AN ABSTRACT OF THE THESIS OF

Ichiro Misumi for the degree of Master of Science in Microbiology presented on July 24, 2003. Title: Immune Responses of Juvenile Chinook Salmon (*Oncorhynchus tshawytscha*) to p,p'-DDE and Tributyltin.

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Carl B. Schreck

In this thesis, we examined the effects of the exposures to anthropogenic pollutants on the fish, primarily juvenile chinook salmon, immune system using newly and recently developed immune assays. In addition, we developed a new assay for measuring immunocompetence of fish. In the first chapter, the Alamar Blue assay was developed to quantify the proliferation of chinook salmon (*Oncorhynchus tshawytscha*) leukocytes. Isolated splenic and pronephric leukocytes were stimulated with different concentration of mitogens (LPS, PWM, and ConA) for various incubation times. Optimum cell culture conditions (cell density, mitogen concentration, and incubation time) for the Alamar Blue assay were evaluated by comparison with flow cytometric analysis. The Alamar Blue dye was non-toxic for leukocytes, and the assay proved to be able to quantify the mitogenic responses using LPS, but PWM and ConA.

In the second chapter, we determined the effects and mechanisms by which p,p'-DDE exposure might affect the immune system of chinook salmon (*Oncorhynchus tshawytscha*). Isolated salmon splenic and pronephric leucocytes were incubated with

different concentrations of p,p'-DDE, and cell viability, induction of apoptosis, and mitogenic responses were measured by flow cytometry and Alamar Blue assay. p,p'-DDE significantly reduced cell viability and proliferation and increased apoptosis. The effect of p,p'-DDE on pronephric leukocytes was more severe than on splenic leukocytes, likely because pronephric leucocytes had a higher proportion of granulocytes, cells that appear more sensitive to p,p'-DDE. The effect of p,p'-DDE on leucocytes appeared to vary between developmental stages or season. The mitogenic response of leukocytes of chinook salmon exposed to p,p'-DDE *in vivo* exhibited a biphasic dose-response relationship. Only leukocytes isolated from salmon treated with 59 ppm p,p'-DDE had a significantly lower percentage of Ig+ blasting cells than controls. Our results support the theory that exposure to chemical contaminants could lead to an increase in disease susceptibility and mortality of fish due to immune suppression.

In the third chapter, we evaluated the direct effects of *in vitro* exposures to tributyltin (TBT), widely used biocide, on the cell mediated immune system of chinook salmon (*Oncorhynchus tshawytscha*). Splenic and pronephric leukocytes isolated from juvenile chinook salmon were exposed for 6, 24, or 96 hr to a concentration range of 0.03 – 0.1 mg TBT l^{-1} in cell cultures. Effects of TBT on cell viability, induction of apoptosis, and mitogenic responses were measured by flow cytometry. Splenic and pronephric leukocytes in the presence of TBT experienced a concentration-dependent decrease in the viability in cell cultures following the induction of apoptosis. In addition, pronephric lymphocytes exhibited a greater sensitivity to TBT exposure than pronephric granulocytes. The functional ability of splenic B-cells to undergo blastogenesis upon LPS stimulation was also significantly inhibited in the presence of 0.05, 0.07, or 0.10 mg

l^{-1} of TBT in the cell cultures. Flow cytometric assay with the fluorescent conjugated monoclonal antibody against salmon surface immunoglobulin was employed for the conclusive identification of B-cell in the chinook salmon leukocytes. Our findings suggest that adverse effects of TBT on the function or development of fish immune systems could lead to an increase in disease susceptibility and its subsequent ecological implications.

Immune Responses of Juvenile Chinook Salmon (*Oncorhynchus tshawytscha*) to p,p'-DDE and Tributyltin.

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CONTRIBUTION OF AUTHORS

Dr. Carl B. Schreck was the major professor and involved in all aspects of this work. Dr. Anthony T. Vella was involved in providing knowledge and direction on the immunological aspects of this work. Lisbeth Siddens helped with sample collecting and preparation of experiments. Dr. Jo-Ann C. Leong shared her knowledge and provided laboratory space, supplies and equipment. Dr. Teruyuki Nakanishi contributed to the vision for developing the Alamar Blue assay. Dr. Takashi Yada was involved in providing ideas for measuring apoptosis of fish. Dr. Grant Feist contributed to data interpretation and analysis.

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General Introduction

A number of studies have reported that fishes are at high risk for contracting infectious diseases in highly contaminated areas (McCain, 1991; Watermann *et al.*, 1992; Arkoosh *et al.*, 2001). Increased mortality from disease can contribute to a major decline of natural fish populations (Gulland, 1995; Arkoosh *et al.*, 1998a). Anthropogenic compounds and mixtures clearly alter the function or development of fish immune systems (Zeeman and Brindley, 1981; Anderson *et al.*, 1984; Arkoosh *et al.*, 1994, 1996, 1998b; Carlson *et al.*, 2002). Potentially impaired immune function consequent to man-made stress could make fish more vulnerable to infectious diseases and tumors. However, it is practically difficult to elucidate the direct link between contaminants and fish diseases in a field study because numerous potential causal factors are involved in the aquatic environment (Grinwis *et al.*, 1998). Therefore, laboratory studies are needed to validate and explain complicated toxicological effects of environmental contaminants (McCain *et al.*, 1988; Danizeau, 1998). Furthermore, the need exists to develop accurate, cost effective and simple techniques to allow comprehensive monitoring of fish immune function and evaluate the impact of man-made stress on fish health.

Objectives of this master's thesis were to develop a new mitogenic-response assay for measuring immunocompetence of fish and to examine the effects of the exposures to anthropogenic pollutants on the fish immune system using those newly developed assays. This thesis consists of three chapters. In chapter 1, Alamar Blue assay was developed and validated to quantify the functional competence of immune system in chinook

salmon (*Oncorhynchus tshawytscha*). Optimum cultural conditions were evaluated with comparisons with the flow cytometric assay. In chapter 2, effects of *in vitro* and *in vivo* exposures to p,p'-DDE, the main metabolite of DDT, on immune systems were examined. Modes of action of p,p'-DDE induced humoral immunosuppression were discussed. In chapter 3, direct effects of *in vitro* exposures to tributyltin (TBT), a biocide widely used as agricultural fungicide and antifouling paint for vessels, on the cell mediated immune system of chinook salmon. The mitogenic-response assay was carried out to examine and discuss a possible suppression of TBT on potential function of B-cell mediated humoral immunity.

Chapter 1

The Development of the Alamar Blue Assay for the Quantification of Mitogenic Response of Chinook Salmon (*Oncorhynchus tshawytscha*) Leukocytes

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Abstract

The Alamar Blue assay was developed to quantify the proliferation of chinook salmon (*Oncorhynchus tshawytscha*) leukocytes. Isolated splenic and pronephric leukocytes were stimulated with different concentrations of mitogens [lipopolysaccharide (LPS), pokeweed mitogen (PWM), and concanavalan A (ConA)] for different incubation times. Optimum cell culture conditions (cell density, mitogen concentration, and incubation time) for the Alamar Blue assay were evaluated by comparison with the flow cytometric assay. The Alamar Blue dye was non toxic for leukocytes, and the assay proved to be able to quantify the mitogenic responses using LPS, but not PWM and ConA.

Keywords- Alamar Blue; chinook salmon; flow cytometry; leukocytes; LPS; mitogenic response

Introduction

Several techniques are available to monitor the immune function of fish (Wester *et al.*, 1994; Anderson, 1996). One of the most frequently used biomarkers to assess the function of the cellular immune system is the mitogenic response of lymphocytes. Cell proliferation is measured *in vitro* after isolated lymphocytes are activated by specific or non-specific mitogens such as concanavalin A (Con A), lipopolysaccharide (LPS), or pokeweed mitogen (PWM).

Recently, a colorimetric assay based on Alamar Blue has received attention to measure the mitogenic response of leukocytes (Ahmed *et al.*, 1994; Nakayama *et al.*, 1997; Byth *et al.*, 2001). This assay is very simple, safe, and rapid compared to the traditional [^{3}H]-thymidine incorporation assay; it also allows cells to remain undamaged for further analysis (Mosmann, 1983; Zhi-Jun *et al.*, 1997). The Alamar Blue assay is considerably less expensive and suitable for measuring large sample size relative to flow cytometric assays described by Chilmonczyk & Monge (1998) and Milston *et al.* (2003). This assay makes use of Alamar Blue dye (oxidation-reduction indicator based test kit) that provides both colorimetric and fluorometric change in response to cellular metabolic activity in culture (Pagé *et al.*, 1993). When Alamar Blue is taken into cells and reduced by cellular activity, it changes from blue (nonfluorescent) to pink (fluorescent). Relative cell proliferation can be measured spectrophotometrically or fluorometrically. While Alamar Blue has been used to assess mammalian lymphocytes (Ahmed *et al.*, 1994; de Fries & Mitsuhashi, 1995), at present only one study has reported the application of Alamar Blue to quantification of mitogenic response in fish (Yagi *et al.*, 2001).

The objective of this study was to develop and validate a reliable, simple, and accurate colorimetric assay to quantify the mitogenic response in chinook salmon (*Oncorhynchus tshawytscha*). Flow cytometric assays developed in our lab oratory allowed comparison and evaluation of results of the Alamar Blue assay.

Material and Methods

FISH

Juvenile spring chinook salmon (Marion Folks stock) weighing 150-200 g were housed at the Fish Performance and Genetics Laboratory, Oregon State University, Corvallis, Oregon, and acclimated for at least 10 months. The fish were maintained in 0.9 m-diameter circular fiberglass tanks supplied with 12-13 °C flow-through water system under natural photoperiod and fed a commercial diet of Semi-Moist Pellets (BioOregon™, Warrenton, OR) twice daily. Sampling was conducted from August to September 2002.

TISSUE CULTURE MEDIUM

Tissue culture medium (TCM) contained 7% heat-inactivated fetal bovine serum, 1% L-glutamine, 200 IU ml^{-1} penicillin, and 0.2 mg ml^{-1} streptomycin in Minimum Essential Media (MEM) buffered with sodium bicarbonate. Isolation-medium was composed of Hank's balanced salt solution and Alserver's solution (0.1 M dextrose, 70 mM sodium chloride, and 30 mM sodium citrate). MEM was purchased from Invitrogen Co. (Carlsbad, CA) and all other regents added into TCM were purchased from Sigma Chem. Co. (St. Louis, MO).

ISOLATION AND CULTIVATION OF LEUKOCYTES

Fish were rapidly netted from their tanks, immediately killed in 200 mg l^{-1} buffered tricaine methane-sulfonate, weighed, and then bled by caudal severance. The

fish were then transported on ice to our immunology laboratory at Oregon State University within 30 min. Spleen and pronephros were aseptically isolated and placed separately into individual conical tubes filled with 1 ml of cold isolation medium. The isolated tissue was placed on a 40 μm nylon cell strainer (Becton Dickinson, Franklin Lakes, NJ) and gently disrupted with the end of a 3 cc syringe plunger. The disrupted tissue was washed through a strainer into a 50 ml polypropylene conical tube (Becton Dickinson) with isolation medium. The homogenized tissue suspension was then centrifuged at 500 g for 7 min at 4°C and the supernatant was aspirated. The pellet was resuspended with 2 ml of ice-cold isolation medium, and clumps were removed. The organ removal and tissue processing were all conducted under aseptic conditions in a laminar flow hood.

In order to separate and purify leukocytes from erythrocytes, hypotonic lysis was used as described by Crippen *et al.* (2001). Briefly, 2 ml of cell suspension were diluted with 9 ml sterile deionized water to lyse the erythrocytes for 20 s, and then 1 ml of sterile 10X PBS was immediately added to stop lysis. Cells were washed twice by centrifugation at 500 g for 7 min at 4°C, the supernatant aspirated, and the cell resuspensed with 2 ml of ice-cold TCM. After the purification of leukocytes, viable cells were counted using a trypan blue exclusion test, and the cell suspension was diluted with ice-cold TCM to a final concentration of viable cells. The cell suspension (100 μl $well^{-1}$) was plated out into flat bottom 96-well plates (Becton Dickinson), and then TCM with or without LPS was added to a final volume of 200 μl $well^{-1}$. The cell cultures were maintained at 17 °C in an incubator culture chamber (C.B.S. Scientific CO., Del Mar, CA)

containing blood gas mixture (10% O_2, 10% CO_2, and 80% N_2) and set a wet paper towel for humidity.

ALAMAR BLUE ASSAY

Alamar Blue dye was purchased from BioSource international, Inc. (Camarillo, CA). According to procedures of the manufacture's protocol and Yagi (2001), 20 μl $well^{-1}$ of Alamar Blue (10% of the final volume of the cell suspension present in each well) was added to the cell culture 24 hr before measurement. Optical densities (OD) at 570 nm (reduced) and 600 nm (oxidized) were then measured with an OPTImax™ Tunable Microplate Reader (Molecular Devices Co., Sunnyvale, CA). The specific absorbance (SA; $OD_{570} - OD_{600}$) was calculated using a computer software, SOFTmax® PRO (Molecular Devices CO.) to calibrate overlap between the two wavelengths (Zhi-Jun *et al.*, 1994). The sensitivity of Alamar Blue assay to mitogenic proliferative response was evaluated by calculating the stimulation index (SI; =SA of stimulated culture / SA of unstimulated culture). SI indicates the level of mitogenic response; a SI of 1 indicates no proliferation induced by mitogen, while a higher SI indicates greater sensitivity to the response.

FLOW CYTOMETRIC ASSAY

The flow cytometric assay measuring functional humoral immunocompetence in salmonids was developed by Milston *et al.* (2003). Following Alamar Blue analysis, flow cytometric assays were performed using the same cell cultures as those used in the Alamar Blue assay in order to check cell viability and morphological changes of

leukocytes after mitogenic response. 200 µl of cell suspensions in 96 well plates were rinsed twice by centrifugation at 300 g for 3 min at 4°C. Supernatants were discarded, and cells were resuspended with 200 µl of ice-cold PBS and 20 µl of propidium iodide (PI; Sigma Chem. Co.) solution (50 µg ml^{-1} in PBS). Cell suspensions were kept on ice for 5 - 10 min in the dark.

Viability and morphological changes of leukocytes were analyzed by flow cytometry (FACScan®; Becton Dickinson). Analysis of forward (a measure for cell size) and side (a measure of cell granularity, complexity) scatter patterns of splenic leukocytes indicated two major populations (Figure1.1 a). One population (smaller cell size) was gated R1, and another population (larger cell size) was gated R2. Cells staining negative for PI were considered to be viable cells, and PI positive cells were considered to be dead cells and excluded from further analysis (Scharsack *et al*, 2000) (Figure 1.1 b). The percentage of viable (PI negative) cells in total leukocytes was calculated using software, Cell Quest (Becton Dickinson). Based on the PI staining analysis, most cells in the population R1 on Figure 1.1 (a) were identified as dead cells. On the other hand, R2 was identified as the viable lymphocyte population based on the microscopic analysis with Wright-Giemsa staining using Hema 3 stain set (Biochemical Sciences, Inc., Swedesboro, NJ) following isolation of population R2 using a cell sorter (MoFlo®, Cytomation, Inc., Fort Collins, CO). Flow cytometry was also used to detect blasting splenic lymphocytes stimulated by mitogens (Figure 1.1 d). The blasting lymphocyte population was distinguished from the resting lymphocyte population based on cell size (Figure 1.1 c).

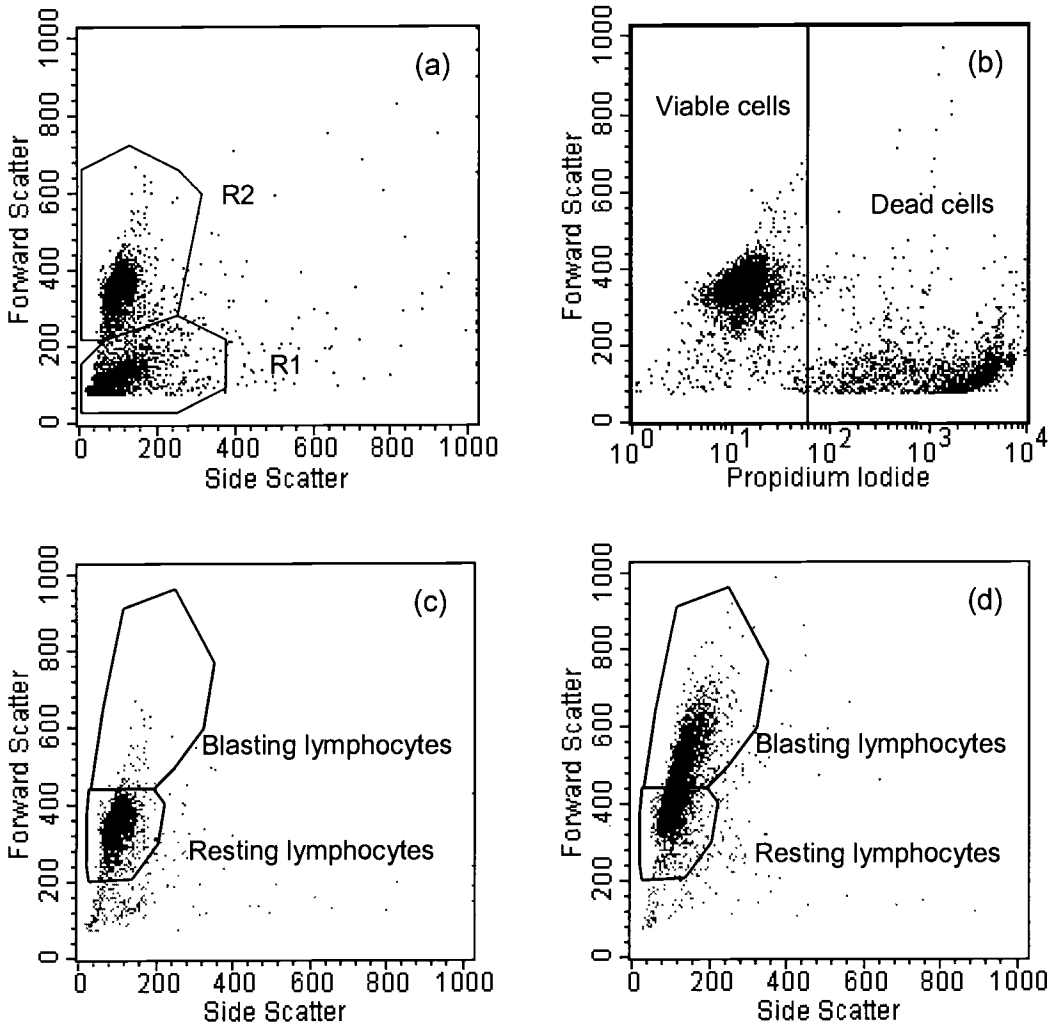

Figure 1.1. Flow cytometric dot plots of splenic leukocytes. Forward and side scatter dot plots of splenic leukocytes (a). PI staining for eliminating dead cells (b). Viable splenic leukocytes incubated without LPS (c). Viable splenic leukocytes incubated with LPS (d). After 4 days in the culture.

Analysis of forward and side scatter patterns of pronephric leukocytes indicated three major populations (R1, R2, and R3) (Figure 1.2 a). Most cells in the cell population R1 were identified as dead cells based on PI staining examination and excluded from further analysis (Figure 1.2 b). R2 was identified as the lymphocyte population, and R3 was identified as the granulocyte population based on same methods used for splenic leukocyte analysis. In contrast to the splenic leukocyte analysis, the pronephric leukocyte analysis involved measurements of relative mean cell size rather than the percentage of blasting cells. The reason for this change is based on our observation that it was very difficult to distinguish between blasting pronephric lymphocytes and non-blasting granulocytes because they overlap in size (Figure 1.2 d).

TOXICITY OF ALAMAR BLUE

Following dilution of cell suspension to $2.5x10^5$ or $5.0x10^5$ per well, leukocytes were incubated with or without Alamar Blue for 24hr. The viability of leukocytes was measured by flow cytometry using PI stain. We used 12 fish for this test.

THE RELATIONSHIP BETWEEN CELL DENSITY AND ABSORBANCE

After the purification of leukocytes, viable cells were counted using the trypan blue exclusion test, and the cell suspension was diluted with ice-cold TCM to a final concentration ($0.625x10^5$ - $20x10^5$ cells per well) of viable cells. The cell suspension (200 μl $well^{-1}$) was plated out into flat bottom 96-well plates and then incubated with Alamar Blue for 24 hours. Following the Alamar Blue assay, viability and cellular morphological change were measured using flow cytometry.

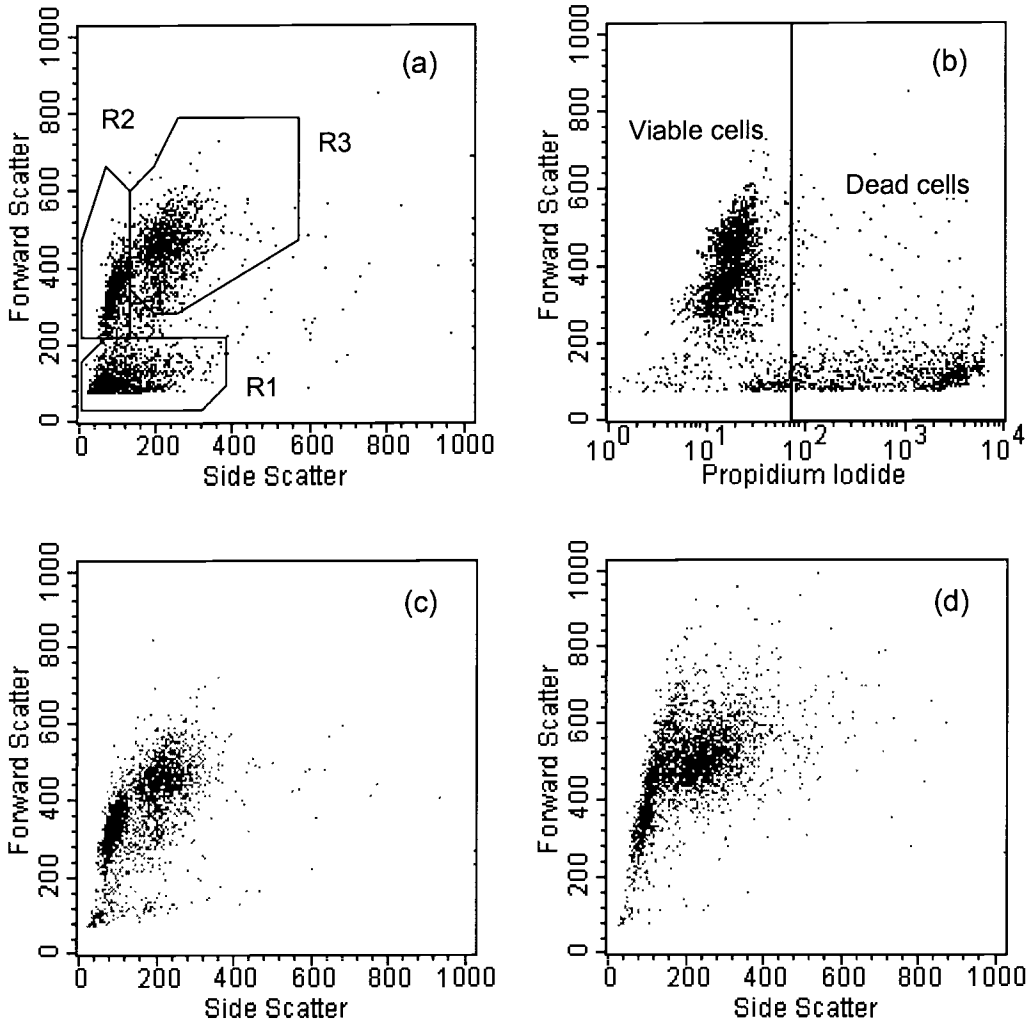

Figure 1.2. Flow cytometric dot plots of pronephric leukocytes. Forward and side scatter dot plots of pronephric leukocytes (a). PI staining for eliminating dead cells (b). Viable pronephric leukocytes incubated without LPS (c). Viable pronephric leukocytes incubated with LPS (d). After 4 days in the culture.

OPTIMUM CELL DENSITY

In order to examine the optimum cell density for the Alamar Blue assay, counted splenic or pronephric leukocytes were resuspended to four different final concentrations (0.625, 1.25, 2.5, and 5.0 $x10^5$ cells per well) and incubated with or without LPS from *Escherichia coli* serotype O 55:B5 (200 mg l^{-1}) for 4 days. The SI was calculated for each treatment. Following the Alamar Blue assay, the viability and the cellular morphological change were measured by the flow cytometric assay.

EFFECTIVE LPS CONCENTRATION AND INCUBATION TIME

Splenic and pronephric leukocytes ($2.5x10^5$ cells $well^{-1}$) were treated with 4 different LPS concentrations (0, 50, 100, 200, or 300 mg l^{-1}), and the SA was measured after exposure to LPS for 2, 3, 4, 5, 6, 7, and 8 days in order to determine the optimal or effective LPS concentration and incubation time. The flow cytometric assay was conducted using same cell suspensions used to measure the SA.

LEUKOCYTE STIMULATION USING OTHER MITOGENS

Splenic and pronephric leukocytes ($2.5x10^5$ cells $well^{-1}$) were incubated with or without PWM (0, 25, 50, 100, or 200 mg l^{-1}) (Sigma) for 3, 4, 5, and 6 days. Splenic and pronephric leukocytes ($2.5x10^5$ cells $well^{-1}$) were incubated with or without ConA (0, 5, 10, 20, or 30 mg l^{-1}) (Sigma) for 1, 2, 3, 4, and 5 days. Following the Alamar Blue assay, the viability and the cellular morphological change were measured by the flow cytometric assay.

STATISTICAL ANALYSIS

For each test, cells were collected from 4 – 18 fish. There was no replication of assays within any individual fish. Data were averaged, and standard error of mean (S.E.M.) was determined. Percent values were transformed by arcsine of the square root of the value for further statistical analysis. For the experiment assessing the toxicity of Alamar Blue dye, a parametric statistical test (Student's t- test) was used. Measurements were considered significant when *P* values were below 0.05. For other experiments, parametric statistical tests (the Student's t-test and ANOVA) and non-parametric statistical tests (the Kruskal-Wallace test and the Mann-Whitney U-test) were used for statistical comparisons of the data. Measurements were considered significant when *P* values of both parametric and non-parametric tests were below 0.05. Tukey's honestly significant difference procedure (HSD) was used to determine which treatments were significantly different from others.

