Expression of immune-regulatory genes in juvenile Chinook salmon following exposure to pesticides and infectious hematopoietic necrosis virus (IHNV)

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A R T I C L E   I N F O

Article history:
Received 2 January 2008
Received in revised form 10 July 2008
Accepted 12 July 2008
Available online 19 July 2008

Keywords:
Cytokine
Gene expression
Pesticide
Infectious hematopoietic necrosis virus
Chinook salmon

A B S T R A C T

Impairment of fish immune function as a consequence of polluted aquatic environments can result in changes in susceptibility to disease. In this study, we investigated the effects of two widely used insecticides, chlorpyrifos (CP) and esfenvalerate (EV), and a pathogen, infectious hematopoietic necrosis virus (IHNV), singly and in combination, on survival and cytokine (Mx protein, IL-1β, TGF-β and IGF-1) expression in juvenile Chinook salmon. Fish were exposed for 96 h to sublethal concentrations of CP (3.7 μg L−1) or EV (0.08 μg L−1), allowed to recover in clean water for seven days, then exposed to IHNV (6.4 × 105 TCID50 mL−1) for 1.5 h. Mortality was recorded daily, and spleen and anterior kidney samples were collected on day 4 (after CP or EV treatment), day 20 and day 60 (after CP or EV treatment and subsequent IHNV exposure) of the experiment.

Significant mortality after 60 days was observed following exposure to EV (17%) or IHNV (20%), and prior insecticide exposure did not synergize the acute effects of pathogen treatment. By day 4, exposure to CP as well as EV led to a significant decrease of Mx protein and IL-1β expression; by day 20, EV-exposed fish significantly overexpressed IL-1β. Mx protein transcription was up-regulated in spleen and kidney of all IHNV-exposed fish groups by day 20. All but one treatment (EV) led to significantly decreased IGF-1 transcription in spleen on days 20 and 60, whereas a short-term increase was seen after CP exposure (day 4). In kidney, decreases of IGF-1 transcription were less pronounced. TGF-β transcription was up-regulated in CP/IHNV and EV/IHNV exposure groups.

Our results indicate that CP and EV alter the expression of cytokines, but this did not negatively affect the ability of fish to survive a subsequent exposure to IHNV. Induced TGF-β transcription indicated that the combined stressors affected fish in a synergistic manner, but the consequences are unknown. Increased transcription of Mx protein was a reliable indicator of virus exposure.

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1. Introduction

Cytokines are pleiotropic, multifunctional mediators of the innate and adaptive immune system. They are capable of initiating and regulating inflammatory processes, thus playing an important role in the vast network of interacting cells and signaling molecules associated with the immune response. Their constitutive expression is generally low, but stimulation by infection or an inflammatory insult can trigger new gene transcription and protein synthesis [1–3]. In recent years significant progress has been made identifying cytokine activity in fish, and an increasing number of cytokine gene sequences from different teleost species are now available [4]. With the cloning of these genes, new tools are accessible to study immune responses of fish to various insults (e.g. pathogens and pollutants) they may encounter during their lifetime.

Pesticide concentrations exceeding guidelines for the protection of aquatic life have been reported in streams and ground water throughout the nation’s agricultural and urban areas [5], and several studies have shown that these contaminants can suppress fish immune responses and reduce host resistance to pathogens [6–9]. Chlorpyrifos and esfenvalerate, members of two large insecticide classes, the organophosphates and pyrethroids, respectively, have been frequently detected in streams and rivers where salmonid rearing occurs [10–12], and results from laboratory experiments indicate that adverse effects on the immune system
may contribute to their toxicity to juvenile Chinook salmon (*Oncorhynchus tshawytscha* Walbaum) [13]. A challenge study performed by Cliford et al. [14] showed significantly induced and earlier onset of mortality in Chinook salmon concurrently exposed to EV and IHNV, suggesting a lethal synergism between the pyrethroid and the salmonid pathogen. Our earlier studies demonstrated that sublethal, environmentally relevant concentrations of CP altered cytokine transcription in kidney and spleen of exposed salmon [13], but the consequences of the observed alterations remained to be elucidated. Suppression of immune responses by aquatic pollutants can lead to an invasion of opportunistic pathogens, increase disease incidence, and exacerbate disease states in fish [15]. To date, little is known about the effects and mechanisms of action of pesticides on the teleost immune response. To study the effects of exposure to pesticides on the immune response, juvenile Chinook salmon previously treated with sublethal concentrations of CP and EV were challenged with IHNV. We then examined changes in the expression of selected cytokine genes at various times post virus challenge. The virus chosen for exposures was IHNV as it is considered the most important viral pathogen of salmonid fishes in North America, and it is present in areas where salmon come in contact with agricultural pollutants [16,17]. Young salmonids respond to IHNV infection with the production of antibody and interferon [16], and Purcell et al. [18] recently demonstrated significant expression changes of important cytokine and related genes following exposure of rainbow trout to IHNV by injection.

