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Potential mechanisms in the early mortality of juvenile Chinook salmon exposed concurrently to Infectious Hematopoietic Necrosis Virus (IHNV) and esfenvalerate

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Replicate groups of juvenile Chinook salmon (*Oncorhynchus tshawytscha*) were exposed to infectious hematopoietic necrosis virus (IHNV), sub-lethal levels of esfenvalerate, or to both agents concurrently. A lethal synergistic effect of concurrent exposure to IHNV and esfenvalerate resulted in 24.1% mortality by 68 h post-virus exposure with no mortality observed in any other treatment groups at this time. Analyses of spleen samples from fish sampled at 68 h following exposure to both IHNV and esfenvalerate was suggestive of a disruption of transcription, and demonstrated a significant decrease in the production of two early, non-specific anti-viral genes (Mx-1 and Vig-8). Analyses of blood serum suggested that osmolality was not a contributing factor to the observed early mortality event. Examinations of stained sections of the gill and anterior kidney from fish in all treatment groups at 68 h did not reveal pathologic microscopic changes. This study suggests that the lethal synergistic effect of exposure to IHNV and esfenvalerate to juvenile Chinook salmon may be related to inhibited transcription of early, non-specific, anti-viral cytokines.

Key words: IHNV, esfenvalerate, synergy, Mx-1, Vig-8, Chinook salmon, pesticides

Exposure to chemical pollutants can disrupt normal physiologic and immunologic processes of fish resulting in greater mortality than exposure to microbial pathogens alone (Hetrick et al. 1979, Dunier 1996, Arkoosh et al. 1998, Clifford et al. 2005). The recent decline in wild salmon populations worldwide has been attributed to many anthropogenic activities including the introduction of agricultural pesticides to aquatic environments. The rivers and Delta region of California's Central Valley (USA) is one area where exposure to pesticides is believed to contribute to recent declines in fish populations (Moyle 1994, Hinton 1998).

California's Central Valley region is one of the most agriculturally productive areas in the world, but one unfortunate consequence is the inadvertent introduction of pesticides into aquatic environments (Hinton 1998, Werner et al. 2002, Weston et al. 2004). The Sacramento and San Joaquin rivers drain the Central Valley and join to form the Sacramento-San Joaquin Delta, which empties into the San Francisco Bay, forming the largest estuary on the west coast of North America. Historically, these waters supported enormous populations of Chinook salmon (*Oncorhynchus tshawytscha*) at an estimated 1–2 million spawning fish annually (Fisher 1994). However, all natural Chinook salmon populations have declined in California, some even to extinction (Fisher 1994).

Pesticides can disrupt immune system functions in fish (Dunier 1996, Banerjee 1999, Eder et al. 2004) and increase mortality when these fish are exposed to microbial pathogens (Clifford et al. 2005). Thus, exposure to Central Valley agricultural pesticides may be contributing to the decline of wild Chinook salmon populations in the Sacramento-San Joaquin Delta. The term "pollution" is used to describe the presence of agents that potentially exert a negative effect on ecosystems and can include hydrocarbons, heavy metals, and pesticides. The use of the word "pollutant" fails to recognize the vastly different effects compounds may, or may not have on fish immune function and overall fish health. Pesticides have many different modes of action affecting both target and non-target species (Miller and Adams 1982). Studies of definite immune effects from specific pesticides on target and non-target species are required to establish direct cause and effect relationships (Austin 1999). Depending on the microbial pathogen involved, immunosuppressive effects suffered from pesticide exposure may or may not influence the incidence and severity of infectious disease. In the current study, we examined in more detail certain features of the physiologic and immunologic effects observed among juvenile Chinook salmon concurrently exposed to sublethal levels of esfenvalerate and to infectious hematopoietic necrosis virus (IHNV). The combination of these two agents induces a reproducible and lethal synergism resulting in mortality by mechanisms of unknown cause (Clifford et al. 2005).