Results

TOXICITY EVALUATION OF ALAMAR BLUE

There was no statistical significant difference in cell viability between leukocytes exposed and unexposed to Alamar Blue at two different cell densities (t-test; $P>0.05$) (Table 1.1).

RELATIONSHIP BETWEEN CELL DENSITY AND SA

The SA increased with cell density following 24 hr incubation of unstimulated cells with Alamar Blue (Figure 1.3). Although both splenic and pronephric leukocytes showed similar trends, the SA of pronephric leukocytes was always higher than that of splenic leukocytes. Splenic and pronephric leukocytes reached a threshold at $12.5x10^5$ and $10x10^5$ cells per well, respectively.

OPTIMUM CELL DENSITY

The SA increased with cell density (Figures 1.4 a, b). By way of exception, SAs of LPS treated pronephric cell cultures with $2.5x10^5$ and $5.0x10^5$ cells per well were same. All cell suspensions treated with LPS had greater SAs than those treated with TCM alone. As shown in Fig.4c, cell suspensions with lower cell densities induced higher SIs. Since a higher SI indicates a higher level of proliferation and greater sensitivity to the response, the optimum cell density for Alamar Blue assay is between 0.5×10^5 and 2.5×10^5 cells per well for splenic leukocytes and 0.5×10^5 cells per well for pronephric leukocytes. LPS alone (without cells) did not affect the color change of

Table 1.1. The mean (±S.E.M) percent of cell viability of splenic and pronephric leukocytes from chinook salmon after exposure to Alamar Blue (AB).

Cell density ($x10^5$cells/well)	Cell viability (%)			
	Spleen		Pronephros	
	No AB	With AB	No AB	With AB
2.5	73.25±3.09	72.22±2.80	81.11±1.35	79.06±1.37
5.0	77.17±2.78	73.74±3.31	84.81±1.29	82.49±1.40

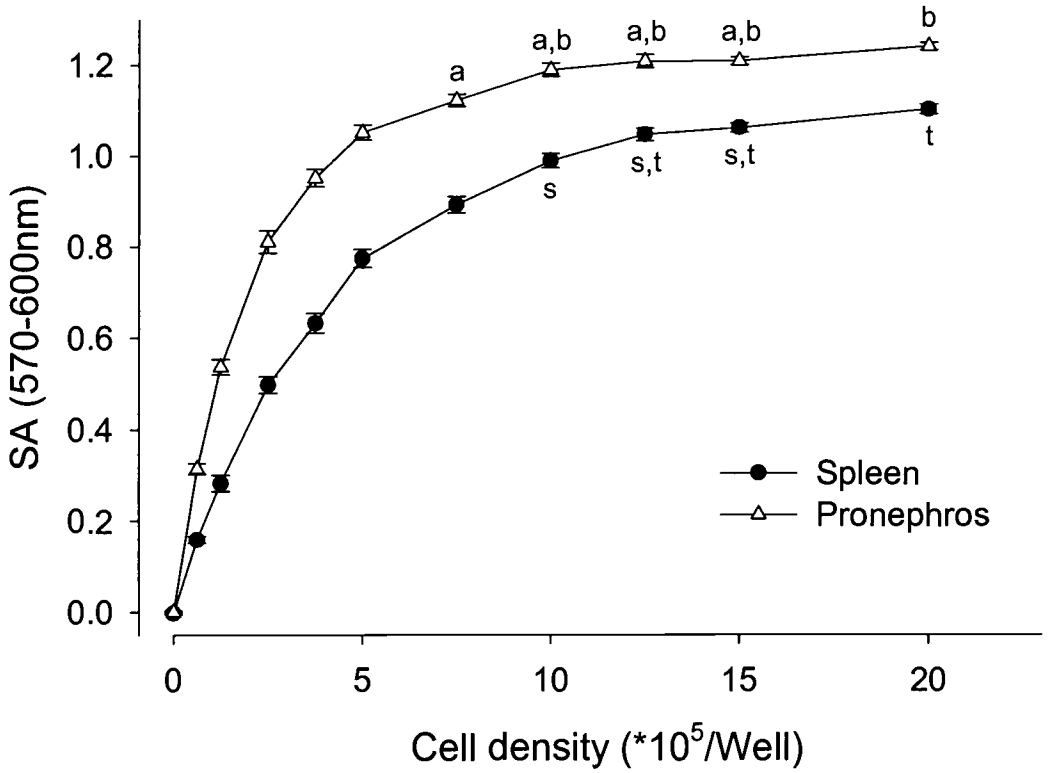

Figure 1.3. The effect of the initial cell density on the specific absorbance (SA: OD 570 nm minus 600 nm) (mean ± S.E.M.) of chinook salmon splenic and pronephric leukocytes cultures in the Alamar Blue assay. Cells were plated at densities of 0.625, 1.25, 2.5, 3.75, 5.0, 7.5, 10.0, 12.5, 15.0, and 20.0 X 10^5 cells per well in 96-well culture plates. For the respective treatments, sample size n=12, 12, 12, 9, 18, 12, 12, 6, 6, and 6. Cells were incubated with 10% of Alamar Blue for 24 hours at 17 C° after isolation. Dots that have at least one same letter are not significantly different (P>0.05, ANOVA and Kruskal-Wallace test).

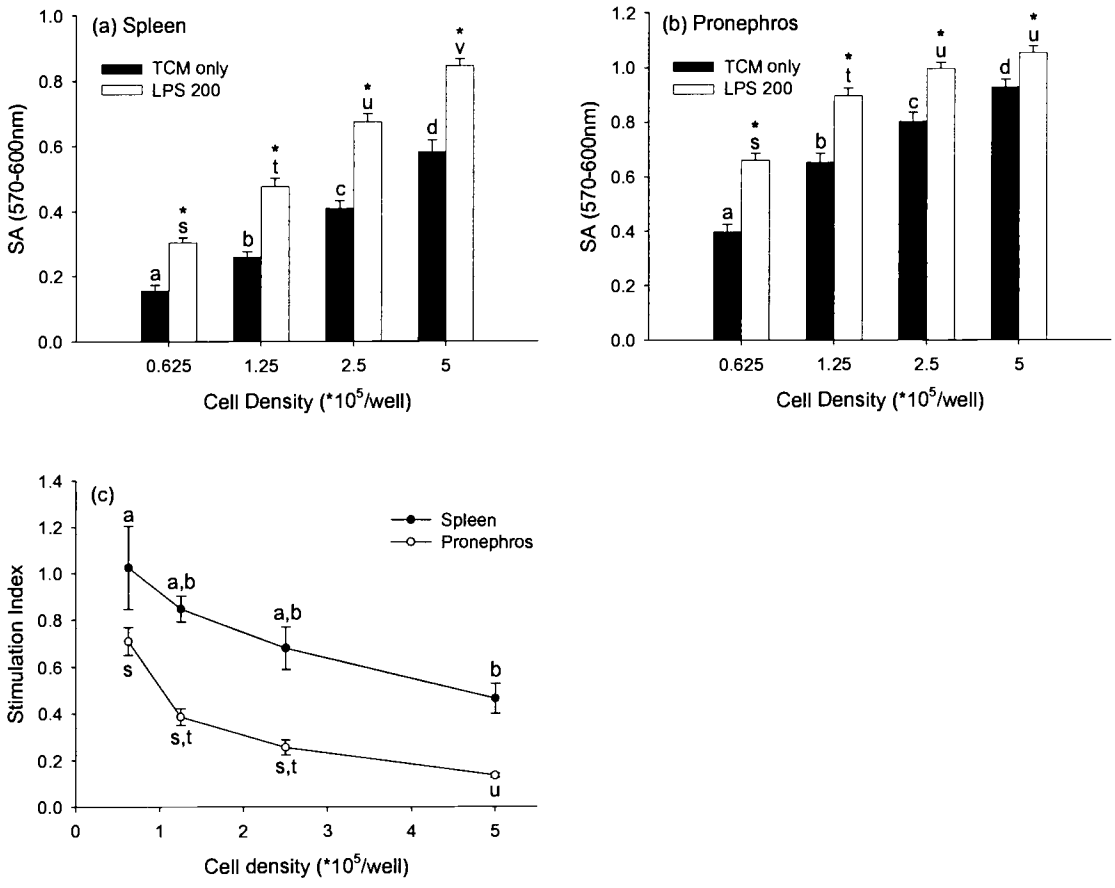

Figure 1.4. The effect of the initial cell density on the specific absorbance (SA: OD 570 nm minus 600 nm) of mitogen stimulated and unstimulated leukocytes of chinook salmon in the Alamar Blue assay. Cells were plated at densities of 0.625, 1.25, 2.5, and 5 X 10^5 cells per well in 96-well culture plates. Splenic (a) and pronephric (b) leukocytes were incubated without or with LPS (200mg/l) for 4 days. The results are expressed as the mean SA $\pm$ S.E.M. of three independent experiments each using three different individual fish (n=9). The columns in bar graphs that have at least one same letter are not significantly different ($P>0.05$, ANOVA and Kruskal-Wallace test). Significant differences between LPS and control (TCM alone) within a same treatment group are denoted * ($P<0.05$, t-test and Mann-Whitney test). The stimulation index (mean $\pm$ S.E.M.) was obtained by dividing mean SA of unstimulated culture by mean SA of LPS-stimulated cultures (c). Dots that have at least one same letter are not significantly different from each other. ($P>0.05$, ANOVA and Kruskal-Wallace test).

the Alamar Blue dye in the culture with TCM.

Cell viabilities of splenic and pronephric leukocytes in the LPS treated cultures were higher than control values at all cell densities (Figures 1.5 a, b). Although no significant difference in the cell viability of splenic leukocytes was observed among control cultures at all cell densities; the cell viability of LPS treated splenic leukocytes increased with cell density (Figure 1.5 a). The cell viability of pronephric leukocytes treated with TCM alone also increased with cell density (Figure 1.5 b). The percentage of blasting cells in LPS treated culture also increased with cell density, but the percentage of blasting cells in control wells did not change with cell density (Figure 1.5 c). Although the mean cell size of pronephric leukocytes did not change with cell density, the LPS treated cells were always larger than those from control cultures (Figure 1.5 d).

EFFECTIVE LPS CONCENTRATION AND INCUBATION TIME

There were significant differences in SA between splenic cell cultures treated with TCM alone and with any LPS concentrations (50 - 300 mg l^{-1}) from day 3 through day 8 (Figure 1.6 a). All concentrations of LPS (50 - 300 mg l^{-1}) induced the same SAs within the groups (Day 2 – Day 8) (Figure 1.6 a). SAs of splenic cell cultures treated with all concentrations of LPS significantly increased from day 2 to day 5. Then, SAs treated with 200 and 300 mg l^{-1} of LPS significantly decreased at day 8. Those treated with 50 and 100 mg l^{-1} of LPS were stable from day 5 through day 8. The SA of splenic cell cultures treated with TCM alone was stable from day 2 through day 7 but significantly decreased from day 5 to day 8.

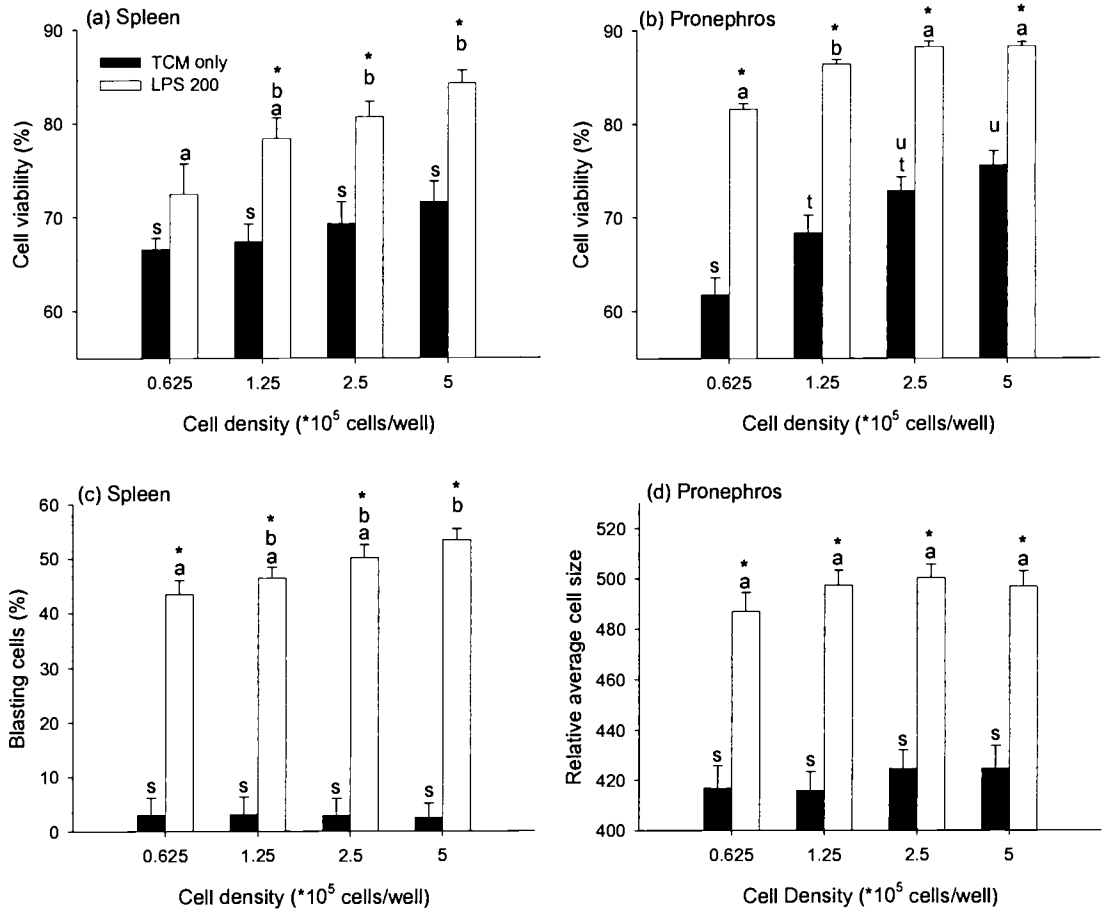

Figure 1.5. The effect of the initial cell density on the cell viability and the blastogenesis of mitogen stimulated and unstimulated leukocytes of chinook salmon. Cells were plated at densities of 0.625, 1.25, 2.5, and 5 X 10^5 cells per well in 96-well culture plates. Splenic and pronephric leukocytes were incubated without or with LPS (200mg/l) for 4 days. The viability of splenic (a) and pronephric (b) leukocytes were measured by the flow cytometric assay. The percentage of blasting splenic leukocytes (c) and the relative size of pronephric leukocytes (d) were also measured by the flow cytometric assay. Results are expressed as the mean $\pm$ S.E.M. of three independent experiments each using three different individual fish (n=9). Columns in bar graphs that have the same letter are not significantly different ($P>0.05$, ANOVA and Kruskal-Wallace test). Significant differences between LPS and control (TCM alone) within a same treatment group are denoted * ($P<0.05$, t-test and Mann-Whitney test).

Figure 1.6. The effect of the incubation time and the LPS concentration on the specific absorbance (SA: OD 570 nm minus 600 nm) (mean ± S.E.M.) of chinook salmon leukocyte suspensions in the Alamar Blue assay. Cells were plated at densities of 2.5×10^5 cells per well in 96-well culture plates. Splenic (a) and pronephric (b) leukocytes were incubated without or with LPS (50, 100, 200, or 300 mg/l) for 8 days. SA was measured after 2, 3, 4, 5, 6, 7, and 8 days after the incubation. For the respective treatments, sample size n=6, 9, 12, 9, 9, 9, and 6. Columns in bar graphs that have at least one same letter are not significantly different within the group treated with same concentration of LPS ($P>0.05$, ANOVA and Kruskal-Wallace test). Significant differences between LPS and control (TCM alone) within a same treatment group are denoted (*$0.01<P<0.05$, **$0.001<P<0.01$, ***$P<0.001$; t-test and Mann-Whitney test).

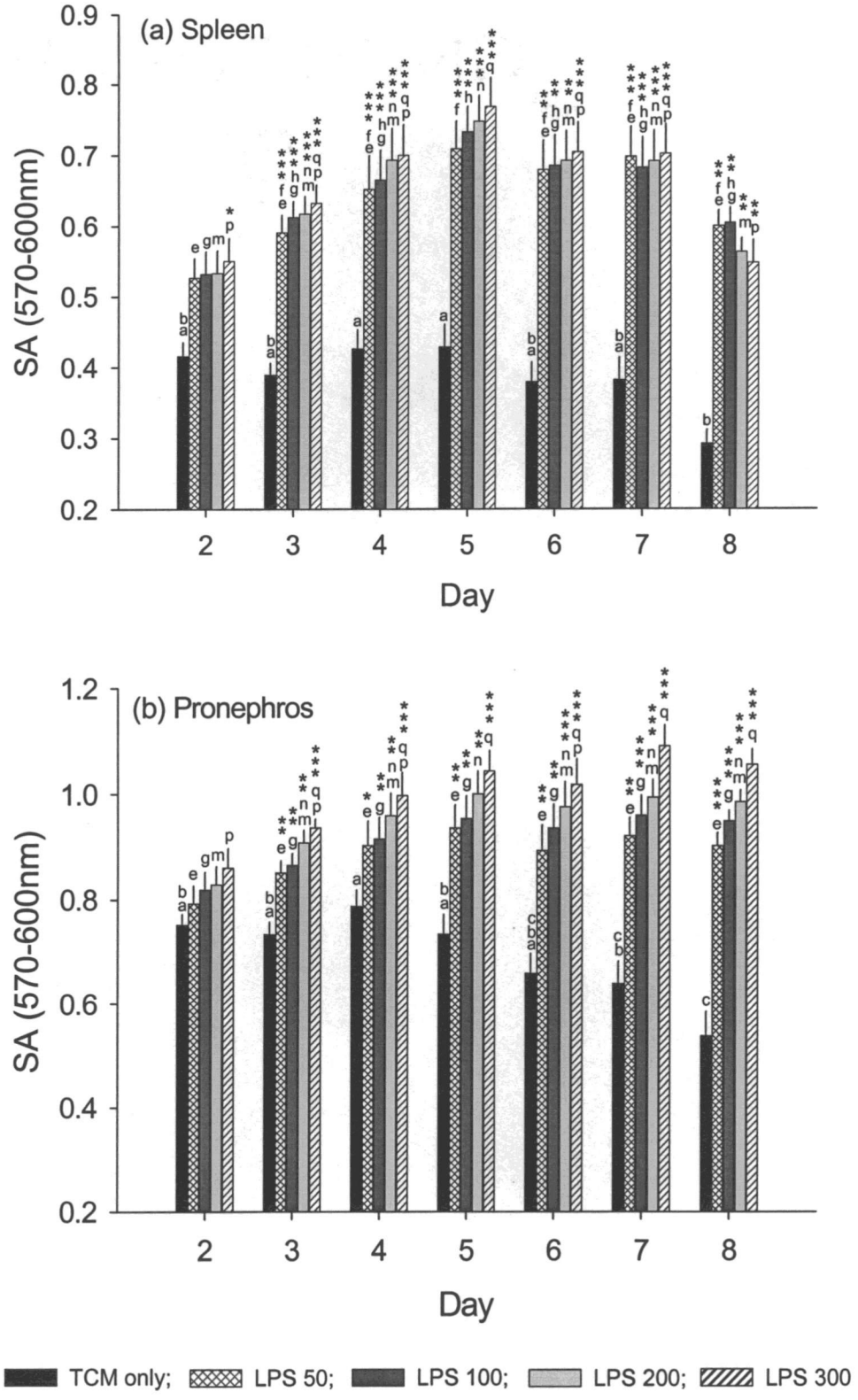
(a) Spleen
SA (570-600nm)
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
2
3
4
5
6
7
8
Day
(b) Pronephros
SA (570-600nm)
1.2
1.0
0.8
0.6
0.4
0.2
2
3
4
5
6
7
8
Day
TCM only;
LPS 50;
LPS 100;
LPS 200;
LPS 300

Figure 1.6.

There were significant differences in SA between pronephric cell cultures treated with TCM alone and with LPS (50 - 300 mg l^{-1}) from day 3 through day 8, but not on day 2 (Figure 1.6 b). The SA of pronephric cell cultures treated with TCM alone was stable from day 2 through day 7, but decreased significantly from day 5 to day 8. SAs of pronephric cell cultures treated with 50 or 100 mg l^{-1} of LPS were stable from day 2 through day 8. The SA of pronephric cell cultures incubated with 200 mg l^{-1} of LPS for 5 days was significantly higher than the SA at day 2, but other SAs of pronephric cell cultures incubated with 200 mg l^{-1} of LPS were not different from the SA at day 2. The SAs of pronephric cell cultures incubated with 300 mg l^{-1} of LPS at day 3, 7, and 8 were significantly higher than those incubated with 50 mg l^{-1} of LPS. Other than those exceptions, concentration of LPS ranging from 50 to 300 mg l^{-1} induced the same SA at each time point.

The SI of splenic and pronephric leukocytes increased with incubation time at least through day 8 (Figure 1.7). There were no differences in SI among LPS concentrations at each time point.

During the 8 days of in vitro incubation, the viability of splenic leukocytes was stable from day 2 through day 7 and declined at day 8 (Figure 1.8 a). The viability of splenic leukocytes treated without or with any concentration of LPS (50 - 300 mg l^{-1}) was similar at each time point. On the other hand, the viability of pronephric leukocytes treated with TCM alone declined markedly, and was significantly lower than those with LPS after day 5 through day 8 (Figure 1.8 b). The viability of pronephric leukocytes treated with 50 - 300 mg l^{-1} LPS was stable from day 2 through day 7 and declined by day 8.

Figure 1.7. The stimulation indexes (SIs; mean ± S.E.M.). Spleen (a) and pronephros (b). SIs were obtained by dividing mean absorbance of unstimulated cell suspensions by the mean absorbance of LPS-stimulated cell suspensions. Columns in bar graphs that have at least one same letter are not significantly different within all treatments ($P>0.05$, ANOVA and Kruskal-Wallace test).

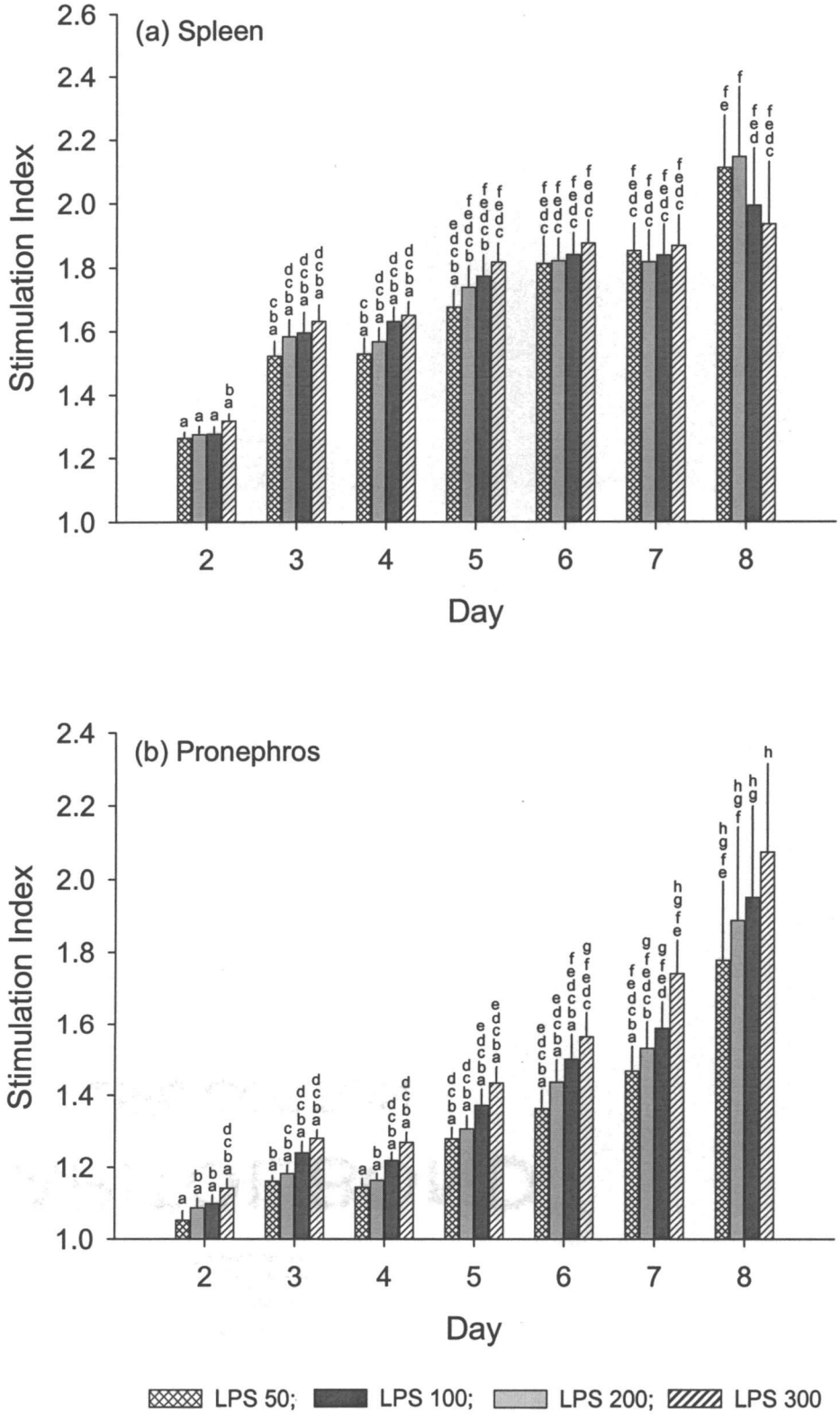
(a) Spleen
Stimulation Index
Day
(b) Pronephros
Stimulation Index
Day
LPS 50;
LPS 100;
LPS 200;
LPS 300

Figure 1.7.

Figure 1.8. The effect of the incubation time and LPS concentration on the cell viability of chinook salmon leukocyte suspensions. Following Alamar Blue analysis, flow cytometric assays were performed using the same cell cultures as those used in the Alamar Blue assay. The viability of splenic (a) and pronephric (b) leukocytes were measured by the flow cytometric assay. Viability was measured after 2, 3, 4, 5, 6, 7, and 8 days of incubation. For the respective treatments, sample size n=6, 9, 9, 9, 9, 9, and 6. The results are expressed in the mean $\pm$S.E.M. Columns in bar graphs that have at least one same letter are not significantly different from other columns within all treatments ($P>0.05$, ANOVA and Kruskal-Wallace test).

