AN ABSTRACT OF THE THESIS OF

Amarisa Marie for the degree of Master of Science in Fisheries Science presented on May 9, 2003.
Title: The Effect of Chronic Exposure of Chinook Salmon to Benzo(a)pyrene and Cortisol on CYP1A1 Induction and Susceptibility to a Microsporidian Parasite, *Loma salmonae*.

Abstract approved Redacted for Privacy
Carl B. Schreck

Wild populations of fish are faced with a multitude of stressors, which may include human interaction, toxins, and disease. Benzo(*a*)pyrene (B*a*P), a known carcinogen and immunotoxin, has been reported in the stomach contents of juvenile chinook salmon, *Oncorhynchus tshawytscha*, in urban waterways. We investigated the impact of chronic dietary exposure of environmentally relevant levels of B*a*P on the immune system and cytochrome P4501A1 (CYP1A1) expression in juvenile chinook salmon.

Two experiments were carried out in which juvenile fish were fed food treated with ethanol (control diet), low or high concentrations of B*a*P, or cortisol. In the first experiment we measured mitogen-stimulated proliferation of splenic leukocytes using flow cytometry and a colorimetric assay using Alamar Blue™. Susceptibility to a microsporidian parasite, *Loma salmonae*, was evaluated in the second experiment by quantification of xenomas in the gills. Hepatic CYP1A1 and plasma cortisol were measured in both experiments.

No significant trends were found in leukocyte mitogen activation or plasma cortisol between treatments or days. However, western blot analysis of CYP1A1

concentration in liver revealed interesting patterns of induction: in cortisol fed groups CYP1A1 was <20% of control on all days, groups fed low levels of BaP were 250% of control values on days 8 and 21 then dropped below control values on day 29, and groups fed high levels of BaP had less CYP1A1 than controls on all days. Similar patterns of CYP1A1 levels were found in the second experiment, and diseased control groups showed about a 55% decrease in CYP1A1 concentration when compared with non-diseased control groups. Susceptibility to *L. salmonae* was significantly higher in groups receiving cortisol. Whereas there was no effect of the high BaP dose, the low BaP dose appeared to increase disease susceptibility. This study supports concerns of stress and toxin induced immune dysfunction in wild populations of fish.

THE EFFECT OF CHRONIC EXPOSURE OF CHINOOK SALMON TO BENZO(A)PYRENE AND CORTISOL ON CYP1A1 INDUCTION AND SUSCEPTIBILITY TO A MICROSPORIDIAN PARASITE, *LOMA SALMONAE*.

by
Amarisa Marie

A THESIS

submitted to

Oregon State University

in partial fulfillment of
the requirements for the
degree of

Master of Science

Presented May 9, 2003
Commencement June 2004

Master of Science thesis of Amarisa Marie presented on May 9, 2003.

APPROVED:

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Major Professor, representing Fisheries Science

Redacted for Privacy

Head of the Department of Fisheries and Wildlife

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Dean of the Graduate School

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Amarisa Marie, Author

ACKNOWLEDGEMENTS

I am grateful to my committee and mentors for their support and guidance. I thank Dr. Carl Schreck, my major professor, for exposing me to the challenges of research and problem solving. With the encouragement of Dr. Michael Kent and Dr. Jo-ann Leong, I came from the islands of Hawaii to Oregon State University. I thank them for introducing me to projects I was involved in both in Oregon and in the islands. Thank you Dr. Selina Heppell for opening my eyes to the ecological perspective and Dr. Chris Bayne for your generous wealth of knowledge. I thank Dr. Nancy Kerkvliet for your critical advice and interpretations. Thanks to Dr. Indira Rajagopal for her knowledge and inspiration in the field of molecular biology. Thank you Dr. Kenneth Johnson for your input as a graduate representative in my committee.

I thank everyone in the Oregon Cooperative Fish and Wildlife Research Unit for their generosity in providing support for this research and other experiments not described. I am indebted to Lisbeth Siddens for her contribution of knowledge in toxicology and chemistry, her long hours of sampling assistance, and her general support. I thank Carisska Anthony for her efficiency, technical and emotional support, and her critical eye. Thank you Ichiro Misumi for your calm assistance in immunology. Thanks to Dr. Grant Feist for his patience, understanding, generous knowledge in chemistry and endocrinology, and general lab assistance. Thank you Rob Chitwood for raising fine stocks of fish and for your expertise in aquaculture. I thank Dr. Molly Webb for her

encouragement, generosity, and knowledge on fish reproductive physiology. Thanks to Nita Campbell, Sarah Lupes, Adam Schwindt, Elaina Snyder, Nathan Truelove, and Denay Tubbs for their assistance in the project. I thank Shaun Clements, David Jepsen, and Mark Karnowski for their time and explanations of computer programs.

I am thankful to the group at The Center for Disease Research for their hospitality and great working environment. Thank you Don Stevens for assistance in setting up the tanks, Harriet Lorz for your support in hard times, and Sarah Solid for caring for my fish when I needed a hand. Thank you Virginia Watral and Chris Whipps for your time and technical assistance.

I'd like to thank Kathi Georgitis for her help in statistics. Thank you Carol Soleau and Sujita Sklenar for keeping me grounded when things were stressful. Thanks to all my friends for their emotional support and nagging to lighten up.

I am thankful to have a supportive family and incredible experiences on this planet. I especially thank my mother, Cynthia Vanderlip, for raising me in such amazing places and inspiring me to care for the environment. I thank my grandfather, Dr. Jack Vanderlip, for introducing me to animal health. And lastly I thank Oregon, the National Sea Grant Program, and the Oregon State University College Sea Grant Program for sharing their wealth of resources with me.

CONTRIBUTION OF AUTHORS

Lisbeth Siddens contributed to the experimental design, assayed plasma cortisol, and provided assistance in moving fish, designing charcoal filters, exposing fish to *Loma salmonae*, and sampling efforts. Dr. Michael Kent assisted with the experimental design, provided expertise in working with *L. salmonae*, histological evaluation, and manuscript editing. Ichiro Misumi contributed the Alamar Blue assay of cell proliferation and assisted in sampling efforts and the analysis of immune data. Dr. Grant Feist assisted in data interpretation and analysis. Dr. Jo-ann Leong contributed to the vision of the project. Dr. Carl Schreck contributed his vision and assisted in experimental design, sampling, analysis, and editing of the manuscript.

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DEDICATION

This thesis is dedicated to all the fish that contribute their lives to scientific exploration.

THE EFFECT OF CHRONIC EXPOSURE OF CHINOOK SALMON TO BENZO(A)PYRENE AND CORTISOL ON CYP1A1 INDUCTION AND SUSCEPTIBILITY TO A MICROSPORIDIAN PARASITE, *LOMA SALMONAE*.

Amarisa Marie, Lisbeth K. Siddens, Michael L. Kent, Ichiro Misumi,

Grant W. Feist, Jo-ann C. Leong, Carl B. Schreck.

ABSTRACT

Wild populations of fish are faced with a multitude of stressors, including human interaction, toxins, and disease. Benzo(*a*)pyrene (B*a*P), a known carcinogen and immunotoxin, has been reported in the stomach contents of juvenile chinook salmon, *Oncorhynchus tshawytscha*, in urban waterways. We investigated the impacts of chronic dietary exposure to environmentally relevant levels of B*a*P on the immune system and cytochrome P4501A1 (CYP1A1) expression in juvenile chinook salmon. Two experiments were carried out in which juvenile fish were fed food treated with ethanol (control diet), low or high concentrations of B*a*P, or cortisol. In the first experiment we measured mitogen-stimulated proliferation of splenic leukocytes. Susceptibility to a microsporidian parasite, *Loma salmonae*, was evaluated in the second experiment. Hepatic CYP1A1 and plasma cortisol were measured in both experiments. Western blot analysis of CYP1A1 concentrations in liver revealed interesting patterns of induction: in cortisol fed groups CYP1A1 was <20% of control on all days, groups fed low levels of B*a*P were 250% of control values on days 8 and 21 then dropped below control values on day 29, and groups fed high levels of B*a*P had less CYP1A1 than controls on all days. Similar patterns of CYP1A1 levels were found in the second experiment, and diseased control groups showed about a 45% decrease in CYP1A1 concentration when compared with non-diseased control groups. Susceptibility to *L. salmonae* was significantly higher in groups receiving cortisol. Whereas there was no effect of the high B*a*P dose, the low

BaP dose appeared to increase disease susceptibility. This study supports concerns of stress and toxin induced immune dysfunction in wild populations of fish.

