 2a).
3.) provide a low-cost means of Vv and Vp detection that can be implemented by state agencies that do not have the resources to conduct real-time PCR assays.
4.) communicate the information to shellfish harvesters and processors in a workshop held at the Division of Aquaculture office in Apalachicola, FL.

References

1. Motes, M.L., DePaola, A., Cook, D.W., Veazey, J.E., Hunsucker, J.C., Garthright, W.E., Blodgett, R.J. & Chirtel, S.J. 1998. Influence of water temperature and salinity on *Vibrio vulnificus* in northern Gulf and Atlantic Coast oysters (*Crassostrea virginica*). *Applied and Environmental Microbiology*, **64**: 1459–1465.
2. Randa, M. A.,. Polz, M. F., and Lim1, E. 2004 Effects of Temperature and Salinity on Vibrio vulnificus Population Dynamics as Assessed by Quantitative PCR. *Applied and Environmental Microbiology*, **70**:5469-5476.
3. Jones, M. K., Warner, E. and Oliver, J.D. 2008. Survival of and In Situ Gene Expression by Vibrio vulnificus at Varying Salinities in Estuarine Environments. *Applied and Environmental Microbiology*, **74**: 182 – 187.
4. Kaspar, C.W. and Tamplin, M.L. 1993. Effects of Temperature and Salinity on the Survival of Vibrio vulnificus in Seawater and Shellfish. *Applied and Environmental Microbiology*, **59**: 2425-2429.
5. Johnson, C.N., Flowers, A.R., Noriea, N.F., Zimmerman, A.M., Bowers, J.C., DePaola, A., Grimes, D.J. 2010. Relationships between environmental factors and pathogenic Vibrios in the Northern Gulf of Mexico. *Applied and Environmental Microbiology*, **76**: 7076 – 7084.
6. Supan, E. W. Cake, E.W. 1982. Containerized-relaying of polluted oysters (Crassostrea virginica [Gmelin]) in Mississippi Sound using suspension, rack and onbottom longline techniques. *J. of Shellfish Research* **2**: 141-151.
7. Lewis M, Rikard S, Arias CR (2010) Evaluation of a Flow-Through Depuration System to Eliminate the Human Pathogen *Vibrio Vulnificus* from Oysters. *J Aquac Res Development* **1**:103. doi:10.4172/2155-9546.1000103
8. Larsen, A.M., Rikard F.S, Walton, W.C., Arias C. R. (2013) Effective reduction of Vibrio vulnificus in the Eastern oyster (Crassostrea virginica) using high salinity depuration. Food Microbiology 34:118-122.
9. Audemard, C., Kator, H. I., Rhodes, M. W., Gallivan, T., Erskine, A. J., Leggett, A. T., Reece, K. S. (2013) High Salinity Relay as a Postharvest Processing Strategy To Reduce *Vibrio vulnificus* Levels in Chesapeake Bay Oysters (*Crassostrea virginica). Journal of Food Protection*: 11, , pp. 1788-1989 , pp. 1902-1907(6)
10. Han, F., Wang, F., and Ge, B. (2011) Detecting Potentially Virulent *Vibrio vulnificus* Strains in Raw Oysters by Quantitative Loop-Mediated Isothermal Amplification. *Applied and Environmental Microbiology*, p. 2589–2595.
11. Chen, S., Ge, B. (2010) Development of a toxR-based loop-mediated isothermal amplification assay for detecting *Vibrio parahaemolyticus*. *BMC Microbiology* 10:41.

Budget					%Source
Vehicle				25,000	
Boat				9,000	
Trailer				1,500	
Salary P.I. (JT,25% effort)				16,349	
	Matching Funds			**51,849**	77.05%
Fuel				1,920	
Oyster bags, anchors				1,000	
Microbiology Reagents				5,000	
LAMP Reagents Vv (2160 samples, $1/ sample)				2,160	
LAMP Reagents Vv (2160 samples, $1/ sample)				2,160	
Freezer (-20oC)				600	
heat block				300	
vortexer				300	
Lab Reagents/Materials				2,000	
	Requested Funds			**15,440**	22.95%
	Total			**67,289**	

The homogenate of 12 oysters will be used for each time point determination and each time point will be collected in triplicate. There will be three time points for each biweekly transfer (D0, D7 and D14), thus 18 time points per month. Collection for eight months will produce a total of 144 samples total, each of which will require that 15 enrichment tubes are assayed (MPN). A total of 2160 samples will be thus be assayed by the assayed by the Vv and the Vp LAMP assays.

