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Bioassay development for ultraviolet radiation exposure of juvenile coho Salmon. (Oncorhynchus Kisutch)

BY

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NWRI Contribution no: 05-307

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AEIRB Contribution Series AEI 05-003

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2004-2005 Completion report for the
BC Forest Science Program

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Project Overview *(background, accomplishments, next steps)*

In the mid-1990's there were significant declines in coho salmon (*Oncorhynchus kisutch*) returning to many rivers and streams in British Columbia. Walters and Ward (1998) speculated that that these declines might be associated with the increasing levels of solar ultraviolet radiation (UVR) that were occurring globally at high latitudes during that same period. Although most evidence now indicates that poor coho runs during the 1990's were likely the result of changes in the open ocean foodwebs, other workers have shown that long term changes in water transparency are actually much more important than stratospheric ozone depletion in increasing UVR exposure of aquatic organisms (Schindler et. al. 1997, Pientiz and Vincent 2000, Leavitt et al. 2003). Likewise, in British Columbia, the removal of riparian cover during clear-cut logging has been shown to increase UVR exposure of streams by 3-5 fold compared to an estimated elevation in UVB of only 6-14% associated with the decadal decrease in stratospheric ozone during the mid 1980-90's (Clare 2000, Kerr et al. 2002).

UVR is potentially harmful to many organisms including fish. UVR is also known to have significant negative effects on the foodweb of fishes rearing in shallow streams (Bothwell et al. 1994, Kiffney et al. 1997). Furthermore, recent studies at the Pacific Biological Station (PBS) in Nanaimo, BC have shown that exposure to high levels of natural solar ultraviolet radiation can have many direct deleterious effects on juvenile coho including fin erosion, incipient cataract formation and altered body morphology (Bothwell, Holtby and others in prep). While some of these effects could potentially retard growth and development of coho, none of them are easily quantified.

In contrast, exposing salmonids to UVR increases the amount of UV absorbing substance(s) in the skin and mucosal layers and these pigments are easily quantified by standard spectrophotometric techniques. Initial tests revealed that sunscreen compounds are also present in the fins of UV-exposed fish. This suggested the possibility that a rapid field assessment of UV exposure might be possible by a simple solvent extraction of excised fins.

We are developing a bioassay that will allow us to determine the amount of UVR juvenile coho are exposed to *in situ*. Once fully calibrated, we will use the bioassay to assess the efficacy of various logging practices in protecting stream biota from exposure to UVR and better understand the relationship between logging and UVR exposure of biota.

Newly emerged juvenile coho from the Big Qualicum Hatchery were held outdoors in a series of modified Capilano troughs at the Rosewall Creek Hatchery on Vancouver Island during the summer of 2004. Covering the troughs with various combinations of UV-transmitting Plexiglas and neutral density filters created different UVR photo-environments. Fish were sub-sampled from each of the flumes twice weekly during the summer for measurement of UV absorbing pigment. The frequent sampling allowed us to determine the time course of pigment development as a function of cumulative sunlight exposure. The results show very high correlation between UVR exposure and the concentration of sunscreen substances until a plateau value is attained.

After sunscreen levels reached a stable plateau, we blocked all light by covering the troughs with black plastic on Day 44 and continued biweekly monitoring to determine the stability/degradation rate of the sunscreen substance(s) in coho skin. Once sunlight exposure ceased, pigment levels declined. The rate of decline was a function of the initial pigment concentration. However, even after a month of darkness, the fish that had been exposed to the highest levels of UVR (i.e. 48% and 88% ambient light) still had significantly greater amounts of pigment than fish not exposed to UVR.

Juvenile coho rearing under natural light conditions in the Little Qualicum Spawning Channels and in the rearing ponds at the Big Qualicum Hatchery were also sampled. Sunscreen levels in these fish were compared to those found in coho held in the experimental troughs at the Rosewall Creek Hatchery. This comparison suggested that factors in addition to UVR might also influence the level of sunscreen in coho skin. In particular, it now seems probable that water temperature and diet are also important factors in sunscreen development. The possibility that genetic differences between strains of coho might influence biosynthesis of sunscreen substances will also be addressed. Experiments to determine the effects of these additional factors on sunscreen pigment production and degradation will be run during the summer of 2005.

Another step in 2005-2006 will be extensive surveys of juvenile coho in streams on Vancouver Island and the lower mainland to document the levels of sunscreen pigmentation in fish in streams with known differences in UVR exposure as determined by the amount of streamside vegetation cover and the concentration of DOC in the water.

Experimental Design

Flume Construction and Layout

A set of Capilano troughs (Shepherd 1984) were used for the experiments at the Rosewall Creek Hatchery. Two of the troughs (#3 and #4; Figure 1) were used as holding tanks into which newly emerged coho fry from the Big Qualicum Hatchery were transferred in April 2004. These two holding troughs were covered with UV-blocking Plexiglas OP-2 (Acrylite, CYRO) and only exposed to visible light (PAR). Trough #4 was also covered with shade screen to further reduce the amount of visible light to ~7% ambient. In addition to serving as a holding tank, the fish in these two troughs served as "controls", i.e. these fish were never exposed to significant UV radiation during their lives. In addition to no UV exposure, the fish in Trough #4 saw only low levels of visible light.

