AN ABSTRACT OF THE THESIS OF

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Carl B. Schreck and Martin S. Fitzpatrick

Environmental factors such as chemical contamination can have immunomodulatory effects on the immune response of fish and may be contributing to the decline in salmonid populations by augmenting disease susceptibility. Xenobiotics can interfere with the immune system at several levels of complexity, and different immune cells and processes have variable sensitivity to pollutants. For this reason, a suite of tests is required to evaluate immunomodulatory mechanisms.

In this thesis, I formulated and calibrated an assay for the detection of humoral immunity for chinook salmon (*Oncorhynchus tshawytscha*). Subsequently, I used this technique in conjunction with other immune and endocrine assays to detect effects of embryonic exposure to *o,p'*-DDE, a known environmental estrogen. The technique combines exposure of whole animals or leukocyte cultures to immunomodulatory agents/conditions with *in vitro* mitogenic activation of B-lymphocytes. The proportion

of leukocytes undergoing blastogenesis following *in vitro* stimulation with lipopolysaccaride (LPS) was quantified by flow cytometric analysis of forward and side scatter properties. In addition, I used a fluorescein isothiocyanate labeled anti-rainbow trout surface immunoglobin monoclonal antibody (anti-RBT SIgM-FITC) to determine the ability of the lymphoblasts to express surface immunoglobin (SIgM) through flow cytometry.

I used the assay to evaluate the effects of short-term exposures to *o,p'*-DDE during early life history stages on the long-term immune competence of fall chinook salmon. Immersion of chinook salmon eggs in 10 ppm *o,p'*-DDE for 1 h at fertilization followed by 2 h at hatch caused significant reductions in the ability of splenic leukocytes to undergo blastogenesis and express SIgM upon *in vitro* stimulation with LPS one year after treatment (ANOVA, $P<0.05$). The concentration of *o,p'*-DDE in fry treated with 10 ppm *o,p'*-DDE was 0.92 $\mu g\ g^{-1}$ lipid one month post first feeding. The chemical persisted through development and, one year after exposure, levels in juvenile muscle tissue were 0.94 $\mu g\ g^{-1}$ lipid. Mortality rate, time to hatch, fish size, sex ratios, gonadal development, plasma estradiol and 11-ketotestosterone concentrations were not affected by treatment with *o,p'*-DDE. In addition, neither plasma lysozyme concentration, nor mitogenic response of splenic leukocytes to concanavallin A or polyinosinic-polycytidylic acid were influenced by the treatment.

A short period of exposure to an estrogenic chemical during early periods of development induced long term effects on humoral immune competence of chinook salmon. I discuss the possibility that the xenobiotic is exerting its activity through steroid-mediated pathways.

Effects of *o,p'*-DDE on the Immune System of Juvenile Chinook Salmon (*Oncorhynchus tshawytscha*).

by

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CONTRIBUTION OF AUTHORS

Dr. Schreck, Dr. Fitzpatrick and Dr. Leong were involved in the design, analysis and editing of both manuscripts. Dr. Gunderson performed the analysis of samples for *o,p'*-DDE concentrations. Dr. Vella and Dr. Crippen were involved in the development of the flow cytometric assay.

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Effects of *o,p'*-DDE on the Immune System of Juvenile Chinook Salmon (*Oncorhynchus tshawytscha*).

INTRODUCTION

This masters thesis consists of two chapters. Chapter 1, entitled "*In vitro* detection of functional humoral immunocompetence in juvenile chinook salmon (*Oncorhynchus tshawytscha*) using flow cytometry", describes a flow cytometric assay formulated and calibrated for measuring humoral immunocompetence in chinook salmon. The assay was validated with respect to the specificity of a fluorescein isothyanate labeled anti-rainbow trout surface immunoglobin monoclonal antibody for chinook salmon leukocyte surface immunoglobin through a series of steps. Using the monoclonal antibody as a marker, the percentage of surface IgM positive leukocytes was found to be highest in the spleen, followed by the blood, and then the pronephros. The optimal time for detection of *in vitro* mitogenic activation of splenic leukocytes was days 4 through 7 of culture with lipopolysaccharide; therefore 4 days of culture is recommended for this assay. The sensitivity of the assay for detecting steroid-induced immunosuppression was demonstrated following *in vitro* exposure to physiologically relevant stress concentrations of cortisol in conjunction with mitogenic stimulation. This assay will be a useful addition to the array of immunoassays available to fish immunologists.

Chapter 2, entitled "Short term exposure of fall chinook salmon (*Oncoryhnchus tshawytscha*) to *o,p'*-DDE during early life history stages affects long-term competence of the humoral immune system" describes the long-term immunological

effects of exposure of chinook salmon during early life history stages to *o,p*'-DDE. When chinook salmon eggs were exposed to 10 ppm *o,p*'-DDE for one hour immediately post-fertilization followed by two hours at hatch, humoral immuno-competence was compromised one year later. Using the assay described in Chapter 1, the percentage of splenic leukocytes undergoing blastogenesis when activated in vitro with lipopolysaccharide was siginificantly lower than control treated fish. Additionally, of those activated cells, the percentage that were surface IgM positive was siginficantly lower in fish treated with 10 ppm *o,p*'-DDE. The concentration of *o,p*'-DDE in one month old treated fry was found to be 0.92 $\mu g\ g^{-1}$ lipid and persisted in muscle tissue at 0.94 $\mu g\ g^{-1}$ lipid one year after exposure.

CHAPTER 1

IN VITRO DETECTION OF FUNCTIONAL HUMORAL IMMUNOCOMPETENCE IN JUVENILE CHINOOK SALMON (*ONCORHYNCHUS TSHAWYTSCHA*) USING FLOW CYTOMETRY.

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Abstract

Environmental factors may have immunomodulatory effects on the humoral response of chinook salmon (*Oncorhynchus tshawytscha*). In this paper, I formulated an assay for the detection of such effects. This technique combines exposure of whole animals or leukocyte cultures to immunomodulatory agents/conditions with *in vitro* mitogenic activation of B-lymphocytes. I quantified the proportion of leukocytes undergoing blastogenesis following *in vitro* stimulation with lipopolysaccharide (LPS) was quantified by flow cytometric analysis of forward and side scatter properties. In addition, I used a fluorescein isothiocyanate labeled anti-rainbow trout surface immunoglobin monoclonal antibody (anti-RBT SIgM-FITC) to determine the ability of the lymphoblasts to express surface immunoglobin (SIgM) through flow cytometry (FCM).

Through a series of calibration steps, the anti-RBT SIgM-FITC was specific for B-lymphocyte SIgM in chinook salmon. The binding of the anti-RBT SIgM-FITC to chinook salmon SIgM positive leukocytes was effectively blocked with salmon serum and an isotype control was established. B-lymphocytes were partially removed from a population of leukocytes through adherence to a nylon wool column resulting in a consequent reduction in anti-RBT SIgM-FITC binding. Using the anti-RBT SIgM-FITC as a marker, I described the distribution of resting lymphocytes expressing SIgM in lymphoid tissues of juvenile chinook salmon. The mean percentages of SIgM positive cells in the spleen, pronephros and blood were 62.1% (± 2.82), 34.8% (± 1.86), and 56.7% (± 4.7) of all lymphocytes, respectively. In a time-course experiment for optimal *in vitro* activation of leukocytes for this assay, I observed blastogenesis

and up-regulation of SIgM expression of splenic leukocytes through flow cytometry by 4 days post *in vitro* stimulation with LPS that continued through 7 days, but was no longer visible by 10 days post stimulation. Using this assay, I detected reduced expression of SIgM in splenic and pronephric B-lymphocytes following *in vitro* exposure to physiologically relevant stress concentrations of cortisol in conjunction with mitogenic stimulation. This technique is a useful addition to the assays already available in the rapidly growing field of fish immunology.

Introduction

Currently, several assays are available for monitoring the immunocompetence of fish, particularly with respect to non-specific cellular immunity (Anderson, 1996). However there are relatively few assays for measuring lymphoid parameters (Wester *et al.*, 1993). Circulating serum immunoglobin level can be measured in either 'naïve' fish or after exposure to a specified antigen by a single radial immunodiffusion (Fuda *et al.*, 1991) or ELISA (Arkoosh and Kaattari, 1990). Incorporation of tritiated thymidine is a widely used technique for the detection of lymphocyte mitogenesis *in vitro* (Chilmonczyk, 1978). In addition, the hemolytic plaque assay (Jerne and Nordin, 1963) is been increasingly used for the measurement of specific antibody producing cells in studies of fish immunocompetence (Tripp *et al.*, 1987).

The value of flow cytometry (FCM) as a tool for measuring immune function has recently been recognized in fish immunology (Voccia *et al.*, 1994, 1997, Chilmonczyk *et al.*, 1997). A suite of assays has been developed for the assessment of phagocytic activity, oxidative burst and natural cytotoxicity using FCM (Chilmonczyk and Monge, 1999). Moritomo *et al.*, (1999) developed a new method for the detection of granulocytes using fluorescence microscopy that they have subsequently adapted to a FCM technique (Moritomo, pers. Comm.). Recently, Crippen *et al.*, (submitted) described the flow cytometric analysis of cell cycle progression following mitogenic stimulation, through propidium iodide labelled DNA of individual cells in salmonids. In addition, monoclonal antibodies (mAbs) have been used to describe surface immunoglobin positive (SIg^+) cell populations through FCM for resting blood cells in white sturgeon (*Acipenser transmontanus)* (Adkison *et al.*, 1996). Seigle *et al.*, (1998)

also used FCM to characterize the SIg^+ cell populations in resting pronephric and spleen cells from rainbow trout (*Oncorhynchus mykiss*). Furthermore, the influence of a stimulatory drug flumequine on *in vivo* SIg^+ mitogenic responses was evaluated by FCM in European eel (*Anguilla anguilla*) blood (van der Heijden *et al.*, 1995).

There are many advantages to the use of FCM in fish immunology. The techniques are very sensitive because they allow the analysis of tens of thousands of cells from each sample. In addition, many samples per day can be processed and assayed using FCM without compromising accuracy. Moreover, eliminating production of radioactive waste is clearly a financial and environmental benefit.

The objective of this paper is to describe and calibrate a method for measuring functional humoral immunocompetence in salmonids that can complement currently available techniques. The approach we have taken is to use FCM to measure B-lymphocyte cell surface immunoglobin M (SIgM) expression following activation by mitogen in tissue culture. *In vitro* stimulation of small resting leukocytes with a B lymphocyte-specific mitogen, lipopolysaccharide (LPS) (Manning and Nakanishi, 1996), results in the activation and consequent increase in cell size. The chromatin in the nuclei becomes less dense, the volume of cytoplasm increases and new RNA and protein synthesis are induced as the nuclei undergo blastogenesis. At this stage the activated cells are called lymphoblasts (Janeway and Travers, 1997). The proportion of lymphoblasts within a leukocyte population that has been stimulated by mitogen can be quantified by analysis of flow cytometric dot plots of forward and side scatter (FSC and SSC). FSC represents cell volume and SSC represents cell granularity. Additionally, when activated, lymphocytes proliferate and the expression of SIgM

may be upregulated. Our approach is to use FCM to quantify the proportion of activated lymphocytes expressing SIgM by staining the cells with a fluorescein conjugated mAb against rainbow trout surface immunoglobin (anti-RBT SIgM-FITC) (DeLuca *et al.*, 1983). The goal of this paper is to formulate and calibrate a FCM approach that provides a useful tool for the measurement of functional humoral immunocompetence in salmonids.

Methods

FISH

Juvenile parr chinook salmon (*O. tshawytscha)* (6 to 12 months old) were raised at Oregon State University's Fish Performance and Genetics Laboratory at Smith Farm, Corvallis, Oregon at densities allowing good growth in 2 m circular tanks at 12.5° C ± 0.5 ° C flow-through well water and natural photoperiod. Fish were fed a commercial diet of Semi-moist Pellets (BioOregon^{TM}) daily.

TISSUE PREPARATION

For each experiment, fish were netted from the holding tanks and immediately immersed in a lethal dose (200 mg l^{-1}) of buffered tricaine methane-sulfonate (MS-222). Blood, spleen or pronephros were taken when required.

When a blood sample was required, blood was drawn from the caudal peduncle with a heparinized vacutainer and 20 gauge needle and placed on ice. When a blood sample was not required for analysis, blood was drained from the fish by severing the

caudal peduncle to minimize erythrocytes in the spleen and pronephric tissue. Fish were held on ice until dissection of spleen and pronephric tissue.

Red blood cells (RBCs) were removed from the blood samples by preferential lysing with distilled water according to the method described by Crippen *et al.*, (in press). Briefly, the osmotic potential of the blood sample was altered by the addition of distilled water for 20 s. The blood sample was immediately returned to isotonic with 10 x phosphate buffered saline (PBS), RBC debris was removed and the blood leukocytes were washed with isolation media. Leukocytes were resuspended in 500 µl RPMI (Becton Dickinson).

For calibration experiments not involving tissue culture, the spleen was removed and immediately placed in 500 µl of ice cold RPMI. The pronephros was then removed and placed in 500 µl of ice cold RPMI. A single cell suspension of splenic leukocytes (containing a trivial number of erythrocytes) was obtained by repeated aspiration with a 1 ml syringe (without needle). Gross debris was allowed to settle out, cell suspensions were drawn off and the connective tissue fragments discarded. This procedure was repeated for pronephric leukocytes. Cell suspensions were held on ice.

Preparation of spleen and pronephric leukocytes for experiments involving tissue culture was carried out in the same way as described above, with the exception that prior to tissue dissection the fish were cleaned with 70% ethanol and the harvesting of the spleen and pronephros were carried out aseptically into sterile, ice cold tissue culture media [TCM: Minimum Essential Media (MEM) supplemented with 5% fetal calf serum (FCS; Invitrogen, Carlsbad, CA), antibiotics (Sigma), sodium bicarbonate (Sigma), essential amino acids, nonessential amino acids and sodium pyruvate (Gibco,

Grand Island, NY)]. Cells were counted for viability using the trypan blue exclusion test and cell suspensions were adjusted to 2 x 10^7 cells ml^{-1} with sterile TCM.

MONOCLONAL ANTIBODY

Mouse hybridomas positive for rainbow trout cell surface IgM (anti-RBT SIgM) were received as a generous gift from Gregory Warr (DeLuca *et al.*, 1983). IgG1 mAbs from these hybridomas were secreted in cell culture at 37° C. Supernatants containing secreted mAb were collected from hybridoma cell culture over a period of about 2 months. Hybridoma cells were removed from supernatants containing the secreted mAb by centrifugation for 15 min at 1400 g. Supernatants were frozen at –20° C until purification. After thawing, supernatants were sterile filtered at 0.2 µm. The mAb was purified from the supernatant by protein G (*Staphylococcus aureus*) affinity chromatography as follows; approximately 1 to 2 ml of protein G was added to a column and the column was wetted with about 50 ml of PBS. The hybridoma supernatant was passed through the column behind the PBS two times at 4° C. Ten ml of 0.1 M glycine (pH 2.8) was added to elute the mAb. The elution media was collected in a series of tubes on ice. The protein concentration of each elution was determined by measuring the optical density at 280 nm. The pH was adjusted to pH 7 with 1M Tris (pH 10) and the mAb was then dialyzed twice for 12 h each time against 4 l PBS at 4° C. After dialysis, the mAb was concentrated with a Centricon Protein concentrator (Centriprep –50 Centrifugal Concentrator 50,000 MW cutoff). Protein concentrations between 5 and 10 mg ml^{-1} were considered acceptable.

The mAb was conjugated to fluorescein isothiocyanate; Isomer I: on celite; 10% FITC (FITC) (Sigma) at a concentration of 1 mg FITC per 1 mg mAb protein. Prior to conjugation, the mAb was dialyzed twice for 12 h against 4 l 0.1 M $NaHCO_3$ (adjusted to pH 9.4 – 9.6 with 0.1 M Na_2CO_3) at 4° C. The mAb was removed from the dialysis tubing and mixed slowly with FITC in a beaker using a stir bar for 30 min, in the dark at room temperature. Excess FITC was removed by centrifugation for 15 min at 1400 g. Any remaining unbound FITC was removed by 3 overnight dialyses against 4 l PBS in the dark at 4° C.

Appropriate anti-RBT SIgM-FITC titer for the assay was determined by FCM analysis of mean channel fluorescence (MCF) and FSC over a serial dilution of α-RBT SIgM-FITC stained splenic and pronephric cell suspensions. The mAb concentration that gave a peak MCF of approximately 10^2 above the negative peak was used in subsequent analyses. We used 10 µl of anti-RBT SIgM-FITC at protein concentration of ~0.5 mg ml^{-1} for our assays.

GENERAL METHOD FOR CELL STAINING WITH ANTI-RBT SIgM-FITC

At least 1 x 10^5 cells of each leukocyte cell suspension were transferred in duplicate into wells of a 96 well plate held on ice (for naïve cell suspensions prepared from fish over 50 g, a volume of 20 µl was sufficient; for cell suspensions that had been in tissue culture the volume transferred was increased to at least 40 µl). The plate was centrifuged at 700 g for 5 min at 4° C. The RPMI/TCM supernatant was discarded (by inverting the plate over a sink with a sharp snap of the hand) and the cells were resuspended in 80 µl of balanced salt solution (BSS) containing 3% FCS and 0.1%

sodium azide. An excess (with respect to anti-RBT SIgM-FITC) of mouse IgG was added to each well (we used 10 µl of 5 mg ml^{-1} mouse IgG in BSS wash buffer) to bind non-specific binding sites. Ten µl of anti-RBT SIgM-FITC at protein concentration of ~ 0.5 mg ml^{-1} were added to one of the two wells. Cells were immediately incubated on ice for 30 min in the dark. For each sample, as a negative control, the duplicate wells were incubated in the absence of anti-RBT SIgM-FITC.

Following incubation, cells were washed twice (200 µl BSS added to each well, plate centrifuged for 5 min at 700 g and 4° C, supernatant discarded, plate vortexed for 1 s and cells immediately resuspended in 200 µl BSS). Following the final wash, cells were resuspended in 200 µl of BSS and transferred to fluorescence-activated cell sorting (FACS) microtubes (Becton Dickinson) containing a further 200 µl of BSS wash buffer. Flow cytometric analyses was either conducted immediately, or cells were fixed for future FCM analysis.

FIXING CELLS FOR FUTURE FLOW CYTOMETRIC ANALYSIS

When time constraints necessitated, cells were fixed in formaldehyde and analysed by FCM up to 4 days after staining with anti-RBT SIgM-FITC. A 2% formaldehyde fixing solution was prepared fresh daily from 10% formaldehyde (methanol free, ultrapure EM grade) (Polysciences Inc.) in BSS. Instead of resuspending the cells in 200 µl in the penultimate step of the staining procedure, cells were resuspended in 200 µl of 2% formaldehyde fixing solution and incubated on ice in the dark for 15 min. Following fixing, the cells were washed twice in BSS as described above and then

resuspended in 200 µl BSS and transferred to FACS microtubes containing 200 µl BSS. FACS microtubes were sealed in a box with parafilm and stored for up to 4 days in the dark at 4° C until FCM analysis. Preliminary studies showed that results were not compromised if the samples were run on the FCM within this time frame.

FLOW CYTOMETRIC ANALYSIS AND GATING

Resting cells

Flow cytometric analysis was carried out using a Becton Dickinson FACSCaliber ™ flow cytometer with Argon-ion laser at 480 nm using Cellquest ™ software. Resting lymphocytes were characterized from dot plots of their forward (FSC) and side scatter (SSC) properties. These populations were gated (Fig 1.1a) and the percent of SIgM positive ($SIgM^+$) cells within the lymphocyte populations from each tissue was assessed from histogram plots of fluorescence intensity and cell counts using "Histogram Stats" (Fig 1.1b).

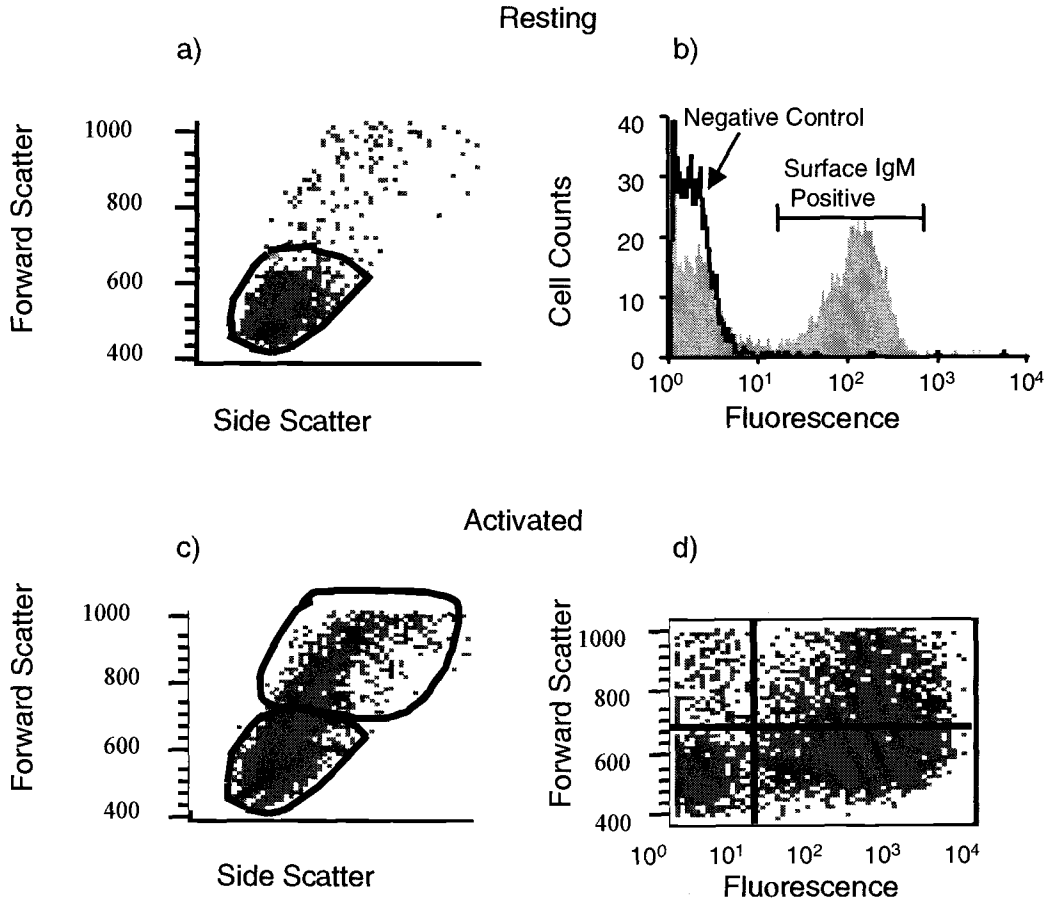

Figure 1.1 Dot plot display of forward and side scatter (FSC and SSC) from resting leukocytes stained with anti-rainbow trout surface immunoglobin monoclonal antibody conjugated to fluorescein isothiocyanate. Resting leukocytes were gated (a) and the % SIgM positive cells were quantified from the corresponding histogram of cell counts and fluorescence intensity (FL1) under 'histogram stats' (b). Unstained leukocytes were used as a negative control. Leukocytes activated with LPS have higher FSC and SSC properties. From the dot plot, the % activated (blasting) cells of the viable cell population were quantified under 'gate stats' (c). When stained with anti-RBT SIgM-FITC, the % SIgM positive blasting cells were quantified under the upper right-hand quadrant of the FSC and FL1 cytogram using 'quadrant stats'(d).

Activated cells

For the experiments involving activated cells, the percent of cells within the population that were undergoing blastogenesis was determined from dot plots of FSC and SSC. Cells with FSC and SSC characteristics of large cells were gated and cells within this gate were calculated as a % of all viable cells using "Gate Stats" (Fig 1.1c). Further analysis of "Quadrant Stats" were then used to analyze the percentage of blasting cells expressing SIgM. Cells falling in the upper right-hand quadrant of a FSC against fluorescence intensity plot (large cells with high fluorescence intensity) were calculated as a % of all viable cells (Fig 1.1d).

ASSAY CALIBRATION EXPERIMENTS

The specificity of anti-RBT SIgM-FITC for chinook salmon IgM was established in the following ways:

Inhibition of α-RBT SIgM-FITC binding

Leukocytes of the pronephros, spleen and blood were incubated for 30 min with α-RBT SIgM-FITC in the presence of an excess (20 µl) of either salmon serum, trout serum, mouse serum or in the absence of serum. Following the incubation, the serum and unbound anti-RBT SIgM-FITC was removed from the leukocyte suspensions by two washes with BSS. The binding of anti-RBT SIgM-FITC to leukocytes surface versus to proteins in the serum was evaluated by the presence or absence of a high fluorescence intensity positive peak using FCM analysis of the leukocyte suspensions.

