PROJECT COMPLETION REPORT

02-02 "Management, Diagnosis, and Prevention of Flounder Infectious Necrotizing Enteritis (FINE)"

Progress Report Period: October 1, 2002 – March 1, 2003, no cost extension until September 2003

NRAC Total Funding: $54,357

Principal Investigators: Marta Gomez-Chiarri, University of Rhode Island, Department of Fisheries, Animal and Veterinary Science.
David R. Nelson, University of Rhode Island.
Roxanna Smolowitz, Marine Biological Laboratories

Participating Investigators/ Cooperative Agencies:

Mr. George Nardi	Great Bay Aqua-farms	New Hampshire
Mr. David Beutel	University of Rhode Island	Rhode Island
Mr. Michael A. Rice	University of Rhode Island	Rhode Island
Dr. Dale Leavitt	SEMAC	Massachusetts

REASONS FOR TERMINATION:

All objectives were completed and funds were terminated, with the exception of funds for publications, which are in the process of being completed.

PROJECT OBJECTIVES:

The main goal of this research was to evaluate the potential negative effect of *Vibrio carchariae* infection and other pathogens in the expansion of summer flounder culture. The particular objectives were to:

1. Monitor disease and the presence of the pathogen in various culture stages of summer flounder as grown under different conditions.

2. Identify the conditions that promote the growth and survival of *Vibrio carchariae* (and other potential pathogens isolated from flounder in objective 1) *in vitro.*

3. Identify the environmental conditions that promote the occurrence of disease and mortality in experi mental challenges with flounder pathogens.

4. Using information in objectives above to establish management protocols that minimize the development of outbreaks.

ANTICIPATED BENEFITS:

<u>This proposal will:</u>

1. Identify and summarize potentially critical, problems in different stages of summer flounder culture (hatchery and nursery) including information on the environmental and culture conditions when those problems occur.
2. Determine the relative importance of FINE and other diseases so researchers can evaluate priorities in the development of diagnostic tools and vaccines.
3. Provide a model for the monitoring of disease problems in other marine fish species with potential for marine aquaculture.
4. Begin the development of a health management plan for summer flounder aquaculture by providing information on the environmental and culture conditions that can trigger epizootics.
5. Provide basic information necessary for the future development of diagnostic tools.
6. Provide the background information necessary for the development of effective methods for prevention (vaccines) and treatment of epizootics.
7. Provide a framework for effective communication of results between researchers including health specialists, pathologists and microbiologists.
8. Establish solid communication and outreach between researchers and industry.
9. Expand communication and collaboration with other

research teams at URI engaged in research on summer flounder aquaculture.

Future products that could be developed using information in this research (diagnostic and prevention tools) will be applicable worldwide to culture systems where Vibrio carchariae and other bacterial pathogens may be a problem, including the land-based and net-pen culture of summer flounder and other marine fish species.

PRINCIPAL ACCOMPLISHMENTS:

Our research indicates that:

1. *V. harveyi* was a major component of the intestinal flora of larval and juvenile summer flounder at GBA, but not at URI.
2. Potential sources of *V. harveyi* include water, algal cultures, rotifers, and artemia.
3. Different strains of *V. harveyi* can be present at a single facility. Further research needs to be done to evaluate the virulence of the different strains.
4. Increased levels of *V. harveyi* in summer flounder are associated with clinical signs of FINE.
5. However, presence of *V. harveyi* in summer flounder is not necessarily correlated with disease outbreaks. Histological signs indicative of FINE were only observed in juvenile fish, and significant mortalities were only observed after transport stress, both in challenge experiments and during the monitoring program. Factors that could influence the occurrence of disease include: age of the fish, genetic susceptibility of the fish, strain of *V. harveyi,* previous exposure to the pathogen, environmental conditions, and the presence of competing bacterial strains.
6. Other potential fish pathogens (*Photobacterium damselae*, subspecies *damselae*, *V. ichthyoenteri*, and *V. scophthalmi*) have been isolated from juvenile summer flounder. Further research needs to be done to evaluate the contribution of these pathogens to mortality and disease. While these species have been documented as pathogens in numerous species including flatfish (*V. ichthyoenteri* in Japanese flounder and *V. scophthalmi* in turbot), they have not yet been identified as pathogens of summer flounder.

IMPACTS:

This research has resulted in the following recommendations:
V. harveyi should be considered a top priority in the health management of summer flounder. Our monitoring program clearly indicates that this bacterial species is ubiquitous, that larval and juvenile summer flounder are prone to colonization with *V. harveyi,* and that infection can result in serious morbidity and mortality. The growth of survivors is impaired by the occurrence of blind sac guts