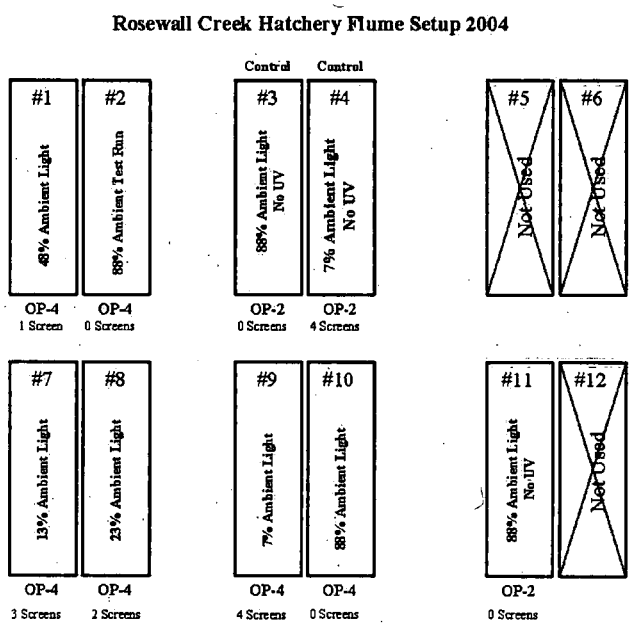

Figure 1. Physical layout of experimental flumes at the Rosewall Creek Hatchery 2004. Troughs are numbered #1 to #12. Troughs #3 and #4 were holding tank controls without any UV. Trough #2 was used for the initial test of UV exposure. Troughs #1 and #7 - #10 were used for different intensities of UV radiation. Trough #11 was used to determine the role of visible light on sunscreen pigment development. Troughs #5, #6 and #12 were not used.

The Capilano troughs used for the UV exposure trials were modified with false bottoms to augment solar exposure and limit shading from the tank sides. The false bottoms were constructed by partially filling the troughs with a rock-sand ballast and covering this with a 10-cm layer of cement producing a smooth surface that raised the bottom to within 25 cm of the tank lip. Aluminum screens were installed at the ends of the troughs to make them all have an equal volume ($0.97m^3$). Water depth in the experimental flumes was 20cm. End screens were removable for ease of cleaning. A perforated horizontal header pipe constructed from 5cm diameter PVC piping was plumbed to the input of each trough

to produce a more laminar flow. The troughs were plumbed to a drainage system that sent all wastewater through the Rosewall wastewater system.

Fish from the Big Qualicum Hatchery were transferred to the Rosewall Creek Facility on April 29, 2004. Using the measured weight of individual alevins as 0.45g (n=20), ~4000 juvenile coho (1.8 kg) were placed in Trough #4 and ~ 6000 (2.7 kg) were placed in Trough #3.

Experimental Conditions

Light and Temperature

During the summer of 2004 we recorded visible light with a Li-190SA quantum sensor mounted on the roof of a building at the Rosewall Creek Hatchery and a Li-100 data logger (Li-Cor Inc., Lincoln Nebraska). Over the summer PAR incident on the site ranged between 5 -60 Einsteins/day with an average of 40 Einsteins/day (Figure 2).

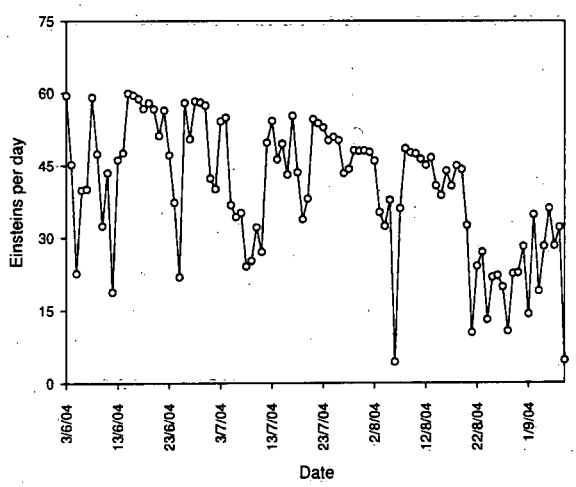

Figure 2. Photosynthetically Active Radiation (PAR) at the Rosewall Creek Hatchery during experimental trials June 3 to September 8, 2004.

Troughs used for UVR exposure were covered with UV-transparent acrylic sheets (OP-4, Acrylite, CYRO). OP-4 passes full spectrum sunlight (PAR+UVA+UVB) although the intensity was reduced to 88% of ambient because of surface imperfections and reflection. Troughs without UVR exposure were covered with OP-2 acrylic sheets (Acrylite, CYRO). OP-2 blocks nearly all radiation shorter than about 390nm. Fiberglas window screen was used as a shade cloth to regulate the intensity of light entering the troughs. Multiple layers of screens were used to achieve the desired shading for each treatment. A Li-Cor PAR sensor was used to quantify the relationship between the number of layers of screen and the transmitted light (Figure 3). Screening of this type is neutral density and does not alter the spectral characteristics of the transmitted radiation.

