

# Warm Springs Fish Technology Center



## Annual Report FY-2012

U.S. Fish and Wildlife Service  
Warm Springs Regional Fisheries Center  
Warm Springs, GA

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Project Leader

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Date

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Center Director

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## Fish Technology Center Overview

The Fish Technology Center (FTC) is a component of the Warm Springs Regional Fisheries Center (RFC) and was developed to improve and enhance fisheries management. We provide consolidated technical operational support to regional fisheries operations and technical assistance to the public. The Fish Technology Center is comprised of a cryopreservation laboratory, conservation genetics laboratory, and the National Fish Strain Registry at Warm Springs, Georgia, and a field station in Wadmalaw Island, South Carolina.

### Goals:

- Provide management support of interjurisdictional coastal and riverine fishes such as robust redhorse, shortnose sturgeon, Gulf sturgeon, and Gulf striped bass.
- Provide conservation genetics support for regional fishery programs.
- Maintain the National Fish Strain Registry for dissemination of information and support of private, state and federal broodstocks.
- Develop cryopreservation techniques for imperiled fish, amphibians, and mussels.
- Develop hatchery product evaluation techniques.

### Conservation Genetics

The Conservation Genetics lab primarily works with biologists and managers of the region to design and implement genetic research on imperiled aquatic organisms.

Current Projects include estimating genetic diversity from: alligator gar, Gulf Coast striped bass, robust redhorse, freshwater mussels, and threatened and endangered species such as spotfin chub.

### Cryopreservation

Cryopreservation is a process in which a living cell is frozen, stored, and thawed and remains viable. Cryopreserved sperm assists reproductive efforts by allowing spawning to take place whenever females are ready, reduces the need to hold males, and can increase flexibility and genetic diversity in spawning protocols.

Currently, the Warm Springs FTC is working on numerous species of fish, including threatened or endangered species. In the near future, the FTC will expand cryopreservation research to include other aquatic species (e.g., freshwater mussels, amphibians) for conservation efforts.

### National Fish Strain Registry

The National Fish Strain Registry (NFSR) is an internet-based program that assembles information on life history, genetics, reproduction, and behavior of wild populations and domestic fish strains throughout the United States. The NFSR database is available for use by public and private producers as well as resource managers of federal, state, and tribal governments through a registration process. Once registered, users are able to search, create new records, edit records, and request information. The NFSR's vision is to provide a broad collaborative program that provides access to data and information on our Nation's aquatic resources.

## Cryopreservation Laboratory

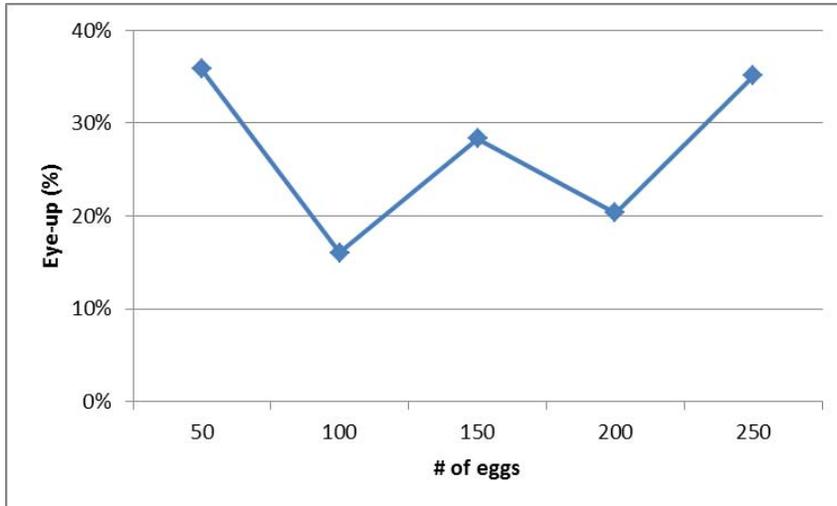
### Rainbow Trout Fertilization Trials

William Wayman and Jaci Zelko returned to Erwin National Fish Hatchery on February 14<sup>th</sup> to perform a fertilization trial with cryopreserved sperm. The trial was done to determine if cryopreserved sperm could be used for production of future broodstock for the station. Sperm that were cryopreserved in February of 2011 were thawed and used to fertilize batches of eggs. The eggs were then combined for disinfection, water hardening, and incubation. Eggs fertilized with cryopreserved sperm produced a 41% eye-up, as compared to 62% for fresh sperm controls. Although the current protocol will meet the needs of the hatchery, it is believed that increasing the cryopreserved sperm:egg ratio will increase the eye-up and hatch rates in future efforts.



(L) Biologists at Erwin NFH strip eggs from a female rainbow trout for use in fertilization trials.  
(R) Frozen sperm is thawed and placed on a small aliquot of eggs for the trial. Credit USFWS

A second week of work was undertaken in August to cryopreserve sperm from 2-year old broodstock. Sperm from 10 males were frozen for 24 hours and then used in a fertilization trial. This project was aimed at determining the optimum number of eggs that can be fertilized with one 0.5-ml straw of frozen sperm. Due to high degree of variation in eye-up percentages within treatment groups, a significant difference was not found among the treatments. This variation was likely caused by variation in the incubation of the eggs in the new research scale incubation system that was developed at Erwin. For instance, eye-up percentages for eggs from the same female ranged from 0% at the 50 egg/straw level to 82% at the 250 eggs/straw level. The study will be repeated in November after the incubation system has been corrected.



Eye-up percentage of rainbow trout eggs fertilized with 0.5-ml of cryopreserved sperm.

### American Shad Cryopreservation

Studies were continued in efforts to develop a cryopreservation method for American shad sperm. The experiments used an extender developed for Indian shad *Tenuulosa ilisha* and 4 cryoprotectant concentrations (MeOH and DMSO). Equilibration motilities were low (25% - 0%). The low motilities were believed to be caused by the extender concentration being too high. Another freezing trial was conducted with same extender diluted by 10X. Equilibration motilities were similar (20%-0%). Sperm from both experiments had 0% post-thaw motility.

### Robust Redhorse Cryopreservation

Jaci Zelko traveled to Augusta, GA in April to assist South Carolina DNR personnel in the spawning of Savannah River robust redhorse. Jaci went over to collect sperm samples for cryopreservation. The sperm were cryopreserved and added to the repository at our lab. This effort focused on freezing sperm to preserve genetic material and for future spawning efforts if needed. Sperm were frozen from 11 males.



Biologists collecting sperm from the robust redhorse. Credit USFWS

### Bonytail Chub Cryopreservation

William Wayman and Jaci Zelko traveled to the Dexter National Fish Hatchery and Technology Center in New Mexico (Region 2) to conduct cryopreservation experiments on sperm of bonytail chub. The bonytail chub is native to the Colorado River, and was listed as endangered in 1980. This species is one of the most imperiled freshwater fish species in the United States and is believed to only persist in the wild through ongoing propagation and stocking efforts. The current hatchery broodstock was created in 1981, and preservation of the remaining genetic diversity is critical due to the aging broodstock population. Multiple experiments were conducted aimed at developing refrigerated storage and cryopreservation techniques for this

species. The refrigerated storage study allowed the refrigerated storage of sperm for up to 10 days without a complete loss of motility. The cryopreservation experiments were successful with hatch rates equaling approximately 70% of fresh sperm. The use of this tool could allow the conservation of the remaining genetic diversity of this endangered species.