Esfenvalerate is a synthetic pyrethroid insecticide derived from compounds produced by certain species of chrysanthemum. All pyrethroids are neurotoxins that affect central nervous system function by disrupting normal voltage-dependent sodium and other ion channels (Miller and Adams 1982, Bradbury and Coats 1989, Burr and Ray 2004). Pyrethroids are used extensively on row crops, orchards, forest spray applications, and in urban areas for structural pest control and pet sprays (Haya 1989, Oros and Werner 2005). These insecticides can enter waterways via agricultural and urban runoff, spray drift, direct application for mosquito control and the release of agricultural tail waters (Oros and Werner 2005). Modeling results indicate that greater than 1% of total pyrethroids applied to agricultural lands alone may be available for transport through the California Delta and into the San Francisco Bay (Oros and Werner 2005). Pyrethroids were detected in 75% of sediment samples taken from small creeks and irrigation canals in the Central Valley of California (Weston et al. 2004) and winter precipitation events were reported to transport

esfenvalerate from agricultural lands to surface waters in February and March of 2003 (Bacey et al. 2005), a time when juvenile Chinook salmon are residing in Central Valley rivers and tributaries.

IHNV is a single-stranded negative-sense RNA virus of the family *Rhabdoviridae* and is a serious pathogen of many species of salmon and trout (Wolf 1988) and is listed as a "catastrophic disease" in the California Code of Regulations (California Code of Regulations, Title 14, Natural Resources, Division 1. Fish and Game Commission. Department of Fish and Wildlife, Section 245). IHNV is endemic to the Pacific Northwest of North America and has been reported in the Sacramento River since the 1940's (Wolf 1988). The virus is associated with significant mortality of hatchery-reared as well as wild salmon populations and the principal target tissues are the hematopoietic organs, the kidney and spleen (Wolf 1988, Drolet et al. 1994). Young fish are most susceptible and succumb to the disease due to kidney failure, anemia or fluid and electrolyte imbalances (Amend and Smith 1974, 1975; Williams and Amend 1976; Wolf 1988; Bootland and Leong 1999). IHNV alone induces mortality among young susceptible salmonids 5–10 days post exposure (dpe) and early survival is likely dependent on innate rather than acquired immune mechanisms (LaPatra 1998, Hattenberger-Baudouy et al. 1995, Cain et al. 1996, Purcell et al. 2004). The early innate immune response to IHNV infection includes increased transcription of two important type-1 interferon regulated genes, Mx-1 and Vig-8 (Purcell et al. 2006a, 2006b).

Horisberger et al. (1983) first reported Mx genes to provide mouse cells resistance to myxovirus infections. Homologues of Mx genes were later discovered and reported in rainbow trout (*Oncorhynchus mykiss*) (Staeheli et al. 1989, Trobridge and Leong 1995). Mx proteins block the transcription of viral mRNA (Caipang et al. 2003) and interfere with the transport of viral proteins to the site of viral assembly (Haller and Kochs 2002). Boudinot et al. (1999) first discovered Vig genes in rainbow trout cell lines infected with viral hemorrhagic septicemia virus (VHSV) and thus their designation as VHSV-induced genes (Vig). Vig genes are upregulated after viral infection and code for proteins that have characteristics of CXC chemokines (O'Farrell et al. 2002) that attract activated T-lymphocytes to the site of viral infection (Laing and Secombs 2004). Both Mx-1 and Vig-8 are transcribed in an early response to virus infection and type 1 interferon production, a vital step in the antiviral cascade (Congleton 1996). Significant increases in both Mx-1 and Vig-8 gene transcription result when rainbow trout and Chinook salmon are exposed to IHNV (Purcell et al. 2004).

Juvenile Chinook salmon undergo an early and unexplained mortality when concurrently exposed to sublethal levels of esfenvalerate and to IHNV (Clifford et al. 2005). In the study presented here, we examined potential causes of this mortality event including blood osmolality, microscopic pathological changes in the gill and anterior kidney and changes in the transcription of selected cytokines (Mx-1 and Vig-8) as indicators of physiologic, pathologic, or immunologic impairments resulting from the lethal synergism of esfenvalerate and IHNV.

