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Development of molecular assays for assessing the impact of environment disturbance on the performance and survival of salmon

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Report of the Period from November 1 2000 to November 1 2001

Background

Natural resource extraction is a major part of the economy of Northern BC and has been shown to alter the natural ecosystem. For example, forest harvesting has been linked to increases in mean stream temperature and greater daily and seasonal fluctuations in temperature have been measured in streams that flow adjacent to harvested blocks. Other land use practices associated with secondary processing of forest products, mining and urbanization, inevitably lead to increased presence of environmental pollutants. Changes in temperature regime and increased presence of environmental toxicants may influence the molecular and cellular processes in organisms living in the aquatic ecosystem, and of interest to this proposal, fish. Environmental disturbances may have an impact on fish physiological function such as metabolic rate, growth rate, and stress response. Additionally, behaviour, migratory performance and ultimately survival of both adult and juvenile populations may be compromised.

Apoptosis, or programmed cell death, plays a central role in development and homeostasis in all species. Molecular and cellular events which accompany apoptosis include but are not limited to depletion of high energy molecules (eg. ATP), increased oxygen radicals, increased mitochondrial membrane permeability and activation of enzymes that degrade DNA. While the significant impact of temperature and genotoxic environmental contaminants on metabolizing enzymes in the liver of salmon and rainbow trout is well established, little is known about their effect on apoptosis in fish. In mammals, apoptosis can be stimulated by the stress hormone cortisol. Cortisol is a stress hormone and circulating levels of this hormone have been shown to change in fish in response to deviations in temperature or exposure to contaminants. Cortisol levels also increases during two critical life history events in salmon; seaward migration of juveniles and spawning migration of adults. However, the role of cortisol in apoptosis in fish during this time is not defined. But what is known is that considerable tissue remodelling (cell growth and cell death) occurs as fish migrate between the freshwater and marine environment. It is likely that apoptosis plays an important role in the physiological effect exerted by cortisol during these events. Therefore, understanding programmed cell death (apoptosis) in fish will likely have broad implications.

Recently, IPNV virus, a virus which causes acute and contagious disease in fish, has been shown to induce apoptosis and downregulate an anti-apoptotic factor called Mcl-1 in chinook salmon embryonic cell line (Hong et al. 1999. *Virus Res* 63:75-83). This suggested that Mcl-1 has a role to play in apoptosis in fish. This proposal examined the impact of daily oscillation in temperature on apoptosis and Mcl-1 gene expression in juvenile Chinook salmon. Morphological changes in cells and fragmentation of DNA were monitored as indication of apoptosis. Changes in Mcl-1 gene expression measured using a quantitative RT-PCR method. To complement the work conducted using cell lines, the effect of temperature on apoptosis and expression of Mcl-1 in a fish cell line was examined.

Planned Objectives

1. Examine the effect of temperature on apoptosis (programmed cell death) and expression of anti-apoptotic gene mcl-1 in fish cell line.
2. Determine the effect of a 10⁰C daily oscillations in temperature on apoptosis and expression of anti-apoptotic gene mcl-1 in juvenile Chinook salmon.
3. Establish rapid and convenient biological assays for evaluating the impact of environment disturbance (eg. temperature, sediment and pollutants) on development and cellular growth of fish of economic, sport and cultural importance.

Technical Activities and Experimental Results

A. Field work and sampling of tissues from juvenile Chinook salmon

Sampling of juvenile Chinook salmon livers and gills were conducted by Mark Shrimpton at Yellow Island Aquaculture Ltd. Briefly, twelve tanks were set up to hold experimental fish and experiments were conducted for a period of 4 months (Mar-Jun) before tissues were sampled. Six tanks were subjected to constant temperature with mean minimum and maximum daily temperatures of 11.8 and 12.8 ⁰C respectively. Another six tanks were subjected to oscillating temperature with mean minimum and maximum daily temperature of 8.8 and 16.1 ⁰C respectively. For the suspended sediment experiment, clay suspension (bentonite) was added to a concentration of 200 mg/L to three of the constant temperature tanks and three of the oscillating temperature tanks. The aim was to make the water turbid, with visibility reduced to less than 10 cm. Gill and liver tissues were removed from six fish in each tank and were quickly frozen in liquid nitrogen. Tissues samples were then stored at -70 ⁰C.

B. Effect of daily oscillation of temperature and increased suspended sediment on apoptosis in juvenile Chinook salmon.

Approximately 0.5 g fish liver tissues from each treatment were subjected to DNA extraction. DNA extracted from rat liver tissue was used as control. Frozen tissues were grounded to small particles in a mortar and the grounded particles homogenized in 0.5 mL of DNAzol. The homogenates were subjected to centrifugation for 30 secs at 12,000 rpm and the supernatant precipitated with 0.5 mL ethanol. DNA pellet was obtained by centrifugation for 1 min at 12,000 rpm. The pellet was washed twice with 70% ethanol and air dried for 15 min at room

temperature. DNA pellet were resuspended in 100 uL of H₂O and quantified using UV spectrophotometer. 5 ug of DNA samples were loaded onto a 1.5% agarose gel and visualization of DNA was performed by ethidium bromide staining. Results are shown in Figure 1.