Isotype control

An isotype control was established using mouse IgG1 anti-KLH-FITC. Cells of the pronephros, spleen and blood were incubated with either anti-RBT SIgM-FITC or IgG1 anti-KLH-FITC. A comparison of the positive staining of the two antibodies to the salmon leukocytes was made by FCM.

B-lymphocyte specificity of the Monoclonal Antibody

To ascertain if B-lymphocytes were in fact the population of leukocytes that were staining positive for SIgM, splenic or pronephric leukocyte cell suspensions were pooled from several fish and were passed through a nylon wool column to remove B-lymphocytes from the cell populations. The effluent cell suspensions, which should have been more T cell rich, were then compared to cell suspensions that had not been passed through a nylon column for the percentage of $SIgM^+$ cells within each population using FCM.

The method employed was modified from mammalian protocol for the removal of B-lymphocytes as described by Julius *et al.* (1973) whereby a population of leukocytes is passed through a nylon wool column, B-lymphocytes adhere to the nylon wool and other leukocytes flow through the column. The columns comprised 3cc syringe barrels with a 20 Gauge needle, packed with 0.12 g of nylon wool. Two ml of warm RPMI (~37° C) were drawn through the column by vacuum and 500 µl of the cell suspension were allowed to drain through to the head of the column. A further 500 µl aliquot of RPMI was then added to the column. The nylon columns were wrapped in foil, the needles were placed in Styrofoam blocks to prevent further

elution and incubated for 2 x 30 min at 37° C. To check for the effect of incubation temperature on efficacy of removal, the procedure was repeated with columns incubated at 17° C (a temperature that is more physiologically relevant for fish cells) for an extended period of 2 x 2.5 h. Between the two incubations, cell suspensions were eluted down the column until the RPMI was at the head of the column. Following the final incubation, the columns were eluted with 4 ml of RPMI. The effluent was washed and resuspended in RPMI then counted for cell viability using the trypan blue exclusion test. The cells in the effluent were stained with α-RBT SIgM-FITC and compared by FCM to cell suspensions which had not been passed through the column for the percentage reduction in cells staining positive for SIgM.

DISTRIBUTION OF $SIgM^+$ CELLS IN LYMPHOID TISSUES OF CHINOOK SALMON

To determine the distribution of resting B-lymphocytes between individual lymphoid tissues of juvenile chinook salmon, single cell suspensions were prepared from leukocytes of the spleen, pronephros and blood of three individual fish. The leukocyte suspensions were stained with anti-RBT SIgM-FITC in duplicate. FCM analysis was used to compare the percentage of $SIgM^+$ cells in each tissue.

TIME-COURSE FOR ACTIVATION

To determine the optimum time for *in vitro* activation of chinook salmon leukocytes with LPS, and to compare the mitogenic response of splenic versus pronephric leukocytes for this assay, cell suspensions from both the pronephros and

the spleen of three individual fish were cultured in either the presence of LPS at a final concentration of 100 μg ml^{-1} or TCM alone. A 0.2 mg ml^{-1} solution of *E. coli* Lipopolysaccharide 055:B5 (LPS) (Sigma) was prepared aseptically in TCM. One hundred μl of this solution was added to 100 μl of 2 x 10^7 viable cells ml^{-1} in a 96 well tissue culture plate (final mitogen concentration of 100 μg ml^{-1} cultured with 2 x 10^6 cells per well). As a control, 2 x 10^6 cells per well were also cultured in 200 μl TCM for each cell preparation. Three tissue culture plates were prepared in this way. The plates were placed in a sealed incubation chamber containing blood gas (10% O_2, 10% CO_2 and 80% N_2) and incubated at 17° C for 4 days, 7 days or 10 days. Pre (Day 0) and post (days 4, 7 and 10) activation, cells were stained with anti-RBT SIgM-FITC and analysed by FCM for blastogenesis and SIgM expression. This experiment was replicated 3 times.

IN VITRO ACTIVATION IN THE PRESENCE OF CORTISOL

To assess the sensitivity of the assay for detecting immunosuppression, leukocyte cell suspensions from both the pronephros and the spleen of three individual fish (mean weight 123 g) were cultured in 96 well tissue culture plates in the presence of; trinitrophenylated-lipopolysaccharide (TNP-LPS) (prepared using *E. coli* Lipopolysaccharide 055:B5 (LPS) as described by Jacobs and Morrison (1975); TNP-LPS plus 100 ng ml^{-1} cortisol; or in TCM alone. Cortisol (hydrocortisone) (Sigma) was prepared from a 2 ng ml^{-1} stock in 100% ethanol. The stock solution was evaporated using a speed vac and reconstituted in TNP-LPS to a concentration of

0.2 ng ml^{-1}. The solution was sterile filtered and 100 µl was added to 100 µl of leukocyte cell suspension at 2 x 10^7 cells ml^{-1}. The cells were placed in a sealed incubation chamber containing blood gas (10% O_2, 10% CO_2 and 80% N_2) and incubated at 17° C for 4 days. On day 4 of tissue culture, cells were stained with anti-RBT SIgM-FITC and analyzed by FCM for blastogenesis and SIgM expression.

STATISTICAL ANALYSIS

Analysis of variance (ANOVA) was used to determine difference between replicates for the time-course experiment. Because variances were not homogenous, we used Kruskal-Wallace non-parametric test with Dunn's post-hoc test. Where differences were not detected, we pooled replicates. The Mann-Whitney U test was applied to detect differences between non-activated and activated cells for % lymphoblasts and % SIgM expression. In addition, the Mann-Whitney U test was applied to detect differences between activated and cortisol suppressed treatments.

Results

ASSAY CALIBRATION EXPERIMENTS

Inhibition of anti-RBT SIgM-FITC binding

The absence of a positive peak indicates that anti-RBT SIgM-FITC bound to immunoglobins in both the trout and salmon serum and was subsequently removed

in the wash step (Figure 1.2a). However, because anti-RBT SIgM-FITC did not bind to proteins in the mouse serum it was able to bind to chinook leukocytes as evidenced by the positive peak for SIgM at high fluorescence intensity (Figure 1.2b).

Isotype control

No positive peak was evident when either splenic (Figure 1.2c) or pronephric leukocytes were incubated with IgG1 anti-KLH-FITC compared to that given by incubation of splenic (Figure 1.2d) or pronephric leukocytes with IgG1 anti-RBT SIgM-FITC.

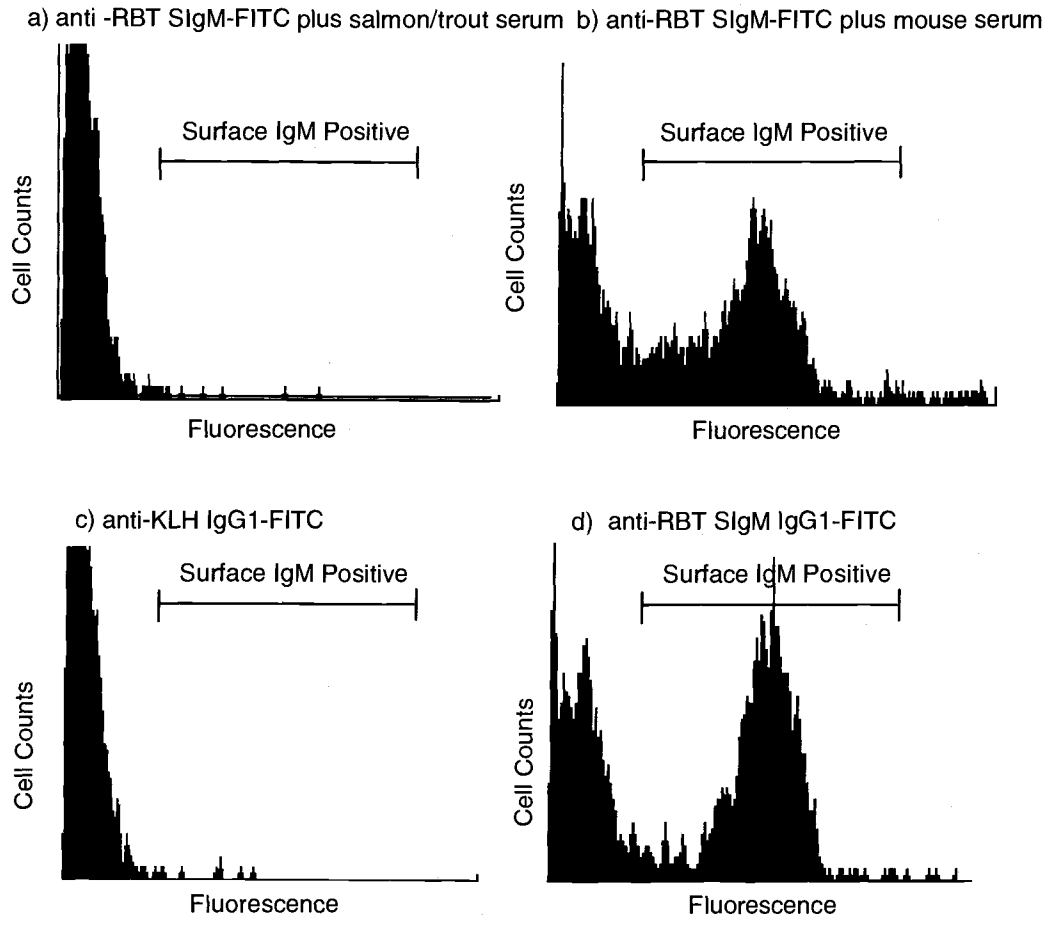

Figure 1.2. Fluorescence histogram showing absence of positive peak for leukocytes incubated with anti-RBT SIgM-FITC in the presence of an excess of salmon or trout serum (a). Leukocytes incubated with anti-RBT SIgM-FITC in the presence of mouse serum stained positively (b). Histogram showing absence of a positive peak when leukocytes were incubated with a FITC conjugated isotype control antibody specific for KLH (c). Leukocytes incubated with anti-RBT SIgM-FITC under the same conditions stained positively (d). Plots are representative of chinook salmon splenic leukocyte populations.

B-lymphocyte specificity of the Monoclonal Antibody

Viable splenic and pronephric cell suspensions that had been passed through the nylon wool column at 37° C showed almost a 50% reduction in positive staining for SIgM when compared to cell suspensions that had not been passed through the column (Figure 1.3). Splenic and pronephric cells that were passed through a nylon column at the more physiologically relevant temperature of 17° C were also lower in $SIgM^+$ cells (37% and 36% respectively). Optimal removal of B-lymphocytes at this lower temperature may require a longer incubation period, however this was not pursued in the present study. The cell viability was higher at 17° C than at 37° C.

DISTRIBUTION OF $SIgM^+$ CELLS IN LYMPHOID TISSUES OF CHINOOK SALMON

Analysis of forward and side scatter patterns of chinook salmon resting blood leukocytes indicated three populations of cells. The population gated I (Figure 1.4a) indicates one primary population of cells with relatively homogenous cell volume and granularity properties of 400 to 550 and 150 to 250 respectively (under the FCM instrument settings used). Further inspection of fluorescence properties of cells under

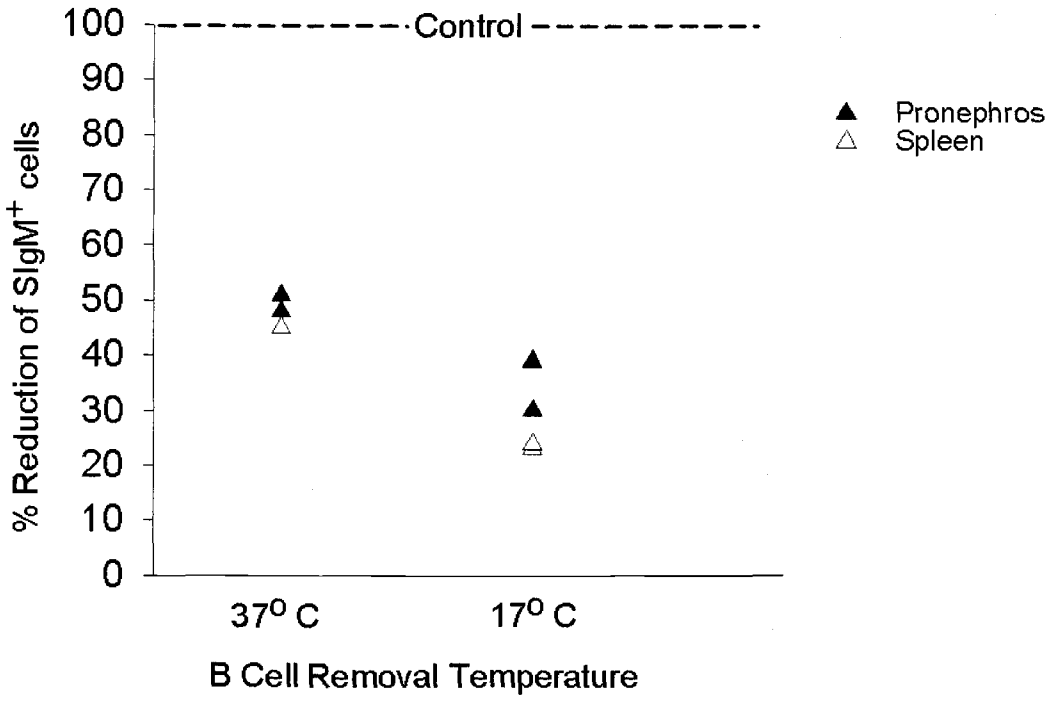

Figure 1.3. The percent reduction in SIgM positive stained cells within leukocyte populations following B cell removal. Leukocyte cell suspensions were passed over a nylon wool column and incubated for either 2 x 2.5 h at 17 °C or for 2 x 0.5 h at 37°C to allow B lymphocytes to adhere to the nylon wool. The non-adherent cells were eluted and the percent cells staining positively for SIgM in the eluate was compared to the percent in the original cell suspension (Control). Each triangle represents a pooled sample of leukocytes from several fish.

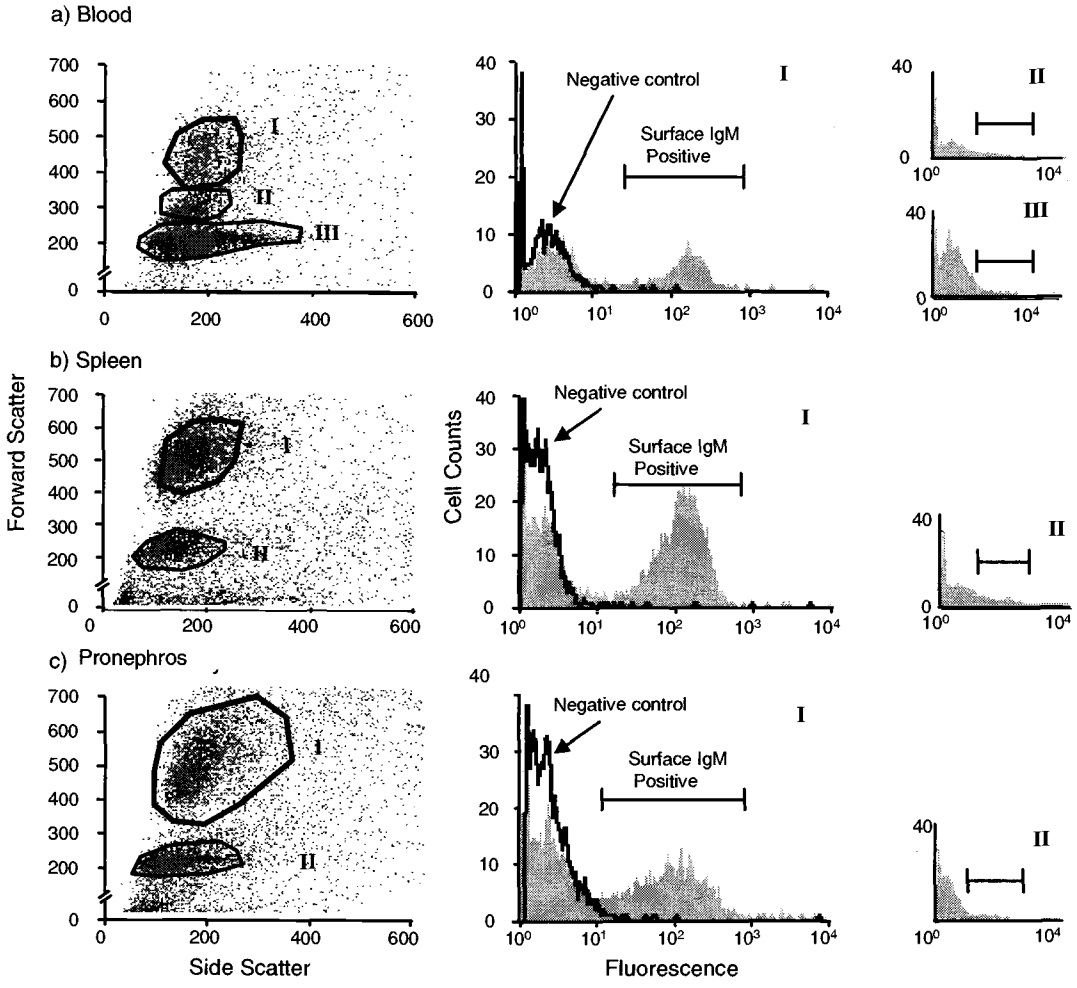

Figure 1.4. Representative FSC and SSC dot plots and corresponding FL1 histograms for resting (a) blood, (b) splenic and (c) pronephric leukocytes stained with anti-RBT SIgM-FITC. Negative controls represent auto-fluorescence of unstained cells. Of the 3 cell populations evident in blood, population I shows positive staining for SIgM. Similarly in spleen and pronephros, only population I shows positive staining for SIgM.

Table 1.1 Percent SIgM positive cells in resting leukocytes from lymphoid tissues of chinook salmon.

Fish	Blood		Spleen		Pronephros	
	1[a]	2[a]	1[a]	2[a]	1[a]	2[a]
1	58.17	55.29	59.95	60.96	38.51	37.19
2	63.96	58.01	58.62	56.24	32.51	39.88
3	47.88	41.19	67.66	70.31	33.49	37.29
Overall Mean	54.08		62.29		36.48	
Overall SE	± 4.831		± 2.613		± 1.295	

[a] For each fish, duplicate leukocyte samples (1 & 2) were run in the assay.

gate I, following staining with anti-RBT SIgM-FITC, showed a positive peak for SIgM cells suggesting that this population is representative of lymphocytes. SIgM positive cells were not found in populations II or III for any of the tissues. Resting splenic leukocytes also indicated a homogeneous population of lymphocytes with respect to FSC and SSC with similar properties to those of blood (Figure 1.4b). Leukocytes of the pronephros were more heterogeneous in nature although a primary population of lymphocytes was evident (Figure 1.4c). The percent of the total lymphocyte population gated in I that were $SIgM^+$ was determined using histogram statistics from a plot of fluorescence intensity against cell count. The percent $SIgM^+$ cells within blood, spleen and pronephros were 62.1% (± 2.82), 34.8% (± 1.86), and 56.7% (± 4.7) (Table 1.1).

TIME-COURSE FOR ACTIVATION

Spleen

There were no significant differences between the three replicate experiments for percent lymphoblasts or percent $SIgM^+$ lymphoblasts within any particular day of incubation (Kruskal Wallace $P > 0.05$). Therefore, data from the same incubation times from the three culture experiments were pooled. There were, however, significant differences in the percent lymphoblasts or percent $SIgM^+$ lymphoblasts in 'naïve' cells prior to incubation (day 0). The percent lymphoblasts after culture with LPS was significantly increased over cells incubated with media alone after 4 days (Mann Whitney U test; $P < 0.0001$) (Figure 1.5a). The increase remained significant

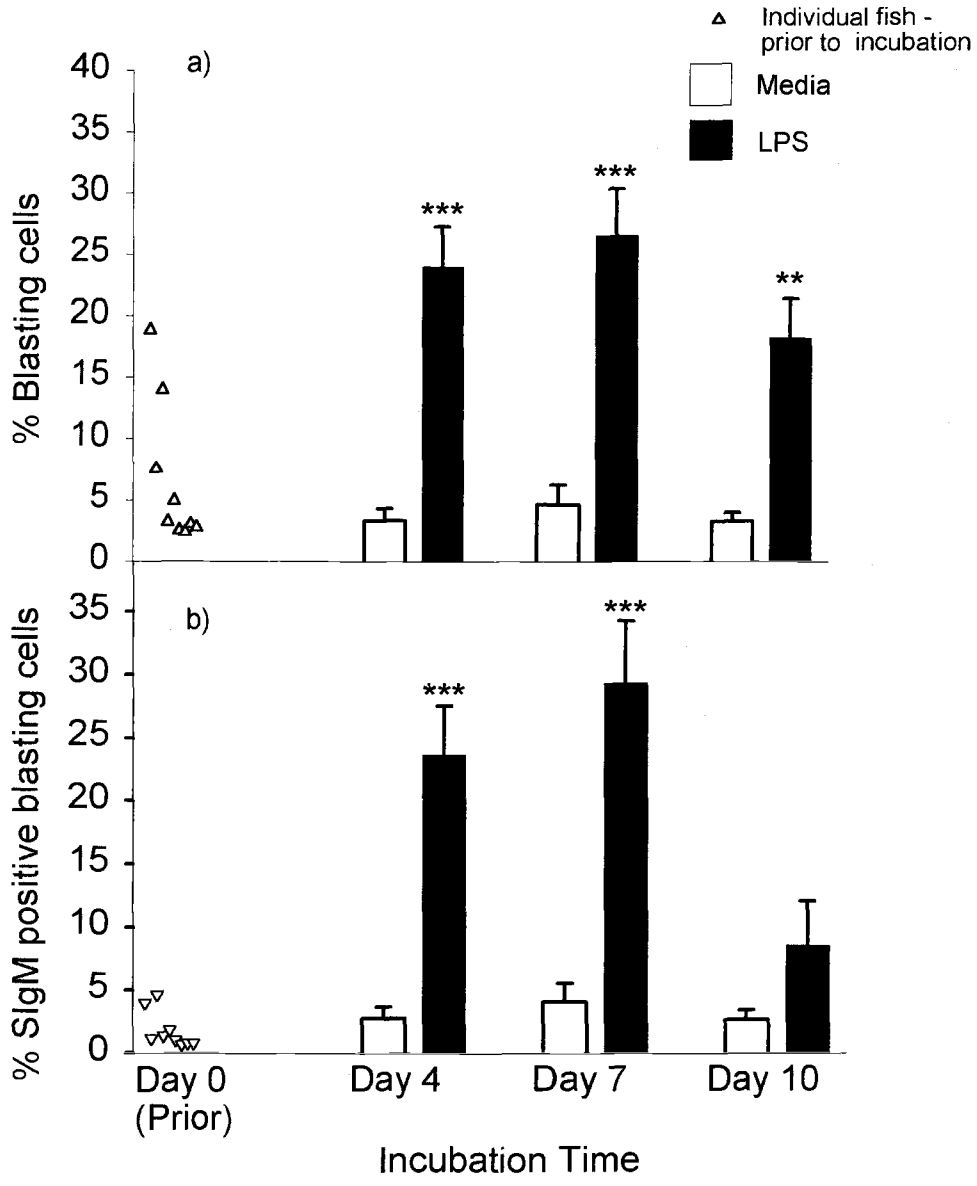

Figure 1.5. Time-course for in vitro activation of splenic leukocytes when cultured with LPS or media alone for either 4, 7 or 10 days. Activation is quantified as percent blasting cells in the population (a) and percent SIgM positive blasting cells in the population (b). Triangles represent resting levels for individual fish prior to tissue culture. For each bar, n = 9 fish. Significant differences between media and LPS treatments are denoted *** ($P < 0.0001$) or ** ($P < 0.001$) or * ($P<0.01$).

by day 7 ($P < 0.0001$) and although to a lesser extent, was still significantly higher than TCM controls by day 10 ($P < 0.0035$).

The percent $SIgM^+$ bearing blasting cells was significantly higher than TCM controls after 4 days ($P < 0.0001$) and remained so by day 7 ($P < 0.0001$); however, the cells no longer expressed elevated levels of SIgM by day 10 ($P = 0.1588$) (Figure 1.5b). Cell viability did not differ between media and LPS treatments.