In this study, the expression of four cytokine genes was examined by quantitative real-time TaqMan PCR: Mx protein, interleukin-18 (IL-18), transforming growth factor-β (TGF-β) and insulin-like growth factor 1 (IGF-1). Mx proteins constitute a family of antiviral proteins induced by the type I interferon (IFN) system, a powerful antiviral defense mechanism of higher vertebrates [19]. Experimental evidence obtained from animal model studies established that Mx alone is sufficient to block virus replication in the absence of other IFN-inducible proteins [20]. In fish, induction of Mx genes by live virus has been demonstrated [18], and a correlation between Mx protein expression and protection against virus infection has been suggested [21–23]. IL-18 is a member of the IL-1 family, prototypic pro-inflammatory cytokines also known to act as immunoadjuvants and regulators of host defense [24]. Studies in salmonids have demonstrated strongly up-regulated expression of the IL-18 gene in response to pesticide exposure and IHNV treatment [13,18]. TGF-β counteracts pro-inflammatory mediators, and numerous studies have demonstrated the importance of TGF-β for the maintenance of immune homeostasis and its critical anti-inflammatory role [25]. Finally we included IGF-1, a protein not only involved in endocrine control of the immune system but also acting as a local growth and differentiation factor, which interacts with other cytokines [26]. The role of IGF-1 in innate and acquired immunity has not been verified for the fish immune system, but the structure and biological potency of fish IGF-1 are very similar to those of mammalian homologues [27].

We examined cytokine gene expression following pesticide exposure, virus treatment, and both stressors combined with the goal of determining potential mechanisms for the immunotoxic effects of CP and EV on the immune response of Chinook salmon to IHNV infection.

2. Materials and methods

2.1. Fish source and maintenance

Fertilized fall-run Chinook salmon eggs were obtained from the Nimbus Salmon and Steelhead Hatchery of the California Department of Fish and Game (CDFG, Rancho Cordova, CA) and transferred to the Center for Aquatic Biology and Aquaculture (CABA), University of California, Davis. Eggs and developing juveniles were maintained at CABA under single pass flow-through conditions with degassed, reoxygenated well water at 8–10 °C. Approximately 5–6-month-old fish with a mean weight of 5.2 g were used in this study. All transfers of juveniles were performed by dip netting individuals between tanks. Juveniles were fed a daily ration of soft-moist salmon diet (3/32" pellet, Rangen Inc., Buhl, ID). Fish on maintenance diets often grow slower but are usually very healthy.

2.2. Chemical treatments

Solid, crystalline CP (99.5% pure, determined by supplier) and solid, crystalline EV (Asana (TM), 98% pure, determined by supplier) were purchased from Chem Service Inc. (West Chester, PA). A 0.015 g L⁻¹ stock solution was prepared by dissolving CP in HPLC grade methanol, and a nominal concentration of 5 µg L⁻¹ was obtained by pipetting 10 mL of stock solution into aquaria filled with 30 L of well water. Esfenvalerate stock solution (0.3 mg L⁻¹) was produced to obtain 0.1 µg L⁻¹ final concentration by following the procedures described for CP. During the 96 h exposure water was changed and dosed with insecticide solution four times at 24 h intervals. Monitoring of CP concentrations in previous studies performed in this laboratory indicated an average loss of 75% of chemical over 24 h. In order to keep the nominal pesticide concentration at or close to 5 µg L⁻¹, we adjusted the amount of stock solution needed after water changes accordingly. For EV, we assumed 100% loss over a 24-h period due to breakdown and absorbance to the aquaria glass. Control groups received HPLC grade methanol at the highest concentration applied to exposure groups (10 mL MeOH in 30 L well water, or 0.033% final concentration). On the fourth day of pesticide exposure, water samples from both treatments were recovered immediately after changing water and applying stock solution (0 h), and just before replacement of test water (24 h). Pesticide concentrations were determined by the Fish and Wildlife Water Pollution Control Laboratory (WWPCL, Rancho Cordova, CA), California Department of Fish and Game, using gas chromatography (GC) with dual electron capture detectors with positive samples confirmed using GC mass spectrometry (MS). Aqueous CP concentrations in exposure aquaria showed ~25% difference between measured and dosed concentrations (dosed: 5 µg L⁻¹; measured: 3.7 µg L⁻¹). The measured EV concentration was 0.08 µg L⁻¹ which was 20% lower than the nominal concentration (0.1 µg L⁻¹). These data agreed with results from previous studies with similar exposure conditions that showed a 20–25% difference between nominal and measured concentrations [13].

2.3. Experimental design and exposure regime

Fish were acclimated by slowly raising the temperature to 12 °C over a period of 48 h, and 16 individuals were randomly selected and sacrificed to serve as untreated controls. Subsequently, 504 juvenile Chinook salmon were transferred to a neighbouring exposure chamber at the Aquatic Toxicology Laboratory, University of California, Davis. Fish were randomly divided into 12 groups of 42 individuals and placed into ~38 L aquaria filled with 30 L of test solution. For each of six different treatments two replicate aquaria were partially submerged in chilled water to maintain a temperature of 12 °C. Water in aquaria was aerated, and 75% of the test solutions were replaced every 24 h to maintain a relatively constant concentration (see Chemical treatments). Upon completion of the pesticide treatments, fish were transferred into clean aquaria identical to those described above, and held in clean well water for seven days before being moved to the flow-through system. This 7-day period in the static system was required to prevent
contamination of the flow-through system. Water in the static system was aerated, exchanged daily and quality was monitored several times per day. Water temperature was 12.2 ± 0.4 °C, dissolved oxygen concentration was 8.05 ± 1.65 mg L⁻¹, pH was 8.55 ± 0.35 and electric conductivity was 709 ± 17 ms/cm. Free ammonia was determined colorimetrically using a commercially available kit (EM Science, Gibbstown, N.J.). To minimize binding of pesticides to organic matter in aquaria, fish were fed a soft-moist salmon diet (3/32” pellet, Rangen Inc., Buhl, ID) 1 h before daily replacement of test solutions. Fish were fed on a daily basis after the pesticide exposure. After a total of 11 days in the static system (L:D = 16:8) exposure groups were transferred to individual 15 L tanks in a flow-through system (12 °C) and exposed for 1.5 h to IHNV by bath exposure (see Virus and cell culture). To observe cumulative mortality, fish were maintained in control water for an additional seven weeks (L:D = 16:8) with supplemental aeration and a daily food ration of approximately 1.5–2.5% of body weight/day. Water temperature and general appearance of fish were monitored one or more times per day. Samples (eight fish per aquarium) were taken directly after pesticide exposure (day 4), nine days after virus exposure (day 20), and at completion of the study (day 60). Fish were sacrificed by decapitation, and tissue samples for gene expression analysis (see Tissue preparation and RNA extraction) were flash-frozen and stored at −80 °C, while tissues for virus sampling were bagged individually, placed on ice and processed immediately (see IHNV challenge and screening). Dead fish accruing during the study were recorded, and those dying after the virus was introduced were examined for presence of IHNV (see IHNV challenge and screening).