1. Management of FINE:
    a) Transport stress continues to be implicated as a major trigger of FINE. Disease could be minimized by decreasing stress conditions during transport (*i.e.* by the addition of anes thetics) or by keeping the fish in environmental conditions that minimize bacterial growth (salinities at or below 10 ppt and/or temperatures at or below 18°C).
    b) Environmental conditions (temperature and salinity) can be manipulated to decrease the risk of disease outbreaks in summer flounder culture. However, low temperatures and salinities may also reduce growth rates. In the case of salinity, it may also be possible to use a low salinity regimen as a short-term treatment when signs of disease are first observed. Such a practice may minimize losses during a disease epizootic with minimal impact on growth.
2. Diagnosis: The combination of biochemical (*i.e.* API20E) and molecular (16S rDNA sequencing) allowed for accurate identification of bacterial species in summer flounder. Other more specific tools that can distinguish between strains of *V. harveyi*, such as diagnostic PCR or ELISA, should be developed in the future. These techniques must be validated with as many strains as possible. This will help insure that the diagnostic method is effective for all strains.
3. Prevention: The most common prevention tools include vaccines, immunostimulants, and probiotics. Vaccine development for the prevention of *V. harveyi* may prove specially chal lenging, due to strain variability. Two potential probiotic strains have been identified in this research and should be further evaluated. While methods to prevent colonization are developed, to best prevention strategy is to maintain the overall health of the fish and reduce stress during transport.

RECOMMENDED FOLLOW-UP ACTIVITIES:

1. Determine the role of *Photobacterium damselae,*

subspecies *damselae*, *V. ichthyoenteri*, and *V. scophthalmi* in disease in summer flounder.
2. Develop tools for the specific diagnosis of *V. harveyi* strains.
3. Evaluate the level of strain variation in *V. harveyi*, the distribution of the different strains, and the ability of each strain to cause disease in summer flounder.
4. Isolate and characterize a conserved antigen from *V. harveyi* strains for vaccine development.
5. Determine the potential of temperature and salinity manipulation during transport stress as a tool to prevent outbreaks of FINE.
6. Determine the effect of the addition of anesthetics to water during transport stress as a tool to prevent outbreaks of FINE.
7. Evaluate the potential of probiotics to prevent FINE and other diseases in summer flounder culture.

PUBLICATIONS, MANUSCRIPTS, OR PAPERS PRESENTED:

Gauger,E.J., Gomez-Chiarri,M., 2002. 16S ribosomal DNA sequencing confirms the synonymy of Vibrio harveyi and V. carchariae. Dis. Aquat. Organ 52, 39-46.

Gauger, E., Smolowitz R.M. Gómez-Chiarri M. Prevalence of *Vibrio harveyi* In Juvenile Cultured Summer Flounder (*Paralicthys dentatus*). Eastern Fish Health Workshop, Pennsylvania, April 2003 (Oral presentation).

DelVescovo L. 2003. The starvation stress response of *Vibrio harveyi* DN01. Master Thesis, University of Rhode Island, pp 42

DelVescovo L, Nelson D. The starvation stress response of *Vibrio harveyi* DN01. Manuscript in preparation for J. Bacteriol.

Gauger E, Leavitt D, Smolowitz, R, Gómez-Chiarri M. Diseased of flatfish. Update to NRAC00-001 (in preparation)

Gauger E, Gómez-Chiarri M. Effect of environmental conditions on the susceptibility of summer flounder to infection with *Vibrio harveyi.* (Manuscript in preparation)

Gauger E, Casey J, Gómez-Chiarri M. Intestinal bacterial community in larval and juvenile cultured summer flounder. (Manuscript in preparation).

Smolowitz R, Gómez-Chiarri M. Histopathological lesions in summer flounder from a commercial run. (Manuscript in preparation).

TECHNICAL ANALYSIS AND SUMMARY:

INTRODUCTION:

Vibrio harveyi (previously known as *V. carchariae)* has been identified as a major pathogen of numerous marine finfish (Austin and Austin, 1999) including the summer flounder (*Paralichthys dentatus*) (Soffientino et al. 1998). The disease caused by *V. harveyi* in summer flounder has been given the name Flounder Infectious Necrotizing Enteritis (FINE). FINE is characterized by severe enteritis with swelling in the abdomen, reddening around the vent and ventral surface, and often hyperpigmentation. The first outbreak in cultured summer flounder occurred in Rhode Island shortly juvenile fish were transported from a hatchery in New Hampshire to the grow-out facility, and coincided with high water temperatures. In order to formulate management strategies for FINE, it is important to know the prevalence of *V. harveyi* and understand the factors that affect its ability to cause disease. This project aimed to address these issues by pursuing the following objectives:

1. Monitoring disease and the presence of the *Vibrio harveyi* various culture stages of summer flounder.
2. Identify the conditions that promote the growth and survival of *Vibrio harveyi in vitro.*
3. Identify the environmental conditions that promote the occurrence of disease and mortality in experimental challenges with *Vibrio harveyi*.
4. Using information in objectives above to establish management protocols that minimize the development of outbreaks.

METHODS AND RESULTS:

Objective 1. Monitoring disease and the presence of the *Vibrio harveyi* various culture stages of summer flounder.

a) Preliminary work: *V. harveyi* is synonymous to *V. carchariae* and constitutes a major finfish and shellfish pathogen.

