IDENTIFICATION, CHARACTERIZATION, AND EVALUATION OF MECHANISMS OF CONTROL OF *BOLBOPHORUS*-LIKE TREMATODES AND *FLAVOBACTERIUM COLUMNARE*-LIKE BACTERIA CAUSING DISEASE IN WARM WATER FISH

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PROJECT OBJECTIVES

1. Identify and characterize all of the life stages of the digenetic trematode (tentatively identified as *Bolbophorus* sp.) that infects channel catfish using both classical and molecular methods.

2. Evaluate integrated methods for snail control in catfish ponds.

    a. Monitor populations of catfish infected with *Bolbophorus* spp. to document the effect of parasite loads on growth and survival of the fish.
    b. Examine the efficacy of chemical control methods on snail populations.
    c. Examine the efficacy of biological control methods (snail-eating fish) on snail populations in ponds.

3. Develop and implement standardized methods for the isolation, culture, and antimicrobial susceptibility testing of strains of columnaris-like bacteria isolated from diseased fish.

4. Characterize archived strains of columnaris-like bacteria based on the following conventional and molecular techniques.

    a. Morphology
    b. Enzyme analysis
    c. Biochemical analysis
    d. Sequencing 16S ribosomal RNA and ribotyping

5. Develop challenge models for columnaris-like bacteria isolated from major warmwater aquaculture species in the southeast.

6. Using the challenge model for each species, correlate virulence with biotype and/or genotype of columnaris-like bacteria.

PROGRESS AND PRINCIPAL ACCOMPLISHMENTS

Objective 1. *Identify and characterize all of the life stages of the digenetic trematode(tentatively identified as* Bolbophorus *sp.) that infects channel catfish using both classical and molecular methods.*

Confirmation of *Bolbophorus* life cycle

Mississippi State University and USDA/ APHIS/WS. Two studies were conducted to confirm the life stages of *Bolbophorus damnificus* in American white pelicans and its snail host, *Planorbella trivolvis*. Three pelicans were pretreated with praziquantel, challenged

with *B. damnificus* metacercaria to establish patent infections, and were subsequently used to artificially infect *P. trivolvis*. Catfish were exposed to these infected snails, metacercaria from this challenge were fed to parasite free pelicans, and patent *B. damnificus* infections were established. Each life stage of this parasite was confirmed to be *B. damnificus* morphologically and molecularly. Data are being analyzed on cercaria and ova shedding.

A second study was conducted to determine potential snail hosts for *B. damnificus* and its life cycle in the snail. Ova from pelicans infected in Study 1 were used to artificially infect several snail species housed in aquaria at 80°F. Snails were checked weekly for cercaria shedding, and checked daily when they were positive. Time and number of cercaria shed was recorded and data are being analyzed.

Collections to Evaluate the Avian Host Range for *Bolbophorus*

USDA/ARS. A total of 106 aquatic birds have been collected and trematodes harvested from their alimentary canals for identification. Some of these trematodes have potential to be transmitted to cultured fish species. In 2003, 25 aquatic birds were collected including 5 pelicans, 10 cormorants, and 10 great egrets. In 2004, 54 birds were collected including 17 great egrets, 12 great blue herons, 11 snowy egrets, 6 cattle egrets, 6 green herons, 4 belted kingfishers, and 1 little blue heron. In 2005, 27 birds were collected including 6 belted kingfishers, 5 white pelicans, 2 great egrets, 9 black-crowned night herons, and 5 little blue herons. It appears that *Bolbophorus* spp. have been recovered only from white pelicans collected in 2003 and 2005. The trematode *Clinostomum* spp., one species of which is responsible for the yellow grub in fish, was found in great egrets, great blue herons, snowy egrets, black-crowned night herons, little blue herons, and cattle egrets. The gill trematode *Centrocestus formosanus* was recovered from green herons and great egrets. Identification of the trematodes is ongoing.

Confirmation of the Definitive Final Host of *Bolbophorus*

North Carolina State University. Adult *Bolbophorus damnificus* and immature *Bolbophorus* sp. type 2 have been recovered and identified from the American white pelican. Mature ovogenous *Bolbophorus* sp. type 2 have not been recovered from any avian species and identification of its definitive host remains a priority.

Mississippi State University. Birds (two each of American white pelicans, double-crested cormorants, great blue herons, great egrets) were live-captured in the Mississippi Delta. They were individually housed in pens with recirculating water tanks and fed catfish ad libitum daily until challenge. Birds were acclimated for at least 1 week. Fecal samples were collected daily starting at 48 hours prior to anti-helmintic treatment and continued until necropsy. At 7 days pre-challenge, birds were administered praziquantel at 34 mg/kg BW per os to eliminate all trematodes. At 7 days post-treatment birds were fed live fish naturally infected with *Bolbophorus damnificus* metacercariae (confirmed by a *B. damnificus*-specific polymerase chain reaction, PCR). Birds were necropsied 21 days post-challenge, intestinal contents of

each bird were examined; all parasites were removed, examined microscopically, identified and enumerated. A sub-sample of each parasite type was processed for electron microscopy and DNA analysis.

The only bird species that shed *B. damnificus* ova (confirmed by PCR) during the trial was the American white pelican. Adult *B. damnificus* were found in pelican 1 (one adult trematode) and pelican 2 (five adult trematodes). All other bird species were negative for *B. damnificus* and other trematodes.

This study confirms that the American white pelican is a host for *B. damnificus*. Results from this study demonstrate that artificial infections of *B. damnificus* could not be established in double-crested cormorants, great blue herons, and great egrets.

Confirmation of Intermediate Hosts of *Bolbophorus* spp.

North Carolina State University, USDA/ARS, Mississippi State University. *Planorbella trivolvis* snails collected from catfish ponds in Mississippi experiencing outbreaks of *Bolbophorus*-associated morbidity/mortality were screened for the shedding of forked-tailed cercariae in snails shipped to North Carolina. Two morphologically distinct types of bolbophorid cercariae were confirmed morphologically and genetically utilizing species-specific PCR. These were *1) Bolbophorus damnificus*, a serious pathogen of channel catfish, *Ictalurus punctatus*, and 2) *Bolbophorus* sp. type 2, a species not recovered from catfish but present in several other fish hosts. Interestingly, several snails were shown to be shedding both bolbophorid species simultaneously or sequentially. This indicated that both species were present in aquaculture ponds and they utilized the same molluscan host. A manuscript "Morphological description of the cercariae of *Bolbophorus damnificus* and *Bolbophorus* sp. with notes on North American Bolbophorids" by J. R. Flowers, M. F. Poore, L. M. Pote, R. W. Litaker and M. G. Levy was submitted to Comparative Parasitology in June 2004. Information in this manuscript will allow identification and speciation of bolbophorid cercariae based on light microscopic details.

RESULTS AT A GLANCE...

★ *Studies on the various life stages of* Bolbophorus damnificus *have revealed that the adult trematode resides in the gut of the American white pelican. The parasite has not been found in wild cormorants, great egrets, great blue herons, snowy egrets, cattle egrets, green herons, belted kingfishers and little blue herons. Attempts to artificially infect cormorants, great blue herons and great egrets failed whereas the white*

North Carolina *Planorbella duryi* snails were sent to Dr. L. Pote at Mississippi State University who was successful in infecting them with *B. damnificus*, indicating that the North Carolina snails are a permissive intermediate host. This indicates that in the presence of a suitable avian host, this infection is capable of further spread to the southeastern United States. Dr. Pote also provided several shipments of *P. trivolvis* positive for *B. damnificus* and *Bolbophorus*-

type 2 to Dr. Michael Levy at North Carolina State University, and has maintained the *P. trivolvis* snail colony which provided negative snails for other cooperators in this project.

The *Bolbophorus* trematode has been found in wild fish species including channel catfish and several centrarchids in Lake Chicot, Arkansas. Metacercarie recovered from a variety of fish demonstrated the following distribution: Only *B. damnificus* was recovered from catfish in aquaculture ponds. *Bolbophorus* species type 2 was recovered from white crappie and longear sunfish and largemouth bass. The fathead minnow was found to harbor both *B. damnificus* and species type 2. This is the first finding of a *B. damnificus* in a fish species other than catfish.