2.4. Virus and cell culture

An isolate of IHNV collected by the CDFG from steelhead trout (Oncorhynchus mykiss) in the Feather River, CA, was used for all virus exposures in this study. The virus isolate was collected February 2002 and passed twice times on a suitable cell line. The virus for fish challenges was propagated in the Chinook salmon embryo cell line (CHSE-214) [28] at 15 °C for 11 days. Virus concentrations were determined by the 50% end-point dilution assay (Tissue Culture Infecting Dose; TCID₅₀) of Rozovzo and Burke [29] utilizing the epithelioma papulosum cyprini (EPC) cell line [30]. Both cell lines were grown in minimal essential media (MEM) supplemented with 7.5% (v/v) foetal bovine serum (FBS), 50 IU penicillin ml⁻¹, 50 µg streptomycin ml⁻¹, and 2 mM l-glutamine (MEM 7.5) as described by Arkush et al. [31]. For virus propagation and the performance of TCID₅₀ assays, we reduced the amount of FBS added to the growth medium to 2% (v/v) (MEM-2). The IHNV isolate was maintained as a frozen stock at −80 °C (first pass). Prior to virus exposures a small aliquot of the virus suspension was removed for determination of IHNV concentration by TCID₅₀ assay.

2.5. IHNV challenge and screening

Shortly after transferring exposure groups to individual 15 L tanks in a flow-through system, fish were challenged with IHNV by bath exposure. For the trial, water flow was interrupted for 1.5 h, and 51 ml of MEM-2 was added to aquaria for control groups. The IHNV exposure groups were challenged by addition of 51 ml of suspension of virus at 6.4 × 10⁶ TCID₅₀ ml⁻¹. After 1.5 h in the static bath, water flow was resumed at approximately 1.5 L/min. Fish were observed daily for mortality. Dead fish were collected, bagged individually, placed on ice, and processed and analyzed for presence of virus as described by Thoensen et al. [32]. Microplates were observed daily for at least three weeks for the presence of cytopathic effects (CPE). Virus concentrations were calculated according to Rozovzo and Burke [29].

2.6. Quantitative real-time PCR

2.6.1. Tissue preparation and RNA extraction

Immediately after fish were sacrificed, the entire spleen and anterior kidney were removed and a small piece of each tissue (<30 mg) stored in 1.5 ml Eppendorf vials and flash-frozen in liquid nitrogen, whereas the rest was used for virus titrations (see IHNV challenge and screening). Samples were stored at −80 °C until RNA extraction and cDNA synthesis were performed. Frozen tissues were transferred to 1.5 ml collection tubes (RNeasy Mini Kit, Qiagen Inc., Valencia, CA), immersed in liquid nitrogen and ground to a fine powder using a mortar and a pestle. Subsequently, 350 ml of RNeasy lysis buffer (RXL, RNeasy Mini Kit, Qiagen Inc.) was added, and lysates were homogenized by using the pestle and by passing it through a pipette tip for 5–10 times. After incubation for several minutes at room temperature, the RNA was extracted according to the manufacturers recommendations (RNeasy Mini Kit, Qiagen Inc.).

2.6.2. cDNA synthesis

Complementary DNA (cDNA) was synthesized using 50 units of SuperScript III (Invitrogen Life Technologies, Carlsbad, CA, USA) containing 10 mIU Revert-Aid H-M Murine Moloney Murine Leukemia Virus reverse transcriptase, 50 mM dNTPs (Promega), 10 mM DTT and 500 ng of mouse β-actin primer. The reaction mixture was incubated for 5 min at 25 °C, followed by 50 min at 42 °C. The final volume was adjusted to 100 µl with RNase-free water, and the reaction was terminated by heating for 5 min to 95 °C and cooling on ice.

2.6.3. Real-time TaqMan® PCR

PCR reactions for each target gene contained 400 nM of each of two primers and 80 nM of the appropriate TaqMan probe. Primers and probes for selected cytokine genes were designed and tested in this laboratory and the sequences are listed in Eder et al. [33]. We used commercially available PCR mastermix (TaqMan Universal PCR Mastermix, Applied Biosystems, Foster City, CA, USA) containing 10 mM Tris–HCl (pH 8.3), 50 mM KCl, 5 mM MgCl₂, 2.5 mM deoxyribonucleotide triphosphates, 0.625 U AmpliTaq Gold DNA polymerase per reaction, 0.25 U AmpErase UNG per reaction and 5 µl of the diluted cDNA sample in a final volume of 12 µl. The samples were placed in 96 well plates and amplified in an automated fluorometer (ABI PRISM 7700 Sequence Detection System, Applied Biosystems). Amplification conditions were 2 min at 50 °C, 10 min at 95 °C, 40 cycles of 15 s at 95 °C and 60 s at 60 °C. Fluorescence of samples was measured every 7 s and signals were considered positive if fluorescence intensity exceeded 10 times the standard deviation of the baseline fluorescence (threshold cycle, C_T). Analytical specificity and sensitivity of all TaqMan systems were confirmed in a previous study by Eder et al. [33].

