February/March/April

Hello all!

Welcome to second winter! Western Wisconsin was hit by our standard April blizzard on the 10th and none of us are happy about it. I had just returned from a conference in Orlando a day before the snow fell and you can bet that all I wanted to do was to get back on the plane and fly back.

The last few months were about preparing for our primary tissue culture phase. What is a primary tissue culture? Well, let's dig into some molecular biology. A cell culture is a group of cells, usually from an animal or human, and they are kept alive outside of the body they came from. Many times, these cells are cultured (grown) in private laboratorys and sold as a way to keep the plethora of university labs using the same cells and to keep things standard across research institutions. These cells require food and a clean environment but they don't have a body to do that for them. As a replacement laboratory technicians, like me, act as the circulatory system of a body. We give them liquid that mimics the original organisms blood to trick the cells into thinking they are still in a body. This liquid needs to be changed every 2-3 days to replenish nutrients and to take away metabolic wastes that the cells produce or else they will starve or poison themselves. It's a lot like taking care of a puppy but these cells don't give us love. Instead, they usually give us endless disappointments, but such is the life of a scientist.

Many things can go wrong when doing cell culture. If you forget to feed them in time, they may starve and die. If you change their liquids too often they won't have enough cell signaling proteins to let them know that other cells are nearby and they'll die. If they aren't kept at the right environmental conditions, they may get too cold or too hot and die. If your gloves aren't completely sterile a bacteria, virus, or fungus may find its way into the culture dish and kill everything. Even the air itself can poison the cells and cause them to die. This happens quite often. The cells we receive from companies are very robust and have been raised to withstand a ton of environmental variability. However, we decided were going to start something a bit riskier. The laboratories that supply cells didn't have fish cell lines so we were going to start a primary tissue culture. The before mentioned laboratory cells came from a sterile environment and they haven't seen their original organism in years. The cells from a primary tissue culture come directly from the source, the fish themselves. There are many steps in the process that are weak points for them to get infected. On top of that, no one has created tissue cultures from black crappies before so we had no idea what they needed for environmental conditions. The most work done on primary tissue cultures of fish came from three papers published around 1970 so we were in for an exciting ride. If you're interested in paging through them, I listed them at the bottom of this report.

After months of literature research and accumulating the appropriate chemicals, I figured we were ready to try. All we had to do was wait for our animal safety committee to approve our fish work. They took two months to review the protocol, but it was finally accepted in March. However, I didn't expect that the weather was going to be so sporadic. Early March it was so snowy that it was hard for anyone to get out on the ice to fish. Late March was so warm that the ice began weakening and it was too dangerous to get out. Last week, I decided that we will wait until it's safe to fish before we did the procedure on black crappies. However, we wanted to make sure that our time wasn't wasting away and decided that we could do some troubleshooting before the fish arrived.

A faculty member at UW-Stout is currently doing genetic work with zebrafish. Zebrafish are two to three inches long, easy to breed, and easy to care for and this makes them a model organism in many genetic labs across the world. A model organism is a species of animal that are standard models for animal work. Other model organisms are mice, rats, and yeast. I got in contact with the professor and she gave me some zebrafish that had died from old age earlier that day. I ran through the proposed protocol and created some tissue cultures using those zebrafish. The verdict is still out about whether or not the cells survived the procedure but there are a couple hopeful troopers. The main goal of this first round was to see if the cultures were contaminated with bacteria from the host fish. Three days after the procedure, everything still looks sterile so that's great news!

Here is a quick breakdown about what happened in each month.