Pronephros

There were no significant differences between the three replicate experiments for percent lymphoblasts or percent $SIgM^+$ lymphoblasts for day 4 and day 7 of culture (Kruskal Wallace; $P > 0.05$). However, because cells died in culture by day 10 (probably due to contamination) in the last replicate experiment, there were significant differences between replicates on day (Kruskal Wallace; $P = 0.01$). Replicate experiments could therefore not be pooled. In all three individual experiments, the percent blasting cells after culture with LPS increased over cells incubated with media alone by day 7 (Mann Whitney U test; $P = 0.05$), but in only one of the experiments was it significantly increased by day 4 (Figure 1.6a). There were no significant increases in percent lymphoblasts by day 10 in any of the three time-course experiments using the pronephros. The percent of $SIgM^+$ bearing blasting cells was significantly higher than TCM controls by day 4 and remained so by day 7 in each individual experiment (Mann Whitney U test; $P = 0.05$); however, the cells no longer expressed significantly elevated levels of SIgM by day 10 (Figure 1.6b). Cell viability was not significantly different in between the media and LPS treatments.

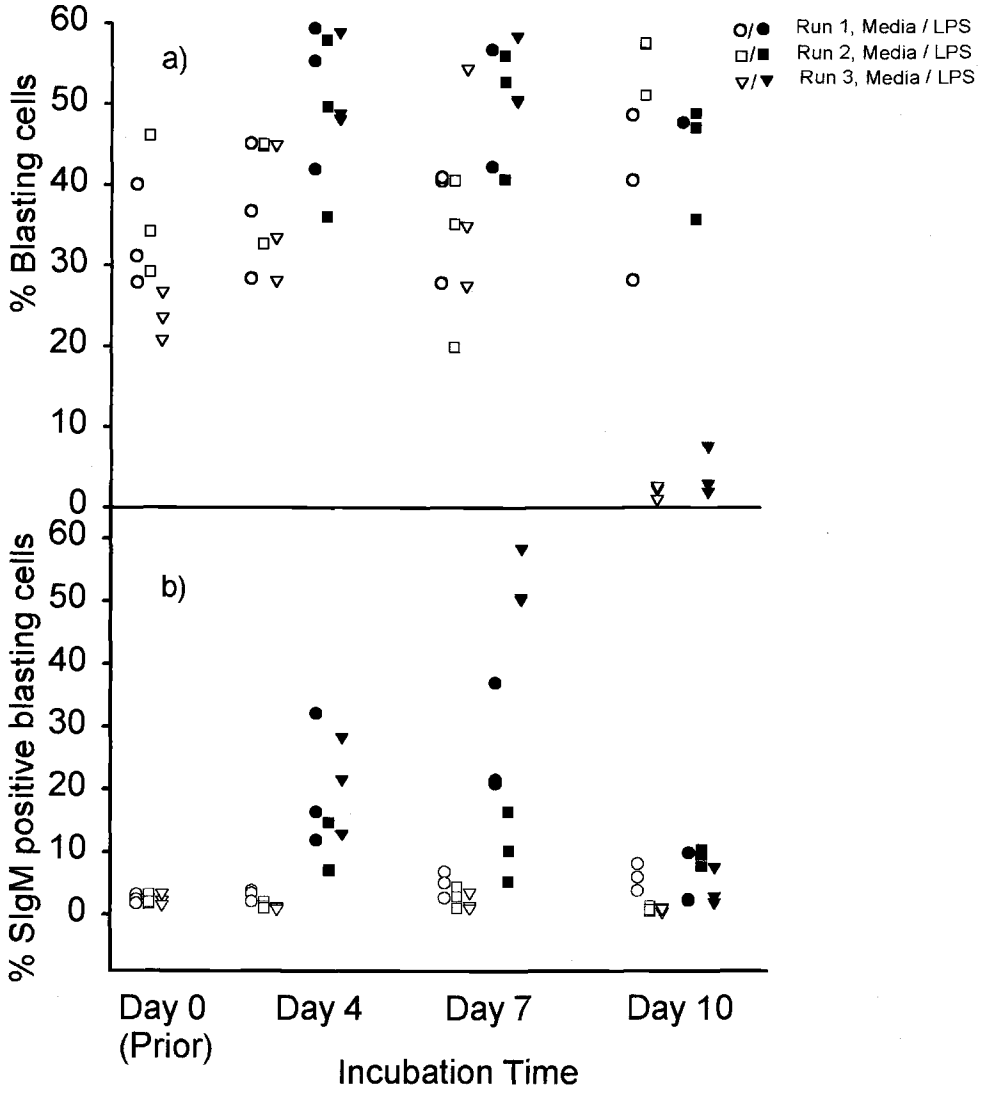

Figure 1.6. Time-course for *in vitro* activation of pronephric leukocytes when cultured with LPS or media alone for either 4, 7 or 10 days. Different symbols represent levels for individual fish from 3 separate experiments (runs). Open symbols represent incubation woth media alone; closed symbols represent incubation with LPS. Activation is quantified as percent blasting cells in the population (a) and percent SIgM positive blasting cells in the population (b).

IN-VITRO ACTIVATION IN THE PRESENCE OF CORTISOL

Spleen

The percent lymphoblasts in the TNP-LPS activated cells was significantly higher than in cells cultured in media alone (Mann-Whitney U-test; $P = 0.05$) (Figure 1.7). In the presence of 100 ng ml^{-1} cortisol, the mean number of cells undergoing blastogenesis, although lower, was not significantly different than the mean for cells activated with TNP-LPS alone.

The mean percent $SIgM^+$ lymphoblasts was significantly increased to > 30 % of all viable cells after activation with TNP-LPS (Mann-Whitney U-test; $P = 0.05$). In the presence of 100 ng ml^{-1} cortisol, this TNP-LPS-induced increase was significantly reduced by over 50% (Mann-Whitney U test; $P = 0.05$).

Pronephros

The percent lymphoblasts in the TNP-LPS activated cells was not significantly higher than in cells cultured in media alone. Nor was blastogenesis inhibited in the presence of cortisol. However, the mean percent $SIgM^+$ lymphoblasts was significantly increased to 15% of the viable cell population after activation with TNP-LPS. Furthermore, in the presence of 100 ng ml^{-1} cortisol this TNP-LPS-induced increase was significantly reduced by over 60% (Mann-Whitney U test; $P = 0.05$). (Data not shown).

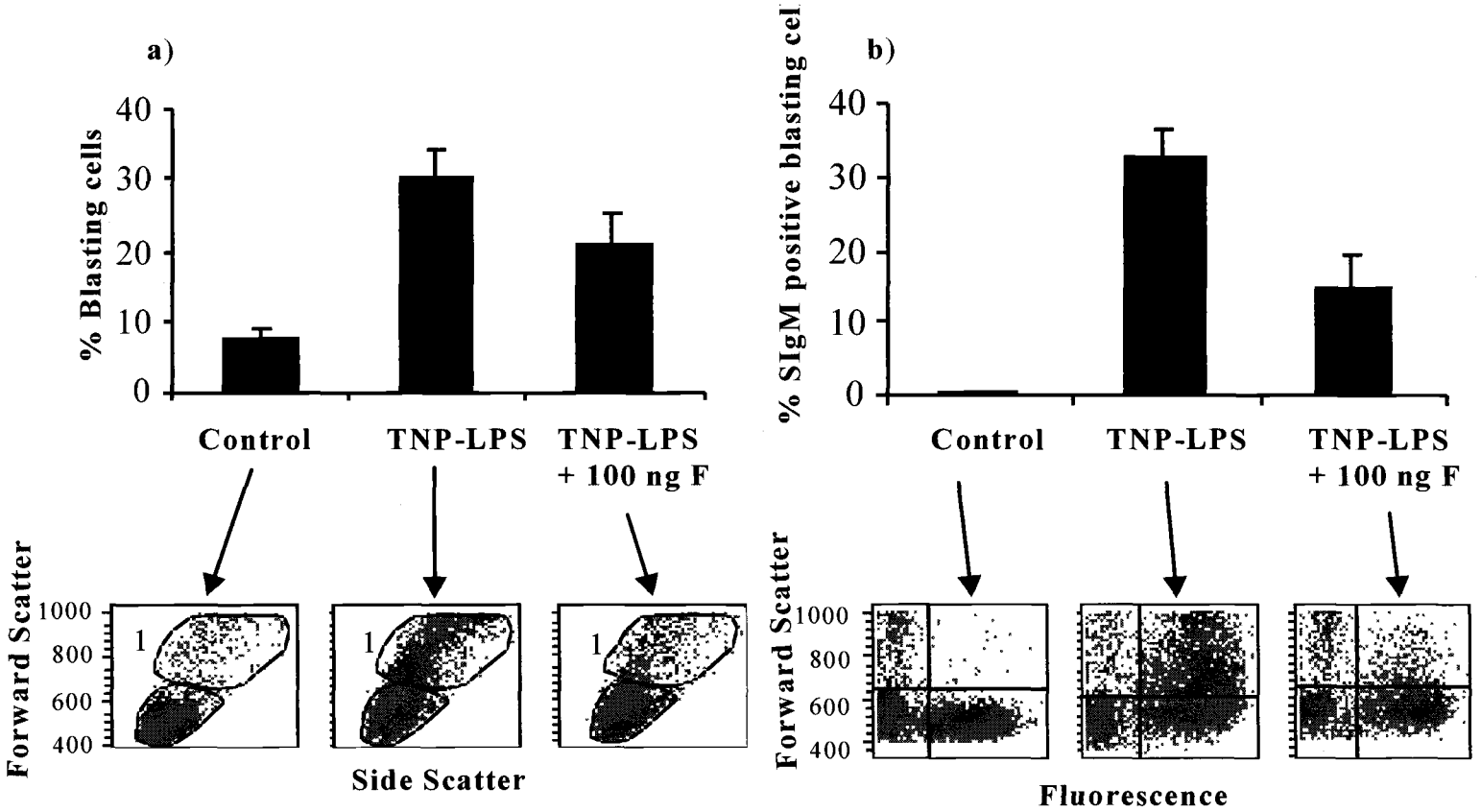

Figure 1.7. Effects of *in vitro* exposure of chinook salmon splenic lymphocytes to mitogen (TNP-LPS) and cortisol (F). (a) Cell activation is quantified as the mean (± SE) percentage of blasting cells within the viable cell population, as shown within gate 1 of the FSC and SSC cytograms. The number of these blasting cells that bound α-RBT SIgM-FITC is shown as the mean (± SE) percentage of all cells (b), n=3 fish.

Discussion

We described and calibrated an approach for measuring humoral immunity in chinook salmon. When chinook salmon leukocytes were incubated with anti-RBT IgM-FITC and analyzed by flow cytometry, we observed a positive peak of high fluorescence intensity. To ascertain that the positive peak represented the specific binding of the mAb to chinook salmon B lymphocyte surface immunoglobin, we conducted a series of validation steps. When chinook salmon leukocytes were incubated with the anti-RBT IgM-FITC in the presence of an excess of salmon or trout serum, then washed, no positive peak of fluorescence was observed. The mAb preferentially bound immunoglobins in the fish serum over the surface immunoglobin and was subsequently removed in the wash steps. However, the mAb did not bind to immunoglobins in the mouse serum, indicating specificity of the mAb to salmonid immunoglobins. In addition, a FITC-conjugated mAb of the same isotype as the anti-RBT IgM-FITC but specific for KLH did not bind to leukocytes of chinook salmon. This indicates that the positive peak observed was not simply an artifact of the fluorescence-conjugated mAb, but due to the anti-RBT IgM-FITC specifically binding to fish leukocytes. To provide evidence that B lymphocytes were the cell type to which the anti-RBT IgM-FITC was binding, the percentage of B-lymphocytes in the population was reduced by an established method (Julius *et al.*, 1973); the result was a consequent reduction in bound anti-RBT IgM-FITC. Taken together, these results indicate that the positive peak observed by flow cytometry when anti-RBT IgM-FITC is incubated with chinook salmon leukocytes is representative of the mAb binding specifically to B-lymphocyte surface immunoglobin.

We described the distribution of B-lymphocytes within the lymphoid tissues of chinook salmon using the anti-RBT IgM-FITC and flow cytometry. The spleen has the highest percentage of B lymphocytes, followed by the blood, then the pronephros. In teleosts, the major site of differentiation and proliferation of B-lymphocytes is the pronephros and is phylogenetically related to the bone marrow of vetebrates (Ellis and de Sousa, 1974; Zappata, 1979a, 1981b; Press *et al.*, 1994). However, because the pronephros is the hematopoietic center, it is not surprising that there are a relatively lower proportion of B-lymphocytes in this tissue, since all other cellular elements of the blood are also represented here. In the acquired immune response, antigens are recognized by the fish's immune system and are transported via the blood to the pronephros and spleen where they are presented to B-lymphocytes and T-lymphocytes. The high percentage of B lymphocytes in the spleen indicates this tissue plays an important role in the humoral immune response of chinook salmon.

Preliminary tests were conducted to assess the influence of splenic and pronephric RBCs on tissue culture, leukocyte activation, and SIgM expression (data not shown). RBCs were removed by preferential lysing in the same way as for the blood samples. Despite producing a cleaner leukocyte preparation there was no observable advantage to tissue culture through the removal of RBCs from these tissues with respect to activation or SIgM expression. Furthermore, it is possible to artificially eliminate RBCs and sub populations of other leukocytes in FCM analysis by setting FSC thresholds and 'gating' according to size and granularity (Secombes, 1996). This provides for a practicable undertaking when sampling and processing large sample numbers of fish in one day. The extent to which the leukocyte preparation is purified

for this assay depends largely on the goal of the experiment for which it is used. For direct comparisons of immunocompetence between animals from different treatments, a preparation including all leukocytes for tissue culture may be more representative of the extant milieu within the tissue of the animal, and therefore may be indicative of the overall *in vivo* response. Notwithstanding, identification of differential sub-populations within the preparations by staining of smears and microscopic examination (Yasutake and Wales, 1983), and physical separation/removal of the different cell types by adherence, panning or density gradients (Sizemore et al., 1984 Secombes, 1990, Lamas and Ellis, 1994a, Moritomo et al., 1999) are recommended to provide further insight into the mechanism of any observed humoral immunomodulation when using this assay.

To verify that the cells being activated by LPS were primarily B-lymphocytes, we carried out some preliminary analysis of flow cytometric data from activated and non-activated cells. We calculated the numbers (as opposed to the percent) of cells from each of the discernable populations following incubation with media or LPS. The numbers of cells with high fluorescence intensity and either low or high FSC properties increased over those in media alone by at least an order of magnitude when cultured with LPS. However, the numbers of cells with low fluorescence intensity and either low or high FSC properties stayed essentially the same when cultured with LPS. These latter cell populations are likely to represent non-lymphocytes such as macrophages/granulocytes, and surface Ig negative lymphocytes (Tcells) respectively. Because non-B-cell populations (as defined by binding of the monoclonal Ab) did not

change in number following culture with LPS, we conclude that the LPS to be B-lymphocyte specific for chinook salmon.

The time-course for activation of chinook leukocytes with LPS indicates that splenic leukocytes are activated by 4 days of immunization. This allows for the assay to be carried out over a relatively short period of time using this tissue. The results are reproducible although it is clear that there is variability between the immune response of individual fish. The pronephros provides less reproducible results with this assay and requires 7 days for optimum activation. This is likely due to the differential stages of maturity of the B lymphocyte population in this tissue as well as the heterogeneous cell population found there.

The assay was shown to be sensitive enough to detect cortisol induced immunosuppression *in vitro*. The degree of immunosuppression observed was comparable with that reported for the reduced production of antibody producing cells using the hemolytic plaque assay (Tripp *et al.*, 1987).

The ability of B lymphocytes to respond to an antigen by expressing and producing antibody against that antigen is paramount to fighting disease. Measurement of IgM levels in circulating serum or resting lymphocytes may be misleading, especially if measured in so called "naïve" fish. The question of whether high serum IgM is indicative of recent exposure to a pathogen or a potentially strong immune capability may be raised. In the assay described in this paper, *in vivo* activation of the immune response is not required; however, the assay may be adapted to incorporate this if desired. The assay described provides information on both the resting levels of SIgM and the ability of those lymphocytes to respond in the face of a mitogen (i.e.,

pathogen). Lymphocyte proliferation assays are useful in that they provide an insight into the ability of an animal's immune cells to become activated in response to a mitogen. However, in terms of immune system function, they tell us little about the cells subsequent ability to fight the invading pathogen. Through this assay, the activation of the B lymphocytes can be quantified both by the percentage of cells undergoing blastogenesis and then ultimately the ability of those cells to express surface immunoglobin; both functions are essential for fighting an invading pathogen.

The assay can allow for non-lethal and/or repeated sampling of fish because blood leukocytes can be used. Therefore, this assay will be useful for detecting immunomodulation as a result of both *in vivo* and *in vitro* exposure to environmental perturbations. For example, application of the assay may include determination of both descriptive and functional humoral immunocompetence of salmonids following subjecting whole organisms to various environmental conditions. Indeed, we have used this approach in our laboratory to detect immunosuppression as a result of *in vivo* exposure of chinook salmon to o,p'-DDE (Milston *et al.*, submitted). Furthermore, the assay repeatedly detected a sexual/maturity dimorphic immune response in Japanese masu salmon (Azuma *et al.*, unpublished data). In addition, the assay can be used as an *in vitro* screening tool for the effect of chemical contaminants on immunocompetence.

Literature cited

Adkison, M.A., Basurco, B. & Hedrick, R.P.,1996. Humoral immunoglobins of the white sturgeon, *Acipenser transmontanus*: partial characterization of and recognition with monoclonal antibodies. Dev. Comp. Immunol., 20: 285-298.

Anderson, D. P., 1996. Environmental factors in fish health: Immunological aspects. In: The fish immune sytsem. Vol 15 ' Fish physiology', (G. Iwama & T. Nakanishi, eds) pp. 289-310. San Diego: Academic Press.

Arkoosh, M. R. & Kaattari, S.L., 1990. Quantitation of fish antibody to a specific antigen by an enzyme linked immunosorbent assay (ELISA) In: Techniques in Fish Immunology (Stolen, J., Fletcher, T.C., Roberson, B.S., van Muiswinkel, W.B., eds) pp. 15-24. New Jersey: S.O.S. Publications.

Azuma, T., Milston, R.H., Heppell, S.A., & Schreck, C.B. Immunological parameters of masu salmon (*Oncorhynchus masou*) raised under various flow regimes. (unpublished data).

Chilmonczyk, S., 1978. In vitro stimulation by mitogens of peripheral blood lymphocytes from rainbow trout (*Salmo gairdneri*). Ann. Inst. Pasteur Imm., 129: 3-12.

Chilmonczyk, S., Voccia, I., Tarazona, J.V. & Monge, D., 1997. Flow cytometric analysis of fish leukocyte populations exposed to pollutants and pathogens: modulatory effects induced by experimental procedures. In: Ecotoxicology: Responses, Biomarkers and Risk Assessment, (J.T. Zelikoff, editor) pp. 171-184.

Chilmonczyk,S & Monge, D., 1999. Flow cytometry as a tool for assessment of the fish cellular immune response to pathogens. Fish Shellfish Immun., 9: 319-333.

Crippen, T., Bootland, L., Leong, J., Fitzpatrick, M. , Schreck, C. & Vella, A., 2001. Analysis of Salmonid leukocytes purified by hypotonic lysis of erythrocytes. J Aquat. Anim. Health., (in press).

Crippen, T., Bootland, L., Vella, A., Fitzpatrick, M., Schreck, C. & Leong. J. Flow cytometric evaluation of the in vitro proliferative response of teleost blood cells. Fish Shellfish Immun., (submitted).

DeLuca, D., Wilson, M. & Warr, G.W., 1983. Lymphocyte heterogeneity in the trout, Salmo gaidneri, defined with monoclonal antibodies to IgM. Eur. J. Immunol., 13: 546-551.

Ellis, A. E., & de Sousa, M., 1974. Phylogeny of the lymphoid system. I. A study of the fate of circulating lymphocytes in plaice. Eur. J. Immunol., 4: 338-343.

Fuda, H., Soyano, K., Yamazaki, F. & Hara, A., 1991. Serum immunoglobin M (IgM) during early development of masu salmon (*Oncorhynchus masou*). Comp. Biochem. Phys., 99A: 637-643.

Jacobs, D.M., & Morrison, D.C., 1975. Stimulation of a T-independent primary anti-hapten response in vitro by TNP-lipopolysaccharide (TNP-LPS). J. Immunol., 144: 360-364.

Janeway, C.A., & Travers, P., 1997. Immunology: the immune system in health and disease. Current Biology Ltd. Garland Publishing Inc., London, New York.

Jerne, N.K. & Nordin, A.A., 1963. Plaque formation in agar by single antibody-producing cells. Science., 140: 405.

Julius, M.H., Simpson, E., & Herzenberg, L.A., 1973. A rapid method for the isolation of functional thymus-derived murine lymphocytes. Eur. J. Immunol., 3: 645-649.

Lamas, J., & Ellis, A.E., 1994a. Atlantic salmon (*Salmo salar*) neutrophil responses to Aeromonas salmonicida. Fish Shellfish Immun., 4: 201-219.

Manning, M.J., & Nakanishi, T., 1996. The specific immune system: cellular defenses. In: The fish immune system. Vol 15 'Fish Physiology', (G. Iwama & T. Nakanishi, eds) pp. 159-205. San Diego: Academic Press.

Milston, R.H., Fitzpatrick, M.S., Vella, A.T., Gundersen, D., Leong, J.C. & Schreck, C.B. Short term exposure of fall chinook salmon (*Oncoryhnchus tshawytscha)* to o,p'-DDE during early life history stages affects long-term competence of the humoral immune system. Aquat. Toxicol. (submitted).

Moritomo, T., Suehiro, M., Kodama, H., Amemori, T., Ohba, S., Nemoto, H., Hasegawa, A., & Yoshida, H., 1999. A new method for the identification and enumeration of carp granulocytes. Fish Pathol., 34: 85-86.

Press, C.M.L., Dannevig, B.H., & Landsverk, T., 1994. Immune and enzyme histochemical phenotypes of lymphoid and non-lymphoid cells within the spleen and head kidney of Atlantic salmon (*Salmo salar L.*). Fish Shellfish Immun., 4: 79-93.

Secombes, C.J., 1990. Isolation of salmonid macrophages and analysis of their killing activity. In: Techniques in Fish Immunology, Vol 1.(J.S. Stolen et al., eds) pp. 137-154. Fair Haven: SOS Publications.

Siegl, E., Nebe, B., Blunk, H. & Rychly, J., 1998. Detection of mitogen induced stimulation of leukocytes from the rainbow trout (*Oncorhynchus mykiss*) by flow cytometric analysis of intracellular calcium. Comp. Biochem. Phys., 119:915-923.

Sizemore, R.G., Miller, N.M., Cuchens, M.A., Lobb, C.L., & Clem, L.W., 1984. Phylogeny of lymphocyte heterogeneity: The cellular requirements for in vitro mitogenic responses of channel catfish leukocytes. J. Immunol., 133: 2920-2924.

Tripp, R.A., Maule, A.G., Schreck, C.B. & Kaattari, S.L., 1987. Cortisol mediated suppression of salmonid lymphocyte responses in vitro. Dev. Comp. Immunol., 11: 565-576.

Van der Heijden, M.H.T., Booms, G.H.R., Tanck, M.W.T., Rombout, J.H.W.M. & Boon, J.H., 1995. Influence of flumequine on in vivo mitogen responses of European eel (*Anguilla anguilla L., 1758*) lymphoid cells. Vet. Immunol. Immunop., 47: 143-152.

Voccia, I., Krzystyniak, K., Flipo, D. & Fuornier, M., 1994. In vitro mercury-related cytotoxicity and functional impairment of the immune cells of rainbow trout (*Oncorhynchus mykiss*). Aquat. Toxicol. 29: 199-203.

Voccia, I., Sanchez-Dardon, J., Dunier, M., Chilmonczyk, S. & Fournier, M., 1997. Contaminants on competence of rainbow trout macrophages. In: Chemically induced alteration in functional development and reproduction of fish. Setac Press, pp. 99-109.

Wester, P.W., Vethaak, A.D. & van Muiswinkel, W.B., 1994. Fish as biomarkers in immunotoxicology. Toxicology. 86: 213-232.

Yasutake, W.T., & Wales, J.H., 1983. Microscopic Anatomy of Salmonids: An Atlas. United States Department of the Interior, Fish and Wildlife Service; 150.