2.6.4. Relative quantitation of cytokine gene transcription

The comparative C_T method was applied to quantify gene transcription of investigated cytokines (User Bulletin #2, Applied Biosystems). Values are reported as relative transcription or the n-fold difference relative to a calibrator cDNA (i.e. lowest target gene transcription of control fish). In brief, the endogenous control ITS-2 was used to normalize the target gene signals (ΔC_T) for the differences in the amount of nucleic acid added to each reaction and RT efficiency. The ΔC_T for each experimental sample was subtracted from the ΔC_T of the calibrator, the lowest target gene signal of control fish. The linear amount of target molecules relative to the calibrator was calculated by 2⁻ΔΔC_T. Therefore, all cytokine gene transcription is expressed as an n-fold difference relative to the calibrator.
2.7. Statistical analysis

Data from treatment groups were compared using the statistical program SigmaStat, Version 2.03 (Systat Software, Inc., Point Richmond, CA). Two Way ANOVA was conducted to test for significant interaction between pesticides and virus. Multiple comparisons were performed using Dunnett’s test to compare against the control group and Tukey’s test for all pairwise comparisons. Data were transformed for some variables to improve normality. Group variances for mortality data were unequal and Welch ANOVA was used for this analysis. Significance was accepted if $p \leq 0.05$.

3. Results

3.1. Mortality and mean day to death

There was no mortality occurring during the 96 h pesticide treatment phase and the following week in control water (static system). However, during the subsequent seven weeks mortality was observed in every treatment group. Only a single fish died in each of the control and CP-exposed groups. Although mortality was relatively low (<20%), fish groups exposed to EV only (EV; 16.8%) and virus only (Control/IHNV; 19.9%) had a significantly greater number of fish dying than in the solvent control (Control; 1.2%) (Fig. 1).

Mean time to death (MTD), a general measure for the susceptibility of organisms to a pathogen, was calculated for fish exposed to IHNV. Although statistically not significant, exposure to both CP and EV appeared to lower the MTD after virus treatment from 25.2 days (Control/IHNV) to 21.2 and 19.5 days, respectively. The MTD for experimental groups with a single mortality was not applicable, EV-exposed fish died on average 32.8 days after treatment.

Growth measured by determining length and weight of each individual was not significantly different from control groups for fish exposed to virus, pesticides or both treatments combined (data not shown).

3.2. Virus concentrations

Each fish that died after virus introduction on day 11 of the 7-week study was examined for presence of IHNV. Furthermore, the presence of virus in live fish was examined at day 9 after virus exposure (day 20) and at the day of completion of the study (day 60). Virus was never detected in fish that were not exposed to IHNV. In fish that died after IHNV exposure, virus was detected in 73% of samples from the virus control group, 71% of the fish treated with CP, and 50% of fish treated with EV. Mean virus concentrations in dead fish determined by end-point dilution assay were significantly higher in the CP/IHNV group than in either Control/IHNV or EV/IHNV groups (Table 1). Virus was isolated from four live control fish sampled nine days after viral challenge, from five juveniles in the CP/IHNV group, and from only one salmon in the EV/IHNV group (Table 1). These 10 fish were grouped together andlabelled ‘Infected’. Mean viral titres in tissues from sampled fish were highest in control fish in comparison to the CP/IHNV group and the single fish with combined EV/IHNV treatment; however, these differences were not statistically significant (Table 1). There was no evidence of virus in live juveniles sampled on the last day of experiment from any treatment group.

3.3. Cytokine transcription

Gene expression changes of four selected cytokines, IL-1β, TGF-β, IGF-1 and Mx protein, were assessed in kidney and spleen of Chinook salmon in response to pesticide treatment, virus exposure, and both treatments combined. On day 4, Mx protein expression was slightly but significantly decreased in kidney of fish treated with CP, but no changes were observed in spleen of these individuals (Table 2). Similarly, exposure to 0.08 μg L⁻¹ EV led to a slight, but significant reduction of Mx protein transcripts in kidney to below basal levels.

Expression of IL-1β protein in kidney and spleen (Fig. 2). Mx protein mRNA levels were between 2.3 and 4 times higher than those of controls nine days after virus exposure in both tissues (day 20). All groups treated with virus had significantly elevated Mx protein transcription in kidney. These changes were also observed in spleen, but to a slightly lesser extent than in kidney. Pesticide treatments had no influence on transcription of the Mx protein gene. Fish with confirmed IHNV infection by end-point dilution assay had the highest Mx protein transcript levels in kidney, significantly differing from all other treatment groups exposed to virus (Fig. 2). Mx protein expression in all groups was near control levels 49 days after exposure to the virus (day 60, Table 2).