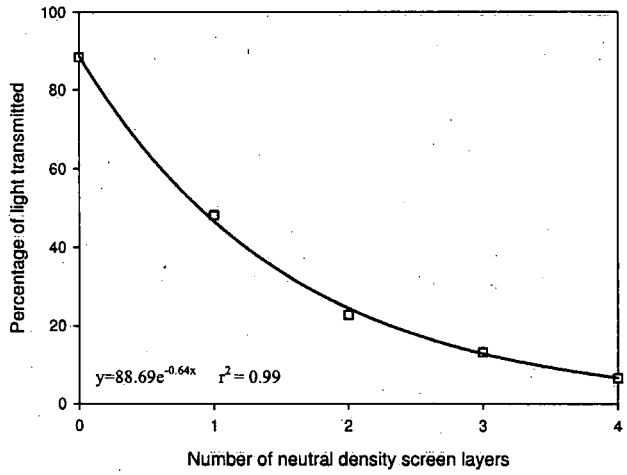

Figure 3. Relationship between the number of layers of neutral density screen and light transmission.

Water temperature was measured continuously with an Onset Stowaway temperature logger placed in the aeration tower at the Rosewall Creek Hatchery. The source of water for the hatchery is groundwater from the Rosewall Creek aquifer. The temperature of the groundwater is very stable, ranging between 7.5 and 9.0°C annually. During our experiments in the summer of 2004 the water temperature averaged around 8.0°C (Figure 4).

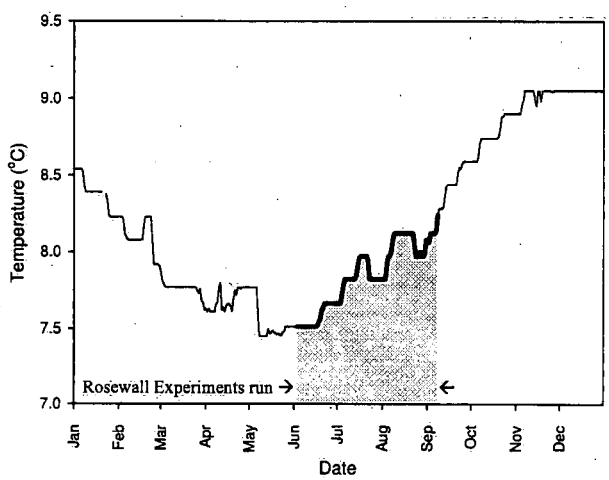

Figure 4. Average daily water temperature in the Rosewall Creek groundwater in 2004. Temperature was recorded using Stowaway Tidbit loggers from Onset Computers. Logger recorded every 15 minutes. Darkened portion of the line indicates time of summer experiments.

Fish Food

Coho fry were started on EWOS #0 crumble (EWOS Canada Ltd.) Poor response of the fry to that particular feed resulted in a switch to a diet of Skretting Nutra-Plus (Skretting

Canada). The fish were fed to satiation using automated belt driven fish feeders. The coho were initially fed with #0 crumble. This was increased to #1 crumble after three weeks. At an average weight of 3 grams the fish were switched to Skretting #2 Nutra-Plus crumble.

Sunscreen Method Development

At the beginning of the project we needed to develop a streamlined approach to analyzing for sunscreen substances in the skin of fish. The method used earlier in our lab was adopted from the literature and was too laborious and time-consuming for routine work. It required surgical excision of an area of skin, careful removal of adhering tissue, freeze drying, weighing, solvent extraction with grinding and centrifugation before final spectrophotometric measurement of the extract. This regime was not amenable to development of a bioassay requiring rapid turnaround of a very large numbers of samples every 2-3 days.

An Agilent 8453 photodiode array spectrophotometer equipped with an auto-sipper cell at the Applied Environmental Research Laboratory (AERL) at Malaspina University-College greatly expedited the extract analysis. However, we still had to develop alternatives to skin excision, weighing, and lengthy extraction with grinding as well as determine the stability of skin samples and extracts. This section outlines trials that eventually lead to a feasible bioassay procedure.

Comparison of sunscreen levels in different areas of the skin

Skin/Fin Comparison

Twenty fish were removed from the 100% ambient no UV control. The upper lobe of the caudal fins were excised, spread, photographed and extracted in 100% methanol. Each fish also had a patch of skin (~1cm^2) surgically removed from the dorsal side of the body, just behind the head, in front of the dorsal fin. The skin patches and fins were spread out and photographed. Skin and fins were handled in the same manner and allowed to extract overnight (~18 hrs). Sub-samples (0.5 mL) of the fin extract were diluted with 2.0 mL of methanol for a dilution of 5 times. Sub-samples of the skin extract (0.25 mL) were diluted with 2.25 mL of methanol for a dilution of 10 times. Samples were run through the photodiode spectrophotometer (Figure 5). Absorbances at 295 nm were corrected for dilution and normalized to the surface area extracted.

The results indicated that there were significantly greater amounts of pigment in the dorsal skin area than on the upper lobe of the caudal fin. However, the amounts present in the caudal fin were easily sufficient for measurement and were also significantly higher than controls. More importantly, the variability in pigment content of the fins was much lower than for samples of dorsal skin (Figure 5). For these reasons and the fact that fins are much easier to sample, we chose to use fin extractions in our bioassay development.