APPENDIX REFERENCES

Steve Otwell, Seafood Specialist

PO Box 110375
Gainesville, Florida 32611-0375
(352) 392-4221
Fax: (352)392-8594
otwell@ufl.edu

APPENDIX PROJECT TEAM STAFFING

All project participants are employees of the Florida Department of Agriculture and Consumer Services, Division of Aquaculture. No employees working on the engagement have ever been convicted of a felony.

Principal Investigator: John L Teem, Ph.D.

Dr Teem is a molecular biologist with extensive experience in detection of aquatic organisms using PCR and LAMP assays. He previously designed and executed preliminary experiments to assess the effect of transferring oysters to high salinity as a means of reducing Vv and Vp. He has made use extensive use of LAMP assays for the detection of reporter genes in aquatic organisms. As the P.I. of the project, he will oversee the collection of samples and the Vv and Vp determinations made using LAMP assays specific for *Vibrio vulnificus* and *Vibrio parahaemolyticus*.

Oyster Collection and Transfer: Joe Shields, John Gunter

Mr. Shields (Enviromental Adminstrator) and Mr. Gunter (Enviromental Specialist III) are both involved in oyster stock assessment surveys conducted in Apalachicola Bay by FDACS. They are experienced in all aspects of the process of collection and transfer of oysters from low salinity to a high salinity environment.

Microbiology: James Smith, Nellrie Lane, Martha Pace

Dr. Smith and his staff (Nellrie Lane and Martha Pace) routinely perform fecal coliform assessments of oyster samples as part of the FDACS regulatory program. They are versed in the microbiology techniques used in assessing microbial contamination of oyster meat and will conduct the MPN and LAMP assays to quantify Vv and Vp in oyster samples.

CURRICULUM VITAE

John L. Teem

PERSONAL DATA:

Business Address: Florida Department of Agriculture and Consumer Services
1203 Governor's Square Blvd.
Tallahassee, FL. 32301

Phone: Office : 850-410-0892
Home : 850-385-1954
Cell : 850-766-6289

PRESENT POSITION:

Biological Scientist IV

PROFESSIONAL EXPERIENCE:

Directed eradication of invasive fish using Trojan fish bearing multiple Y chromosomes. Sterile-release technology development for invasive apple snails. DNA fingerprinting of invasive microalgae *Caulerpa taxifolia*. Detection of the rat lungworm human pathogen in Giant African Land Snails and invasive apple snails. Development of sterile ornamental species for the aquarium industry. Flow cytometric ploidy analysis of aquatic organisms. Reduction of *Vibrio vulnificus* in oysters by high-salinity post harvest transfer.

Risk assessment of aquatic invasive species for FDACS Division of Aquaculture. National Aquaculture Association representative to the Gulf and South Atlantic Regional Panel (GSARP) of the Aquatic Nuisance Species Task Force (ANSTF). Member of the Aquatic Organisms Screening Work Group and the Pathways Workgroup of National Invasive Species Council Prevention Committee. GSARP chair of Research and Development Workgroup. FDACS representative to the Invasive Species Working Group. Grant reviewer for NOAA Invasive Species grant program.

EDUCATION:

1984 Ph.D. in Biology, Brandeis University, Waltham, Massachusetts

1978 B.S. in Microbiology, Ohio State University, Columbus, Ohio

TRAINING:

1995-2002 Assistant Professor, Department of Biological Science, Florida State University

1992-1994 Research Scientist, University of Iowa (Dr. Michael Welsh)
Department of Internal Medicine, Iowa City, Iowa

1990-1992 Postdoctoral Fellow, Hospital for Sick Children (Dr. Lap-Chee Tsui)
Department of Medical Genetics, Toronto, Ontario

1984-1989 Postdoctoral Fellow, Whitehead Institute for Biomedical Research and Department of Biology (Dr. Gerald Fink), Massachusetts Institute of Technology, Cambridge, Massachusetts

TEACHING EXPERIENCE:

1980 Introductory Biology, Brandeis University
1996-2001 Mammalian Biochemistry and Genetics, FSU

1996-2001 Genetics, FSU

RECENT GRANT AWARDS:

1999-2002 NIH International Cooperative in Biodiversity Group
1999-2001 FSU Program Enhancement Award
2003-2004 USDA
2005-2007 US Fish and Wildlife Service (administered through PSMFC)
2006-2008 US Fish and Wildlife Service (administered through GSMFC)
2011-2012 US Fish and Wildlife Service (administered through GSMFC)
2011-2015 US Fish and Wildlife Service

RECENT PUBLICATIONS:

Teem, JL, Gutierrez, JB. (2013) Combining the Trojan Y Chromosome and Daughterless Carp Eradication Strategies. Biological Invasions (in press), online DOI 10.1007/s10530-013-0476-1.