Exposure to 300 μg L⁻¹ CP and 0.08 μg L⁻¹ EV led to significantly lower expression of IL-1β in spleen tissue in samples collected immediately after pesticide treatment (day 4, Table 2). Esfenvalerate, however, induced significantly higher expression of IL-1β in kidney (eightfold) and spleen (threefold) in comparison to control fish on day 20 of the experiment (nine days after virus was administered, Fig. 3). Exposure to IHNV alone did not alter IL-1β mRNA levels, however, expression in kidney from fish with confirmed virus infection was significantly increased (4.3-fold) on day 20 (Fig. 3). Analysis of tissues sampled at day 60 showed, that

<table>
<thead>
<tr>
<th>Table 1</th>
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<tbody>
<tr>
<td>Percent of samples with virus present and mean titers observed in kidney/spleen of juvenile Chinook salmon after exposure to IHNV only or pesticide/IHNV</td>
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<tr>
<td>Treatment</td>
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<tr>
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</tr>
<tr>
<td>IHNV</td>
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<tr>
<td>CP + IHNV</td>
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<td>EV + IHNV</td>
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<td>IHNV</td>
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<td>CP + IHNV</td>
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<td>EV + IHNV</td>
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</table>

Samples were taken from fish that died during the study (“Mortality”) and random individuals (“Random sample”; n = 16) collected on day 20 of the study.
expression generally decreased in tissues of fish exposed to pesticides, virus or both stressors combined on day 20 of the experiment. Results represent the means ± standard error expressed as an n-fold difference relative to the calibrator in kidney and spleen measured at day 4 (three treatment groups), day 20 (seven treatment groups) and day 60 (six treatment groups).

### Table 2

Mean cytokine gene transcription ± standard error expressed as an n-fold difference relative to the calibrator in kidney and spleen measured at day 4 (three treatment groups), day 20 (seven treatment groups) and day 60 (six treatment groups)

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Treatment</th>
<th>Day 4 Kidney</th>
<th>Day 4 Spleen</th>
<th>Day 20 Kidney</th>
<th>Day 20 Spleen</th>
<th>Day 60 Kidney</th>
<th>Day 60 Spleen</th>
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</thead>
<tbody>
<tr>
<td>Mx protein</td>
<td>C</td>
<td>2.3 ± 0.4</td>
<td>3.6 ± 0.8</td>
<td>Fig. 2</td>
<td>Fig. 2</td>
<td>2.3 ± 0.3</td>
<td>1.8 ± 0.2</td>
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<td></td>
<td>CP</td>
<td>–0.9 ± 0.6*</td>
<td>2.9 ± 0.7</td>
<td>Fig. 2</td>
<td>Fig. 2</td>
<td>5.1 ± 0.9</td>
<td>3.2 ± 0.2</td>
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<tr>
<td></td>
<td>EV</td>
<td>–0.3 ± 0.6*</td>
<td>1.8 ± 0.5</td>
<td>Fig. 2</td>
<td>Fig. 2</td>
<td>4.1 ± 1.0</td>
<td>0.9 ± 1.0</td>
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<tr>
<td>C/IHVNV</td>
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<td>–</td>
<td>Fig. 2</td>
<td>Fig. 2</td>
<td>4.3 ± 1.3</td>
<td>2.2 ± 0.2</td>
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<tr>
<td>CP/IHVNV</td>
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<td>Fig. 2</td>
<td>Fig. 2</td>
<td>3.9 ± 1.1</td>
<td>4.0 ± 0.8</td>
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<tr>
<td>EV/IHVNV</td>
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<td>–</td>
<td>Fig. 2</td>
<td>Fig. 2</td>
<td>5.1 ± 1.2</td>
<td>3.3 ± 0.7</td>
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<td>Infected</td>
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<td>–</td>
<td>Fig. 2</td>
<td>Fig. 2</td>
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<tr>
<td>IL-1β</td>
<td>C</td>
<td>14.1 ± 4.0</td>
<td>27.5 ± 7.1</td>
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<td>Fig. 3</td>
<td>2.7 ± 0.5</td>
<td>2.8 ± 0.5</td>
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<td>CP</td>
<td>10.5 ± 2.6</td>
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<td>Fig. 3</td>
<td>1.8 ± 0.8</td>
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<td>Fig. 3</td>
<td>Fig. 3</td>
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<td>CP/IHVNV</td>
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<td>Fig. 3</td>
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<td>3.8 ± 1.5</td>
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<td>EV/IHVNV</td>
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<td>Fig. 3</td>
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<td>2.3 ± 0.7</td>
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<tr>
<td>Infected</td>
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<td>Fig. 3</td>
<td>Fig. 3</td>
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<tr>
<td>IGF-1</td>
<td>C</td>
<td>4.7 ± 2.3</td>
<td>4.6 ± 0.9</td>
<td>11.8 ± 2.3</td>
<td>Fig. 4</td>
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<td>6.8 ± 2.1</td>
<td>Fig. 4</td>
<td>6.3 ± 1.5</td>
<td>Fig. 4</td>
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<tr>
<td></td>
<td>EV</td>
<td>10.3 ± 2.6</td>
<td>7.3 ± 1.2</td>
<td>7.1 ± 1.7</td>
<td>Fig. 4</td>
<td>16.0 ± 4.7</td>
<td>Fig. 4</td>
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<tr>
<td>C/IHVNV</td>
<td>–</td>
<td>–</td>
<td>1.7 ± 1.1*</td>
<td>Fig. 4</td>
<td>12.1 ± 2.9</td>
<td>Fig. 4</td>
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<tr>
<td>CP/IHVNV</td>
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<td>–</td>
<td>17.4 ± 5.0*</td>
<td>Fig. 4</td>
<td>8.4 ± 2.6</td>
<td>Fig. 4</td>
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<tr>
<td>EV/IHVNV</td>
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<td>–</td>
<td>5.4 ± 1.7</td>
<td>Fig. 4</td>
<td>5.8 ± 1.7*</td>
<td>Fig. 4</td>
<td></td>
</tr>
<tr>
<td>Infected</td>
<td>–</td>
<td>–</td>
<td>5.6 ± 1.8</td>
<td>Fig. 4</td>
<td>–</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td>TGF-β</td>
<td>C</td>
<td>3.3 ± 0.8</td>
<td>2.5 ± 0.3</td>
<td>3.3 ± 0.5</td>
<td>2.6 ± 0.7</td>
<td>2.4 ± 0.6</td>
<td>10.6 ± 1.7</td>
</tr>
<tr>
<td></td>
<td>CP</td>
<td>1.5 ± 1.2</td>
<td>2.7 ± 0.3</td>
<td>1.7 ± 0.7</td>
<td>0.7 ± 0.6</td>
<td>1.7 ± 0.3</td>
<td>17.1 ± 3.7</td>
</tr>
<tr>
<td></td>
<td>EV</td>
<td>2.2 ± 0.5</td>
<td>2.6 ± 0.5</td>
<td>5.9 ± 1.3</td>
<td>1.0 ± 0.4</td>
<td>2.1 ± 0.4</td>
<td>14.0 ± 3.3</td>
</tr>
<tr>
<td>C/IHVNV</td>
<td>–</td>
<td>–</td>
<td>2.9 ± 0.6</td>
<td>1.1 ± 0.3</td>
<td>1.8 ± 0.1</td>
<td>13.9 ± 2.6</td>
<td></td>
</tr>
<tr>
<td>CP/IHVNV</td>
<td>–</td>
<td>–</td>
<td>6.4 ± 1.2*</td>
<td>–0.4 ± 0.5*</td>
<td>1.7 ± 0.4</td>
<td>19.1 ± 2.3*</td>
<td></td>
</tr>
<tr>
<td>EV/IHVNV</td>
<td>–</td>
<td>–</td>
<td>6.4 ± 1.2</td>
<td>1.1 ± 0.5</td>
<td>2.0 ± 0.3</td>
<td>29.1 ± 6.2*</td>
<td></td>
</tr>
<tr>
<td>Infected</td>
<td>–</td>
<td>–</td>
<td>9.4 ± 1.5*</td>
<td>0.4 ± 0.6</td>
<td>–</td>
<td>–</td>
<td></td>
</tr>
</tbody>
</table>