Both patent and pre-patent infections in infected snails were identified using PCR. Using PCR we also identified snails shedding either *B. damnificus* or type 2 exclusively. Cercariae were then fixed in hot 10% neutral buffered formalin. Ten cercariae of each type were examined for body length, body width, tail-stem length and width, furcae length and width, and oral sucker size. An additional large number of living cercariae were held under a cover slip and examined for the following characteristics: penetration glands, flame cells, organ primordial and tegumental spine arrangements. Differences between the two species strongly suggest that cercariae have distinguishing morphologic characteristics. Confirmation of these observations will be accomplished by examining additional cercariae during the coming season in order to rule out individual snail variation.

Fish Challenge Trials with *Bolbophorus* spp.

North Carolina State University. The potential pathogenic effect of both trematode species was investigated in a series of preliminary experiments. Hybrid striped bass (*Morone saxitalis* × *M. chrysops*), and channel catfish fingerlings were obtained from commercial farms in North Carolina where *Bolbophorus* is not known to be present. Snails were divided into two groups based on PCR identification of the *Bolbophorus* species that they shed. Infection rates were based on available numbers of cercariae less than 2 hours after emergence from the snails. Catfish were 2- to 3-inch fingerlings and hybrid striped bass were 1.5-inch fingerlings. An aliquot of cercariae was retained from each infection time and the challenge species reconfirmed using PCR. These results were not available until after challenge was completed due to the time involved in running the PCR assay.

Five groups of five bass were infected with 300, 500, or 550 *B. damnificus*, and two groups of bass were infected with either 40 or 285 *Bolbophorus* sp. type 2. Eight groups of five catfish were infected with 175, 350, 637, or 700 *B. damnificus* cercariae/fish. Three groups were infected with 300, 700, or 1,000 *Bolbophorus* species type 2. One group of fish was infected with 700 cercariae of a mixture of the two species due to a "switch" in the species shed by one or more snails in this group. All fish were necropsied and metacercariae removed and identified as to type using PCR.

All catfish infected with any dose of

B. damnificus developed the typical hemorrhagic lesions and most died beginning on day 4 post-infection. Several fish exposed to the lowest numbers of cercariae survived and were euthanized 6 weeks post-infection. Although catfish exposed to only *Bolbophorus* sp. type 2 failed to exhibit obvious signs of infection such as hemorrhagic lesions typical of a *B. damnificus* challenge, exposure to B. sp type 2 cercariae did result in these fish going 'off feed' for several weeks. A few degenerate metacercariae, none containing intact immature adult worms, were recovered. These were identified as type 2 by PCR.

Hybrid striped bass challenged with type 2 cercariae exhibited hemorrhagic lesions similar to those observed with *B. damnificus*-challenged catfish and mortality rates were similarly high. No morbidity or mortality was observed with hybrid striped bass challenged with *B. damnificus*. Only *Bolbophorus* species type 2 metacercariae were recovered from hybrid bass.

In Year 2 experimental infection of fish was continued with the two bolbophorid species. The potential pathogenic effect of both trematode species was investigated in a series of additional experiments complementing those performed in Year 1.

Laboratory-reared hybrid striped bass fingerlings and raceway-reared channel catfish fingerlings were purchased from commercial fisheries. Snails that had identified bolbophorid infections were placed in separate groups by species of cercariae as determined by species-specific PCR and cercariae were allowed to escape from their snail host into the water column. Samples of cercariae from each snail group were counted and cercarial yields were calculated. Within 4 hours of escape from the snails, cercariae were added to fish tanks containing experimental fish.

Groups of ten hybrid striped bass were separately exposed to *Bolbophorus damnificus* cercariae and *Bolbophorus* sp. cercariae at the following exposure rates: 500, 250, or 100 cercariae/fish. Groups of ten channel catfish were also separately exposed to *Bolbophorus damnificus* cercariae and *Bolbophorus* sp. cercariae at the following exposure rates: 250, 100, or 25 cercariae/fish.

At 5-days post-exposure, hemorrhagic lesions, lethargy, and decreased appetite were noted in the hybrid striped bass exposed to the *Bolbophorus* sp. cercariae at the rates of 500, 250, and 100 cercariae/fish. Mortality of the hybrid striped bass exposed to 500 cercariae/fish began at 6 days post-infection and all fish were dead by 11 days post-exposure. Hemorrhagic lesions in hybrid striped bass exposed to 250 and 100 cercariae per fish disappeared by 19 days post-exposure; however, large bumps under the skin were noted. None of the hybrid striped bass exposed to *Bolbophorus damnificus* developed lesions.

Conversely, channel catfish reacted to the *Bolbophorus damnificus* cercarial exposures. Hemorrhagic lesions and bumps were observed on catfish exposed to *B. damnificus* cercariae at the rates of 250 and 100 cercariae/fish in the morning of day 9 post-exposure. In the afternoon of day 9 post-exposure, three catfish exposed to 250

cercariae/fish and one catfish exposed to 100 cercariae/fish had died. Later, one catfish exposed to 250 cercariae/fish and one exposed to 100 cercariae/fish died at 14 and 23 days post-exposure, respectively. Some of the catfish exposed to 250 cercariae/fish developed exophthalmia and abdominal distension. None of the catfish exposed to the *Bolbophorus* sp. cercariae developed lesions. Also, lesions were not seen in the catfish exposed to *Bolbophorus damnificus* cercariae at the rate of 25 cercariae/fish.

The pathological consequences of *B. damnificus* infection in channel catfish were less severe compared with those seen in past experiments. This may be due to the larger size of fingerlings or the presence of fewer non-trematode pathogens. In previous challenges, fingerlings were collected from ponds and may have harbored other pathogens, whereas for this experiment we collected swim-up fry hatched in well water and laboratory-reared the fish.

These results demonstrate a high degree of specificity for the intermediate hosts for these two bolbophorid trematodes. *Bolbophorus damnificus* caused lesions only in catfish where *bolbophorus* sp. caused lesions only in hybrid bass.

Fish growth rates at the different parasite-exposure rates are currently being statistically analyzed. The number of encysed metacercariae for each fish (for each challenge parasite species and number) will also be determined. Development of methods for quantification and estimation of parasite loads in molluscan populations are also in progress.

Objective 2. *Evaluate integrated methods for snail control in catfish ponds.*

Objective 2a. *Monitor populations of catfish infected with* Bolbophorus *sp. to document the effect of parasite loads on growth and survival of the fish.*

Mississippi State University. Laboratory and field studies were conducted to evaluate the effects of sub-lethal trematode infections on growth, performance and disease resistance of channel catfish fingerlings. Trematode infections were established in populations of fish stocked in four, 0.1-acre ponds. A reservoir of trematode-infected snails was maintained in recirculating 300 gallon tanks located on the bank of each pond. Pond water was recirculated through each tank at a rate of 2 gallons per minute. The effluent (containing *Bolbophorus* cercariae) from the tank was directed back into the pond and served as the source of infection. Four additional ponds were used as control ponds. After 40 days, each population of fish was sampled to evaluate health status and 120 fish from each pond were transferred to 30-gallon aquaria to evaluate growth rates under controlled laboratory conditions using well water free of *Bolbophorus* cercariae. Only fish containing visible cysts (1 to 5 cysts per fish) were selected and used to evaluate growth potential. Fish were acclimated to laboratory conditions at 31 to 32°C for 3 weeks before

the start of the study. Following the conditioning period, fish were fed once daily for 9 weeks. Total weight gain, percent weight gain, specific growth rate, and feed efficiency were used to evaluate growth.

Evaluation of health and growth of channel catfish continually exposed to the cercarial stage of *Bolbophorus damnificus* throughout a production cycle

Mild trematode infections were established in pond populations of experimental fish by exposing fish to trematode cercaria. The percent of infected fish in each pond ranged between 20.4% and 1.6%. Mortalities directly related to trematode infections were not observed. *Edwardsiella ictaluri* and *Flavobacterium columnare* infections were diagnosed from all populations of fish and no differences in mortality were observed between trematode infected and non-infected fish. At the end of the production cycle, trematode infected fish consumed approximately 40% less feed compared to fish in control ponds.