(L) Sperm stripped from a bonytail chub male were used in cryopreservation and fertilization trials.  
(R) Eggs were divided into small aliquots for the experiments. Credit USFWS

### **Striped Bass Sperm Cryopreservation**

It was decided to end the captive striped bass spawning research at Warm Springs. Due to the unusually warm temperatures this spring, initiating spawning conditions for the striped bass was held off until July due to other commitments. This is far outside their normal spawning window. Seven males were implanted with Ovaplant pellets at a rate of 40-60  $\mu\text{g}/\text{kg}$  body weight. None of the males were spermiating at implantation. After 48 hours, one male was spermiating. Sperm was collected from this male and cryopreserved. The sperm will be stored with other striped bass cryopreserved sperm until a fertilization trial can be developed to test the success of the cryopreservation procedure. All of the remaining striped bass broodstock were euthanized.

### **Lake Sturgeon Cryopreservation**

Dr. James Candrl of the USGS Columbia Environmental Research Center requested any surplus lake sturgeon cryopreserved sperm that might be available for use in experiments. No reproductive males were able to be collected during their collection efforts. Surplus cryopreserved sperm from our initial studies in 2001 were shipped to Columbia for use in the experiments. Unfortunately, the females produced poor quality eggs and did not result in any fertilization.

### **Pallid Sturgeon Cryopreservation**

#### *Middle Basin*

No males were collected this year from the middle basin for recovery production efforts. This was likely due to the unusually warm spring and early onset of spawning. In order to spawn the two females that were collected, cryopreserved sperm were used. Sperm from 3 males that were cryopreserved in 2011, and 1 male that was cryopreserved in 2010 were shipped to

Neosho National Fish Hatchery in Missouri. Only one of the females was successfully spawned and produced approximately 15,000 eggs. Hatch rates for eggs fertilized with cryopreserved sperm ranged from 30-50%.

#### *USGS Study*

Dr. James Candrl of the USGS Columbia Environmental Research Center requested excess cryopreserved pallid sturgeon sperm for use in some Corps of Engineers funded research projects. They had collected 6 females and 3 males for the projects, but none of the males produced any sperm. Cryopreserved sperm were sent from samples that were left over from previous research projects. The cryopreserved sperm allowed the completion of the studies.

#### *Upper Basin*

##### *Genetic Analysis*

Every pallid sturgeon collected for the recovery efforts are typically PIT tagged and fin clipped when collected. The fin clips are used to provide a genetic analysis of the fish. The information is then used during spawning to ensure that only distantly related fish are spawned together in an effort to maintain genetic diversity. Fin clips from 3 males were either not collected or lost prior to genetic analysis over the past years of sampling. Fortunately, sperm were cryopreserved from each of these fish and could possibly serve as a source of DNA for the analysis. A blind study was set up to see if the Lamar Fish Technology Center could successfully extract DNA from sperm from known fish, analyze the DNA, and get back results consistent with the previous genetic analyses. The results turned out perfectly. Sperm from the 3 unknown males were sent to Lamar, and the genetic analysis performed. These results will allow these 3 additional males to become part of the cryopreserved broodstock for future spawning efforts.

#### *Cryopreservation Research and Archival Freezing*

William Wayman and Chester Figiel traveled to Garrison Dam National Fish Hatchery in Riverdale, ND (Region 6) to cryopreserve endangered pallid sturgeon sperm. Sperm from four males were collected, cryopreserved, and added to the cryopreserved sperm repository, which is held at Warm Springs FTC, Garrison Dam NFH, and Gavins Point NFH. Additionally, sperm were cryopreserved for three experiments. The first experiment was to determine how long cryopreserved sperm remains viable once thawed. The second and third experiments were to determine if 0.5- or 5-ml straws and at which extender:sperm dilution ratio produced highest fertilization rates. Unfortunately, due to the single female releasing very small amount of eggs, the fertilization experiments were not able to be conducted. They will be tried again next year.

#### **Freshwater Mussel In-Vitro Trials**

Studies are currently underway on the cryopreservation of freshwater mussel glochidia, a potential method for the preservation and recovery of imperiled unionid species. We are also investigating the effectiveness of transforming glochidia in a culture media, without the use of a host fish. The studies conducted this year used a common mussel species, the paper pondshell. Although, the glochidia collected were not used in our cryopreservation studies because of clumping, the glochidia were transformed for use in evaluating the hatchery's new juvenile rearing system. The transformed mussels' average growth was 42.5 microns over a 30 day

period equaling ~ 1.42 microns of growth/day. The overall survivability across the two treatments was low with 180 out of 1068 in-vivo juveniles and 21 out of 1524 in-vitro juveniles surviving to the 30 day assessment. On July 30, 2012, a 60 day assessment was conducted with no live juveniles being found from either treatment.



Checking glochidia viability. Credit USFWS

## Conservation Genetics Laboratory

### Environmental DNA (eDNA) as a Detection Technique for Invasive Species

CGL is collaborating with Dr. Edgardo Diaz-Ferguson, UGA postdoctoral researcher, on the application of molecular techniques to detect rare and invasive species. For this project, we are currently testing new primers and developing DNA extraction techniques for the detection of environmental DNA of aquatic invasive species inhabiting Florida and Georgia ecosystems. A filtration system was set up to successfully extract DNA from water samples. This step is critical to reaching our final goal of detecting genetic material of invasive species from water samples of natural systems in Florida and Georgia.

### Environmental DNA (eDNA) Protocol Enhancements

A preservation method for environmental water samples using sodium acetate and 95% ethanol was successfully tested. This method allows the preservation of water samples collected from local ponds for more than 15 days. This is a key step towards the final goal of detecting genetic material from natural systems. We also tested our designed primers and probes for *H. letourneuxi* (African jewel fish) and set up new reaction conditions for quantitative PCR analysis using the 7500 Fast Real-Time PCR system. This new qPCR analysis allows us to run a preliminary standard curve for the detection of our probes. The eDNA project began phase II in March and April by conducting the first qPCR experiments for optimization of our designed primers and probes using tissue and water samples. We also collected 30 African jewel fish in the canals adjacent to the Loxahatchee National Wildlife Refuge in order to start our density controlled experiments. Through this controlled experiment we compared different densities of fish using 1 to 6 fish per treatment and then processing water for eDNA from the tanks. Our goal was to determine the minimum amount or threshold level of DNA required for detection in natural freshwater systems.



