The Expression of the Heat Shock 70 Protein in Shark Blood

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Project # S69

OFFICIAL ABSTRACT and CERTIFICATION

Developing methods for the analysis of exhaustion-exposed shark blood

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Sharks are a crucial and offen under-appreciated part of the oceanic ecosystem. Sharks are also a massively understudied group of species. While many species of shark are endangered and very near extinction, the two species used in this study, the blue shark (Prionace Glauca) and Mako shark (Isurus Oxyrinchus) are in relatively plentiful. The HSP70 protein is part a group of proteins called chaperones. Chaperones are key indicators of bodily stress in most organisms, sharks included. This study observed the tendencies of sharks during capture and used gel electrophoresis to measure the amount of the HSP70 protein in blood samples. Samples were taken from live caught sharks and stored at -80 degrees Celsius before being prepared and run through gel electrophoresis. This technique had been used before but still had a number of imperfections. Through trial and error as well as information from past studies, these methods were improved greatly.

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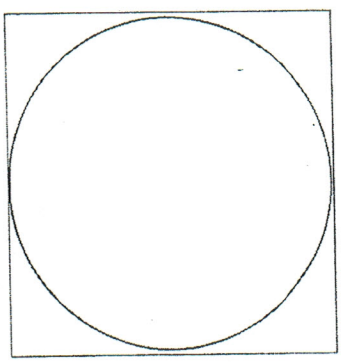

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Abstract

Sharks are a crucial and often underappreciated part of the oceanic ecosystem. Sharks are also a massively understudied group of species. While many species of shark are endangered and very near extinction, the two species used in this study, the blue shark (Prionace Glauca) and Mako shark (Isurus Oxyrinchus) are relatively plentiful. The HSP70 protein is part a group of proteins called chaperones. Chaperones are key indicators of bodily stress in most organisms, sharks included. This study observed the tendencies of sharks during capture and used gel electrophoresis to measure the amount of the HSP70 protein in blood samples. Samples were taken from live caught sharks and stored at -80 degrees celsius before being prepared and run through gel electrophoresis. This technique had been used before but still had a number of imperfections. Through trial and error as well as information from past studies, these methods were improved greatly. The results of this study will allow improved gel electrophoresis techniques to be used to measure the amount of the HSP70 protein in shark blood samples so as to assess the impact of human interaction on stress levels

Introduction

Since the predatorial sharks make up the top of the food chain, their population is very important. Without the top of the food chain, the population of medium sized fish such as tuna and the mackerel would explode, causing a decrease in the population of organisms beneath those fish and an overall dangerous imbalance in all the oceans of the world. Because of this, all new information leading to the continued survival of sharks is vitally important.

The heat shock 70 protein (HSP 70) is part of a group of proteins called chaperones. Chaperones essentially work as a quality control system in the body of an organism. They do this by, in times of stress, performing ATP to bind to proteins and assist in the folding and destruction of ineffective cells (Feder et al., 1999). These chaperones are in the bodies of every living organism and any new information is crucial to the understanding of many life functions.

The heat shock 70 protein is a little known protein, considering it is in nearly every multi cellular living organism. The purpose of the heat shock 70 protein is to regulate body temperature in order to stop enzyme denaturing. If it weren't for the heat shock 70 protein, every time an organism's internal temperature increased, all of its enzymes would denature causing massive bodily failure and essentially imminent death.

In the past it has been discovered that Marine elasmobranches, such as sharks, gain molecular chaperones through their diet (Tredberg and Driedzic et al., 2006). However we still don't know if these molecular chaperones exist naturally in sharks. This study used methods that would allow only proteins created by the body of the shark to be expressed. This made it possible to distinguish whether or not sharks do in fact create their own molecular chaperones. A study done by Kolhatkar et al., in 2014 looked at the chemical chaperones in the blood of sharks. In said study, samples were calmed and then excited in order to remove all chaperones obtained through diet and only gain data from naturally present chaperones.

Fishing and human interaction have been proven to affect sharks and other species of fish in a wide variety of ways. Any amount of added stress and exposure can dangerously affect sharks in such a competitive environment (Frick et al., 2010). Any time spent out of water can lead to chemical problems involving the content of the blood and the brain (Bernal et al., 2012). Through the analysis of the HSP70 protein, we can determine if and how we can help stop these stress-related deaths (Iwama et al., 1998).