Teem, JL, Gutierrez, JB, Parshad, RD. (2013) A Comparison of the Trojan Y Chromosome and Daughterless Carp Eradication Strategies. Biological Invasions (in press), online DOI 10.1007/s10530-013-0475-2.

Thresher, RE, Hayes, K, Bax, NJ, Teem, J, Benfey, TJ, Gould, F. (2013) Genetic control of invasive fish: technological options and its role in integrated pest management. Biological Invasions, (in press), online DOI 10.1007/s10530-013-0477-0.

Teem, JL, Qvarnstrom, Y, Bishop, H, da Silva, AJ, Carter, J, White-Mclean, J, Smith, T.(2013) The Occurrence of the Rat Lungworm *Angiostrongylus cantonensis* in Nonindigenous Apple Snails in the Gulf of Mexico Region of the United States. Hawaii Journal of Medicine and Public Health, June 2013, Volume 72, No. 6, Supplement 2, ISSN 2165-8218.

Teem, JL, Gutierrez, JB. (2011) A Theoretical Strategy for Eradication of Asian Carps Using a Trojan Y Chromosome to Shift the Sex Ratio of the Population. In Invasive Carps in North America, Duane C. Chapman and Michael H. Hoff, editors. American Fisheries Society, AFS Symposium 74. June, 2011. ISBN: 978-1-934874-23-3.

Gutierrez, JB, Hurdal, MK, Parshad, RD, Teem, JL. Analysis of the Trojan Y Chromosome Model for Eradication of Invasive Species in a Riverine System. J Math Biol. 2012 Jan;64(1-2):319-40.DOI: 10.1007/s00285-011-0413-9. Epub 2011 Mar 4.

Qvarnstrom Y, da Silva AC, Teem JL, Hollingsworth R, Bishop H, Graeff-Teixeira C, da Silva AJ. (2010) Improved molecular detection of *Angiostrongylus cantonensis* in mollusks and other environmental samples with a species-specific internal transcribed spacer 1-based TaqMan assay. Appl Environ Microbiol. 2010 76:5287-9. Epub 2010 Jun 11.

Gutierrez, JB, Teem, JL. (2006) A Model Describing the Effect of Sex-Reversed YY Tilapia in an Established Wild Population: the Use of a Trojan Y Chromosome to Cause Extinction of an Introduced Exotic Species. J Theoretical Biol 241: 333-341.

(a) Featured in Nature News. Louis Buckley. Sex change wipes out invasive species. Nature, July 2007. London, UK. http://dx.doi.org/10.1038/news070723-9 [Online; accessed 7-November-
2008. Requires subscription]. BioEd Online hosts free copy at http://www.bioedonline.org/news/news.cfm?art=3484 [Online; accessed 7-November-2008]

(b) Featured in Featured in Trends in Ecology & Evolution. Samuel Cotton and Claus Wedekind
(Switzerland). Control of introduced species using Trojan sex chromosomes. Trends in Ecology & Evolution 22(9), pp. 441-3, 09-2007.

(c) Featured in Scienceline. Rachel Mahan. Supermales to the rescue. Scienceline, Jan 2008. New York, NY. http://scienceline.org/2008/01/11/env-mahan-invasives/ [Online; accessed 7-
November-2008].
(d) Featured in NCR Hansdelsblad. Sander Voormolen. Vrouwtjes verdrijven (Females away). *NCR* Hansdelsblad, 2007. Rotterdam, Netherlands.
http://www.nrc.nl/wetenschap/article1828576.ece/Vrouwtjes_verdrijven [Online; accessed 7-November-2008].
(e) Featured in Conservation Magazine. Cynthia Mills (WA, USA). Operation Sex Change. Conservation Magazine, a publication of the Society for Conservation Biology, Sep 2009. http://www.conservationmaga

APPENDIX: COMPANY OVERVIEW

Official registered name:	Florida Department of Agriculture and Consumer Services Division of Aquaculture
Address:	600 S. Calhoun St., Suite 217, Tallahassee, FL 32399
Dun & Bradstreet Number:	809558919
Primary and secondary SIC numbers: NA	
Phone:	850-617-7600
Fax:	850-617-7601

Key contact name:	Dr. John L. Teem Biological Scientist IV Florida Department of Agriculture and Consumer Services Division of Aquaculture 600 S. Calhoun St., Suite 217, Tallahassee, FL 32399
Phone:	850-617-7616
Fax:	850-617-7601

Person authorized to contractually bind the organization for any proposal against this RFP: Mike Gresham, FDACS Office of Administration

Information Security Testing: FDACS Information Security Testing Policy available by request

Possible conflicts of interest: None