Zapata, A.G., 1979. Ultrastructural study of the teleost fish kidney. Dev. Comp. Immunol., 3: 55-65.

Zapata, A.G., 1981. Lymphoid organs of the teleost fish. II. Ultrastructure of renal lymphoid tisue in Rutilus rutilus and Gobio gobio. Dev. Comp. Immunol. 5: 685-690.

CHAPTER 2

SHORT TERM EXPOSURE OF FALL CHINOOK SALMON (*ONCORYHNCHUS TSHAWYTSCHA*) TO *O,P'*-DDE DURING EARLY LIFE HISTORY STAGES AFFECTS LONG-TERM COMPETENCE OF THE HUMORAL IMMUNE SYSTEM

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Abstract

We evaluated the effects of short term exposures to *o,p'*-DDE during early life history stages on the long term immune competence of fall chinook salmon (*Oncoryhnchus tshawytscha*). Immersion of chinook salmon eggs in *o,p'*-DDE for 1 h at fertilization followed by 2 h at hatch caused significant reductions in humoral immune competence at one year of age. The ability of splenic leukocytes to undergo blastogenesis and express surface immunoglobin M (SIgM) upon *in vitro* stimulation with lipopolysaccharide was significantly reduced in cells from exposed fish 1 year after treatment with 10 ppm *o,p'*-DDE. There was no treatment effect on resting SIgM expression of unstimulated splenic leukocytes. The concentration of *o,p'*-DDE in fry treated with 10 ppm *o,p'*-DDE was 0.92 μg g^{-1} lipid 1 month after first feeding. The chemical persisted and 1 year after exposure, levels in juvenile muscle tissue were 0.94 μg g^{-1} lipid. Mortality rate, time to hatch, fish length and weight were unaffected by treatment with *o,p'*-DDE. Similarly, sex ratios, gonadal development, plasma estradiol and 11-ketotestosterone concentrations were not affected by the treatment. In addition, we found no evidence that plasma lysozyme concentrations or the mitogenic response of splenic leukocytes to concanavalin A or polyinosinic-polycytidylic acid were influenced by the treatment.

In this experiment, a short period of chemical exposure during early development was able to induce long term effects on humoral immune competence of chinook salmon. Such immunosupression may increase susceptibility to disease, which may be critical to regulating the population.

Introduction

In many fish populations, including salmonids, substantial mortality occurs during the early life history stages. Incidences of disease can be critical in population regulation at these stages (Freeland, 1983), and increased susceptibility to disease due to immunosupression may contribute to the decline of fish populations exposed to chemical contaminants (Arkoosh, 1998). One such contaminant is the organochlorine 1,1,1-trichloro-2, 2-bis (p-chlorophenyl) ethane (DDT), a widely used pesticide in the United States until 1972 (Hayes, 1991). Despite evidence that DDT impairs developmental and reproductive success in fish and wildlife species (Guillette et al., 1994, 1996; Fry, 1995; Donohoe and Curtis, 1996), it is still used in some countries. Furthermore, global atmospheric transport results in ubiquitous contamination in the environment. Dichlorodiphenyldichloroethylene (DDE) is the primary metabolite of DDT. Its lipophilic nature is such that it tends to accumulate in sediments and it has been measured in several estuaries on the Oregon Coast and detected in fish in the Pacific Northwest (Oregon Department of Environmental Quality, 2000, Foster et al., 2001). Two isomers of DDE are present in the environment: *p,p'*-DDE and *o,p'*-DDE. The former has been reported to act as an anti-androgen in rats (Kelce et al., 1995), whereas *o,p'*-DDE has estrogenic activity in tissue and biological assays (Colburn et al., 1993; Soto et al., 1995; Donahoe and Curtis, 1996). Displacement of the estrogen receptor by *o,p'*-DDE has been demonstrated in a number of vertebrate organisms including humans and fish (Nelson, 1974, 1978; Chen et al., 1997; Kramer and Giesy, 1999; Matthews et al., 2000).

The immune effects of DDT and its metabolites are well documented in mammals. Lahvis et al. (1995) demonstrated an inverse correlation between the lymphocyte responses to concanavalin A (CON A) and blood concentrations of *p,p'*-DDT, *p,p'*-DDE and *o,p'*-DDE in free ranging bottlenose dolphins (*Tursiops truncatus*). In addition, exposure of beluga whale (*Delphinapterus leucas*) leukocytes to 25 to 100 ppm *p,p'*-DDT *in vitro* caused a significant reduction in leukocyte proliferation when stimulated with phytohemagglutinin (DeGuise, 1998). In rats, humoral immunosuppression occurred after oral administration of DDT (Koner, 1998) and in humans, prenatal exposure to DDE has been correlated to incidences of disease (Dewailly et al., 2000). Furthermore, in epidemiological studies, DDE is increasingly linked to the risk of cancer (Wolff, 1993; Porta et al., 1999; Cocco et al., 2000; Romieu et al., 2000), although the findings are often contradictory and inconclusive.

The mechanism for chemically induced immunomodulation is unclear. One possible mode of action is from direct toxicity to the immune cells and organs. Alternatively, immunomodulation may result from interactions between the immune and endocrine systems (Dunier, 1996). Interactions between the immune system and the hypothalamus-pituitary-gonadal (HPG) axis in mammals (Waltman et al., 1971; Yohn, 1973; Ablin et al., 1974; Blair, 1981; Grossman, 1984) and in fish (Pickering and Christie, 1980; Cross and Wolloughby, 1989; Slater and Schreck, 1993), suggest that exposure of fish to a contaminant with estrogenic activity such as *o,p'*-DDE may induce effects on the immune system via the endocrine system. It is widely accepted that endogenous estrogen suppresses cell-mediated immunity, but paradoxically stimulates the humoral response (Grossman, 1984). Similarly, xenobiotics can

interfere with the immune system at a number of levels of complexity, and different immune cells and processes have variable sensitivity to pollutants. For this reason, a suite of tests is required to evaluate the immunomodulatory mechanisms involved.

The goal of this study was to determine if brief exposures to *o,p'*-DDE during embryonic development can have long term effects on immune function in chinook salmon (*Oncoryhnchus tshawytscha)*. We chose to expose fish to the compound immediately post fertilization (during the water hardening period) and then subsequently during hatch, because a) these life history stages are particularly sensitive to environmental perturbations (Rosenthal and Alderdice, 1976), b) development of the immune system occurs during and subsequent to these stages (Ellis, 1977; Grace & Manning, 1980; Tatner, 1996), c) developing fish embryos are particularly sensitive to hormonal signals during these stages, evidenced by manipulation of sex ratios by exposure to exogenous estrogens or androgens (Piferrer and Donaldson, 1989; Feist et al. 1995), d) eggs may receive high contaminant loads by transgenerational exposure through maternal transfer of mobilized lipids and e) these fish are in closest contact with potentially contaminated sediments during these periods of development.

Methods

FISH GAMETES

We obtained fall chinook salmon gametes from Fall Creek Hatchery, Oregon in November 1998. Five mature females and four mature males were removed from their

holding pen and killed by a blow to the head. Eggs were stripped from the abdomen of the females, pooled, placed in an insulated plastic bag and kept on ice. Milt was extracted and pooled from the males and placed in O_2 enriched plastic bags on ice and held in the dark. Eggs and milt were transported to the Fish Performance and Genetics Laboratory (FPGL) at Oregon State University within 5 h.

EXPERIMENTAL DESIGN

On arrival at FPGL, pooled eggs were randomly assigned to one of four triplicated treatment groups immediately prior to fertilization: (1) *water control*-- 2.5 L of well water; (2) *vehicle control*-- 1 ml dimethylsulfoxide (DMSO) in 2.5 L of well water; (3) *10 ppm o,p'-DDE*-- stock solution of *o,p'*-DDE (AccuStandard) prepared at 250 mg ml^{-1} by dissolving 900 mg of *o,p'*-DDE in 3,600 ml of DMSO. A treatment dose of 10 ppm *o,p'*-DDE was prepared by making a ten fold dilution of stock solution and pipetting 1 ml of this into 2.5 L of well water; and, (4) *100 ppm o,p'-DDE*-- 1 ml of stock solution was pipetted into 2.5 L of well water for a treatment dose of 100 ppm DDE. On addition of the 100 ppm stock solution to the water bath, a white precipitate formed.

Within each treatment group, eggs were separated into three replicates. Each replicate consisted of 90 g of eggs weighed in a glass beaker (weight equivalent to approximately 250 to 300 eggs per replicate). In random order, individual replicates were transferred to a dip-net and fertilized by the addition of 1 ml of pooled milt followed by a 2 min immersion in a bath of water at 11 ± 0.5^{o} C.

Immediately post-fertilization, the eggs were transferred to a 3 L glass treatment bath and immersed for 1 h in one of the four treatment solutions.

All immersion solutions were mixed thoroughly before, and periodically throughout the exposure by gentle, repeated aspiration with a Pasteur pipette. Replicates were treated sequentially in a random order allowing 4 min for preparation, fertilization and transfer to immersion bath for each replicate. The fertilization bath water was renewed between each replicate. The temperature of the immersion solutions was $11 \pm 0.5^{\circ}$ C before and after the exposures.

Following the immersion, each replicate was transferred to a Heath tray. The eggs were maintained undisturbed in darkness until 240° C Temperature Units (CTU) had accumulated. The Heath trays were supplied with a constant flow of water (12.5 ± 0.5 $^{\circ}$ C at 9 L min^{-1}). In accordance with standard hatchery practice, after 240 CTU, eggs were treated twice a week with 230 ml formalin at 0.5 ml min^{-1} to control fungus. Subsequent to the eyed stage (between 340 and 360 CTU), mortalities were removed and recorded on approximately alternate days. To identify unfertilized eggs, water was sprayed on the eggs at 420 CTU. Eggs that turned opaque were judged unfertilized and were removed. A random sub-sample of the remaining viable eggs (n = 200 eggs per replicate) was placed in a mono-layer in holding containers (10 cm diameter x 5 cm high PVC pipe with mesh base) within the Heath trays. These eggs continued to be maintained in the Heath trays and mortality and time to hatch were recorded for each group.

When 50% of the water control group had hatched, each replicate of each treatment was exposed by immersion to the same treatment as at fertilization, with the

exception that the immersion period was for 2 h. Fry were transferred in the holding container to 3 L glass baths containing 2.5 L treatment solution. Throughout the immersion oxygen was supplied to each bath for 5 s every 15 min. Following the immersion the fry were returned to Heath trays.

Fry were raised in Heath trays until the yolk sac was absorbed; then, a random sub-sample of fish from each replicate of each treatment (n = 55 fry per replicate) was placed in individual 0.6 m-diameter circular tanks as the experimental group. The remaining fry were maintained under the same conditions as the experimental groups. These fry were used to replace mortalities in the experimental groups in order to keep rearing densities standard. At the onset of exogenous feeding, fish were fed several times daily with semi moist pellet (BioOregon^{TM}). Mortalities were recorded and replaced for approximately 1 month after first feeding.

At 1 month after first feeding, a random sample of fry was netted from each replicate and killed by a lethal dose (200 mg l^{-1}) of tricaine methane-sulfonate buffered with sodium bicarbonate (MS-222). Fry from each replicate were pooled within a treatment and frozen at –20° C until analysis for *o,p'*-DDE tissue concentration.

The fish were raised under regular hatchery conditions of 12.5 ± 0.5° C flow-through well water and natural photoperiod and fed a commercial diet of Semi-moist Pellets, BioOregon^{TM} daily. When size and density dictated, the fish were transferred to and maintained in 1 m-circular tanks until sampling for immunocompetence approximately 1 year post first-feeding.

SAMPLING

We sampled fish exposed to the four treatment groups for plasma steroid levels, sex, gonadal histology, plasma lysozyme levels, splenic leukocyte blastogenesis and surface immunoglobin M expression in response to lipopolysaccharide April 2000, approximately 1 year after first feeding. We sampled again in May 2000 to assess the mitogenic response of splenic leukocytes to concanavalin A and polyinosinic-polycytidylic acid.

We conducted sampling over 3 days for both sampling events. For the first sampling event, eight fish from one replicate (chosen randomly) of each treatment were randomly sampled on the first day. This was repeated on the second and third day for the other two replicates (n = 24 fish per treatment). Fish were netted from the holding tanks and immediately immersed in a lethal dose (200 mg l^{-1}) of buffered MS-222. We recorded weight and fork length of the fish and noted any external abnormalities. We collected blood from the caudal peduncle using EDTA-coated vacuum tubes with a 21 gauge needle; samples were centrifuged at 1800 g for 5 min. Plasma was collected and stored at –80° C until assayed for plasma 17 β-estradiol (E_2), 11 ketotestosterone (11-KT), and plasma lysozyme levels. We drained residual blood from the fish by severing the caudal peduncle, and returned them to the laboratory on ice for dissection of the spleen.

For the second sampling event, six fish from one replicate (chosen randomly) of each treatment were randomly sampled on the first day. This was repeated on the second and third day for the other two replicates (n = 18 fish per treatment). Fish were netted from the holding tanks and immediately immersed in a lethal dose (200 mg l^{-1})

of buffered MS-222. We recorded weight and fork length of the fish and noted any external abnormalities. Blood was drained from the fish by severing the caudal peduncle, and returned fish to the laboratory on ice for spleen dissection and visual identification of sex.

ASSAY PROCEDURES

Concentration of o,p'-DDE in the fish

We analyzed whole fry 1 month after first-feeding for concentration of *o,p'*-DDE in tissue by gas chromatography, following the methods of Gunderson et al. (1998) to a detectable limit of 0.01 ppm. In addition, a sample of muscle and liver tissue was taken from one juvenile fish per treatment selected randomly from the replicates, 1 year after the onset of exogenous feeding and analyzed for tissue concentration of *o,p'*-DDE.

Blastogenesis and surface immunoglobin expression

We determined ability of splenic leukocytes to undergo blastogenesis and express surface immunoglobin M (SIgM) upon *in vitro* mitogenic stimulation with lipopolysaccharide. The method used quantifies the fluorescein isothiocyanate-labeled anti-rainbow trout surface immunoglobin antibody (anti-RBT IgM-FITC) (DeLuca et al., 1983) bound to individual cells by flow cytometry. The assay was optimized and validated for juvenile chinook salmon by Milston *et al.*, (submitted). Briefly, splenic leukocytes were cultured with either tissue culture media (TCM) or *Escherichia coli*

lipopolysaccharide 055:B5 (LPS) (Sigma) (final concentration 100 μg ml^{-1}) at 17° C for 4 days under blood gas. Pre-(day 0) and post-(day 4) incubation, we incubated cells with anti-RBT IgM-FITC on ice, in the dark for 30 min. We removed unbound antibody incubation, resuspended cells in balanced salt solution (BSS) supplemented with 3% fetal calf serum and 0.1% sodium azide and transferred cells to FACS microtubes for flow cytometric analysis. Flow cytometric analysis was carried out using a Becton Dickinson FACSCaliber ™ flow cytometer with Argon-ion laser at 480 nm using Cellquest ™ software. A minimum of 10,000 events was acquired for each sample. For fish within each exposure treatment, the percentage of the cell population undergoing blastogenesis was determined from flow cytometric plots of side and forward scatter using Cellquest™ software. We determined percentage SIgM positive blasting cells within the cell populations for non-stimulated and stimulated cells by analysis of quadrant statistics from fluorescence intensity and forward scatter plots. The viability of cells within stimulated and non-stimulated cultures was determined using the Trypan blue exclusion test.

Plasma steroid concentrations

We assayed plasma concentrations of E_2 and 11-KT by radio-immunoassay following the methods of Sower & Schreck (1982) as modified by Fitzpatrick et al. (1986).

Sex determination and gonadal histology

We visually determined sex of each fish by examining gross gonadal morphology under a dissecting microscope. In addition, we inspected gonads for developmental abnormalities by histological analysis. Gonad samples were fixed in 10% buffered formalin and imbedded in paraffin, sectioned (10 μm transverse sections) and stained in eosin and hematoxin. We examined the sections under a light microscope and compared them to descriptions for normal reproductive development as described by Piferrer and Donaldson (1989) and Feist et. al. (1990).

Lysozyme Activity

Lysozyme activity in plasma was measured following a modified method of Litwack (1955), as applied by Sankaran and Gurnani (1972). Briefly, we established ability of plasma lysozyme to break down the peptidoglycan layer of the gram positive bacterium *Micrococcus lysodeikticus*. We incubated 10 μl of plasma in triplicate in a 96 well microplate with 200 μl of *Micrococcus lysodeikticus* (0.025% w/v suspension in 0.02 M acetate buffer) for a 20 min period at room temperature. We measured the change in optical density over the incubation period at 450 nm and compared it to a standard curve of hen egg white lysozyme over the linear range 3-15 mg ml^{-1}.

Mitogenic response to concanavalin A or polyinosinic-polycytidylic acid

We carried out a second sampling 1 month after the first to assess the ability of leukocytes from fish from the different exposure treatments to be stimulated in response to T cell specific mitogens. We followed the methods described by Crippen

et al. (submitted) whereby cell cycle progression is determined and quantified by flow cytometric analysis of propidium iodide (PI) (Sigma) labeled DNA of individual cells. Briefly, splenic leukocytes were stimulated *in vitro* with concanavalin A (250 μg ml^{-1}), polyinosinic-polycytidylic acid (500 μg ml^{-1}), or in TCM alone for 4 days at 17° C under blood gas. Following incubation, cells were fixed with methanol, washed, then resuspended in PBS plus EDTA and RNAse A, and then transferred to FACS tubes. Cells were incubated with PI for at least 30 min at room temperature prior to flow cytometric analysis. Analysis of the cells was performed on a Becton Dickinson FACSCaliber™ flow cytometer at wavelength 488 nm. A minimum of 10,000 events was acquired from each sample. We determined the percentage of cells within the population that were undergoing mitosis, as defined by DNA content reflected by PI fluorescence using Cellquest™ company software. We calculated stimulation index as the ratio of the percentage of cells in S and G_2M phases in mitogen-stimulated cultures divided by the percentage of cells in S and G_2M phases in unstimulated cultures.

STATISTICAL ANALYSES

We used analysis of variance (ANOVA) to determine differences between replicates, followed by ANOVA of treatment groups. If there were no differences between replicates, data were pooled within a treatment. Groups were tested for normality and variance homogeneity and, where detected, parametric statistical analyses were applied (Tukey's multiple comparison test). If variances were not homogenous, we applied non-parametric tests (Kruskal-Wallis or Mann-Whitney U

test) with Dunn's post test where differences were detected. Differences were considered significant below P=0.05.

Percent values were transformed by arcsine of the square root of the value for further analysis by ANOVA and subsequent multiple range tests. Differences were considered significant below P=0.05.

Treatment effects on the discrete responses of mortality and hatching times were analyzed using SAS™, version 6.4 (SAS Institute Inc., Cary NC, USA). We used chi-squared test using the PROC FREQ procedure to determine differences between treatments in mortality and hatch rates over several time points. Differences were considered significant below P=0.05.

Results

CONCENTRATION OF O,P'-DDE IN TISSUES

The concentration of *o,p'*-DDE in fry treated with 10 ppm *o,p'*-DDE was 0.92 μg g^{-1} lipid 1 month after first feeding (Table 2.1). One year later, levels in juvenile muscle tissue were 0.94 μg g^{-1} lipid. The level of *o p'*-DDE in fry was below the minimum detection limit for the 100 ppm treated fish and for controls. Concentrations of *o,p'*-DDE in 1 year-old juvenile liver tissue were non-detectable in all treatments.

Table 2.1. Concentration of *o,p'*-DDE in pooled whole fry (1 month post first-feeding) or individual juvenile muscle tissue (1 year post first-feeding) of chinook salmon following a 1 h immersion at fertilization and a 2 h immersion at hatch with either water, 0.04% dimethylsulfoxide vehicle, 10 ppm *o,p'*-DDE or 100 ppm *o,p'*-DDE (<MDL= Below minimum detection limit of 0.01 μg g^{-1} lipid, n = 1 for each treatment).

	Fry		Juvenile	
	μg DDE g-1 Lipid	% Lipid	μg DDE g-1 Lipid	% Lipid
Water Control	< MDL	2.4	0.34	11.7
DMSO Control	< MDL	3.3	0.19	15.9
10 ppm Treatment	0.92	3.8	0.94	13.6
100 ppm Treatment	< MDL	5.1	0.29	10.3

PERCENTAGE OF CELLS UNDERGOING BLASTOGENESIS FOLLOWING 4 DAY IN VITRO ACTIVATION WITH TCM OR LPS

For all treatments, cells immunized *in vitro* with LPS had significantly more cells undergoing blastogenesis than those that were cultured in the presence of TCM alone (Mann-Whitney U-test $P < 0.001$) (Figure 2.1). By day 4 of *in vitro* activation with LPS the percentage of cells undergoing blastogenesis was significantly lower in the 10 ppm *o,p'*-DDE treatment than all other treatments (one-way ANOVA; $P = 0.0032$, followed by Tukey's multiple comparison test). There were no significant differences between treatments for the percentage of cells undergoing blastogenesis after incubation with TCM alone (one-way ANOVA, $P > 0.05$). Cell viability did not differ between cells incubated with LPS or with TCM alone.

PERCENTAGE SIGM$^+$ BLASTING CELLS

For all treatments, cells immunized *in vitro* with LPS had significantly higher percentage of SIgM positive blasting cells within the population than those that were cultured in the presence of TCM alone (Mann-Whitney U-test $P < 0.05$) (Figure 2.2). Following 4 day *in vitro* activation with LPS, the 10 ppm *o,p'*-DDE treatment had significantly lower percentage of SIgM positive blasting cells than the water control (one-way ANOVA $P = 0.0327$, followed by Tukey's multiple comparison test ($P < 0.05$). Although the means for the vehicle control and the 100 ppm *o,p'*-DDE treatment tended to be lower than the water control, these differences were not statistically significant. The percentage surface IgM positive blasting cells within the

leukocyte population did not differ significantly between treatments for cells incubated in TCM alone (one-way ANOVA, $P > 0.05$).

FISH SIZE

At 16 months and 18 days after fertilization (blastogenesis and SIgM assay), the mean weights of the fish were 144.4 g (± 7.3), 129.6g (± 5.7), 116.2g (± 4.7) and 131.1g (± 5.6) for the water control, vehicle control, 10 ppm *o,p'*-DDE and 100 ppm *o,p'*-DDE treatments, respectively. Despite the median weight from the 10 ppm *o,p'*-DDE treatment being lower than that of the controls for this sampling event ($P = 0.0394$), there were no weight differences between treatments for the second sampling event (mitogenic assay) 3 months and 10 days later (mean weights: 212.7 (± 18.66), 199.6 (± 13.05), 205.9 (± 10.62), 221.2 (± 9.46) for the water control, vehicle control,

10 ppm *o,p'*-DDE and 100 ppm *o,p'*-DDE treatments, respectively. A linear regression of weight at the first sampling against percent blasting cells and percent SIgM positive blasting cells showed no relationship ($R^2 = 0.0086$ and 0.003 respectively) between weight and the immune parameters. There were no significant differences between fish lengths for either sampling event.

There was no effect of *o,p'*-DDE on mortality, time to hatch, sex ratio, gonadal development, plasma lysozyme concentration or leukocyte mitogenesis in response to CON A or Poly I:C, (Table 2.2) or plasma E_2 and 11-KT concentration (Figures 2.3a & 2.3b).