The species of shark looked at in this study were the blue shark (Prionace glauca) and the Mako shark (Isurus oxyrinchus). These two species are very similar in behavior, size, and distribution. The blue shark grows to about 250 pounds and 12 feet in length. The Mako grows to about 300 pounds and 13 feet in length. They are commonly found in the North Atlantic but can be found in almost any ocean in the world and are known for their migrations as well as speed. The Mako and Blue shark are some of the fastest moving creatures in the ocean. This means that their bodies are rapidly pumping blood through their bodies at a higher rate than a slower or less active fish. Because of this, the heat shock protein will be introduced to the body in less time making them ideal candidates for this study.

By understanding how different species of shark react to shock, it may be possible to differentiate between the breeds of sharks that must be protected from rod and reel capture. If scientists know in the future that a specific species of sharks has a lower or higher tolerance to rod and reel capture, they will change their methods in order to allow for the highest survival rates.

Gel electrophoresis can be used to identify levels of HSP70 in shark blood samples. However, past attempts to do so have been unsuccessful. The purpose of this study was to…

Methodology

Acrylamide gels for electrophoresis (6, 8, and 10%) were created using standard recipes (See Table 1) for resolving and stacking gels.

Table 1: The ingredients and quantities required for the production of resolving and stacking gel.

*Temed and APS must be added once gel holders are prepared. Temed and APS when combined form a hardening agent. Therefore if the Temed and APS are combined before preparation is complete they may harden before the gels are created.

Ingredients	6%	8%	10%	Stacking gel
30% Acrylamide	10.0 ml	13.3 ml	16.7 ml	1.7 ml
HPLC H20	26.5 ml	23.2 ml	19.8 ml	6.8ml
1.5M Tris pH 8.8	12.5 ml	12.5 ml	12.5 ml	
1.0M Tris pH 6.8				1.25 ml
10% SDS (Sodium dodecyl disulfate)	0.5 ml	0.5 ml	0.5 ml	0.1 ml
10% APS*	0.5 ml	0.5 ml	0.5ml	0.1 ml
Temed*	0.04 ml	0.03 ml	0.02 ml	0.01 ml

Bio Rad gel holders were assembled and the bottom seal was checked before the TEMED and APS (Ammonium persulfate) were added to only the resolving gel. Resolving gel was pipetted in and double distilled water (DDH_20) was added on top. Fifteen to twenty minutes were allowed. After hardening the DDH_2O was removed and filter paper was used to remove any remaining moisture. TEMED and APS were then added to the stacking gel. Stacking gel was then carefully pipetted on top of the resolving gel. A comb was then inserted to form sample wells (See Figure 1). Twenty minutes were then allowed for hardening.

Samples were stored at -80°C. Samples were thawed and centrifuged at 10,000rpms for 10 minutes. The pellet and supernatant were then separated into different 2 ml aliquots. The pellet was then placed back at -80°C. Dilutions of the supernatant were made using High pressure liquid chromatography (HPLC) H_20. 1:100 and 1:150 dilutions were created. Laemmli dye was then added to the dilutions at a 1:3 ratio (1 part dye 3 parts sample). Once the dilutions were made, they were put through a process of 5 minutes of boiling and 5 minutes of freezing in order to activate the proteins.

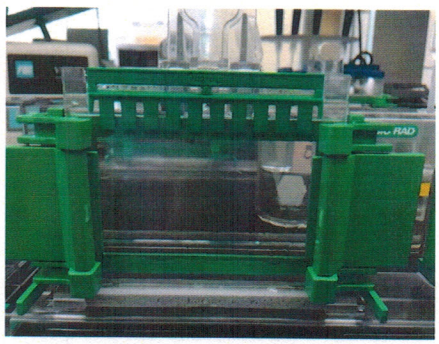

Figure 1: Completed gel inside Bio Rad Gel holder

Gels were then transferred to Bio Rad electrophoresis container and filled with 1X running buffer. 1X running buffer was created by making a 1:4 dilution of 5X running buffer. Samples were then pipetted in using a 2-20ul micropipette and loading tips. 5ul of Kaleidoscope ladder was also added for comparison. See Table 2 for completed gel outline.

Table 2: Completed gel outline

5ul Kaleidoscope ladder	5ul 1:100 sample	10 ul 1:100 sample	15ul 1:100 sample	20ul 1:100 sample	5ul 1:10 standard	5ul 1:20 standard
5ul Kaleidoscope	5ul 1:150 sample	10ul 1:150 sample	15ul 1:150 sample	20ul 1:150 sample	5ul 1:10 standard	5ul 1:20 standard

Once all samples, standards, and ladders were added, the gels were attached to the Biorad Powerpac HC electrophoresis machine and run at 125V for approximately 1.5 hours. After running for the initial 10 minutes, the entire system was placed on ice. After one hour running, the buffer was replaced.