INTRODUCTION

Benzo(*a*)pyrene (B*a*P), a ubiquitous polyaromatic hydrocarbon (PAH) formed by the partial combustion of fossil fuels, has been found in the stomach contents of juvenile chinook salmon, *Oncorhynchus tshawytscha,* collected in the Puget Sound (Varanasi et al., 1993). Contaminant exposure of chinook salmon in these urban waterways led to the induction of aryl hydrocarbon hydroxylase activity (AHH) and to increased hydrophobic compound binding to DNA (Stein et al., 1995). Juvenile chinook salmon collected from PAH and polychlorinated biphenyl (PCB) contaminated estuaries also exhibited immunosuppression and increased disease susceptibility when exposed to *Vibrio anguillarum* (Arkoosh et al., 1998a). Arkoosh et al. (1998b) argued that pollution of estuaries, which alters the host susceptibility to naturally occurring pathogens, might increase the probability of diseases in fish populations.

Our first objective was to determine if long-term dietary exposure to environmentally relevant doses of BaP affects hepatic cytochrome P4501A1 (CYP1A1) protein levels in juvenile chinook salmon. Our second objective was to determine if B*a*P exposure affects the ability of fish to resist disease. *Loma salmonae*, a microsporidian parasite and common pathogen in the Pacific Northwest, was used to evaluate disease susceptibility. We used cortisol treatment to serve both as a positive control and to learn more about the effects of this corticosteroid. A further objective was to test the possibility that B*a*P

affected the immune system of fish using flow cytometric evaluation of the mitogen-stimulated proliferative response of splenic leukocytes.

CYP1A1 is a mixed function monooxygenase, which catalyzes aryl hydrocarbon hydroxylase (AHH) and ethoxyresorufin *O*-deethylase (EROD) activity (Buhler and Wang-Buhler, 1998; Stegeman and Hahn, 1994) and thereby plays a central role in the bioactivation of BaP to more toxic forms (Pelkonen and Nebert, 1982). Several studies have evaluated the use of CYP1A as a biomarker of PAH exposure (Schlenk and Di Guilio, 2002; Wirgin and Theodorakis, 2002; Willett et al., 1997). Our study differs from many laboratory studies in that the doses of BaP are environmentally relevant and administered in the diet over a long time period rather than by acute intraperitoneal injection.

Loma salmonae was selected as a means to evaluate the health of fish after long-term BaP exposure. Although parasitic diseases are seldom used to investigate the effect of contaminant exposure, they may provide additional indications of impaired immune function, since parasitic organisms stimulate several immune mechanisms (Jones, 2001). Susceptibility to *L. salmonae* is also an excellent endpoint for understanding the potential outcome of BaP induced changes in the physiology of fish. The formation of spore-laden xenomas within the endothelial and pillar cells of the gills during the final stage of development of the parasite allows easy diagnosis and a source of infection. Outbreaks of *L. salmonae* have caused substantial problems for aquaculture of chinook salmon in seawater netpens (Kent et al., 1995).

The immunotoxic effect of BaP has been documented in several fish species. Japanese medaka, *Oryzias latipes*, exhibited significantly suppressed T- and B- cell mitogen-stimulated proliferation 48 hours after exposure to 2 mg BaP /kg, and at higher doses phagocyte mediated O_2^- production and host resistance to bacterial, *Yersinia ruckeri,* infection were reduced (Carlson et al., 2002). Holladay et al. (1998) found apoptotic bodies and significantly reduced leukocyte cell counts in the pronephros of Nile tilapia, *Oreochromis niloticus*, exposed to BaP for five days. Tilapia exposed to BaP exhibited significantly reduced humoral response as indicated by plaque formation (Smith et al., 1999). These disruptions in the fish immune system may subsequently reduce resistance to disease after contaminant exposure.

Cortisol is the major corticosteroid in fish and is not only important in the primary stress response but also functions as an osmoregulatory hormone (Wendelaar Bonga, 1997). Many stimuli including handling, exposure to toxins, disease, and social interactions of fish may activate the stress or hypothalmo-pituitary-interrenal axis (Schreck, 1981). The effect of stress and glucocorticoids on the immune system of fish is the most completely documented endocrine-immune interaction (Weyts et al., 1999; Besedovsky and Del Ray, 1996; Schreck, 1996). The numbers of corticosteroid receptors on leukocytes within the head kidney or spleen are influenced by cortisol, which may result in reduced proliferation of leukocytes (Maule and Schreck, 1991). Cytokines produced by

leukocytes may in turn influence the corticotropic actions of hormones from the pituitary and may have inhibitory effects (Schreck and Maule, 2001).

We used dietary exposure to cortisol as a positive control for immunosuppression and CYP1A1 induction. The dose administered was based on results of a study in which 2 weeks of dietary exposure of masu salmon, *O. masou*, to 1 mg cortisol/g food showed a reduction of plasma IgM concentration (Nagae et al., 1994). Prior to our study, it was unknown whether cortisol treatment would affect chinook salmon susceptibility to *L. salmonae*. Cortisol induced changes in susceptibility or metabolic capacity in fish is important considering the multitude of stressors in the wild.

MATERIALS AND METHODS

Fish and Maintenance

Spring chinook salmon fry obtained from Marion Forks Hatchery, Oregon were raised in Oregon State University's (OSU) Fish Performance and Genetics Laboratory (FPGL). Yearling salmon (17 months old) were used in the first experiment and 7 month old fish were used in the second experiment. Stock fish were held under a natural photoperiod in approximately 1 or 1.5 m diameter circular tanks with flow through, aerated, pathogen free, 12 ± 1°C well water and fed a diet of Bio-Oregon semi-moist pellets at ~2% body weight per day.

Food Preparation and Treatment Doses

BaP (Sigma) and cortisol (Sigma) were dissolved in ethanol and sprayed evenly at a rate of 0.1 ml/g food over a thin layer of food under a hood. Control food was sprayed with the same volume of ethanol. All preparations involving BaP were performed under yellow light to prevent photodegradation. A fresh stock solution of 400 μg BaP/ml was prepared for each new batch of food. After spraying, the food was resprayed with a rinse of the bottle to deliver any residual chemicals. Food was dried overnight to evaporate ethanol, wrapped in foil, and stored at 4°C. Fish were fed known amounts of ration by weighing the quantity of food to be administered each day.

In the second experiment the low dose of 0.1 μg BaP/g food corresponds to the concentration of BaP in stomach contents of chinook salmon found in the Puget Sound and the high dose of 10 μg BaP/g food corresponds to the total concentration of PAHs in stomach contents found by Varanasi et al. (1993). In the first experiment the low and high doses were 0.15 μg BaP/g food and 15 μg BaP/g food, respectively. Doses per g of fish per day were calculated based on the weight of fish at the beginning and end of the treatment period. Estimated doses ranged from 0.0010 to 0.0015 μg BaP/g fish/day (low BaP) and from 0.10 to 0.15 μg BaP/g fish/day (high BaP).

The concentration of cortisol was 1 mg/g food in the first experiment resulting in an estimated dose was 10 μg cortisol/g fish/day. In the second experiment the cortisol concentration was changed from 665 to 335 μg cortisol/g

food in response to decreased appetite during the course of the experiment. The estimated average dose per g fish was calculated based on the weight of fish at the beginning and end of the experiment and daily average grams of food consumed by fish in a tank. This estimation resulted in two ranges of doses for the cortisol treatment. During the period in which food concentration was 665 μg cortisol/g food the estimated average dose per day of cortisol administered was from 5.7-10 μg cortisol/g fish. After the reduction in concentration to 335 μg cortisol/g food, the estimated average dose per day of cortisol administered was from 2.8-4.5 μg cortisol/g fish.