Evaluation of health and growth of fish that have been infected with *Bolbophorus damnificus* cercariae by a single-pulse exposure

At the start of the acclimation period, trematode infected fish were significantly smaller compared to fish collected from control ponds. No differences in any of the measured parameters were observed between trematode-infected and non-infected fish at the end of 9 weeks. Although the final weight of trematode-infected fish was numerically smaller than control fish, percent weight gain and specific growth rate demonstrated a tendency towards compensatory growth of trematode-infected fish. Feed efficiency (0.86) was identical between treatment groups. Data indicates that once fish are removed from the source of infection, chronic trematode infections do not affect the growth potential of channel catfish.

Evaluation of health status and growth potential of channel catfish fingerlings infected with *Bolbophorus damnificus* under controlled laboratory conditions

Trematode infections were established under laboratory conditions by placing fingerlings in triplicate tanks containing *Planorbella trivolvis* snails shedding cercariae. Fish were left in the tank for 24 hours and snails were shedding cercariae at a rate of 770 ± 82 per 24 hours. Unexposed fish were maintained in three tanks under similar conditions. From each tank, trematode-infected or non-infected fish were transferred to six aquaria (30 fish/aquaria). Three aquaria from each replicate treatment tank received 7.5×10^5 CFU *E. ictaluri*/mL of water for 30 minutes (*Bolbophorus*-ESC and ESC-only groups). Fish in the remaining three aquaria were not exposed to *E. ictaluri* and served as *Bolbophorus*-only and negative control groups. The later two treatment groups were used in a second study and were challenged with *E. ictaluri* 28 days after exposure to *Bolbophorus* sp. cercariae.

No mortalities were observed in the *Bolbophorus*-only and negative control groups. Twenty-one days following exposure to *E. ictaluri*, the percent cumulative mortalities were 84.1 ± 16.2% in the *Bolbophorus*-ESC treatment and 45.9 ± 3.2% in the ESC-only

treatment. Mortalities were significantly different between the two groups. In the second study, when *E. ictaluri* exposure was delayed 28 days following *Bolbophorus* sp. infection, there was no difference in mortalities between the ESC-only (17.8 ± 4.0%) and combined *Bolbophorus*-ESC (21.5 ± 1.7%) exposed groups. Apparently, once fish are removed from the source of additional infections, chronic trematode infections do not increase the susceptibility of fish to ESC.

RESULTS AT A GLANCE...

★ *Mild, sub-lethal trematode infections can significantly reduce catfish growth by reducing feed consumption and increasing mortality associated with concurrent bacterial infections.*

Findings in these studies have significant implications for management strategies to control losses associated with trematode infections. Data collected from laboratory and field trials indicated that mild sub-lethal active trematode infections, commonly observed in channel catfish production systems, can significantly reduce production by reducing feed consumption and increasing losses associated with ESC. These studies also indicated that the presence of fully developed metacercariae does not compromise growth and health status of fish. These data support the contention that the deleterious effects of trematode infection are associated with penetration of the parasite and initial stages of encystment. Findings point to the need for increased surveillance for this disease and the benefit of initiating management protocols at the earliest stages of infection. In addition, breaking the trematode's life cycle by moving fish to non-infested water or by eradicating snails in the pond will eliminate the adverse effects associated with this disease.

RESULTS AT A GLANCE...

★ *The presence of fully developed* Bolbophorus *metacercariae does not affect growth or health of catfish. The deleterious effects of this infectious agent are therefore associated with penetration of the parasite and initial stages of encystment.*

Evaluation of the farm-wide economic impact of *Bolbophorus damnificus* infections of channel catfish

The economic impact of trematode infections was evaluated by conducting a disease-monitoring and production-efficiency study on a commercial catfish operation with ponds containing trematode-infected fish. Fish were sampled from each food fish production pond and examined for the presence of cysts that contain the metacercariae. Each pond was placed into one of four categories based on the percentage of infected fish in the sample. Ponds with trematode-infected catfish were placed into categories of light, moderate, or severe when the percentage of trematode-infected fish in the sample ranged from 0% to 33%, 33% to 66% or 66% to 100%, respectively. Ponds that did not contain trematode-infected fish were categorized as negative. Production records were grouped by infestation level for analysis. Of the 40 pond populations sampled, 17 were categorized as

negative, 6 as light, 6 as moderate, and 11 as severe. Fish from trematode-positive ponds consumed significantly less feed compared to fish from ponds that were categorized as trematode-negative. Fish from ponds in the trematode-negative category consumed on average 73.4 pounds/acre per day, and fish from ponds categorized as light, moderate and severe consumed 62.2, 47.5, and 47.2 pounds/acre per day, respectively. Similarly, production decreased as severity of infection increased. Compared to trematode-negative ponds, ponds in the light, moderate and severe categories produced 16.8%, 36.4%, and 44.5% fewer pounds of fish per acre, respectively. Net returns from ponds in the light category were reduced by 80.8% and production from ponds in the moderate and severe categories were not shown to cover variable costs of production. Ponds in the moderate category produced a net loss of $506 per acre and severe ponds produced a net loss of $631 per acre. These data from a commercial setting support results from experimental ponds and demonstrate that *Bolbophorus* infestations, regardless of severity, are a significant risk to commercial production of channel catfish.

Obective 2b. *Examine the efficacy of chemical control methods on snail populations.*

USDA/ARS. A total of seven trials were conducted from 2003 through 2005 to test the effectiveness of pond-shoreline treatments in controlling aquatic snails. Initially, four trials were conducted to compare a slurried-hydrated lime treatment with an established copper sulfate treatment. Copper sulfate and hydrated lime were applied at 4 pound and 80 pounds, respectively, per 100 feet of shoreline in a 6-foot swath. Trials were run under conditions of variable wind speed (0 to 16 mph) and treatment temperature (24 to 32°C). Both treatments effectively lowered the snail populations in the test cages. It appears that copper sulfate was more effective than lime in most trials, that hydrated lime treatments appeared to increase in effectiveness at higher temperatures (32°C vs 25°C), and that strong winds negatively impacted both treatments. Snail survival under all conditions in the four trials ranged from an average of 3.4% to 27.8% and 10.4% to 41.5% for copper sulfate and hydrated lime, respectively.

The goal of the second part of the study changed from comparing lime and copper sulfate to optimizing the hydrated lime treatment. Trials 5, 6, and 7 were conducted using only hydrated lime treatments at temperatures of 24 to 26°C and under low wind conditions. In Trial 5, the rate of hydrated lime was increased to 175 pounds per 100 feet of shoreline in a 6-foot swath. At that rate, effectiveness was increased and snail survival was less than 2%. Further optimization of the hydrated lime treatment was made by narrowing the treatment swath to 3 feet and reducing the amount of lime to 80 and 100 pounds per 100 feet of shoreline. Average snail survivals for the 80 and 100 pound treatments were 13.3% and 2.7%, respectively. The optimum pond shoreline treatment with slurried hydrated lime is

100 pounds of lime per 100 feet of shoreline applied in a 3-foot swath.

RESULTS AT A GLANCE...

★ *A shoreline treatment with slurried hydrated lime applied in a 3-foot swath at 100 pounds of lime per 100 feet of shoreline reduced snail populations by over 95%.*

Mississippi State University. The toxicity of copper sulfate to ram's horn snails was evaluated by establishing the 24-hour LC50 using Spearman-Karber analyses. Tests were conducted in 300-mL glass containers containing 200 mL of pond water (alkalinity = 235 mg/L as $CaCO_3$, hardness = 300 mg/L as $CaCO_3$, pH = 7.5, temperature = 23°C). Test concentrations of copper were arranged in a geometrically spaced dilution series. Each test concentration consisted of 4 replications with 5 snails per replication. Copper sulfate granules were dissolved in distilled water and delivered as a solution. After 24 hours, snails were removed from the test solution and placed in fresh, untreated water. End-points for the tests were death of the snails as determined by an additional 96-hours post-test observation period to confirm mortality. The effect of temperature (15, 20, 25, and 30°C) and alkalinity (0, 50, 100, and 200 mg/L $CaCO_3$) on the toxicity of copper to snails were also evaluated.