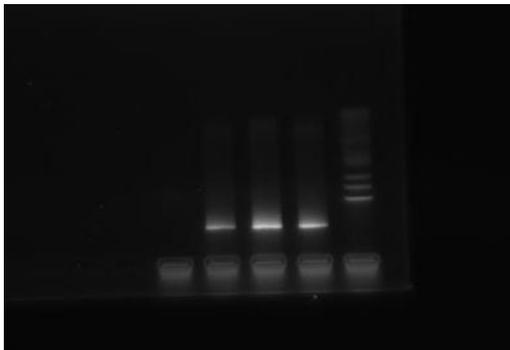
(L) qPCR amplification plot of African jewel fish tissue samples.  
(R) Density experiments for the African jewel fish. Credit: USFWS



Edgardo Diaz-Ferguson, Tom Sinclair and Andy Jackson electro-fishing at the Loxahatchee National Wildlife Refuge. Credit: USFWS

During May and June we conducted experiments to determine temperature effects on DNA persistence using water samples collected from density tanks where the African jewel fish was present. This experiment showed that DNA is degraded at temperatures higher than 30°C, which is an important finding for our future field work with eDNA. Also during this period, we presented our preliminary results for the African jewel fish eDNA analysis at the International Wetlands Conference in Orlando Florida.

Finally, in June, we set up aquaria for density trials and eDNA detection of our second target species, the bullseye snakehead (*Channa marulius*). Through this controlled experiment we compared different densities of fish using from 1 to 6 fish per treatment and then processed water for eDNA from these density tanks. From these data, we will determine the minimum amount or threshold level of DNA required for detection in natural freshwater systems.

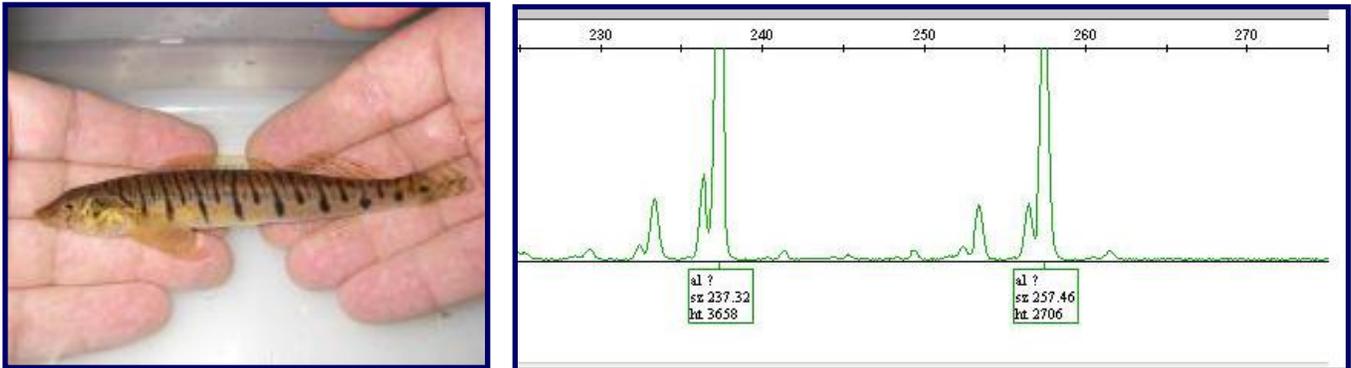


(L) Gel image of DNA extracted from water samples after 8 days submitted to 4 different temperature treatments. Lane 1(from right to left)-molecular weight marker (100bp), line 2 DNA extracted from 8°C treatment (+), line 3 DNA extracted from 15°C treatment (+), line 4 DNA extracted from 25°C treatment (+) and line 5 (-) (No band resolution from DNA extracted from waters samples at 33°C). (R) Density experiments and water collection for the bullseye snakehead (*Channa marulius*). Credit USFWS

### What to do with the Logperch?

Conservation Fisheries, Inc. (CFI), Tennessee Aquarium Conservation Institute, and USFWS-Athens have asked the Conservation Genetics Lab to assist in their efforts to monitor the genetic diversity of a breeding program for the Conasauga logperch. CFI sent samples of the broodstock and progeny to the lab to conduct parentage analysis. Preliminary genetic analysis from the broodstock and progeny indicated that two of the broodstock were females, and all males contributed offspring, though in different proportions. The effective population size of

the offspring was five and nearly identical to theoretical expectations. The partners have used these data to assist the next steps in potential augmentation efforts.

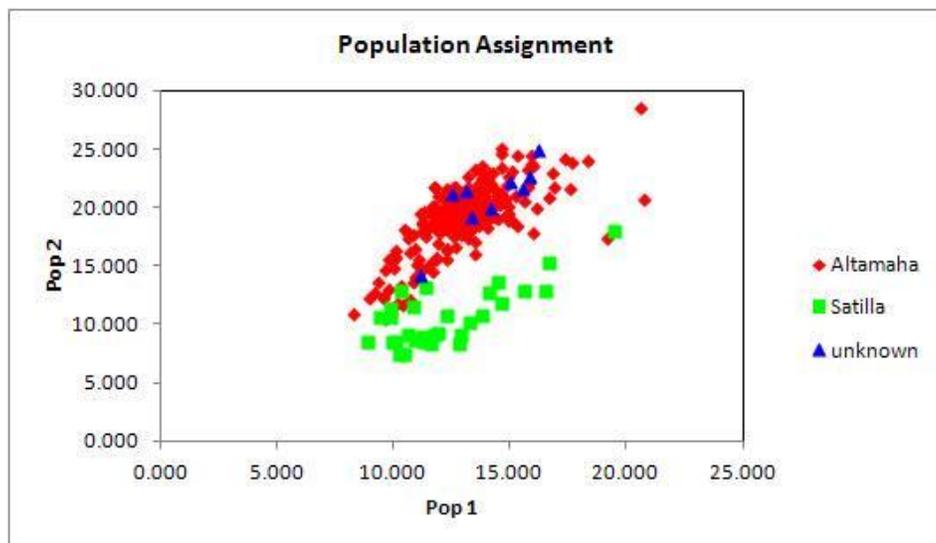


Conasauga logperch Credit: Conservation Fisheries Inc.; Conasauga logperch marker Credit: USFWS

**Genetic Identification of Atlantic Sturgeon Origins in the Altamaha River**

There has been interest in the restoration of Atlantic Sturgeon in South Carolina and other states in the southeastern US. As a result, the St. Mary’s Fishery Restoration Committee was formed. Bears Bluff National Fish Hatchery plays a key role in stocking enhancement to re-introduce Atlantic sturgeon from the Altamaha River into the St. Mary’s River and has obtained nine broodstock for potential augmentation or reintroductions. To determine where each individual originated from, the Conservation Genetics Lab was asked to provide genetic identification for each broodstock Atlantic sturgeon collected from the Altamaha River. A multiplex of 10 microsatellite markers known to amplify in Atlantic sturgeon (Henderson-Arzapalo and King, 2002; King et al., 2001) and lake sturgeon (May et al., 1997) was used to genotype each individual along with a baseline of genetic data from juveniles captured in the Satilla and Altamaha Rivers. Based on genetic analysis, the broodstock fish were identified as originating from the Altamaha River or a river system close to the Altamaha, given that we only had baseline data from the Altamaha.