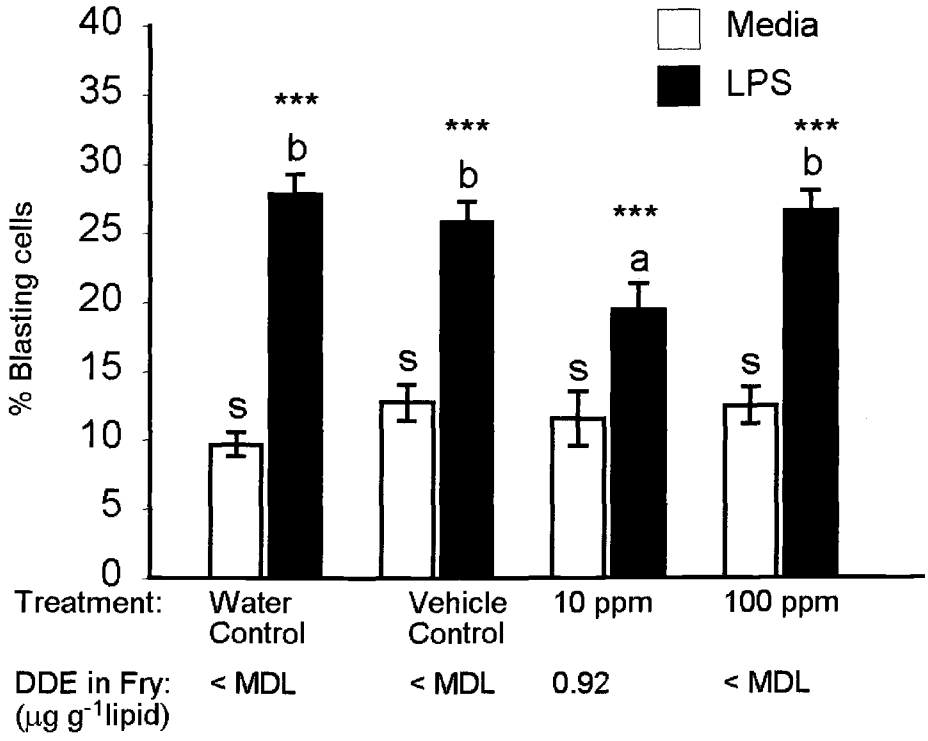

Figure 2.1. The percentage (mean ± S.E.) of the population of splenic leukocytes undergoing blastogenesis following a 4 day *in vitro* incubation with media or LPS. Cells were harvested from chinook salmon 1 year after a 1 h immersion at fertilization and a 2 h immersion at hatch with either water, 0.04% dimethylsulfoxide vehicle, 10 ppm *o,p'*-DDE or 100 ppm *o,p'*-DDE. For the respective treatments, n = 14, 23, 16, and 21. The concentrations of *o,p'*-DDE found in treated fry 1 month post first feeding were <MDL, <MDL, 0.92, <MDL μg g^{-1} lipid (<MDL = below minimum detectable limit of 0.01μg g^{-1}) respectively. Columns that have the same subscripts are not significantly different (P > 0.05, ANOVA). Significant differences between LPS and media within an immersion treatment are denoted *** (P < 0.001, Mann-Whitney U test).

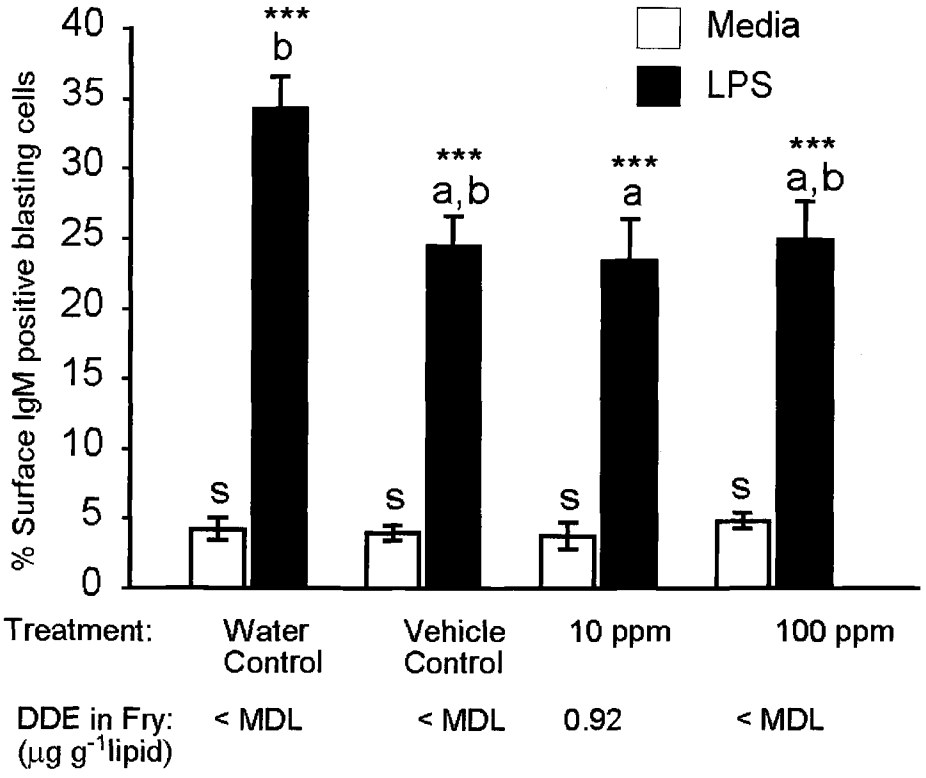

Figure 2.2.The percentage of blasting splenic leukocytes expressing surface immunoglobin M (IgM) following a 4 day *in vitro* incubation with media or LPS. Cells were harvested from chinook salmon 1 year after a 1 h immersion at fertilization and a 2 h immersion at hatch with either water, 0.04% dimethylsulfoxide vehicle, 10 ppm *o,p'*-DDE or 100 ppm *o,p'*-DDE. For the respective treatments, n = 14, 23, 16, and 21. The concentrations of *o,p'*-DDE found in treated fry one month post first feeding were <MDL, <MDL, 0.92, <MDL μg g^{-1} lipid (<MDL = below minimum detectable limit of 0.01μg g^{-1}) respectively. Columns that have the same subscripts are not significantly different ($P > 0.05$, ANOVA). Significant differences between LPS and media within an immersion treatment are denoted *** ($P < 0.001$, Mann-Whitney U test).

Table 2.2. Individual experimental replicates (Rep 1, 2 and 3) of parameters with no significant differences between treatments.

Treatment		Plasma Lysozyme[a] ($\mu g ml^{-1}$)	Poly I:C SI[b]	Con A SI[b]	% Hatched[d]	% Mortality Period 1[e]	# Mortalities Period 2[f]	% Female[g]
Water Control	Rep 1	41.88 (±1.42)	2.38 (±0.34)	2.03 (±0.34)	39	35	41	71
	Rep 2	39.14 (±1.56)	[c]	[c]	30	36	18	57
	Rep 3	36.26 (±1.20)	1.49 (±0.12)	1.74 (±0.13)	29	26	8	50
Vehicle Control	Rep 1	45.95 (±1.58)	2.14 (±0.27)	2.10 (±0.30)	47	28	12	29
	Rep 2	40.37 (±1.12)	[c]	[c]	48	39	7	43
	Rep 3	37.10 (±1.33)	1.58 (±0.20)	1.34 (±0.04)	40	32	0	43
10 ppm *o,p'*-DDE	Rep 1	41.19 (±1.14)	1.77 (±0.37)	1.69 (±0.22)	36	35	1	50
	Rep 2	43.65 (±0.91)	[c]	[c]	34	37	4	64
	Rep 3	36.34 (±0.85)	1.96 (±0.38)	1.72 (±0.5)	50	41	3	64
100 ppm *o,p'*-DDE	Rep 1	40.89 (±2.49)	2.78 (±0.33)	2.31 (±0.27)	64	42	2	57
	Rep 2	36.93 (±1.79)	[c]	[c]	33	40	2	43
	Rep 3	34.59 (±1.76)	1.73 (±0.30)	1.86 (±0.14)	50	23	1	57

[a] For each replicate, n = 8.

[b] SI = mean stimulation index (± standard error), calculated: ratio of the percentage of the splenic leukocyte population in S and G2/M phases of cell cycle after culture with mitogen [polyinisinic-polycytidylic acid (500 $\mu g\ ml^{-1}$) or concavallin A (250 $\mu g\ ml^{-1}$)] to the percentage of the splenic leukocyte population in S and G2/M phases of cell cycle after incubation with media. For each replicate, n = 6.

[c] Following cell culture there were insufficient viable leukocytes to conduct analysis.

[d] % hatched at 40 days post fertilization based on n = 300 per replicate.

[e] % mortality between fertilization and hatch based on n = 300 per replicate.

[f] Number of mortalities removed between button-up and one month post first-feeding. Initial n = 55 per replicate, mortalities were replaced with like-treated fish to keep densities the same.

[g] For each replicate, n = 14

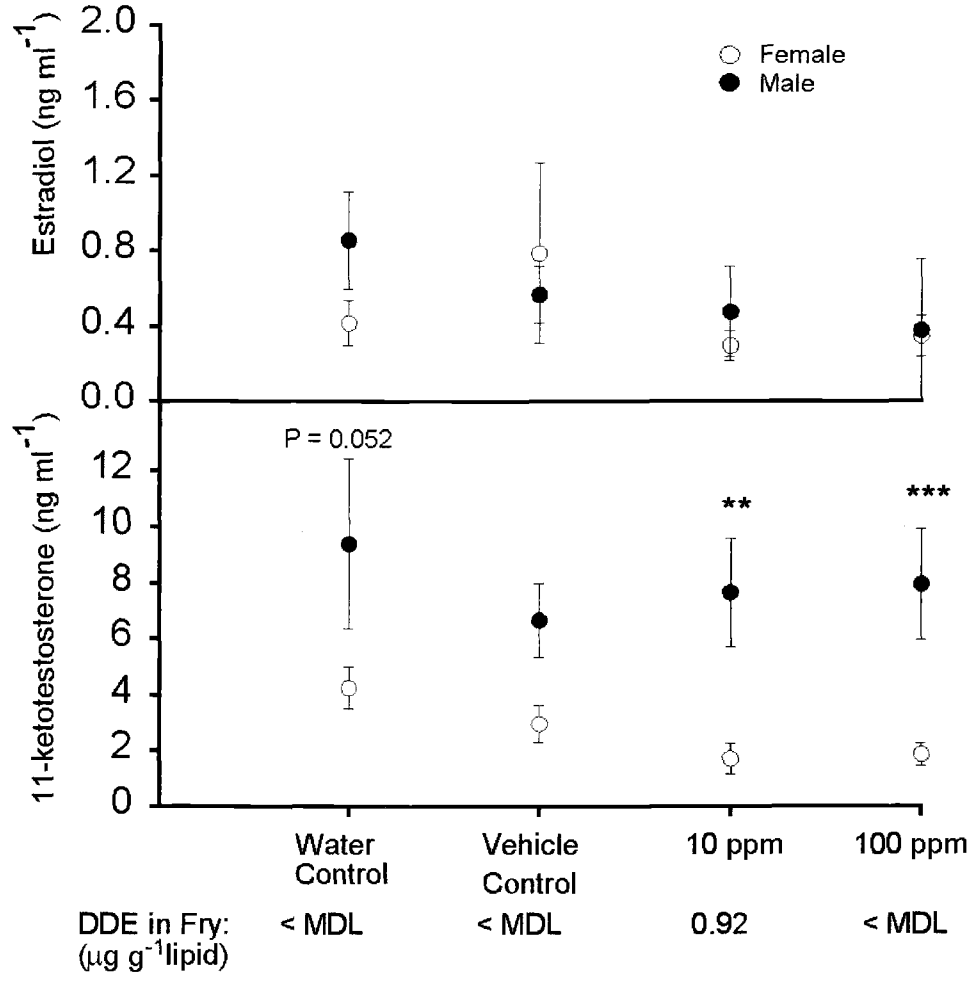

Figure 2.3a and b. Means (± S.E.) plasma estradiol and 11- ketotestosterone concentration of juvenile chinook salmon. Plasma was taken from fish 1 year after a 1 h immersion at fertilization and a 2 h immersion at hatch with water, 0.04% dimethylsulfoxide vehicle, 10 ppm *o,p'*-DDE or 100 ppm *o,p'*-DDE. There were no significant differences between treatments (ANOVA). Significant differences between males and females are denoted ** or *** (Mann-Whitney U test), n = 24 for each treatment.

Discussion

This study provides evidence that very brief exposures to low doses of *o,p'*-DDE at early life stages can be sufficient to cause long term effects on the immune function in chinook salmon. When chinook salmon eggs were exposed to a dose of 10 ppm *o,p'*-DDE by immersion for 1 h at fertilization followed by 2 h at hatch a significant reduction in the humoral response of the juveniles was observed. The ability of splenic leukocytes to undergo blastogenesis, when stimulated in vitro for 4 days with lipopolysaccharide, was significantly reduced in treated fish 1 year after *o,p'*-DDE exposure. Furthermore, of those cells that did undergo blastogenesis, significantly fewer expressed surface IgM in fish treated with 10 *o,p'*-DDE when compared to water control. The immunosuppressive effect of the vehicle DMSO may contribute wholly or in part to this suppression of SIgM expression. Fry treated with 10 ppm had 0.92 $\mu g\ g^{-1}$ lipid *o,p'*-DDE 1 month post first-feeding, whereas those treated with 100 ppm *o,p'*-DDE had non-detectable levels. During the 100 ppm immersions, following addition of the stock solution to the water bath, the compound precipitated out of solution, which is likely attributable to the low solubility of the compound in water. The precipitate was conceivably too large to be absorbed, and thus coated the membrane of the eggs, therefore impeding uptake of the compound into the fish. The absence of *o,p'*-DDE in fry tissue of the 100 ppm treatment would explain the lack of a response with respect to SIgM and blastogenesis for this treatment. The precipitate was not observed during the preparation of the 10 ppm treatments.

The concentration of *o,p'*-DDE in muscle of juveniles was elevated over the levels found in whole fry. The source of this *o,p'*-DDE is unclear, but because the compound

is considered ubiquitous, it is possible that it may be present in the fish feed. We did not test this in the present study, but feel that it would be an interesting avenue to pursue.

Despite the primary metabolite of DDT being recognized as the *p,p'* isomer of DDE, DeVault (1985) found high ratios of *o,p'*- DDE to *p,p'*- DDE in fish from the Great Lakes harbors and tributaries. Composite fish samples of indigenous fish samples from various sites had concentrations of *o,p'*- DDE ranging from 0.1 to 0.32 mg/kg. In our study, fry with whole body lipid concentrations of *o,p'*- DDE of the same order exhibited long term humoral immunosuppression.

In natural environments, one route of exposure to lipophilic contaminants is through maternal transfer of mobilized lipids. Similar observations have been made in mammals; Rehana and Rao (1992) found that dietary exposure of female mice to DDT led to humoral immunosuppression in the form of reduced number and proliferation of specific antibody producing cells. Interestingly, offspring of these mice also exhibited immunosuppression, without further exposure, suggesting that the immunosuppression occurred during development of the immune system as a result of maternally transferred DDT.

Based on the results of our study, we hypothesize that exposure to the compound *o,p'*-DDE during embryonic development causes permanent organizational effects on the immune system. This hypothesis supports the theory proposed by Guillette et al., (1995) for the long-term effects of endocrine disrupting chemicals in wildlife.

In many non-mammalian vertebrates, estrogens are apparently essential for sexual differentiation and early exposure to endocrine disrupting chemicals can influence this

process (Bull & Gutzke, 1986; Guillette et al., 1994; 1995; 1996; Fry, 1995). In fish, steroid hormones are produced by the salmonid embryo at the period around hatching (Feist et al., 1990) and exposure to exogenous estrogens or androgens around this period can alter the sex of the individuals (Piferrer and Donaldson, 1989; Feist et al., 1995). These observations suggest that the developing embryo is particularly sensitive to hormonal signals (Guillette et al., 1996). Organizational roles of steroids during early development may thus affect not only cells designed to have reproductive function, but other steroid dependent systems such as the immune system as well.

It is during this period of early development that the appearance of immune organs and the onset of functional maturity occur. Generally in fish, the thymus is the first lymphoid organ to develop, followed by the pronephros. Both tend to develop prior to hatching whereas the spleen and the gut associated lymphoid tissue tend to develop after hatching. Cytoplasmic IgM has been detected 12 days pre-hatch, while surface IgM was detected 8 days pre-hatch in rainbow trout, *O. mykiss* (Castillo, 1993). In Atlantic salmon, *Salmo salar,* IgM was demonstrated at 41 days post-hatch, around the time of first feeding (Ellis, 1977). Chemically induced disruption, such as exposure to *o,p'*-DDE, during the ontogeny of the immune system may compromise the fishes' long term immune capabilities.

The mechanism for observed developmental affects of DDT and its metabolites may be receptor mediated. The presence of estrogen receptors on lymphoid cells and endocrine-immune interaction has been suggested as a possible mechanism for estrogen-induced immunomodulation in humans (Cutolo et al., 1995). Furthermore, Grossman (1984) reviewed a number of studies suggesting that gonadal steroids play a

significant role in the regulation of the mammalian immune function. In mammals estrogen has pleiotropic effects on immune function. Whilst estrogen appears to suppress cell-mediated immunity, it is thought to augment humoral-based immunity (Nikolaevich et al., 1991; Kanda and Tamaki, 1999; McMurray, 2001). Studies with broiler chickens show that estrogen's stimulatory role in humoral immunity can be blocked with the anti-estrogen tamoxifen (Leitner et al, 1996). In mice, LPS induced blastogenesis and IgM levels were significantly reduced by a 14-day exposure to the highly estrogenic synthetic hormone diethylstilbesterol (DES); Bick et al., 1984. Similarly, *in utero* murine exposure to DES did not reduce the response to the T-dependent mitogen sheep red blood cells, but did suppress their response to LPS, a T-independent mitogen (Luster et al., 1978).

Donahoe and Curtis (1996) demonstrated the estrogenic activity of *o,p'*-DDE by inducing vitellogenesis in juvenile rainbow trout. Furthermore, *o,p'*-DDE displayed antagonist interaction with the human androgen receptor (Maness et al., 1998). Soto et al. (1994) found DDE and its isomers, including *o,p'*-DDE, to have estrogenic activity using the E-SCREEN method of detecting estrogen induced increases in the number of human breast MCF-7 cells. Moreover, *o,p'*-DDE displaced estradiol from the rat uterus cytosolic estrogen receptor (Nelson, 1974, 1978), whereas *p,p'*-DDE did not. However, in a recent study with the human estrogen receptor, *o,p'*-DDE did not display agonist behavior (Sheeler et al., 2000). Receptors on salmonid lymphocytes for androgens (Slater et al., 1995) and glucocorticoids (Maule and Schreck, 1989) have been characterized. In addition, the presence of estrogen receptors on the leukocytes of channel catfish has been detected in preliminary studies (R. Patino, pers.

comm.) although further work is required for them to be fully isolated and characterized. If present, this may provide a route for the immunomodulatory behavior of *o,p'*-DDE.

Despite the presence of glucocorticoid receptors on leukocytes, it is unlikely that the immunosuppression we observed was a result of a stress response to the treatment. In a study aimed at determining when the stress response first arises in chinook salmon, Feist (2000) found that fry did not begin to elicit a corticosteroidogenic stress response until 1 week after hatch. In our experiment, any perceived stressor was over before this time.

The results of our study support the many studies showing immunosuppressive effects of organochlorines through *in vitro*, laboratory and environmental exposures. What is particularly alarming about our results is that such a short period of exposure was able to induce long term effects on humoral immune competence. In recent decades, with increases in urban, industrial and agricultural development, contaminated effluent and run off is flowing directly into watercourses. Chemical exposure of fish during early periods of their life can lead to immunosupression, increasing their susceptibility to disease. In the event of an epizootic, recruitment of juveniles would likely be reduced, which may be critical to population regulation.

Literature Cited

Ablin, R.J., Bruns, G.R., Guinan, P. and Bush, I.M., 1974. The effect of estrogen on the incorporation of ^{3}H-thymidine by PHA-stimulated human peripheral blood lymphocytes. J. Immunol., 113: 705-707.

Ablin, R.J., Bhatti, R.A., Guinan, P. and Khin, W., 1979. Modulatory effects of estrogen on immunological responsiveness. II. Suppression of tumor-associated immunity in patients with prostatic cancer. Clin. Exp. Immunol., 38: 83-91.

Arkoosh, M.R., Casillas, E., Clemons, E., Kagley, A.N., Olson, R., Reno, P. and Stein, J.E., 1998. Effect of pollution on fish diseases: potential impacts on salmonid populations. J. Aquat. Anim. Health., 10: 182-190.

Bick, P.H., Tucker, A.N., White, K.L. Jr., and Holsapple, M.P., 1984. Effects of subchronic exposure to diethylstilbestrol on humoral immune function in adult female (C3B6) miceF1 mice. Immunopharmacology., 7:27-39.

Blair, P., 1981. Immunologic consequences of early exposure of experimental rodents to dithylstilbesterol (DES) in pregnancy (A. Herbst and H. Bern, eds). New York: Thieme-Stratton, pp.167-178.

Bull, J.J., Gutzke, W.H. and Crews, D., 1988. Sex reversal by estradiol in three reptilian orders. Gen. Comp. Endocrinol., 70: 425-428.

Castillo, A., Sanchez, C., Dominguez, J., Kaattari, S. and Villena, A.J., 1993. Ontogeny of IgM and CIgM bearing cells in Rainbow trout. Dev. Comp. Immunol., 17: 425-437.

Cocco, P., Kazerouni, N. and Zahm, S.H., 2000. Cancer mortality and environmental exposure to DDE in the United States. Environ. Health Perspect., 108:1-4

Colburn, T., vom Seal, F.S. and Soto, A.M., 1993. Developmental effects of endocrine-disrupting chemicals in wildlife and humans. Environ. Health Perspect., 101: 378-384.

Cross, M.L. and Willoughby, L.G., 1989. Enhanced vulnerability of rainbow trout (*Salmo gairdneri*) to Saprolegnia infection, following treatment of the fish with an androgen. Mycol. Res., 93: 379-383.

Crippen, T., Bootland, L., Leong, J., Fitzpatrick, M., Schreck, C. and Vella, A., 2001. Analysis of Salmonid leukocytes purified by hypotonic lysis of erythrocytes. J. Aquat. Anim. Health., (in press).

Crippen, T., Bootland, L., Vella, A., Fitzpatrick, M., Schreck, C. and Leong. J., Flow cytometric evaluation of the *in vitro* proliferative response of teleost blood cells. Fish and Shellfish Immunology (submitted).

Cutolo, M., Sulli, A., Seriolo, B., Accardo, S., and Masi, A.T., 1995. Estrogens, the immune response and autoimmunity. Clin. Exp. Rheumatol., 13: 217-226.

De Guise, S., Martineau, D., Beland, P. and Fournier, M., 1998. Effects of *in vitro* exposure of beluga whale leukocytes to selected organochlorines. J. Toxicol. Env. Health., 55: 479-493.

DeLuca, D., Wilson, M. and Warr, G.W., 1983. Lymphocyte heterogeneity in the trout, *Salmo gaidneri*, defined with monoclonal antibodies to IgM. Eur. J. Immunol., 13: 546-551.

DeVault, D.S., 1985. Contaminants in fish from Great Lakes harbors and tributary mouths. Arch. Environ. Contam. Toxicol., 14: 587-594.

Dewailly, E., Ayotte, P., Bruneau, S., Gingras, S., Belles-Isles, M. and Roy, R., 2000. Susceptibility to infections and immune status in Inuit infants exposed to organochlorines. Environ. Health Perspect., 108: 205-11.

Donahoe, R.M., and Curtis, L.R., 1996. Estrogenic activity of chlorodecone, o,p'-DDT and o,p'-DDE in juvenile rainbow trout: Induction of vitellogenesis and interaction with hepatic estrogen binding sites. Aquat. Toxicol., 36: 31-52.

Dunier, M., 1996. Water pollution and immunosuppression of freshwater fish. Ital. J. Zool., 63: 303-309.

Ellis, A.E., 1977. Ontogeny of the immune response in *Salmo salar*. Histogenesis of the lymphoid organs and appearance of membrane immunoglobin and mixed leukocyte reactivity. In: Solomon J.B., Horton, J.D. (eds), Developmetal Immunobiology. Elsevier/North-Holland Biomedical Press, Amsterdam; New York, pp. 225-231.

Feist, G., Schreck, C.B., Fitzpatrick. M.S. and Redding, J.M., 1990. Sex steroid profiles of coho salmon (*Oncoryhynchus kisutch*) during early development and sexual differentiation. Gen. Comp. Endocrinol., 80: 299-313.

Feist, G., Yeoh, C., Fitzpatrick, M.S. and Schreck, C.B., 1995. The production of functional reversed male trout with 17α-methyltestosterone and 11β-hydroxyandrostenedione. Aquaculture., 131: 145-152.

Feist, G., 2000. Early development of the endocrine system in salmonids: Regulation of sex differentiation and ontogeny of the stress response. In: Ph.D Thesis. Oregon State University, pp. 90-115.

Fitzpatrick, M.S., Van der Kraak, G. and Schreck, C.B., 1986. Plasma profiles of sex steroids and gonadotropin in coho salmon (*Oncorhynchus kisutch*) during final maturation. Gen. Comp. Endocrinol., 62: 437-451.

Freeland, W.J., 1983. Parasites and the coexistence of animal host species. Am. Nat., 121: 223-236.