Laboratory tests showed copper sulfate crystals had a 24-hour LC50 of 0.6 mg/L Cu and, based on the alkalinity of the test water, was below the level considered toxic to fish. Alkalinity at the levels tested (0 to 200 mg/L $CaCO_3$) was not shown to effect to the toxicity of copper to snails. The LC50 concentration at an alkalinity of 0 mg/L $CaCO_3$ was 0.52 mg/L Cu versus 0.67 mg/L Cu at an alkalinity of 200 mg/L. Although there appeared to be a trend in the LC50 values toward decreasing toxicity with increasing alkalinity, these differences were not statistically significant. Analysis of data from this study showed a significant linear relationship between temperature and LC50 values for copper. As temperature increased from 15°C to 30°C, the LC50 values decreased from 1.1 mg/L to 0.18 mg/L Cu, representing a ten-fold increase in toxicity. This would be an important consideration when treating ponds with copper sulfate with respect to both snail and fish toxicity.

Three dose-titration trials were performed to determine the copper concentration required to kill snails under field conditions. Two trials were conducted in plastic tanks containing 200 gallons of pond water. Fish and snails were placed in three replicate tanks and dosed with a solution of copper sulfate at 0 (control) 1.25, 2.5, 5.0, and 10.0 mg/L Cu during the first trial and 0 (control), 0.375, 0.75, 1.25, and 2.50 mg/L Cu during the second trial. Each tank consisted of 20 snails confined in wire mesh cages and 10 channel catfish fingerlings to evaluate the toxicity of the treatment dose to fish.

The third trial was a non-replicated study conducted in 0.25-acre ponds containing approximately 1 acre-foot of water. In each pond, three sample sites were evenly distributed along the center long axis of the pond. At each sample site, 20 snails were confined in cages located at the surface and bottom of the pond. The toxicity of copper to

fish was evaluated in the 0.25-acre ponds by confining 10 channel catfish fingerlings in floating net-pens in close proximity to each snail sampling site. Each pond received a dose of 0 (control), 1.25, 2.5, 5.0, and 10.0 mg/L Cu by evenly applying the copper sulfate solution (200 gallons/pond) around the margins of the pond. Twenty-four hours after treatment, test snails were transferred to 1-L containers (containing the appropriate test water) and transported to the laboratory and observed for 72 hours. Twice daily, dead snails were removed and placed in a separate container containing untreated pond water. Dissolved oxygen concentrations in the test water and fish mortality were observed for 96 hours.

Titration trials in tanks and ponds were comparable, with laboratory toxicity trials and indicated the minimum effect dose (snail mortality >90%) of copper sulfate ranged between 0.75 and 1.25 mg/L Cu. Fish mortality was not observed at or below 1.25 mg/L Cu in the pond tank studies. Fish mortality (average mortality 5.3%), however, was observed at one of the three sample locations in one of the replicate ponds following treatment with 1.25 mg/L Cu.

Based on the results of the dose-titration trials, triplicate 0.25-acre and duplicate 10.0-acre experimental ponds were treated with 0.75 and 1.25 mg/L Cu to verify the minimum effective dose. Sample site configurations and treatment applications for tests conducted in the 0.25-acre ponds are described above. Sample site configurations for tests conducted in 10-acre ponds varied to accommodate pond size. Each 10-acre experimental pond contained 12 uniformly distributed sample sites consisting of 20 snails confined at the surface and bottom of the pond. An additional 20 snails were confined along the pond bank at equal intervals. Each 10.0-acre pond was managed as a commercial production pond and contained approximately 3,500 (average estimated size = 1 pound) fish per acre. No mortality or signs of disease were noted before any of the tests were conducted. Toxicity of the copper treatment to fish was evaluated by observing the pond stock for behavioral indicators of toxicosis and mortality. For each test conducted, a non-treated pond contained similar sample site configurations and served as a control.

Replicate single-dose pond treatments verified that treatment doses of 0.75 and 1.25 mg/L Cu were effective in killing snails. Average snail mortality in trials conducted in the 0.25-acre ponds ranged between 98.0% and 95.5% at the low treatment doses and was 100% at the high treatment dose. Fish mortality was observed in 1 of the 3 replicate ponds at each treatment dose. Similar results with respect to snail toxicity were observed in the single dose toxicity trials conducted in 10-acre ponds. Average snail mortality of the replicate trials at each sample location ranged between 92 and 98% following treatment, with 0.75 mg/L and between 98 and 100% following treatment with 1.25 g/L Cu. In contrast to the 0.25-acre pond trials, no fish mortality or behavioral signs of toxicosis were observed following treatment. In all pond trials, dissolved oxygen depletions were not observed for up to 168 hours after treatment. Fish mortality in the 0.25 acre ponds may be caused by exposure of confined fish to high concentrations of the applied chemical before it was completely mixed with the pond water.

Treatment efficacy was then evaluated in a 13.0-acre commercial channel catfish production pond. Moderate numbers of snails were

observed along the margins of the pond. Seven sample sites (surface and bottom cages containing 20 snails each) were placed a minimum of 50 yards from the pond bank and were distributed randomly across the pond. An equal number of cages containing 20 snails each were placed along the margins of the pond. In addition, natural snail populations along the margins of the test pond were sampled at five locations before and after treatment. A 20-foot section of pond bank consisting of uniform vegetation and levee slope was marked and divided into 2 equal sections. Snails were collected from a 10-foot section of the sample site before the pond was treated, and the remaining section was sampled 24 hours after the pond was treated. Snails collected from each section were placed in 4-L containers and transported to the laboratory for observation. Live snails were counted to estimate viable snail numbers and used to determine the number of snails per foot of pond bank in the sampled area. Production fish were monitored for behavioral signs of toxicosis and mortality. Snails confined in a similar configuration in an adjoining pond served as a control.

Results of the commercial field trial were comparable to tests conducted in the experimental ponds where application of copper sulfate at 1.25 mg/L was shown to be effective in killing snails. Average mortality of snails confined in cages ranged between 95.4 and 97.7%. The treatment was also shown to be effective against natural populations of snails along the margins of the pond. The average number of snails per foot of pond bank decreased from 21.5 snails to 0.18 snails 24 hours after treatment, representing a 99% reduction in viable snail populations in the habitat along the pond bank.

Treatment of the commercial pond resulted in changes in fish behavior and mortality that was likely related to the copper treatment. Within 4 hours of treatment application, an increase in the number of moribund fish were observed. Affected fish appeared lethargic or exhibited a spiraling swimming pattern. However, it is not thought that these observations were solely related to the chemical treatment. Prior to treatment, moribund and dead fish were present in the pond that was diagnosed with bacterial (*Edwardsiella ictaluri* and *Flavobacterium columnare*) and parasitic infections (*Bolbophorus* sp.). Moribund fish also exhibited clinical symptoms consistent with visceral toxicosis of catfish. Mortality rates in the pond were characterized as chronic and were estimated to be 150 to 200 fish per day. Farm management estimated total mortality in excess of 20,000 pounds. Following treatment, fish mortality increased within the first 24 hours. It was estimated that approximately 2,000 pounds of fish were lost following treatment, however, a majority of the fish had clinical signs of ESC. In addition to infectious disease, analysis of water quality 2 hours after treatment revealed low chloride to nitrite ratios and examination of fish gills and blood indicated acute nitrite toxicosis. On the

RESULTS AT A GLANCE...

★ *A low-dose, full-pond treatment with copper sulfate was developed that safely eradicates the trematode's intermediate host—the ram's horn snail.*

day of treatment, salt was added to the pond water and, after the first 24 hours, the daily mortality rate returned to pretreatment levels.

Objective 2c. *Examine the efficacy of biological control methods (snail-eating fish) on snail populations in ponds.*

Effect of diet conditioning on prey selection by blue catfish and redear sunfish

Mississippi State University, Southern Illinois University. Four aquaria were stocked with 300 juvenile blue catfish and four additional aquaria were stocked with 300 juvenile redear sunfish. The fish were given an initial "conditioning diet" which consisted of only one of the following: fish food – insect larvae, ram's horn snails or red-rimmed melania snails. After 2 weeks of feeding the "conditioning diets," 100 fish of each species were stocked into eight separate aquaria (16 aquaria total) and offered a known amount of their conditioning diet and a known amount of one of the other conditioning diets used above. Prey selection was determined by the frequency of selection of the various diets. A Chi-square contingency test was used to determine the influence of conditioning on food selection. A two-tailed test of binomial proportion was used to examine the significance of food preference in each conditioning experiment. Because fish that consume large amounts of food may bias results, the percentage of the total number of prey items the percentage of food for each fish was determined either by video or by examination of the stomach contents. For video analysis, a video camera was used to record a 2-hour segment of feeding. The data obtained was analyzed using the Observer (1997), a computer program specifically programmed for behavior analysis. Data obtained from this program was analyzed using a Chi-square contingency test.