Groups represent the mean of 10–12 individual fish except for the group ‘Infected’, where n = 8–9 (C/IHVNV – 4, CP/IHVNV – 5, EV/IHVNV – 1); significant changes (p ≤ 0.05) in comparison to control are shown in bold with an asterisk.

* Significantly different from C/IHVNV.

b Significantly different from CP.

**IL-1β** mRNA transcripts of all groups had returned to basal levels (Table 2).

Spleen from fish treated with 3.7 μg L⁻¹ CP showed significantly increased IGF-1 expression at day 4 (Table 2). Thereafter, IGF-1 gene expression generally decreased in tissues of fish exposed to pesticides, virus, and both stressors combined. Significantly reduced IGF-1 transcription in kidney and spleen was observed in fish nine days after virus exposure (day 20). In spleen, all treatment groups with the exception of EV-exposed fish showed a significant reduction of IGF-1 transcription in comparison to controls, however, the lowest expression was observed in fish with confirmed virus infection (Fig. 4). IGF-1 transcript levels in spleen stayed below basal levels for the entire study, leading to

![Fig. 2](image1.png)

**Fig. 2.** Mx protein profiles in kidney and spleen from juvenile Chinook salmon treated with pesticides, virus or both stressors combined on day 20 of the experiment. Results represent the means ± SE of Mx protein transcription expressed as n-fold calibrator value. n = 11–12, except for infected fish where n = 9 (C/IHVNV – 4, CP/IHVNV – 4, EV/IHVNV – 1); *significantly different from Control; **significantly different from all other HHNV-exposed groups in pairwise comparisons; significance level p ≤ 0.05.

![Fig. 3](image2.png)

**Fig. 3.** IL-1β profiles in kidney and spleen from juvenile Chinook salmon treated with pesticides, virus or both stressors combined on day 20 of the experiment. Results represent the means ± SE of IL-1β transcription expressed as n-fold calibrator value. n = 10–12, except for infected fish where n = 9 (C/IHVNV – 4, CP/IHVNV – 4, EV/IHVNV – 1); *significantly different from Control; **significantly different from all other HHNV-exposed groups in pairwise comparisons; significance level p ≤ 0.05.
concentration used in our study was not acutely lethal but led to significant mortality over an extended period of time (mean day to death = 32.8). Most of the symptoms seen in acute lethality tests with pyrethroids point to central nervous system (CNS) toxicity, but adverse effects on respiratory surfaces and renal ion regulation have also been reported [35]. Analyses of several organs from fish utilized in the present study demonstrated cellular stress in gills and muscle [36], and inflammatory processes in the hematopoietic tissues of kidney and spleen in response to EV treatment. In addition, slow elimination of the pyrethroid may have prolonged adverse effects, thus contributing to the delay of mortality.