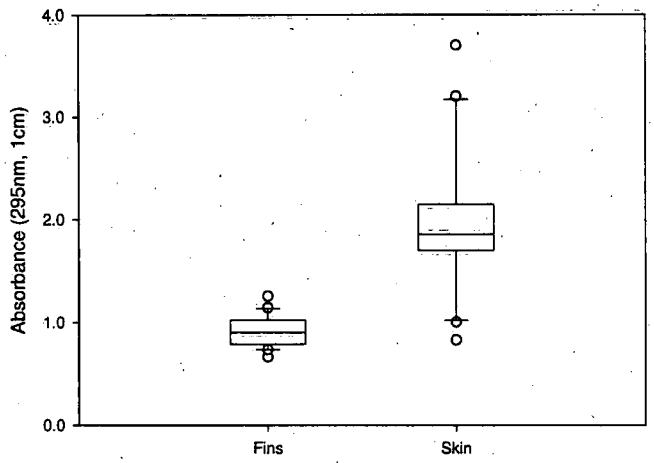

Figure 5. Difference in absorbance between skin and caudal fin samples. Absorbance has been corrected for dilution and normalized to area of tissue extracted.

Fin Comparison

Twenty fish were taken each from the 100% ambient light treatment and the 10% ambient light, No UV control. Fish were anaesthetized and samples were taken of the left pectoral, right pectoral, anal, adipose, dorsal, and both the upper and lower lobes of the caudal fin (Figure 6). Fins were photographed, placed into individual micro-centrifuge tubes and extracted in 1.9 mL of 100% methanol overnight (approximately 18 hours). Extracted samples from all fins, except the adipose fins, were diluted 5 times. Due to their relatively small size adipose fin extracts were undiluted.

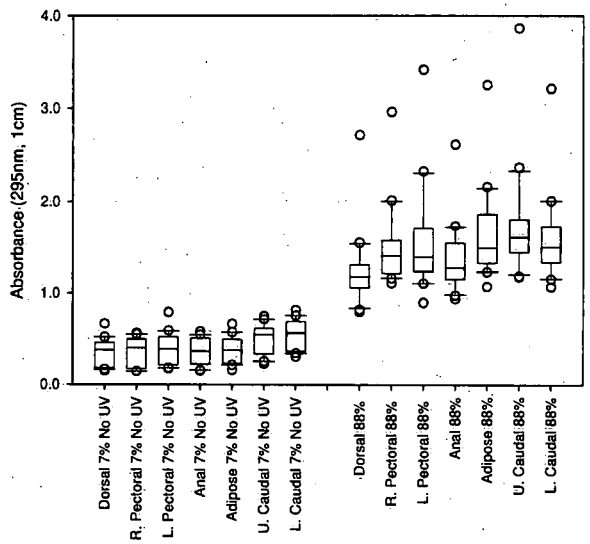

Figure 6. Comparison of absorbance from different fins. Fins taken from coho in two different UV treatments. Absorbance has been corrected for dilution and normalized to fin area.

Kinetics of Pigment Extraction

Trial 1

Ten coho were removed from the 100% ambient no UV control and transported to the AERL at Malaspina University-College. Fish were humanely killed on site and the upper lobes of their caudal fins were removed. Five fins were placed into each of two test tubes containing 40 mL of 100% methanol. Timing commenced immediately upon placement of the fins into the solvent. The tubes were vigorously shaken immediately prior to the withdrawal of each sub-sample to ensure thorough mixing. Sub-samples of 0.5 mL were withdrawn from the tubes after 1, 2, 5, 10, 15, 30, 60, and 120 min intervals. A final sub-sample was withdrawn after extracting overnight in the refrigerator (~ 19 hrs). Each sub-sample was diluted with 2.0 mL of methanol, i.e. a 5 fold dilution. The samples were immediately run through the photodiode spectrophotometer.

Trial 2

Twenty coho were removed from the 100% ambient no UV control treatment and transported to the AERL at Malaspina University College. The fish were humanely killed on site and the upper lobes of their caudal fins excised. Five fins were placed into each of four test tubes containing 40 mL of 100% methanol. Timing commenced immediately upon placing the fins into methanol. Tubes were shaken vigorously prior to withdrawing each sub-sample. Sub-samples (0.5 mL) were withdrawn at 15 sec intervals for the first two min; followed by samplings at 30 sec intervals till 5 min had elapsed then at 30, 60, and 120 minutes. A final sub-sample was withdrawn after extracting overnight in the refrigerator (~ 19 hrs). Sub-samples were diluted five fold and scanned in the spectrophotometer.

From these two trials it was apparent that solvent action alone was sufficient to extract the surface localized UV absorbing sunscreen compound(s) and nearly 90% extraction occurred within 20 minutes (Figure 7).

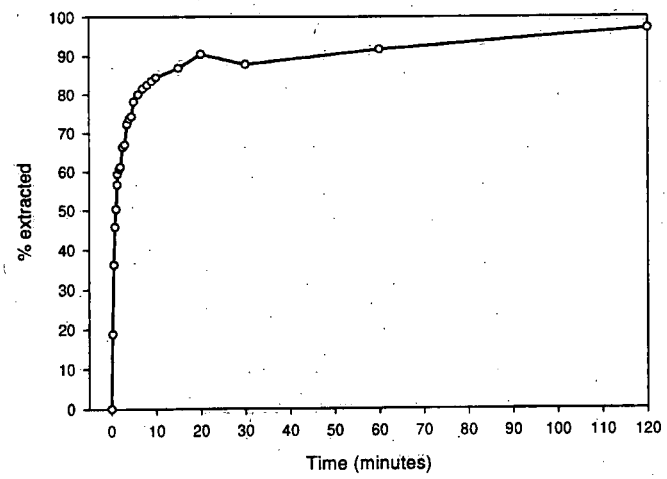

Figure 7. Percent of sunscreen substance (absorbance 295nm, 1cm) extracted as a function of contact time with 100% methanol. 100% extraction assumed after 19h.