Prey selection studies with the blue catfish revealed that regardless of the training or conditioning diet, blue catfish readily converted to catfish feed when it was offered. Redear sunfish preferentially consumed snails when available. Even redear sunfish trained to eat commercial fish food readily consumed snails and chironomids when available.

RESULTS AT A GLANCE...

★ *Ponds stocked with redear sunfish had significantly fewer snails than ponds without sunfish. Redear sunfish preferentially consumed snails and midge larvae when available, even when trained on pelleted diets prior to stocking ponds.*

Determination of the ability of redear sunfish to withstand conditions of commercial catfish culture

Southern Illinois University. In the spring, redear sunfish (small, medium, and large) were stocked at a rate of 300 fish/acre into four, 0.0-acre experimental ponds (12 ponds total) stocked with channel catfish at a production rate of 8,000 pounds/acre. Water quality variables such as dissolved oxygen,

temperature, alkalinity, carbon dioxide, ammonia, and pH were measured on a daily basis. The number of dead fish (catfish and sunfish) was recorded. In September, the ponds were seined and harvest size channel catfish removed. The number of surviving individuals of each species was recorded. The ponds were under stocked with 5-inch fingerlings to replace the fish removed for market. Survivability of redear sunfish was analyzed to determine if any correlation with water quality variables exist. The number of redear sunfish that died due to seining was recorded. In the spring of 2004, the ponds were seined and all redear were counted and weighed. Harvest size catfish were removed and 5-inch fingerlings were stocked to replace those removed.

In the fall of 2004, redear sunfish and channel catfish were removed from ponds. The number and type of snails within a one meter transect were counted and recorded. One hundred channel catfish from each pond were sampled and analyzed for the presence of trematodes on gills and in the flesh. This study was repeated in 2005.

In the 2004 study, ponds containing redear sunfish had significantly fewer snails than ponds without sunfish. The number and type of snails remaining in the ponds did not differ significantly when medium size or large sunfish were stocked. Redear sunfish trained to eat snails did not remove significantly higher numbers of snails than fish not trained or conditioned to snail diets. Survival of the redear sunfish was 100 percent in all ponds. The incidence of trematode infestation was still evident on channel catfish. Approximately, 25% of the catfish had trematodes on the gills and in some cases within the flesh.

In the 2005 study, the water temperatures in the ponds at Southern Illinois University averaged 5°C higher than in 2004. Catfish survival in all ponds was greater than 98%. Survival of redear sunfish was strongly correlated with the decreased dissolved oxygen and increased temperature in the ponds. Ponds that routinely had dissolved oxygen concentrations of 3 mg/L and temperatures of 32°C at sunrise had significantly higher mortality rates than ponds with higher morning dissolved oxygen concentrations. No significant correlations between alkalinity, carbon dioxide, ammonia, nitrite, or pH on survival of redear sunfish were observed. The incidence of trematode infestation was still evident on channel catfish. Approximately, 31% of the catfish had trematodes on the gills and in some cases within the flesh. The increase observed in the number of channel catfish with trematodes visible on the gills or in the flesh is likely due to the decrease in redear sunfish numbers in the ponds due to mortalities.

Objective 3. *Develop and implement standardized methods for the isolation, culture, and antimicrobial susceptibility testing of strains of columnaris-like bacteria isolated from diseased fish.*

Louisiana State University. Various agar media were evaluated for optimum primary isolation and maintenance of *Flavobacterium columnare*. Media under investigation included

both selective and non-selective cytophaga agar (CA), Hsu-Shotts (HS), Shieh (S), tryptone yeast extract (TYE), dilute Mueller Hinton (DMH) and Flavobacterium columnare growth medium (FCGM). Media were made selective by the addition of 5 µg/mL neomycin and 200 units/mL polymixin B. For primary isolation the media were prepared as agar plates and for maintenance the media were prepared as 20-mL slants in 50-mL tubes with 1 mL of saline added to preserve moisture. For the evaluation of primary isolation media, a standardized mixture of *F. columnare*, *Edwardsiella tarda*, *E. ictaluri*, *Aeromonas hydrophila*, and *Streptococcus difficilis* was prepared. This mixture was designed to mimic the mixture of aquatic bacteria that might be present in contaminated external sites such as the gills and skin of diseased fish. The mixture was inoculated onto the various test media to evaluate their ability to produce pure colonies of *F. columnare* while inhibiting contaminating bacteria.

Selective cytophaga agar (SCA) performed the best as a primary isolation medium for isolation of columnaris from a mixed inoculum of aquatic bacteria. The remainder of the media were ranked as follows: (2) SS (3) SHS, (4) DMH and (5) FCGM. Both DMH and FCGM produced no isolated *F. columnare* colonies.

For maintenance following isolation, TYE slants performed the best with some cultures maintaining viability as long as 84 days. The remaining media were ranked as follows: (2) CA, 52 days (3) DMH, 47days (4) HS, 34 days (5) S, 32 days and (6) FCGM, 23 days.

Some of the above-mentioned media were evaluated as broths for batch culture of *F. columnare*. A 40-mL volume of media was inoculated with 200 µL of a McFarland #5 standard inoculum and growth performance measured by colony forming units (cfu) per mL and absorbance at 600 nm following 24 hours of incubation at 28°C. *Flavobacterium columnare* growth medium (FCGM) out-performed other formulations tested producing mean absorbances of 0.2377 and colony counts of 2.2×10^9/mL. The clumping of cells, which is a problem in other broth media, was avoided in FCGM. The remaining broth media were ranked as follows: (2) Shieh (3) cytophaga and (4) DMH. For a summary of broth culture results see Table 1.

For disk-diffusion antimicrobial susceptibility testing, dilute Mueller Hinton (DMH) plates prepared with different levels of agar and nutrient were evaluated for clarity and consistency of zone size. To insure uniformity, one lot of each of five anti-microbial agents, one lot of Mueller Hinton medium, and one lot of equine serum were used in all disk diffusion test evaluations. The anti-microbial disks (BBL) chosen were Sulfamethoxazole : trimethoprim (SXT 25 μg), Sulfadimethoxine : ormetoprim

RESULTS AT A GLANCE...

★ *Selective cytophaga agar SCA has performed the best as a primary isolation medium in preliminary tests in isolation of Flavobacterium columnare from contaminated sites such as the gills and skin. For maintenance following isolation, tryptone yeast extract TYE medium as a moist slant, held cultures viable for as long as 84 days. For large batch broth culture, FCGM outperforms other formulations tested.*

Table 1. Growth of *F. columnare* in various broth media at 28°C for 24 hours. Data is presented as absorbance at 600nm and by colony forming units (cfu) per mL.

Test Medium	Mean Absorbance (600nm)	Mean Colony counts (CFU/mL)
DMH*	0.0858	1.3×10^7
Cytophaga*	0.1646	6.3×10^7
Shieh*	0.2078	3.1×10^8
FCGM*	0.2377	2.2×10^9

* Indicates significance at P = 0.05

(PRI-MOR 25 Fg), Oxolinic acid (OA 2 μg), Oxytetracycline (T 30 μg), and Florfenicol (FFC 30 μg). The basal MH broth used was (Difco) lot # 3126187, and the degranulated agar used was (Difco) lot #3265229. The concentrations of MH broth evaluated were 3 g, 3.5 g, 4 g, and 5 g/L. The agar concentrations that were evaluated were 9 g, 12 g, 15 g, and 17 g/L. Varying concentrations of MH broth were used to determine the optimum amount of nutrients for *F. columnare* growth. The varying agar levels were evaluated for their effects on zone appearance by limiting the gliding motility of the bacterium. Each medium was evaluated for thickness of growth, distinct zones, and uniformity of zone margins. Once the optimum concentration of broth to agar was determined, 5% equine serum lot #ANE18713 (Hyclone, Logan, Utah) was evaluated as a replacement for the more expensive fetal calf serum as a growth supplement for *F. columnare* cultures on MH agar plates.