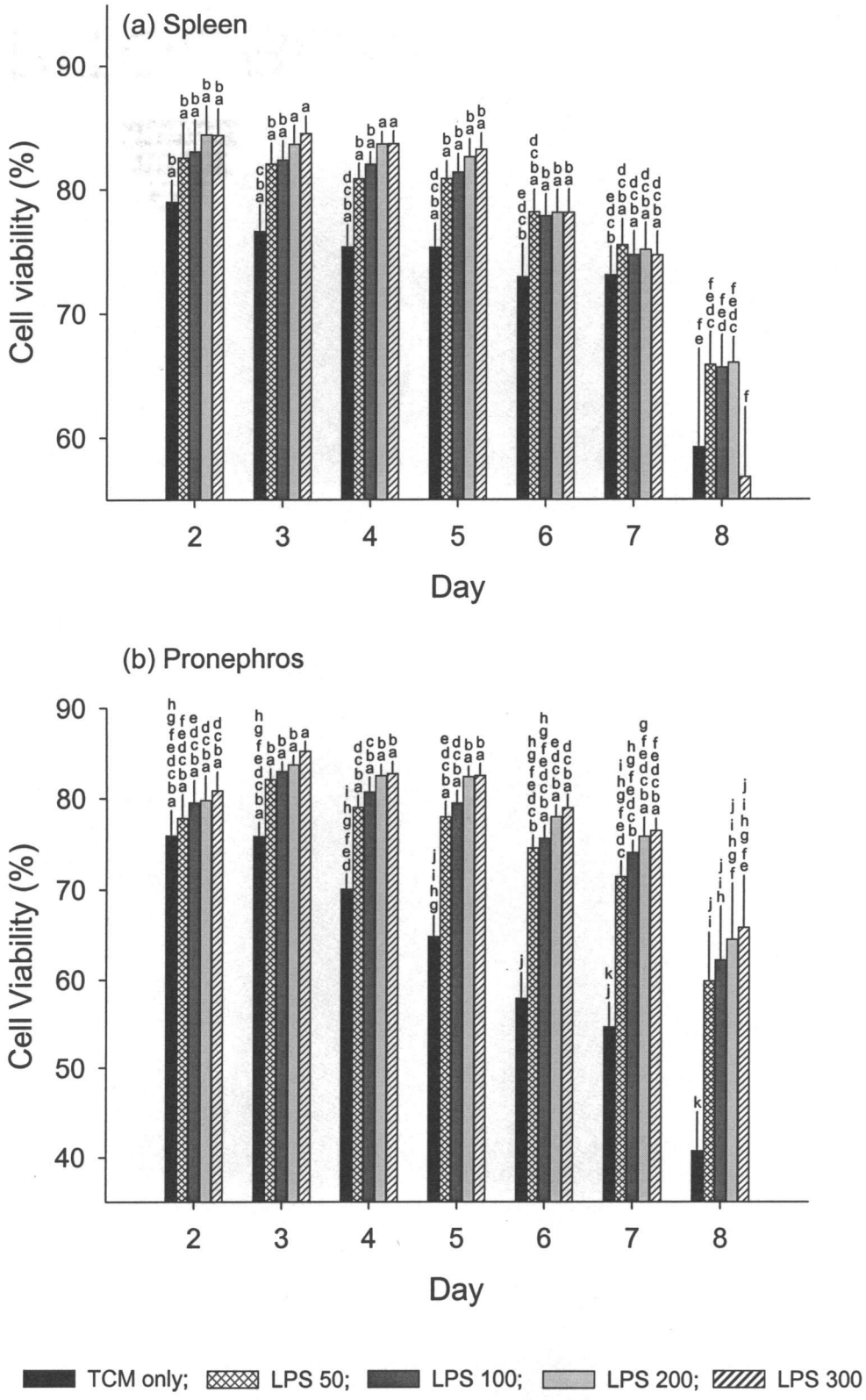
(a) Spleen
Cell viability (%)
90
80
70
60
2
3
4
5
6
7
8
Day
(b) Pronephros
Cell Viability (%)
90
80
70
60
50
40
2
3
4
5
6
7
8
Day
TCM only;
LPS 50;
LPS 100;
LPS 200;
LPS 300

Figure 1.8.

For all treatments, splenic leukocytes activated with LPS had a significantly higher percentage of blasting cells in the viable leukocyte population than those that were cultured in the presence of TCM alone from day 3 through day 8, but not day 2 (Figure 1.9 a). The percentage of blasting splenic leukocytes cultured with TCM alone was stable from day 2 through at least day 8. The percentage of blasting splenic leukocytes treated with 50 or 100 mg l^{-1} of LPS was also stable from day 2 through at least day 8. On the other hand, the percentage of blasting splenic leukocytes treated with 200 or 300 mg l^{-1} of LPS increased from day 2 through day 4 and then remained stable until day 8.

Viable pronephric leukocytes activated with LPS were significantly larger than those cultured in the presence of TCM alone from day 3 through day 8 (except those treated with 50 mg l^{-1} of LPS for 3 days), but not day 2 (Figure 1.9 b). The mean size of pronephric leukocytes cultured with TCM alone declined with incubation time through at least day 8. However, when cells were incubated with LPS, the mean cell size was stable from day 2 through at least day 8.

LEUKOCYTE STIMULATION USING OTHER MITOGENS

The SAs of splenic cell cultures treated with PWM or ConA were never significantly higher than those treated with TCM alone (Figures 1.10 a, 1.11 a). The SAs of pronephric cell cultures treated with PWM were similar to those treated with TCM alone at day 3, 4, and 6, but the SAs of PWM-treated cultures were significantly higher than those with TCM alone at day 5 (Figure 1.10 b). The SAs of pronephric cell cultures treated with ConA were similar to those treated with TCM alone at day 1, 2, 3, and 4, but, like PWM, were significantly higher than those exposed to TCM alone at

Figure 1.9. The effect of the incubation time and LPS concentration on the blastogenesis of chinook salmon leukocyte suspensions. Following Alamar Blue analysis, flow cytometric assays were performed using the same cell cultures as those used in the Alamar Blue assay. Levels of blastogenesis of splenic leukocytes (a) and pronephric leukocytes (b) were measured by the flow cytometric assay. Blastogenesis was measured after 2, 3, 4, 5, 6, 7, and 8 days of incubation. For the respective treatments, sample size n=6, 9, 9, 9, 9, 9, and 6. Results are expressed in the mean $\pm$S.E.M. Columns in bar graphs that have at least one same letter are not significantly different from other columns within all treatments ($P>0.05$, ANOVA and Kruskal-Wallace test).

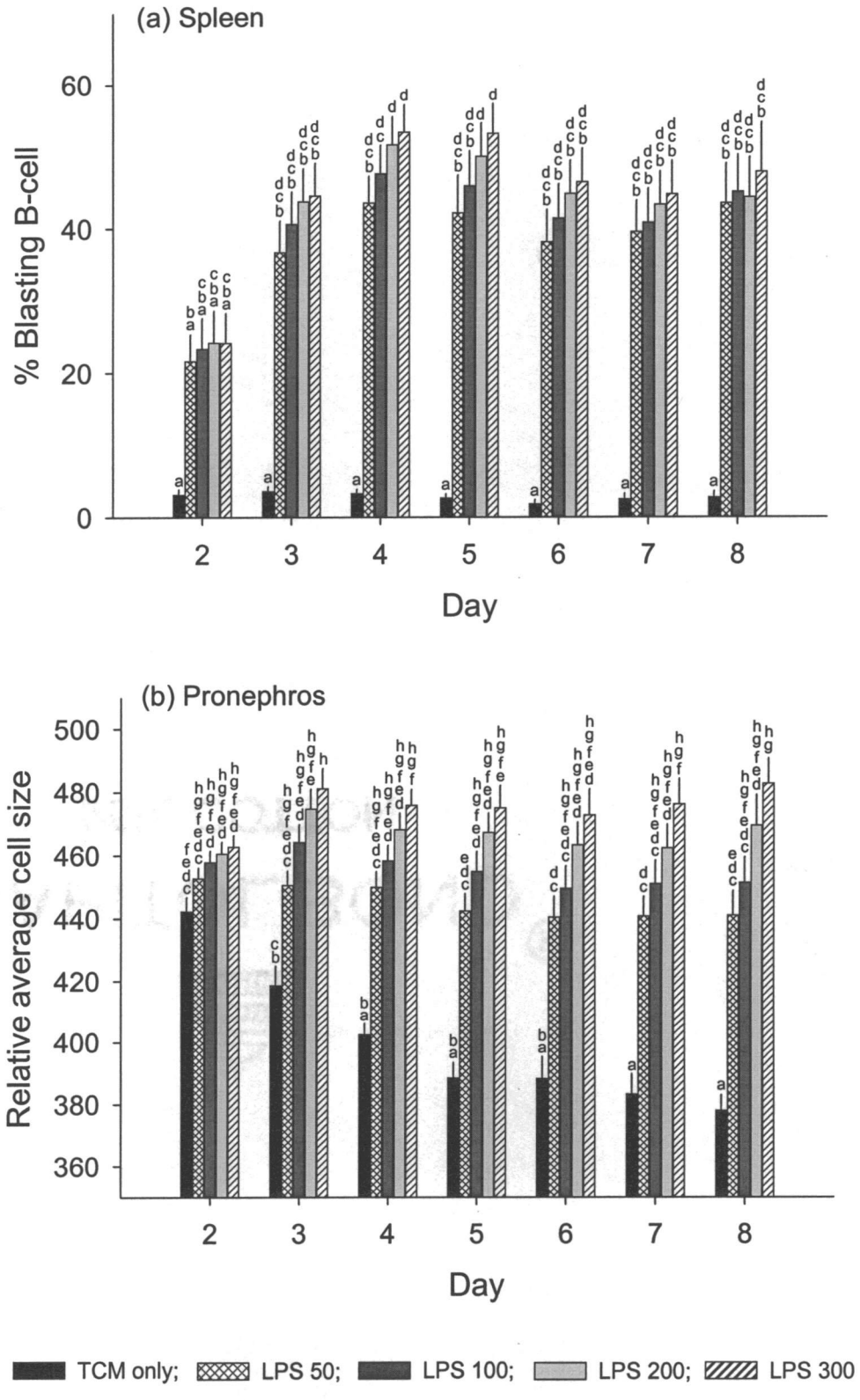
(a) Spleen
% Blasting B-cell
0
20
40
60
2
3
4
5
6
7
8
Day
(b) Pronephros
Relative average cell size
360
380
400
420
440
460
480
500
2
3
4
5
6
7
8
Day
TCM only;
LPS 50;
LPS 100;
LPS 200;
LPS 300

Figure 1.9.

Figure 1.10. The effect of the incubation time and the PWM concentration on the specific absorbance (SA: OD 570 nm minus 600 nm) of chinook salmon leukocyte suspensions in the Alamar Blue assay. Cells were plated at densities of 2.5 X 10^5 cells per well in 96-well culture plates. Splenic (a) and pronephric (b) leukocytes were incubated without or with PWM (25, 50, 100, or 200 mg/l) for 3, 4, 5 and 6 days. The effect of the incubation time and PWM concentration on cell viability and blastogenesis of chinook salmon leukocyte suspensions. Cells were plated at densities of 2.5 X 10^5 cells per well in 96-well culture plates. The stimulation indexes of spleen (c) and pronephros (d) were obtained by dividing mean SA of unstimulated cell suspensions by the mean SA of PWM-stimulated cell suspensions. The viability of splenic (e) and pronephric (f) cells were measured by the flow cytometric assay. The level of blastogenesis of splenic leukocytes (g) and pronephric leukocytes (h) were also measured by the flow cytometric assay. Results are expressed as the mean $\pm$S.E.M. of one experiment using 4 different individual fish (n=4).

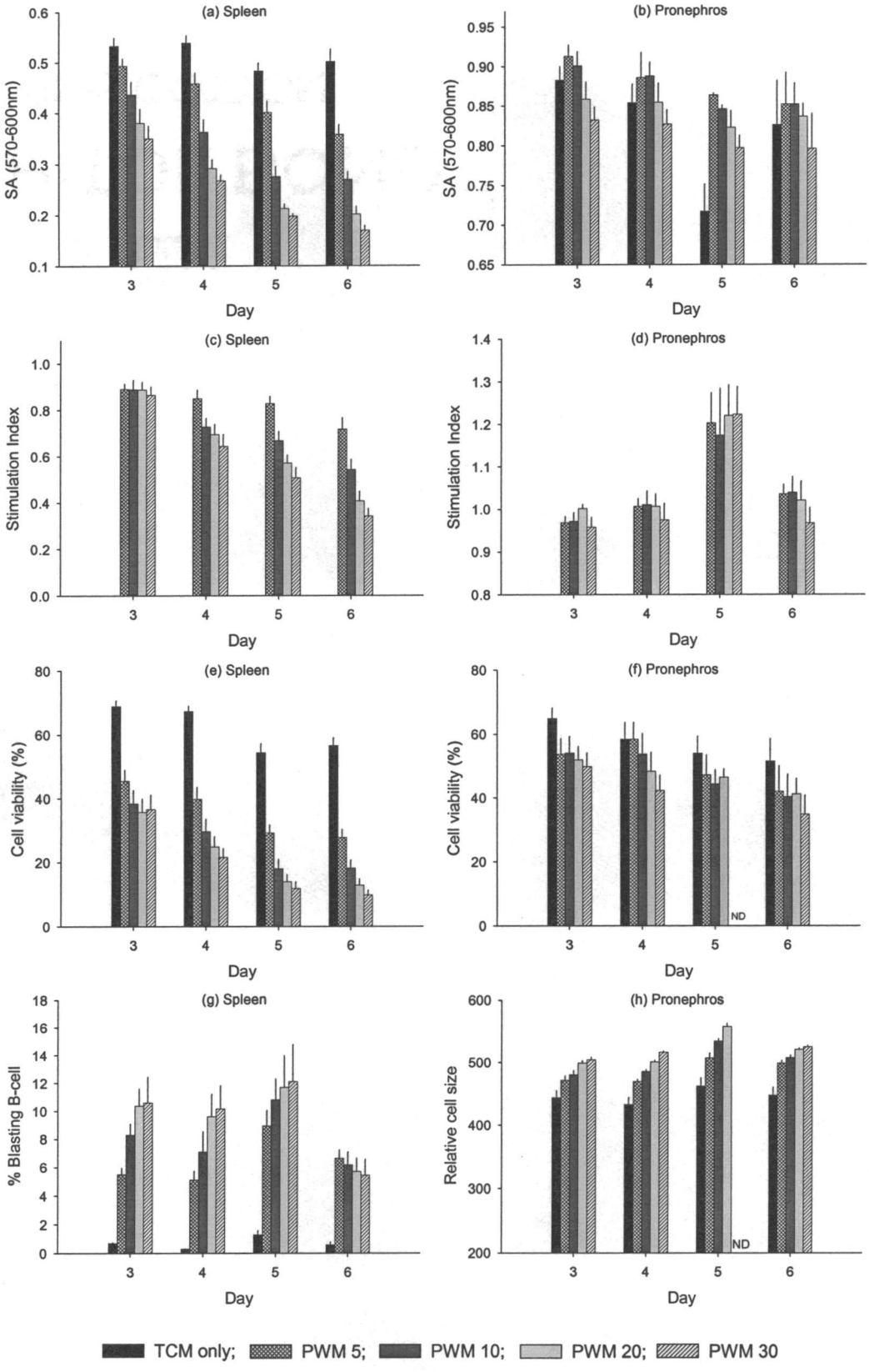
(a) Spleen
SA (570-600nm)
Day
(b) Pronephros
SA (570-600nm)
Day
(c) Spleen
Stimulation Index
Day
(d) Pronephros
Stimulation Index
Day
(e) Spleen
Cell viability (%)
Day
(f) Pronephros
Cell viability (%)
ND
Day
(g) Spleen
% Blasting B-cell
Day
(h) Pronephros
Relative cell size
ND
Day
TCM only;
PWM 5;
PWM 10;
PWM 20;
PWM 30

Figure 1.10.

Figure 1.11. The effect of the incubation time and the ConA concentration on the specific absorbance (SA: OD 570 nm minus 600 nm) of chinook salmon leukocyte suspensions. Cells were plated at densities of 2.5 X 10^5 cells per well in 96-well culture plate. Splenic (a) and pronephric (b) leukocytes were incubated without or with ConA (5, 10, 20, or 30 mg/l) for 1, 2, 3, 4, and 5 days. The effect of the incubation time and the ConA concentration on the cell viability and the blastogenesis of chinook salmon leukocyte suspensions. Cells were plated at densities of 2.5 X 10^5 cells per well in 96-well culture plates. The stimulation indexes of spleen (c) and pronephros (d) were obtained by dividing mean SA of unstimulated cell suspensions by the mean SA of ConA-stimulated cell suspensions. The viability of splenic (e) and pronephric (f) leukocytes were measured by the flow cytometric assay. The level of blastogenesis of splenic leukocytes (g) and pronephric leukocytes (h) were also measured by flow cytometric assay. The results are expressed as the mean $\pm$ S.E.M. of one experiment using 4 different individual fish (n=4).

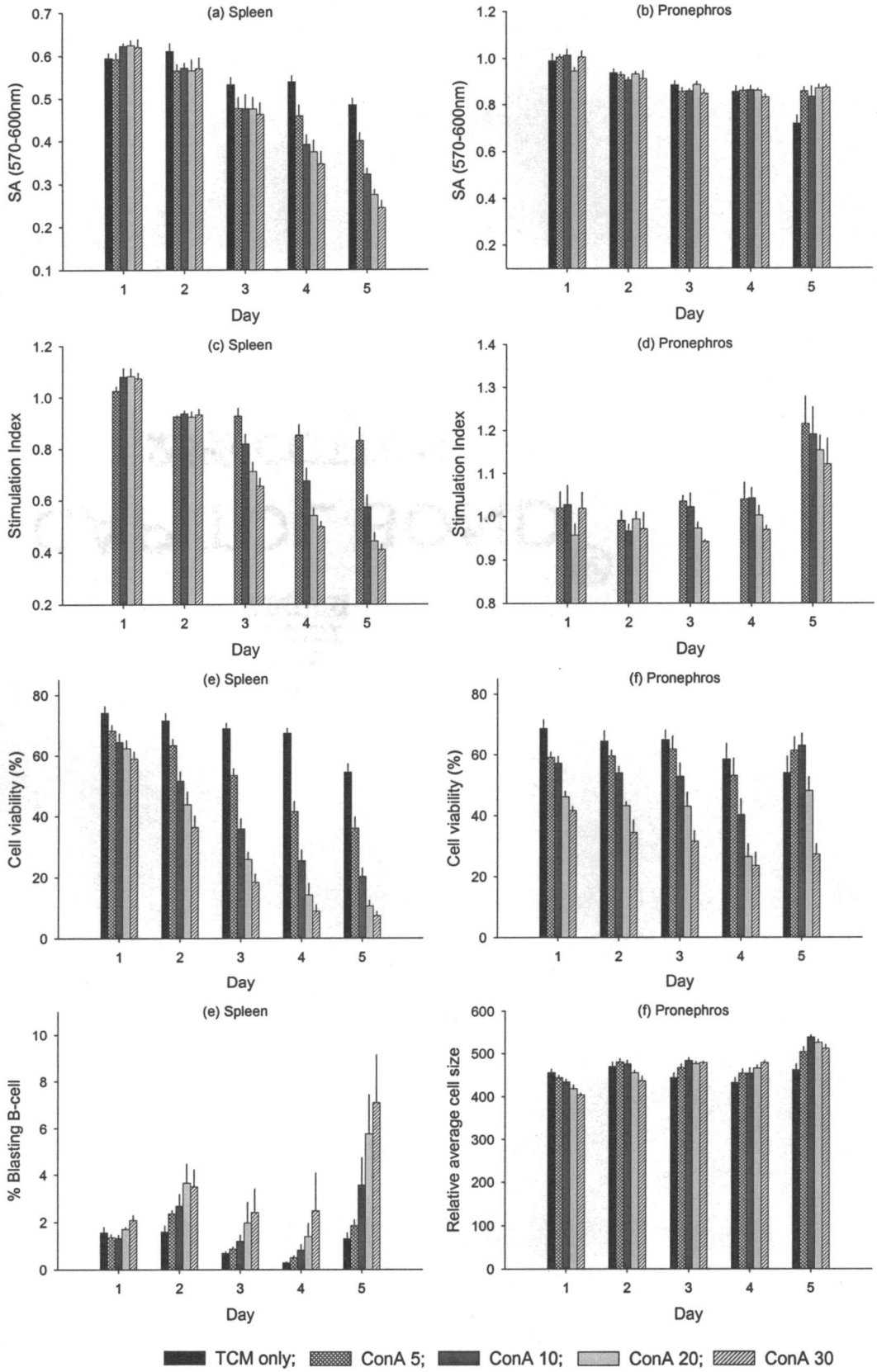
(a) Spleen
SA (570-600nm)
Day
(b) Pronephros
SA (570-600nm)
Day
(c) Spleen
Stimulation Index
Day
(d) Pronephros
Stimulation Index
Day
(e) Spleen
Cell viability (%)
Day
(f) Pronephros
Cell viability (%)
Day
(e) Spleen
% Blasting B-cell
Day
(f) Pronephros
Relative average cell size
Day
TCM only;
ConA 5;
ConA 10;
ConA 20;
ConA 30

Figure 1.11.

day 5 (Figure 1.11 b). The SIs determined by the Alamar Blue assay of cells exposed to PWM (Figure 1.10 c, d) or ConA (Figure 1.11 c, d) were much lower than with LPS. PWM significantly reduced cell viability of splenic leukocytes but not pronephric leukocytes (Figure 1.10 e, f). Cell viabilities of splenic and pronephric leukocytes treated with ConA were lower than those treated with TCM alone (Figure 1.11 e, f). PWM induced blastogenesis in splenic and pronephric leukocytes (Figure 1.10 g) while ConA induced blastogenesis in splenic and pronephric leukocytes on day 5 but not from day 1 through day 4.

Discussion

We developed an Alamar Blue assay to quantify mitogenic responses of splenic and pronephric leukocytes of chinook salmon. This assay is very simple, safe, and inexpensive relative to others. In summary, cultural conditions for the Alamar Blue assay using LPS are; 1) optimum cell density for splenic and pronephric leukocytes: between 0.5×10^5 and 2.5×10^5 cells per well, 2) effective LPS concentration for splenic and pronephric leukocytes: between 50 and 300 mg l^{-1}, 3) effective incubation time with LPS for splenic leukocytes: between 3 and 8 days, effective incubation time with LPS for pronephric leukocytes: day 7 and day 8.

While the manufacturer of Alamar Blue dye has not revealed its chemical identity (Horobin, 2001), O'Brien *et al.* (2000) have identified Alamar Blue as resazurin. After Alamar Blue is taken into cells, nonfluorescent resazurin (non-reduced Alamar Blue) is reduced to fluorescent resorufin (reduced Alamar Blue) by intracellular reductases (Rasmussen, 1999; Andrews *et al.*, 1997). There is a possibility that resorufin may damage nuclear membranes and DNA due to its chemical structure (O'Brien *et al.*, 2000). However, we did not observe any adverse effects on viability of fish leukocytes in culture with 10% Alamar Blue dye for up to 24 hr. Zhi-Jun *et al.* (1997) and Nociari *et al.* (1998) reported that Alamar Blue did not affect other analyses of apoptosis by flow cytometry with PI-staining, lymphokine mRNA expression by RT-PCR, or the phenotypic characteristic (expression of cell surface antigens) using antibodies. The Alamar Blue assay thus allows cell culture to be used for further multiple analyses on the same cells.

We observed that the SA of Alamar Blue increased with cell density, reaching a threshold at high densities (Figure 1.3). This suggests that the ability of Alamar Blue to

detect differences at high cell density is limited. We also observed that the SA of pronephric leukocytes was always higher than that of splenic leukocytes, suggesting that the pronephros is the major haemotopoietic organ in teleost and contains granulocytes, and therefore pronephric leukocytes may have a higher metabolism than splenic leukocytes.

Pronephric leukocytes treated with TCM alone had a shorter life span than splenic leukocytes (Figure 1.8), and cell sizes of the former decreased with incubation time (Figure 1.9). However, pronephric leukocytes treated with LPS lived longer and were in size. We suspect that this phenomenon can be explained by the fact that neutrophils are relatively abundant in the pronephros yet are short-lived when incubated with TCM alone, thereby decreasing the overall average cell size of the pronephric leukocyte population.

Although a higher SI indicates a higher level of proliferation and greater sensitivity to the response, it does not directly denote higher mitogenic response. For example, despite the fact that the viability and the percentage of blasting cells in LPS treated cultures increased when cell density was increased (Figure 1.5), the SI decreased with cell density (Figure 1.4 c). Because the absorbance of stimulated cultures approached a threshold with increased cell density, the difference of absorbance between stimulated and un-stimulated cells diminished. Consequently, the ratio of absorbance of LPS treated culture to absorbance of untreated cultures decreased with cell density.

Currently, several mitogens such as PWM for T- and B-cells, PHA for T-cells, ConA for T-cells, and LPS for B-cells are used to stimulate fish leukocytes (Warr *et al.*, 1983; Kaattari & Yui, 1987; Kehrer *et al.*, 1998). We found that LPS yielded better results in the Alamar Blue assay than the other mitogens because PWM and ConA

significantly reduced cell viability and resulted in low SIs. Although PWM and ConA induced blastogenesis in leukocytes, the absolute number of cells did not increase likely due to toxic effects of PWM and ConA. Daly et al. (1995) also reported that the lack of mitogenic responses of peripheral blood lymphocytes of brook trout by ConA comparing to LPS. To our knowledge, the toxic effects of PWM and ConA on fish leukocytes have not reported. These results suggest that PWM and ConA are not practical mitogens for the Alamar Blue assay under our culture conditions.

In conclusion, the Alamar Blue assay is suitable for the quantification of LPS-stimulated responses of chinook salmon leukocytes. By appropriate choice of culture conditions, the sensitivity of the assay could be altered. While we calibrated and determined the optimum or effective cell density, concentration and type of mitogens, and incubation time to achieve optimum cell proliferation for the Alamar Blue assay, other culture conditions such as the purity of leukocytes, presence of serum, osmolarity, temperature, pH, and incubation atmosphere are also known to affect cell proliferation in the culture (Arkoosh & Kaattari, 1992).