Fry, D.M., 1995. Reproductive effects in birds exposed to pesticides and industrial chemicals. Environ. Health Perspect., 103: 165-171.

Grace, M.F. and Manning, J.J., 1980. Histogenesis of the lymphoid organs in rainbow trout, *Salmo gaidneri Richardson 1836*. Dev. Comp. Immunol., 4: 225-264.

Grossman, C.J., 1984. Regulation of the immune system by sex steroids. Endocr. Rev., 5: 435-449.

Guillette, L.J. Jr., Gross, T.S., Masson, G.R., Matter, J.M., Percival, H.F. and Woodward, A. R., 1994. Developmental abnormalities of the gonad and abnormal sex hormone concentrations in juvenile alligators from contaminated and control lakes in Florida. Environ. Health Perspect., 102: 680-688.

Guillette, L.J. Jr., Crain, D.A., Rooney. A.A. and Pickford, D.B., 1995. Organization versus activation: the role of endocrine-disrupting contaminants (EDCs) during embryonic development in wildlife. Environ. Health Perspect., 103: 157-164.

Guillette, L.J. Jr., Arnold, S.F. and McLachlan. J.A., 1996. Ecoestrogens and embryos-is there a basis for concern? Anim. Reprod. Sci., 42: 13-24.

Gunderson, D.T., Krahling, M.D., Donosky, J.J., Cable, R.G., Mims, S.D., 1998. Polychlorinated biphenyls and chlordane in the gonads of paddlefish, *Polyodon spathula*, from the Ohio river. Bull. Environ. Contam. Toxicol., 61:650-657.

Hayes, W.J. Jr., 1991. Handbook of pesticide toxicology. San Diego: Academic Press.

Kanda, N. and Tamaki, K., 1999. Estrogen enhances immunoglobin production by human PBMCs. J. Allergy Clin. Immunol., 103:282-288.

Kelce, W.R., Monosson, E., Gamscik, M.P., Laws, S.C. and Gray, L.E., 1994. Environmental hormone disruptors: evidence that vinclozolin developmental toxicity is mediated by antiandrogenic metabolites. Toxicol. Appl. Pharm., 126: 276-285.

Koner, B.C., Banerjee, B.D. and Ray, A., 1998. Organochlorine pesticide-induced oxidative stress and immune suppression in rats. Indian J. Ex. Biol., 36: 395-398.

Lahvis, G.P., Wells, R.S., Kuehl, D.W., Stewart, J.L., Rhinehart, H.L. and Via, C.S., 1995. Decreased lymphocyte responses in free-ranging bottlenose dolphins (*Tursiops truncatus*) are associated with increased concentrations of PCBs and DDT in peripheral blood. Environ. Health Perspect., 103: 67-72.

Leitner, G., Landsman, T., Blum, O., Zaltsmann, N. and Heller, E.D., 1996. Effects of gonadal steroids and their antagonists on the humoral response of immune-selected broiler chickens. Poult. Sci. 75: 1373-1382.

Litwack, G., 1955. Photo determination of lysozyme activity. Soc. Exp. Biol. Med., 89: 401-403.

Luster, M.I., Faith, R.E. and McLachlan, J.A., 1978. Alterations in antibody response following *in utero* exposure to diethylstilbesterol. Bull. Environ. Contam. Toxicol., 20:433-437.

Maness, S.C., McDonnell, D.P. and Gaido, K.W., 1998. Inhibition of androgen receptor-dependent transcriptional activity by DDT isomers and methoxychlor in HepG2 human hepatoma cells. Toxicol. Appl. Pharm., 151: 135-42.

Maule, A.G. and Schreck, C.B., 1989. Glucocorticoid receptors in leukocytes and gill of juvenile coho salmon (*Oncorhynchus kisutch*). Gen. Comp. Endocrinol., 77: 448-455.

McMurray, R.W., 2001. Estrogen, prolactin, and autoimmunity: actions and interactions. Int. Immunopharmacol.,1: 995-1008.

Nelson, J.A., 1974. Effects of dichlorodiphenyltrichloroethane (DDT) analogs and polychlorinated biphenyl (PCB) mixtures on 17 β–[^{3}H] estradiol binding to rat uterine receptor. Biochem. Pharmacol., 23: 447-451.

Nelson, J.A., Struck, R.F. and James, R., 1978. Estrogenic activities of chlorinated hydrocarbons. Toxicol. Environ. Health, 4: 325-339.

Nikolaevich, K.N., Ivanovich, S.J. and Victotovich, S.S., 1991. Major reproductive hormones as regulators of cell to cell interactions in humoral immune responses. Brain Behav. Immun., 5:149-161.

Oregon Department of Environmental Quality., 2000. Human health risk assessment of chemical contaminants in four fish species from the middle Willamette River, Oregon. EVS Environment Consultants, Inc. EVS Project No. 2/839-01.

Pickering, A.D. and Christir. P., 1980. Sexual differences in the incidence and severity of ectoparasitic infestation of the brown trout, *Salmo trutta L.* J. Fish Biol., 16: 669-683.

Piferrer, F. and Donaldson, E.M., 1989. Gonadal differentiation in coho salmon, *Oncoryhynchus kisutch*, after a single treatment with androgen or estrogen at different stages during ontogenesis. Aquaculture., 77: 251-262.

Porta, M., Malats, N., Jariod, M., Grimalt, J.O., Rifa, J., Carrato, A., Guarner, L., Salas, A., Santiago-Silva, M., Corominas, J.M., Andreu, M. and Real, F.X., 1999. Serum concentrations of organochlorine compounds and K-ras mutations in exocrine pancreatic cancer. PANKRAS II Study Group. Lancet (North American edition)., 354: 2125-2129

Rehana, T and Rao, P.R., 1992. Effect of DDT on the immune system in swiss albino mice during adult and prenatal exposure: humoral responses. Bull. Environ. Contam. Toxicol., 48: 535-540.

Romieu, I., Hernandez-Avila, M., Lazcano-Ponce, E., Weber, J.P. and Dewailly, E., 2000. Breast cancer, lactation history, and serum organochlorines. Am. J. Epidemiol., 52: 363-370.

Rosenthal, H. and Alderdice., 1976. Sublethal effects of environmental stressors, natural and pollutional, on marine fish eggs and larvae. J. Fish Res. Can., 33: 2047-2065.

Sankaran, K. and Gurnani, S., 1972. On the variation in the catalytic activity of lysozyme in fishes. Indian J. Biochem. Bio., 9: 162-165.

Sheeler, C.Q., Dudley, M.W. and Khan, S.A., 2000. Environmental estrogens induce transcriptionally active estrogen receptor dimers in yeast: activity potentiated by the coactivator RIP140. Environ. Health Perspect., 108: 97-103.

Slater, C.H. and Schreck, C.B., 1993. Testosterone alters the immune response of chinook salmon (*Oncorhynchus tshawytscha*). Gen. Comp. Endocrinol., 89: 291-298.

Slater, C.H., Fitzpatrick, M.S. and Schreck, C.B., 1995. Characterization of an androgen receptor in salmonid lymphocytes: possible link to androgen-induced immunosuppression. Gen. Comp. Endocrinol., 100: 218-225.

Soto, A.M., Chung, K.L. and Sonnenschein, C., 1994. The pesticides endosulphan, toxaphene and dieldrin have estrogenic effects in human estrogen–sensitive cells. Environ. Health Perspect., 102: 380-383.

Sower, S.A. and Schreck, C.B., 1982. Steroid and thyroid hormones during sexual maturation of coho salmon (*Oncorhynchus kisutch*) in saltwater or freshwater. Gen. Comp. Endocrinol., 47: 42-53

Tatner, M.F., 1996. Natural changes in the immune system of fish. In: Iwama, G., Nakanishi, T., (eds). The fish immune system, organism, pathogen, and environment. Academic Press, San Diego, pp. 255-287.

Waltman, S.R., Burde, R.M. and Berios, J., 1971. Prevention of corneal homograft rejection by estrogens. Transplantation., 1: 194-196.

Wolff, M.S., Toniolo, P.G., Lee, E.W., Rivera, M. and Dubin, N., 1993. Blood levels of organochlorine residues and risk of breast cancer. J. Natl. Cancer I., 85: 648-652.

Yohn, D.S., 1973. Sex related resistance in hamsters to adenovirus oncogenesis. Prog. Exp. Tumor Res., 18: 138-165.

GENERAL CONCLUSIONS AND SPECULATIONS

In this thesis, we demonstrated that a flow cytometric method can be used to detect modulation of the immune system in juvenile chinook salmon. The first chapter of the thesis validated a flow cytometric assay for the detection of humoral immunity in chinook salmon. We showed that this assay can be useful for *in vitro* detection of immune modulation by steroids, and may be adapted for use as a technique for screening the effect of environmental contaminants both *in vitro* and *ex vivo*. The assay can be run successfully with leukocytes from blood, so could therefore be used as a non-lethal bio-indicator.

In the second chapter, the assay was used in conjuction with other techniques in an experiment to determine the immune effects of a laboratory exposure of chinook salmon to a known anthropogenic estrogen. In this experiment, two acute exposures to *o,p'*-DDE (a ubiquitous metabolite of the pesticide DDT) during embryogenesis and early postnatal development were sufficient to cause suppression of the humoral immune response in the juvenile fish 1 year later. The cell-mediated and innate immune parameters tested were not observed to be affected by the chemical exposure.

It is likely that the xenobiotic is exerting its activity through steroid-mediated pathways and the mechanism for observed developmental affects of DDT and its metabolites may be hormone receptor mediated. Recent studies have shown the presence of estrogen receptors on lymphoid cells and endocrine-immune interaction has been suggested as a possible mechanism for estrogen-induced immunomodulation in humans (Cutolo et al., 1995). Furthermore, Grossman (1984) reviewed a number of studies that show evidence suggesting that gonadal steroids play a significant role in

the regulation of the mammalian immune function. It is widely recognized in mammals that estrogen has pleiotropic effects on immune function. Whilst estrogen appears to supress cell-mediated immunity, it is thought to augment humoral-based immunity (Nikolaevich et al., 1991; Kanda and Tamaki, 1999; McMurray, 2001). Studies with broiler chickens show that estrogen's stimulatory role in humoral immunity can be blocked with the anti-estrogen tamoxifen (Leitner et al, 1996). In mice, LPS induced blastogenesis and IgM levels were significantly reduced by a 14-day exposure to the highly estrogenic synthetic hormone diethylstilbesterol (Bick et al., 1984). Similarly, *in utero* murine exposure to DES did not reduce the response to the T-dependent mitogen sheep red blood cells, but did suppress their response to LPS, a T-independent mitogen (Luster et al., 1978). Because DES has been shown to elicit estrogenic activity in many situations, including displacing estrogen from the ER and induction of vitellogenisis, it is of interest that the compound appears to be acting as an anti-estrogen in the context of the immune system. Similarly, *o,p'*-DDE has been shown to be 'weakly estrogenic' as evidenced by its ability to displace estrogen from the trout ER (Matthews et al., 2000), the human estrogen receptor (Chen et al., 1997) and the calf uterine receptor (Kramer and Giesy, 1999) amongst others. It has also been shown to induce vitellogenesis in trout (Donohoe and Curtis,1996).

We know that sex steroids exert their activity and achieve their response by binding to intracellular receptors within cells of different tissues of the body including the uterus, liver and gonads and immune tissues. Depending on its location within the cell, the receptor-steroid complex either translocates to the nucleus, or if already located in the nucleus it binds to the DNA, inducing translation of mRNA coding for

the transcription of a particular protein. This protein could be any number of a myriad of proteins vital to initiating a healthy humoral immune response. For example, it is known in mammalian immunology that estrogen binds to receptors on both T and B lymphoctes and is involved in the regulation of fas ligand – a membrane protein involved in apoptosis (Mor et al. 2000). Similarly, estrogen may be involved in initiating the synthesis of any number of enzymes required for cell signalling during B cell activation (Janeway and Travers, 1997). It is also possible that estrogen plays a part in the transcription of cytokines and even the immunoglobin itself.

A compound that is able to bind to the estrogen receptor, may or may not initiate gene transcription. In our experiment, the humoral immune response was suppressed by a compound that had been shown to bind to the estrogen receptor, thus I propose that the *o,p'*-DDE is acting as an antagonist in this situation, preventing the binding of estrogen to its receptor, but not exerting the same biological activities of the endogenous steroid. The chemical contaminant may therefore be depriving the humoral immune system of estrogen's stimulatory influence.

Another route of disruption that must be considered is that *o,p'*-DDE may be binding to the plasma binding globulins, thus releasing endogenous E_2 freely into the plasma, which may then signal to the pituitary to reduce production of endogenous E_2, through the negative feedback loop. However, we did not observe a dramatic difference in plasma E_2 of juvenile fish between control and treatment groups. This may or may not be because levels were low anyway since the fish were sexually immature, and significant differences may have been difficult to detect. I speculate that during spawning, when endogenous estrogen levels are high, the endogenous

estrogen may be acting to enhance the fishes' ability to fight disease during this period via the humoral arm of the immune system. Disruption by a compound such as *o,p'*-DDE at this critical stage may therefore bear much higher consequences.

Alternatively, it could be argued that the *o,p'*-DDE would be less likely to bind to plasma binding globulins than endogenous E_2. If this were the case, *o,p'*-DDE would be more bioavailable than E_2 and would be free to pass across cell membranes to reach the intracellular E_2 receptors, thus exerting a stronger biological impact despite its lower affinity for the estrogen receptor.

Other modes of action of *o,p*-DDE induced humoral immunosuppression that must be considered are direct toxicity to immune cells. Because the embryo is much more sensitive to hormone signals than the adult, this is a possibility. Alternatively, intereference by the contaminant with enzyme systems such as the biosynthesis or metabolism and excretion pathways of estradiol may have been involved with the immunosuppression observed. In particular, o,pDDE may stimulate cytochrome p450 clearance mechanisms for estrogen and hence reduce the number of moles of E_2 available at any one time.

We also observed humoral immunosuppression in the DMSO vehicle groups. This is interesting but not unexpected because currently DMSO is marketed in many countries for relief of pain, inflammation, allergies and auto-immmune diseases such as systemic lupus erythematosus and rheumatoid arthritis. It may be that auto antibodies are suppressed by the application of DMSO in humans. However, what is alarming that only 0.04% DMSO was required to elicit immunosuppression in fish, whilst topical application of up to 70 to 100% pure DMSO is recommended for the

relief of pain in humans. In the United States, DMSO is currently not FDA approved and cannot legally be marketed as a medication, it is however easily available as a 'solvent' and there is pressure from many to have it approved for medical use. There is a paucity of research on the affects of the substance, but clearly more studies are required on its mode of action before it is released for general use.

BIBLIOGRAPHY

Ablin, R.J., Bruns, G.R., Guinan, P. and Bush, I.M., 1974. The effect of estrogen on the incorporation of ^{3}H-thymidine by PHA-stimulated human peripheral blood lymphocytes. J. Immunol., 113: 705-707.

Ablin, R.J., Bhatti, R.A., Guinan, P. and Khin, W., 1979. Modulatory effects of estrogen on immunological responsiveness. II. Suppression of tumor-associated immunity in patients with prostatic cancer. Clin. Exp. Immunol., 38: 83-91.

Adkison, M.A., Basurco, B. and Hedrick, R.P., 1996. Humoral immunoglobins of the white sturgeon, *Acipenser transmontanus*: partial characterization of and recognition with monoclonal antibodies. Dev. Comp. Immunol., 20: 285-298.

Anderson, D. P., 1996. Environmental factors in fish health: Immunological aspects. In: The fish immune sytsem. Vol 15 ' Fish physiology', (G. Iwama and T. Nakanishi, eds) pp. 289-310. San Diego: Academic Press.

Arkoosh, M.R., Casillas, E., Clemons, E., Kagley, A.N., Olson, R., Reno, P. and Stein, J.E., 1998. Effect of pollution on fish diseases: potential impacts on salmonid populations. J. Aquat. Anim. Health., 10: 182-190.

Arkoosh, M. R. and Kaattari, S.L., 1990. Quantitation of fish antibody to a specific antigen by an enzyme linked immunosorbent assay (ELISA) In: Techniques in Fish Immunology (Stolen, J., Fletcher, T.C., Roberson, B.S., van Muiswinkel, W.B., eds) pp. 15-24. New Jersey: S.O.S. Publications.

Azuma, T., Milston, R.H., Heppell, S.A., and Schreck, C.B. Immunological parameters of masu salmon (*Oncorhynchus masou*) raised under various flow regimes. (unpublished data).

Bick, P.H., Tucker, A.N., White, K.L. Jr., and Holsapple, M.P. 1984. Effects of subchronic exposure to diethylstilbestrol on humoral immune function in adult female (C3B6) miceF1 mice. Immunopharmacology., 7:27-39.

Blair, P., 1981. Immunologic consequences of early exposure of experimental rodents to dithylstilbesterol (DES) in pregnancy (A. Herbst and H. Bern, eds). New York: Thieme-Stratton, pp.167-178.

Bull, J.J., Gutzke, W.H. and Crews, D., 1988. Sex reversal by estradiol in three reptilian orders. Gen. Comp. Endocrinol., 70: 425-428.

Castillo, A., Sanchez, C., Dominguez, J., Kaattari, S. and Villena, A.J., 1993. Ontogeny of IgM and CIgM bearing cells in Rainbow trout. Dev. Comp. Immunol., 17: 425-437.

Chen, C.W., Hurd, C., Vorojeikina, D.P., Arnold, S.F. and Notides, A.C., 1997. Transcriptional activation of the human estrogen receptor by DDT isomers and metabolites in yeast and MCF-7 cells. Biochem. Pharmacol., 53: 1161-1172.

Chilmonczyk, S., 1978. *In vitro* stimulation by mitogens of peripheral blood lymphocytes from rainbow trout (*Salmo gairdneri*). Annales D'Immunolgie., 129: 3-12.

Chilmonczyk, S., Voccia, I., Tarazona, J.V. and Monge, D.,1997. Flow cytometric analysis of fish leukocyte populations exposed to pollutants and pathogens: modulatory effects induced by experimental procedures. In Ecotoxicology: Responses, Biomarkers and Risk Assessment, (J.T. Zelikoff, editor) pp. 171-184.

Chilmonczyk,S and Monge, D., 1999. Flow cytometry as a tool for assessment of the fish cellular immune response to pathogens. Fish Shellfish Immun., 9: 319-333.

Cocco, P., Kazerouni, N. and Zahm, S.H., 2000. Cancer mortality and environmental exposure to DDE in the United States. Environ. Health Perspect., 108:1-4.

Colburn, T., vom Seal, F.S. and Soto, A.M., 1993. Developmental effects of endocrine-disrupting chemicals in wildlife and humans. Environ. Health Perspect., 101: 378-384.

Cross, M.L. and Willoughby, L.G., 1989. Enhanced vulnerability of rainbow trout (*Salmo gairdneri*) to Saprolegnia infection, following treatment of the fish with an androgen. Mycol. Res., 93: 379-383.

Crippen, T., Bootland, L., Leong, J., Fitzpatrick, M., Schreck, C. and Vella, A., 2001. Analysis of Salmonid leukocytes purified by hypotonic lysis of erythrocytes. J Aquat. Anim. Health., (in press).

Crippen, T., Bootland, L., Vella, A., Fitzpatrick, M., Schreck, C. and Leong. J., Flow cytometric evaluation of the in vitro proliferative response of teleost blood cells. Fish Shellfish Immun., (submitted).

Cutolo, M., Sulli, A., Seriolo, B., Accardo, S., and Masi, A.T., 1995. Estrogens, the immune response and autoimmunity. Clin. Exp. Rheumatol., 13: 217-226.

De Guise, S., Martineau, D., Beland, P. and Fournier, M., 1998. Effects of in vitro exposure of beluga whale leukocytes to selected organochlorines. J. Toxicol. Env. Health., 55: 479-493.

DeLuca, D., Wilson, M. and Warr, G.W., 1983. Lymphocyte heterogeneity in the trout, *Salmo gaidneri*, defined with monoclonal antibodies to IgM. Eur. J. Immunol., 13: 546-551.

DeVault, D.S., 1985. Contaminants in fish from Great Lakes harbors and tributary mouths. Arch. Environ. Contam. Toxicol., 14: 587-594.

Dewailly, E., Ayotte, P., Bruneau, S., Gingras, S., Belles-Isles, M. and Roy, R., 2000. Susceptibility to infections and immune status in Inuit infants exposed to organochlorines. Environ. Health Perspect., 108: 205-11.

Donahoe, R.M. and Curtis, L.R., 1996. Estrogenic activity of chlorodecone, o,p'-DDT and o,p'-DDE in juvenile rainbow trout: Induction of vitellogenesis and interaction with hepatic estrogen binding sites. Aquat. Toxicol., 36: 31-52.

Dunier, M., 1996. Water pollution and immunosuppression of freshwater fish. Ital. J. Zool., 63: 303-309.

Ellis, A. E., and de Sousa, M., 1974. Phylogeny of the lymphoid system. I. A study of the fate of circulating lymphocytes in plaice. Eur. J. Immunol., 4: 338-343.

Ellis, A.E., 1977. Ontogeny of the immune response in Salmo salar. Histogenesis of the lymphoid organs and appearance of membrane immunoglobin and mixed leukocyte reactivity. In: Solomon J.B., Horton, J.D. (eds), Developmetal Immunobiology. Elsevier/North-Holland Biomedical Press, Amsterdam; New York, pp. 225-231.

Feist, G., Schreck, CB., Fitzpatrick. M.S. and Redding, J.M., 1990. Sex steroid profiles of coho salmon (*Oncoryhynchus kisutch*) during early development and sexual differentiation. Gen. Comp. Endocrinol., 80: 299-313.

Feist, G., Yeoh, C., Fitzpatrick, M.S. and Schreck, C.B., 1995. The production of functional reversed male trout with 17α-methyltestosterone and 11β-hydroxyandrostenedione. Aquaculture., 131: 145-152.

Feist, G., 2000. Early development of the endocrine system in salmonids: Regulation of sex differentiation and ontogeny of the stress response. In: Ph.D Thesis. Oregon State University, pp. 90-115.

Fitzpatrick, M.S., Van der Kraak, G. and Schreck, C.B., 1986. Plasma profiles of sex steroids and gonadotropin in coho salmon (*Oncorhynchus kisutch*) during final maturation. Gen. Comp. Endocrinol., 62: 437-451.

Freeland, W.J., 1983. Parasites and the coexistence of animal host species. Am. Nat., 121: 223-236.

Fry, D.M., 1995. Reproductive effects in birds exposed to pesticides and industrial chemicals. Environ. Health Perspect., 103: 165-171.

Fuda, H., Soyano, K., Yamazaki, F. and Hara, A., 1991. Serum immunoglobin M (IgM) during early development of masu salmon (*Oncorhynchus masou*). Comp Biochem. Phys., 99A: 637-643.

Grace, M.F. and Manning, J.J., 1980. Histogenesis of the lymphoid organs in rainbow trout, *Salmo gaidneri Richardson 1836*. Dev. Comp. Immunol., 4: 225-264.

Grossman, C.J., 1984. Regulation of the immune system by sex steroids. Endocr. Rev., 5: 435-449.

Guillette, L.J. Jr., Gross, T.S., Masson, G.R., Matter, J.M., Percival, H.F. and Woodward, A. R., 1994. Developmental abnormalities of the gonad and abnormal sex hormone concentrations in juvenile alligators from contaminated and control lakes in Florida. Environ Health Perspect., 102: 680-688.

Guillette, L.J. Jr., Crain, D.A., Rooney. A.A. and Pickford, D.B., 1995. Organization versus activation: the role of endocrine-disrupting contaminants (EDCs) during embryonic development in wildlife. Environ. Health Perspect., 103: 157-164.

Guillette, L.J. Jr., Arnold, S.F. and McLachlan. J.A., 1996. Ecoestrogens and embryos-is there a basis for concern? Anim. Reprod. Sci., 42: 13-24.

Gunderson, D.T., Krahling, M.D., Donosky, J.J., Cable, R.G., Mims, S.D., 1998. Polychlorinated biphenyls and chlordane in the gonads of paddlefish, *Polyodon spathula*, from the Ohio River. Bull. Environ. Contam. Toxicol., 61:650-657.

Hayes, W.J. Jr., 1991. Handbook of pesticide toxicology. San Diego: Academic Press.

Jacobs, D.M. and Morrison, D.C., 1975. Stimulation of a T-independent primary anti-hapten response in vitro by TNP-lipopolysaccharide (TNP-LPS). J. Immunol., 144: 360-364.