Stability of Samples and Extracts

Substance stability under refrigerated conditions

Surplus sample extracts from the 48% ambient light and 88% ambient light treatment fish were held in the dark at 4°C and re-run 27 days, 63 days, 218 days, and 240 days after the initial sampling. Data were compared to initial absorbance readings (Figure 8). The absorbance at 295nm had declined by 18%.

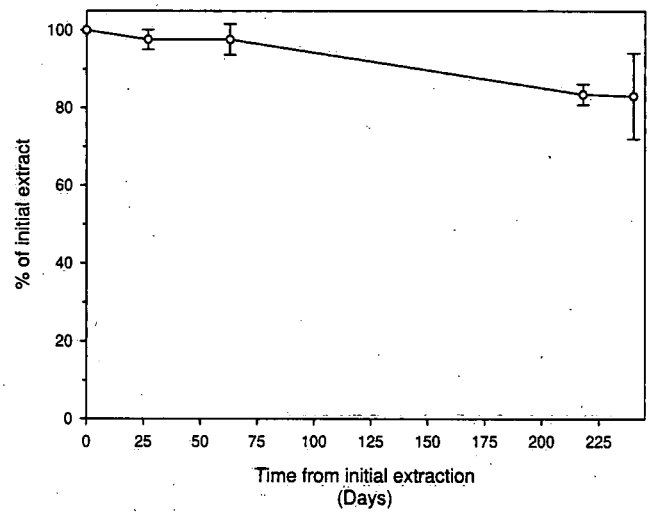

Figure 8. Stability of the sunscreen substance after storage in darkness at 4°C. Samples were re-run 27 days, 63 days, 218 days, and 240 days after initial sampling.

Stability of frozen, non-extracted fins

Seventy-five coho were removed from the 88% ambient no UV control. Fish were anaesthetized and the upper lobes of their caudal fins were excised. Fins were laid out on duksbak^{tm} waterproof paper and photographed in three groups of twenty-five. One group was immediately placed into micro-centrifuge tubes and extracted in 100% methanol. The fins from the other two groups were placed into individually labelled micro-centrifuge tubes and frozen at -10°C. Extracts from the first group analyzed the next morning (~18 hours later). After six months the frozen fins were extracted and run to see if their absorbance differed significantly from the original group. The absorbance at 295nm had declined by 38%.

Preliminary Trial

In a preliminary trial, ~ 200 juvenile coho were moved from the control Trough #3 into Trough #2 with full spectrum sunlight at 100% ambient intensity on June 4th (Figure 1). Thirteen days later, 12 fish were removed for sampling from both Troughs #2 and #3. The fish were anaesthetized, weighed and measured. Clippings were taken from the caudal, dorsal and adipose fins, and skin was excised from the dorsal area of each fish. All skin and fin samples were weighed on an analytical balance and then placed into a micro-centrifuge tube with 2.0 mL of 100% spectral grade methanol. Samples were

shaken and placed into a dark cooler for transport. Spectrophotometric analysis of the extracts was made at the AERL. All samples contained readily measurable amounts of the sunscreen component as determined by absorbance at 295nm. At the end of this test, the upper lobe of the caudal fin was chosen as the body part for all future sampling because it was easily excised, it contained large amounts of sunscreen pigmentation, and the variance among replicates was low (see Figure 5).

The best procedure for normalizing pigment to the amount of tissue extracted was the next consideration. Because the pigment is present on the surface of the skin, normalizing to surface area rather than to the weight of the tissue seemed logical. Using an appropriate image analyzing (Motic Images Advance Software) program and digital photography, it should also be a simpler procedure than weight measurements on air or freeze-dried tissue.

Following this initial test, the protocol outlined below was adopted and used throughout the summer of 2004 to quantify the sunscreen substance on the skin (fins) of juvenile coho.

1. Twelve fish were removed from each tank.
2. The fish were anaesthetized and the upper lobe of the caudal fin was excised.
3. The caudal fin lobes from each tank were photographed with a Nikon Coolpix 4500 digital camera and fin area was calculated using Motic Images Advanced software calibrated to a graphic image of known size. A calibration image was taken with each set of photographs.
4. Each fin lobe was then placed in a micro-centrifuge tube with 1.9 mL of spectral grade 100% methanol.
5. Fins were allowed to extract overnight in the dark at 4C.
6. The absorbance at 295nm was measured with a photodiode array spectrophotometer and then normalized to the total surface area of the fin lobe extracted, i.e. the 2D measured area multiplied by two.