Experimental Design- Experiment 1

The first experiment was carried out at the FPGL using approximately 1 m diameter circular tanks with flow through, aerated, pathogen free, 12 ± 1°C well water. Duplicate tanks for treatments were randomly assigned and randomly stocked with eight fish each. The following treatments were applied: low BaP, high BaP, cortisol, and control. On August 7, 2002 fish were weighed and then acclimated for three weeks during which time fish were fed control ration at 1% body weight. Fish were sampled on days 8, 21, and 29 after the onset of receiving the treated diets. Fish were not fed the day before sampling. To minimize the effects of density, tagged fish were stocked into tanks to replace those removed during sampling. The mean (± S.E.M.) fish weight recorded on day 29 was 170.1 ± 16.2 g.

Experimental Design- Experiment 2

Triplicate tanks for treatments were randomly assigned and randomly stocked on August 20, 2002. The following treatments were applied: low BaP plus *L. salmonae*, high BaP plus *L. salmonae*, cortisol plus *L. salmonae*, control plus *L. salmonae*, and control. Groups of fifteen fish each were transferred from the FPGL to the Salmon Disease Laboratory (SDL), OSU, Corvallis. The 100 L tanks used at this facility were supplied with flow through, aerated, pathogen free, 12 ± 1°C well water. Total fish weights per tank were recorded, and fish were acclimated for two weeks. Fish were fed at 1.5% body weight until day 34 when the food was increased to 2% of the original fish weight, thereby maintaining approximately similar doses across the experiment. Fish were fed twice a day except on days when tanks were cleaned. Tanks were cleaned every two weeks after a morning feeding in which fish were given their entire ration for the day. All treatment tanks were exposed to *L. salmonae* except one set of non-exposed fish, which were fed control food. Charcoal filters were placed beneath tanks treated with BaP to decontaminate the effluent. Water temperature for all tanks was raised from 13°C to 15°C over a period of two weeks beginning day 19 to enhance infection. The mean (± S.E.M.) fish weight at the end of the experiment was 15.79 ± 0.27 g.

Cortisol treatment was adjusted throughout the second experiment in response to decreased appetite in the fish. The concentration of cortisol in food was decreased from 665 to 335 μg cortisol/g food on day 21. The feeding

regime went as follows: first 5 days cortisol was fed daily, food was withheld on days 6-8 during the *L. salmonae* exposure, on days 9-15 cortisol was fed every 2nd day, and day 16 until the end of the experiment cortisol was fed every 3rd day. Control food was fed on days between cortisol treatment. During each change in the feeding regime fish seemed to regain appetite, but the response was short lived. Fish were fed until satiation, which was generally below the 1.5 or 2% diet consumed by the other treatment groups.

Transmission of Disease

Gill tissue from a heavily infected fish generously donated by Dr. S. Jones at the Pacific Biological Station, Fisheries and Oceans, Canada, Nanaimo, B.C. was used to infect juvenile chinook salmon averaging 150 g weight at the SDL. These fish served as a reservoir for the pathogen. After 10 weeks fish were lethally sampled and infected gills were removed from gill arches, diluted with declorinated fresh water, and ground into a slurry using a Polytron™ tissue homogenizer. Gill slurries were prepared on ice and then stored at 4°C in capped falcon tubes for up to two days before use.

Fish were exposed to the slurry after 5 days of consuming treated food followed by a day of fasting. After light anesthetization with tricaine methanesulfonate the slurry (200 μl/fish) was delivered into the stomach of each fish by intubation using a 1cc syringe with a 16 g needle tipped with tubing. The

concentration of *L. salmonae* spores was approximately 2 x10^6 spores per ml as determined by a hemocytometer. Fish were not fed the day of or after intubation.

Blood and Tissue Collection

In both experiments fish were sampled by rapid netting, transferred to a lethal concentration (200 mg l^{-1}) of tricaine methanesulfonate buffered (pH 7) with sodium bicarbonate, and then weighed. Blood was collected via caudal severance, and the fish were allowed to exsanguinate on ice. In the second experiment blood was collected only in control, control plus *L. salmonae* (only one replicate), and cortisol plus *L. salmonae* treated fish (six fish per replicate). Plasma was stored at –80°C until assayed for cortisol according to a method modified from the one described in Redding et al. (1984).

In the first experiment, harvest and preparation of spleens was performed in a laminar flow hood using sterile technique and ice to keep samples cold. Leukocytes were harvested from the spleens as described by Crippen et al. (2001). Cell suspensions were stored on ice until aliquots were removed for cell counts using a hemocytometer.

After 47 days of *L. salmonae* exposure, fish were sampled. The left second gill arch was removed, placed in 10 ml phosphate buffer solution (PBS), and stored at 4°C until examination the following day. A few gill samples were fixed in buffered formalin for histology until processed by the Veterinary Diagnostic Laboratory, OSU. Livers were collected from 10 fish per tank, snap

frozen in cryovials using liquid nitrogen, and later stored at -80°C. Due to limited time, livers were not collected from the fish of two in the cortisol treated groups.

Proliferation Assays- Experiment 1

The proliferative response of leukocytes to lipopolysaccharide (LPS) stimulation was measured by flow cytometric evaluation of blastogenesis using a modified protocol of Milston et al. (in press). Briefly, splenic leukocyte suspensions diluted with tissue culture media (TCM: 7% heat-inactivated fetal bovine serum, 1% L-glutamide, 200 units penicillin ml^{-1}, and 0.2 mg streptomycin ml^{-1} in Minimum Essential Media buffered with sodium bicarbonate) were transferred in duplicate to 96 well plates to give 5×10^5 cells $well^{-1}$ and incubated in either TCM or LPS (200 µg/ml) (Sigma) for four days at 17°C in blood gas (10% O_2, 10% CO_2, 80% N_2). After four days incubation cells were washed, resuspended in 200 µl PBS, and transferred to FACS tubes. Prior to analysis, 20 µl of propidium iodide (PI: 50µg/ml) (Sigma) in PBS were added to tubes, which were then incubated at room temp in the dark for 15 min. Viability and blastogenesis were assessed using a Becton Dickinson FACSCaliber™ flow cytometer with Argon-ion laser at 480 nm using Cellquest™ software. Low PI fluorescing viable cells were gated (selected for analysis) and the percentage of blasting cells in this population was quantified using dot plot displays of forward scatter vs. PI fluorescence and 'quadrant stats'. Stimulation of leukocytes was

calculated as a ratio of the percent of blasting cells from stimulated and nonstimulated cell cultures [stimulated (nonstimulated)$^{-1}$].

The proliferative response was also measured by a fluorometric assay using Alamar Blue™ (Nakayama et al., 1997). The use of Alamar Blue™ for evaluating the proliferative response of chinook salmon leukocytes has been validated in our lab (I. Misumi, Department of Microbiology, OSU). Splenic leukocyte suspensions diluted with TCM were transferred in duplicate to 96 well plates to give 2.5 x 10^5 cells well^{-1} and incubated in either TCM or LPS (200 µg/ml) (Sigma) at 17°C in blood gas. Wells containing TCM and no cells were used for background fluorescence. After three days of incubation 20 µl of Alamar Blue™ (BioSource International) were added to cultures under dark conditions. Absorbences were read on day four at 570 nm and 600 nm using a Molecular Devices V max™ kinetic microplate reader. The specific absorbance was calculated using SOFT Max® PRO software to calibrate overlap between the two wavelengths. Stimulation of leukocytes was calculated as a ratio using absorbance values of stimulated and nonstimulated cell cultures [stimulated (nonstimulated)$^{-1}$].

L. salmonae Infection Evaluation

Prior to sampling, infected gill integrity was assessed over time to determine the maximum time gills could be stored in PBS in which xenomas could be visualized in wet mount preparations. Wet mounts of gills were

examined at 100 X magnification over a period of two days. The intensity of infection was defined as the number of xenomas per 10 gill filaments. The first five full-length filaments on each side of the arch were used for this assessment. Ten gills were examined from each tank.

Isolation of Liver Microsomes for CYP1A1 Western Blots

The entire procedure was performed on ice or at 4°C following the methods of Gilroy et al. (1993). Livers were weighed and pooled per treatment replicate. The final microsomal fraction was stored in 0.5 ml aliquots at -80°C.