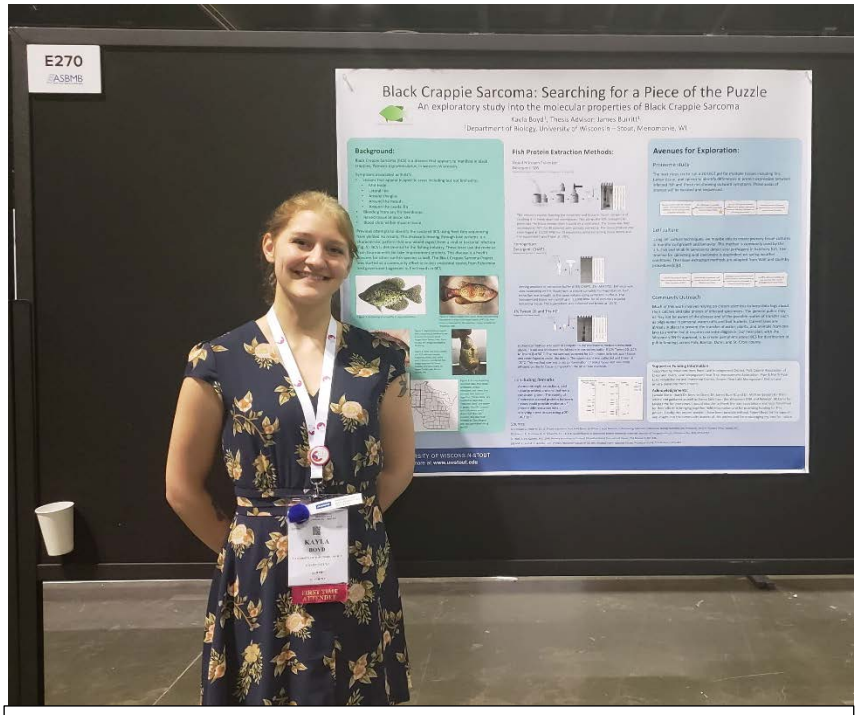
<u>February</u>

January and February were all about getting our protocols approved by various groups to ensure that we didn't violate any university policies or state laws. As much as I would like to go to fish jail, I really don't. University of Wisconsin-Stout requires that any work on live animals needs to be approved by our animal safety committee (Institutional Animal Care and Use Committee). While they were approving our methods, I ordered the materials necessary to work on the tissue cultures as soon as fish arrived. The grocery list consisted of a special liquid called Minimal Essential Medium Eagle which would mimic fish blood, antibiotics and antimycotics to kill any invading bacteria or fungus, and tricaine which would be used to humanely kill the fish. In total, the bill was approximately $200, which wasn't too shabby. Everything ordered was paid for by donations to the Black Crappie Sarcoma Project. Our next order will consist of materials that the university won't be able to supply after May. This includes plates to grow the cells in, more medium, a machine called a micro-pipetter that will measure out very small volumes of liquid, and the big purchase which is a microscope. With these materials, I'll be able to monitor cell behavior for the rest of the summer and the hope is that growing these cells in culture for long periods of time will allow a virus or bacteria to make

itself seen and it will show proof of the pathogen causing Black Crappie Sarcoma. I should have images from the cell culture by the end of the month to share with you. How exciting!!

March/April

March consisted of waiting for committees to approve our protocols and setting up the lab for fish surgery. The majority of my time was spent working on my thesis and creating a poster to bring to a conference. This conference, called Experimental Biology 2019, was the place where experts in their fields came together to discuss the major areas of research for the next year. The main topics were about biochemistry and molecular biology. Researchers talked about Alzheimer's causes, mitochondrial DNA, and the opioid crisis. During the conference, there was a poster competition that my research was submitted for and I used the opportunity to talk to many professors about the importance of ecological research and asked if there was any way they could help with this work. I found a professor in Louisiana who was willing to shoulder some of the pathology work and I'll be inquiring about it within in the next month or so. Everyone I talked to began to understand the importance of doing work like this and hopefully we'll see an increase in people taking the reins for local environmental research.



Kayla Boyd is pictured here presenting the current work done on Black Crappie Sarcoma at EB 2019. The full poster is at the end of this report.