Routine Sampling Procedure

On each sampling day, 12 coho were netted from each trough. Fish were killed with MS 222 (100mg/L) one trough at a time. Excess MS 222 was rinsed off, the fish placed on a cutting board and the upper lobe of the caudal fin was severed with surgical scissors. The twelve fins from each trough were placed onto one sheet of duksbak^{tm} waterproof paper (R.D. Penhall Ltd.) (Figure 9). The fins were spread out with long needle and photographed using a tripod mounted Nikon Coolpix 4500 digital camera (image size 2272 x 1704; focal length 7.8mm). After photographing the fins were transferred to individually labelled micro-centrifuge tubes (VWR international) containing 1.9 mL of methanol (OmniSolv). The tubes were closed and shaken vigorously for 10 sec then transported to the PBS in a dark cooler. At PBS the tubes were shaken again for 10 sec and placed in a refrigerator for overnight extraction (15-18 hours).

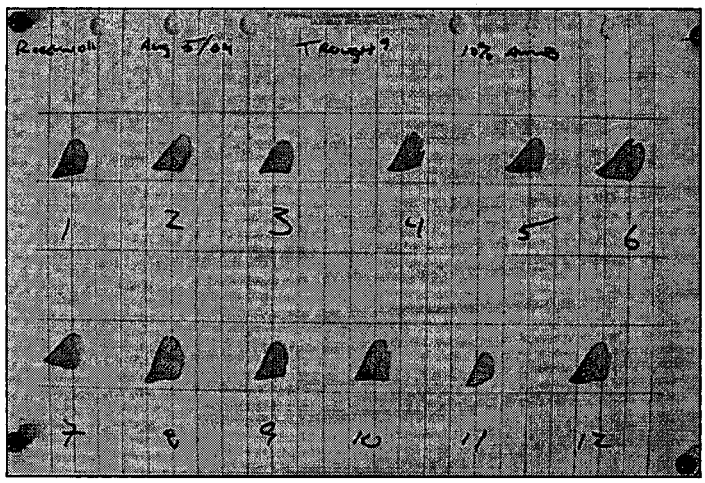

Figure 9. Excised dorsal fin lobes from twelve juvenile coho in Trough #9 on August 5, 2004. The images of each fin were digitally analyzed for area and perimeter.

Analysis of Extracted Skin Pigment

Sample extracts were analysed on an Agilent 8453 photodiode spectrophotometer in the Applied Environmental Research Laboratory (AERL) at Malaspina University-College. Photometer lamps were warmed for one hour prior to use. The instrument was blanked with 100% methanol. All samples were diluted 5-fold (0.5 mL sample plus 2.0 mL methanol) before measurement to ensure readings were in the linear range of absorbance (Absorbance 0.0 - 0.4 in a 1-cm cell). The Agilent 8453 was equipped an automatic sipper cell programmed to draw sample for 10 seconds, followed by a 5 second rinse with 100% methanol. For each extract, triplicate scans were made at 2 nm intervals from 190nm to 1100nm. Samples waiting for analysis were in the dark at 4°C. All scans were converted to Microsoft Excel format for analysis.

Timecourse Experiment

On July 5, 2004, ~1500 coho (individual mean weight ~ 2.55g) were moved from the holding control Trough #4 into each of the following full spectrum sunlight treatments: 88% ambient, 23% ambient, 13% ambient, 7% ambient, and 88% ambient/No UV. Fish were sampled from each of these photo-treatment troughs and the control (Trough #4) twice every week following the procedures #1-6 outlined above. The time course of sunscreen pigment production was followed until 19 August (44 days). After that date light was blocked from all of the experimental troughs with opaque black plastic. Sampling continued until 19 September to determine the rate of disappearance of the pigment.

The rate of sunscreen development in the fins of juvenile coho was directly proportional to the amount of sunlight exposure. Increasing radiation between 7% and 88% of full spectrum sunlight nearly doubled the concentration of sunscreen substances in ~ 25 days (Figure 10). During the first two weeks, sunscreen production increased rapidly then

slowed and reached a plateau after about 20 - 30 days. Similar kinetic patterns were observed under most treatments. The plateau concentration of sunscreen was also a function of sunlight intensity (Figure 10).

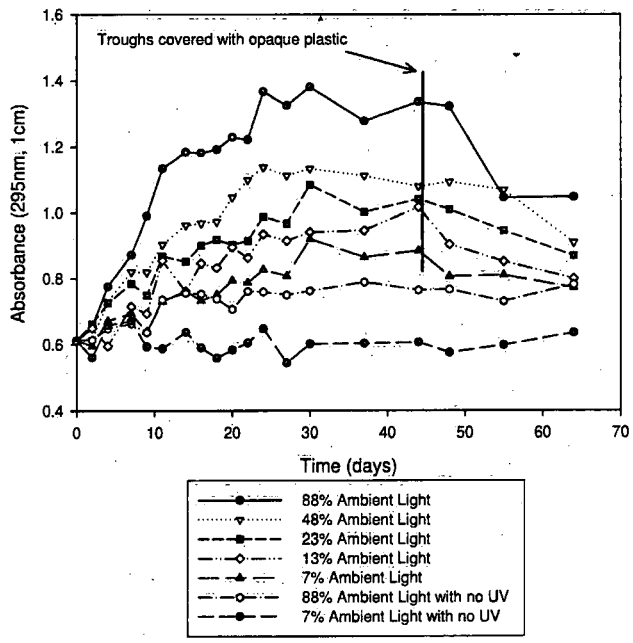

Figure 10. Rosewall Creek Hatchery timecourse experiment July 5 - September 19, 2004. Absorbance (295nm) of methanol extracts of caudal fins normalized to fin surface area. Vertical black line indicates when UV exposed troughs were covered with opaque plastic (Day 44).