This graph demonstrates the 9 unknown broodstock are putatively from the Altamaha River. Credit: USFWS



### **Prioritizing Conservation Units for the Federally Endangered Bluemask Darter (*Etheostoma akatulo*)**

The USFWS has a recovery plan to downlist the bluemask darter once viable populations are restored throughout a portion of its historical range. The plan defines a viable population as a reproducing population large enough to maintain sufficient genetic variation to evolve and respond to natural habitat changes. A joint effort among the Conservation Genetics Lab, Cookeville Ecological Services, and the Tennessee Valley Authority sought to establish and prioritize units of conservation for this species. A pilot study was initiated using samples from the Caney Fork River system. Tests for the amplification of nineteen microsatellite markers that have been known to amplify in related *Etheostoma* species (Tonnis, 2006; Beneteau et al., 2007; Khudamrongsawat et al., 2007; Gabel et al., 2008) and estimation of general population genetic parameters for this species was conducted. Of the nineteen loci used in this study, seventeen produced reliable PCR products. Ten of seventeen (59%) of the loci were monomorphic with four of seventeen loci deviating from HWE. The average number of alleles and genetic diversity were low (2.29 and 0.20, respectively). Two major conclusions from the study were that existing microsatellite markers can be used to amplify bluemask darter DNA and that the Caney Fork population appears to harbor limited genetic diversity indicative of an event that has bottlenecked the genetic diversity.



A Bluemask Darter. Credit: USFWS

### **Lake Sturgeon Molecular Tagging Study**

The Tennessee River Lake Sturgeon Reintroduction Working Group (TRLRSG) has been working towards the goal of restoring lake sturgeon. The plan called for the marking or tagging of all individuals to track success of restoration. Conventional tags for long-lived species are prone to failure because of battery life, tissue regeneration or loss of external tag. The Tennessee Wildlife Resource Agency contacted the Conservation Genetics Laboratory (CGL) to explore the utility of molecular tags, which do not suffer from the inadequacies of conventional tags and are a part of the fish until death. Ashantye' Williams attended the annual TRLRSG Meeting held in Chattanooga, TN on January 17-18, 2012. She presented the importance of molecular tags for the lake sturgeon mark/recapture study and reminded the group about the importance of sampling procedures when collecting tissue samples used to determine the genetic characteristics of the adult broodfish. A preliminary study was conducted using simulations on a suite of eleven microsatellite markers used in sturgeon. Results indicate that six markers are necessary for accurate allocation of closely related progeny (brothers and sisters) to their respective parents. Once developed, molecular tags could be used to accurately perform mark/recapture studies for estimation of survival and census size of lake sturgeon in the Tennessee River.

### Genetic Assessment of Hybridization Among Black Bass Species

The Conservation Genetics Lab (CGL) assisted the Tennessee Wildlife Resource Agency (TWRA) and the Alabama Department of Natural Resources in the management and conservation of black bass subspecies in various systems throughout Tennessee and Alabama. Florida bass and northern largemouth bass co-exist in a natural zone of introgression that extends through the southeastern and mid-Atlantic coastal states. While genetic distinctiveness has been identified with several allozyme markers, subsequent introgression makes the detection of backcrossed individuals difficult with these markers. This uncertainty poses problems for state hatcheries that assume purity of strain based on morphological comparisons for hatchery augmentation or reintroduction efforts. Stocking activities and subsequent introgression have led to an extended range for each subspecies, and an expansion of the hybrid zone, which has created difficulties for TWRA in quantifying the success of stocking events or resolving the identity of individuals. CGL established a rapid screening technique to identify several black bass species with the use of genetic markers. The objective is to evaluate the ability of genetic markers developed from Florida bass to generate successful amplification in shoal bass, spotted bass and their hybrids. The genetic markers would aid TWRA in genetically distinguishing samples into their respective categories of hybrids.

### Preliminary Genetic Analysis of Grass Carp Broodstock

Grass carp (*Ctenopharyngodon idella*) which is native to Siberia and northern China, were introduced into the U.S. in the 1960s as an aquatic weed control. The species is known for reducing vegetation density, altering nutrient cycles, and reducing native plankton-eating species. This species is currently labeled an invasive species due to its ability to reproduce and establish wild populations potentially causing a reduction in food supply and shelter for native species. The Warm Springs Conservation Genetics Laboratory (CGL) has been asked to participate in collaboration with Regions 3 and 4 grass carp producers to ask the question “can progeny from grass carp broodstock be assigned to their respective parents with a high degree of accuracy?” Greg and Ashantyé began a pilot study to investigate possible polymorphic microsatellites to answer this question. They started with 24 loci developed from grass carp and silver carp along with samples housed by Warm Springs Fish Health Center and the facility in Stuttgart, AR. A preliminary analysis indicated 17 microsatellites may answer this question with high accuracy. Findings were presented at the December 2012 National Triploid Grass Carps Inspectors Meeting.



(L) Greg and Ashantyé collect fin clips for genetic analysis of grass carp. (R) A juvenile grass carp. Credit USFWS

**Mississippi River Lower Basin Scaphirhynchus Genetic Data Standardization Study**

The pallid sturgeon (*Scaphirhynchus albus*) was described as a morphological variant of the shovelnose sturgeon (*S. platyrhynchus*) based on morphometric measurements, differing geographic distributions, and spawning habitat preferences. Pallid sturgeon prefer larger river channels with swift, turbid flows and rocky or sandy substrate, whereas shovelnose sturgeon prefer slower flows and shallow pools associated with sandbars and channel edges. Morphologically intermediate forms identified as putative hybrids are found in more than half of the pallid sturgeon's range and are based on morphologic intermediacy or the inconsistency of some characters. To determine the genetic origin of individual sturgeon that are morphologically intermediate to pallid and shovelnose sturgeon, scientists from the USFWS, St. Louis University, and Southern Illinois University Carbondale have come together to resolve this issue using microsatellites and mtDNA genetic markers. The Warm Springs CGL is a repository for sturgeon species within the southeastern US, and has become the housing facility for this multi-lab genetic standardization initiative. Recently, Ashantyé has compiled an extensive tissue database of over 250 samples collected along the lower basin of the Mississippi River. Approximately 130 samples have been sent to the university labs to start the multi-lab genetic standardization. The goal is to provide the scientific community with data explaining the genetic identification of pallid, shovelnose and/or intermediate sturgeon observed in the lower Mississippi River.

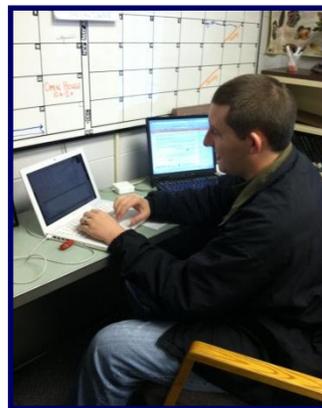
**Lake Sturgeon Management Plan Updated**

Lake sturgeon is considered an endangered species in Tennessee and many populations have suffered from overfishing, construction of dams, destruction of habitats, and others. The Tennessee Lake Sturgeon Reintroduction Working Group (TLSRWG) has been working towards the goal of restoring lake sturgeon in the Tennessee River basin. In 2007 a management plan with a genetic component was developed for the reintroduction of lake sturgeon in the Upper Tennessee River. The management plan's goal is to create and maintain genetic diversity in the stocked population. Warm Springs CGL conducted an initial assessment this year of the genetic diversity of the broodstock being reintroduced into the river system. Based upon preliminary data, CGL determined that the genetic diversity available in the Wolf River lake sturgeon source population appears to have been successfully captured by TLSRWG's reintroduction program in the Upper Tennessee River. The working group met September 20, 2012 to update the management plan to include all work done thus far and the direction in which the group will go in the near future.