CYP1A1 Western Blots

Total protein concentration of microsomal preparations was determined using the Coomassie Protein Assay Reagent Kit (Pierce) microplate procedure. Small aliquots of microsomal samples were brought up to 20 µl with 0.5 M Tris (pH 6.8) followed by the addition of 180 µl sample buffer (2.5 ml 0.5 M Tris, 2 ml 20% SDS, 2 ml glycerol, 1 ml 2-mercaptoethanol, 0.2 ml 0.1% bromophenol blue, brought to 10 ml with Millipure water and filtered with a 0.2 µm acrodisc filter on a 10 ml syringe) to bring the final protein concentration to 1 µg/µl. Samples were denatured in a 100°C water bath for 1 min and then loaded into either Tris-HCl Ready Gels (7.5%resolving gel, 4% stacking gel, BioRad, Hercules, California) for the first experiment or a hand poured SDS-polyacrylamide (4%) stacking gel overlaying a SDS-polyacrylamide (7.5%) resolving gel. While 10 µg protein/well

were loaded for unknowns, only 1 μg protein/well was loaded for the β-naphthoflavone (BNF) induced microsomal preparation (CYP1A1 positive marker generously gifted from Dr. D. Buhler, Department of Environmental and Molecular Toxicology, OSU). Microsomal pools collected on days 8, 21, 29 were run on separate gels. All of the pooled microsomal samples from the second experiment were loaded on one gel. Using a BioRad Modular Electrophoresis System (Hercules, California) gels were run at 200 V for 45 - 50 min in running buffer (25 mM Tris, 192 mM glycine, 1 g/L SDS, pH 8.3).

Proteins were blotted onto nitrocellulose membranes using the method described by Towbin et al. (1979). Membranes were rinsed twice with wash buffer (80 mM dibasic sodium phosphate, 20 mM monobasic sodium phosphate, 100 mM sodium chloride, 0.1% Tween 20, pH 7.5) and then blocked overnight at 4°C in a 5% powdered milk solution of wash buffer. After a series of washes anti-trout CYP1A1 primary antibody (1° Ab) from rabbits (generously gifted from Dr. D. Buhler, OSU) was diluted with 2% BSA wash buffer to 20 μg/ml and membranes were incubated for 1 hr. The 1° Ab working solution was then stored at -20°C for later use. Membranes were again washed and incubated for 1 hour in a secondary antibody (2° Ab), anti-rabbit Ig from donkey linked to horseradish peroxidase (Amersham, UK) diluted 1:25,000 (first experiment) or 1:6,250 (second experiment) in wash buffer. ECL Western Blotting Detection Reagents (Amersham, UK) were used to visualize 2° Ab binding. After several washes membranes were incubated 1 min in the ECL solution just prior to developing on

Kodak Scientific Imaging Film (Rochester, New York). Film was scanned and band density was measured by ImageQuant™ software.

Statistics

The analysis of data from the first experiment was a repeated measures design. The experimental unit was the tank with fish within being the observational unit. The covariance structure in this design was modeled various ways: split plot in time, compound symmetry, and unstructured. The second experiment followed a completely randomized design with sub-sampling. Again, the experimental unit was the tank, while the fish within tanks were the observational unit. An F-test was followed by all pairwise treatment comparisons using Least Square Means to test differences in infection intensity. Sample sizes were unequal due to the loss of fish in one control tank in the first experiment and in one control plus *L. salmonae* tank in the second experiment. The significance level for all tests was 5%. No statistics were applied to western blot analysis due to limited sample size. Results were expressed as the mean ± standard error (S.E.M.).

RESULTS

CYP1A1 Protein Levels

Cortisol, high BaP, and *L. salmonae* infections resulted in decreased concentrations of CYP1A1 protein as measured by band density of western blots (Fig. 1 and 2A). In the first experiment, cortisol and high BaP treatments resulted in an average decline to 10% of control and 24% of control respectively (Fig. 3). The single measurement of CYP1A1 protein in the cortisol plus *L. salmonae* treatment was 10% of control (Fig. 2C), and diseased control fish exhibited a mean decline to 55% of control (Fig. 2B). The high BaP plus *L. salmonae* treatment resulted in a decline to 13% of control (Fig. 2C).

Low BaP treatment induced CYP1A1 protein on days 8 and 21 to levels 250% of control. On day 29 CYP1A1 declined to 67% of control in the low BaP treatment (Fig. 2). The effect of low BaP and disease on CYP1A1 levels in the second experiment was variable with one tank induced, while the other two were suppressed (Fig. 3B). The mean of the three groups receiving this treatment was 80% of control.

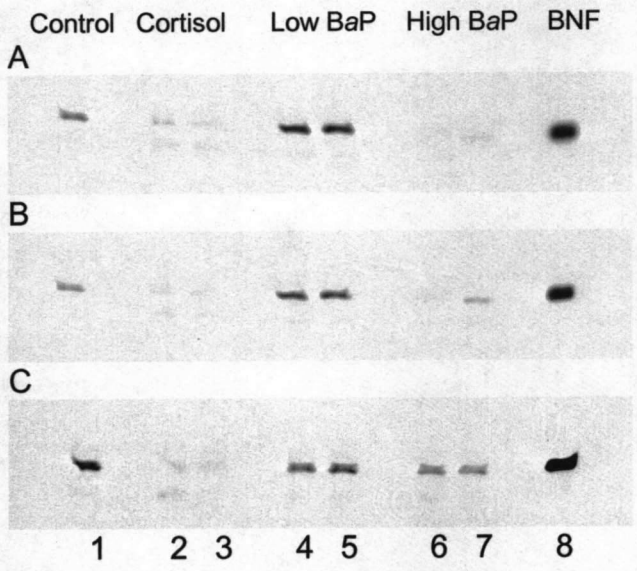

Fig. 1. Western blots of CYP1A1 protein detected by antibodies in control (lane 1), cortisol (lanes 2,3), low BaP (lanes 4,5), and high BaP (lanes 6,7) pooled chinook salmon liver microsomes after 8 (A), 21 (B), and 29 (C) days of dietary exposure. Each band represents 10 µg microsomal protein/pooled treatment replicate. Pools consisted of two fish on days 8 and 29 and three fish on day 21. The band in lane 8 represents 1 µg of BNF induced microsomal protein.