With the final goal of developing diagnostic tools that would assist in the process of disease monitoring, we analyzed the biochemical profiles and 16S rDNA sequences of 17 bacterial isolates that have been shown to be very similar or undistinguishable to *V. carchariae* (*V. harveyi* and *V. campbellii*). These isolates have been responsible for disease and mortality in a wide variety of finfish and shellfish species worldwide, from oysters and seabass in Europe to shrimp and grouper in Asia. 16S

rDNA sequencing confirmed the homogeneity and synonymy of *V. harveyi* and *V. carchariae* (Gauger & Gómez-Chiarri, 2002). Therefore, we refer to *V. carchariae* as *V. harveyi* from now on. Analysis of biochemical profiles revealed that they are insufficient by themselves to differentiate *V. harveyi* and *V. campbellii* strains. 16S rDNA sequencing, however, could be used in conjunction with biochemical techniques to provide a reliable means of distinguishing *V. harveyi* from other closely related species. This work confirms that *V. harveyi* is a major pathogen of finfish and shellfish worldwide, and stresses the importance of vaccine development for this bacterial species.

b) Gross, histological, and microbiological examination.

The presence of *V. harveyi* and other bacterial species was monitored at a total of eight collection periods through the hatchery/nursery/early grow-out phases of culture. Collections were conducted at 12, 25, 50, 100, &155 days post hatch (dph) on a production run of summer flounder at Great Bay Aquaculture (GBA) in New Hampshire, with an additional collection (215 dph) conducted on the same production run after fish were transported to the University of Rhode Island (URI). Two collections were also conducted at 17 and 46 dph at the University of Rhode Island Flounder Facility. Animals were sampled from a maximum of 4 tanks (depending on collection date/animal age), and gross, histological, and microbiological examinations were performed. Samples of rotifers, artemia (live feed used in hatchery and nursery stages), and water from the tanks were also collected for microbiological analyses. For each collection, identification of bacterial isolates was accomplished in three steps:

1. Bacterial colonies were typed by color, size, and morphology (morphotypes). When possible, the numbers of each morphotype were recorded in order to estimate relative abundance. Several colonies of each morphotype were suspended in 20% glycerol and frozen at –70°C for further identification.
2. Examining the biochemical profile (phenotype) of the isolates using the API 20E system. This system consists of 20 biochemical reactions that are scored as positive or negative and translated into a unique 7-digit code that identifies a bacterium, generally at the species level. One or more isolates with each unique 7-digit code were then analyzed by DNA sequencing for confirmation.
3. For selected isolates, partial sequences of the 16S rRNA gene were determined and compared to genetic databases. This analysis enabled positive identification of the isolates to the genus and usually species level (genotype).

Using this three-step approach allowed us to efficiently and accurately identify the major components of the culturable flora associated with summer flounder culture.

A total of 33 bacterial species were identified from the collections at GBA and URI (Table 1). At GBA, a shift in the bacterial flora was observed between the larval (feeding on rotifers and artemia) and juvenile stages (after metamorphosis, feeding on pellet diet). *V. harveyi* was the only bacterial species that was isolated from summer flounder at all stages of the production cycle, as well as from algal cultures and live feed. In fact, *V. harveyi* was the most common bacterial species isolated from flounder, and was detected at each of the first four collection periods (12 – 100 dph) at steadily increasing numbers. By the fourth collection (100 dph), *V. harveyi* constituted 94% of the culturable intestinal flora of the juvenile flounder. A parallel increase in the presence of histological lesions in summer flounder was observed. Gross and histological examinations showed no visible parasites or other abnormalities in 12 day-old summer flounder larvae. At 50 days post hatch (dph), a few of the smaller juveniles showed mild fin erosions in one of the tanks, which may represent stress/overcrowding/mild cannibalism. No other major abnormalities were observed. At 100 dph, most fish were healthy-looking animals and showed no external parasites or significant gross lesions. However, about 75% of the animals examined showed the occurrence of moderate to abundant numbers of inflammatory cells in the tissues of the anterior intestine. The presence of inflammatory cells was associated with lesions of the intestinal tissues (enteritis) (Fig. 1).

Additionally, in two animals, these expansive areas of subacute to chronic enteritis extended to the peritoneum, resulting in localized peritonitis (inflammation of the peritoneum). In some foci potential short thick rods (potentially *V. harveyi)* were noted in macrophages in the epithelium and underlying inflamed tissue. A slight but unusual increase in mortalities (0.1-0.2%/day) was observed in juvenile summer flounder between 100 and 155 dph. Histological examination of affected animals showed the presence of peritonitis, but not enteritis. Sections of lesions from were stained with tissue gram stain and showed red stained rods (gram negative bacteria). Two animals collected at about 155 dph showed moderate to severe peritonitis microscopically, as well as dilation of the posterior intestine/colon with mucus and fluid and granularity/adhesions of the serosa of the intestine/colon to the peritoneal wall. Other animals showed mild

to moderate degrees of enteritis in the intestine, colon, and stomach (at the pyloric/intestine junction). Mild to severe peritonitis was present in animals with only mild enteritis. In many animals, the peritonitis appeared to be more severe around the true ventrum of the abdominal cavity (cloacal/rectal area-not just the ventro-lateral side of the fish). These histological lesions are consistent with the signs observed in previous outbreaks of FINE, as well as animals experimentally infected with *V. harveyi.* However, *V. harveyi* was not isolated from fish collected at 155 dph. The main species isolated at this time-point were *Photobacterium damselae*, subspecies *damselae*, *V. ichthyoenteri*, and *V. scophthalmi*, which have been associated with disease in other marine finfish species, including flatfish (Austin & Austin 1999). Further work needs to be done to determine if the presence of these bacterial diseases is associated with pathological lesions.