**Making Strategic Habitat Conservation (SHC) and Landscape Conservation Cooperatives (LCC) a Priority**

One of the primary missions of the U.S. Fish and Wildlife Service is to conserve our living natural resources and their habitats. CGL is currently restructuring its research program to better serve the Service's move to SHC and LCC designs. CGL's approach is to place more emphasis on aspects of the SHC model by primarily focusing efforts on the characterization of populations, designation of critical habitat for such units, and prediction of global climate change scenarios on these units and their habitat. CGL in collaboration with post-doc Dr. John Robinson developed a project seeking to meet these goals by helping to identify important areas for

landscape protection, with genetic diversity in mind. Using previously published population genetic datasets, we are looking for patterns of genetic diversity and divergence across the South Atlantic Landscape Conservation Cooperative (SALCC) research area. These patterns will most likely be different for terrestrial species and those associated with aquatic environments. Therefore, we are considering datasets from these two habitat types separately. Areas with unusually high diversity or divergence would be good candidates for future conservation efforts. We will also be able to determine the extent to which these hotspots of diversity overlap with presently defined priority conservation areas. To this end, John Robinson has compiled datasets from a total of 28 species, including fish, amphibians, insects, and mollusks. We calculated genetic diversity and pairwise divergence for the populations sampled within the SALCC study region from each of these datasets. This information will be used, along with the geographical locations of the samples, to generate a GIS layer depicting hotspots of genetic diversity and genetic divergence in the region. These data will allow us to assess how well presently defined conservation areas protect genetic diversity, and to identify new areas of importance across the southeastern United States. In addition to identifying areas of conservation importance for terrestrial and aquatic taxa, this research will also help to uncover gaps in our knowledge of population structure across species in the southeastern United States.



*(left)* John is sampling Collins River. *(right)* John compiling datasets. Credit: USFWS

### **GIS Training**

May 21-25<sup>th</sup>, 2012 Greg and Ashantye attended the 2012 Region 4 GIS training seminars in Cookeville, TN. During their attendance they brought awareness and excitement about the role CGL plays in collaborating with the LCCs. With the training received, the goal is to assist with the knowledge and expertise of integrating genetic diversity with geographical information in the South Atlantic Landscape Conservation Cooperative (SALCC) research projects.

## National Fish Strain Registry

The National Fish Strain Registry (NFSR) works under the guidance of several management plans including the Southeast Region Strategic Plan, Conserving America's Fisheries and the National Broodstock Policy and Implementation Guidelines. For example, the NFSR has information on self-sustaining populations of native fish and other aquatic resources that maintain species diversity, provide recreational opportunities for the American public, and meet the needs of tribal communities. In addition, this database provides information on gametes and fry of species and strain that best meet the biological requirements of the specific resource management programs.

Historically, the NFSR was developed as a salmonid database to keep track of the various fish strains, their environmental tolerances, behavior, and health. Data were focused on aquaculture issues, such as feed conversion efficiency, swim-up time, egg hatch rates, water quality tolerances, and other production information. Additional modules were added in the 1990s to incorporate catfish, sturgeon, pike and perch. Within the last five years, we have expanded the scope of the NFSR to include threatened and endangered fishes as we incorporate fish that inhabit and reproduce within a specific area. This concept is usually referred to as a "stock"; and includes fish populations that inhabit specific areas without migration, such as lakes. This has enhanced the biological relevance of the database with a more complete scope, and allows comparisons between cultured fish, stocked fish, and native fish. For example, we can compare performance of razorback suckers in the lower basin of the Colorado River – a distinct reproductive population between Hoover and Davis Dams, to the performance of a captive broodstock held at Dexter NFH&TC.

Currently there are 1156 strain records representing over 40 species, however, the records in the database are predominantly trout (Salmonidae) (825 records), catfish (Ictaluridae) (65 records), pike (Esocidae) and perch (Percidae) (78 records), and sturgeon (Acipenseridae) and paddlefish (Polodontidae) (75 records).

The NFSR was transferred to the *Environmental Conservation Online System* (ECOS) in October 2011. This permits every Fish and Wildlife Service (FWS) employee that has access to ECOS, to be able to view, search and create records in the database. Additional FWS personnel and people from other federal or state agencies or private entities can request access to the database with no breach in security (however, the system will have to be refined so that sensitive information will have restricted access). Special thanks to Jon French ASRC Management Services and Eric Rank from ECOS for making this occur. Chester is currently working with ECOS for the deployment of a public access site of the Registry.

The database is primarily used as a management support tool that provides assistance for fisheries in Federal Water Project reservoirs, to support fish stockings on DOD and tribal waters, and to a lesser extent, to support interjurisdictional fisheries. According to the National Broodstock Managers, this use is selecting the "right" strain for the appropriate resource. The

database should also be regarded as a management tool for maintaining diverse, self-sustaining fish and other aquatic resource populations. The potential for captive rearing and propagation of native wild fish including distinct population segments suggest a strong need to document performance, culture requirements, behavior, and biological aspects - characteristics that will aid in their restoration and recovery efforts.

## Other Projects:

### Aquatic Species Conservation & Management

#### *Batrachochytrium dendrobatidis* “Chytrid” Surveys

Populations of many amphibians have declined dramatically over the last 25 years which raises concerns about the state of our aquatic ecosystems and the possible impact on other wildlife populations. The invasive chytrid fungus, *Batrachochytrium dendrobatidis*, (hereafter Bd) has been implicated in these declines and has been cited to cause death in close to 100 amphibian species. The fungus grows in the keratinized epidermal cells in amphibians and is likely spread through colonization by free-swimming zoospores. Little is known how widespread the fungus is in habitats frequented by amphibians or the various ways the fungus is spread. For example, these fungi can remain virulent for 7 days in contaminated water, thus offering the potential for disease transmission even without direct contact with infected amphibians.

Additionally, many species are vulnerable to climate change impacts and species with very restricted distributions such as amphibians are believed to be at greatest risk. It is important to establish baseline occurrence and distribution maps of Bd and infected amphibian species so current habitat requirements can be determined and monitored into the future. Understanding which species are vulnerable, and why, is key to developing effective adaptation strategies. Further, the relative vulnerability of species and the distribution of Bd can be used to set goals and determine management priorities. Thus, the objectives of this research are to document the distribution of Bd fungus and determine what amphibian species are potentially positive for the Bd fungus. This project is in alignment with the Southeast Aquatic Resources Partnership approach to protect freshwater resources through focused habitat assessments of the nation’s economically and socially significant aquatic habitats.

Chester R. Figiel, Jr., (Warm Springs Fish Technology Center) Brian Hickson (Warm Springs Fish Health Center), Betsie Rothermel (Archbald Field Station, Florida), Christine Densmore, Casey Densmore, and Frank Panek from the U.S. Geological Survey (Leetown Science Center, West Virginia) surveyed multiple streams in the Southern Appalachians to compare chytrid prevalence in water and salamanders in low- and high-order streams.