The optimum media formulation for susceptibility testing was determined to contain 4 grams M-H broth and 17 grams of agar per liter with 5% equine serum. This medium gave the highest bacterial growth and allowed for better definition of zones of inhibition. Equine serum improved the growth of *F. columnare* cultures on dilute M-H agar, but not significantly different from fetal calf serum. Because of availability and cost of equine serum compared to fetal calf serum it was determined to be a suitable enrichment factor for the improved medium.

RESULTS AT A GLANCE...

★ *An improved medium has been developed for antimicrobial susceptibility testing of* Flavobacterium columnare.

Objective 4. *Characterize archived strains of columnaris-like bacteria based on conventional and molecular techniques.*

Morphologic and biochemical analysis of columnaris-like bacteria

Louisiana State University. Forty-nine strains of columnaris-like bacteria archived in the LADL and UAPB collections were analyzed using conventional biochemical testing in test tubes, the API 20E system, the API NE system

and the API ZYM system. These strains were obtained from a larger pool of yellow pigmented columnaris-like isolates that were subjected to screening procedures that involved a species-specific PCR (Bader et al. 2003), the five point characteristics of Griffin (1992) and the physiological characteristics of Bernardet and Grimont (1989). To conform to the Griffin screen, the bacterium must be shown to satisfy the following requirements: production of flat, spreading, yellow, and rhizoid colonies on cytophaga agar, growth in the presence of neomycin and polymixin B, production of gelatin degrading enzymes, binding of congo red dye to the colony, and production of chondroitin sulfate A degrading enzymes. Isolates were also tested for the presence of flexirubin-type pigments using the potassium hydroxide (KOH) method outlined in Bergey's Manual of Determinative Bacteriology 9th Edition (1994). The goal of this part of the project is to determine if *F. columnare* can be identified using conventional and commercially available biochemical testing schemes by labs that may not have molecular capabilities. Prior to the start of our study, 48 of 49 archived strains of columnaris-like bacteria conformed to the Griffin screen and were confirmed by PCR as *F. columnare.*

Results indicate that *F. columnare* should be shown morphologically to be a long, thin, gram negative rod (3 to10 micrometers length and 0.3 to 0.5 micrometers in width) with gliding and flexing motility, and form rhizoid, yellow-pigmented colonies on agar plates. Morphology of the cells and colony appearance is slightly variable among strains and is influenced by growth conditions and age of the culture. The isolate should be a strict aerobe, should not produce acid from carbohydrates, and should be cytochrome oxidase and catalase negative. Negative reactions were obtained in the GMD and TSI agar tests due to the lack of acid production from carbohydrates. The API 20E, API NE and API ZYM systems (bioMérieux-Vitek) were examined for usefulness in the identification of *F. columnare*. The API 20E and API ZYM systems were determined to be inadequate due to the lack of positive reactions on the strips. The API NE was very useful producing an adequate number of positive reactions. Isolates of *F. columnare* uniformly gave reactions resulting in the API NE code number 0441455 at 24 hours. The positive reactions in the API NE strip were as follows: esculin, D-glucose, L-arabinose, potassium gluconate, capric acid, malic acid, and citrate.

Adhesion to plastic, cultured cells, and isolated gills

Auburn University. Six types of plastic multiwell plates (BD Biosciences, Franklin Lakes, NJ) were compared for use in a bacterial adhesion assay. Two hours after washed *F. columnare* cells were added to the wells, there were significant differences among the plates. The same results were obtained with two isolates. Adhesiveness of *F. columnare* was greater for bacteria grown in Hsu-Shotts broth rather than in Shieh broth. The addition of calcium and magnesium to water used in the adhesion assay increased the adhesiveness of one isolate of *F. columnare* (PL-04-02) but had no effect on another isolate (PB-02-110). Other waters tested, which had high concentrations of NaCl, tended to reduce the adhesiveness of the isolates tested.

Isolates of *F. columnare* tested with the multiwell plate assay had a wide range of adhesiveness to plastic. As additional isolates are obtained for testing, these results will be compared to other types of results obtained by investigators at other institutions to determine if there is a relationship between adhesiveness and other characteristics. Attempts to quantify the adhesion of *F. columnare* to cultured cells was hindered by the adhesion of the bacteria to the glass or plastic substrate used for cell culture and by problems with accurate counting of bacteria stained by conventional methods. To overcome these problems, antibodies against *F. columnare* were made in rabbits as a first step in development of antibody-based methods for bacterial quantification.

Three types of assays for adhesiveness of *F. columnare* (isolated fish gills, larval fish, and cultured cells) were developed and then compared with a multi-well plastic plate assay. The assay with plastic plates was previously found to be useful for quantification of *F. columnare* adhesiveness.

For the gill assay, gills were dissected from channel catfish, bluegill, and common carp. For each fish, one section of gill was used as a control and two sections were exposed to *F. columnare*. After a 10-minute exposure to bacteria, gills were rinsed twice and homogenized. Plate counts of serial dilutions of the gill homogenate were used to quantify the *F. columnare* adhering to gills. There was a significant difference among *F. columnare* isolates adhering to for gills from bluegill (8 isolates) and common carp (3 isolates). Only two isolates were tested with normal channel catfish gills; however, there was a significant increase in the number of bacteria adherent to gills of channel catfish with proliferative gill disease or with *Aeromonas* infection.

A larval zebrafish assay for adhesiveness of *F. columnare* was developed. Whole fish were exposed to *F. columnare* for 1 hour, rinsed for 2 minutes, and then homogenized. The adherent *F. columnare* were enumerated by plate counts. There were significant differences in adhesiveness of the 11 isolates of *F. columnare* evaluated with this assay. The CFU/mg of fish varied over a 150-fold range for the isolates tested.

A cultured-cell assay was used to examine adhesiveness to EPC cells. The cell culture medium was removed from the cells and replaced by a suspension of *F. columnare* in either phosphate-buffered saline (PBS) or well water. After incubation at 30°C for 10 minutes, plate counts were used to determine the number of adherent bacteria. Seven isolates of *F. columnare* were tested for adhesiveness in hypotonic conditions (well water), and there was no significant differences among isolates. Four isolates were tested in PBS, and one isolate had significantly reduced adhesiveness. The assay with EPC cells was not satisfactory because the high concentration of sodium chloride in PBS reduces the adhesiveness of *F. columnare*. The use of fresh water during the incubation of EPC cells with *F. columnare* resulted in swelling of the EPC cells because of the hypotonic conditions. The variation in adhesiveness among the *F. columnare* isolates tested was small for the cultured-cell and gill assays.

Based on adhesion to plastic, the mean adhesion of 11 isolates of *F. columnare* isolated from external organs was higher than the mean for

11 isolates from internal organs. The virulence of 13 isolates (as reported by Thomas-Jinu and Goodwin in 2004) was not correlated with adhesion to plastic. Adhesion of 11 isolates of *F. columnare* to larval zebrafish was also not correlated with the virulence reported by Thomas-Jinu and Goodwin (2004). The lack of correlation between virulence and adhesion could be the result of additional passages in culture between the determination of virulence and the adhesion evaluations. There could also be differences in virulence or adhesion related to species of fish. Additional experiments will evaluate adhesion and virulence in simultaneous experiments.

Molecular identification of columnaris-like bacteria using rapid sequence analysis of a portion of the 16S ribosomal gene and the 16S-23S intergenic spacer region

Mississippi State University. Isolates of columnaris-like bacteria obtained from LSU and MSU were cultured, and DNA isolated using Purgene DNA isolation Kit (Gentra Systems, Inc., Minneapolis, Minnesota). A portion of the 16S and the entire 16S-23S intergenic spacer of one isolate was PCR-amplified using primers to regions of the 16S and 23S ribosomal sequences that are conserved among the gram negative bacteria. One predominant product was obtained and cloned into pPCR4 TOPO cloning vector (Invitrogen) and sequenced. This was an intergenic sequence containing the tRNA for alanine and the tRNA for isoleucine. Several products were expected, representing different ribosomal operons, but as of yet only this ITS product was found. Alignment of these sequences with the tRNA sequences from related organisms were used to identify conserved sequences, and primers were developed to allow direct PCR of the specific ITS and direct sequencing of the products. These PCR products have been produced and both strands of both products sequenced for all isolates.