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Chapter 2

The Effect of *in vitro* and *in vivo* Exposures to p,p'-DDE on the Immune Competence of Chinook Salmon (*Oncorhynchus tshawytscha*) Leukocytes

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Abstract

p,p'-DDE, the main metabolite of DDT, is still detected in aquatic environments throughout the world. We determined the effects and mechanisms by which p,p'-DDE exposure might affect the immune system of chinook salmon (*Oncorhynchus tshawytscha*). Isolated salmon splenic and pronephric leucocytes were incubated with different concentrations of p,p'-DDE, and cell viability, induction of apoptosis, and mitogenic responses were measured by flow cytometry and Alamar Blue assay. p,p'-DDE significantly reduced cell viability and proliferation and increased apoptosis. The effect of p,p'-DDE on pronephric leukocytes was more severe than on splenic leukocytes, likely because pronephric leucocytes had a higher proportion of granulocytes, cells that appear more sensitive to p,p'-DDE. The effect of p,p'-DDE on leucocytes appeared to vary between developmental stages or seasonal differences. The mitogenic response of leukocytes of chinook salmon exposed to p,p'-DDE *in vivo* exhibited a biphasic dose-response relationship. Only leukocytes isolated from salmon treated with 59 ppm p,p'-DDE had a significantly lower percentage of Ig+ blasting cells than controls. Our results support the theory that exposure to chemical contaminants could lead to an increase in disease susceptibility and mortality of fish due to immune suppression.

Keywords- Alamar Blue, apoptosis, cell proliferation, chinook salmon, DDE, flow cytometry, granulocytes, immune response, leukocytes, LPS, mitogenic response, seasonal change,

Introduction

Exposure to chemical contaminants can lead to an increase in disease susceptibility and mortality of fish due to immune suppression and be associated with declines of populations (Gulland, 1995; Arkoosh *et al.*, 1998a). Recent evidence clearly proves that anthropogenic contaminants and mixtures can alter the function or development of fish immune systems (Zeeman & Brindley, 1981; Anderson *et al.*, 1984; Arkoosh *et al.*, 1994, 1996, 1998b; Carlson *et al.*, 2002). 1,1,1-trichloro-2,2-bis(*p*-chlorophenyl)ethane (DDT) has been used on a global extent and was examined in the first immunotoxicological study of organochlorine insecticides (Anderson, 1996). Currently, there is considerable evidence that DDT has adverse effects on the immune system of vertebrates (Zeeman & Brindley, 1981; Exon *et al.*, 1987; Barnett & Rodgers, 1994). Although most countries have limited or banned the use of DDT since the 1970's, its metabolites are still detected in aquatic environments throughout the world because of their chemical stability and lipophilic nature (Simonich & Hites, 1995; Kumblad *et al.*, 2001; Wainwright *et al.*, 2001). 1,1-dichloro-2,2-bis(*p*-chlorophenyl)ethylene (p,p'-DDE) is the main metabolite of DDT and is also the most consistently found organochlorinated compound in aquatic biota and bed sediment in contaminated rivers and lakes (Brown, 1997; Wentz *et al.*, 1998; Clark *et al.*, 2001; Schmitt, 2002).

A number of studies on mammals have reported that p,p'-DDE as well as DDT is immunosuppressive and can increase the susceptibility to a number of diseases. In the wild, incidents of high mortality due to infectious diseases of beluga whales (*Delphinapterus leucas*) and bottlenose dolphins (*Tursiops truncatus*) exposed to high

level of organochlorines including p,p'-DDE were reported by De Guise *et al.* (1995) and Lahvis *et al.* (1995). In experimental animals, Banerjee *et al.* (1996a) reported that rats fed on a diet containing 200 ppm of p,p'-DDE for 6 weeks showed humoral and cellular immune suppression. In addition, p,p'-DEE has been also identified in human blood samples and associated with immune suppression. People living near the pesticide dumpsite in North Carolina, U.S.A., had higher plasma p,p'-DDE level and showed lower mitogenic response of lymphocytes (Vine *et al.*, 2001). In addition, p,p'-DDE is considered a carcinogen (Cocco *et al.*, 2000; Romieu *et al.*, 2000). Little is known about the mechanism of p,p'-DDE's immunosuppressive action in vertebrates. Since the humoral and cell-mediated immune responses are exceptionally complicated, there are difficulties in determining whether the mode of action is from direct or indirect toxicity (Banerjee *et al.*, 1996b).

In spite of the fact that p,p'-DDE is an immunosuppressant and a carcinogen for mammalian species and is highly persistent in the aquatic environment, knowledge regarding the effects of p,p'-DDE on the immune system of fish is lacking. Basic structural and functional characters of fish and mammalian immune system are quite similar (Bernstein *et al.*, 1998). That p,p'-DDE seems to suppress fish immune systems should therefore not seem surprising. The objective of this study was to determine if p,p'-DDE had adverse effects on the immune system of chinook salmon (*Oncorhynchus tshawytscha*) by direct action on leukocytes.

Materials and Methods

FISH CARE

Yearling spring chinook salmon (Marion Folks stock) weighing 100-200 g were housed at the Fish Performance and Genetics Laboratory, Oregon State University, Corvallis, Oregon. The fish were maintained in 0.9 m-diameter circular fiberglass tanks supplied with 12-13 °C flow-through water under natural photoperiod and fed a commercial diet of Semi-Moist Pellets (BioOregon™, Warrenton, OR) twice daily.

CHEMICALS AND REAGENTS

Tissue culture medium (TCM) contained 7% heat-inactivated fetal bovine serum, 1% L- glutamine, 200 Iu ml^{-1} penicillin, and 0.2 mg ml^{-1} streptomycin in Minimum Essential Media (MEM) buffered with sodium bicarbonate. Isolation-media was composed of Hank's balanced salt solution and Alservers solution (0.1 M dextrose, 70 mM sodium chloride, and 30 mM sodium citrate). p,p'-DDE (Chem Service, Inc., West Chester, PA) was dissolved in 100% ethanol and diluted in TCM at most 2h before each experiment started. The final concentration of ethanol in each p,p'DDE treated culture and vehicle control was always 0.075%. However, we do not know the actual dose of dissolved p,p'-DDE to which the cells were exposed in cell cultures because we could not determine the actual quantity of p,p'-DDE that could be dissolved in TCM. Lipopolysaccharide (LPS; Sigma, St. Louis, MO) from *Escherichia coli* serotype O 55:B5 was dissolved in TCM to a final concentration of 200 $\mu g\ ml^{-1}$ in each well. MEM

was purchased from Invitrogen Co. (Carlsbad, CA), and all other regents added into TCM were purchased from Sigma.

ISOLATION AND CULTIVATION OF LEUKOCYTES

Fish were rapidly netted from their tanks, immediately killed in 200 mg l^{-1} buffered tricaine methane-sulfonate (MS 222), weighed, and then bled by caudal severance. The fish were then transported on ice to our immunology laboratory at Oregon State University within 30 min. The spleen and pronephros were aseptically isolated and placed separately into individual conical tubes filled with 1 ml of cold isolation medium. The isolated tissue was placed on a 40 μm nylon cell strainer (Becton Dickinson, Franklin Lakes, NJ) and gently disrupted with the end of a 3 cc syringe plunger. The disrupted tissue was washed through a strainer into a 50 ml polypropylene conical tube (Becton Dickinson) with isolation medium. The homogenized tissue suspension was then centrifuged at 500 g for 7 min at 4°C and the supernatant was aspirated. The pellet was resuspended with 2 ml of ice-cold isolation medium, and clumps were removed. The organ removal and tissue processing were all conducted under asceptic conditions in a laminar flow hood. In order to purify and separate leukocytes from erythrocytes, hypotonic lysis was used as described by Crippen et al. (2001). Briefly, 2 ml of cell suspension were diluted with 9 ml sterile deionized water to lyse the erythrocytes for 20 s, and then 1 ml of sterile 10X concentrated phosphate-buffered saline (PBS) was immediately added to stop lysing. Cells were washed twice by centrifugation at 500 g for 7 min at 4°C, the supernatant aspirated, and the cell resuspended with 2 ml of ice-cold TCM. After the purification of leukocytes, viable cells were counted using trypan blue exclusion test, and the cell suspension was diluted with

ice-cold TCM to final concentration of 5 x 10^6 viable cells ml^{-1}. We did not pool each cell suspension from each individual fish. The cell suspension (100 µl $well^{-1}$) was plated out into flat bottm 96-well plates (Becton Dickinson), and then p,p'-DDE solution, LPS solution, and / or TCM were added to a final volume of 200 µl $well^{-1}$. The cell cultures were maintained at 17 °C in an incubator culture chamber (C.B.S. Scientific CO., Del Mar, CA) containing blood gas mixture (10% O_2, 10% CO_2, and 80% N_2) and set on a wet paper towel for humidity.

FLOW CYTOMETRIC ANALYSIS FOR CELL VIABILITY

Fish were sampled in spring [March 2002: 134.1 ± 12.3 [mean ± the standard error of mean (S.E.M.)] g, 10 months old], summer (August 2002: 157.0 ± 7.4 g, 15 months old), and winter (December 2002 and January 2003: 22.8 ± 2.8 g, 7 to 8 month old). Cell suspensions with or without p,p'-DDE, were incubated for various times (0, 2, 6, 17, 24, or 48hr). Control cultures contained 0.075% ethanol as a vehicle control. Following incubation, 50 µl of each cell suspension was washed once with PBS and resuspended in 200 µl of PBS. Ten µl of propidium iodide solution (PI; 50 µg ml^{-1} in PBS) were added, and cells were incubated for 10 min at room temperature.

After incubation, cells were analyzed by flow cytometry (FACScan®; Becton Dickinson). Cells that stained negative for PI were determined as viable cells, and PI positive cells were determined as dead cells and excluded from further analysis. The percentage of PI negative cells in total leukocytes was calculated using software, Cell Quest (Becton Dickinson). Analysis of forward (a measure for cell size) and side (a measure for cell granularity, complexity) scatter patterns indicated one major population

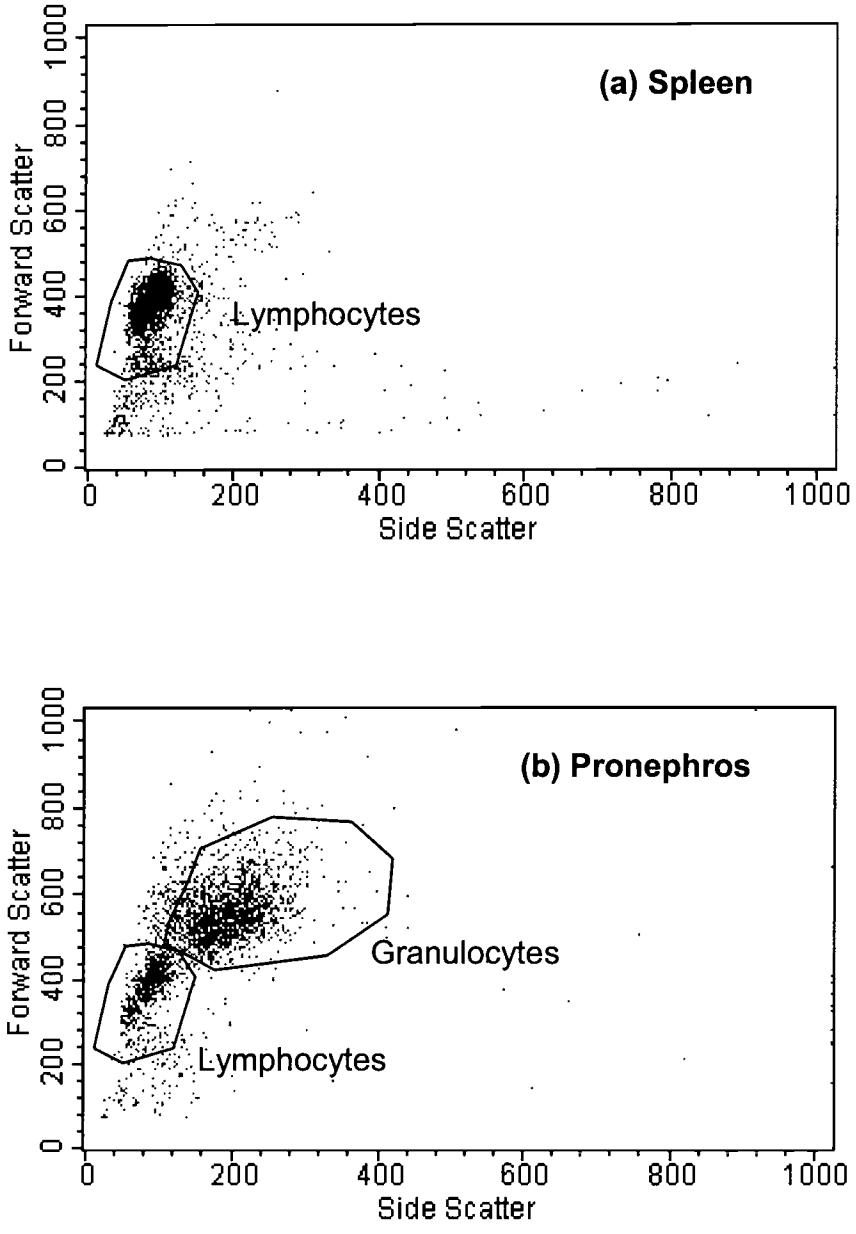

Figure 2.1. Flow cytometric forward and side scatter dot plots. Viable splenic (a) and pronephric (b) leukocytes. Dead cells were eliminated by the PI staining method.

in the splenic cell suspension (Figure 2.1 a) and two major populations in the pronephric cell suspension (Figure 2.1 b). Those cell populations were already identified as lymphocytes and granulocytes by Misumi *et al.* (2003) based on the microscopic analysis with Wright-Giemsa staining using Hema 3 stain set (Biochemical Sciences, Inc., Swedesboro, NJ) following isolation of populations using a cell sorter (MoFlo®, Cytomation, Inc., Fort Collins, CO).

APOPTOSIS ASSAY

To investigate the possible involvement of apoptosis in p,p'-DDE-mediated inhibition of cell viability, we performed flow cytometric analysis. Currently, several methods have been developed to assess the induction of apoptosis in cells of teleost fish (Evans *et al.*, 2001). In this study, we used Annexin-V assay to detect phosphotidylserine inversion on the membrane of apoptotic cells. Apoptotic cells were determined using the ApoAlert Annexin V-FITC Apoptosis kit (BD Biosciences Clontech, Palo Alto, CA). Fish were sampled on April, 2002. Splenic and pronephric cell suspensions were incubated with or without p,p'-DDE for 2 or 6 hr. Control culture contained 0.075% ethanol as a vehicle control. Following incubation, 50 μl of cell suspensions were centrifuged in microtubes (Bio-Rad Laboratories, Inc., Hercules, CA) at 500 g for 7 min at 4°C and resuspended in 40 μl of Annexin V Binding Buffer containing 1 μl of Annexin V-FITC and 2 μl of PI. Cells were incubated for 10 min at room temperature according to the manufacture's protocol (ApoAlert Annexin V-FITC Apoptosis kit; BD Biosciences Clontech). Apoptotic cells (Annexin V positive and PI negative) and necrotic cells (Annexin V negative and PI positive) were detected and distinguished by flow cytometry.

ALAMAR BLUE ASSAY

This assay was performed to determine if p,p'-DDE suppressed the mitogenic proliferative response of salmon leukocytes. Fish were sampled on April, 2002. Cell suspensions with or without p,p'-DDE were incubated for 3 days with or without LPS. According to the manufacture's protocol and Misumi *et al.* (2003), 20 μl $well^{-1}$ of Alamar Blue dye (10% of the final volume of the cell suspension present in each well) was added to the cell culture 24 hr before measurement. Alamar Blue dye was purchased from BioSource international, Inc. (Camarillo, CA). Optical densities (OD) at 570 nm (reduced) and 600nm (oxidized) were then measured with OPTImax™ Tunable Microplate Reader (Molecular Devices Co., Sunnyvale, CA). The specific absorbance (SA; $OD_{570} - OD_{600}$) was calculated using a computer software, SOFTmax® PRO (Molecular Devices Co.) to calibrate overlap between the two wavelengths (Zhi-Jun *et al.*, 1994). The sensitivity of the Alamar Blue assay to mitogenic proliferative response was evaluated by calculating the stimulation index (SI; =SA of stimulated culture / SA of unstimulated culture). SI indicates the level of the cell proliferation; a SI of 1 indicates no proliferation induced by mitogen, while a higher SI indicates greater response to mitogen.

FLOW CYTOMETRIC ANALYSIS FOR B-CELL BLASTOGENESIS

This experiment was performed to determine if p,p'DDE suppressed the blastogenesis of surface IgM presenting leukocytes (B-cells) isolated from the spleen. The flow cytometric assay for B-cell blastogenesis measuring functional humoral immunocompetence in chinook salmon was developed by Milston *et al.* (2002).

Following the cell proliferating analysis mentioned above, flow cytometric assays were performed using the same cell cultures as those used in the Alamar Blue assay. 100 µl of splenic cell suspensions were removed into new 96 well plates and rinsed twice by centrifugation at 300 g for 3 min at 4°C, the supernatant was discarded, and the cell resuspended with 200 µl of ice-cold PBS. The cell suspension was again centrifuged at 300 g for 3min at 4 °C and resuspended with 10 µl of diluted Anti-Salmon & Trout Ig Mab Biotin Labeled solution (DiagXotics Inc., Wilton, CT) and 90 µl of ice-cold PBS. Following incubation on ice for 30 min, the cells were washed twice with PBS and then resuspended in 100 µl of ice-cold 1X PBS containing 1 µl of 1 mg ml^{-1} streptavidin-FITC conjugates buffered with PBS (Biosource international). The splenic leukocytes were incubated on ice for 30 min in the dark and washed twice with ice-cold PBS. Following the final wash, cells were resuspended with 150 µl of ice-cold PBS including 10 µl of 50 µg ml^{-1} PI solution, and cell suspensions were transferred into microtubes. Flow cytometry was used for detecting Ig+ blasting (larger) B-cells stimulated by LPS. Unstained leukocytes were used as negative control. Dead cells were eliminated employing the same method as for the viability assay using PI before the percentage of blasting B-cells in the total number of viable splenic leukocytes was calculated.

IN VIVO P,P'-DDE EXPOSURE

Spring chinook salmon [167.1 ± 7.1 (mean ± S.E.M.) g, 13 – 14 months old] were fed p,p'-DDE to elucidate effects of *in vivo* exposure on humoral immunity. This feeding experiment was conducted from June through July, 2001. Individuals were anesthetized with buffered MS-222, weighed, and randomly placed in each of eight tanks (20 per tank).

Contaminated diets were prepared by pipetting p,p'DDE solubilized in ethanol onto commercial semi-moist pellets (BioOregon^{TM}) fish food and allowing the ethanol vehicle to evaporate. Control diets were prepared in the same manner using ethanol alone. Fish were fed pellets containing 0, 0.11, 0.23, or 0.46 mg p,p'DDE per gram food, ad libitum twice daily, for 30 days. Daily rations were weighed before and after each feeding to estimation total of the consumption per tank at the end of the experiment. This resulted in fish receiving an average total dose of 0, 23, 46, 59, 88, and, 105 mg p,p'DDE per kg wet weight of fish over the course of the experiment. Fish were fasted for 24 hours before sampling. Eight individuals were randomly sampled from each tank. The flow cytometric assay for B-cell blastogenesis measuring functional humoral immunocompetence was then conducted.

STATISTICAL ANALYSIS

Eight to 16 fish were used for each test. There was no replication of assays within any individual fish. Data were averaged, and S.E.M. was determined. Percent values were transformed by arcsine of the square root of the value for further statistical analysis. Parametric statistical tests (the Student's t-test and ANOVA) and non-parametric statistical tests (the Kruskal-Wallace test and the Mann-Whitney U-test) were used for statistical comparisons of data. Measurements were considered significant when P values of both parametric and non-parametric tests were below 0.05. Tukey's honestly significant difference procedure (HSD) was used to determine which treatments are significantly different from others.

Results

FLOW CYTOMETRIC ANALYSIS FOR CELL VIABILITY

Incubation of salmon leukocytes in the presence of p,p'-DDE led to a time - and concentration dependent decrease in the percentage of viable cells in cultures. There were no significant differences in viability between TCM-alone control and vehicle control (Figure 2.2). There was a significant decline of viability of splenic and pronephric leukocytes 2 and 6 hours after addition of p,p'-DDE to the cell suspensions. Pronephric leukocytes were more sensitive to p,p'-DDE than splenic leukocytes (Figure 2.3). Viability of splenic leukocytes was about 50% of its vehicle control at about 15 mg l^{-1}, while pronephric leukocytes required only 11 mg l^{-1} to achieve that percentage.

APOPTOSIS ASSAY

p,p'-DDE induced apoptosis in in direct dose-dependent manner (Figure 2.4). The percentage of apoptotic cells due to p,p'-DDE was always greater with pronephric leukocytes than those from the spleen. A greater percent of necrotic cells was detected as well as apoptotic cells in p,p'-DDE treated cell suspensions than non-treated cell suspensions.

SENSITIVITY BETWEEN TWO DIFFERENT TYPES OF LEUKOCYTES

Pronephric lymphocyte viability was unaffected by exposure to p,p'-DDE (Figure 2.5). However, viability of granulocytes was inhibited in a dose-dependent manner. In the presence of p,p'-DDE, the percentage of viable lymphocytes was always significantly

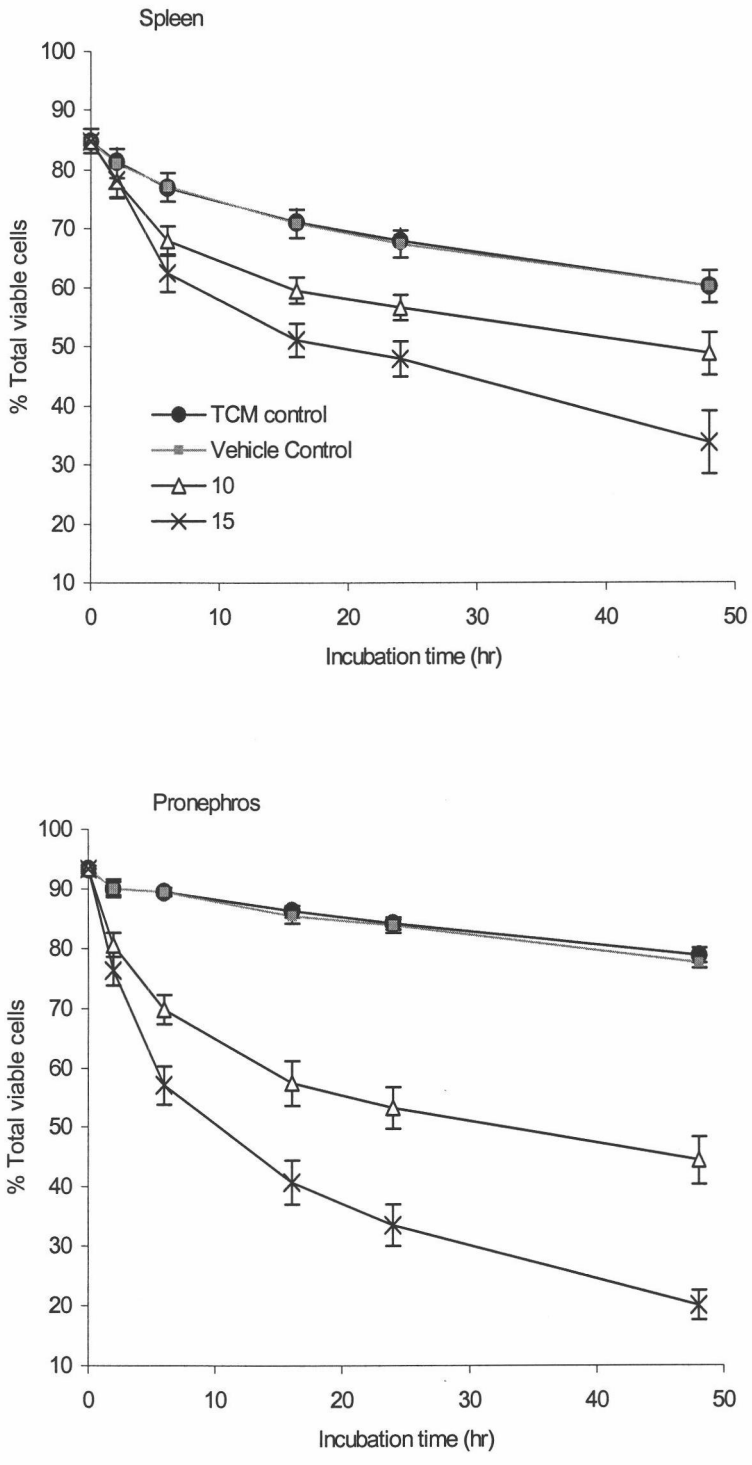

Figure 2.2. The effect of *in vitro* exposures to p,p'-DDE on percent total viable splenic and pronephric leukocytes. Mean (±S.E.M.) percent total viable splenic and pronephric leukocytes following 2, 6, 17, 24, and 48 hr incubation with p,p'-DDE (N=9). The viability was measured by flow cytometry. Fish were sampled from March to April, 2002.

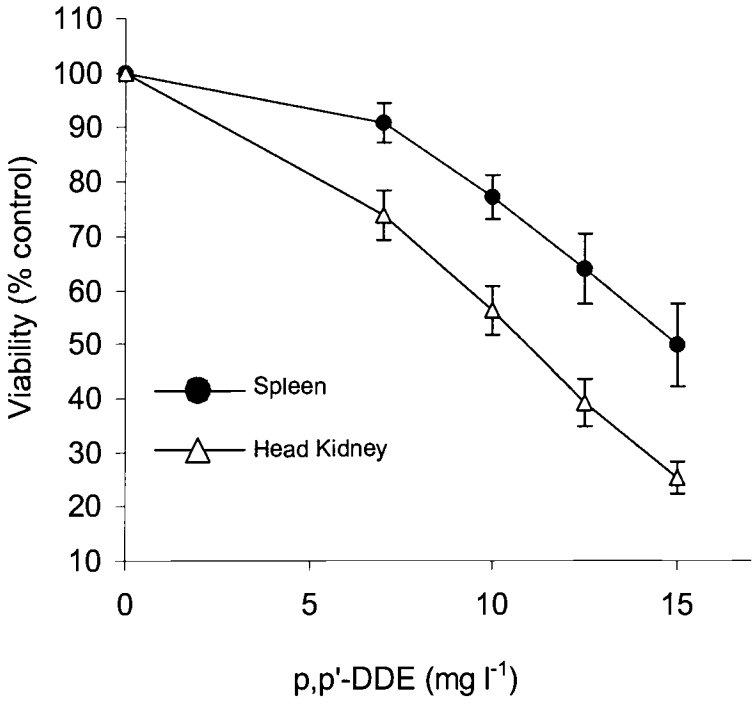

Figure 2.3. Mean (±S.E.M) viability (% TCM control) of splenic and pronephric leukocytes following different concentrations of p,p'-DDE for 48h in the culture (N=9).