Even though Mx protein expression patterns demonstrated antiviral activity in all fish exposed to IHNV alone, we observed relatively low cumulative mortality (20%) over an extended period of time (mean day to death = 25.2) compared to the more typical time frame of 5 days to 2 weeks [16]. Virus concentrations up to 10^5 plaque forming units (PFU) have been detected in the natural environment of salmonids [37] and are less than the 6.4 × 10^6 TCID50 mL^{-1} challenge dose in our study. Although immersion challenge with IHNV at higher concentrations has been demonstrated to induce infections in both juvenile and adult salmonids [38,39], subsequent mortality may be highly variable due to a range of factors dependent on the host, environment, and virus strain [40]. Fish age is one critical factor, and fish, particularly those younger than two months are considered the most susceptible to the virus [40]. Furthermore, the virus utilized in the present study was isolated from steelhead trout, and it is possible that virulence of this particular IHNV strain for juvenile Chinook salmon is relatively low [40]. Mortality rates in fish groups treated with pesticides and IHNV were generally lower than in IHNV-exposed control fish. Synergistic effects between EV and IHNV resulting in higher mortality than either stressor observed by Clifford et al. [14] could not be verified in our study. This may be explained by a key difference in experimental design, as in their study fish were exposed to both stressors concurrently while in our study fish experienced a 7-day period in clean water prior to virus challenge.

Sublethal pesticide concentrations have been shown to stimulate the cellular repair system and/or immune system, increase the metabolic rate and/or cause other physiological changes [13,41–43]. These physiological alterations may help stronger, less susceptible individuals to resist infection, whereas weaker fish may be rendered more susceptible. In this study, fish exposed to CP or EV and IHNV died sooner than juveniles subjected to IHNV alone, suggesting that exposure to insecticides accelerated the deleterious effects of IHNV infection. This early onset of mortality in fish concurrently subjected to EV and IHNV was also observed by Clifford et al. [14].

The present work describes the applicability of a highly sensitive TaqMan PCR system to indicate viral exposure by analyzing Mx gene expression. To our knowledge, this is the first study to report induced mRNA transcription of Mx protein in juvenile Chinook salmon following the natural route of infection with IHNV. Mx genes have been identified in a number of fish species [44–47], and their induction and expression linked to protection against virus infection [18,21–23,48]. In this study, juvenile salmon expressed significantly increased levels of Mx transcripts in anterior kidney and spleen following waterborne exposures to IHNV concentrations of 6.4 × 10^6 TCID50 mL^{-1}. In comparison, TCID50 assays applied to anterior kidney and spleen of the same individuals resulted in a small percentage (between 6% and 31%) of fish identified as infected with IHNV, clearly demonstrating the greater sensitivity of the TaqMan PCR method, and thus the applicability of Mx protein gene transcription as an indicator of virus exposure. Significantly higher expression levels of Mx mRNA in kidney among fish with IHNV detected by TCID50 assay than in those without detectable virus further strengthens this correlation. Our findings are consistent with results from a study by Purcell et al. [18], where live IHNV
and an IHNV DNA vaccine both elicited a significant induction of Mx protein in rainbow trout. Several other studies injecting live virus in vivo [21,48] or immunization with viral DNA [22,23,49] also describe up-regulation of Mx protein. It was suggested that Mx protein induction correlates with an early nonspecific antiviral protection mediated by type I-IFN, leading to increased resistance against viral insults [21–23], which might have contributed to lower the mortality rates in our virus challenge. Mx protein gene transcription reverted to control levels 60 days after virus exposure, thus supporting the notion of an early and transient nonspecific antiviral phase through type I-IFN. Although a temporary, slight decrease of Mx protein gene expression after pesticide exposure was detected, further analysis demonstrated that CP and EV did not significantly influence the transcription of this antiviral protein.

In contrast to Mx protein, expression of IL-1β and TGF-β mRNA in kidney and spleen was affected by exposure to both insecticides, CP and EV. Whereas gene expression was depressed immediately after exposure to both insecticides, strong induction of IL-1β transcription was observed on day 20 in kidney (eightfold) and spleen (threefold) of EV-exposed fish, the treatment group experiencing significant mortality. Expression of IL-1β, a pro-inflammatory cytokine, has been confirmed in fish [50] and increased transcription in juvenile salmon exposed to pesticides has been reported [13]. Up-regulated IL-1β levels in both tissues on day 20 may be caused by sublethal deleterious effects of EV resulting in cellular damage and associated inflammatory processes. Besides acting as a voltage-dependent sodium-channel agonist, pyrethroids have been shown to affect respiratory surfaces, renal ion regulation and the metabolism of fish [35]. Furthermore, histopathological examination of gills of rainbow trout treated with another pyrethroid, fenvalerate, demonstrated lamellar aneurysms, epithelial separation and numerous necrotic cells [51]. Surprisingly, transcription of IL-1β was not significantly induced in fish subjected to both, EV and IHNV. Initial suppression of IL-1β transcription by the pesticide may have affected the response to IHNV, or resulted in different timing and magnitude of IL-1β expression. Although results from IL-1β analyses presented here suggest that EV affected components of the salmon immune system, they provide no specific mechanisms for synergistic effects between the pathogen and EV observed by Clifford et al. [14]. However, virus concentration determinations in the latter study revealed that the observed increase and earlier onset of mortality were not due to a more rapid spreading of the virus, but instead to an unknown mechanism of toxicity. A synergistic effect of IHNV and EV was also seen in expression levels of several members of the heat-shock protein (hsp) family in muscle and gills of the fish used in the study presented here [36].

Although head kidney and spleen are among the primary sites of infection and inflammation during IHNV infection [39,52], significant up-regulation of IL-1β transcription was detected in the anterior kidney of IHNV-infected salmon, but not in the spleen. Thus, findings of Purcell et al. [18], who recently reported induction of IL-1β in spleen after injecting rainbow trout with live IHNV, could not be confirmed. However, induced transcription of IL-1β is transient [18], and IHNV infection of kidney and spleen does not occur simultaneously [52]. In this study, sampling was performed when IHNV progression was expected to peak, i.e. between 5 and 14 days [16], but it is possible that a period of temporally induced IL-1β transcription in spleen took place before tissues were preserved for analysis. Transcript levels determined in anterior kidney sampled on days 20 and 60 show that IL-1β expression changes were, indeed, transient in this organ.