During the running process polyvinyl difluoride membranes and filter paper were cut. The membranes were then soaked in methanol for 15 minutes. Filter papers were soaked in 1X transfer buffer for 15 minutes. After the running process ends, the gels were carefully transferred to membranes. Three pieces of filter paper were then added to each side. This was done for both gels. The sandwiches were then moved into the Thermo Pierce G2 fast blotter machine and run at MW for 7 minutes. After 7 minutes the sandwiches were then removed and separated. The gels and filter paper were disposed of while the membranes were transferred to casein. The membranes were then allowed to soak overnight in casein.

Figure 2: From left to right: Resolving Gel Buffer (1.5M Tris pH 8.8), Stacking Gel Buffer (1.0M Tris pH 6.8), 10% SDS, DDH20, 30% Acrylamide.

Table 3: pH levels for all used buffers

Buffer	pH
Stacking Gel Buffer	6.8
Resolving Gel Buffer	8.8
TBS	7.4
TBS-T	8.8

After the overnight casein wash, the membranes were washed in TBS-T 3 times for 10 minutes each. After the three washes, the membranes were placed in 10 to 15ml of a 1:10,000 dilution of 1* antibody for 1.5 hours. After the 1.5 hours, the antibody was removed and replaced with the first of three TBS-T washes. The antibodies were then saved for future use.

The completed membranes are then soaked in a mix of CLR Buffer, Coumaric acid, and Luminol, a solution required for activating the reaction. Due to the light sensitivity of Luminol and Coumaric, they were added last. The membranes then soaked for 3-5 minutes in this solution. After 3-5 minutes, the membranes were moved into the Gel Doc XR+ machine where they were scanned for 1 hour. This machine produced 1 image every minute for one hour. These images were then compared to previous scans. These scans determined whether the process was successful at showing the presence of the HSP-70 protein.

Results

Luminol and Coumaric:

Luminol and Coumaric are light sensitive and as such should be stored in complete darkness. It was found that even when stored in almost complete darkness, new Luminol and Coumaric acid must be prepared before each use. This was discovered after multiple runs produced blank images. Luminol and Coumaric are the key element in the reaction between the protein and the Gel xr+ machine (Figure 3). They produce the glow that is picked up in the images. Without new Luminol and Coumaric each time, the image will always appear blank.

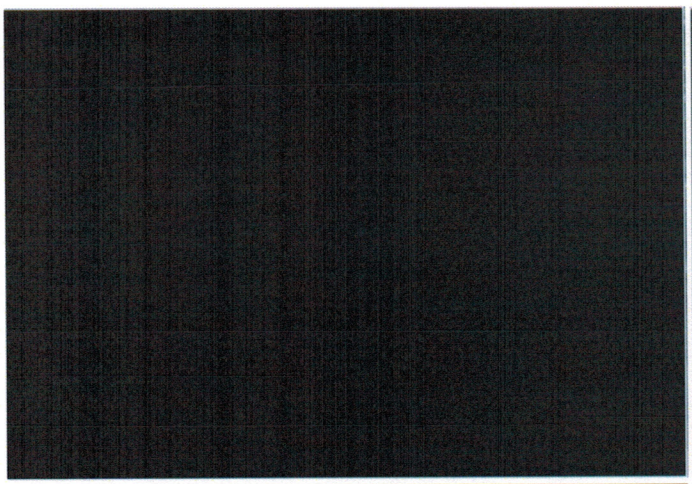

Figure 3: Membrane image post scanning containing 50% hydrogen peroxide

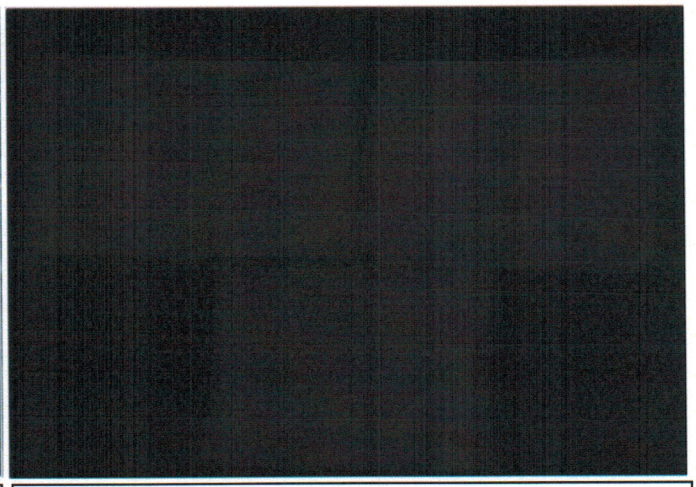

Figure 4: Membrane image post scanning containing 30% hydrogen peroxide

Hydrogen Peroxide:

It was found that although previously unspecified, when creating a CLR buffer, 30% hydrogen peroxide is far preferable to 50% hydrogen peroxide. Figure 5 shows that 50% H_20_2 creates a blurry image and sometimes blocks it entirely. As shown in Figure 6, membranes produced using 30% were much clearer.