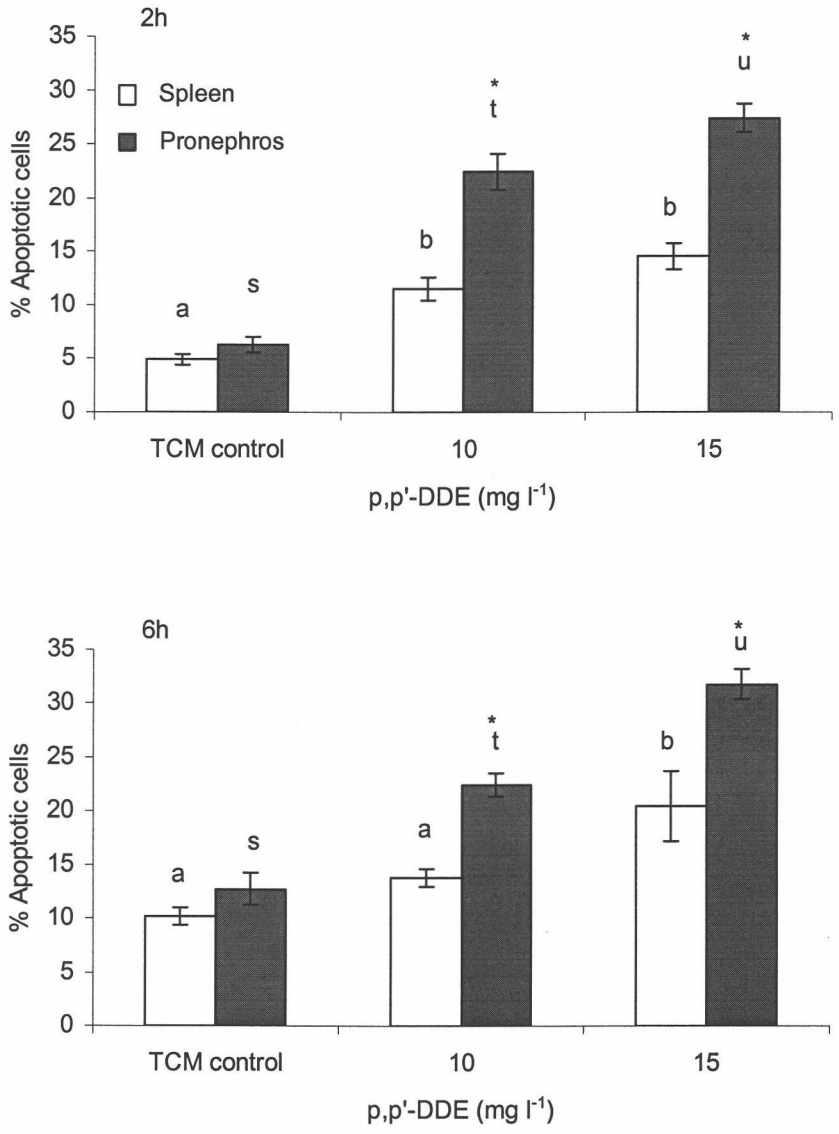

Figure 2.4. Mean percent (±S.E.M.) splenic and pronephric apoptotic leukocytes following incubation with p,p'-DDE for 2h and 6h (N=12). Columns that have the same subscripts are not significantly different ($P>0.05$, Kruskal-Wallis test and ANOVA). Significant differences between spleen and pronephros are denoted * ($P<0.05$, Mann-Whitney U-test and Stutent's t-test).

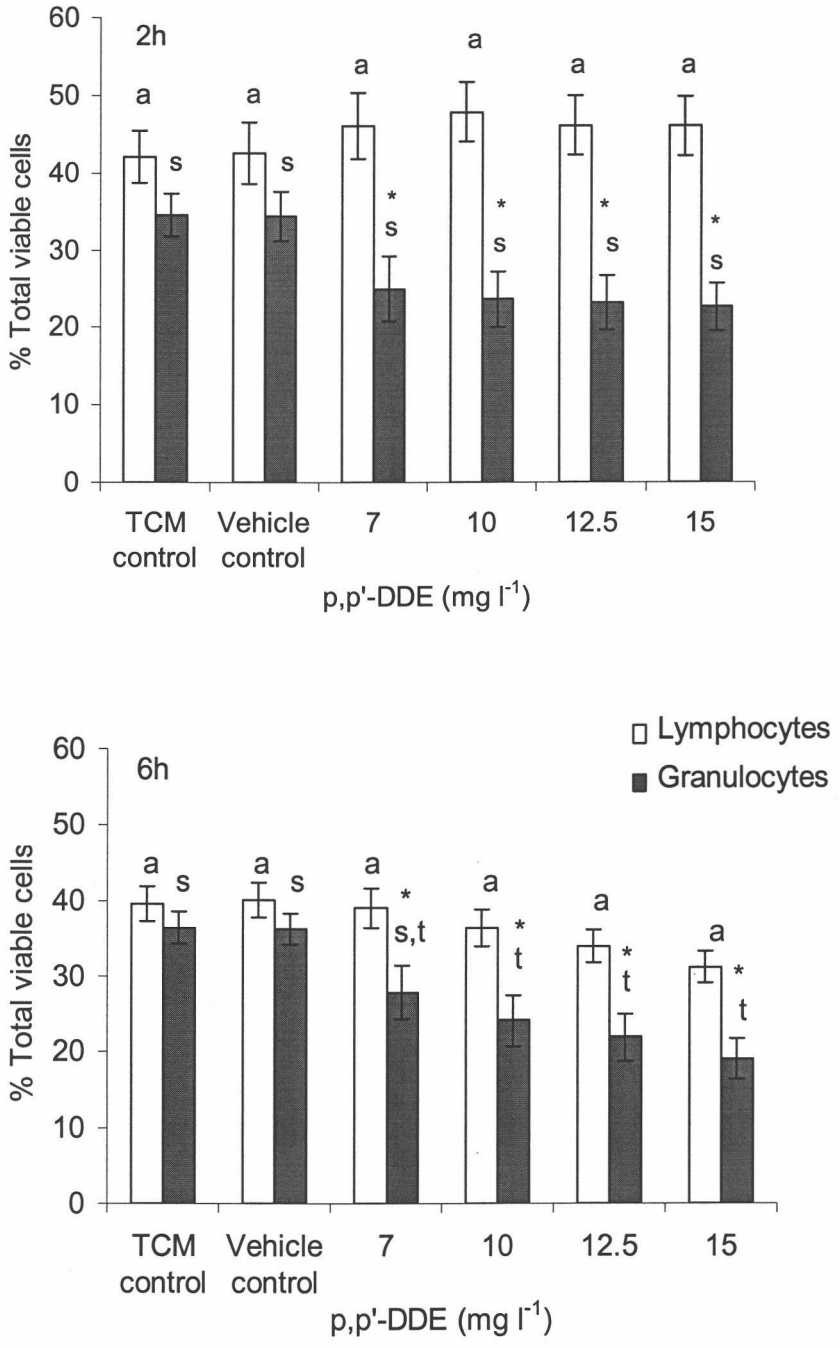

Figure 2.5. Mean (±S.E.M.) percent total viable pronephric lymphocytes and granulocytes following *in vitro* incubation with p,p'DDE for 2 and 6hr. Viability was measured by flow cytometry. Columns that have the same subscripts are not significantly different ($P>0.05$, Kruskal-Wallis test and ANOVA). Significant differences between lymphocytes and macrophage are denoted * ($P<0.05$, Mann-Whitney U-test and t-test) (N=12).

higher than that of granulocytes.

We observed that p,p'-DDE induced significantly more apoptotic cells in the granulocyte population than in the lymphocytes population (Figure 2.6). Without p,p'-DDE, the percentage of apoptotic cells in the granulocyte population was significantly lower than in the lymphocyte population. However, in the presence of p,p'-DDE, the percentage of apoptotic granulocytes did not differ from those in the lymphocyte population or was significantly higher than that of apoptotic lymphocytes

MITOGENIC RESPONSES

Incubation of salmon leukocytes in the presence of 10 mg l^{-1} of p,p'-DDE in the splenic leukocyte culture and 7 and 10 mg l^{-1} of p,p'-DDE in the pronephric leukocyte culture led to significant inhibition of the cell proliferation (Figure 2.7). The percentage of blasting splenic-B-cells stimulated by LPS was also significantly inhibited in the presence of 10 mg l^{-1} of p,p'-DDE in the culture (Figure 2.8).

SEASONAL-RELATED DIFFERENCES IN SENSITIVITY

Incubation of salmon leukocytes in the presence of p,p'-DDE led to a concentration – dependent decrease in the percentage of viable cells in cultures in winter, spring, and summer (Figure 2.9). Seasonal-related differences occurred at concentrations higher than 5 mg/l. Leukocytes isolated from fish in summer were more sensitive to DDE than from fish in winter.

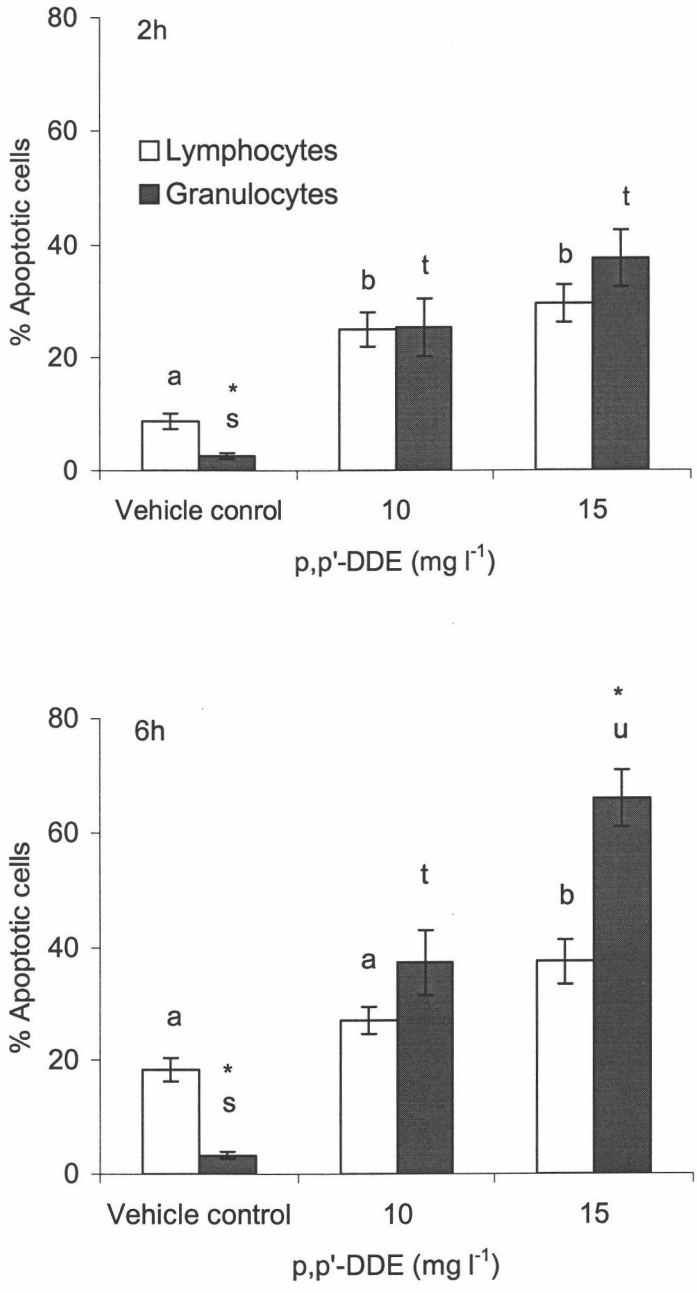

Figure 2.6. Mean (±S.E.M.) percent apoptic cells of pronephric lymphocytes and granulocytes following *in vitro* incubation with p,p'-DDE for 2 and 6 hr (N=12). Apoptosis was measured by flow cytometry. Columns that have the same subscripts are not significantly different ($P>0.05$, Kruskal-Wallis test and ANOVA). Significant differences between lymphocytes and granulocytes are denoted * ($P<0.05$, Mann-Whitney U-test and t-test).

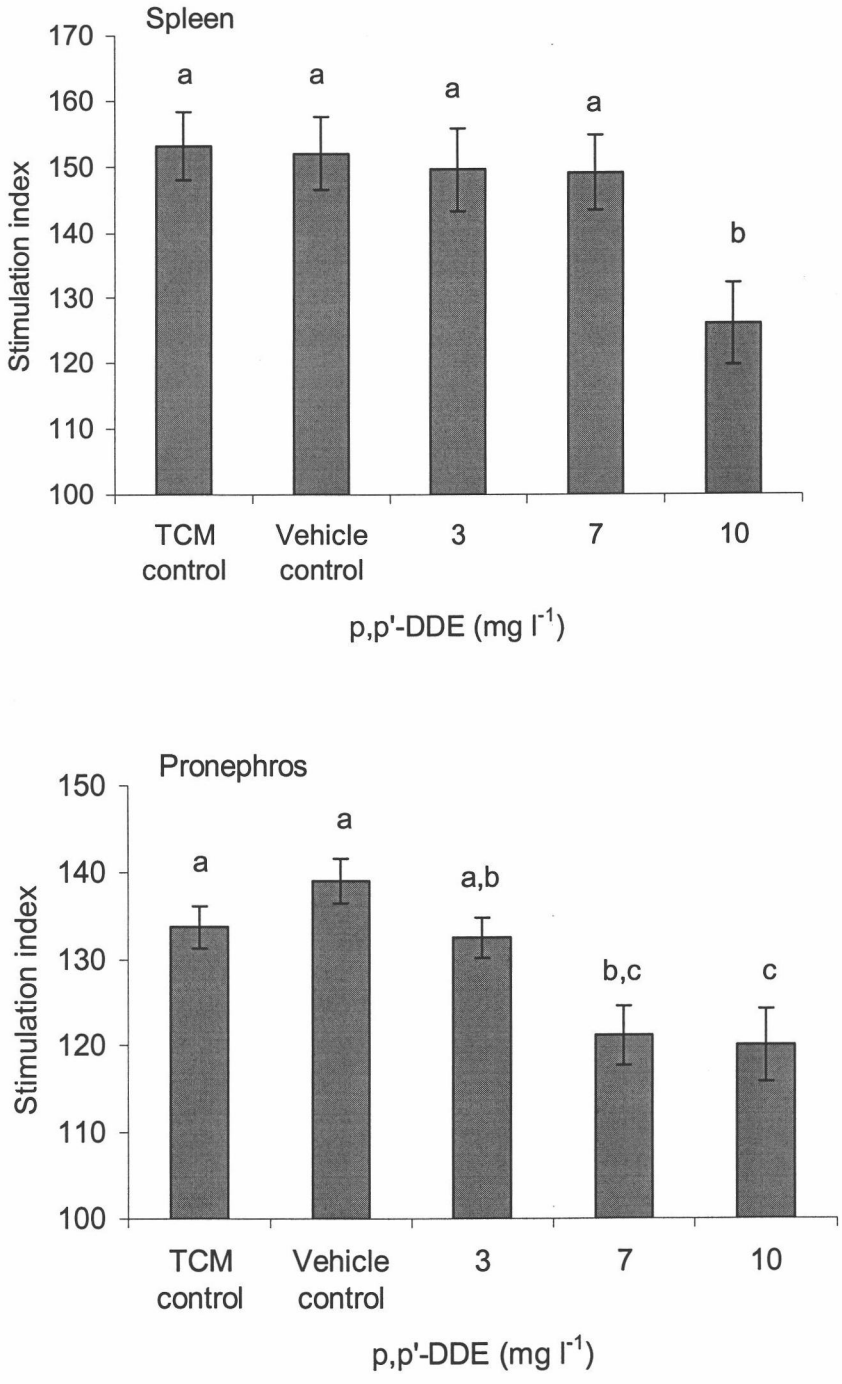

Figure 2.7. Mean (±S.E.M.) stimulation index of mitogen L.P.S. stimulated splenic and pronephric leukocyte proliferation following *in vitro* incubation with p,p'-DDE for 4 days (N=12). The proliferation was analyzed by the Alamar Blue assay. Columns that have the same subscripts are not significantly different ($P>0.05$, Kruskal-Wallis test and ANOVA).

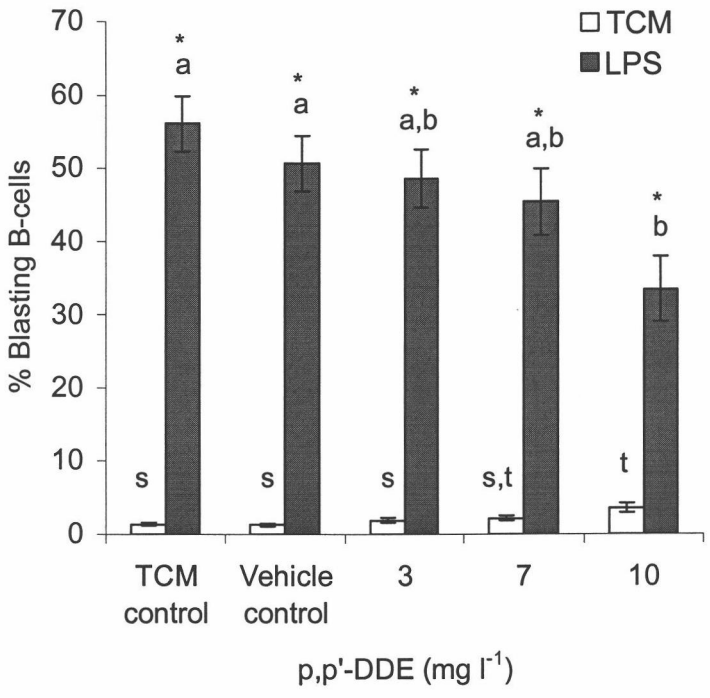

Figure 2.8. Mean (±S.E.M.) percent of blasting splenic B-cells expressing surface immunoglobin when cultured in the presence of p,p'-DDE with TCM alone or LPS for 4 days (N=12). The percentage of blasting leukocytes was measured by flow cytometry. Columns that have the same subscripts are not significantly different ($P>0.05$, Kruskal-Wallis test and ANOVA). Significant differences between LPS and media are denoted with * ($P<0.05$, Mann-Whitney U-test and t-test).

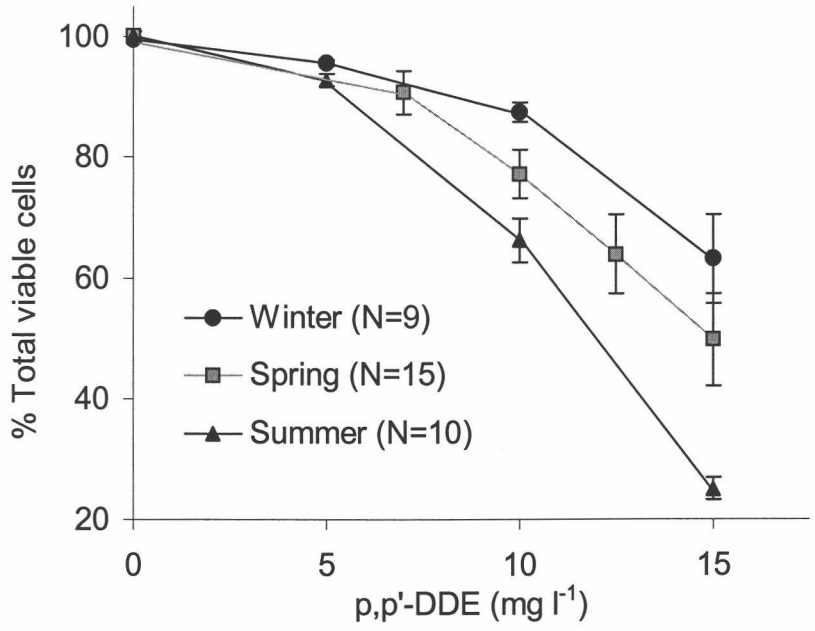

Figure 2.9. Mean (±S.E.M.) percent total viable splenic leukocytes following incubation with different concentrations of p,p'-DDE for 48 hr. Each representative value depicted is the percent of the TCM control. The viability was measured by flow cytometry.

MITOGENIC RESPONSE: *IN VIVO* EXPOSURE

For all treatments, splenic leukocytes, isolated from fish fed p,p'-DDE, treated with LPS had significantly higher percentage of viable Ig+ blasting B-cells than those incubated with TCM alone (Figure 2.10). There were no significant differences between treatments for the percentage of cells undergoing blastogenesis after incubation with TCM alone (Tukey's HSD test). By day 4 of *in vitro* activation with LPS the percentage of Ig+ blasting cells was significantly lower in the 59 ppm p,p'-DDE treatment than 0 ppm treatment (ANOVA; p=0.018, followed by Tukey's HSD test). However, there was a decrease in the percentage of Ig+ blasting cells that was not dose dependent, only leukocytes in salmon treated with 59 ppm DDE showed significantly lower percentage of Ig+ blasting cells than 0 ppm treatment.

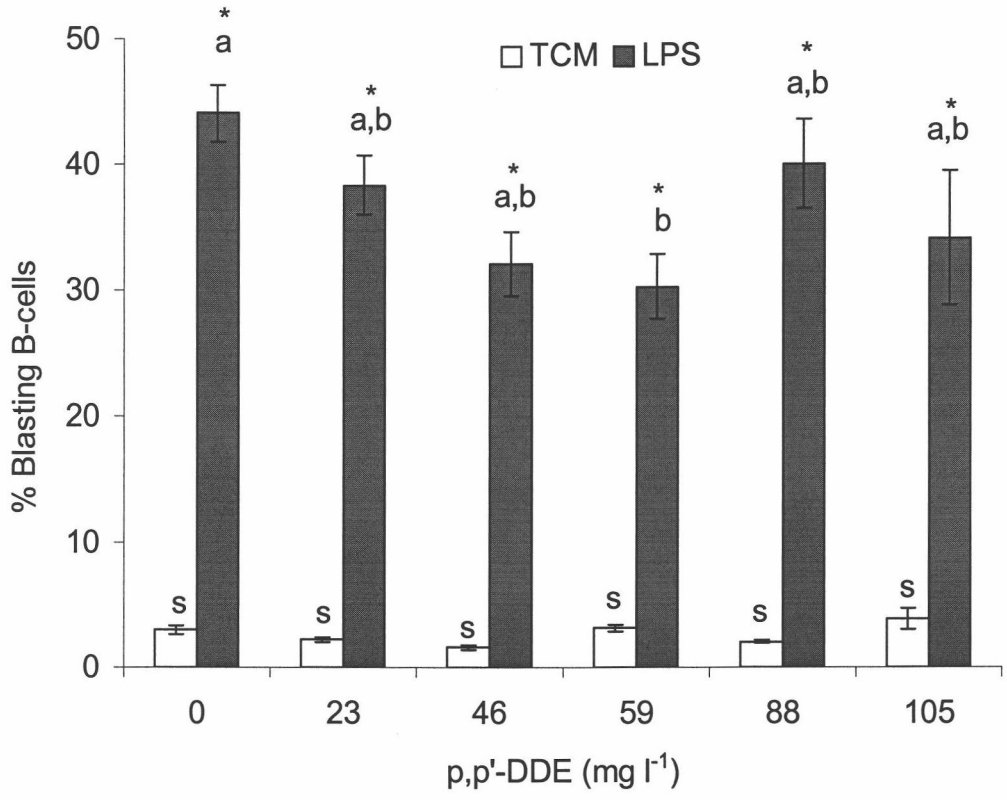

Figure 2.10. Mean (±S.E.M.) percent of Ig+ blasting splenic B-cells expressing surface immunoglobin when cultured with TCM alone or LPS for 4 days following *in vivo* p,p'-DDE exposure (N=16 or 8). The percentage of blasting leukocytes was measured by flow cytometry. Columns that have the same subscripts are not significantly different ($P>0.05$, Kruskal-Wallis test and ANOVA). Significant differences between LPS and media are denoted with* ($P<0.05$, Mann-Whitney U-test and t-test).

Discussion

We examined the specific effects that p,p'-DDE had on chinook salmon leukocytes. In vitro exposure to p,p'-DDE significantly reduced the viability and suppressed the mitogenic responses of salmon leukocytes. In addition, we demonstrated involvement of apoptosis in p,p'-DDE-mediated cell killing. The *in vitro* data leads us to conclude that p,p'-DDE exerts its immunosuppressive effects by direct action on salmonid leukocytes. In addition, after *in vivo* exposure to a total dose of 59 ppm of p,p'-DDE, the capability of lymphocytes to undergo mitogenic response was significantly reduced. Those results give credence to the theory that exposure to chemical contaminants leads to an increase in disease susceptibility and mortality of fish due to immune suppression.

Our data suggests that abnormal induction of apoptosis due to DDE exposure may disturb the natural mechanism of cellular proliferation and lead to immunosuppression. We provide the first direct evidence that DDE mediates apoptosis in salmon leukocytes. Apoptosis (programmed cell death) is distinguished from necrosis (accidental cell death) based on morphological and biochemical patterns of cell degradation (Vermes and Haanen 1994). Apoptosis is a normal and important physiological process, which is frequently found in positive and negative selection of thymocytes, morphogenetic and embryonic development, and maintenance of normal tissue (Vermes *et al.*, 1994; Janeway *et al.*, 1999). Induction of apoptosis is triggered by various signals such as cortisol, anthropogenic chemicals, and radiation and leads to immunosuppression (Weaver *et al.*, 1996; Illidge, 1998; Weyts *et al.*, 1998). Tebourbi *et al.* (1998) reported

that DDT induced excess apoptosis and caused atrophy of rat thymocytes. The mechanism for the induction of apoptosis by DDE is still unclear. Possible mechanisms of action of other organochlorinated pesticides inducing apoptosis is suggested by Kannan *et al.* (2000).