Swabbing a frog. Credit: Amphibian Ark



Tallulah River in Northeast Georgia. Credit USDA Forest Service

**Baseline Population Inventory of Amphibians at Mountain Longleaf NWR**

Gregory Scull and Chester R. Figiel, Jr. began an amphibian inventorying and monitoring study on the Mountain Longleaf National Wildlife Refuge. We are collaborating with Sarah Clardy – Refuge manager; and Mark Meade, Professor, Jacksonville State University in this baseline survey that involves screening amphibians for the invasive fungus *Batrachochytrium dendrobatidis*. The USFWS Refuge Southeast Inventory and Monitoring Network supports this yearlong study. Little to nothing is known about the forest dwelling amphibians associated with the Refuge’s ecologically unique seeps and shrub bogs. Arguably, these areas might even be considered to be “extreme wetlands” because they only occur under specific conditions related to their source of water and position along the Refuge’s landscape. The capacity in which the Refuge’s seeps and shrub bogs serve as refugia for rare, uncommon or highly specialized salamanders is also not well understood. Due to the unique forest type associated with the Refuge, fire was a historical disturbing force that frequently removed the leaf litter and the herbaceous layer that serves as habitat for many forest dwelling amphibian species during the non-breeding season. Fire also removes down woody debris, another important refugia for many species. Due to the ecology of amphibians, it is reasonable to assume that many species would temporarily utilize these moist seeps and shrub bogs for continued survival after the removal of their primary non-breeding habitat by fire to prevent desiccation.

Because these springs and seeps can serve as a hotspot for amphibians from the surrounding forest, the concern for disease may be an issue. Populations of many amphibians have declined dramatically over the last 25 years (Houlahan et al 2000; Mendelson et al 2006) which raises concerns about the state of our aquatic ecosystems. The chytrid fungus (*Batrachochytrium dendrobatidis*) has been identified as a major culprit in the decline in numbers of many species of amphibians in North America. It is reasonable to assume that these pristine headwaters lacking the elements of pollution and other human caused events would be ideal habitat for persistence of most species. However, the spread and distribution of the Chytrid fungus is poorly known. This multi-faceted proposal would be an initial assessment of the amphibian communities associated with the spring seepages and shrub bogs of the Mountain Longleaf NWR and the status of chytrid in these waters.



*Pseudotriton ruber*. Credit USFWS

**A Science Emphasis for Imperiled Crayfish—a Collaborative Effort for Conservation Needs**

Warm Springs FTC and Chester R. Figiel, Jr. hosted our science partners on May 9, 2012 to discuss and establish conservation objectives for crayfish species (and their habitats) in southeast Georgia. In attendance were James Stoeckel and Brian Helms - Auburn University; Troy Keller and George Stanton - Columbus State University; and Chris Skelton - Georgia College

and State University. Our approach targets conservation activities on a broader spatial scale as increasing demands for water impact these aquatic organisms.

The Southeastern United States is the center of crayfish biodiversity with nearly two-thirds of crayfish species endemic to the region. However, many of these crayfishes have limited geographic distributions and loss of habitat is a primary factor in population decline. Although much work has focused on select species, there is still a considerable amount of information to learn about the general biology, habitat requirements, and ecology of the majority of our native fauna. This is particularly true of burrowing species, which are notoriously difficult to observe. The Piedmont blue burrower (*Cambarus (Depressicambarus) harti*), a state-endangered, primary burrowing species native to Georgia, is found in only a few localities in Meriwether county, including woodlands adjacent to the Warm Springs Regional Fishery Center. It has been found in complex burrows in wooded areas with sandy, humus soils and high water tables, often near streams and springs. Except for general habitat associations, virtually nothing is known about the biology, ecology, or natural history of this animal. Also, given this species restricted range to seepage areas in the Pine Mountain ridge, there is particular concern as to the response of this crayfish to habitat destruction, degradation, groundwater fluctuations and other conditions associated with climate change.

For example, previous sites where individuals were captured have been altered due to logging operations, and the species may be gone from that location. Additionally, this species is an obligate burrowing crayfish and may be susceptible to climate changes related to drought. Preliminary sampling efforts during 2007 and 2008 were made during drought-like conditions and it is unknown if this factor affected crayfish foraging behavior, growth, body size, and reproduction. Reduced rainfall may have resulted in changes in soil moisture, prey availability, burrowing ability, or crayfish movement. Because of its highly specialized life history and because of the small natural range of this species, understanding the threats that disrupt natural processes is critical to conserving this species.



*Cambarus harti* Credit USFWS

### **Nothing But Net!!!**

Since the Deepwater Horizon oil platform and BP's Macondo well exploded last year, the USFWS Baton Rouge Fish and Wildlife Conservation Office (BRFWCO), as part of the Natural Resource Damage Assessment and Restoration Program (NRDAR), has dedicated its efforts to protecting the Gulf Coast ecosystem. To aid in this effort, William Wayman and Chad Shirey

(Warm Springs National Fish Hatchery) (1 week in September) and Ashantyé Williams (3 weeks in October) traveled to Covington, LA and assisted with Gulf sturgeon sampling on the Pearl River. The objective was to place gill nets along the portions of the Pearl River where sturgeon have been known to congregate. Morphometric data and blood samples were collected from any captured sturgeon to check for impacts from the oil spill. Unfortunately despite numerous attempts, only two Gulf sturgeon were captured during the four week effort. Ashantyé' did capture an alligator gar, paddlefish, trout, and even a sheepshead. Although unsuccessful with capturing many sturgeon, we were able to work with some great people like Cedric Doolittle (BRFWCO), Kayla Dibenedetto Kimmel (BRFWCO) and Alan Brown (Welaka National Fish Hatchery). It is always a rewarding experience when we can step outside our labs and be of assistance to other offices.



Ashantyé is checking the net for sturgeon (*left*) and with an alligator gar (*right*) Credit: USFWS



Chad Shirey, William Wayman, Cedric Doolittle, and Kayla Dibenedetto Kimmel displaying one of the Gulf sturgeon collected during the sampling effort. Credit: USFWS

## Meetings

### South Carolina Field Office

On October 5<sup>th</sup>, William Wayman, Brian Hickson (Warm Springs Fish Health Center), Haile Macurdy (Warm Springs National Fish Hatchery) and Vince Mudrak (Warm Springs Regional Fisheries Center) met with Jay Herrington and Amanda Hill from the Ecological Services South Carolina Field Office at the Bears Bluff National Fish Hatchery on Wadmala Island, SC. The meeting involved the possible development of a research program aimed at restoring sturgeon populations along the southeast Atlantic coast.

### Fish Technology Centers Director's Meeting

During the week of October 17<sup>th</sup>, William Wayman traveled to Burlington, Vermont to participate in the annual Fish Technology Centers Director's Meeting. The meeting focused on updating current research priorities for each Center, examination of possible budgetary issues, and discussing collaborative ideas with Fish Health Centers. The meeting was also co-located with the Fisheries ARD meeting. The final day included a combined meeting, which focused on how the Service's science capacity supports Strategic Habitat Conservation and identifying areas of common priorities and emerging needs for which increased capacity is needed.