The fragment of DNA between the 16S and 23S ribosomal RNA encoding (ITS) of a total of 50 *Flavobacterium columnare* case isolates were amplified by polymerase chain reaction using the common 16S-tRNA and tRNA-23S primer sets reported in year one of the project. The products were cloned and sequenced. The sequences consist of a total of 748 base pairs (bp) and include a 100 bp portion of the 16S fragment and 200 bp overlapping region. In sequence comparisons the 16S region was useful in identifying isolates that were not actually *F. columnare*. The ITS demonstrated substantial variation, however, at least 3 distinct clusters of similar sequences were identified. These clusters demonstrated 5-10% sequence differences in the ITS region but less than 2% divergence within a cluster. This suggests that the isolates represent at least 3 different strains. We are evaluating an additional 20 isolates and comparing sources to see if sequence data correlates with host or season. Also, the conserved sequences will be evaluated for differentiating diagnostic PCR. All sequencing data will be submitted to GenBank so that other diagnostic and research labs can use this information.

Ribotyping techniques to differentiate isotypes of columnaris-like bacteria

University of Tennessee. A number of *Flavobacterium columnare* isolates from fish

disease cases were acquired, as well as several *Flavobacterium columnare*-like bacteria, which share a close taxonomic relationship to the target organism, including *Flavobacterium hydatis, F. succinicans,* and *F. psychrophilum*. Those isolates are currently being typed using ribotyping methodologies, and assay components and procedural variations that provide the greatest fingerprint definition between the various isolates are being determined. Once established, the optimal methodology will be used to generate a fingerprint database of the above control isolates, which will then form the basis for comparison of wild type isolates obtained from other investigators involved with this project.

We have replicated and validated ribotyping methods developed to differentiate various isolates of *Flavobacterium columnare* (ATCC strains, wild type strains isolated from infected fish), *F. hydatis, F. resiovorum, F. aquatile, F. flevense,* and suspected *Flavobacterium spp.* obtained from various sources and other diverse species of bacteria that might inhabit aquatic environments (*Citrobacter freundii, Brochothrix thermosphacta, Aeromona veronii, Sphingomonas capsulate, Vibrio Cholerae, Pseudomonas stutzeri, Micrococcus luteus, Glaciecolia pallidula*). While the Qualicon database is quite extensive across many species of bacteria, the database for *Flavobacterium spp*. is rather limited, and thus as a part of this process, we are building a riboprint database, which will become available to other users of that system. In that analysis, we have noted good homology and yet acceptable separation of riboprints between various *Flavobacterium* species and good separation from and between most of the diverse isolates, including *Flavobacterium*-like bacteria.

We have also progressed in studies to determine the efficacy of pulsed-field gel electrophoresis (PFGE) analysis for identification and differentiation of a number of the above species, and including various strains of pathogenic *Flavobacterium columnare*. The first phase of this work has involved the development of culture methods and PFGE protocols, based on restriction digests, to obtain optimal fingerprints for identifying and separating various bacterial isolates. Because *F. colmnare* colonies cannot be readily separated from solid growth media, obtaining an appropriate mass of cells for PFGE assays via picks or loops, as is done with other bacterial species, was found to be difficult. Thus, isolates were grown in liquid culture, followed by centrifugation to obtain a cell pellet. Varying cell densities/concen-trations were used for DNA isolation procedures, following by restriction enzyme digests prior to running digested DNA through PFGE gels in conjunction with CHEF-mapper system (Bio-Rad Laboratories, Hercules, California). Test growth media have included ATCC Nutrient Broth 3, 1839 Harpo's HTYE, 1750 Anacker and Ordal Medium. Separate trials were conducted to test various restriction enzymes, including *Spe*I, *Xba*I, *Bam*HI, *Sma*I, *Apa*I. Additionally, varying densities of SeaKem Gold Agarose (Fisher Scientific, Far Lawn, New Jersey) were used for PFGE separation of bands.

For the PFGE analysis, *Spe*I, *Xba*I, *Bam*HI restriction enzymes produced small and numerous bands from DNA extracted from *Flavobacterium spp.* and some *Flavobacterium*-

like bacteria, and those patterns were found to be unsuitable for PFGE type analysis; whereas suitable fingerprints were generated for other gram-negative species, including *Salmonella choleraesuis* Braenderup control (ATCC BAA-664). We believe that restriction sites in *Flavobacterium* genome are too numerous for the three restriction enzymes above. *SmaI* and *ApaI* digests produced fewer but less distinct bands in *Flavobacterium spp*. and some Flavobacterium-like bacteria, whereas those enzymes again produced suitable fingerprints for other bacteria including control strains. Varying densities of PFGE gels did not provide better resolution of banding patterns.

Currently, we are implementing additional protocols and alternate restriction enzymes to develop more definitive PFGE typing patterns. Such protocols will likely require more lengthy DNA extraction procedures, which will in turn require longer turn-around times for sample analyses.

To date, our studies indicate that ribotyping may offer a more efficient and timesaving option for identification and differentiation of *Flavobacterium*, *Flavobacterium*-like, and non-*Flavobacterium* isolates.

RESULTS AT A GLANCE...

★ *Molecular (PCR) and conventional (API system) methods may be used for the identification of F. columnare. Molecular methods such as ribotyping, RAPD analysis, and sequencing of the intergenic spacer region between the 16S-23S ribosomal RNA genes can allow for discrimination between different genotypic strains.*

Determine the presence of unique outer membrane proteins of various strains of columnaris-like bacteria

Clemson University. Several outer membrane proteins (OMP) from *Flexibacter columnare* have been isolated and are consistently found in all *F. columnare*. Over the last two years we reported that a 30 kDa OMP isolated from *F. columnare* is a potent inducer of type II nitric oxide synthase (iNOS) and inducible prostaglandin H2 synthase (cyclooxygenase-2; COX-2) in isolated catfish phagocytes, and that these activities can be blocked using specific antibodies against the OMP.

The proteomics facility within the Clemson University Genomics Institute (CUGI) helped us to identify *F. columnare* OMPs, with an initial focus on the 30 kDa, 40 kDa, and larger OMPs. Protein fragments between 12 and 15 amino acid residues were isolated from each band, yet were not identified because of a lack of homology with any known proteins in current data banks. These fragments, how-ever, may be useful for generating PCR primer sets to identify larger coding regions of the OMP genes. Very recently, we constructed a cDNA library from a virulent Clemson University strain of *F. columnare* using a commercially available kit (Ambion) to remove 18S and 20S RNA, thereby enriching mRNA that was subsequently used to generate a long-PCR-based library (Clonetech). Our in-hand antibodies against OMPs recognized recombinant proteins expressed in the library, and those positive plasmids are being sequenced at the moment. Over the course of

the next 5 months recombinant OMP proteins will be expressed and purified, then screened for both *in vitro* and *in vivo* activity in channel catfish and tilapia phagocytes, and finally will be used as potential vaccines against our virulent strain of *F. columnare* The most important product of this study will be the availability of OMP-specific antibodies and recombinant OMPs for general use by the cooperators in this project.

Objective 5. *Develop challenge models for columnaris-like bacteria isolated from major warmwater aquaculture species in the southeastern United States.*

Internal genetic labeling of columnaris-like bacteria for use in the development of an effective challenge model

Mississippi State University and Auburn University. Our objectives for this project are to 1) ligate a *Bacteroides* consensus promoter sequence upstream of *gfp* mut3a to allow expression of green fluorescent protein in Flavobacterium *columnare,* 2) ligate the *gfp* gene and promoter into shuttle vector pCP11, 3) transfer the pCP11-gfp plasmid into *Flavobacterium columnare* for expression of green fluorescent protein, and 4) use the fluorescent-labeled *F. columnare* to develop an effective challenge model (in cooperation with Joe Newton at Auburn University).