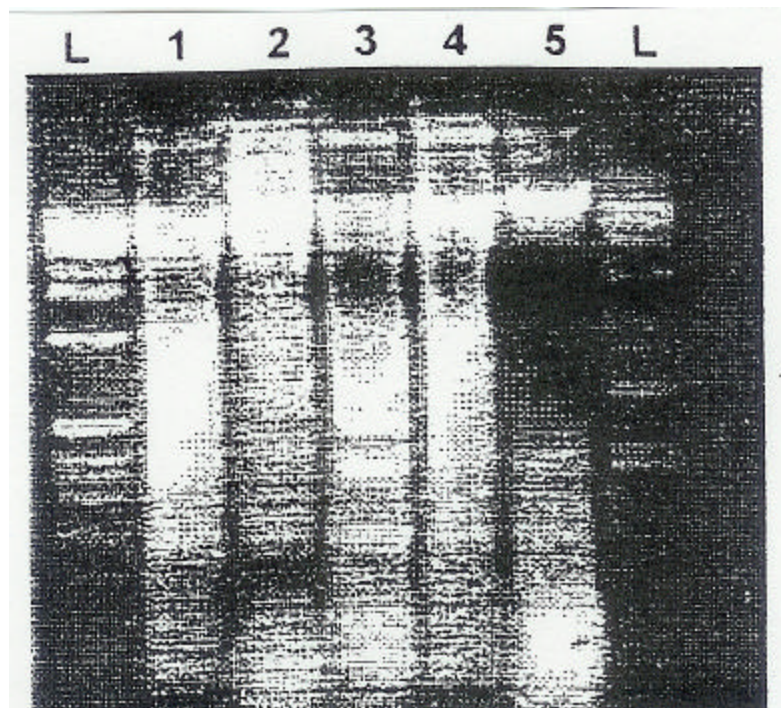


Figure 1. 1.5% agarose gel. DNA fragment analysis of juvenile Chinook salmon hepatocytes using rat liver as a control (Lane 5). Lane 1 sample taken from variable temperature with sediment. Lane 2 sample taken from variable temperature with no sediment. Lane 3 sample taken from constant temperature with no sediment. Lane 4 sample taken from constant temperature with no sediment. L represents the 1-kb ladder from life technologies used in the gel for sizing fragments from 500 bp to 1kb.

An intact high molecular weight genomic DNA with little or negligible fragmentation/laddering was observed from rat liver sample (lane 5). This indicated that our method of extracting genomic DNA is valid and does not lead to degradation of DNA which can be a problem when interpreting DNA laddering. Lanes 1-4 were genomic DNA extracted from liver of juvenile salmon subjected to various parameters as described above. As clearly shown in Figure 1, all lanes from salmon liver exhibited an intact high molecular weight DNA followed by laddering of smaller fragments of DNA. Further samples of liver as well as gill from fish subjected to the different parameters were analyzed. Unfortunately, similar results were observed. All samples exhibited pattern of DNA laddering regardless of the type of environment they were subjected to. Such results indicated that tissues (livers and gills) of juvenile salmon are apoptotically active,

NLUI Progress Report - *Molecular assays for assessing impact of environment on fish*

and suggests that any further external effects (temperature, sediment) on apoptosis will be difficult to detect, at least using DNA laddering method.

C. Effect of daily oscillation of temperature and increased suspended sediment on expression of mcl-1 in juvenile Chinook salmon.

Approximately 1 g of frozen livers (rat, salmon) was placed in mortar and pestle and grounded until finer particles were obtained. 6 mL of Trizol reagent was added and the resulting solution was homogenized with a polytron for 1 min. 0.6 mL of chloroform was added and the contents mixed. The solution was let sit at room temperature for 5 min before centrifugation at 4000 x g for 15 min. The aqueous layer was transferred into a 15 mL tube. RNA was precipitated by addition of 1.5 mL isopropanol and 0.1 mL 3 M sodium acetate. RNA pellet was obtained by centrifugation at 3000 x g for 20 min. The supernatant was removed and RNA pellet was washed with 1 mL 70% ethanol. The pellet was air dried and resuspended in 200 uL H₂O before quantification was performed. Before we proceed to measure the expression of Mcl-1 using quantitative RT-PCR, it is important that the integrity of RNA be checked first. Approximately 5 ug of total RNA extracted from livers of salmon