After transport of flounder to URI (200 dph), fish began showing reddening of the anus and swollen bellies (clinical signs of FINE), as well as reddening and erosion of the mouth and eyes. Fish mortalities were also observed, to a total of 30% of cumulative mortalities in 14 days. *V. harveyi* was isolated from the intestine, kidney, peritoneum, and some deep surface lesions of several moribund as well as apparently healthy fish. Several animals surviving the outbreak were collected at 215 dph. Many of these animals had developed blind ended guts. Tissues from one of the six animals examined showed a distinctive blind ended intestine (atresia). Microscopically, all six animals showed apparently unassociated severe lesions and inflammation of the pharynx and associated mandible and gill tissues (Figure 2). One animal showed severe acute inflammation and lesions in the kidney and similar inflammatory changes in an eye rete. Another animals showed a chronic inflammation of the kidney.

Figure 2. Histopathological lesions in flounder (215 dph). Pharyngitis.

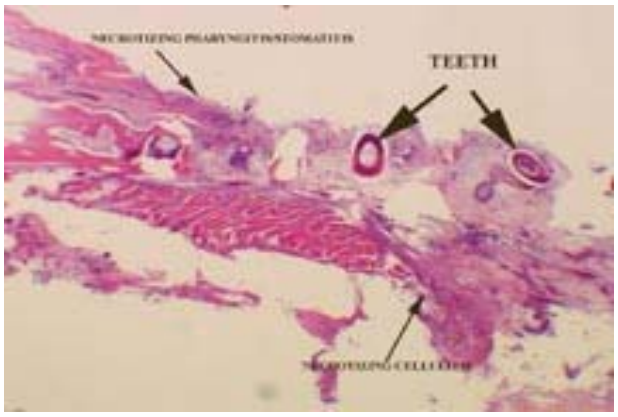
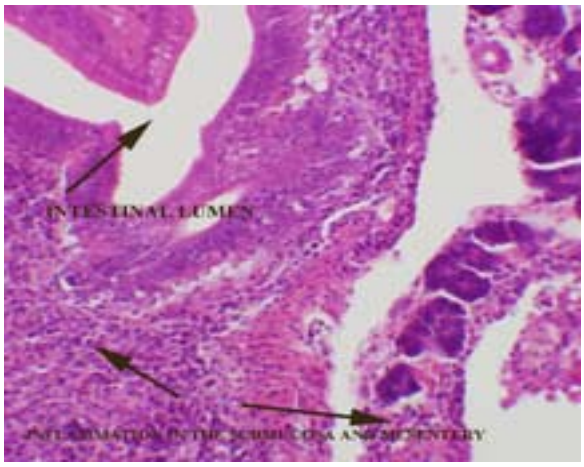


Figure 1. Histopathological lesions in summer flounder (155 dph). Left: peritonitis (25x). Right: enteritis and peritonitis (100x). *Notice areas of blue staining indicative of inflammation and presence of bacterial cells*



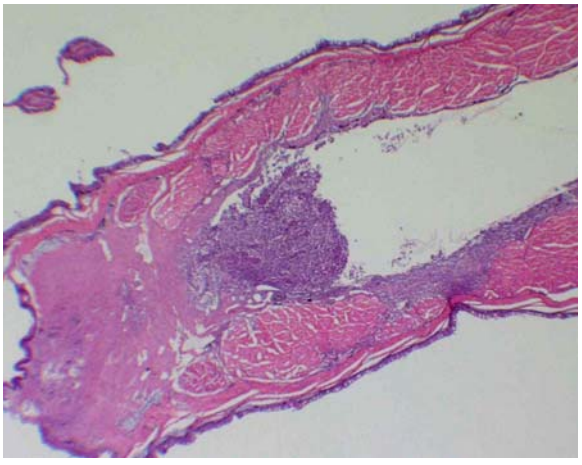
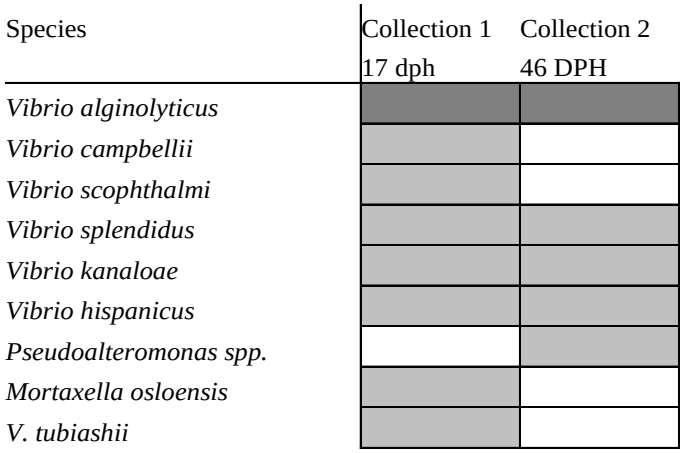


Table 1. Bacterial species collected from the summer flounder monitoring program at GBA and temporal relationship to pathological changes. Box shading indicates the isolation of the bacterium in fish at that time point. Intensity of shading is proportional to the relative abundance of the bacterium in fish.