Materials and Methods

Fish.—Fall-run Chinook salmon were obtained as fertilized eyed eggs and hatched at the Fish Health Laboratory (University of California, Davis) and maintained for experimental purposes. Fish were approved for research purposes under an Institutional

Animal Care and Use Protocol. Hatched fish were reared in fiberglass tanks receiving 12º C, aerated, single-pass well water. This is a temperature at which both IHNV and esfenvalerate demonstrate effects on fish (LaPatra 1998, Kumaraguru and Beamish 1981) and is within the normal range of wild juvenile salmon habitat. Fish received a commercial diet at approximately 3–5% body weight per day. At 12 weeks post-hatch, 14 fish were randomly selected for each of 4 replicates per treatment group (control, esfenvalerate only, IHNV only and esfenvalerate/IHNV). Fourteen fish were used per replicate to achieve an approximate ratio of 0.008 µg esfenvalerate per g of fish, which proved critical in preliminary studies regarding the propensity of esfenvalerate to be bound by organic material (see discussion section). Replicate groups were held in 16-L static, glass aquaria provided with aeration and partially submerged in 12º C chilled water baths as described elsewhere (Clifford et al. 2005). These aquaria provided environments in which fish could be exposed to nominal amounts of esfenvalerate and IHNV. Every 24 hours 75% of water and any uneaten food or fecal material was siphoned out of aquaria and replaced with fresh 12º C well water. The mean fork length and weight of fish was 4.85 cm and 0.96 g, respectively. During esfenvalerate exposures, fish in all treatment groups were not fed for ≥1 hour before water changes to minimize esfenvalerate binding to organic material in the tanks. Following esfenvalerate and IHNV exposure in static aquaria, all treatment groups were transferred to 15-L flow-through tanks receiving aerated, single pass 12º C well water. Fish were held in the 15-L flow-through tanks for the remainder of the experiment (21 days) and total cumulative mortality was recorded. Water temperature and appearance of fish were monitored one or more times per day.

Esfenvalerate exposures.—Fish transferred to 16-L static-system aquaria were held for 24 h, after which the first water change took place. Upon completing the first water change, the first esfenvalerate test and control treatments were administered to aquaria. Water changes and esfenvalerate dosing were conducted 4 times at 24-h intervals to complete a 96-h pesticide exposure time. Methanol served as the solvent for esfenvalerate and thus also for the control (non-pesticide) groups (10 ml per aquarium per dosage). Solid, crystalline and 98% pure esfenvalerate (ChemService, West Chester, PA, USA) was diluted in methanol to make a stock solution of 160.0 mg/L. Ten ml of this stock solution was added to the 16-L static-system aquaria for a final nominal concentration of 0.1 mg/L. Because 100% of esfenvalerate was assumed to breakdown or adsorb to glass after 24 h, 10.0 ml of stock solution was added after each subsequent water change to keep concentrations as close to 0.1 mg/L as possible for a period of 96 h. Composite water samples from all esfenvalerate groups were taken on the fourth day of pesticide dosing immediately after administration, and again 24 h later (just prior to water change) to record actual esfenvalerate concentrations in aquaria. These water samples were analyzed by the Fish Wildlife Water Pollution Control Laboratory of the California Department of Fish and Wildlife using gas chromatography with dual electron capture detectors, and with positive samples confirmed using gas chromatography mass spectrometry. Esfenvalerate concentrations were 0.06 µg/L at initial dosing and 0.02 µg/L 24 h later. After 96 h of pesticide exposure, water in all 16 L static-system aquaria was changed for 1 additional day, allowing fish to remain in clean and pesticide-free water after which all groups of fish were transferred to 15-L flow-through aquaria receiving 12º C well water supplied with aeration for the remainder of the experiments.