In accordance with results obtained in an earlier study [13], TGF-β up-regulation appears to be connected to inflammatory processes and thus, the transcription of other immune system mediators. Pesticide treatments, exposure to live IHNV or IHNV DNA vaccine, and parasitic infection have been shown to be among the factors altering TGF-β expression [13,18,53,54]. In this study, induction of IL-1β and Mx protein in kidney coincided with significantly increased TGF-β levels. In individuals where results from TCID50 assays and Mx protein expression suggested a cryptic infection, TGF-β transcription increases were more modest but nevertheless different from controls, supporting the notion that TGF-β functions as an immunosuppressive agent antagonizing pro-inflammatory events, possibly down-regulating expression of other cytokines and cytokine-induced effects in order to re-establish immune homeostasis [25,55,56]. Up-regulation of this gene was less pronounced than that of IL-1β or Mx protein, confirming results of other studies in fish [18] where only modest changes of TGF-β gene transcription were detected in response to treatments with IHNV DNA vaccine, poly I:C and a control plasmid, or injection of live IHNV. However, Lindenstrom et al. [53] observed a more rapid induction of TGF-β transcription in secondary parasitic infections, and it can be speculated, that the extent of the immune response determines TGF-β gene expression and its immunoregulatory function. Interestingly, significant alterations of TGF-β transcription were detected in spleen samples taken at the end (day 60) of this study. Both pesticide/IHNV treatment groups had significantly higher TGF-β transcript levels than groups exposed to solely a pesticide. When individual TGF-β levels in these groups were analyzed, it appeared that differences were mostly due to a few individuals with 3–4 times higher TGF-β gene expression than the average of all other fish. Consistent with results discussed above, these individuals also showed higher than average Mx protein transcript levels, indicating higher virus titers and a correlation between IHNV concentration and TGF-β gene expression.

While little is currently known about the function of IGF-1 in fish under physiological and pathophysiological conditions, our data demonstrate that both pesticides and IHNV can depress IGF-1 gene expression in spleen of juvenile salmon for an extended amount of time. Although less pronounced, decreased IGF-1 mRNA levels were also detected in kidney in response to both pesticides and virus. One of the prominent aspects of IGF-1 is its participation in the regulation of immunity and inflammation [57]. Regulation takes place mainly on an autocrine and paracrine level, and nearly all cells of the immune system are susceptible to the effects of IGF-1. Thus, this endocrine mediator induces hematopoiesis, lymphopoiesis, and direct effector functions of cells of the innate and acquired immune system, it increases pro-inflammatory cytokine production, and it has beneficial effects on survival in challenge experiments [57]. These functions have not been verified for the fish immune system, but structure and biological potency of fish IGF-1 are very similar to those of mammalian homologues [27]. Considering the important roles of IGF-1 in immune system development and as local growth and differentiation factor, such long-term reduction of IGF-1 may result in permanent alteration of immune functions and compromised immune responses.

Lowest IGF-1 gene expression was observed in spleen of fish with verified infection, and to a lesser extent in kidney and spleen of individuals subjected to virus but without confirmed disease. As discussed earlier, IGF-1 can prime the response of immunocompetent cells to deal with infection, and consequently trigger release of pro-inflammatory cytokines such as IL-1β, leading, in turn, to a decrease in circulating IGF-1 levels [57]. The detection of significantly lower levels of IGF-1 transcripts in IHNV-infected fish that showed strongly induced IL-1β gene expression suggests cytokine regulation of IGF-1 [57].
In conclusion, results from this study emphasize the high toxicity of EV to fish, and demonstrate that sublethal concentrations of this insecticide can lead to delayed mortality not reflected by acute toxicity tests. Elevated gene expression of the pro-inflammatory cytokine IL-1β indicates that adverse effects in response to EV exposure may elicit inflammatory processes. Juvenile Chinook salmon subjected to IHNV showed induced Mx protein transcription independent of pesticide treatment, supporting the notion of a non-specific antiviral protection mediated by type 1-IFN, and demonstrating the applicability and sensitivity of Mx protein as an indicator of virus exposure. Alterations in transcription of all cytokines investigated were greatest in infected fish, suggesting a correlation between severity of infection and expression of these genes. IGF-1 transcription in spleen decreased in response to both stressors over an extended period of time, and further studies will need to examine the consequences and potentially serious long-term effects on fish growth and development and their immune system. Changes of TGF-β gene expression were the least conclusive of all cytokine alterations observed in this study, however, highest transcript levels were found in fish where antiviral and inflammatory processes were indicated by the expression of other cytokines.

Acknowledgments

This study was supported by the CALFED Ecosystem Restoration Program, Project #99-N08. The authors would like to thank Nimbus Fish Hatchery for fish eggs, the Center for Aquatic Biology & Aquaculture and Paul Lutes for maintaining the fish, the Aquatic Toxicology Laboratory staff for sharing their facility, and CDFG for providing IHNV isolates. Thanks also to Gina Lee, the Aquatic Toxicology Laboratory staff for sharing their facility, and CDFG for providing IHNV isolates. Thanks also to Gina Lee, Terry McDowell and Susan Yun for help and guidance in the laboratory.

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