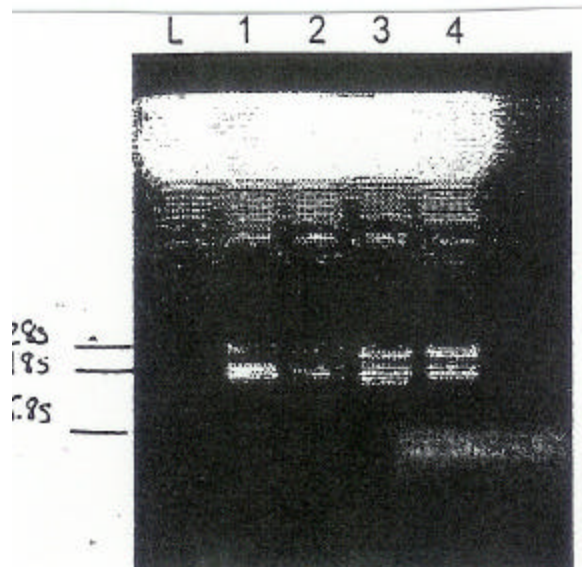


Figure 2. Visualization of total RNA from juvenile Chinook salmon hepatocytes. L indicated the 0.24 – 9.49 kb RNA marker from life technologies. Lane 1 sample from variable temperature with sediment. Lane 2 sample from variable temperature with no sediment. Lane 3 sample from constant temperature no sediment. Lane 4 sample from constant temperature with sediment. All sample lanes show 2 bright bands indicating 28s and 18s subunits. As well there is a fainter band in all the samples indicating the 5.8s subunit.

and rat were run on a 1% formaldehyde gel and integrity of RNA was visualized by ethidium bromide staining. Results shown in Figure 2 indicated that total RNA extracted from rat and salmon livers were intact. This is indicated by the integrity of the two intense 28 S and 18 S ribosomal RNA bands. With the good quality RNA in hand, we then proceeded to quantify the expression of Mcl-1 using quantitative RT-PCR. Since the Mcl-1 primers are of human origin and actin primers are of rat origin, we decided to first test/optimize the assay using rat liver RNA samples. Briefly, 1 ug total RNA from rat liver was subjected to reverse transcription using AMV reverse transcriptase and oligodT primer. PCR was then performed using the following parameter:

94⁰C for 30 sec, 50⁰C for 30 sec, 72⁰C for 45 sec - 15 cycles (for actin), 30 cycles (for Mcl-1)
 5 uL of PCR products were ran on a 2% agarose gel and possible bands visualized by ethidium bromide staining. A sample result of quantitative RT-PCR is shown in Figure 3.

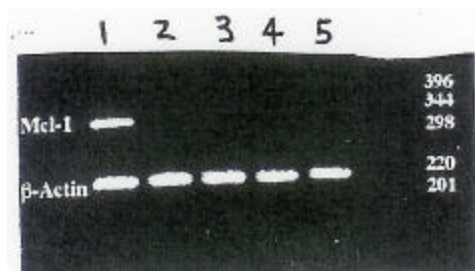


Figure 3. Expression of Mcl-1 as determined by quantitative RT-PCR. 5 ug total RNA from rat liver and juvenile salmon liver samples were subjected to RT-PCR as described in the text. Lane 1 sample is from rat liver. Lanes 2-5 are samples from juvenile salmon liver. Lane 2 is sample from variable temperature with no sediment; lane 3 is sample from variable temperature with sediment; lane 4 is sample from constant temperature with no sediment; lane 5 is sample from constant temperature with sediment.

Lane 1 in Figure 3 shows detection of the expected 334 base pairs RT-PCR product of Mcl-1 from rat liver. Using similar parameters, no products were seen with various liver (lane 2-5, Fig.3) and gill samples (data not shown) from juvenile Chinook salmon. On the other hand, PCR-amplified β-actin transcripts (210 base pairs) were detectable from all samples (Fig.3). Thus, the failure to detect Mcl-1 transcripts from tissues of juvenile salmon could be due to the following: (i) Mcl-1 mRNA is not expressed, and (ii) Mcl-1 primers used which are of human origin, are not homologous to fish Mcl-1 and therefore unable to amplify fish Mcl-1 mRNA.

D. Effect of temperature on apoptosis and expression of Mcl-1 in rainbow trout hepatocyte cell line.

A rainbow trout hepatocyte cell line has been purchased from American Type Culture Collection (ATCC) and was maintained at 21⁰C in culture conditions recommended by ATCC. Preliminary NLUI Progress Report - *Molecular assays for assessing impact of environment on fish*

experiments on the effect of temperature on apoptosis and expression of Mcl-1 in the cell line have been conducted. At sub-confluent density (0 h), temperature was switched to 24⁰C and cells maintained for 4, 8 and 12 h. At these time points including the 0 h time point, DNA and RNA were extracted using standard protocols as described in section B and C. Preliminary results showed that temperature changes had no effect on apoptosis in rainbow trout hepatocyte cell line, as indicated by the lack of DNA laddering pattern (data not shown). Similar to results in section C, no Mcl-1 transcripts were detected in the cell line using quantitative RT-PCR as described in section C (data not shown).

Conclusion

The following have been established from this investigation:

1. We have developed a simple molecular assay to assess apoptosis in fish.
2. We have developed a quantitative RT-PCR method to detect Mcl-1 expression. However, design of new primers for detecting Mcl-1 mRNA in fish would be required.
3. We showed that tissues from juvenile Chinook salmon actively undergo apoptosis, and concluded that future studies on the effect of environment may require adult Chinook salmon.
4. Our preliminary results showed that changes in temperature had no effect on apoptosis in rainbow trout hepatocyte cell line.