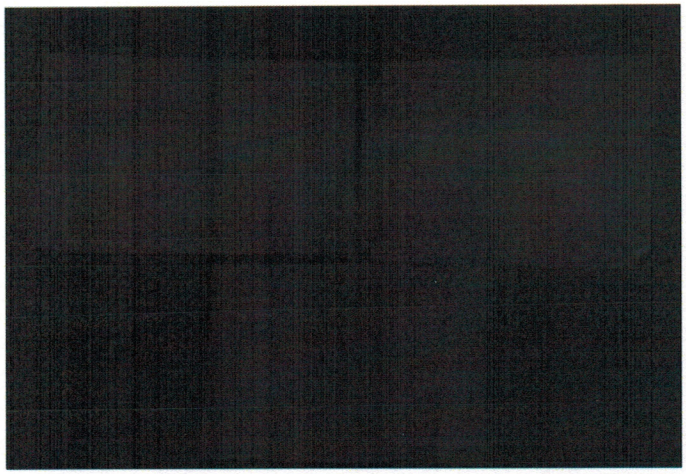

Figure 5: Membrane scan with reused Luminol and Coumaric acid.

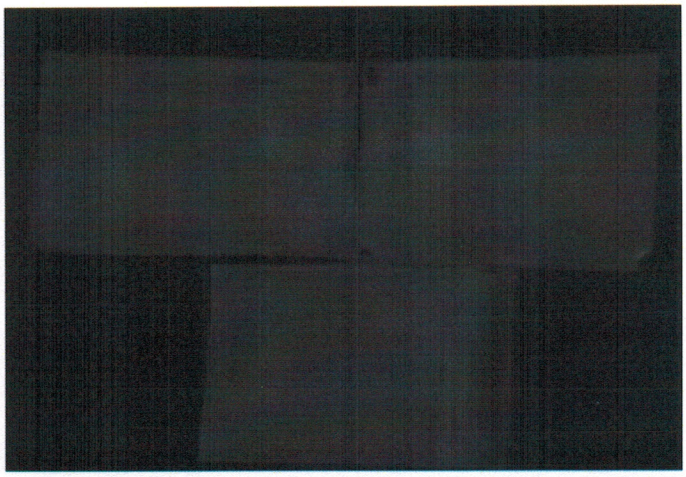

Figure 6: Membrane scan using fresh Luminol and Coumaric acid.

TEMED and APS:

It was found that when creating lower percentage gels (6%) the APS and TEMED levels should be increased in order to avoid leakage. The amounts must be increased evenly in order for the process to work. This was found after a number of unsuccessful 6% gels. Because the gels are so close to a liquid, if the seal is not perfect they will drip right out of the bottom.

Discussion

Images presented in the results section (Figures 3,4,5 and 6) were applicable to the hypothesis in that discernible changes in gel clarity were shown. The progression from Figure 3 to 6 shows a very evident change in visibility that will lead to an extreme increase in the ability of these images to be analyzed and discussed. In previous studies many aspects of the results were not specified. These specifics were found to be crucial to the results of these studies. This study is similar to the study done by Kolhatkar et al., in 2014, in that it analyzed the Chemical chaperones in the blood of sharks. The differences were in not only the species but the methods. Spiny dogfish hold very little similarity to blue and Mako sharks both physically and molecularly. In that study the samples were allowed to settle and then re-agitated in order to activate the chaperones. The results of this study don't necessarily confirm or differ from the findings of any other studies. While similar studies have obtained similar results, the approach of this study was different from those. This study was looking to improve previously determined methods rather than discover new information. The ultimate purpose of this study was to develop and improve on methods for comparing the levels of heat shock protein in blood, specifically that of sharks.

Conclusion

Images of the scans showed that in accordance to the problem statement, these methods were in fact imperfect and were improved. Through a combination of observation and trial and error, these methods were able to be improved greatly and produce a much clearer image than was previously attainable. A newly created CLR buffer containing unaffected Luminol and Coumaric in conjunction with a 30% H_2O_2 and raised levels of TEMED and APS produced the ideal image for scanning gels for electrophoresis. In the future these methods should be applied to both new and old questions related to the HSP70 and other proteins in the blood. Some new questions that could be asked are: Do morphometrics (length, weight, girth, sex) affect stress levels?, Does location effect stress levels?, Do certain methods of capture affect stress levels?.