Since p,p'-DDE induced more necrosis as well as apoptosis in cell suspensions than those treated with TCM alone, we suggest that in vitro exposure to DDE induces both pathways of cell death in salmon leukocytes. The induction of both apoptosis and necrosis by *in vitro* exposures other organochlorine contaminants has been reported by a study with lake trout (*Salvelinus namaycush*) (Sweet *et al*, 1998). The exposure to lower concentration of organochlorine contaminant may induce apoptosis, and necrosis may be induced at higher concentrations (Kannan *et al.*, 2000).

The induction of apoptosis is one of the indicators of *in vitro* exposure of salmon leukocytes to DDE and can be used as a biomarker. However, there are difficulties in measuring apoptosis following in vivo exposure due to following reason: 1) single apoptotic cells are scattered, 2) apoptotic cells are rapidly ingested by phagocytic cells, 3) the entire process of apoptotic cell death takes only a few hours, 4) there is no inflammatory reaction (Vermes *et al.*, 1994; Sweet *et al.*, 1998).

Our data showed that granulocytes seem to be more sensitive to DDE than lymphocytes. This result suggests that the nonspecific cellular-immune system involving granulocytes is more vulnerable to p,p'DDE exposure than the specific immune system involving lymphocytes. The mechanism of p,p'-DDE's immunosuppressive action to salmon leukocytes is unclear. One possibility is the direct action of p,p'-DDE on salmonid leukocytes (Banerjee *et al.*, 1996). Kaminski (1986) reported that the cellular

ingestion of lipoprotein-bound DDT by murine phagocytic cells was a potential route into the cells and resulted in immunotoxicity. Thus, if fish granulocytes actively ingest lipoprotein-bound DDE, this action may lead to the death of a significant number of these granulocytes compared to lymphocytes that granulocytes do not ingest.

Our results indicate that the fish sampled during summer were more sensitive to DDE than younger fish sampled during winter or spring. The age- or seasonal-related variations in sensitivity of immune system to toxicants have been reported by studies with fish and rats (Smialowicz *et al.*, 1989; Ottinger and Kaattari, 1998; Duffy *et al.*, 2003). DDE's immunosuppressive action may at least in part be receptor mediated. DDE is a potent antiandrogen that binds to androgen receptors and prevents the normal effect of androgen (Kelce *et al.*, 1995; Sharpe, 1995). Recently, Thomas (2000) and Baatrup & Junge (2001) have reported that DDE showed an antiandrogenic action in fish. The presence of androgen receptors in salmonid lymphocytes and a possible mechanism for androgen-induced immunosuppression has been suggested (Slater *et al.*, 1995). Furthermore, the abundance of androgen receptors of leukocyte changes seasonally and increases in the sexually mature fish (Slater *et al.*, 1998). Maule *et al.* (1993) reported seasonal changes in the affinity and number of hormonal receptor in leukocytes of coho salmon (*O. kisutch*) during the parr-to-smolt transformation. Further work is required to demonstrate the androgen receptors binding affinity for p,p'-DDE in salmon leukocytes. Thereby, it may provide an explanation of the mechanism for the immunomoduratory behavior of p,p'-DDE and the age or seasonal-related variation in sensitivity.

Another possible explanation of the cause for the age- or seasonal-related differences in sensitivity is the natural change in distribution of sub-populations of

salmon splenic leukocytes. Splenic leukocytes used in our study were not homogeneous. Although the major cell type of splenic leukocytes is the B-cells (Milston *et al*, 2002), the presence of T-like lymphocytes in splenic tissue is also considerable (Manning and Nakanishi, 1996). It is known that the composition of leukocytes in fish alters with age or development (Maule *et al.*, 1987; Tatner, 1996). If each sub-population of splenic leukocyte had a different sensitivity to p,p'-DDE, then the sensitivity of splenic leukocytes to DDE could be changed with time.

The mitogenic assay was used to verify effects of p,p'-DDE on functional humoral immunocompetence *in vivo*. This assay was used as a sensitive biomarker measuring potential capability of lymphocytes to respond to antigenic challenge. Following the *in vivo* exposure to 59 ppm p,p'-DDE, the mitogenic response of salmon splenic leukocytes was significantly suppressed. However, exposures to other concentration (23, 46, 88, and 105 ppm) of p,p'-DDE did not affect the mitogenic response. The reason we observed this results may be explained by the principle of homeostasis (Davis and Svendsgaard, 1990). Cragg and Rees (1984) reported that increasing exposure concentration of organic pollutants induces extra metabolism to detoxify and stimulates body growth. It is possible that the immune system may be recovered or stimulated due to extra metabolism by the process of detoxification of p,p'-DDE.

Understanding the mechanism for toxic effects of p,p'-DDE is confounded by the complexity of the immune system. In this study we conclude that the mode of action of *in vitro* exposure is due to direct toxicity to the immune cells. However, it is also possibility that p,p'-DDE might affect the immune system of fish indirectly, by disruption

of the natural interaction between the immune and endocrine systems. p,p'-DDE has been identified as endocrine disruptor in fish through their antiandrogenic activity (Baatrup and Junge, 2001). It is also well established that endocrine factors modulate the fish immune system (Schreck, 1996; Schreck & Maule 2001; Balm, 1997; Weyts *et al.*, 1998; Harris and Bird, 2000). When p,p'-DDE alters the endocrine system, the immune system is likely also affected. Future study must be conducted to determine if p,p'-DDE modulates the immune system via endocrine system.

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Chapter 3

The Effect of *in vitro* Exposure to Tributyltin on the Immune Competence of Chinook Salmon (*Oncorhynchus tshawytscha*) Leukocytes

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Abstract

We evaluated the direct effects of *in vitro* exposures to tributyltin (TBT), a widely used biocide, on the cell mediated immune system of chinook salmon (*Oncorhynchus tshawytscha*). Splenic and pronephric leukocytes isolated from juvenile chinook salmon were exposed for 6, 24, or 96 hr to a concentration range of 0.03 – 0.1 mg TBT l^{-1} in cell cultures. Effects of TBT on cell viability, induction of apoptosis, and mitogenic responses were measured by flow cytometry. Splenic and pronephric leukocytes in the presence of TBT experienced a concentration-dependent decrease in the viability in cell cultures following the induction of apoptosis. In addition, pronephric lymphocytes exhibited a greater sensitivity to TBT exposure than pronephric granulocytes. The functional ability of splenic B-cells to undergo blastogenesis upon LPS stimulation was also significantly inhibited in the presence of 0.05, 0.07, or 0.10 mg l^{-1} of TBT in the cell cultures. Flow cytometric assay using a fluorescent conjugated monoclonal antibody against salmon surface immunoglobulin was employed for the conclusive identification of B-cell in the chinook salmon leukocytes. Our findings suggest that adverse effects of TBT on the function or development of fish immune systems could lead to an increase in disease susceptibility and its subsequent ecological implications.

Introduction

Tributyltin (TBT) has been widely used as agricultural fungicide and antifouling paint for vessels and released into aquatic environment since 1960s (Fent, 1996; Chau *et al.*, 1997). TBT and its metabolites accumulate and persist for prolonged periods in water, aquatic sediment, and aquatic organisms (Becker-van Slooten & Tarradellas, 1995; Chau *et al.*, 1997; Kannan & Falandysz, 1997; Morcillo *et al.*, 1997; Oshima *et al.*, 1997; Ueno *et al.*, 1999). Although TBT is a very effective biocide, a number of studies have reported that TBT exposure causes serious environmental and health problems in non-target aquatic organisms (Short & Thrower, 1986; Alzieu *et al.*, 1989; Maguire, 1987; Wax & Dockstader, 1995; Sumpter, 1998; Axiak *et al.*, 2000). Currently, TBT has been recognized as one of the most toxic anthropogenic chemicals released into the aquatic environment (Mee & Fowler, 1991).

TBT is known to induce immunoaltering effects in fish. Wester & Canton (1987) reported that long-term (3 months) *in vivo* exposure of guppies (*Poecilia reticulata*) to TBT (0.032 – 10 $\mu g\ l^{-1}$) caused thymus atrophy and increase in circulating granulocytes. Schwaiger *et al.* (1994) also reported the elevation of the number of circulating granulocytes in rainbow trout (*Oncorhynchus mykiss*) following *in vivo* exposures to 2 and 4 $\mu g\ l^{-1}$ of TBT for 28 days and 6 $\mu g\ l^{-1}$ of TBT for 10 days, while the number of lymphocytes was significantly reduced. A lymphocytic depletion has been reported in spleen of rainbow trout after *in vivo* exposures to 0.6 – 4.0 $\mu g\ l^{-1}$ of TBT for 28 days (Schwaiger *et al.*, 1992). In channel catfish (*Ictalurus punctatus*), *in vitro* exposure to TBT suppressed non-specific cytotoxic cell activity and humoral immune response

against heat-killed *Edwardsiella ictaluri* (Rice *et al.*, 1995). Brief *in vitro* TBT exposure of kidney macrophages isolated from oyster toadfish (*Opsanus tau*), hogchoker (*Trinectes maculatus*) and Atlantic croaker (*Micropogonias undulatus*) suppressed phagocytic function (Wishkovsky *et al.*, 1989). Although, the effects of TBT on fish immune systems are basically very similar to those in mammals, studies examining the effects of TBT on the fish immune system are more limited (Fent, 1996). Rice *et al.* (1995), O'Halloran *et al.* (1998), and Regala (2001) have reported adverse effects of TBT on fish humoral immune systems, and in addition, Grinwis *et al.* (2000) have concluded that the high prevalence of lymphocystis virus infections in fish observed in the field studies could be related to the TBT exposure. However, basic knowledge regarding the mechanism of action of TBT induced humoral immunosuppression is still lacking. Additional laboratory experiments using more precise techniques such as an *in vitro* lymphocyte cell culture system coupled with a sensitive assay such as the flow cytometric assay are needed to fully evaluate and explain the cause-and-effect relationships between TBT exposure and the cell mediated humoral immunosuppression in fish.

The present study was undertaken to investigate the direct effects of TBT on the immune competence of chinook salmon (*Oncorhynchus tshawytscha*). To elucidate the mechanism of toxic action, the effect of TBT on cell viability and induction of apoptotic cells in leukocytes were analyzed. Moreover, the sensitivity to TBT was compared between pronephric lymphocytes and granulocytes to identify the leukocyte more susceptible to toxicity. Finally, the mitogenic response assay was carried out to examine a possible suppression of function of B-cell mediated humoral immunity by TBT.

Materials and Methods

FISH CARE

Yearling spring chinook salmon (Marion Folks stock) weighing 127.46 ± 7.30 [mean ± standard error of mean (S.E.M.)] g were housed at the Fish Performance and Genetics Laboratory, Oregon State University, Corvallis, Oregon. The fish were maintained in 0.9 m circular fiberglass tanks supplied with 12-13 °C flow-through water under natural photoperiod and fed a commercial diet of Semi-Moist Pellets (BioOregon™, Warrenton, OR) twice daily.

CHEMICALS AND REAGENTS

Tissue culture medium (TCM) contained 7% heat-inactivated fetal bovine serum (Sigma, St. Louis, MO), 1% L- glutamine (Sigma), 200 Iu ml^{-1} penicillin (Sigma), and 0.2 mg ml^{-1} streptomycin (Sigma) in Minimum Essential Media (MEM; Invitrogen Co., Carlsbad, CA) buffered with sodium bicarbonate. Tri-n-butyltin methaoxide (TBT; Chem Service, Inc., West Chester, PA) was dissolved in 100% ethanol and diluted to working solution in TCM at most 2h before starting the incubation with cells. The final concentration of ethanol in each TBT treated culture and vehicle control was always 0.003%. Lipopolysaccharide (LPS; Sigma) from *Escherichia coli* serotype O 55:B5 was dissolved in the TCM to a final concentration of 200 μg ml^{-1} in each cell culture. Isolation medium was composed of Hank's balanced salt solution and Alserver's solution (0.1 M dextrose, 70 mM sodium chloride, and 30 mM sodium citrate).

TISSUE SAMPLE

Fish were rapidly netted from their tanks, immediately killed in 200 mg l^{-1} buffered tricaine methane-sulfonate (MS 222), weighed, and then bled by caudal severance. The fish were then transported on ice to our immunology laboratory at Oregon State University within 30 min. The spleen and pronephros were aseptically isolated and placed separately into individual conical tubes filled with 1 ml of cold isolation medium.

ISOLATION AND CULTIVATION OF LEUKOCYTES

The isolated tissue was replaced on a 40 μm nylon cell strainer (Becton Dickinson, Franklin Lakes, NJ) from conical tubes and gently disrupted with the end of a 3 ml syringe plunger. The disrupted tissue was washed through a strainer into a 50 ml polypropylene conical tube (Becton Dickinson) with isolation medium. The homogenized tissue suspension was then centrifuged at 500 g for 7 min at 4°C and the supernatant was aspirated. The pellet was resuspended with 2 ml of ice-cold isolation medium, and clumps were removed. Hypotonic lysis was used as described by Crippen *et al.* (2001) in order to purify and separate leukocytes from erythrocytes. Briefly, the 2 ml of cell suspension were diluted with 9 ml sterile deionized water to lyses the erythrocytes for 20 s, and then 1 ml of sterile 10X phosphate buffered saline (PBS) was immediately added to stop lysing. Cells were washed twice by centrifugation at 500 g for 7 min at 4°C, the supernatant aspirated, and the cells resuspended in 2 ml of ice-cold TCM. After the purification of leukocytes, viable cells were counted using a trypan blue exclusion test, and the cell suspension was diluted with ice-cold TCM to final

concentration of 5 x 10^6 viable cells ml^{-1}. We did not pool each cell suspension from each individual fish. The cell suspension (100 µl $well^{-1}$) including 5 x 10^6 viable leukocytes was plated out into flat bottm 96-well plates (Becton Dickinson), and then TBT solution, LPS solution, and/or TCM were added to a final volume of 200 µl $well^{-1}$. The cell cultures were maintained at 17 °C in an incubator culture chamber (C.B.S. Scientific CO., Del Mar, CA) containing blood gas mixture (10% O_2, 10% CO_2, and 80% N_2).

FLOW CYTOMETRIC ASSAY OF CELL VIABILITY

Splenic and pronephric leukocytes were exposed to various concentration of TBT (0, 0.1, 0.2, 0.3, 0.4, 0.5, and 0.6 mg l^{-1}) for 24 hours. TCM alone control contained 100 µl of cell suspension and 100 µl of TCM. Vehicle control contained 100 µl of cell suspension and 100 µl of concentrated ethanol (0.006%) with TCM. Cell viability was analyzed by flow cytometry (FACScan®; Becton Dickinson), as described previously (Misumi *et al.*, 2003a). Flow cytometry was also used for distinguish among lymphocytes, granulocytes, and other cells in the pronephric leukocyte suspension based on the cell size and granularity as described previously (Misumi *et al.*, 2003a).

FLOW CYTOMETRIC ASSAY OF APOPTOSIS

Splenic and pronephric leukocytes were exposed to TBT (0, 0.3, and 0.6 mg l^{-1}) in the cell cultures for 6 hours. The percentage of apoptotic cells (Annexin V positive and PI negative) was measured using ApoAlert Annexin V-FITC Apoptosis kit (BD Biosciences Clontech, Palo Alto, CA) with flow cytometry as previously described

(Misumi *et al.*, 2003 b). Control culture contained 0.003% ethanol as a vehicle control. Following incubation, 50 µl of cell suspensions were centrifuged in microtubes (Bio-Rad Laboratories, Inc., Hercules, CA) at 500 g for 7 min at 4°C and resuspended in 40 µl of Annexin V Binding Buffer containing 1 µl of Annexin V-FITC and 2 µl of PI. Cells were incubated for 10 min at room temperature according to the manufacture's protocol (ApoAlert Annexin V-FITC Apoptosis kit; BD Biosciences Clontech). Apoptotic cells (Annexin V positive and PI negative) were detected and distinguished by flow cytometry.

FLOW CYTOMETRIC ASSAY OF MITOGENIC RESPONSE

Splenic and pronephric leukocytes we exposed to TBT (0, 0.03, 0.05, 0.07, and 0.1 mg l^{-1}) with or without LPS for 96 hours. TBT and LPS were present for the whole duration in the cell culture. Flow cytometry was used for measuring the percentage of Ig+ blasting (larger) B-cells in the viable cell population, as previously described (Milston *et al.*, 2002; Misumi *et al.*, 2003 b). Anti-salmon and trout Ig Mab Biotin Labeled solution was purchased from DiagXotics Inc. (Wilton, CT) and used to detect B-cells expressing Ig on their cell surface. At same time, the cell viability was also measured to determine if cell death might relate to the inhibition of blastogenesis by TBT.

STATISTICAL ANALYSIS

The mean and S.E.M. were determined for the 9 individual fish samples for each immune test. There was no replication of assays within any individual fish. Percent values were transformed by arcsine of the square root of the value for further statistical analysis. Parametric statistical tests (the Student's t-test and ANOVA) and non-

parametric statistical tests (the Kruskal-Wallace test and the Mann-Whitney U-test) were used for statistical comparisons of data. Measurements were considered significant when *P* values of both parametric and non-parametric tests were below 0.05. Tukey's honestly significant difference procedure (HSD) was used to determine which treatments are significantly different from others.

Results

CELL VIABILITY

Chinook salmon leukocytes in the presence of TBT experienced a concentration-dependent decrease in the viability in cultures (Figure 3.1). There was no difference between splenic and pronephric leukocytes in viability. There was no difference in viability between the medium alone control and vehicle control (data not shown).

APOPTOSIS

A significantly higher percent of apoptotic cells was observed in the splenic leukocyte culture exposed to 0.6 mg l^{-1} TBT than other splenic leukocytes cultures (Figure 3.2). Pronephric leukocyte cultures exposed to TBT led to a concentration-dependent increased in the percent apoptotic cells. The pronephric leukocyte culture exposed to 0.3 mg l^{-1} TBT concentration resulted in significantly higher percent of apoptotic cells than the splenic leukocyte culture exposed to same concentration of TBT.

TWO DIFFERENT SUBPOPULATIONS OF PRONEPHRIC LEUKOCYTES

The rate of viable lymphocytes in the pronephric leukocyte culture significantly decreased with concentrations of TBT after 96 hr incubation. On the other hand, the rate of viable granulocytes in the pronephric leukocyte culture significantly increased with concentrations of TBT (Figure 3.3 a, b, and c). The rate of viable lymphocytes was significantly higher than that of granulocytes in the presence of 0 mg l^{-1} of TBT and significantly lower in the presence of 0.1 mg l^{-1} of TBT (Figure 3.3 c). Although each rate of viable lymphocytes and granulocytes varied toward opposite directions, at this

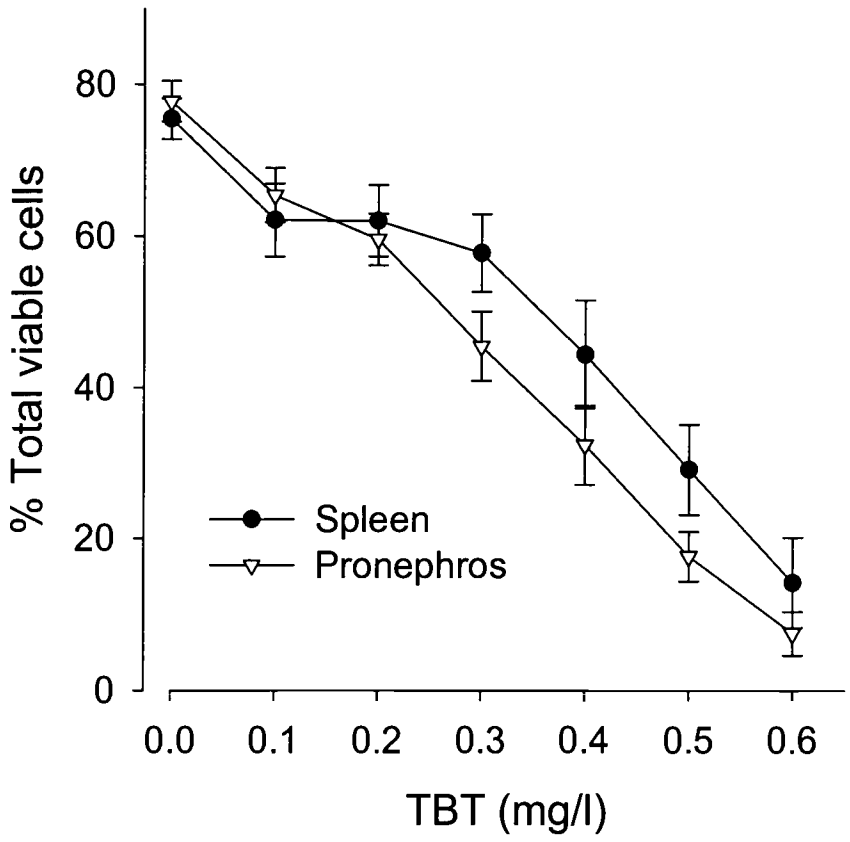

Figure 3.1. Mean percent (±S.E.M.) total viable splenic and pronephric leukocytes following exposure to TBT (0, 0.1, 0.2, 0.3, 0.4, 0.5, and 0.6 mg l^{-1}) for 24 hr (N=9). The percentage of viable leukocytes in the cell culture was measured by the flow cytometry with PI staining.

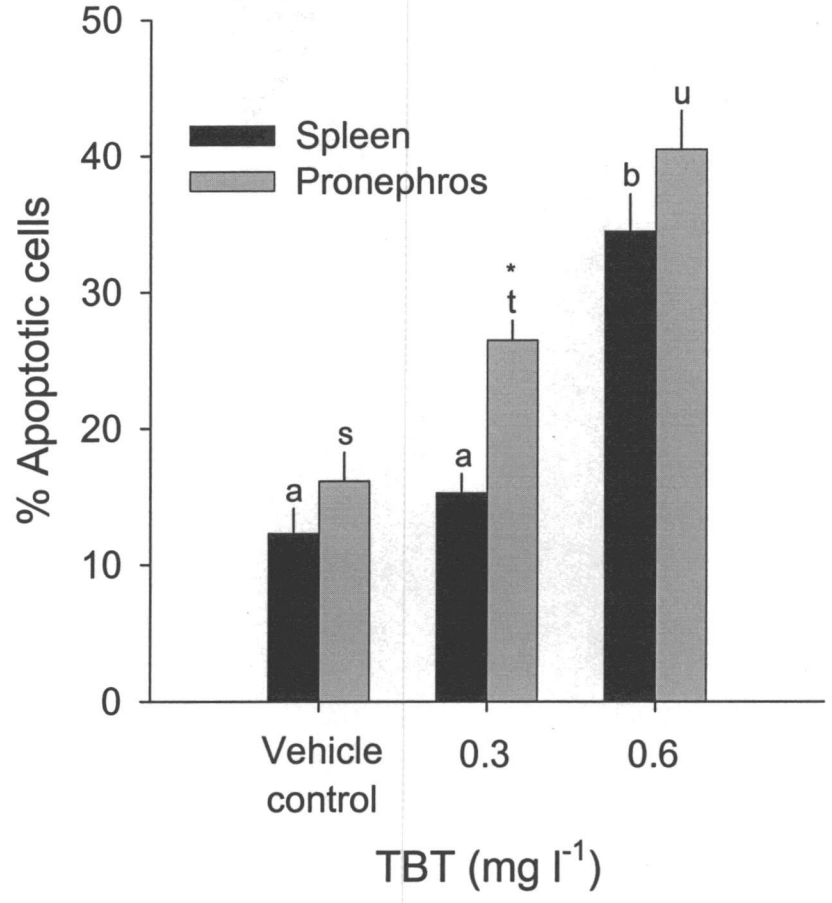

Figure 3.2. Mean percent (±S.E.M.) splenic and pronephric apoptotic leukocytes following exposure to TBT (0, 0.3, and 0.6 mg l^{-1}) for 6 hr (N=9). Columns that have the same superscripts are not significantly different ($P>0.05$, Kruskal-Wallis test and ANOVA). Significant differences between spleen and pronephros are denoted * ($P<0.05$, Mann-Whitney U-test and Stutent's t-test).