Besides the tissue culture work mentioned above, the protein piece of the study is still going. We're currently talking with a laboratory in Madison that can provide us some data that we can't do here. What they're going to do is take tumor tissue and healthy tissue and compare the protein expression. Cells are constantly producing proteins, which at the bare bones are machines that do work in the cell. Proteins are like thneeds, if I can borrow the idea from Dr. Seuss' The Lorax. Proteins do anything from building the cell, to doing house chores inside the cell, to making more cells, to talking to other cells in the area. It's a really interesting molecule and if you would like to learn more about proteins feel free to check out this video about it -> https://www.mometrix.com/academy/proteins/ . Cells produce certain proteins in certain conditions and you can read their 'mood', if you will, by the different amount of protein produced. We're going to use this to take a look at the cell's health. If there is a virus inside the

tumor cells, it will change its protein expression. Some virus types create very specific protein changes. If this happens, we might be able to see what family the virus is from (if it is a virus).

Thank you to those who have send in pictures and additional information about the experiences you've had with Black Crappie Sarcoma! Much of this work wouldn't have been possible without your help! Together, hopefully we can figure out what's going on with the crappies and get some answers.

Have a wonderful spring!

Kayla Boyd
Lead researcher

Some papers to look at:

Primary Monolayer Culture of Fish Cells Initiated From Minced Tissues by Ken Wolf and M.C, Quimby

Crappies by Paul Holtan, Wisconsin Department of Natural Resources, Bureau of Fisheries Management and Habitat Protection

Observed Differences in CO2 Requirements Between Mammalian and Salmonid Fish Cell Lines by Kleeman, Fryer, and Pilcher

Black Crappie Sarcoma: Searching for a Piece of the Puzzle

An exploratory study into the molecular properties of Black Crappie Sarcoma

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Background:

Black Crappie Sarcoma (BCS) is a disease that appears to manifest in black crappies, *Pomoxis nigromaculatus*, in western Wisconsin.

Symptoms associated with BCS:

- Lesions that appear in specific areas including but not limited to:
  - Mid-body
  - Lateral line
  - Around the gills
  - Around the mouth
  - Around the caudal fin
- Bleeding from any fin membrane
- Raised tissue at lesion site
- Blood clots within muscle tissue

Previous attempts to identify the cause of BCS using Next Gen sequencing have yielded no results. This disease is moving through lake systems in a characteristic pattern that one would expect from a viral or bacterial infection (Fig. 5). BCS is detrimental to the fishing industry. These lakes use the revenue from tournaments for lake improvement projects. This disease is a health concern for other sunfish species as well. The Black Crappie Sarcoma Project was started as a community effort to collect anecdotal stories from fishermen and government agencies to find trends in BCS.

Figure 1. A Drawing of a healthy *P. nigromaculatus*.

Figure 2. A Black crappie from Lower Turtle Lake with BCS lesions in areas of the body typical of BCS. The lower tumor is observed on this specimen. Image provided by Shawnon Dill.

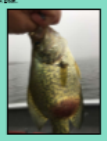


Figure 3. (right) A Black crappie with raised tissue believed to be a neoplastic tumor. Lower Turtle Lake, Dunn county, WI. Image provided by Bill Daley.

Figure 4. (left) The cross section of a BCS infected crappie with BCS-like cells which aren't found in uninfected fish. Image provided by Aaron Cole, Lindsey, and was taken in Lower Turtle Lake, Barron county, WI.

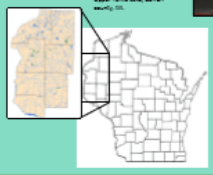


Figure 5. A map depicting reported lakes that show symptoms of BCS infections and rivers that connect lake systems together. "Green lakes" are marked by blue and "infected lakes" are shown in green. Smaller streams and tributaries aren't shown but they are present. BCS lake map created by Tom Meyers and was generated using ArcGIS.