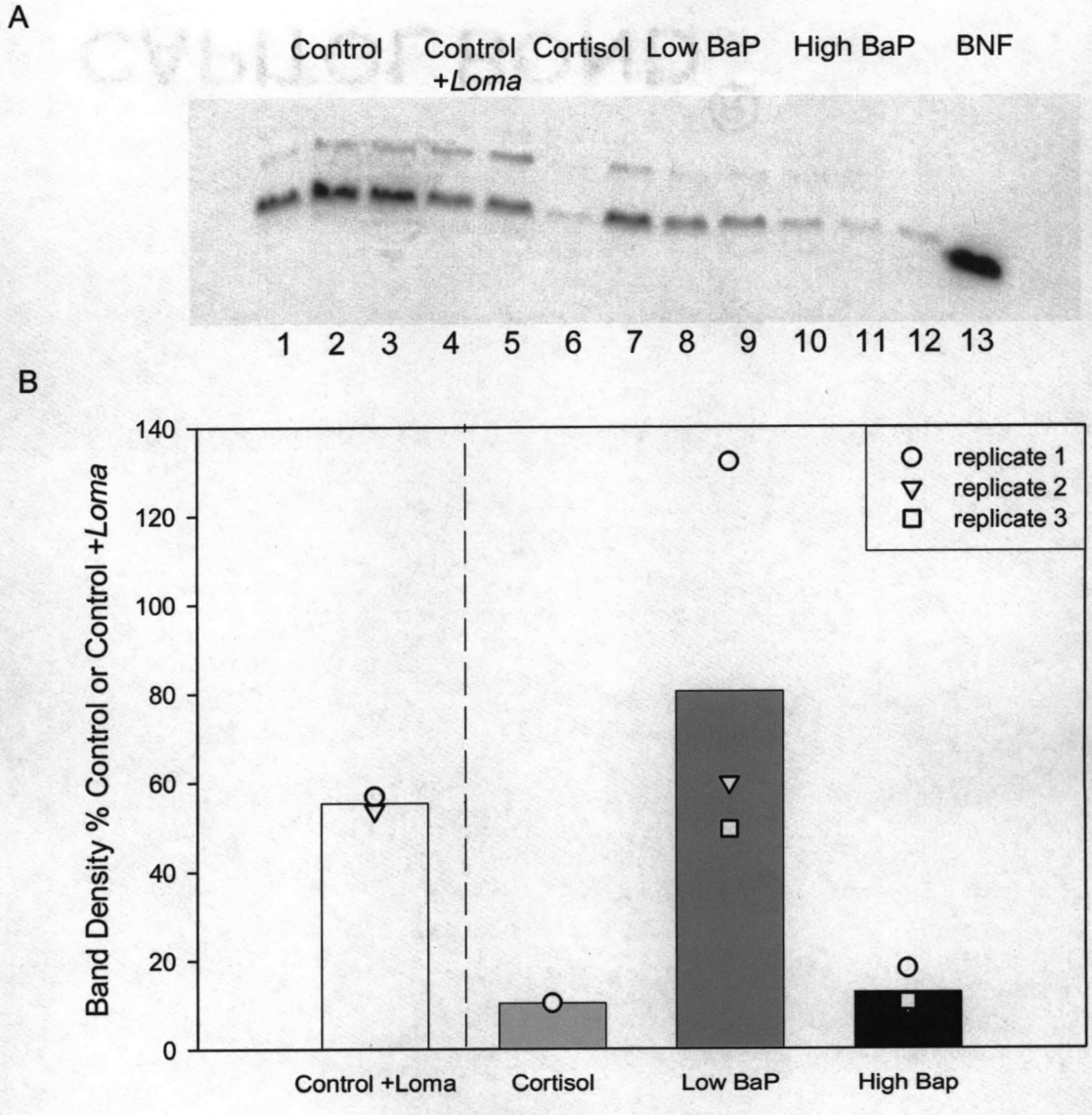

Fig. 2. A. Western blot of CYP1A1 protein detected by antibodies in chinook salmon not exposed *Loma salmonae* (controls; lanes 1-3), controls exposed to *L. salmonae* (lanes 4,5), and exposed to either cortisol (lane 6), low BaP (lanes 7-9), or high BaP (lanes 10-12). Fish were exposed to compounds for 54 days and to *L. salmonae* for 47 days. Each band represents liver microsomes pooled from 10 fish per replicate. The band in lane 13 represents 1 µg of BNF induced microsomal protein. B. Band densities expressed as either percent of control mean (left of dashed line) or as percent of control plus *L. salmonae* mean (right of dashed line). Symbols represent treatment replicates and bars represent overall treatment means.

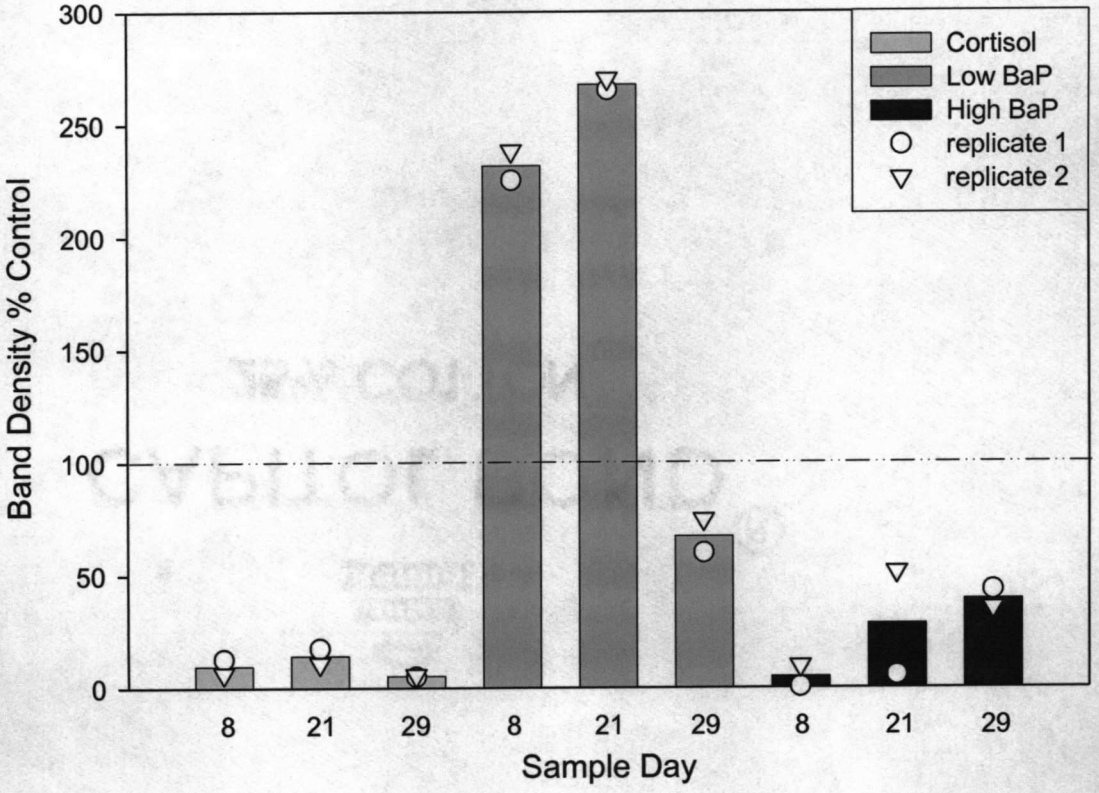

Fig. 3. Percent of control (for corresponding day) band density of CYP1A1 protein from pooled chinook salmon liver microsomes after 8, 21, and 29 days of cortisol, low BaP, and high BaP dietary exposure in the first experiment as detected by western blot analysis. There were two replicates per treatment each representing a pool of microsomes consisting of two fish on days 8 and 29 and three fish on day 21. Bars represent treatment means.

Disease Intensity

Susceptibility to *L. salmonae* as measured by the number of xenomas per 10 gill filaments was highly variable within each replicate (Fig. 4A). Many fish exhibited an extremely dense occurrence of xenomas (Fig. 5) with signs of inflammation and tissue degradation (Fig. 6). During the last week of the experiment some of the fish treated with cortisol died (replicate 1: five mortalities,

and replicate 2: one mortality). There was one mortality in the low BaP treatment (rep 2). Moribund fish exhibited apparent signs of hypoxia and severe *L. salmonae* infection of the gills.

There was a significant difference between treatments in their intensity of *L. salmonae* infection (F statistic = 4.76; p = 0.041) (Fig. 4B). Cortisol treated fish had significantly more xenomas than did controls (p = 0.020) and fish receiving the high BaP treatment (p = 0.021) but not those given the low BaP treatment (p = 0.790). The fish in the low BaP treatment exhibited suggestive but not significant difference with controls (p = 0.056) and the high BaP treatment (p = 0.065). The number of xenomas in fish in the high BaP treatment did not differ significantly from those of controls (p = 0.744).

Fig. 4. A. Variability of *O. tshawytscha* susceptibility to *Loma salmonae* as determined by evaluation of the number of xenomas per 10 gill filaments. Symbols represent individual fish (10 fish per replicate) and bars represent the mean of each replicate. B. Effect of cortisol, low BaP, and high BaP on intensity of *Loma* infection. Only fish given cortisol were significantly different from control plus *Loma* ($p = 0.02$). Symbols represent tank replicates and bars represent overall treatment means. The numbers of replicates are given within the bars.