Sunscreen production was primarily driven by exposure to UVR. However, a small but significant amount of the substance was also stimulated by visible radiation. Throughout most of the experiment there were significantly higher levels of sunscreen substance in 88% ambient/No UV than in 7% ambient/No UV (Figure 10). Nevertheless, adding UV to the spectrum greatly accentuated the sunscreen response. Comparing sunscreen increases between 7% and 88% ambient light with and without UV indicates that UV radiation results in 2-3 fold more sunscreen production than visible radiation.

The amount of sunscreen substance in the fins of juvenile coho was correlated with the cumulative amount of light received over time by the fish. When the data from spectrally comparable treatments are plotted together, there was a remarkably strong relationship between cumulative light energy and sunscreen substance concentration (Figure 11). A plateau seems to be reached at around 800 Einsteins. Further, increasing the amount of accumulated light by extending the duration of exposure did not result in higher concentrations of sunscreen pigmentation.

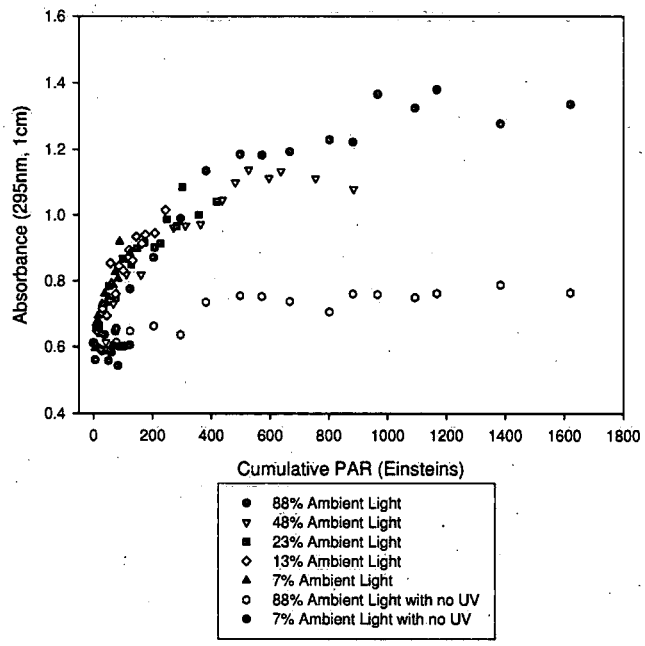

Figure 11. Absorbance (295nm) of methanol extracts of caudal fins normalized to fin area versus PAR accumulated during the UV exposures at Rosewall in 2004.

After light was blocked, sunscreen pigment in the fins of juvenile coho declined over the next month (Figure 10). However in fish that had been exposed to the highest levels of UVR, i.e., 48 and 88% ambient, there was at least a 4 day lag in the loss of pigment. After Day 48 the rate of decrease was proportional to the concentration of the pigment (Figure 10).

Sunscreen Substances in Wild Coho

Little Qualicum wild coho

Gee traps were positioned at six sites along the Little Qualicum spawning channel. These sites were chosen to represent three different photo-environments in the channel; heavy cover (closed), light cover (open), and intermediate cover (intermediate). Traps were baited with salted salmon roe and left in place overnight. The following morning 15 fish were taken from the closed site trap (fish in the other heavy cover trap were released), and each of the open site traps. The contents of the two intermediate site traps were combined to produce a total of 15 fish. Fish were anaesthetized one group at a time. They were weighed, measured; the upper lobe of the caudal fin was removed, placed into a labelled micro-centrifuge tube and extracted in 1.9 mL of methanol. Samples were shaken for approximately 15 seconds and placed into a cool container for transport to Malaspina University-College for analysis the same day. Samples were diluted 5 times (0.5 mL sample with 2.0 mL methanol) and run through the photodiode spectrophotometer (Figure 12). Remaining samples were diluted and run a second time the following day to ensure that results obtained the previous day were accurate.