Species	GBA 7/10/03 12 DPH	GBA 7/25/02 25 DPH	GBA 8/20/02 50 DPH	GBA 10/8/02 100 DPH	GBA 11/22/02 150 DPH	URI 1/25/03 215 DPH
Alteromonas macleodi	Algae				Wall	
Alteromonas spp.	Rotifers					
Bacillus benzoevorans						
Bacillus thuringiensis						
Exiguobacterium spp.						
Listonella anguillarum	Rotifers					
Marinomonas spp.				Wall		
Microbacterium spp. 1&2						
P. damselae subsp. damselae					K/P	P
Photobacterium phosphoeum					P	
Pseudoalteromionas atlantica					Wall	
Pseudoalteromonas spp.						
Pseudoalteromonas spp. 2		Wall		Wall		
Pseudomonas anguilliseptica	Rotifers					
Pseudomonas spp.	Wall					
Shewanella fidelis						
Shewanella saccharophilus	Rotifers					
Shewanella spp.	Wall				P	
Staphylococcus succinus						
Vibrio aestuarinus						
Vibrio alginolyticus	Algae/Wall	Wall				
Vibrio campbellii	Wall					
Vibrio corralilyticus	Rotifers					
Vibrio fluvialis	Wall	Artemia				
Vibrio harveyi	Algae	Artemia				MS/K/P
Vibrio ichthyoenteri					Wall	P
Vibrio neptunis						
Vibrio splendidus		Wall	Wall			
Vibrio spp.						
Vibrio parahaemolyticus			Wall			
Vibrio pelagius		Wall	Wall			
Vibrio proteolyticus		Artemia				
Vibrio scophthalmi					P	MS
Vibrio splendidus						MS
Vibrio vulnificus	Rotifers/ Wall					
Pathology	Frayed fins and eye injuries Cannibalism			Inflammation colon/cloacea	Enteritis, peritonitis	FINE 30% mort

Examination of flounder from URI's Flounder Facility showed no significant lesions and no unusual mortalities. *V. harveyi* was not isolated from either of the two collections at URI's Flounder Facility (17 and 46 dph). The most common bacterium isolated was *V. alginolyticus* (Table 2).

Table 2. Bacterial species collected from the summer flounder monitoring program at the Flounder Aquaculture Facility, URI. Box shading indicates the isolation of the bacterium in fish at that time point. Intensity of shading is proportional to the relative abundance of the bacterium in fish