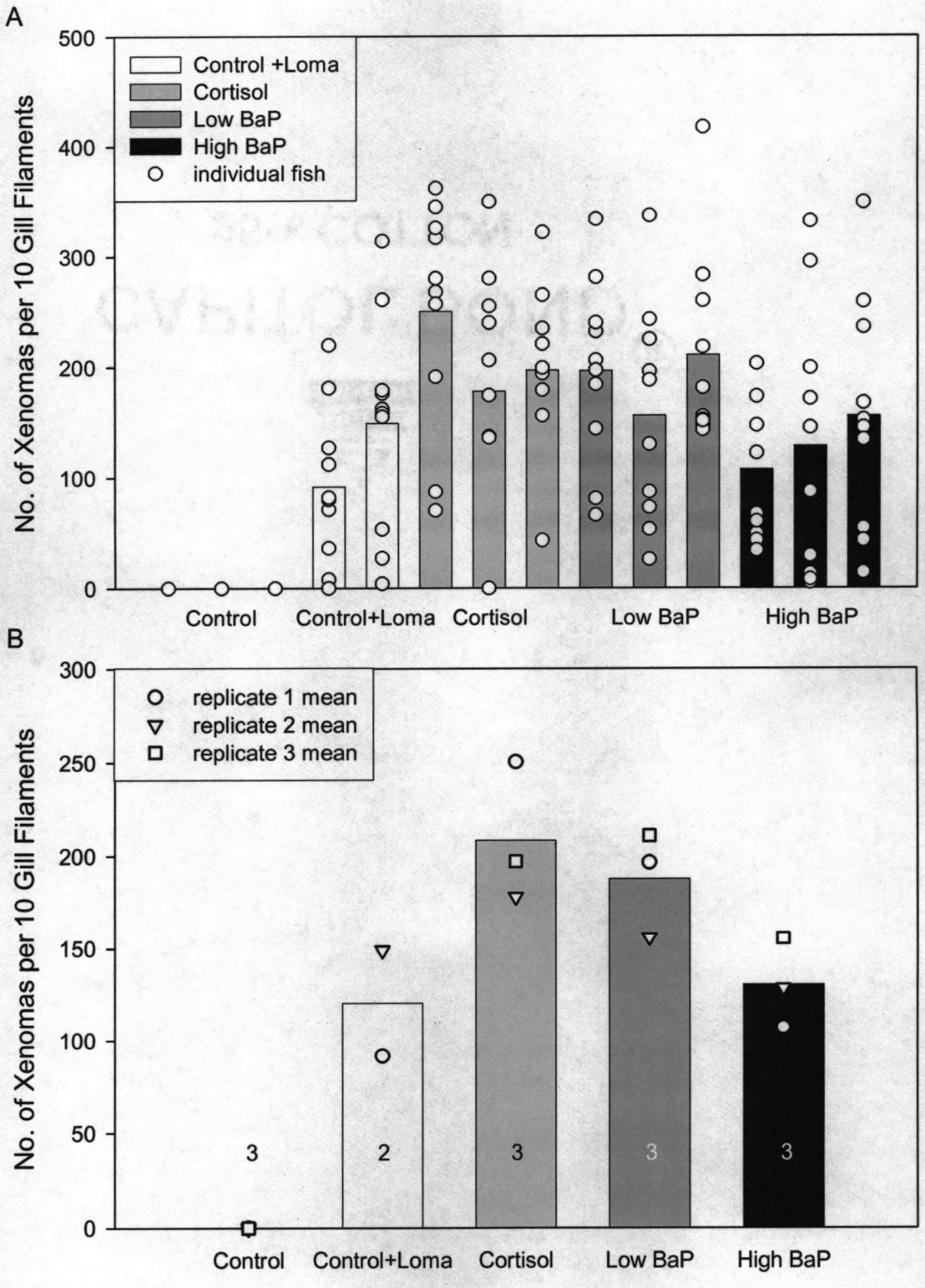

Fig. 4. A. Variability of *O. tshawytscha* susceptibility to *Loma salmonae.* B. Effect of cortisol, low BaP, and high BaP on intensity of *Loma* infection.

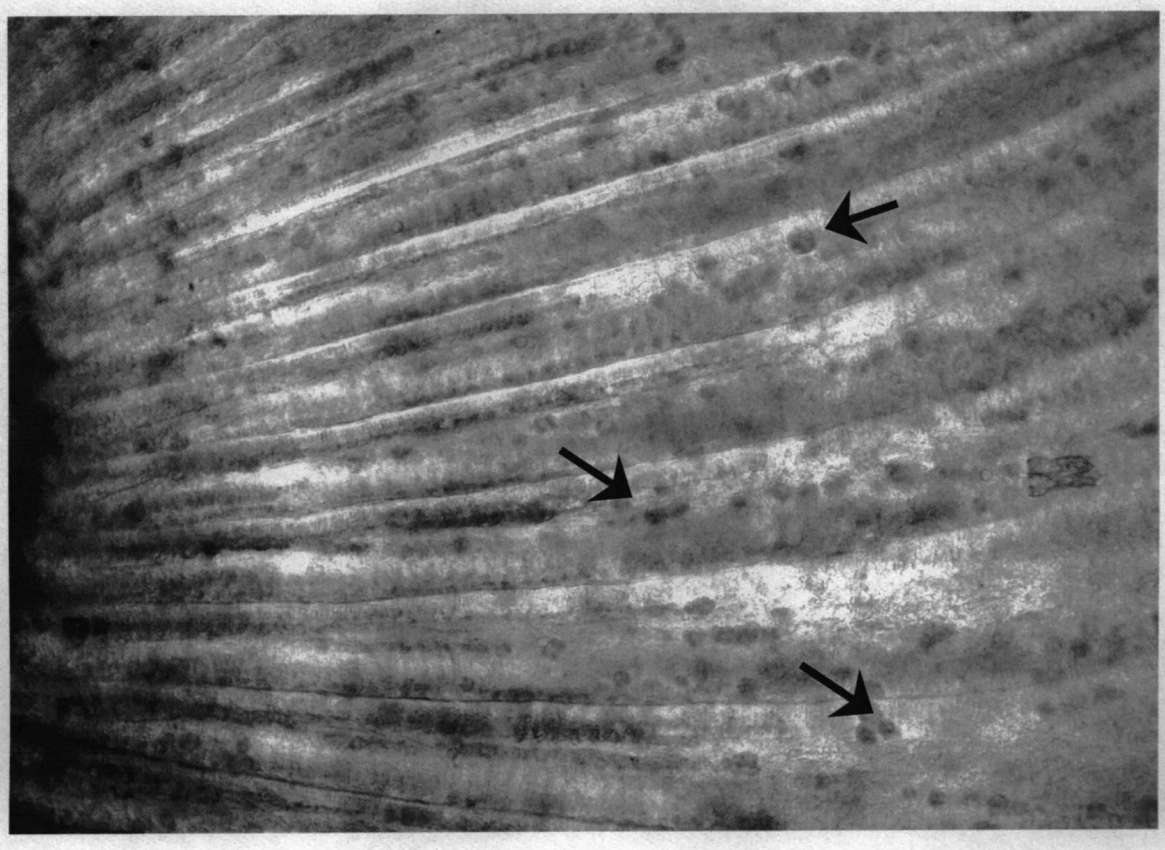

Fig. 5. Massive infection of *Loma salmonae* in fresh gill filaments in chinook salmon after 47 days. Arrows point to xenomas.