### RRCC Information Technology Working Group

Jaci Zelko attended the 2011 Robust Redhorse Conservation Committee (RRCC) Annual Meeting. The meeting was held at Mt. Morrow State Park near Albemarle, NC. The RRCC is a voluntary stakeholder partnership charged with the overall responsibility for directing the recovery of the robust redhorse (*Moxostoma robustum*). Jaci has been the Chair of the Information Technology Technical Working Group since 2003. She updated the participants on several topics including updates needed for the capture database, status of the annual meeting reports, and the robust redhorse website ([www.robustredhorse.com](http://www.robustredhorse.com)).

### Appalachian LCC Conservation Science Priorities Workshop

William Wayman participated in the Appalachian Landscape Conservation Cooperative's Conservation Science Priorities Workshop on November 29-30<sup>th</sup>. The workshop gathered scientists and managers from throughout the geographic area to work on determining the science priority needs for addressing the conservation challenges and opportunities across the LCC. The participants discussed and ranked priority science needs based on 5 thematic areas (GIS/Information Management, Climate Change, Human Dimensions, Terrestrial Species, and Aquatic Species). The workshop generated a report that will be used to assist the LCC Steering Committee in determining future conservation and science-support funding opportunities.



SHC Elements: Biological Planning, Conservation Design, Delivery, Monitoring, and Research. Credit: USFWS.

### Annual Morone Meeting

Greg Moyer and Ashantye' Williams attended the annual *Morone* meeting held February 8 – 10, 2012 in Crawfordville, FL. The ACF Striped Bass Restoration and Evaluation Plan was developed to restore and maintain a self-sustaining population of native Gulf Coast striped bass. Ashantye' presented results of Gulf coast striped bass broodstock screening and assessed the genetic status of broodstock lakes. The ACF river system has continually supported a naturally reproducing population of striped bass (*Morone saxatilis*) of the Gulf of Mexico lineage. Smith Lake (Alabama), and Lake Talquin (Florida) have been designated as suitable broodstock repositories for the Gulf Coast strain of striped bass. Genetic monitoring of the stocking strategies of the broodstock lakes is crucial for the successful reestablishment of striped bass and the maintenance of its genetic integrity. Using microsatellites, results indicate that while there is ample genetic diversity in the broodstock lakes, the variation differs significantly among lakes. Ashantye' recommended hatchery managers create more unique crosses of striped bass and stock their progeny throughout ACF river system.

### Alabama Annual Fish/Mollusk/Crayfish Meeting

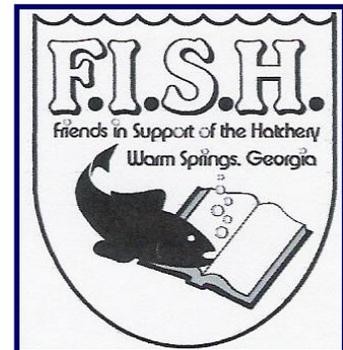
Jaci Zelko attended the Alabama Division of Wildlife and Freshwater Fisheries annual non-game fish, mollusk, and crayfish meeting January 17-19, 2012 at Guntersville State Park, Guntersville, Alabama. The meeting is held for managers, academics, public utilities, consultants, and other researchers to discuss ongoing projects and activities related to non-game freshwater species in Alabama. Jaci gave a quick update on the current activities at the Tech Center including freshwater mussel studies involving cryopreservation and in vitro techniques.



The Alabama state listed holiday darter (*Etheostoma brevirostrum*) was one of many aquatic species discussed at the 2012 Alabama Fish/Mollusk/Crayfish meeting. Credit: Jacksonville State University

### F.I.S.H. Friends Group Meeting

Jaci Zelko and Greg Scull attended the March F.I.S.H. Friends group meeting. Greg, who will be conducting outreach for the Tech Center, was introduced to the members. Topics of discussion included staffing of upcoming large outreach events, membership drives, and assisting the hatchery with various projects.



### **National Broodstock Meeting**

Chester Figiel attended the USFWS National Broodstock Meeting held at the White Sulphurs Springs NFH April 24 to April 27, 2012. This annual meeting is conducted to discuss broodstock egg requests, fish health and nutrition matters and database concerns for each region. Each station gave an update on fish strains being raised for egg requests to production hatcheries. Chester provided an update of the National Fish Strain Registry and received valued feedback from meeting attendees.

## Outreach

### Carnivorous Plant Display

Jaci Zelko and Chester Figiel assisted Fish Health Center employee Allison Hernandez and Regional Fisheries Center employee Rosla Plant with the carnivorous plant display annual maintenance. The display was overrun by invasive plants since the last time it was cared for a year ago. The plants were dug up and divided by species. New peat moss and sand were added to each section of the boat and mixed in a one to one ration. Landscape cloth was added and the carnivorous plants were replanted. Great care was taken to remove as many invasive plants, roots, runners, and leaves as possible. An additional layer of sand was added to help hold down the cloth. Even though it was a long and dirty day, the group hopes the changes will minimize the growth of invasive plants for a long time, so that the public can view and appreciate the native carnivorous plant display.



(from left to right) the boat before the invasives are removed, Jaci and Allison mix new soil, the boat after the carnivorous plants are re-planted. Credit: USFWS

### Bringing the Outdoors to the Rutledge Center

Jaci Zelko visited the Rutledge Center in Newnan, GA on November 1, 2011. Established in 1968, the Rutledge Center is a non-profit organization, and its mission is to serve and promote the general welfare of individuals with developmental disabilities that live in Newnan and Coweta County, GA. Jaci shared information with the clients about the importance of aquatic animal and habitat conservation and the role of the Warm Springs Regional Fisheries Center. They talked about the function of healthy rivers and the importance of the freshwater fish and mussels that live in the rivers. Everyone was given the opportunity to touch turtles, crayfish, and a juvenile American alligator. Over 70 clients and staff thoroughly enjoyed the visit and have already expressed interest in future events. This is the third year Jaci has visited the Rutledge Center and she hopes this great relationship continues for many years.



Jaci Zelko assisted Rosla Plant, Warm Springs RFC Park Ranger, with a school group tour on March 9, 2012. The forty 3rd graders from Cain Danforth Elementary School in Macon, GA were divided up into two groups and given a tour of the public aquarium, boardwalk, and public use areas. Rosla and Jaci highlighted the various ways that the Regional Fisheries Center works with native species.

Greg Scull and Bill Bouthillier conducted a BiT (Biologist in Training) class on March 15, 2012 for 11 students from Crossroads Christian School in Fayetteville, GA. The students were given a tour of the Aquarium, completed classroom assignments from the BiT book, and then completed a stream assessment of a stream on site. The students checked pH, D.O. and water temperatures and then conducted surveys for aquatic invertebrates to assess the stream. All the students, their teacher and three parents enjoyed themselves in the stream looking through leaf packs and turning over rocks.

A group of 25 senior adults from Cottondale Baptist Church, Cottondale, FL were given a tour of the Aquarium and public use area on March 27, 2012 by FWS staff Rosla Plant and Greg Scull.