Janeway, C.A. and Travers, P., 1997. Immunology: the immune system in health and disease. Current Biology Ltd. Garland Publishing Inc., London, New York.

Jerne, N.K. and Nordin, A.A., 1963. Plaque formation in agar by single antibody-producing cells. Science. 140: 405.

Julius, M.H., Simpson, E. and Herzenberg, L.A., 1973. A rapid method for the isolation of functional thymus-derived murine lymphocytes. Eur J. Immunol., 3: 645-649.

Kanda, N. and Tamaki, K., 1999. Estrogen enhances immunoglobin production by human PBMCs. J. Allergy Clin. Immunol., 103:282-288.

Kelce, W.R., Monosson, E., Gamscik, M.P., Laws, S.C. and Gray, L.E., 1994. Environmental hormone disruptors: evidence that vinclozolin developmental toxicity is mediated by antiandrogenic metabolites. Toxicol. Appl. Pharm., 126: 276-285.

Koner, B.C., Banerjee, B.D. and Ray, A., 1998. Organochlorine pesticide-induced oxidative stress and immune suppression in rats. Indian J. Ex. Biol., 36: 395-398.

Kramer, V.J. and Geisy, J.P.,1999. Specific binding of hydroxylated polychlorinated biphenyl metabolites and other substances to bovine calf uterine estrogen receptor: structure-binding relationships. Sci. Total Environ., 233:141-161.

Lahvis, G.P., Wells, R.S., Kuehl, D.W., Stewart, J.L., Rhinehart, H.L. and Via, C.S., 1995. Decreased lymphocyte responses in free-ranging bottlenose dolphins (*Tursiops truncatus*) are associated with increased concentrations of PCBs and DDT in peripheral blood. Environ. Health Perspect., 103: 67-72.

Lamas, J. and Ellis, A.E., 1994. Atlantic salmon (*Salmo salar*) neutrophil responses to *Aeromonas salmonicida*. Fish Shellfish Immun., 4, 201-219.

Leitner, G., Landsman, T., Blum, O., Zaltsmann, N. and Heller, E.D., 1996. Effects of gonadal steroids and their antagonists on the humoral response of immune-selected broiler chickens. Poult. Sci., 75: 1373-1382.

Litwack, G., 1955. Photo determination of lysozyme activity. Soc. Exp. Biol. Med., 89: 401-403.

Luster, M.I., Faith, R.E. and McLachlan, J.A., 1978. Alterations in antibody response following *in utero* exposure to diethylstilbesterol. Bull. Environ. Contam. Toxicol. 20:433-437.

Maness, S.C., McDonnell, D.P. and Gaido, K.W., 1998. Inhibition of androgen receptor-dependent transcriptional activity by DDT isomers and methoxychlor in HepG2 human hepatoma cells. Toxicol. Appl. Pharm., 151: 135-42.

Manning, M.J. and Nakanishi, T., 1996. The specific immune system: cellular defenses. In The fish immune system. Vol 15 'Fish Physiology', (G. Iwama and T. Nakanishi, eds) pp. 159-205. San Diego: Academic Press.

Maule, A.G. and Schreck, C.B., 1989. Glucocorticoid receptors in leukocytes and gill of juvenile coho salmon (*Oncoryhynchus kisutch*). Gen. Comp. Endocrinol., 77: 448-455.

Matthews, J., Celius, T., Halgren, R. and Zacharewski, T., 2000. Differential estrogen receptor binding of estrogenic substances: a species comparison. J. Steroid Biochem. Mol. Biol., 74:223-234.

Milston, R.H., Fitzpatrick, M.S., Vella, A.T., Gundersen, D., Leong, J.C and Schreck, C.B. Short term exposure of fall chinook salmon (*Oncoryhnchus tshawytscha*) to o,p'-DDE during early life history stages affects long-term competence of the humoral immune system. Aquat. Toxicol. (submitted).

Mor, G., Kohen, F., Garcia-Velasco, J., Nilson. J., Brown, W., Song, J. and Naftolin, F., 2000. Regulation of fas ligan expression in breast cancer by estrogen: functional differences between estradiol and tamoxifen. J. Steroid Biochem. Mol. Biol., 73:185-194.

Moritomo, T., Suehiro, M., Kodama, H., Amemori, T., Ohba, S., Nemoto, H., Hasegawa, A. and Yoshida, H., 1999. A new method for the identification and enumeration of carp granulocytes. Fish Pathol. 34: 85-86.

McMurray, R.W. 2001. Estrogen, prolactin, and autoimmunity: actions and interactions. Int. Immunopharmacol.,1: 995-1008.

Nelson, J.A., 1974. Effects of dichlorodiphenyltrichloroethane (DDT) analogs and polychlorinated biphenyl (PCB) mixtures on 17 β–[^{3}H] estradiol binding to rat uterine receptor. Biochem. Pharmacol., 23: 447-451.

Nelson, J.A., Struck, R.F. and James, R., 1978. Estrogenic activities of chlorinated hydrocarbons. Toxicol. Environ. Health., 4: 325-339.

Nikolaevich, K.N., Ivanovich, S.J. and Victotovich, S.S. 1991. Major reproductive hormones as regulators of cell to cell interactions in humoral immune responses. Brain Behav. Immun., 5:149-161.

Oregon Department of Environmental Quality, 2000. Human health risk assessment of chemical contaminants in four fish species from the middle Willamette River, Oregon. EVS Environment Consultants, Inc. EVS Project No. 2/839-01.

Pickering, A.D. and Christir. P., 1980. Sexual differences in the incidence and severity of ectoparasitic infestation of the brown trout, *Salmo trutta L.* J. Fish Biol., 16: 669-683.

Piferrer, F. and Donaldson, E.M., 1989. Gonadal differentiation in coho salmon, *Oncoryhynchus kisutch*, after a single treatment with androgen or estrogen at different stages during ontogenesis. Aquaculture., 77: 251-262.

Porta, M., Malats, N., Jariod, M., Grimalt, J.O., Rifa, J., Carrato, A., Guarner, L., Salas, A., Santiago-Silva, M., Corominas, J.M., Andreu, M. and Real, F.X., 1999. Serum concentrations of organochlorine compounds and K-ras mutations in exocrine pancreatic cancer. PANKRAS II Study Group. Lancet (North American edition)., 354: 2125-2129.

Press, C.M.L., Dannevig, B.H. and Landsverk, T., 1994. Immune and enzyme histochemical phenotypes of lymphoid and non-lymphoid cells within the spleen and head kidney of Atlantic salmon (*Salmo salar L.*). Fish Shellfish Immun., 4, 79-93.

Rehana, T and Rao, P.R., 1992. Effect of DDT on the immune system in swiss albino mice during adult and prenatal exposure: humoral responses. Bull. Environ. Contam. Toxicol., 48: 535-540.

Romieu, I., Hernandez-Avila, M., Lazcano-Ponce, E., Weber, J.P. and Dewailly, E., 2000. Breast cancer, lactation history, and serum organochlorines. Am. J. Epidemiol., 52: 363-370.

Rosenthal, H. and Alderdice., 1976. Sublethal effects of environmental stressors, natural and pollutional, on marine fish eggs and larvae. J. Fish Res. Bd. Can., 33: 2047-2065.

Sankaran, K. and Gurnani, S., 1972. On the variation in the catalytic activity of lysozyme in fishes. Indian J. Biochem. Bio., 9: 162-165.

Secombes, C.J., 1990. Isolation of salmonid macrophages and analysis of their killing activity. In: Techniques in Fish Immunology, Vol 1.(J.S. Stolen et al., eds) pp. 137-154. Fair Haven: SOS Publications.

Sheeler, C.Q., Dudley, M.W. and Khan, S.A., 2000. Environmental estrogens induce transcriptionally active estrogen receptor dimers in yeast: activity potentiated by the coactivator RIP140. Environ. Health Perspect., 108: 97-103.

Siegl, E., Nebe, B., Blunk, H. and Rychly, J., 1998. Detection of mitogen induced stimulation of leukocytes from the rainbow trout (*Oncorhynchus mykiss*) by flow cytometric analysis of intracellular calcium. Comp Biochem. Phys., 119, 915-923.

Sizemore, R.G., Miller, N.M., Cuchens, M.A., Lobb, C.L., and Clem, L.W., 1984. Phylogeny of lymphocyte heterogeneity: The cellular requirements for in vitro mitogenic responses of channel catfish leukocytes. J. Immunol., 133: 2920-2924.

Slater, C.H. and Schreck, C.B., 1993. Testosterone alters the immune response of chinook salmon (*Oncorhynchus tshawytscha*). Gen. Comp. Endocrinol., 89: 291-298.

Slater, C.H., Fitzpatrick, M.S. and Schreck, C.B., 1995. Characterization of an androgen receptor in salmonid lymphocytes: possible link to androgen-induced immunosuppression. Gen. Comp. Endocrinol., 100: 218-225.

Soto, A.M., Chung, K.L. and Sonnenschein, C., 1994. The pesticides endosulphan, toxaphene and dieldrin have estrogenic effects in human estrogen–sensitive cells. Environ. Health Perspect., 102: 380-383.

Sower, S.A. and Schreck, C.B., 1982. Steroid and thyroid hormones during sexual maturation of coho salmon (*Oncorhynchus kisutch*) in saltwater or freshwater. Gen. Comp. Endocrinol., 47: 42-53.

Tatner, M.F., 1996. Natural changes in the immune system of fish. In: Iwama, G., Nakanishi, T (eds), The fish immune system, organism, pathogen, and environment. Academic Press, San Diego, pp. 255-287.

Tripp, R.A., Maule, A.G., Schreck, C.B. and Kaattari, S.L., 1987. Cortisol mediated suppression of salmonid lymphocyte responses in vitro. Dev. Comp. Immunol., 11: 565-576.

Van der Heijden, M.H.T., Booms, G.H.R., Tanck, M.W.T., Rombout, J.H.W.M. and Boon, J.H., 1995. Influence of flumequine on in vivo mitogen responses of European eel (*Anguilla anguilla L,* 1758) lymphoid cells. Vet. Immunol. Immunop., 47: 143-152.

Voccia, I., Krzystyniak, K., Flipo, D. and Fuornier, M., 1994. In vitro mercury-related cytotoxicity and functional impairment of the immune cells of rainbow trout (*Oncorhynchus mykiss*). Aquat. Toxicol. 29: 199-203.

Voccia, I., Sanchez-Dardon, J., Dunier, M., Chilmonczyk, S. and Fournier, M., 1997. Contaminants on competence of rainbow trout macrophages. In: Chemically induced alteration in functional development and reproduction of fish. Setac Press, pp. 99-109.

Waltman, S.R., Burde, R.M. and Berios, J., 1971. Prevention of corneal homograft rejection by estrogens. Transplantation., 1: 194-196.

Wester, P.W., Vethaak, A.D. and van Muiswinkel, W.B., 1994. Fish as biomarkers in immunotoxicology. Toxicology., 86: 213-232.

Wolff, M.S., Toniolo, P.G., Lee, E.W., Rivera, M. and Dubin, N., 1993. Blood levels of organochlorine residues and risk of breast cancer. J. Natl. Cancer I., 85: 648-652.

Yasutake, W.T. and Wales, J.H., 1983. Microscopic anatomy of salmonids: An atlas. United States Department of the Interior, Fish and Wildlife Service; 150.

Yohn, D.S., 1973. Sex related resistance in hamsters to adenovirus oncogenesis. Prog. Exp. Tumor Res., 18: 138-165.

Zapata, A. G., 1979. Ultrastructural study of the teleost fish kidney. Dev. Comp.Immunol., 3: 55-65.

Zapata, A. G., 1981. Lynphoid organs of the teleost fish. II. Ultrastructure of renal lymphoid tisue in *Rutilus rutilus* and *Gobio gobio*. Dev. Comp. Immunol., 5: 685-690.

APPENDIX

Isolation and Characterization of an Estrogen Receptor in the Lymphoid Tissue of Chinook Salmon: Steps Taken to Optimize Cytosol Preparation and Receptor Assay Conditions.

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Purpose

1. To isolate and characterize an estrogen receptor (ER) in the lymphoid tissue of chinook salmon.

A) Tissue preparation

General Method Followed:

Yearling spring chinook salmon (n=20, approximate weight 100 – 200 g/fish) raised at the FPGL were netted from their tanks and killed by immersion in a lethal dose of 200 mg/L MS-222 buffered with 500 mg/L sodium bicarbonate. Fish of this age and size were used in an effort to attain sufficient tissue for receptor binding assays. The fish were bled from the caudal vasculature to reduce red blood cell counts in the pronephros tissue. Cytosol was isolated from the leukocytes of the pronephros following a modified method described by Slater et al. (1995). Individual pronephric tissues were removed and harvested in 1 ml RPMI + Alsevers solution. A single cell suspension was prepared by repeated aspiration with a 1 ml syringe. Gross debris was removed by straining through a 70 μm cell strainer and the cell suspensions was pooled then centrifuged at 500 g for 10 minutes at 4°C. The supernatant was discarded and the cell pellet will be reconstituted in 2 ml TEMS buffer at pH 7.4 (containing anti-protease). Both red and white blood cells were counted using a hemacytometer. If possible, red blood cells were removed. Cells were disrupted using a sonifier and then centrifuged for 20 minutes at 2000 x g at 4 °C. Endogenous

steroids were removed by incubating with 0.5% dextran-5% charcoal solution in TEMS buffer for 10 minutes. Charcoal was removed by centrifuging at 2000 x g for 20 minutes at 4 °C. The supernatant was ultra-centrifuged at 100,000 x g for 1 hour. The supernatant was considered the cytosol. The cytosol was stored at -80°C until assayed for receptor binding.

Key Points for optimizing preparation:

1. **Need about 20 fish ~ 100 g /fish, Kidney weight ~ 0.3 g**
2. **Kidney coagulates easily, harvest into RPMI/Alsever mix or isolation media (HBSS/Alsever mix).**
3. **Method of making SS suspension – through 70 um mesh with syringe plunger wash into 50 ml tube. (probably 10 kids per tube, ie 2 x 50 ml)**
4. **Spin**
5. **Reconst in 2 mls Tems + proteiase inh, wash 1 into the other => ~ 8 mls**
6. **Sonicate 5 sec on , 10 sec off (10 x)**
7. **Add 3ml DC**
8. **Total cytosol yeild = 8ml**
9. **Protein conc = 5.5- 9 mg/ml**

Protein Analysis

Protein content of the cytosol was determined using the Bradford Protein Assay (Bradford, 1976) and adjusted if necessary to achieve binding of less than 10% of the added radioligand.

B) Receptor Assay

Specific binding of tritiated estradiol in cytosol was calculated by the difference between total binding and non-specific binding following the methods developed for testosterone and cortisol by Slater et al., (1995) and Maule and Schreck, (1990) respectively. Total binding was found by incubation of the cytosol with tritiated estradiol or testosterone (5nM); non-specific binding will be found by incubating cytosol with tritiated estradiol and an excess of unlabeled estradiol 500nM. Unbound ligand was removed by incubation with a dextran-charcoal solution. An aliquot was removed to a scintillation vial and counted by spectrophotometry.

TRIAL 1.

Objective

To ascertain that the receptor assay was working by looking at the binding of testosterone to liver cytosol.

Methods and Results

Liver protein concentration ~ 10 mg ml^{-1}

2hr Incubation at $4^{o}C$

Charcoal 2.5%

Dextran 0.25%

		DPMS	Mean
Total Counts	50 µl 5 nM ^{3}HT + 700 µl TEMS	22383	22777
Total Counts	50 µl 5 nM ^{3}HT + 700 µl TEMS	23171	
Total Binding	50 µl 5 nM ^{3}HT + 150 µl cytosol + 50 µl TEMS	1608	1719
Total Binding	50 µl 5 nM ^{3}HT + 150 µl cytosol + 50 µl TEMS	1829	
Non Specific Binding	50 µl 5 nM ^{3}HT + 150 µl cytosol + 50 µl 2500 nM cold T	686	844
Non Specific Binding	50 µl 5 nM ^{3}HT + 150 µl cytosol + 50 µl 2500 nM cold T	1001	

% Total Binding = 1719/22777

= 7.5 %

Specific binding = Total Binding – Non Specific Binding

= 1719 – 844

= 876

% Specific Binding

(as % of Total Binding) = 876/ 1719

= 51 %

TRIAL 2

Objective

I) to prepare cytosol from pronephric tissue and to see if get binding of Estradiol, TA or Testosterone.

II) To compare incubation times and temperatures

Methods and Results

Date of Experiment:3/23/99

Prepared as above.

18 chinook, 15 Female, 3 Male

Mean fish weight = 130 g

Prior to Sonication had : ~ 7 mls of 9.6×10^7 cells/ml WBC

and 1.1×10^7 cells/ml RBC

Made 8 mls of cytosol

Protein concentration = 5.5 mg/ml

Results

#	Inc Temp And Time			DPM	Mean
1	4°C, 2 h	TC	50 µl 5 nM $^3E_2$ + 700 µl TEMS	51145	52158
2		TC	50 µl 5 nM $^3E_2$ + 700 µl TEMS	53170	
3		TB	50 µl 5 nM $^3E_2$ + 150 µl cytosol + 50 µl TEMS	1212	1112
4		TB	50 µl 5 nM $^3E_2$ + 150 µl cytosol + 50 µl TEMS	1240	
5		TB	50 µl 5 nM $^3HE_2$ + 150 µl cytosol + 50 µl TEMS	1124	
6		NSB	50 µl 5 nM $^3HE_2$ + 150 µl cytosol + 50 µl 2500 nM cold E_2	999	996
7		NSB	50 µl 5 nM $^3HE_2$ + 150 µl cytosol + 50 µl 2500 nM cold E_2	984	
8		NSB	50 µl 5 nM $^3HE_2$ + 150 µl cytosol + 50 µl 2500 nM cold E_2	1006	
9	4°C, 2 h	TC	50 µl 5 nM ^3HTA + 700 µl TEMS	9803	9309
10		TC	50 µl 5 nM ^3HTA + 700 µl TEMS	8815	
11		TB	50 µl 5 nM ^3HTA + 150 µl cytosol + 50 µl TEMS	136	126
12		TB	50 µl 5 nM ^3HTA + 150 µl cytosol + 50 µl TEMS	120	

13		TB	50 µl 5 nM ^{3}HTA + 150 µl cytosol + 50 µl TEMS	122	
14		NSB	50 µl 5 nM ^{3}HTA + 150 µl cytosol + 50 µl 2500 nM cold TA	106	109
15		NSB	50 µl 5 nM ^{3}HTA + 150 µl cytosol + 50 µl 2500 nM cold TA	109	
16		NSB	50 µl 5 nM ^{3}HTA + 150 µl cytosol + 50 µl 2500 nM cold TA	113	
17		TC	50 µl 5 nM ^{3}HT + 700 µl TEMS	25636	25120
18		TC	50 µl 5 nM ^{3}HT + 700 µl TEMS	24603	
19	4°C, 2 h	TB	50 µl 5 nM ^{3}HT + 150 µl cytosol + 50 µl TEMS	2313	2142
20		TB	50 µl 5 nM ^{3}HT + 150 µl cytosol + 50 µl TEMS	2032	
21		TB	50 µl 5 nM ^{3}HT + 150 µl cytosol + 50 µl TEMS	2080	
22		NSB	50 µl 5 nM ^{3}HT + 150 µl cytosol + 50 µl 2500 nM cold T	562	586
23		NSB	50 µl 5 nM ^{3}HT + 150 µl cytosol + 50 µl 2500 nM cold T	589	
24		NSB	50 µl 5 nM ^{3}HT + 150 µl cytosol + 50 µl 2500 nM cold T	607	
25		TC	50 µl 5 nM ^{3}HT + 700 µl TEMS	24616	24793
26		TC	50 µl 5 nM ^{3}HT + 700 µl TEMS	24969	
27	12°C, 2 h	TB	50 µl 5 nM ^{3}HT + 150 µl cytosol + 50 µl TEMS	1692	1505
28		TB	50 µl 5 nM ^{3}HT + 150 µl cytosol + 50 µl TEMS	1388	
29		TB	50 µl 5 nM ^{3}HT + 150 µl cytosol + 50 µl TEMS	1434	
30		NSB	50 µl 5 nM ^{3}HT + 150 µl cytosol + 50 µl 2500 nM cold T	521	546
31		NSB	50 µl 5 nM ^{3}HT + 150 µl cytosol + 50 µl 2500 nM cold T	572	
32		NSB	50 µl 5 nM ^{3}HT + 150 µl cytosol + 50 µl 2500 nM cold T	546	
33		TC	50 µl 5 nM ^{3}HT + 700 µl TEMS	27011	25825
34		TC	50 µl 5 nM ^{3}HT + 700 µl TEMS	24638	
35	4°C, 4 h	TB	50 µl 5 nM ^{3}HT + 150 µl cytosol + 50 µl TEMS	1739	1719
36		TB	50 µl 5 nM ^{3}HT + 150 µl cytosol + 50 µl TEMS	1725	

37		TB	50 µl 5 nM ^{3}HT + 150 µl cytosol + 50 µl TEMS	1694	
38		NSB	50 µl 5 nM ^{3}HT + 150 µl cytosol + 50 µl 2500 nM cold T	554	552
39		NSB	50 µl 5 nM ^{3}HT + 150 µl cytosol + 50 µl 2500 nM cold T	550	
40		NSB	50 µl 5 nM ^{3}HT + 150 µl cytosol + 50 µl 2500 nM cold T	error	
41	Free Tritium $^3HE_2$		50 µl 5 nM $^3HE_2$ + 200 µl TEMS + 500 µl charcoal	694	

Summary Table

Incubation Time and Temp	**Steroid**	**% Total Binding**	**% of TB that is Specific Binding**
4°C, 2 h	E_2	2.1	10
4°C, 2 h	TA	1.4	13
4°C, 2 h	T	8.5	73
12°C, 2 h	T	6.1	64
4°C, 4 h	T	6.7	68

Conclusion

No Binding for TA or E_2. Probably TA total counts concentration erroneously too low. This shows that the binding of T is as expected, so the assay and cytosol prep must be ok.

Optimum temp and incubation time = 4° C for 2 h for T. Ideally between 5 to 10% total binding, and between 50 to75% specific binding. Therefore, 8.5% and 73% respectively is perfect.

TRIAL 3.Repeat Prep

Objective

To try to optimise receptor assay for E_2 binding.

I) Can the concetration of charcoal used in the receptor assay reduce the NSBs for E_2 ?

II) Does Incubation Temperature and time increase total and specific binding for E_2?

Methods and Results

Date of Cytosol Prep:4/28/99

Date of Receptor Assay: 5/4/99

Cytosol prepared as above.

20 chinook, 19 Female, 1 Male

Mean fish weight = 205 g

Prior to Sonication had : ~ 8 mls of 8.4×10^7 cells/ml WBC

and 1.4×10^7 cells/ml RBC

Add 3mls dextran-charcoal

Made 8 mls of cytosol

Protein concentration = 9.5 mg/ml

Ran E_2 receptor assay at 2 concetrations of charcoal and 2 different temperatures and 2 different incubation times.