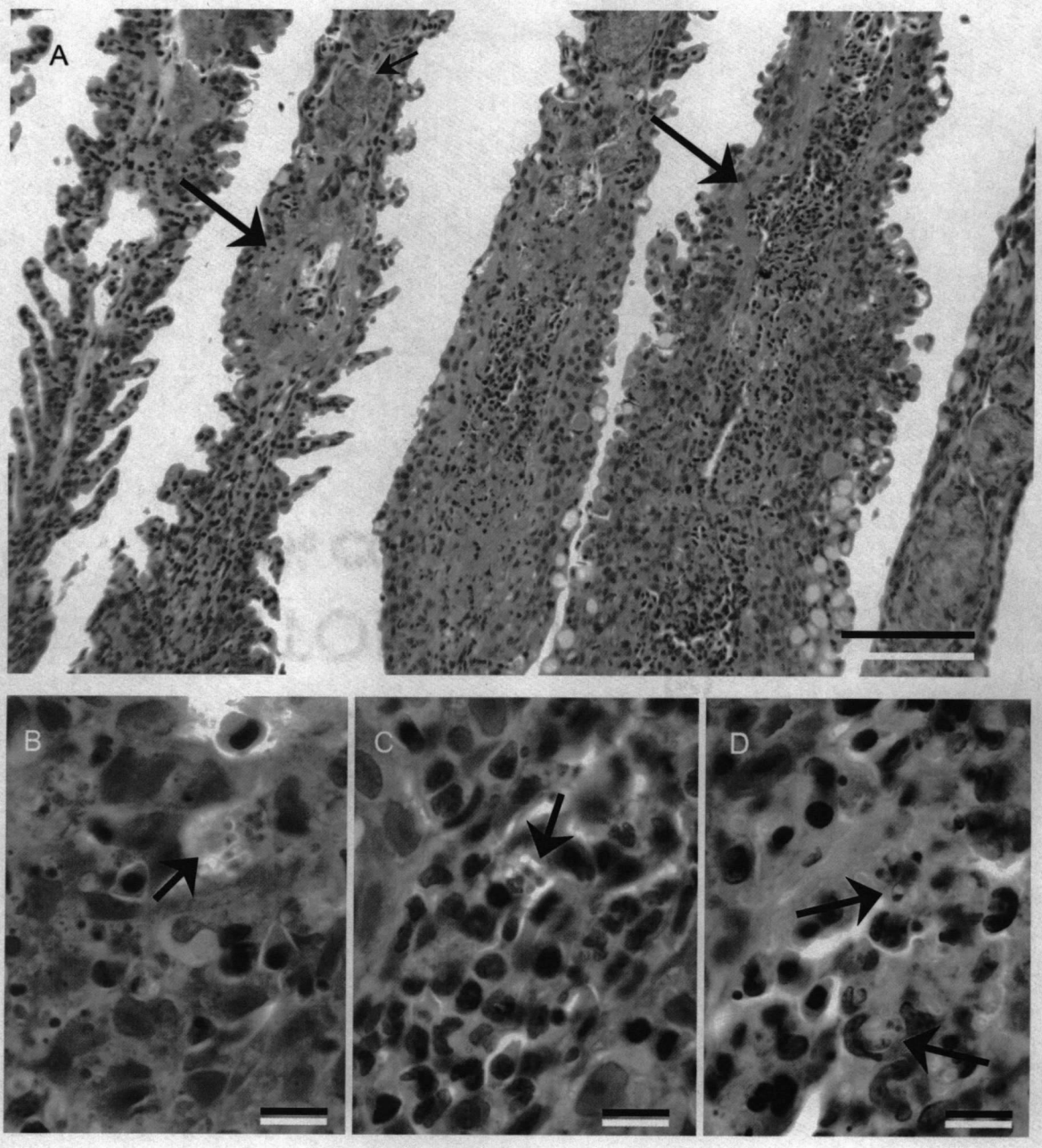

Fig. 6. Severe, chronic vasculitis, perivasculitis, and hemorrhage of the gill filaments due to *Loma salmonae* infection (47 d post-exposure). A. Low magnification showing inflammation of vessel walls and severe congestion (large arrows). Bars = 50 µm. Small arrow = intact xenomas. B-D. High magnification (bars = 10 µm) showing free or phagocytosed spores within granulomatous lesions in perivascular areas.

Other Measurements

There were no trends in cell viability (unstimulated or stimulated) or LPS mitogen activation of leukocytes as assessed by the stimulation index from flow cytometry and the Alamar Blue assay associated with any of the treatments (Table 1).

There were no significant differences in plasma cortisol at the time of sampling in either the first experiment (Table 1) or the second experiment [control 8 ± 3 ng/ml (n=18), control plus *L. salmonae* 40 ± 26 (n=6), cortisol plus *L. salmonae* 27 ± 10 (n=18)]. Even though treatments sometimes appear to be different, with so much variation inherent in controls, we are not able to speculate if the differences are real or artifactual due to sample size.

Although fish were all approximately the same size at the start of the experiment, fish exposed to *L. salmonae* and fed cortisol weighed significantly less than all other treatments ($p<0.05$ for all pair-wise comparisons). Therefore, the steroid in combination with *L. salmonae* infection had an adverse effect on growth. *L. salmonae* in the absence of cortisol treatment did not affect growth in the fish ($p=0.895$).

Table 1. Immunological data and plasma cortisol concentration of chinook salmon in the mitogen experiment collected on days 8, 21,and 29 from fish exposed to control, cortisol, low BaP, or high BaP diets. Immune data presented as mean ± S.E.M. calculated from individual fish pooled among replicates for each treatment. Controls on day 8, which consist of two fish, are given as actual data. Plasma cortisol given as treatment mean; actual data.

	Day 8	Day 21	Day 29
Cell Viability (%) – Nonstimulated (Flow Cytometry)			
Control	60.52, 63.67	61.58 ± 2.62	60.88 ± 2.99
Cortisol	49.25 ± 4.09	58.22 ± 3.56	52.78 ± 7.22
Low BaP	49.99 ± 3.94	57.51 ± 4.69	57.95 ± 1.67
High BaP	43.48 ± 4.81	54.86 ± 7.72	60.48 ± 4.17
Cell Viability (%) – Stimulated with LPS (Flow Cytometry)			
Control	68.90, 74.81	70.29 ± 0.93	68.08 ± 4.28
Cortisol	62.87 ± 4.17	63.26 ± 3.90	62.19 ± 6.73
Low BaP	65.79 ± 2.62	64.94 ± 4.79	67.49 ± 2.48
High BaP	58.73 ± 0.04	62.99 ± 3.76	71.06 ± 2.76
Stimulation Index - Flow Cytometry			
Control	13.26, 29.60	18.28 ± 4.13	31.80 ± 8.00
Cortisol	11.64 ± 5.67	14.23 ± 1.80	17.94 ± 2.77
Low BaP	18.76 ± 2.86	18.72 ± 3.34	28.21 ± 1.55
High BaP	6.48 ± 1.16	14.61 ± 3.50	27.18 ± 2.16
Stimulation Index - Alamar Blue Assay			
Control	1.53, 1.60	1.75 ± 0.09	1.53 ± 0.07
Cortisol	1.85 ± 0.19	1.55 ± 0.04	1.61 ± 0.04
Low BaP	1.93 ± 0.04	1.66 ± 0.09	1.53 ± 0.09
High BaP	1.74 ± 0.06	1.57 ± 0.11	1.47 ± 0.09
Cortisol (ng/ml)			
Control	1; 1,2	34; 1,24,27	50; 15,35,99
Cortisol	24; 0,3,12,80	15; 7,15, 16,18,18,20	27; 7,10,13,19,86
Low BaP	48; 9,32,51,100	60; 12,54,55,66,70,103	11; 1,2,2,4,13,17,38
High BaP	33; 4,23,64,41	62; 20,33,46,48,71,155	44; 25,37,44,58

DISCUSSION

The ability of fish to metabolize toxins and resist disease is important for the survival of populations of fish. Delayed and non-lethal effects of toxicant exposure in the wild are difficult to evaluate due to the multitude of interactions within biological systems. We found that dietary exposure of juvenile chinook salmon to cortisol and BaP exposure reduced the PAH metabolizing capacity of the liver by reducing levels of CYP1A1 and increased susceptibility to infection by *L. salmonae*.

The reduction in the level of CYP1A1 protein by cortisol treatment is interesting. This finding is supported by other in vivo studies involving fish. Rainbow trout injected with dexamethasone (DEX), a synthetic glucocorticoid, had reduced hepatic CYP1A1 protein and mRNA levels (Lee et al., 1993). Daily stress, which induced high levels of plasma cortisol in Atlantic charr, *Salvelinus alpinus*, resulted in apparent, though not significant, decrease in baseline CYP1A levels, but when combined with BaP treatment resulted in significantly lower CYP1A protein levels than in controls (Jørgensen et al., 2001). Because the cortisol-BaP immune interaction was not tested in our study, there is a possibility that cortisol may act differently on CYP1A1 when combined with BaP.

In vitro studies in fish have yielded conflicting evidence concerning the glucocorticoid-CYP interaction. Experiments using trout hepatocytes have found both glucocorticoid mediated down regulation in CYP1A protein level and EROD activity (Dasmahapatra and Lee, 1993) and potentiation in EROD activity when

combined with BNF treatment (Devaux et al., 1992). While no difference was found with DEX alone, when combined with BNF or 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) there was potentiation of both CYP1A protein level and EROD activity in a *Poeciliopsis lucida* hepatoma cell line (Celander et al., 1997). The contradictory results of these studies may be due to differences in cell culture conditions (Dasmahapatra and Lee, 1993), or possibly lack of sensitivity in the assays.

Our finding of suppression of CYP1A1 protein in the fish given the high B*a*P dose is not unusual. Inhibition of CYP1A1 has been recognized in other dose-response studies involving higher doses of B*a*P (Goddard et al., 1987) and BNF (Haasch, 1993). Reduction of CYP1A mRNA, protein, or activity has also been documented in fish from sites highly contaminated with PAHs and PCBs (Nacci et al., 2002; Bello et al., 2001; Elskus et al., 1999). Altered CYP1A responsiveness has been linked to lower DNA adduct formation in these chemical stressor "resistant" fish and may reflect low rates of PAH metabolism (Nacci et al., 2002). Depressed levels of CYP1A1 in hepatic lesions may also represent an adaptive response of a wild population to carcinogen exposure (Van Veld et al., 1992).