#### **A New Touch Screen at the Aquarium**

A new Turn-Key Kiosk System has been installed in the Aquarium. This multimedia computer system with an integrated LCD-touch monitor replaces a 15-year old interactive kiosk. The original touch screen was out-of-date in regards to technology and content. Jaci Zelko and the Outreach Team worked on re-designing the content and outreach messages to be included in the new program. The new kiosk has integrated microphone that detects the level of ambient noise in the area around the speaker system and automatically matches the output volume to the ambient level, so that visitors get the best sound quality while interacting with the kiosk. The new program includes information on our Fisheries Programs, Facility History, our Mission, and much more!

Main Screen of the new interactive touch screen.  
Credit USFWS



### Kids Fish for Fun Day

The Warm Springs RFC and Friends Group hosted the 2012 Kids Fish for Fun Day to teach children the enjoyment of fishing. Five FTC employees and one SCA intern helped staff this event held on June 9, 2012. This year, 291 children participated and had the opportunity to catch and take home five catfish. Greg Moyer, Greg Scull, Ashantye Williams, and Chester Figiel, Jr. greeted and registered participants at the Pre-Registration and On-Site Registration tables. SCA intern Ashley Zrubek also assisted in this area. Jaci Zelko and Fish Health Center employee Allison Hernandez recorded the number of catfish each participant caught. This year, members of the Ft. Benning Bass Club were on hand to teach the kids knot tying and casting skills. Thanks to the Friends Group for providing food, beverages, and prizes for this event.



(L) Ashantye Williams places a wrist band on a participant. (R) A father teaches his son to reel in the line.



Greg Moyer registers participants for the 2012 Kids Fish for Fun Day. Credit: USFWS

### Chinese Delegation Tour

An informational meeting between the USFWS and the 2012 Delegation of Chinese Scientists was held in September at the Warm Springs Regional Fisheries Center, Warm Springs, GA. The delegation was part of the Protocol on Cooperation and Exchanges in the Field of Conservation of Nature which is coordinated by the USFWS Division of International Affairs. Presentations on program activities were given by several staff. Tours were given by Project Leaders and each

discussed their accomplishments with aquatic species conservation. Tom Sinclair, Deputy Assistant Regional Director for Fisheries, reported that the participants truly enjoyed the experience and the trip stimulated a variety of meaningful questions.

### **Steve Harvey Foundation Fishing Event**

Warm Springs hosted a fishing event for the Steve & Marjorie Harvey Foundation Atlanta Chapter in September. The event was provided specifically for the Steve Harvey Mentoring Program for young men. Approximately 40 young men ages 13 to 18 years old and their mentors fished and enjoyed the event. In addition to learning how to fish, this event provided a chance for the participants to experience the hatchery and the outdoors. All participants were accompanied by a mentor from the Steve & Marjorie Harvey Foundation and a leader from U.S. Fish and Wildlife Service who assisted with the fishing activities. The participants learned how to tie knots, safely assemble a rod and reel, types of casts, fish handling techniques, and participated in an aquarium tour. After catching fish, they watched a fish cleaning demonstration and were provided the opportunity to taste a few fish that were cooked on site. The young men also learned about the Regional Fisheries Center's scientific activities. Fish Tech Center staff had demonstrations on the cryopreservation and genetics work that we do. It was a fun and educational experience for everyone involved.



(L) Participants in the fishing event. (R) Ashantye and Jaci are ready to get their science on!!! Credit: USFWS

## Workforce Management

### Warm Springs FTC Welcomes New Biologist!

Gregory Scull joined the staff of the FTC on February 13, 2012 as a biologist for the National Fish Strain Registry. Greg brings valued federal experience from working with the USDA Forest Service as the district Wildlife Biologist at the Kootenai National Forest (Montana) where he performed duties related to the protection and improvement of habitat within the framework of landscape level processes. Greg completed his masters degree at Jacksonville State University (Jacksonville, AL) where he worked on fish assemblages of the Choccolocco Creek watershed (northeast Alabama). We are happy to have his service.



Gregory Scull Credit: USFWS

### Welcome Summer!

The Tech Center has welcomed a new intern, Summer Molnar. Summer is a senior at Harris County High School in Hamilton, GA. Her internship is part of an honors internship class. She is interested in DNA and genetics and hopes this experience will help prepare her for college. Summer will be working in the Conservation Genetics Lab.

“Working as a volunteer at the Warm Springs has been an exciting experience so far. I have learned many things that have prepared me for my future career. My main focus of work takes place in the genetics lab. There I use the centrifuge, NanoDrop, electrophoresis equipment, and various other pieces of equipment. This opportunity has advanced my understanding of science and has allowed me to grow as an individual. It has been a pleasure interning at the Warm Springs Fish Technology Center. – Summer Molnar”

### New SCA Intern for the Warm Springs FTC

The Cryopreservation Lab has recently added a new intern to their team to assist with sperm cryopreservation for the summer. Having graduated from Texas A&M University–Corpus Christi in December 2011, Ashley Zrubek received a Bachelor of Science degree in Biology and is pursuing her career in wildlife biology. Much of her upper level coursework in college centered around field work and sampling techniques (salt marsh sampling, seining, gillnetting, electrofishing, trawling, and tagging) often used in the typical duties of a wildlife biologist. She volunteered in a laboratory with her ecology professor a few times during her senior year to collect mud and stone crab, performed water quality work for the environmental microbiology

laboratory at Texas A&M University–Corpus Christi, and is currently a member of the Texas Marine Mammal Stranding Network in Corpus Christi, Texas.

Ashley’s prior experience before coming into the program was working in fisheries and conducting fisheries-based research. Ashley’s primary goal is to serve as a constituent for natural resource conservation, whether as a researcher or laboratory analyst. By serving as an intern, she hopes to get her foot in the door and gain access to a myriad of opportunities in biology and learn more about the progressions in her field. We believe that her background and strengths will benefit our goals and help fulfill our vision for fisheries management.



SCA Intern Ashley Zrubek conducting water quality and performing PCR on 4 black bass species. Credit: USFWS

## News & Notes

### **Environmental Management System and Safety**

Several documents were submitted in October for inclusion in the Warm Springs Regional Fisheries Center Environmental Management Plan (EMP) as part of the annual review. These documents are updated and annually submitted to the Regional Office. These documents include Employee Safety Training Records, Hazardous Chemical Inventories, and FY2011 Recycling Log. Nora David, RO Engineering, visited on Nov 12<sup>th</sup> to conduct the EMP annual review and a Safety Inspection. Jaci Zelko demonstrated and explained the documentation, safety measures and standard protocols that are in place in the FTC area.

### **Safety Site Visit by Washington Office Staff**

Washington Office Safety staff conducted an on-site visit in May. The visit focused on record keeping and supervisory understanding of role and responsibilities. It included interviews, an inspection of our existing written plans, safety self-inspection documents, and examining supporting documents we have on file such as hazardous chemical inventory lists. This was followed by a short walking tour to the Alkalinity building, Fish Health Center, Tech Center and related facilities in that area, and Aquarium building. Several suggestions were made and were incorporated into our safety program.