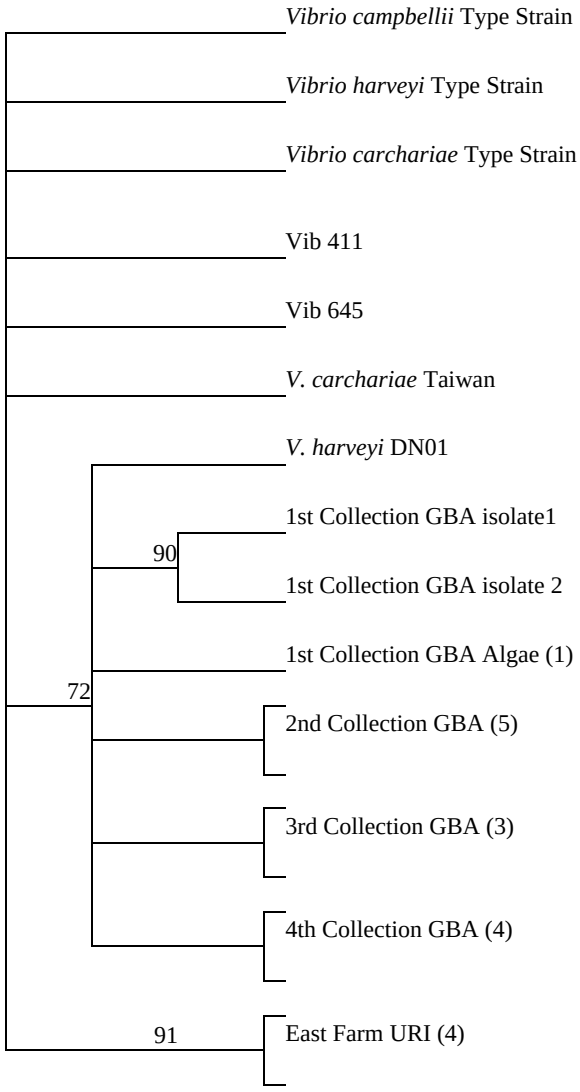


c) Evaluation of strain variation in *V. harveyi* collected at different stages of the monitoring program. A large number of *V. harveyi* isolates was obtained throughout the monitoring program from healthy and diseased fish, as well as algae, and live feed. It is conceivable that several strains of *V. harveyi* may be present in these collections. To address this issue, we conducted DNA fingerprinting on a number of the isolates from each collection period using a technique called Random Amplification of Polymorphic DNA (RAPD). This technique uses small random primers to amplify regions of DNA using the technique of Polymerase Chain Reaction (PCR). Small differences in DNA sequences result in different numbers and sizes of amplified DNA fragments that can be separated and visualized on an agarose gel. The patterns of these "bands" can then be compared for the different isolates, along with several reference strains, and phylogenetic relationships can be determined. The banding patterns obtained for the *V. harveyi* isolates from the GBA and URI were compared along with several reference strains using the phylogenetic software PAUP (Swofford, 1998). RAPD analysis of the *V. harveyi* isolates from collections at GBA and URI showed that all isolates taken from GBA showed identical genetic profiles, while the isolates isolated from GBA fish after transportation to URI showed a different profile, indicating that it is a different strain (Fig. 3). The origin of the *V. harveyi* isolate detected in moribund fish at URI is unknown, but it is reasonable to speculate that it was present in the water at URI's facility. The presence of numerous strains of *V. harveyi* will complicate the development of diagnostic tools and vaccines. The virulence of two of the *V. harveyi* isolates (one isolated from GBA at 50 dph, when no mortalities were occurring, and a reference strain isolated from the original outbreak of FINE) was evaluated by experimental infection of summer flounder by intraperitoneal (IP) injection. The *V. harveyi* isolated at GBA caused similar levels of mortality as the reference strain (not shown). Although this experiment indicates that both *V. harveyi* isolates are virulent, results must be interpreted with caution. Challenges using intraperitoneal injection do not mimic a natural infection, since natural protection barriers of the fish (such as skin and intestinal mucus) are bypassed. Differences in the ability of strains to bypass these barriers, or differences in the ability to colonize the intestine, could result in differences in pathogenicity that would not be detected using an intraperitoneal challenge. Alternatively, differences in previous exposure, as well as in the immune responses of flounder to the isolates could account for differences in pathogenicity. Further research would need to be done to evaluate these issues.

Objective 2. Identify the conditions that promote the growth and survival of *Vibrio carchariae in vitro.*

The optimal growth conditions of *V. harveyi* include salt concentrations ranging from 10 – 30 ppt and temperatures ranging from 10 – 30°C. Growth is increased by the inclusion of intestinal salmon in the growth media. Because bacteria from the genus *Vibrio* can withstand starvation-stress for extended periods of time, the ability of *V. harveyi* to adapt to starvation conditions and exhibit a starvation-stress response was examined. Cells were grown in various media, including rich media and minimal media lacking selected nutrients. *V. harveyi* cells grown in both rich and minimal media reached maximum cell density within 12-24 h at either 19°C or 27°C. The stationary phase cells exhibited no decline in viability over a one-week incubation at either temperature. During stationary phase, the motile, rod-shaped exponential phase cells exhibited a morphological change to a rod, non-motile form. Starved cells converted into round, non-motile cells. Cells starved for nitrogen, carbon, and phosphorus exhibited no decline in cell numbers over 1 wk incubation at 27°C. Only cells starved for carbon exhibited a decline of 1 $\log_{10}$. These observations demonstrate that starved or stationary phase *V. harveyi* cells are well adapted to coping with nutrient limiting conditions, able to adapt to and survive long periods of starvation. Exponential phase and stationary phase *V. harveyi* cells were tested for their ability to withstand various stress conditions (for 60 min) including heat shock (43°C), ethanol shock (10%), oxidative stress – H_2O_2 (10 mM), and sodium hypochlorite (bleach). Exponential phase cells were extremely sensitive to exposure to stress conditions with heat shocked cells declining by 3.5 $\log_{10}$; ethanol shocked cells declining by 4.5 $\log_{10}$; and H_2O_2 exposed cells declining by 3.5 $\log_{10}$. In contrast, stationary phase cells were more resistant to stress conditions. Stationary phase cells exposed to heat shock for 1 h exhibited decrease in cell density of 1.5 $\log_{10}$; ethanol shocked cells declined by 3.5 $\log_{10}$; and H_2O_2 exposed cells decrease in viability by <1 $\log_{10}$. These observations show that *V. harveyi* cells become resistant to multiple environmental stresses when in stationary phase. Concentrations of sodium hypochlorite higher than 3µM result in killing of *V. harveyi.* Increased resistance to sodium hypochrorite is seen when cells are at high densities. Concentrations of bleach commonly used to disinfect facilities and equipment (10 - 100 ppt) efficiently kill *V. harveyi* cells.

Figure 3. Phylogenetic tree of isolates from GBA, URI, and reference strains based on RAPD profiles. Type Strains as listed, VIB 411 isolated from fish in South Africa, VIB 645 Isolated from Sea Bass in Tunisia, *V. carchariae* Taiwan isolated from grouper, *V. harveyi* DN01 isolated from summer flounder in Rhode Island, remaining isolates from monitoring program, the number in parentheses indicates the number of isolates evaluated from each collection.