Two 65 base-pair DNA oligonucleotides containing a consensus promoter sequence from *Bacteroides fragilis* were synthesized and hybridized. The double stranded DNA containing the promoter was then digested with *Eco*RI and *Sac*I and ligated upstream of *gfp* mut3a in plasmid pFPV25. Expression of the *gfp* gene from this plasmid (designated pFCgfp) in *E. coli* was confirmed using a fluorescence plate reader.

A *Spe*I-*Eco*RV fragment from pFCgfp was ligated into pCP11, and the resulting plasmid was designated pMWFCgfp. Expression of *gfp* from this plasmid in *E. coli* was also confirmed using a fluorescence plate reader. Another plasmid was also constructed by amplifying the *ermF* (erythromycin resistance) gene from pCP11 by PCR and cloning it into the *Eco*RI site of the broad host range plasmid pBBR1MCS4. The resulting plasmid, pBBRermF, will allow selection in *F. columnare* based on erythromycin resistance. We then transferred the *gfp* gene with the *Bacteroides* promoter from pMWFCgfp into pBBRermF on a *Sma*I/*Spe*I fragment to construct pBBRFCgfp.

Objective 3 has not been successfully completed despite numerous attempts to transfer pMWFCgfp and pBBRFCgfp into multiple *F. columnare* isolates. We have been utilizing a conjugation technique using *E. coli* SM10 l*pir* as a donor strain to attempt transfer of the plasmids into *F. columnare*. We have been using 25 columnaris strains that were collected from John Hawke (LSU-SVM) as well as an additional 20 isolates received from Joe Newton. This year, we have phenotypically characterized all the isolates to enable optimal growth conditions for *F. columnare* during the conjugation experi-

ments. We have also conducted experiments that resulted in adjustment of the erythromycin concentration used on the selection plates. In addition, we have obtained two additional plasmids, pCP23 and pCP29, from Mark McBride at University of Wisconsin, Milwaukee that will enable us to utilize other antibiotic selection markers. pCP23 carries a tetracycline resistance gene that is expressed in flavobacteria, and pCP29 carries a cefoxitin gene. Experiments are ongoing using our optimized growth/antibiotic conditions to transfer pMWFCgfp, pBBRFCgfp, pCP23, and pCP29 into the *F. columnare* isolates from our panel.

A nalidixic acid resistant *F. columnare* mutant (spontaneous–not recombinant) has been isolated that can be used as a tagged organism (if it is still virulent) until the *gfp* gene in *F. columnare* is successfully expressed. Several *F. columnare* isolates (including the nalidixic acid isolate) have been used in challenge experiments following the procedures of Andy Goodwin and S. Thomas-Jinu at the University of Arkansas at Pine Bluff. To date it has not been possible to cause columnaris disease in these experiments using their procedures.

Challenge models for channel catfish and golden shiners

University of Arkansas at Pine Bluff. In the first part of our study we compared the biochemistry, DNA sequence (by RAPD) and pathogenicity of a large group of columnaris strains. Channel catfish and golden shiners were subjected to temperature shock and then immersed in a bath of columnaris bacteria at a concentration sufficient to cause 60 to 70% mortality in 2 days using the more pathogenic of archived columnaris strains for the respective host. Each experiment was performed in triplicate with 20 fish per tank. Moribund fish were necropsied and the cause of death verified. Columnaris bacteria were re-isolated and identified by biochemical (tube tests) and molecular (randomly amplified polymorphic DNA, RAPD, Promega) techniques to verify that the fish died from infection by the challenge bacteria. In this work, we found that the catfish and cyprinid fish isolates fell in different clades, but that there was no correlation between these genetic and biochemical results, and any other measure including fish species of origin or pathogenicity to catfish and golden shiners.

Another way to look at differences between columnaris isolates is to challenge fish and then look for differences in response of the infections to practical disease treatments. Columnaris disease was produced in channel catfish, *Ictalurus punctatus* (Rafinesque) by bath exposure to 4 highly virulent isolates of *Flavo-bacterium columnare*. In untreated controls, mortality began 20 hours after exposure and was 100% by 48 hours after exposure. Mortality in channel catfish given antibiotic treatments with oxytetracycline (OTC) or a combination of sulfadimethoxine and ormetoprim (SOR) in feed prior to bacterial challenge was 0% with all four strains of *F. columnare*. Diquat was the most effective bath treatment; mortality with all four strains was 0%. With potassium permanganate, chloramine-T, hydrogen peroxide, and copper sulfate bath treatments, efficacy varied significantly among bacterial

isolates tested, pathogenicity in channel catfish and shiners was similar. There was no correlation between biochemical characteristics or RAPD genogroup and pathogenicity.

Hybrid striped bass

Strains identified by the RAPD grouping sysytem of Farmer 2004 were used in challenge studies. Representative isolates from each RAPD group and host species were obtained from our collection of archived strains at LSU. Strains used in the challenges were: LADL 04-046 isolated from channel catfish (RAPD Group I), LADL 04-066 isolated from large-mouth bass (RAPD Group I), PB-02-12 isolated from fathead minnow (RAPD Group II), and LADL 94-141 isolated from channel catfish (RAPD Group III). Strains LADL 04-066 largemouth bass (Group I) and PB-02-12 fathead minnow (Group II) were virulent in hybrid striped bass causing 100% mortality in 96 hours. Strains LADL 94-141 channel catfish (Group III) and LADL 04-046 channel catfish (Group I) were non-virulent in hybrid striped bass. Thus far virulence appears to be correlated more with host source than with RAPD group. Additional strains are currently being tested.

PUBLICATIONS, MANUSCRIPTS, OR PAPERS PRESENTED

Publications in print

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Theses

Zhang, Y. 2004. Adhesiveness of *Flavobacterium columnare*: comparison of different methods and isolates. M.S. thesis. Auburn University, Auburn, Alabama.

strains and among treatments. Bath treatments with chloramine-T and potassium permanganate reduced mortality from 100% to 75% and 69%, respectively, but copper sulfate and hydrogen peroxide treatments were not effective.

Based on our results, oral antibiotics prevented columnaris disease but, of the bath treatments, only Diquat produced a dramatic reduction in the mortality of acutely infected fish. Diquat is labeled for aquatic use as an herbicide in the United States but in large ponds it is prohibitively expensive.

Challenge models for hybrid striped bass

Louisiana State University. Strains of *Flavobacterium columnare,* archived in the LSU Aquatic Diagnostic Laboratory repository, are being used in virulence studies in hybrid striped bass. Methods that produce uniform mortality rates of 75% or greater following exposure are classified as virulent strains of *F. columnare.* These criteria will be adopted for use to compare virulence of archived strains from various locations and various species outlined in Objective 6. Hybrid striped bass (20 g mean weight) were acclimated and held in the Aquatic Pathobiology Building at the LSU School of Veterinary Medicine. The strains (isolates) were evaluated for virulence in a standardized challenge procedure where scales were removed and the skin scarified in a 1 cm^2 area. Cultures of the *F. columnare* bacteria were swabbed on the scarified area rather than using the immersion method. This was done due to difficulties with clumping of the bacteria and difficulty enumerating bacteria in the challenge bath. Virulent strains of *F. columnare* colonized the scarified skin readily and caused infection and disease whereas avirulent isolates did not cause infection. Mortality was evident 96 hours after infection with virulent strains.

Objective 6. *Use challenge models for each fish species to correlate virulence with biotype and or genotype of columnaris-like bacteria.*

Channel catfish and golden shiners

University of Arkansas at Pine Bluff. Variability in *Flavobacterium columnare* pathogenicity makes disease treatment difficult because there is currently no way to easily recognize those strains that warrant aggressive treatments. In order to identify suitable markers, 17 isolates of *F. columnare* were cultured from six different fish species. The DNA from all isolates was analyzed using randomly amplified polymorphic DNA (RAPD). Bootstrap analysis of the RAPD data produced a tree with three major groups supported by scores of 80% to 100% similarity.

The remaining objective was to complete challenge assays to see if there were any stain differences in the virulence of columnaris isolates using the golden shiner as a host. Of the strains tested, two of catfish origin that were highly virulent in channel catfish (100% mortality) produced much lower mortality (30% to 40%) in golden shiners. For the other

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