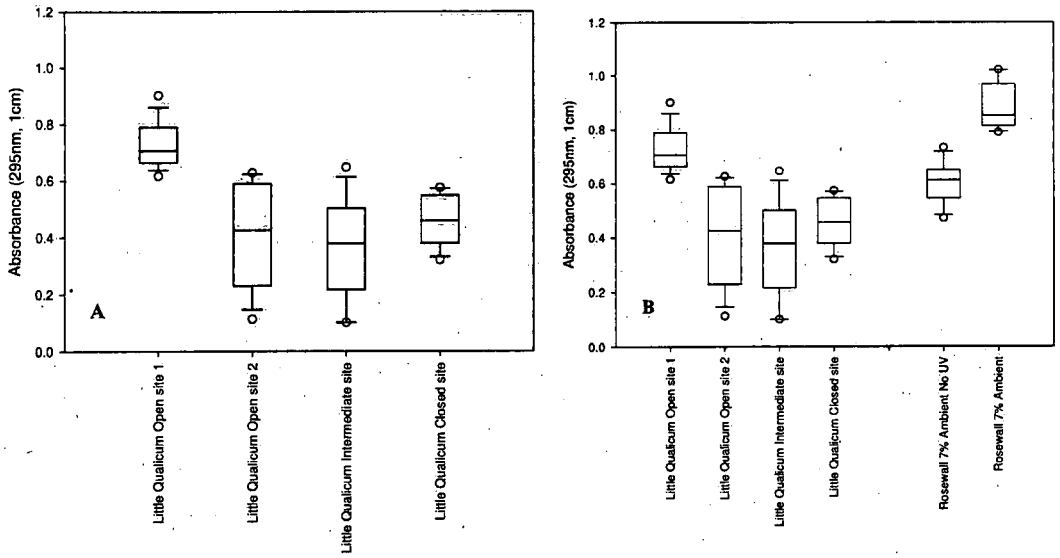

Figure 12. **A.** Sunscreen levels in juvenile coho taken from the Little Qualicum Spawning Channel on September 23, 2004. **B.** Sunscreen levels in Little Qualicum coho compared to coho from the Rosewall Creek experimental flumes on August 19, 2004.

Big Qualicum Earthen Pond

Hatchery personnel at the Big Qualicum hatchery captured 52 coho from an earthen holding pond on October 6, 2004. Fish were anaesthetized and the upper lobe of their caudal fins excised. Fins were photographed and placed into labelled micro-centrifuge tubes with 1.9 mL methanol. The tubes were shaken for approximately 15 seconds and placed into a cool dark container for transport. The tubes were shaken once again and allowed to extract in the refrigerator overnight (~18 hours). The following morning the tubes were shaken prior to sampling and 0.5 mL was with drawn diluted with 2.0 mL of methanol and scanned in the spectrophotometer (Figure 13).

Coho in different locations in the Little Qualicum Spawning Channel had different levels of sunscreen (Figure 12A). Fish from one of the open sites had significantly higher sunscreen levels than fish from the other sites. But most of the Little Qualicum fish exposed to ambient solar radiation had lower levels of sunscreen than fish at Rosewall Creek which were either not exposed to UV at all or had only to very low levels of UV exposure (Figure 12B). Likewise, coho from the Big Qualicum rearing pond had levels of sunscreen similar to the control fish in the Rosewall Creek trial (Figure 13B). Both Big Qualicum and Little Qualicum coho were in the same range (Figure 13A). One possible explanation for this discrepancy might be the higher temperatures in the Little Qualicum River and the Big Qualicum pond. In both cases, summer temperatures run between 15-20 C, much higher that those at Rosewall ~8C. Higher temperatures may influence the turnover time of these compounds.

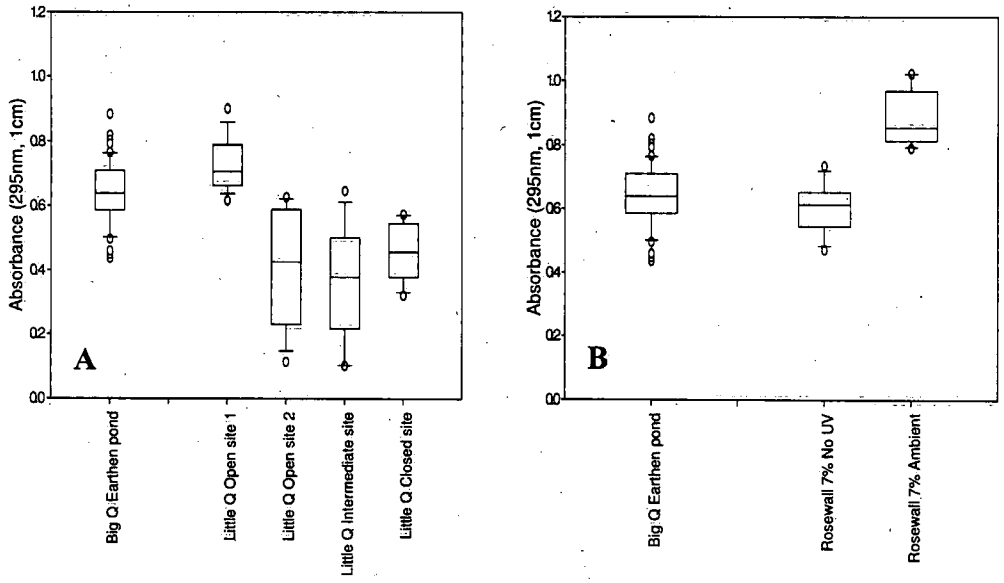

Figure 13. **A.** Absorbance of samples from coho at Big Qualicum earthen holding pond on October 6, 2004 compared with coho from the Little Qualicum Spawning channel on September 23, 2004. **B.** Big Qualicum samples compared to samples from the Rosewall Creek experimental flumes on August 19, 2004.

Data Analysis

Data was exported from the Agilent software into Microsoft Excel for manipulation. Average absorbance at 295nm was taken for the fish in each treatment/trough. Averaged absorbances were corrected for fin area and dilution factor. Statistical analysis of data was done using SPSS 12.01 statistical software. Data were analyzed with one way ANOVA as well as post hoc tests (Student-Neuman-Keuls) at a 0.05 confidence level. Output was displayed as Sigmaplot box plots, charts, and summary tables. Data over time was also compared using the same analysis.

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PRINTED IN CANADA ON RECYCLED PAPER
IMPRIMÉ AU CANADA SUR DU PAPIER RECYCLÉ

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