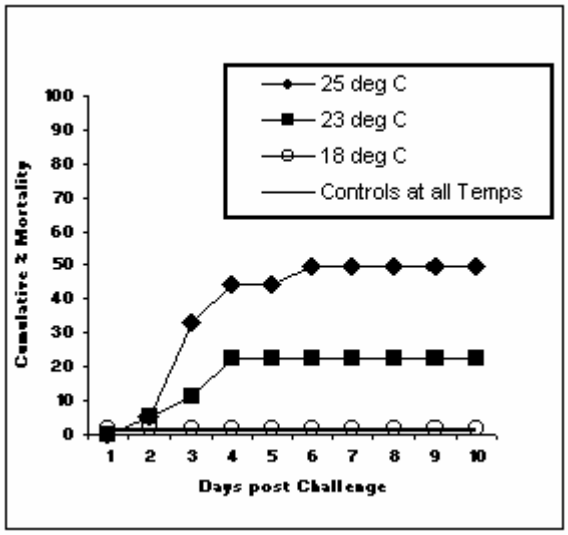
We have also evaluated protease activity of *V. harveyi.* Proteases are common virulence factors in intestinal pathogens such as vibrios. Protease activity was only detected when *V. harveyi* is grown in the presence of intestinal mucus, indicating that proteases could be involved in the pathogenicity of *V. harveyi*

Objective 3. Identify the environmental conditions that promote the occurrence of disease and mortality in experimental challenges with *Vibrio harveyi*.

Bacterial challenge experiments were conducted in order to determine the effect of temperature, salinity, and stress on the virulence of *V. harveyi* to juvenile summer flounder. For the challenge experiments, fish were anesthetized and inoculated with bacterial solutions suspended in Nine Salts Solution (NSS) or sterile NSS (negative controls). After inoculation, fish were held 10 gal glass aquaria equipped with standard aquarium filters and airstones and observed for 10 days. For determining the effect of temperature and salinity on virulence, the experimental design was 3 replicates of 6 fish injected IP with an estimated LD_{50} dose and 1 control replicate. Challenges were conducted with fish held at 18, 22, and 25°C with a constant salinity of 28ppt, and 10 and 28ppt salinity at a constant temperature of 22°C. The effect of stress on the susceptibility of summer flounder to infection by *V. harveyi* was assessed by comparing the ability of *V. harveyi* to cause mortality via different routes of infection under different stressors. Juvenile fish were inoculated by IP injection, intramuscular injection, gastric intubation, anal intubation, immersion, and cohabitation in stressed and non-stressed conditions. The stressors that were evaluated are: 1) high stocking density (0.5 kg/l) and 2) a combination of high stocking density and simulated transport (4 hours held in plastic bag with heavy aeration).

The results of our challenge experiments indicate that temperature, salinity, and stress can all have an effect on the virulence of *V. harveyi* in summer flounder. The challenges where temperature was varied from 18 to 25° C (constant salinity of 28 ppt) showed that increasing temperature results in increased virulence (Fig. 5). More importantly, no mortalities were observed at 18°C, indicating that this may represent a minimum temperature at which *V. harveyi* can kill juvenile summer flounder. When salinity was varied (10 and 28 ppt, constant 22°C), no mortalities were observed at the 10 ppt

Figure 5. Effect of temperature (left) and salinity (right) on the virulence of V. harveyi



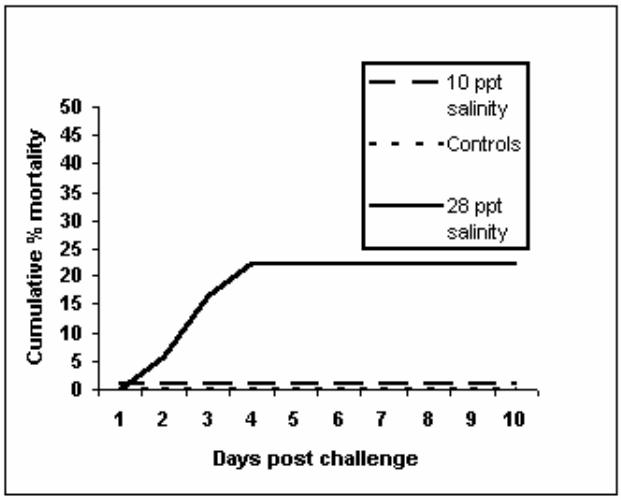


Table 3. Effect of stress on the virulence of V. harveyi to summer flounder

Challenge method	**Low density**	**High Density**	**High Density plus simulated transport**
Intraperitoneal injection	Mortalities	Mortalities	Mortalities
Intramuscular injection	Mortalities	Mortalities	---
Gastric intubation	No Mortalities	No Mortalities	Mortalities
Anal intubation	No Mortalities	---	---
Immersion	No Mortalities	No Mortalities	Mortalities
Cohabitation	---	---	Mortalities

salinity group (Fig. 5). This indicates that a reduced salinity may help the juvenile flounder combat the infection. We have observed that it is difficult to cause mortalities with *V. harveyi* in healthy summer flounder by methods of experimental infection other than IP injection. *V. harveyi* can only cause disease in non-stressed fish if the route of infection bypasses primary epithelial defenses. For routes of infection that do not bypass the epithelial layer, some stressor is required to initiate infection and cause mortality (Table 3).

Objective 4. Using information in objectives above to establish management protocols that minimize the development of outbreaks.