The variation in CYP1A1 levels over time in the first experiment, with elevated CYP1A1 levels in the first two sample dates and reduction in levels at the end of the experiment, is similar to trends found by others (Gravato and Santos, 2002; Levine and Oris, 1997; Goddard et al., 1987). Temporal

differences in CYP1A1 levels make comparisons between studies difficult, especially because levels depend on dose, length of exposure, and time of sampling.

The reduction in hepatic CYP1A1 level could also be attributed to the exposure route and site-specific induction. Whereas aqueous BaP exposure causes more widespread CYP1A1 induction, dietary BaP mainly affects the gut mucosal epithelium (Van Veld et al., 1997). Induction in the gut may result in BaP biotransformation prior to entering the liver (Van Veld et al., 1997) as evidenced by higher and more persistent levels of DNA adducts in the gut (Ericson and Balk, 2000) and intestinal epithelium modification (Lemaire et al., 1992). Inhibition of hepatic CYP1A1 found in our study may have been due to upregulation of CYP1A1 in the intestine.

Our documentation of the suppressive effect of *L. salmonae* on hepatic CYP1A1 is consistent with decreases in CYP450 levels frequently observed during parasitic infections and may be caused by damage to the endoplasmic reticulum by migrating parasites, release of toxic substances or immune factors, liver damage, and/or increased lipid peroxides due to malabsorption of nutrients as discussed by Tekwani et al. (1988). Depression of CYP450 associated with inflammation and other types of infection has been linked to the release of cytokines during activation of the immune system (Krzanowski, 1991). Juvenile carp, *Cyprinus carpio*, treated with a bacterial endotoxin (LPS) showed reduced basal CYP450 levels in liver and spleen, and when combined with a CYP1A1

inducer, 3-methylcholanthrene, CYP450 protein content dropped below basal levels in liver and head kidney (Marionnet et al., 1998). Co-treatment of mice with interferon and BaP resulted in a significant reduction of CYP1A1 and cytogenetic response as compared to that of mice receiving BaP alone, suggesting impaired metabolic ability (Hrelia et al., 1994).

The down regulation of CYP1A1 in fish exposed to *L. salmonae* may be due to cytokine release during inflammation or possibly a stress response to the disease. Infection of chinook salmon by *L. salmonae* can cause severe inflammation and tissue damage in gills, spleen, and kidney after release of spores from xenomas (Kent et al., 1989). Inflammatory response following a cytokine cascade is well known in mammals and a number of cytokines have been described in fish (Secombes et al., 2001). There is also a possibility that *L. salmonae* infection resulted in a modulation of the stress response as documented in rainbow trout exposed to an ectoparasite, *Argulus foliaceus* (Ruane et al., 1999), although we were unable to detect differences in plasma cortisol between infected and noninfected fish.

We speculate that the increased susceptibility to *L. salmonae* consequent to low BaP exposure can be attributable to effects on macrophages. In various fish species BaP exposure has been shown to reduce phagocytic capacity (Walczak et al., 1987), alter pronephric macrophage superoxide production (Carlson et al., 2002) and H_2O_2 production (Holladay et al., 1998), and totally inhibit respiratory burst in splenic macrophages (Lemaire-Gony et al., 1995).

Ladics et al. (1992) found that murine macrophages were capable of producing 7,8-dihydroxy-9,10-epoxy-7,8,9,10-tetrahydro-B*a*P, a highly reactive metabolite of B*a*P, which may bind nucleophilic targets (RNA, DNA, and proteins) in macrophages, thereby affecting their immune response. Environmental stress and disease susceptibility are reviewed by Rice and Arkoosh (2002).

The fact that the low B*a*P dose but not the high B*a*P dose affected susceptibility to *L. salmonae* may be due to inhibited B*a*P metabolism at high doses. In fish a CYP1A inhibitor, α-naphthoflavone (ANF), suppressed the effects of BaP on antibody forming cell numbers (Carlson et al. 2002b) and mitogen stimulated proliferative response (Faisal and Huggett, 1993), suggesting the CYP1A-mediated metabolism of B*a*P is responsible for BaP immunotoxicity. It is possible that in our study the higher dose may have caused B*a*P to become inhibitory to CYP1A metabolism in our fish. Another possible explanation for no high B*a*P dose effect on disease susceptibility may be due to toxicity of B*a*P directly on *L. salmonae*.

Difficulties in detecting differences between treatments may also be due to the high infection rate also present in control fish. There is a possibility that treatment differences in infection may have been more distinct had the fish been sampled earlier. Sample bias toward surviving fish may also have lowered our estimated infection intensity in some groups because fish that died had high levels of infection and were not part of the population sampled on a later date.

The effect of cortisol treatment on susceptibility to *L. salmonae* was not surprising. Glucocorticoid induced changes in the immune system are numerous and may affect susceptibility to the parasite. Cortisol induced apoptosis or reductions in mitogen-stimulated proliferation, cell numbers, phagocytosis and antibody production as observed in several fish species (Weyts et al., 1999) could potentially affect multiple stages of infection. Rainbow trout exposed to cortisol were more susceptible to a myxosporean caused proliferative kidney disease (Kent and Hedrick, 1987) and infection by a ciliate, *Ichthyophthirius multifiliis* (Robertson et al., 1963) and a flagellate, *Cryptobia salmositica* (Woo, 1987). Diseases caused by microsporidia have also been associated with immune suppression in humans especially in the case of AIDS patients (Kotler and Orenstein, 1999).

A number of disease challenge biomarkers have been developed, but they use death as an endpoint. The use of *L. salmonae* has the additional advantage of easy quantification of infection rates, thereby perhaps providing a more robust indication of toxicant effect.

While elevations in levels of circulating cortisol were not evident in fish fed the steroid, it is clear from the biological response in CYP1A1 and *L. salmonae* susceptibility that the hormone entered the fish. The exogenous steroid was likely cleared during the 38 hours between the last feeding and the time of sampling.

Although mitogen-stimulated proliferation of leukocytes has been established as a biomarker of immunotoxicity in fish (Zelikoff et al., 2000), we found no differences between treatments. It is interesting that we were unable to detect the immunosuppressive effects of cortisol treatment as shown by others (Weyts et al., 1999). Cortisol treatment has been associated with migration of leukocytes out of the spleen (Maule and Schreck, 1990), which may have been a confounding factor in our experiment. The high variability found in mitogen response may be attributed to the limited sample size or long-term exposure compared to most studies that involve more acute exposures.

Long-term exposure to BaP, cortisol, and infection by a microsporidian suppressed hepatic CYP1A1 protein levels in juvenile chinook salmon. The consequences of reduced levels of hepatic CYP1A1 protein are unknown. Nacci et al. (2002) discussed the possibility that reduction of AH receptor-regulated enzymes may lead to decreased metabolism and elimination, bioaccumulation, increased maternal transfer, and selection of PAH resistant fish in urban waters. Alternatively CYP1A1 may be upregulated in another organ such as the intestine and result in alternative toxic effects (Ericson and Balk, 2000).

Multiple levels of biological organization are affected by environmental stress. The ecological significance of indicators from the lower levels is difficult to interpret (Johnson and Collier, 2002). The coupling of biochemical indicators such as CYP1A1 concentration and disease challenges using pathogens such as

L. salmonae allows for more confidence with interpretations of the consequences of contaminant exposure at the population level.

Pollution effects on fish populations may also involve changes in parasite prevalence. While parasite abundance in snails was found to decrease with increasing distance from sewage outfall (Siddall et al., 1993), prevalence and abundance of parasites in fish increased with distance down current of a PCB contaminated site (Khan, 1999).

Our finding of increased susceptibility to *L. salmonae* after prolonged elevation in cortisol concentration suggests that fish exposed to stressors, which result in prolonged elevation in cortisol may also experience higher susceptibility to infection. We suspect that low doses of BaP also have similar effects. The implication of cortisol and toxicant induced susceptibility to pathogens in fish in the wild is alarming considering the implications of disease and reduced metabolism of toxins on fish populations.

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