Results

#	Temp	Charcoal %			DPM	Mean
1	4°C 2 h	0.25 + 0.025	TC	50 µl 5 nM $^3HE_2$ + 700 µl TEMS	44647	43897
2			TC	50 µl 5 nM $^3HE_2$ + 700 µl TEMS	43146	
3			TB	50 µl 5 nM $^3HE_2$ + 150 µl cytosol + 50 µl TEMS	2560	2486
4			TB	50 µl 5 nM $^3HE_2$ + 150 µl cytosol + 50 µl TEMS	2407	
5			TB	50 µl 5 nM $^3HE_2$ + 150 µl cytosol + 50 µl TEMS	2490	
6			NSB	50 µl 5 nM $^3HE_2$ + 150 µl cytosol + 50 µl 2500 nM cold E_2	1693	1748
7			NSB	50 µl 5 nM $^3HE_2$ + 150 µl cytosol + 50 µl 2500 nM cold E_2	1921	
8			NSB	50 µl 5 nM $^3HE_2$ + 150 µl cytosol + 50 µl 2500 nM cold E_2	1629	
9	4°C 2 h	2.5 + 0.25	TC	50 µl 5 nM $^3HE_2$ + 700 µl TEMS	44647	43897
10			TC	50 µl 5 nM $^3HE_2$ + 700 µl TEMS	43146	
11			TB	50 µl 5 nM $^3HE_2$ + 150 µl cytosol + 50 µl TEMS	999	999
12			TB	50 µl 5 nM $^3HE_2$ + 150 µl cytosol + 50 µl TEMS	1022	
13			TB	50 µl 5 nM $^3HE_2$ + 150 µl cytosol + 50 µl TEMS	977	
14			NSB	50 µl 5 nM $^3HE_2$ + 150 µl cytosol + 50 µl 2500 nM cold E_2	553	555
15			NSB	50 µl 5 nM $^3HE_2$ + 150 µl cytosol + 50 µl 2500 nM cold E_2	548	
16			NSB	50 µl 5 nM $^3HE_2$ + 150 µl cytosol + 50 µl 2500 nM cold E_2	563	
17	12°C 2 h	0.25 + 0.025	TC	50 µl 5 nM $^3HE_2$ + 700 µl TEMS	36514	36103
18			TC	50 µl 5 nM $^3HE_2$ + 700 µl TEMS	35692	
19			TB	50 µl 5 nM $^3HE_2$ + 150 µl cytosol + 50 µl TEMS	1755	1726
20			TB	50 µl 5 nM $^3HE_2$ + 150 µl cytosol + 50 µl TEMS	1774	
21			TB	50 µl 5 nM $^3HE_2$ + 150 µl cytosol + 50 µl TEMS	1648	
22			NSB	50 µl 5 nM $^3HE_2$ + 150 µl cytosol + 50 µl 2500 nM cold E_2	1851	1495
23			NSB	50 µl 5 nM $^3HE_2$ + 150 µl cytosol + 50 µl 2500 nM cold E_2	1326	
24			NSB	50 µl 5 nM $^3HE_2$ + 150 µl cytosol + 50 µl 2500 nM cold E_2	1307	
25	12°C, 2 h	1.5 + 0.25	TC	50 µl 5 nM $^3HE_2$ + 700 µl TEMS	36514	36103
26			TC	50 µl 5 nM $^3HE_2$ + 700 µl TEMS	35692	
27			TB	50 µl 5 nM $^3HE_2$ + 150 µl cytosol + 50 µl TEMS	867	749
28			TB	50 µl 5 nM $^3HE_2$ + 150 µl cytosol + 50 µl TEMS	590	
29			TB	50 µl 5 nM $^3HE_2$ + 150 µl cytosol + 50 µl TEMS	789	
30			NSB	50 µl 5 nM $^3HE_2$ + 150 µl cytosol + 50 µl 2500 nM cold E_2	583	534
31			NSB	50 µl 5 nM $^3HE_2$ + 150 µl cytosol + 50 µl 2500 nM cold E_2	520	
32			NSB	50 µl 5 nM $^3HE_2$ + 150 µl cytosol + 50 µl 2500 nM cold E_2	499	
33	4°C 4 h	0.25 + 0.025	TC	50 µl 5 nM $^3HE_2$ + 700 µl TEMS	44169	51982
34			TC	50 µl 5 nM $^3HE_2$ + 700 µl TEMS	59795	
35			TB	50 µl 5 nM $^3HE_2$ + 150 µl cytosol + 50 µl TEMS	2928	2609
36			TB	50 µl 5 nM $^3HE_2$ + 150 µl cytosol + 50 µl TEMS	2473	
37			TB	50 µl 5 nM $^3HE_2$ + 150 µl cytosol + 50 µl TEMS	2425	
38			NSB	50 µl 5 nM $^3HE_2$ + 150 µl cytosol + 50 µl 2500 nM cold E_2	2433	2067
39			NSB	50 µl 5 nM $^3HE_2$ + 150 µl cytosol + 50 µl 2500 nM cold E_2	1898	
40			NSB	50 µl 5 nM $^3HE_2$ + 150 µl cytosol + 50 µl 2500 nM cold E_2	1870	
41	4°C 4 h	2.5 + 0.25	TC	50 µl 5 nM $^3HE_2$ + 700 µl TEMS	44169	51982
42			TC	50 µl 5 nM $^3HE_2$ + 700 µl TEMS	59795	
43			TB	50 µl 5 nM $^3HE_2$ + 150 µl cytosol + 50 µl TEMS	1364	1213
44			TB	50 µl 5 nM $^3HE_2$ + 150 µl cytosol + 50 µl TEMS	1152	
45			TB	50 µl 5 nM $^3HE_2$ + 150 µl cytosol + 50 µl TEMS	1124	
46			NSB	50 µl 5 nM $^3HE_2$ + 150 µl cytosol + 50 µl 2500 nM cold E_2	778	773
47			NSB	50 µl 5 nM $^3HE_2$ + 150 µl cytosol + 50 µl 2500 nM cold E_2	767	
48			NSB	50 µl 5 nM $^3HE_2$ + 150 µl cytosol + 50 µl 2500 nM cold E_2	984	
49	4°C 24 h	0.25 + 0.025	TC	50 µl 5 nM $^3HE_2$ + 700 µl TEMS	43908	43901
50			TC	50 µl 5 nM $^3HE_2$ + 700 µl TEMS	43894	
51			TB	50 µl 5 nM $^3HE_2$ + 150 µl cytosol + 50 µl TEMS	3323	3394
52			TB	50 µl 5 nM $^3HE_2$ + 150 µl cytosol + 50 µl TEMS	3440	
53			TB	50 µl 5 nM $^3HE_2$ + 150 µl cytosol + 50 µl TEMS	3420	
54			NSB	50 µl 5 nM $^3HE_2$ + 150 µl cytosol + 50 µl 2500 nM cold E_2	3155	3169
55			NSB	50 µl 5 nM $^3HE_2$ + 150 µl cytosol + 50 µl 2500 nM cold E_2	3127	
56			NSB	50 µl 5 nM $^3HE_2$ + 150 µl cytosol + 50 µl 2500 nM cold E_2	3224	
57			TC	50 µl 5 nM $^3HE_2$ + 700 µl TEMS	43908	43901
58			TC	50 µl 5 nM $^3HE_2$ + 700 µl TEMS	43894	

59	4°C 24 h	1.5 + 0.25	TB	50 µl 5 nM $^3HE_2$ + 150 µl cytosol + 50 µl TEMS	1261	1263
60			TB	50 µl 5 nM $^3HE_2$ + 150 µl cytosol + 50 µl TEMS	1299	
61			TB	50 µl 5 nM $^3HE_2$ + 150 µl cytosol + 50 µl TEMS	1230	
62			NSB	50 µl 5 nM $^3HE_2$ + 150 µl cytosol + 50 µl 2500 nM cold E_2	1133	1111
63			NSB	50 µl 5 nM $^3HE_2$ + 150 µl cytosol + 50 µl 2500 nM cold E_2	1109	
64			NSB	50 µl 5 nM $^3HE_2$ + 150 µl cytosol + 50 µl 2500 nM cold E_2	1092	

Summary Table

Incubation Time and Temp	Charcoal %	Steroid	% Total Binding	% of TB that is Specific Binding
4°C, 2 h	**0.25 + 0.025**	**E_2**	**5.7**	**29.7**
4°C, 2 h	**2. 5 + 0.25**	**E_2**	**2.3**	**44.4**
12°C, 2 h	0.25 + 0.025	E_2	4.8	13.4
12°C, 2 h	2. 5 + 0.25	E_2	4.1	28.7
4°C, 4 h	0.25 + 0.025	E_2	5.0	20.8
4°C, 4 h	2. 5 + 0.25	E_2	2.3	36.3
4°C, 24 h	0.25 + 0.025	E_2	7.7	6.6
4°C, 24 h	2. 5 + 0.25	E_2	7.2	12.0

Conclusions

From these results, the optimum conditions for maximizing specific binding are: temperature = 4° C, incubation time = 2h, charcoal/dextran conc = 2.5% charcoal + 0.25% dextran.

However, optimum conditions for maximizing total binding are: temperature = 4° C, incubation time = 2h, charcoal/dextran conc = 0.25% charcoal + 0.025% dextran.

Suggest trying a concentration of dextran-charcoal somewhere in between e.g 1.25% charcoal +0.125% dextran.

TRIAL 5.

Objective

I) To compare binding of E_2 to RBC cytosolic prep with binding of E_2 to WBC+RBC head kidney cytosol prep.

Methods

Prep date = 5/6/99

40 fish, mean weight 100g

Took head kidney and blood

Kindey Prep

Head kidney prep as above: gave 4 x 50 ml tubes, reconstitute in 2 mls of TEMS = 8mls, pooled and washed tubes gave 18 ml total.

WBC = 1.06×10^8 cells/ml RBC = 7.2×10^6 cells/ml

Added 5 ml dextran-charcoal

Total yield cytosol = 11 x 1.5 = 16.5 ml

Protein conc = 16 mg/ml

Blood Prep

Blood spun, plasma removed, white blood cell layer removed with pipette.

Blood prepared in exactly same way as kidney.i.e gave 4 x 50 ml tubes, reconstitute in 2 mls of TEMS = 8mls, pooled and washed tubes gave 18 ml total.

RBC = 1.85 x 10^9 cells/ml

Total yield cytosol = 11 x 1.5 = 16.5 ml

Protein conc = 75 mg/ml

Receptor Assay

Dilute head kidney cytosol to 10 mg/ml

Dilute RBC cytosol to 10 mg/ml

Dilute RBC cytosol to same RBCs/ml as was in head kidney cytosol prep (i.e 7.2 x 10^6 cells/ml).

Incubation = 4°C, 2h

Dextran-Charcoal = 2.5%+ 0.25%

Results

#			DPM	Mean
1	TC	50 µl 10 nM $^3HE_2$ + 700 µl TEMS	99650	101736
2	TC	50 µl 10 nM $^3HE_2$ + 700 µl TEMS	103822	
3	TB	50 µl 10 nM $^3HE_2$ + 150 µl blood cytosol @ 10 mg/ml protein + 50 µl TEMS	855	764
4	TB	50 µl 10 nM $^3HE_2$ + 150 µl blood cytosol @ 10 mg/ml protein + 50 µl TEMS	719	
5	TB	50 µl 10 nM $^3HE_2$ + 150 µl blood cytosol @ 10 mg/ml protein + 50 µl TEMS	717	
6	NSB	50 µl 10 nM $^3HE_2$ + 150 µl blood cytosol @ 10 mg/ml protein + 50 µl 5000 nM cold E_2	592	596
7	NSB	50 µl 10 nM $^3HE_2$ + 150 µl blood cytosol @ 10 mg/ml protein + 50 µl 5000 nM cold E_2	591	
8	NSB	50 µl 10 nM $^3HE_2$ + 150 µl blood cytosol @ 10 mg/ml protein + 50 µl 5000 nM cold E_2	604	
9	TB	50 µl 10 nM $^3HE_2$ + 150 µl blood cytosol @ 7.2 x 10^6 RBC/ml + 50 µl TEMS	1131	1111
10	TB	50 µl 10 nM $^3HE_2$ + 150 µl blood cytosol @ 7.2 x 10^6 RBC/ml + 50 µl TEMS	1030	
11	TB	50 µl 10 nM $^3HE_2$ + 150 µl blood cytosol @ 7.2 x 10^6 RBC/ml + 50 µl TEMS	1172	
12	NSB	50 µl 10 nM $^3HE_2$ + 150 µl blood cytosol @ 7.2 x 10^6 RBC/ml + 50 µl 5000 nM cold E_2	880	903
13	NSB	50 µl 10 nM $^3HE_2$ + 150 µl blood cytosol @ 7.2 x 10^6 RBC/ml + 50 µl 5000 nM cold E_2	948	
14	NSB	50 µl 10 nM $^3HE_2$ + 150 µl blood cytosol @ 7.2 x 10^6 RBC/ml + 50 µl 5000 nM cold E_2	880	
15	TB	50 µl 10 nM $^3HE_2$ + 150 µl head kidney cytosol @ 10 mg/ml protein & 7.2 x 10^6 RBC/ml + 50 µl TEMS	1585	1471
16	TB	50 µl 10 nM $^3HE_2$ + 150 µl head kidney cytosol @ 10 mg/ml protein & 7.2 x 10^6 RBC/ml + 50 µl TEMS	1437	
17	TB	50 µl 10 nM $^3HE_2$ + 150 µl head kidney cytosol @ 10 mg/ml protein & 7.2 x 10^6 RBC/ml + 50 µl TEMS	1392	
18	NSB	50 µl 10 nM $^3HE_2$ + 150 µl head kidney cytosol @ 10 mg/ml protein & 7.2 x 10^6 RBC/ml + 50 µl 5000 nM cold E_2	1010	

19	NSB	50 µl 10 nM $^3HE_2$ + 150 µl head kidney cytosol @ 10 mg/ml protein & 7.2 x 10^6 RBC/ml + 50 µl 5000 nM cold E_2	1063	1023
20	NSB	50 µl 10 nM $^3HE_2$ + 150 µl head kidney cytosol @ 10 mg/ml protein & 7.2 x 10^6 RBC/ml + 50 µl 5000 nM cold E_2	997	

Summary Table

Cytosol	Steroid	% Total Binding	% of TB that is Specific Binding
Blood @ 10 mg/ml protein	E_2	0.75	22.0
Blood @ 7.2 x 10^6 RBCs/ml	E_2	1.1	18.7
Head Kidney cytosol @ 10 mg/ml protein and 7.2 x 10^6 RBCs/ml	E_2	1.4	30.5

TRIAL 6.

Objective

Time course for E_2 binding

Methods and results

Experiment Date :5/11/99

Diluted cytosol to 10 mg/ml

2nM $^3HE_2$ with 1000 nM cold E_2 in final volume

% dextran-charcoal = 2.5% + 0.25%

Incubated for 1, 1.5, 2, 4, or 8h at either 4 deg C or 12 deg C

Results

#	Temp Time			DPM	Mean
1	4°C 0.5 h	TC	50 µl 10 nM $^3HE_2$ + 700 µl TEMS	101982	101797
2		TC	50 µl 10 nM $^3HE_2$ + 700 µl TEMS	101612	
3		TB	50 µl 10 nM $^3HE_2$ + 150 µl cytosol + 50 µl TEMS	1200	1190
4		TB	50 µl 10 nM $^3HE_2$ + 150 µl cytosol + 50 µl TEMS	1134	
5		TB	50 µl 10 nM $^3HE_2$ + 150 µl cytosol + 50 µl TEMS	1236	
6		NSB	50 µl 10 nM $^3HE_2$ + 150 µl cytosol + 50 µl 5000 nM cold E_2	852	844
7		NSB	50 µl 10 nM $^3HE_2$ + 150 µl cytosol + 50 µl 5000 nM cold E_2	824	
8		NSB	50 µl 10 nM $^3HE_2$ + 150 µl cytosol + 50 µl 5000 nM cold E_2	855	
9	4°C 1 h	TC	50 µl 10 nM $^3HE_2$ + 700 µl TEMS	99899	99526
10		TC	50 µl 10 nM $^3HE_2$ + 700 µl TEMS	99152	
11		TB	50 µl 10 nM $^3HE_2$ + 150 µl cytosol + 50 µl TEMS	1418	1369
12		TB	50 µl 10 nM $^3HE_2$ + 150 µl cytosol + 50 µl TEMS	1371	
13		TB	50 µl 10 nM $^3HE_2$ + 150 µl cytosol + 50 µl TEMS	1317	
14		NSB	50 µl 10 nM $^3HE_2$ + 150 µl cytosol + 50 µl 5000 nM cold E_2	939	918
15		NSB	50 µl 10 nM $^3HE_2$ + 150 µl cytosol + 50 µl 5000 nM cold E_2	895	
16		NSB	50 µl 10 nM $^3HE_2$ + 150 µl cytosol + 50 µl 5000 nM cold E_2	920	
17	12°C 1.5 h	TC	50 µl 10 nM $^3HE_2$ + 700 µl TEMS	96051	101044
18		TC	50 µl 10 nM $^3HE_2$ + 700 µl TEMS	106037	
19		TB	50 µl 10 nM $^3HE_2$ + 150 µl cytosol + 50 µl TEMS	1540	1486
20		TB	50 µl 10 nM $^3HE_2$ + 150 µl cytosol + 50 µl TEMS	1480	
21		TB	50 µl 10 nM $^3HE_2$ + 150 µl cytosol + 50 µl TEMS	1437	
22		NSB	50 µl 10 nM $^3HE_2$ + 150 µl cytosol + 50 µl 5000 nM cold E_2	1040	1063
23		NSB	50 µl 10 nM $^3HE_2$ + 150 µl cytosol + 50 µl 5000 nM cold E_2	1099	
24		NSB	50 µl 10 nM $^3HE_2$ + 150 µl cytosol + 50 µl 5000 nM cold E_2	1050	
25	12°C, 2 h	TC	50 µl 10 nM $^3HE_2$ + 700 µl TEMS	96966	95929
26		TC	50 µl 10 nM $^3HE_2$ + 700 µl TEMS	94891	
27		TB	50 µl 10 nM $^3HE_2$ + 150 µl cytosol + 50 µl TEMS	1740	1643
28		TB	50 µl 10 nM $^3HE_2$ + 150 µl cytosol + 50 µl TEMS	1576	
29		TB	50 µl 10 nM $^3HE_2$ + 150 µl cytosol + 50 µl TEMS	1612	
30		NSB	50 µl 10 nM $^3HE_2$ + 150 µl cytosol + 50 µl 5000 nM cold E_2	1051	1073
31		NSB	50 µl 10 nM $^3HE_2$ + 150 µl cytosol + 50 µl 5000 nM cold E_2	1086	
32		NSB	50 µl 10 nM $^3HE_2$ + 150 µl cytosol + 50 µl 5000 nM cold E_2	1083	
33	4°C 4 h	TC	50 µl 10 nM $^3HE_2$ + 700 µl TEMS	99938	99990
34		TC	50 µl 10 nM $^3HE_2$ + 700 µl TEMS	100042	
35		TB	50 µl 10 nM $^3HE_2$ + 150 µl cytosol + 50 µl TEMS	1841	1779
36		TB	50 µl 10 nM $^3HE_2$ + 150 µl cytosol + 50 µl TEMS	1812	
37		TB	50 µl 10 nM $^3HE_2$ + 150 µl cytosol + 50 µl TEMS	1685	
38		NSB	50 µl 10 nM $^3HE_2$ + 150 µl cytosol + 50 µl 5000 nM cold E_2	1131	1178
39		NSB	50 µl 10 nM $^3HE_2$ + 150 µl cytosol + 50 µl 5000 nM cold E_2	1216	
40		NSB	50 µl 10 nM $^3HE_2$ + 150 µl cytosol + 50 µl 5000 nM cold E_2	1187	
41	4°C 8 h	TC	50 µl 10 nM $^3HE_2$ + 700 µl TEMS	102082	100604
42		TC	50 µl 10 nM $^3HE_2$ + 700 µl TEMS	99125	
43		TB	50 µl 10 nM $^3HE_2$ + 150 µl cytosol + 50 µl TEMS	1982	1961
44		TB	50 µl 10 nM $^3HE_2$ + 150 µl cytosol + 50 µl TEMS	1978	
45		TB	50 µl 10 nM $^3HE_2$ + 150 µl cytosol + 50 µl TEMS	1924	
46		NSB	50 µl 10 nM $^3HE_2$ + 150 µl cytosol + 50 µl 5000 nM cold E_2	1314	

47		NSB	50 µl 10 nM $^3HE_2$ + 150 µl cytosol + 50 µl 5000 nM cold E_2	1271	1287
48		NSB	50 µl 10 nM $^3HE_2$ + 150 µl cytosol + 50 µl 5000 nM cold E_2	1275	

Summary Table

Incubation Time and Temp	Steroid	% Total Binding	% of TB that is Specific Binding
4°C, 0.5 h	E_2	1.2	29.1
4°C, 1.0 h	E_2	1.4	32.9
4°C, 1.5 h	E_2	1.5	28.5
4°C, 2.0 h	**E_2**	**1.7**	**34.7**
4°C, 4.0 h	E_2	1.8	33.8
4°C, 8.0 h	E_2	1.9	34.4

Conclusion

From this timecourse, the maximum specific binding was found at 4°C, 2h. However, total binding is too low.

TRIAL 7

Objective:

To find optimum cytosol dilution for E_2 receptor assay

Methods

Experiment date 5/12/01

Used head kidney cytosol prep from 5/6/01

Diluted cytosol with TEMS to :

Neat (16mg/ml)

10 mg/ml

7.5 mg/ml

5 mg/ml

Incubation Time = 2 h

Incuabtion Temp = 4°C

Dextran-charcoal = 2.5% and 0.25%

Results

#	Cytosol Dilution Mg/ml			DPM	Mean
1	16	TC	50 µl 10 nM $^3HE_2$ + 700 µl TEMS	96408	96450
2		TC	50 µl 10 nM $^3HE_2$ + 700 µl TEMS	96492	
3		TB	50 µl 10 nM $^3HE_2$ + 150 µl cytosol + 50 µl TEMS	1559	1579
4		TB	50 µl 10 nM $^3HE_2$ + 150 µl cytosol + 50 µl TEMS	1566	
5		TB	50 µl 10 nM $^3HE_2$ + 150 µl cytosol + 50 µl TEMS	1611	
6		NSB	50 µl 10 nM $^3HE_2$ + 150 µl cytosol + 50 µl 5000 nM cold E_2	1084	1090
7		NSB	50 µl 10 nM $^3HE_2$ + 150 µl cytosol + 50 µl 5000 nM cold E_2	1109	
8		NSB	50 µl 10 nM $^3HE_2$ + 150 µl cytosol + 50 µl 5000 nM cold E_2	1078	
9	10	TC	50 µl 10 nM $^3HE_2$ + 700 µl TEMS	96408	96450
10		TC	50 µl 10 nM $^3HE_2$ + 700 µl TEMS	96492	
11		TB	50 µl 10 nM $^3HE_2$ + 150 µl cytosol + 50 µl TEMS	2034	2071
12		TB	50 µl 10 nM $^3HE_2$ + 150 µl cytosol + 50 µl TEMS	2071	
13		TB	50 µl 10 nM $^3HE_2$ + 150 µl cytosol + 50 µl TEMS	2108	
14		NSB	50 µl 10 nM $^3HE_2$ + 150 µl cytosol + 50 µl 5000 nM cold E_2	1310	1305
15		NSB	50 µl 10 nM $^3HE_2$ + 150 µl cytosol + 50 µl 5000 nM cold E_2	1281	
16		NSB	50 µl 10 nM $^3HE_2$ + 150 µl cytosol + 50 µl 5000 nM cold E_2	1325	
17	7.5	TC	50 µl 10 nM $^3HE_2$ + 700 µl TEMS	96408	96450
18		TC	50 µl 10 nM $^3HE_2$ + 700 µl TEMS	96492	
19		TB	50 µl 10 nM $^3HE_2$ + 150 µl cytosol + 50 µl TEMS	1905	1882
20		TB	50 µl 10 nM $^3HE_2$ + 150 µl cytosol + 50 µl TEMS	1851	
21		TB	50 µl 10 nM $^3HE_2$ + 150 µl cytosol + 50 µl TEMS	1890	
22		NSB	50 µl 10 nM $^3HE_2$ + 150 µl cytosol + 50 µl 5000 nM cold E_2	1176	1189
23		NSB	50 µl 10 nM $^3HE_2$ + 150 µl cytosol + 50 µl 5000 nM cold E_2	1171	
24		NSB	50 µl 10 nM $^3HE_2$ + 150 µl cytosol + 50 µl 5000 nM cold E_2	1219	
25		TC	50 µl 10 nM $^3HE_2$ + 700 µl TEMS	96408	96450
26		TC	50 µl 10 nM $^3HE_2$ + 700 µl TEMS	96492	

27	5	TB	50 µl 10 nM $^3HE_2$ + 150 µl cytosol + 50 µl TEMS	1039	1049
28		TB	50 µl 10 nM $^3HE_2$ + 150 µl cytosol + 50 µl TEMS	1093	
29		TB	50 µl 10 nM $^3HE_2$ + 150 µl cytosol + 50 µl TEMS	1014	
30		NSB	50 µl 10 nM $^3HE_2$ + 150 µl cytosol + 50 µl 5000 nM cold E_2	795	806
31		NSB	50 µl 10 nM $^3HE_2$ + 150 µl cytosol + 50 µl 5000 nM cold E_2	833	
32		NSB	50 µl 10 nM $^3HE_2$ + 150 µl cytosol + 50 µl 5000 nM cold E_2	789	

Summary Table

Head Kidney Cytosol Dilution mg/ml	Steroid	% Total Binding	% of TB that is Specific Binding
16	E_2	1.6	31.0
10	E_2	2.1	37.0
7.5	E_2	1.95	36.8
5	E_2	1.1	23.2