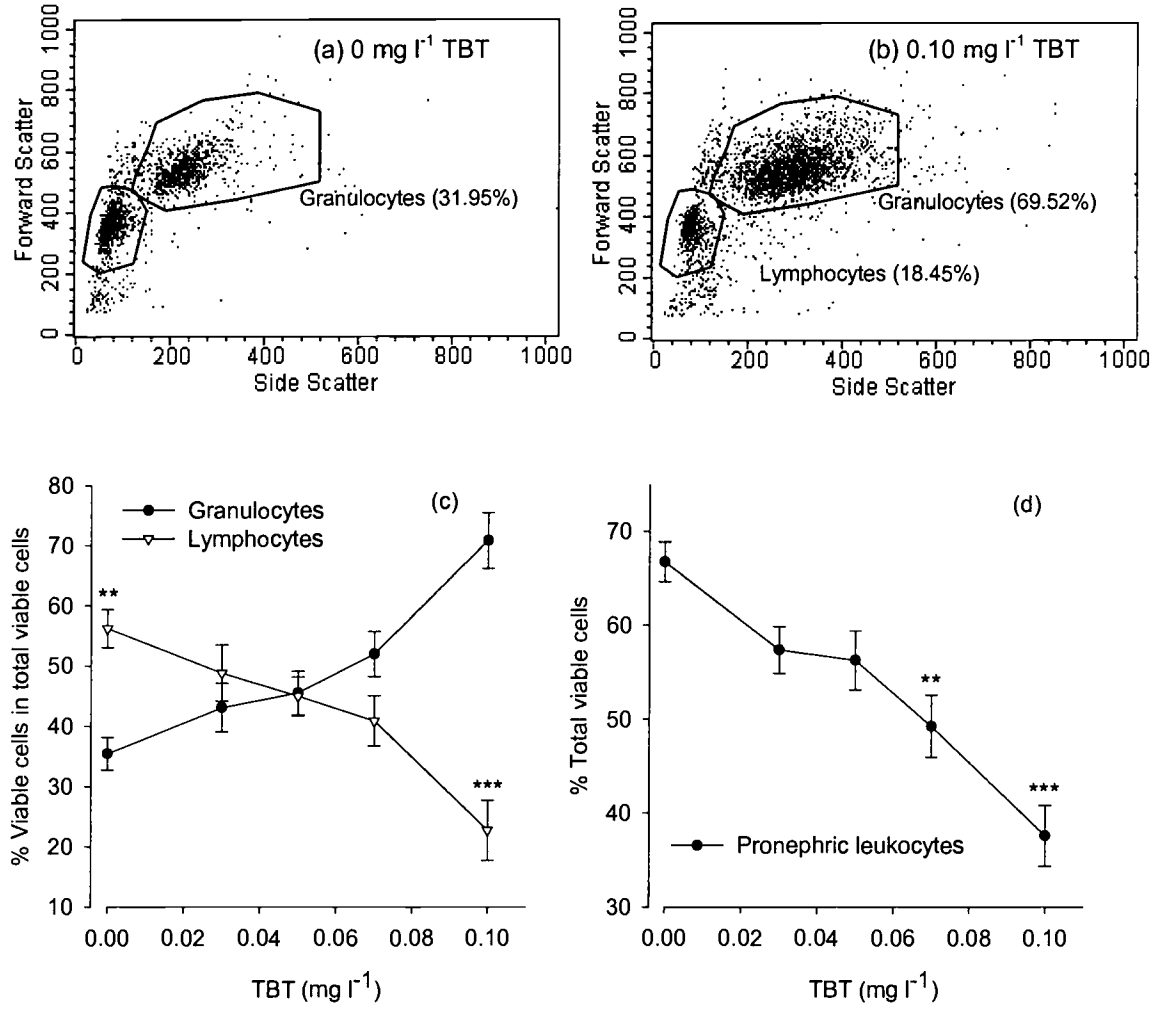

Figure 3.3. Effects of exposure of pronephric leukocytes to TBT. Representative flow cytometric forward and side scatter dot plots following incubation for 96 hr without TBT (a) and with TBT (0.1 mg l^{-1}) (b). Mean (±S.E.M.) percent viable lymphocytes and granulocytes in the pronephric cell culture exposed to TBT (0, 0.03, 0.05, 0.07, and 0.1 mg l^{-1}) for 96 hr (N=9) (c). Significant differences between lymphocytes and granulocytes are denoted adobe plots of lymphocytes *** (P<0.001, Mann-Whitney U-test and Stutent's t-test), ** (P<0.01), or * (P<0.05). Mean (±S.E.M.) percent viable pronephric leukocytes in the cell culture exposed to TBT (0, 0.03, 0.05, 0.07, and 0.1 mg l^{-1}) for 96 hr (N=9) (d). Any TBT treatments, which are significantly different from vehicle control (0 mg l^{-1} TBT) are denoted *** (P<0.001, Mann-Whitney U-test and Stutent's t-test) or ** (P<0.01).

point we did not know which cell type was the driving force (granulocytes increased or lymphocytes decreased). Therefore, we examined whether total viable cells increased or decreased. Then, we observed the percent of total viable cells in the pronephric cell culture significantly decreased with concentrations of TBT (Figure 3.3 d); besides the decrease in the number of viable pronephric cells after TBT exposure was observed under the microscope (data not shown). That is to say, it seems that the percentage of granulocyte increased as the result of the depression of the number of lymphocytes in the pronephric cell culture. Lymphocytes exhibited a greater sensitivity to TBT exposure than granulocytes.

MITOGENIC RESPONSE

The functional ability of splenic B-cells to undergo blastogenesis upon LPS stimulation was significantly inhibited in the presence of 0.05, 0.07, or 0.10 mg l^{-1} of TBT in the cell cultures (Figure 3.4 a). There were no significant differences among treatments for the percentage of blasting B-cells in the cell cultures incubated without LPS (TCM alone). For all treatments, splenic leukocytes treated with LPS had significantly higher percentage of blasting B-cells than those incubated with TCM alone.

The cell viability was significantly suppressed in the unstimulated cell cultures exposed to 0.10 mg l^{-1} of TBT (Figure 3.4 b). In the LPS stimulated cell cultures, significant suppression in viability was observed at 0.07 and 0.10 mg l^{-1} of TBT. The cell viabilities in the LPS stimulated cell cultures were always higher than those in unstimulated cell cultures except in the culture exposed to the highest concentration of TBT tested, 0.10 mg l^{-1}.

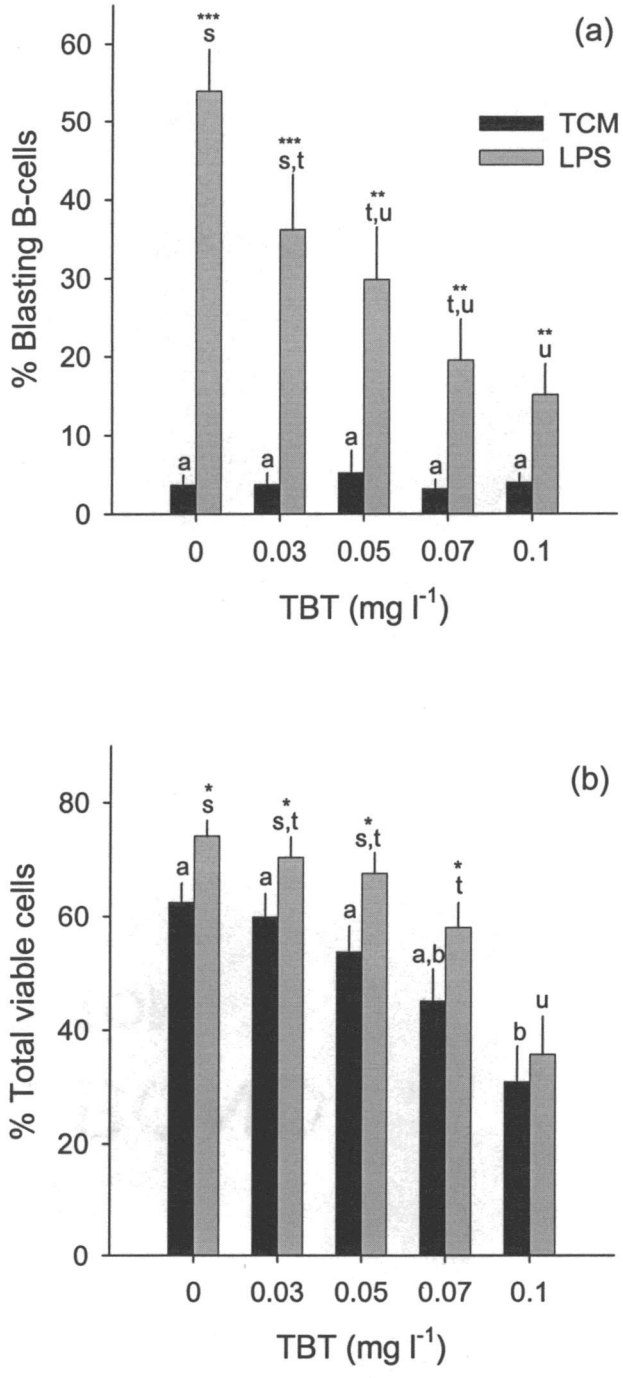

Figure 3.4. The effect of *in vitro* exposures to TBT on blastogenesis of splenic B-cells. Mean (±S.E.M.) percent of blasting splenic B-cells expressing surface Ig (a) and percent viable cells (b) in the splenic cell culture following exposure to TBT (0, 0.03, 0.05, 0.07, and 0.1 mg l^{-1}) with TCM alone or LPS for 96 hr (N=9). Columns that have the same superscripts are not significantly different ($P>0.05$, Kruskal-Wallis test and ANOVA). Significant differences between LPS and media are denoted with * ($P<0.05$, Mann-Whitney U-test and t-test).

Discussion

In vitro exposure to TBT induced the immunosuppression in splenic and pronephric leukocytes isolated from chinook salmon and significantly reduced cell viability following the induction of apoptosis. Furthermore, splenic leukocytes cultured with LPS in the presence of TBT demonstrated a dose-dependent inhibitory effect on the ability of mitogen-stimulated B-cells to undergo blastogenesis. The findings suggest that adverse effects of TBT on the function or development of fish immune systems could lead to an increase in disease susceptibility and its subsequent ecological implications.

Apoptosis (programmed cell death) is distinguished from necrosis (accidental cell death) based on morphological and biochemical pattern of cell degradation (Fawthrop *et al.*, 1991; Vermes & Haanen, 1994). Apoptosis is a normal and important physiological process for proliferating cells frequently found in positive and negative selection of thymocytes, morphological development of embryos, or maintenance of the normal tissue (Abelli *et al.*, 1998; Vermes & Haanen, 1994; Janeway *et al.*, 1999). Therefore, the disorganization of the apoptotic process by TBT could lead to serious health problems and disorders in immunoregulation, causing hypersensitivity and autoimmunity (O'Halloran *et al.*, 1998). Following TBT exposures, histopathologic alterations included thymic atrophy in guppy (Wester & Canton, 1987) and splenic lymphocytic depletion associated with a decrease in circulating lymphocytes in rainbow trout (Schwaiger *et al.*, 1992, 1994). Several studies using rats have also reported the thymic atrophy and splenic lymphocyte depletion following TBT exposures (Funahashi *et al.*, 1980; Vos *et al.*, 1984; Snoeij *et al.*, 1988). Although a direct link between apoptosis and thymic atrophy and

splenic depletion in fish had not been reported, Raffray & Cohen (1993) and Raffray *et al.* (1993) concluded that the TBT triggered apoptosis in thymocytes, resulting in thymic atrophy in the rat. Our study demonstrated for the first time that TBT induced apoptosis in fish splenic and pronephric leukocytes. This suggests a direct link between apoptosis and the results of histopathologic alterations in fish exposed to TBT.

Pronephric lymphocytes exhibited a greater sensitivity to TBT exposure than pronephric granulocytes. This finding is of particular interest because a previous study (Schwaiger *et al.*, 1994) in the rainbow trout has demonstrated the elevation of the number of circulating granulocytes in rainbow trout following prolonged *in vivo* exposure to sublethal concentrations of TBT, while the number of lymphocytes was significantly reduced. Increase of granulocytes following TBT exposure has also been reported in the guppy (Wester & Canton, 1987) and rat (Krajnc *et al.*, 1984; Raffray & Cohen, 1993). Those results suggest that specific immune systems involving lymphocytes are more vulnerable to TBT exposure than nonspecific cellular immune systems involving granulocytes.

We found that the cell population profiles in cell cultures were different between splenic and pronephric leukocytes. Most splenic leukocytes were lymphocytes (>90%), while pronephric leukocytes consisted of not only lymphocytes (57%) but also granulocytes (35%). Because our findings indicate that lymphocytes were more sensitive to TBT than granulocytes, we would hypothesize that splenic leukocytes populations might exhibit a greater sensitivity than pronephric leukocytes populations. However, we actually observed there were no statistically significant differences in sensitivity to TBT exposures between splenic and pronephric leukocytes. Those findings might relate to the

fact that the teleost pronephros is the primary site of hematopoiesis (Yasutake & Wales 1983) and pronephric lymphocytes consist of relatively undifferentiated cells (e.g. stem cell or pro-B-cells) (Etlinger *et al.*, 1976; Milston *et al.*, 2002). Undifferentiated young lymphocytes could be more sensitive to TBT than differentiated, matured cells. The sensitivity of lymphocytes to TBT possibly depends on the level of the cell development.

Our data showed that TBT suppressed the cell blastogenesis of surface IgM presenting B-cells. Our findings support O'Halloran *et al.* (1996) who reported that TBT selectively suppressed the mitogenic activity of LPS-stimulated leukocytes. The mitogenic cellular responses are potentially sensitive indicators commonly used to evaluate effects of exposure to xenobiotics on lymphocyte function (Faisal 1991; Arkoosh et al., 1996). Following stimulation with LPS (B-cell mitogen), resting B-cells enlarge their size, increase their rate of new RNA and protein synthesis, and develop into B-lymphoblasts (Janeway *et al.*, 1999). The B-lymphoblasts then differentiate into mature plasma cells which can secrete significant amount of antibodies (Janeway *et al.*, 1999). Therefore, we suggest that when blastogenesis is suppressed by TBT, the amount of circulating antibody can be reduced in fish. We then speculated that the suppression of the humoral immune responses could lead to an increase in disease susceptibility and mortality in fish.

Weyts (1997) concluded that the cortisol induced suppression of mitogenic responses of carp lymphocytes was due to actual cell death involving apoptosis. We, therefore, investigated cell viability in the cell cultures which were used for the mitogenic response assay to determine if inhibition of mitogenic responses by TBT was related to actual cell death. Although the cell viability was significantly suppressed in the

unstimulated cell cultures exposed to 0.1 mg l^{-1} of TBT, there were no differences in the percent blasting B-cells among all unstimulated cell cultures. On the other hand, in the LPS-stimulated cell cultures, the viability was suppressed when those cultures were exposed to 0.07 and 0.1 mg l^{-1} of TBT, and the percent blasting B-cells in the viable cells was suppressed at 0.05, 0.07, and 0.1 mg l^{-1}. In fact, in the unstimulated cell cultures, cell death was not related to the percent blasting B-cells. In the LPS-stimulated cell cultures, the suppression of mitogenic response in the cell culture exposed to 0.05 mg l^{-1} was also not relevant to cell death. This suggests that the cytotoxic effect of TBT on blastogenesis of salmon B-cell could result in an inhibition of mitosis of resting B-cells without cell death. The TBT-induced inhibitions of mitogenic responses in the cell cultures with 0.07 and 0.1 mg l^{-1} TBT might be associated with cell death. Misumi *et al.* (2003a) reported that lower density of viable cells causes lower induction of blasting B-cell in the culture following 4 days incubation with LPS.

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GENERAL CONCLUSIONS AND SPECULATIONS

We examined the effects of the exposures to anthropogenic pollutants on the fish, primarily juvenile chinook salmon, immune system using newly and recently developed immune assays. In addition, we developed a new assay for measuring immunocompetence of fish. In the first chapter of the thesis, the Alamar Blue assay was calibrated for quantifying the proliferation of chinook salmon splenic and pronephric leukocytes following stimulating with mitogens. Optimum or effective cultural conditions for the Alamar Blue assay using LPS were: 1) optimum cell density for splenic and pronephric leukocytes: between 0.5 x 10^5 and 2.5 x 10^5 cells per well; 2) effective LPS concentration for splenic and pronephric leukocytes: between 50 and 300 mg l^{-1}; and 3) effective incubation time with LPS for splenic leukocytes: between day 3 and day 8; effective incubation time with LPS for pronephric leukocytes: day 7 and day 8.

We demonstrated that the Alamar Blue assay can be useful for assessing fish immune function. At present the three assays most frequently used for the quantification of the mitogenic response of fish are: 1) [^{3}H]-thymidine incorporation (DeKoning & Kaattari, 1991); 2) colorimetric assay (Daly *et al.*, 1995); and 3) flow cytometric assay (Chilmonczyk & Monge, 1998, Milston *et al.*, 2003). The traditional and most used assay is [^{3}H]-thymidine incorporation (Etlinger *et al.*, 1976; Sizemore *et al.*, 1984). This assay is very sensitive, but it requires handling of radioactive material and cell lysis to determine the uptake of ^{3}[H]-thymidine (Pechhold & Kabelitz, 1998). Moreover, production of radioactive waste is clearly an environmental and financial disadvantage. The flow cytometric assay does not require radioactive materials and is very accurate to

measure blastogenesis (Milston *et al.*, 2003). However, the flow cytometry is very expensive. Therefore, we developed an Alamar Blue assay, one of the colorimetric assays, as an alternative. This assay is safer and easier than the traditional [^{3}H]-thymidine incorporation assay, and allows cells to remain undamaged for further analysis (Mosmann, 1983; Zhi-Jun *et al.*, 1997). And the Alamar Blue assay is considerably less expensive and suitable for measuring large sample size comparing to the flow cytometric assay. In conclusion, Alamar Blue assay is very simple, safe, and inexpensive relative to others.

While the manufacturer of Alamar Blue dye has not revealed its chemical identity (Horobin, 2001). O'Brien *et al.* (2000) have identified Alamar Blue as resazurin. After Alamar Blue is taken into cells, nonfluorescent resazurin (non-reduced Alamar Blue) is reduced to fluorescent resorufin (reduced Alamar Blue) by intracellular reductases (Rasmussen, 1999; Andrews *et al.*, 1997). Resazurin has been used to assess bacterial contamination of milk and evaluate quality of bovine sperm for more than 50 years (Rasmussen, 1999). Although we have not tried resazurin instead of Alamar Blue, if resazurin produces comparable results to Alamar Blue for assessing mitogenic response, then the cost of the assay could be further reduced because resazurin is much cheaper than Alamar blue.

In the second chapter, effects of *in vitro* and *in vivo* exposures to p,p'-DDE on chinook salmon leukocytes were examined. *In vitro* exposure to p,p'-DDE significantly reduced cell viability, and we demonstrated the involvement of apoptosis in p,p'-DDE-mediated cell killing. In addition, the Alamar Blue assay and the flow cytometric assay determined that *in vitro* exposure to p,p'-DDE causes suppression of the humoral

immune responses in chinook salmon leukocytes. In addition, in *vitro* exposure significantly suppressed mitogenic responses of salmon leukocytes. Those *in vitro* results lead us to conclude that p,p'-DDE exerts its immunosuppressive effects by direct action on salmonid leukocytes. After *in vivo* exposure to a total dose of 59 ppm of p,p'-DDE, the capability of lymphocytes to undergo mitogenic response was significantly reduced. Those *in vitro* and *in vivo* results give credence to the theory that exposure to p,p'-DDE leads to an increase in disease susceptibility of fish due to immune suppression.

In the third chapter, effects of *in vitro* exposure to TBT on the cell-mediated immune system of chinook salmon was examined. *In vitro* exposure to TBT significantly reduced the viability and suppressed blastogenesis of surface IgM presenting B-cells. In addition, we found that involvement of apoptosis in TBT-mediated cell killing. We also found that pronephric lymphocytes exhibited a greater sensitivity to TBT exposure than pronephric granulocyte. The result supports a previous study (Schwaiger *et al.*, 1994) that demonstrated the elevation of the number of circulating granulocytes in rainbow trout following prolonged *in vivo* exposure to sublethal concentrations of TBT, while the number of lymphocytes was significantly reduced. Based on those results, we concluded that TBT exerts its immunosuppressive effects by direct action on salmonid leukocytes. In conclusion, our finding that anthropogenic chemicals can negatively affect the immune system of fish suggests that exposures to such chemicals in the wild would reduce the ability of the fish to avoid infection or pathogenesis. Consequently, exposures to contaminants can lead to an increase in disease susceptibility and mortality of fish due to immune suppression and be associated with declines of populations.

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APPENDICES

APPENDIX A:

Developing mitogenic response assays for common carp (*Cyprinus carpio*) and assessing effects of in vitro exposures to p,p'-DDE and TBT on splenic and pronephric leukocytes.

Objective

To calibrate the flow cytometric assay and the Alamar Blue assay to quantify the mitogenic response in common carp leukocytes.

General Methods

Common carp weighing 1.5 – 2.3 kg were captured from the Willamette River, Oregon, and housed at the Fish Performance and Genetics Laboratory, Oregon State University, Corvallis, Oregon. The fish were maintained in a rectangular (7.0 x 1.0 m) fiberglass tank supplied with 12-13 °C flow-through water under natural photoperiod and fed a commercial diet of Semi-Moist Pellets (BioOregon™) twice daily. Sampling was conducted on October and November, 2002.

Fish were rapidly netted from their tank, immediately killed in 200 mg l^{-1} buffered tricaine methanosulfonate, weighed, and then bled by caudal severance. The fish were then transported on ice to our immunology laboratory at Oregon State University within 30 min. Tissue culture medium (TCM) and isolation-medium was exactly the same as the one developed for the mitogenic assays for chinook salmon (chapter 1 of this thesis). The spleen and pronephros were aseptically isolated and placed separately into individual Falcon tubes (12ml) filled with 5 ml of cold isolation medium. The isolated tissue was placed on a 40 μm nylon cell strainer (Becton Dickinson) and gently disrupted with the end of a 3 cc syringe plunger. The disrupted tissue was washed through a strainer into a 50 ml polypropylene conical tube (Becton Dickinson) with isolation medium. The homogenized tissue suspension was then centrifuged at 500 g for 7 min at 4°C and the supernatant was aspirated. The pellet was resuspended with 2 ml of ice-cold isolation

medium, and clumps were removed. The organ removal and tissue processing were all conducted under aseptic conditions in a laminar flow hood. In order to eliminate erythrocytes from the leukocyte suspension, hypotonic lysis was used as described in chapter 1 of this thesis. After the hypotonic lysis, viable cells were counted using the trypan blue exclusion test, and the cell suspension was diluted with ice-cold TCM to a final concentration of viable cells. The cell suspension (100 μl $well^{-1}$) was plated out into flat bottom 96-well plates (Becton Dickinson), and then TCM with or without LPS was added to a final volume of 200 μl $well^{-1}$. The cell cultures were maintained at 17 °C in an incubator culture chamber (C.B.S. Scientific CO.) containing blood gas mixture (10% O_2, 10% CO_2, and 80% N_2) and set on a wet paper towel for humidity.

Alamar Blue (10% of the final volume of the cell suspension present in each well) was added to the cell culture 24 hr before measurement. Optical densities (OD) at 570 nm (reduced) and 600 nm (oxidized) were then measured with OPTImax™ Tunable Microplate Reader (Molecular Devices CO.). Flow cytometric assays were performed using the same cell cultures as those used in the Alamar Blue assay. 200 μl of cell suspensions in 96 well plates were rinsed twice by centrifugation at 300 g for 3 min at 4°C. The supernatants were discarded, and the cells were resuspended with 200 μl of ice-cold PBS and 20 μl of propidium iodide (PI, Sigma) solution (50 μg ml^{-1} in PBS). Cell suspensions were kept on ice for 5~10 min in the dark. Cell viability and the level of blastogenesis were measured using the flow cytometry (FACScan®, Becton Dickinson). Setting of the flow cytometry was adjusted for carp leukocytes.

Sample date	Sex	Weight (kg)	Hematocrit (%)
10/3/2002	Female (matured)	2.1	-
10/23/2002	Male (matured)	1.9	-
10/29/2002	Female (matured)	2.3	35.9
10/29/2002	Male (matured)	1.5	69.3

Table. A.1. Summary of fish sex, weight, and percentage of hematocrit.

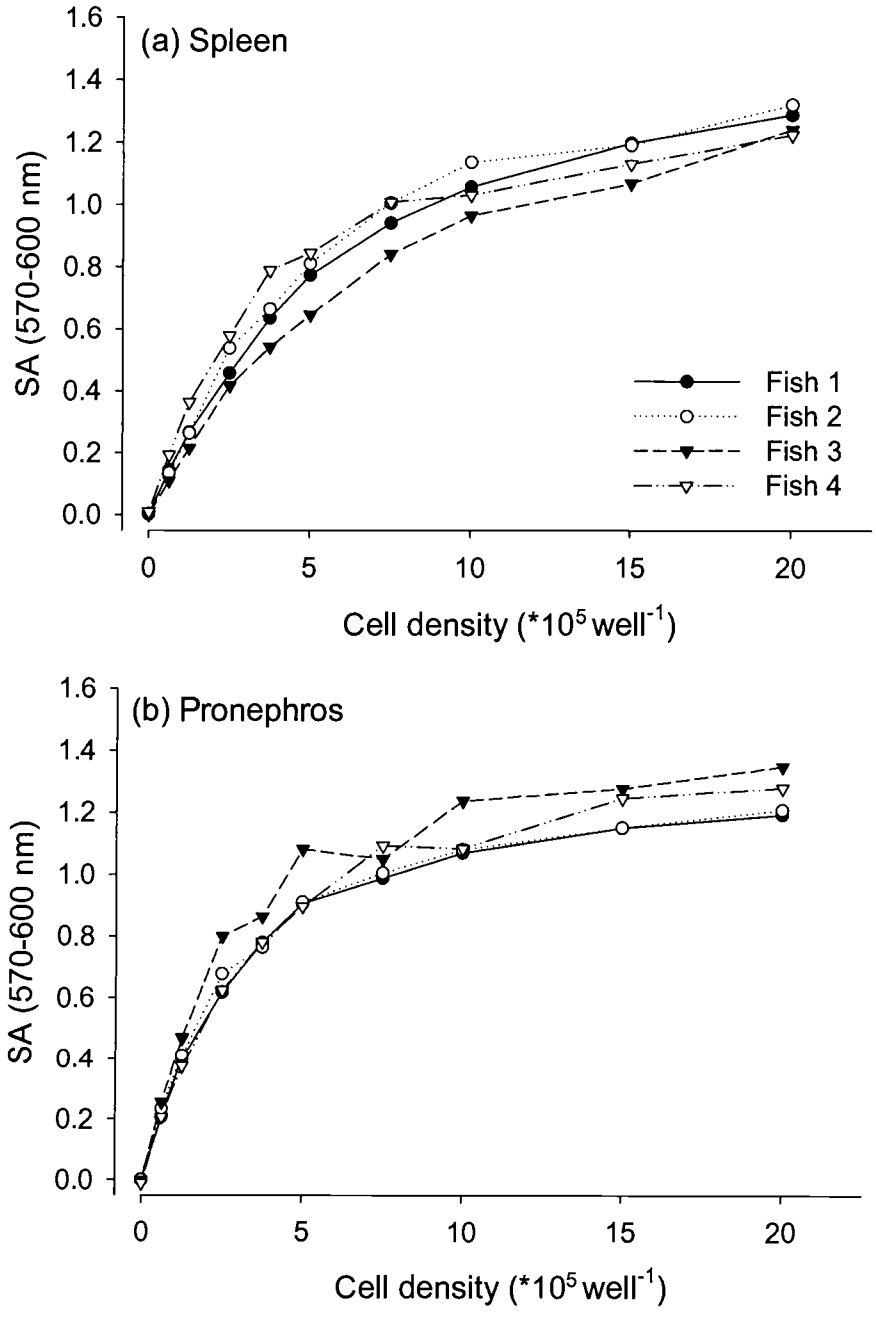

Figure A.1. The relationship between the initial cell density and the specific absorbance (SA: OD 570 nm minus 600 nm) of common carp splenic (a) and pronephric (b) leukocytes cultures with Alamar Blue. Each symbol in the graph represents a single datum point (no replication).