Fish Protein Extraction Methods:

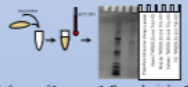
Liquid Nitrogen Pulverizer
Detergent: SDS

This process requires freezing the pulverizer and tissue in liquid nitrogen and crushing it to break apart cell membranes. This allows the SDS detergent to penetrate the tissue deeper than it could on a solid piece. The tissue was then incubated at 70°C for 30 minutes with periodic vortexing. The tissue mixture was centrifuged at 13,000 RPM for 20 minutes to pellet remaining tissue debris and the supernatant was frozen at -20°C.

Homogenizer
Detergent: CHAPS

Varying amounts of extraction buffer A (1% CHAPS, 25mM HEPES, 1M urea) was used depending on the tissue type to ensure complete homogenization. Each extraction was brought to the same volume using extraction buffer A. The homogenized tissue was centrifuged (15,000 RPM for 30 minutes) to pellet remaining tissue. The supernatant was collected and frozen at -20 °C.

1% Tween 20 and Tris-HCl



A chemical method was used to compare to the mechanical methods described above. Tissue was incubated for 24 hours in extraction buffer B (1% Tween 20, 0.25 M Tris-HCl) at 30°C. The mixture was vortexed for 10 minutes to break apart tissue and centrifuged to pellet the debris. The supernatant was collected and frozen at -20°C. This method saw less cross contamination of tissue types and was more efficient on the fin tissue compared to the other two methods.

Concluding Remarks

Across the eight extractions, each tissue provided proteins that were consistently seen. The variety of Coomassie stained proteins between tissues could provide evidence of protein differentiation when analyzing these tissues using a 2D DIGE gel.

Sources

[a] Bressen C., Wolfe M., (2011) Protein Extraction from Solid Tissue. In: Oliver J. (eds) 1D Methods in Biochemistry. 1D Methods in Molecular Biology, (1D Methods and Protocols), vol 673. Humana Press, Totowa, NJ

[b] Brant, J. O., Bradshaw, G. D., Schroeder, J. M., & et al. Quantitation of differential protein expression in the MT ventricle of the aged male rat. J Proteome Res, 2(6), 6210–6218.

[c] Wolf, K. and Quimby, M. C. 1970. Primary monolayer cultures of fish cells initiated from minced tissues. TCS Manual 2: 663–668.

[d] Wolf, K., and M. C. Quimby. 1977. Primary monolayer cultures of fish cells initiated from trypsinized tissues. ProseCure 01343. TCS Manual 3: 658–666.

Avenues for Exploration:

Proteome study

The next steps are to run a 2D DIGE gel for multiple tissues including fins, tumor tissue, and spleen to identify differences in protein expression between infected fish and those not showing outward symptoms. Those areas of interest will be isolated and sequenced.

Cell culture

Using cell culture techniques, we may be able to create primary tissue cultures to monitor cell growth and behavior. This method is commonly used by the U.S. Fish and Wildlife Services to detect viral pathogens in hatchery fish. The timeline for collecting wild specimens is dependent on spring weather conditions. The tissue extraction methods are adapted from Wolf and Quimby procedures[c][d].

Community Outreach

Much of this work involves relying on citizen scientists to keep data logs about their catches and take photos of infected specimens. The general public may or may not be aware of the disease and of the possible routes of transfer such as bilge water in personal watercrafts and bait buckets. Current laws are already in place to prevent the transfer of water, plants, and animals from one lake to another but it requires extreme diligence. Our next plan, with the Wisconsin DNR's approval, is to create pamphlets about BCS for distribution at public landings across Polk, Barron, Dunn, and St. Croix county.

Support or Funding Information:
Supported by donations from Bone Lake Management District, Polk County Association of Lakes and Rivers, Lake Wapogasset/Bear Trap Improvement Association, Pipe & North Pipe Lake Rehabilitation and Protection District, Beaver Dam Lake Management District and private donations from citizens.

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Poster presented at EB 2019