An interesting observation during the 5th collection at GBA (155 dph fish) led us to explore two potential management tools. This was the only time at which *V. harveyi* was not isolated from summer flounder. We also observed that there were lower numbers of bacteria overall, as compared to other collections. This collection was conducted approximately one week after a one-hour formalin treatment was used to control external parasites. Saltwater fish drink in order to maintain their osmotic balance so we hypothesized that during the one-hour treatment, enough formalin may have been ingested to reduce the intestinal bacterial load. In order to test this hypothesis we determined the ability the effect of a formalin dip on the levels of *V. harveyi* colonizing the intestine of flounder after challenge by anal intubation. Formalin treatment resulted in an increase in the levels of *V. harveyi* in the intestine after challenge, indicating that formalin may act as an stressor resulting in increased bacterial infection. Another possible explanation for the lack of *V. harveyi* and reduced overall bacterial load is that one of the several species, uniquely isolated at 155 dph, had an inhibitory, or probiotic effect on *V. harveyi*. We tested the ability of several probiotic candidates isolated from the monitoring program using a zone inhibition assay. Two species, *Photobacterium phosphoreum* and *Staphylococus succinus*, showed some ability to inhibit the growth of *V. harveyi*, but this could not be confirmed on subsequent tests. Conditions for efficient inhibition of *V. harveyi* need to be tested further.

In summary, our research indicates that:

- *V. harveyi* was a major component of the intestinal flora of larval and juvenile summer flounder at GBA, but not at URI.
- Potential sources of *V. harveyi* include water, algal cultures, rotifers, and artemia.
- Different strains of *V. harveyi* can be present at a single facility. Further research needs to be done to evaluate the virulence of the different strains.
- In general, increased levels of *V. harveyi* are associated with clinical signs of FINE.
- However, presence of *V. harveyi* in summer flounder is not necessarily correlated with disease. Histological signs indicative of FINE were only observed in juvenile fish, and significant mortalities were only observed after transport stress, both in challenge experiments and at the monitoring program. Factors that could influence the occurrence of disease include: age of the fish, genetic susceptibility of the fish, strain of *V. harveyi*, previous exposure to the pathogen, environmental conditions, and the presence of competing bacterial strains.
- Other potential fish pathogens (*Photobacterium damselae*, subspecies *damselae*, *V. ichthyoenteri*, and *V. scophthalmi*) have been isolated from juvenile summer flounder. Further research needs to be done to evaluate the contribution of these pathogens to mortality and disease. While these species have been documented as pathogens in numerous species including flatfish (*V. ichthyoenteri* in Japanese flounder and *V. scophthalmi* in turbot), they have not yet been identified as pathogens of summer flounder.

IMPLICATIONS:

- *V. harveyi* should be considered a top priority in the health management of summer flounder. Our monitoring program clearly indicates that larval and juvenile summer flounder are prone to colonization with *V. harveyi* and that infection can result in serious morbidity and mortality. The growth of survivors is impaired by the occurrence of blind-sac guts.
- Management of FINE after transport stress: Transport stress continues to be implicated as a major trigger of FINE. The occurrence of mortalities after fish were transported to URI reinforces the view that the stress related to transport can impair the juvenile flounder's ability to fight the disease. There are several ways in which this problem can be addressed. Currently, there is research being conducted at URI, by Dr. Jennifer Specker, which aims to determine the effectiveness of using anesthetics to reduce the stress of transport. This research may aid in developing transport protocols that decrease the incidence of FINE. Our research may also be useful in addressing this problem. We have

observed that under conditions of low salinity (10 ppt) or low temperature (18°C), challenging fish with normally lethal concentrations of bacteria does not cause mortalities. Applying these observations to transport protocols may reduce the risk of FINE. Transporting fish at 10 ppt salinity may aid in the fishes ability to tolerate transport. Reducing osmotic stress during transport is already a standard practice with many freshwater fish; in this case it is accomplished by adding salt to transport tanks. In addition, it may advisable to control temperature during transport and the two following weeks.

- Management of FINE in farms: Because low salinity and low temperature increase the ability of summer flounder to resist *V. harveyi*, environmental conditions in the farm can be manipulated to decrease the risk of disease outbreaks. However, low temperatures and salinities may also reduce growth rates. If the loss of growth outweighs the gains in fish health, these recommendations may not be practical. Ultimately, a balance must be reached between the costs and benefits of using low temperature and low salinity to improve fish health. In the case of salinity, it may also be possible to use a low salinity regimen as a short-term treatment when disease is first observed. Such a practice may minimize losses during a disease epizootic and only result in a temporary reduction in growth. Additional research would be needed to assess the effectiveness of this practice.
- Diagnosis: The majority *V. harveyi* isolates collected during our monitoring program gave identical or nearly identical API 20E profiles, and no other species identified gave the same profiles. However, due to the variability of these profiles, we recommend using an additional diagnostic tool, such as 16S rDNA sequencing. Other more specific tools that can distinguish between strains of *V. harveyi*, such as diagnostic PCR or ELISA, should be developed in the future. These techniques must be validated with as many strains as possible. This will help insure that the diagnostic method is effective for all strains.
- Prevention: The most common prevention tools include vaccines, immunostimulants, and probiotics. Vaccine development for the prevention of *V. harveyi* may prove specially challenging, due to strain variability. Two potential probiotic strains have been identified in this research and should be further evaluated. While methods to prevent colonization are developed, to best prevention strategy is to maintain the overall health of the fish and reduce stress during transport.

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