(a) Spleen

Fish #	Culture condition	SA (570-600 nm)	Mean
1	TCM only	0.7851	
2	TCM only	0.6121	0.6451
3	TCM only	0.6592	
4	TCM only	0.5242	
1	LPS	1.1006	
2	LPS	0.9621	0.9875
3	LPS	0.9027	
4	LPS	0.9847	

Fish #	Stimulation Index	Mean
1	1.4019	
2	1.5718	1.5554
3	1.3694	
4	1.8785	

(b) Pronephros

Fish #	Culture condition	SA (570-600 nm)	Mean
1	TCM only	0.9091	
2	TCM only	0.7416	0.7363
3	TCM only	0.7177	
4	TCM only	0.5767	
1	LPS	1.2641	
2	LPS	1.1848	1.1874
3	LPS	1.0772	
4	LPS	1.2237	

Fish #	Stimulation Index	Mean
1	1.3905	
2	1.5976	1.6527
3	1.5009	
4	2.1220	

Table A.2. The mitogenic response of splenic (a) and pronephric (b) leukocytes isolated from common carp measured by the Alamar Blue assay. Cells were initially plated at the density of 2.5 x 10^5 cells per well in 96-well culture plates. Leukocyte cell suspensions were incubated with LPS (200 mg/l) or TCM alone for 4 days at 17 °C. Each SA represents averaged duplicate sample for each fish.

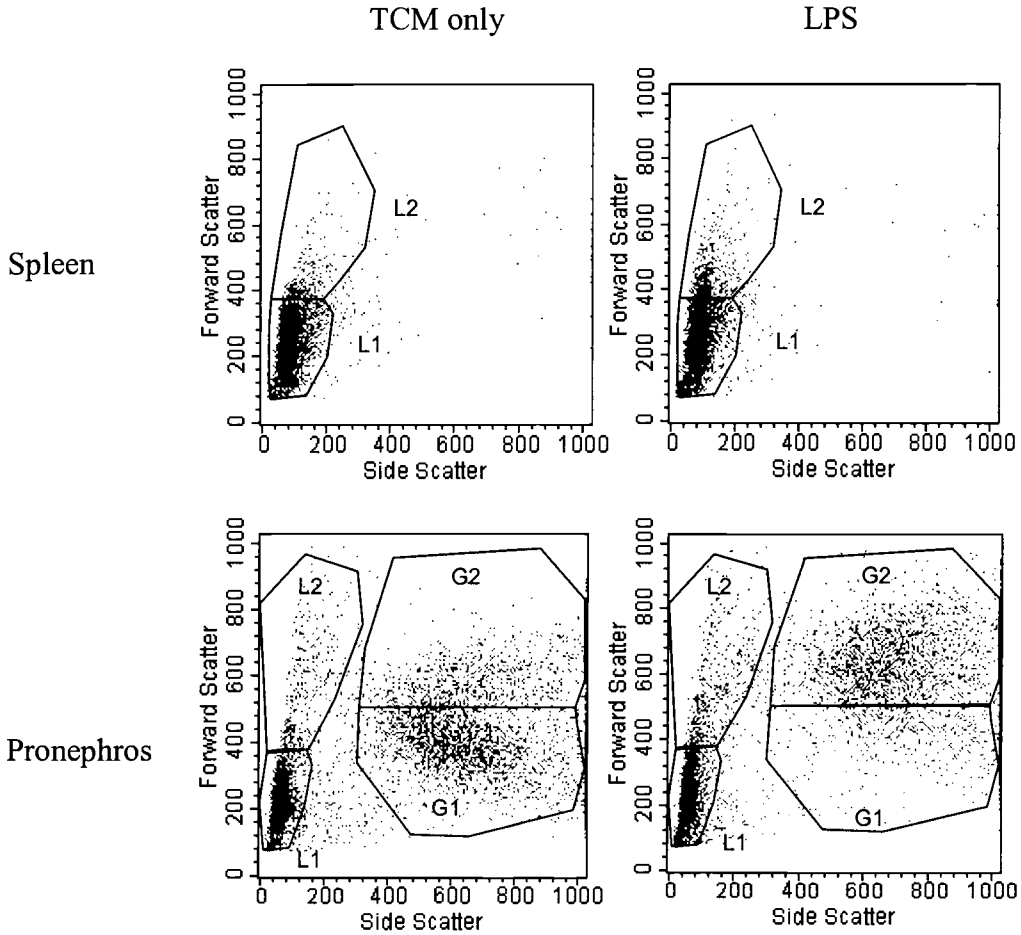

Figure A.2. Representative flow cytometric forward and side scatter dot plots of splenic and pronephric viable leukocytes following incubation for 4days with TCM alone or with LPS (200 mg/l). Cells were initially plated at the density of 2.5 x 10^5 cells per well in 96-well culture plates. Dead cells were excluded by PI staining. L1 and L2 were identified as the lymphocyte population based on the microscopic analysis with Wright-Giemsa staining using Hema 3 stain set following isolation of each population using a cell sorter. On the other hand, G1 and G2 were identified as the granulocyte population based on same methods.

(a) Spleen

TCM only

Fish #	L1	L2
1	95.44	4.56
2	96.77	3.23
3	96.17	3.83
4	95.27	4.70
Mean	95.91	4.08

LPS

Fish #	L1	L2
1	89.51	10.49
2	92.99	7.01
3	93.40	6.60
4	90.64	9.36
Mean	91.64	8.365

(b) Pronephros

TCM only

Fish #	L1	L2	G1	G2
1	47.31	7.67	23.43	15.36
2	42.29	11.71	25.48	10.69
3	38.98	6.85	29.46	12.66
4	39.67	5.59	32.34	13.33
Mean	42.06	7.96	27.678	13.01

LPS

Fish #	L1	L2	G1	G2
1	49.48	11.04	11.44	20.77
2	49.02	12.80	10.20	20.16
3	37.42	10.38	13.44	24.40
4	47.98	8.49	7.93	28.25
Mean	45.98	10.678	10.753	23.40

Table A.3. Summary tables of flow cytometric forward and side scatter dot plots of splenic and pronephric viable leukocytes of carp following incubation for 4 days with TCM alone or with LPS. Each number represents the percentage of each gated cell population in the cell culture, n = 1 (no replication).

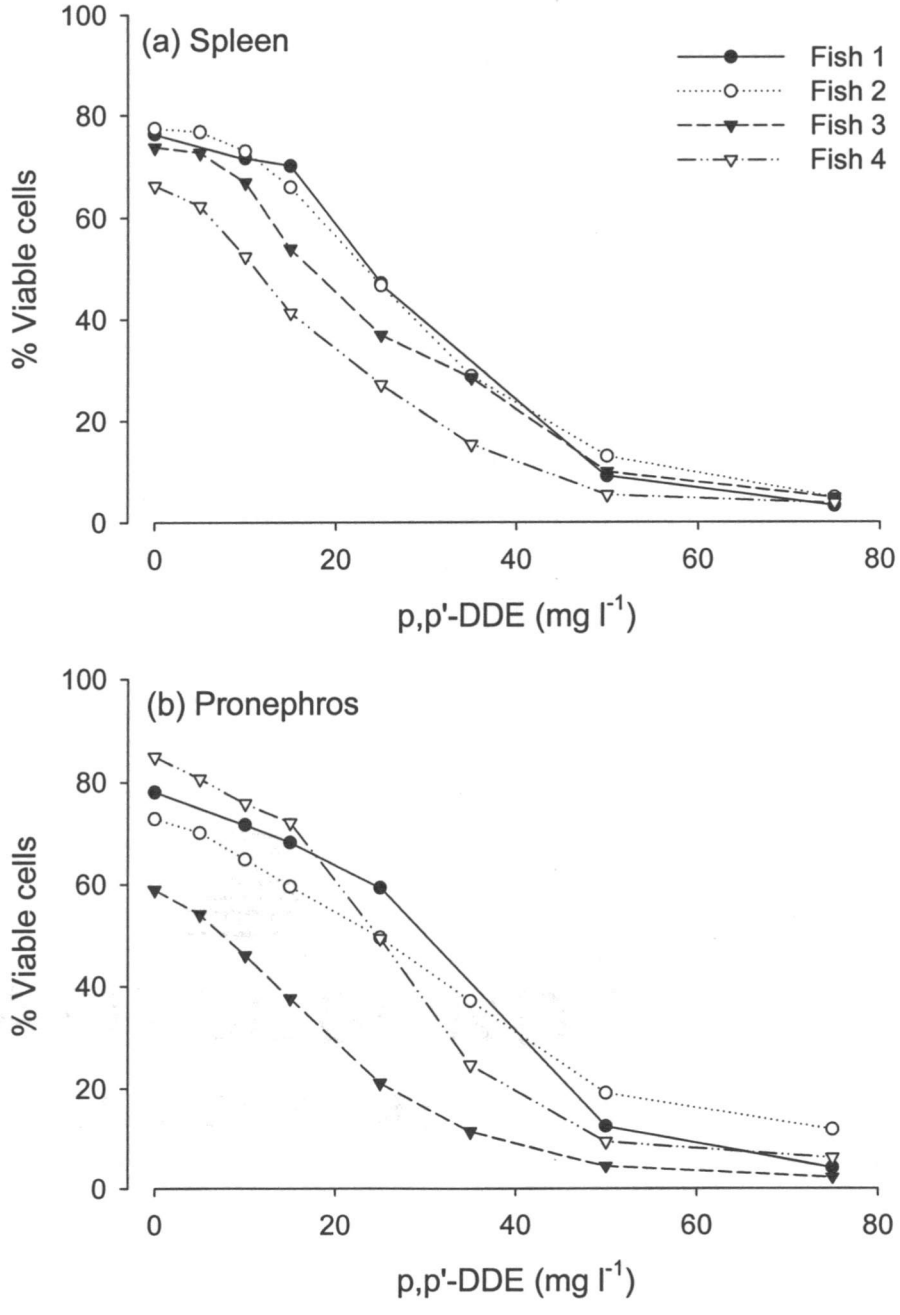

Figure A.3. Percent total viable splenic (a) and pronephric (b) leukocytes following exposure to p,p'-DDE for 48 hr. Concentrations of p,p'-DDE were 0, 5, 10, 15, 25, 35, 50, or 75, mg l^{-1}. Cells were initially plated at the density of 2.5 x 10^5 cells per well in 96-well culture plates. The percentage of viable leukocytes in the cell culture was measured by the flow cytometry. Vehicle control contained 100 µl of cell suspension and 100 µl of concentrated ethanol (1.0%) with TCM. There was no difference in viability between the medium alone control and vehicle control (data not shown) (n=1; no replication).

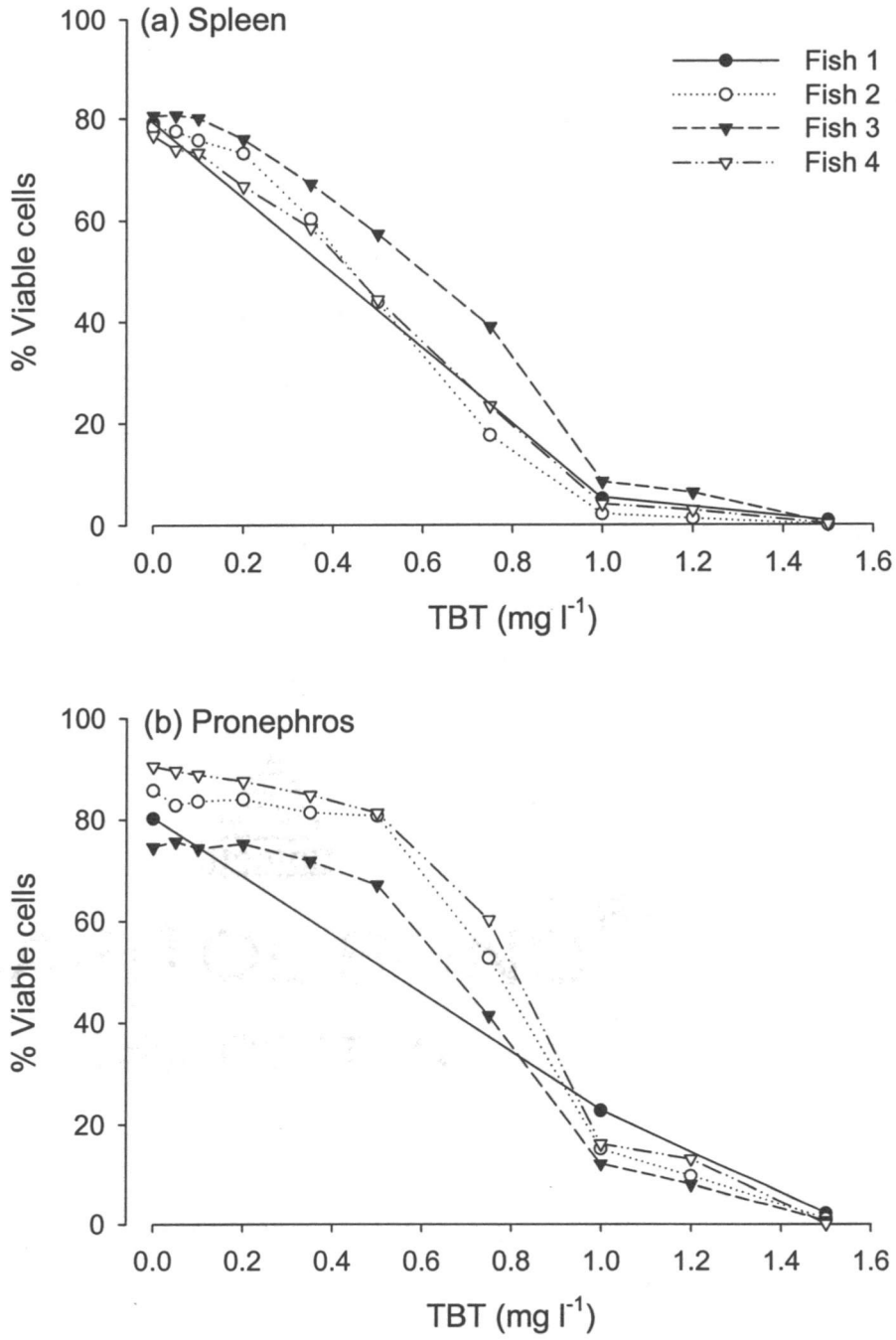

Figure A.4. Percent total viable splenic (a) and pronephric (b) leukocytes following exposure to TBT for 24 hr. Concentrations of TBT were 0, 0.05, 0.10, 0.20, 0.35, 0.50, 0.75, 1.00, 1.20, or 1.50mg l^{-1}. Cells were initially plated at the density of 2.5 x 10^5 cells per well in 96-well culture plates (n=1; no replication). The percentage of viable leukocytes in the cell culture was measured by the flow cytometry. Vehicle control contained 100 µl of cell suspension and 100 µl of concentrated ethanol (0.015%) with TCM. There was no difference in viability between the medium alone control and vehicle control (data not shown).

APPENDIX B:

Effects of *in vitro* exposure to extracts of river sediments on the immune system of chinook salmon (*Oncorhynchus tshawytscha*) leukocytes.

Objective

To determine if *in vitro* exposure to river sediment extracts suppresses the immune system of chinook salmon.

Methods

Bottom sediment was collected from the Willamette River (a known contaminated site in an area zoned for heavy industrial uses) and from the Mary's River (a presumed uncontaminated site) to obtain "clean" reference. The sampling site of the Willamette River was located between River Mile 7 and 8, off shore of McCormick and Baxter Co. (a former wood treatment facility). The sampling site of the Mary's River was located at the confluence of the West Fork and the East Fork, approximately 0.2 miles past the intersection of Mary's River Road and Hoskins Road in the Northwest corner of Benton County, Oregon. All sediments were transported in plastic zip-lock bags on ice wrapped in the black plastic box to eliminate light. Samples were stored in glass jars at –20 °C until ready for extraction. Sediments were extracted based on the methods described by Schiewe *et al.* (1991). Briefly, sediment sample was mixed with 3X sodium sulfate and extract with 4X methylene chloride. Solvent from extractions was pooled and concentrated to 10 ml in a 60 °C water bath. Three fractions were separated using column chromatography (MacLeod *et al.*, 1985). All works was done under yellow light. About 20 mg of dry extract of the sediment was dissolved in 1 ml of icy cold 100 % acetone and then diluted in TCM at most 2h before each experiment started. The final concentration of acetone in each culture and vehicle control was always 1%. Cell viability, % blasting splenic leukocytes, and % apoptotic cells were measured by the flow

cytometry. Fish numbers in tables indicates identification number of fish. Same fish numbers identical with same individual fish.

References

MacLeod, W.D., Brown, D.W., Friedman, A. J., Burrow, D.G., Mayes, O., Pearce, R.W., Wigren, C.A. & Bogar, R.G. (1985). Standard analytical procedures of the NOAA National Analytical Facility 1985-1086. Extractable Toxic Organic Compounds. 2nd Ed. U.S. Dept.of Commerce, NOAA/NMFS. NOAA Tech. Memo. NMFX F/NWC-92.

Schiewe, M. H., Weber, D. D., Myers, M. S., Jacques, F. J., Reichert, W. L., Krone, C. A., Malins, D. C., McCain, B. B., Chan, S-L., Varanasi, U. (1991). Induction of foci of cellular alteration and other hepatic lesions in English sole (*Parophrys vetulus*) exposed to an extract of an urban marine sediment. *Can J Fish Aquat Sci* ***48*** 1750-1760

Table B-1. The percentage of viable splenic leukocytes isolated from chinook salmon following *in vitro* exposure to sediment extracts from the Willamette River.

	Fish number																
Dose	S1 [g]	S2	S3	S4	S5	S6	S7	S8	S9	S10	S11	S12	S13	S14	S15	S16	S17
0 [a]	72.54 [h]	76.60	79.29						74.57	44.12	41.29	62.78	63.51	38.33	70.83	63.87	52.66
Ace 1% [b]	70.98	77.70	80.26	84.55	84.51	85.31	72.37	75.87	74.55	40.40	48.13	65.75	61.79	30.65	65.75	56.9	48.37
10 [c]			76.40							10.98	22.59			21.18	6.08		
20 [d]	43.08	46.74	63.24						57.88	0.77	5.78	39.90	29.00	13.42	33.11	41.01	36.34
30 [e]			3.32	76.25	80.80	79.76	72.95	56.83	37.10		13.57			13.28	39.16	38.79	32.5
40 [f]	0.18	0.96	1.23	76.55	76.97	78.75	69.27	52.28	20.09	9.31		5.34	7.61	6.94	23.56	32.77	28.39

[a] 0 = the cell culture including TCM alone. [b] Ace 1% = vehicle control (1 % Acetone). [c] 10 = including about 10 µg of the sediment extract from the Willamette River in the culture. [d] 20 = including about 20 µg of the sediment extract from the Willamette River in the culture. [e] 30 = including about 30 µg of the sediment extract from the Willamette River in the culture. [f] 40 = including about 40 µg of the sediment extract from the Willamette River in the culture. [g] S 1 = splenic leukocytes isolated from fish (identification # 1). [h] All numbers represent the percentage of viable leukocytes isolated from chinook salmon in the cell culture (no replication).

Table B-2. The percentage of viable splenic leukocytes isolated from chinook salmon following *in vitro* exposure to sediment extracts from the Marry's River.

	Fish number					
Dose	S12	S13	S14	S15	S16	S17
0			38.33	70.83	63.87	52.66
Ace 1%			30.65	65.75	56.9	48.37
10 [i]			25.38	60.95		
20	38.74	23.94	16.79	50.41	45.60	38.44
30			19.22	45.15	34.26	33.07
40	16.40	11.01	14.86	42.83	24.47	26.31

[i] 10 = including about 10 µg of the sediment extract from the Mary's River in the culture.

Table B-3. The percentage of viable pronephric leukocytes isolated from chinook salmon following *in vitro* exposure to sediment extracts from the Willamette River.

	Fish number																
	P1[j]	P2	P3	P4	P5	P6	P7	P8	P9	P10	P11	P12	P13	P14	P15	P16	P17
0	77.36	87.00	86.59						88.84	73.22	72.98	64.95	72.19	58.96	52.8	35.9	51.07
Ace 1%	82.02	89.46	90.50	91.23	91.31	90.15	90.09	82.07	89.12	73.48	74.58	71.00	75.06	56.51	44.43	34.17	44.74
10			82.13							65.17	61.30			31.96	30.31		
20	61.62	67.10	65.52						73.26	8.72	36.21	52.20	63.21	15.21	33.64	21.83	33.53
30			16.19	87.27	87.92	86.81	88.61	64.64	44.64		59.05			9.19	12.53	22.57	31.47
40	0.10	0.42	2.81	83.60	83.56	84.83	83.47	54.12	56.65	58.22		35.67	51.73	7.37	13.97	19.61	30.47

[j] P1 = pronephric leukocytes isolated from fish (identification #1)

Table B-4. The percentage of viable pronephric leukocytes isolated from chinook salmon following *in vitro* exposure to sediment extracts from the Marry's River.

	Fish number					
	P12	P13	P14	P15	P16	P17
0	64.95	72.19	58.96	52.8	35.9	51.07
Ace 1%	71.00	75.06	56.51	44.43	34.17	44.74
10			46.33	42.00		
20	53.57	65.30	35.96	38.21	25.59	37.81
30			40.23	33.36	27.31	40.54
40	23.70	32.60	25.51	30.70	27.29	40.27

Table B-5. The percentage of blasting splenic leukocytes in the cell culture following *in vitro* exposure to sediment extracts.

	Fish number			
Dose	S 14	S 15	S 14	S 15
	TCM [k]	TCM	LPS [l]	LPS
0	1.34 [m]	4.80	63.27	52.91
Ace 1%	1.21	3.64	56.26	59.61
Will [n] 10	1.23	1.95	63.92	67.09
Will 20	1.59	2.92	67.57	68.29
Will 30	2.18	4.50	70.93	48.23
Will 40	1.48	2.98	68.34	70.50
Mary [o] 10	1.53	5.52	66.81	64.50
Mary 20	1.32	3.43	69.18	68.21
Mary 30	3.97	3.81	71.89	71.98
Mary 40	4.00	3.43	69.67	70.28

[k] TCM = the culture without any mitogen, TCM alone. [l] LPS = the culture containing 200 mg/l of LPS. [m] All numbers represent the percentage of blasting splenic leukocytes in the cell culture (no replication). [n] Will = the sediment extract from the Willamette River. [o] Mary = the sediment extract from the Marr's River.

Table B-6. The percentage of apoptotic cells in the splenic and pronephric cell culture *in vitro* exposure to sediment extracts.

	Fish number									
Dose	S 4	S 5	S 6	S 7	S 8	P 4	P 5	P 6	P 7	P 8
Control	4.09 [p]	8.12	8.10	22.37	7.94	10.18	9.69	8.36	12.42	9.00
Will 75	6.37	8.99	10.94	25.51	22.01	13.47	13.50	11.64	16.27	24.82
Will 100	7.49	11.45	12.25	26.43	21.01	17.47	16.37	13.47	19.19	20.93

[p] All numbers represent the percentage of apoptotic cells in the cell culture (no replication).

APPENDIX C:

Instrument settings for the flow cytometry (FACSCalibur)

Table C.1. Setting of FACSCalibur for the cell viability assay and the mitogenic response assay for chinook salmon spleen & pronephros.

Detectors/Amps:

Parameter	Detector	Voltage	AmpGain	Mode
P1	FCS	E00	1.30	Lin
P2	SSC	480	1.00	Lin
P3	FL1	551	1.00	Log
P4	FL2	551	1.00	log
P5	FL3	150	1.00	Log
P6	FL1-A		1.00	Lin
P7	FL1-W		1.00	Lin

Primary parameter: FSC
Value: 52

Compensation:
FL1- 0.8% FL2
FL2 - 16.1% FL1
FL2 - 0.0% FL3
FL3 - 14.3% FL2

OR

Detectors/Amps:

Parameter	Detector	Voltage	AmpGain	Mode
P1	FCS	E00	1.30	Lin
P2	SSC	480	1.00	Lin
P3	FL1	619	1.00	Log
P4	FL2	600	1.00	log
P5	FL3	150	1.00	Log
P6	FL1-A		1.00	Lin
P7	FL1-W		1.00	Lin

Primary parameter: FSC
Value: 52

Compensation:
FL1- 19.0% FL2
FL2 - 19.5% FL1
FL2 - 0.0% FL3
FL3 - 0.0% FL2

Table C.2. Setting of FACSCalibur for detecting apoptosis using ApoAlert AnnexinV-FITC Apoptosis kit for chinook salmon spleen & pronephros.

Detectors/Amps:

Parameter	Detector	Voltage	AmpGain	Mode
P1	FCS	E00	1.60	Lin
P2	SSC	480	1.00	Lin
P3	FL1	625	1.00	Log
P4	FL2	595	3.00	log
P5	FL3	723	1.28	Log
P6	FL1-A		1.00	Lin
P7	FL1-W		1.00	Lin

Primary parameter: FSC
Value: 52

Compensation:
FL1- 0.8% FL2
FL2 - 16.1% FL1
FL2 - 0.0% FL3
FL3 - 14.3% FL2

Table C.3. Setting of FACSCalibur for the cell viability assay and the mitogenic response assay for carp spleen

Detectors/Amps:

Parameter	Detector	Voltage	AmpGain	Mode
P1	FCS	E00	1.68	Lin
P2	SSC	480	1.00	Lin
P3	FL1	504	1.00	Log
P4	FL2	609	1.00	log
P5	FL3	150	1.00	Log
P6	FL1-A		1.00	Lin
P7	FL1-W		1.00	Lin

Primary parameter: FSC
Value: 52

Compensation:
FL1- 19.0% FL2
FL2 - 19.5% FL1
FL2 - 0.0% FL3
FL3 - 0.0% FL2

Table C.4. Setting of FACSCalibur for the cell viability assay and the mitogenic response assay for carp pronephros.

Detectors/Amps:

Parameter	Detector	Voltage	AmpGain	Mode
P1	FCS	E00	1.40	Lin
P2	SSC	452	1.00	Lin
P3	FL1	619	1.00	Log
P4	FL2	609	1.00	log
P5	FL3	150	1.00	Log
P6	FL1-A		1.00	Lin
P7	FL1-W		1.00	Lin

Primary parameter: FSC
Value: 52

Compensation:
FL1- 19.0% FL2
FL2 - 19.5% FL1
FL2 - 0.0% FL3
FL3 - 0.0% FL2