IHNV propagation.—IHNV was propagated in the CHSE-214 line as previously described (Clifford et al. 2005). The IHNV isolate used in this study originated from adult

winter-run Chinook salmon in the upper Sacramento River in July 2001 and was passed 4 times on the CHSE-214 cell line and is designated as Type Q from the L genogroup by Kelley et al. (2006). Virus in culture medium was cleared of cell debris by centrifugation at 1,300**g** for 10 min at 10º C. The resulting supernatant was kept on ice until used for the fish exposures. Because viral culture media (minimal essential media, MEM) contains organic material that can bind esfenvalerate, the virus was further purified by ultracentrifugation at 30,000**g** for 1 h at 10º C. The virus pellet was re-suspended in 4º C double-distilled water. Concentrations of virus used in the exposure studies were determined by plaque titration as previously described (Clifford et al. 2005).

IHNV Exposures.—Virus was added to the static-system aquaria during the daily water change beginning after the first 24 h of esfenvalerate exposure. Virus groups were exposed to 3.2 x 10^5 plaque forming units/ml/day while control groups received 37 ml of double distilled water. Groups were exposed to treatments for 1 h (at 12º C), after which aquaria were replenished to 16 L with fresh water and the toxicant or methanol control dosages resumed as described. All trials were conducted for 21 days to observe total cumulative mortality of non-sampled tanks.

Tissue sample timing and numbers.—Four replicates were used for each treatment group: two for obtaining tissue samples at a designated time point and two for observing total cumulative mortality. For baseline measurements, 10 fish were randomly selected from the original stock tank used to hold related juvenile Chinook salmon prior to selecting fish for replicate treatment and control groups. These fish were euthanized with an overdose of anesthetic (100 ppm benzocaine, no more than 3 fish at a time), and immediately weighed, measured and dissected aseptically to obtain blood, gill, anterior kidney and whole spleen samples. Fish were visually inspected during the procedure for external and/or internal signs of disease and observations were recorded. This same procedure was followed to obtain tissue samples from 10 fish in each replicate aquarium designated for sampling at 68 h post-virus exposure. This time point was chosen as it is just prior to the anticipated early mortality event (72 h post-virus exposure) induced by concurrent exposure to esfenvalerate and IHNV as reported by Clifford et al. (2005).

Total blood serum osmolality.—As described above, 10 fish from each replicate designated for tissue sampling for each treatment group, were euthanized at 68 h post-virus (or control) exposure. Immediately following euthanasia and recording weight and fork length, fish were bled by caudal severance and blood collected in 20 µl capillary tubes without heparin (Drummond Microcaps, Drummond Scientific Company, Broomall, PA, USA). Blood was centrifuged in a micro-hematocrit centrifuge (Clay Adams, Benton Dickson and Company, Parsippany, NJ, USA) for 15 min and serum collected in autoclaved 0.5 ml microcentrifuge tubes and frozen at -20º C until analyzed (approximately 1 week). All serum samples were thawed and analyzed within a 3-h period. Serum samples were centrifuged briefly to collect contents, kept on ice and then diluted in RNase-free autoclaved sterile water (Ultra Pure, USB Corporation, Cleveland, OH, USA) at a ratio of 2.5 µl serum to 7.5 µl water just prior to testing osmolality. The dilution was necessary as the vapor pressure osmometer (Vapro model 5520, Westcor Inc., Logan, Utah, USA) requires 10 µl of sample and many blood samples did not yield that volume of serum. In trial studies, serial dilutions of catfish serum and water demonstrated a consistent linear relationship in blood osmolality (M. Clifford, California Department of Fish and Wildlife [CDFW], unpublished data).

Histopathology.—A sagittal incision from posterior of the operculum to the base of dorsal fin was made on all euthanized fish. This section included both the gills and anterior kidney, while preserving the spleen for subsequent cytokine transcription analyses. The anterior portion of the fish was placed whole into Davidson's fixative for 24–48 h, after which the fixative was removed and replaced with 70% ethanol. Fixed samples were divided into two along a midsagittal plane and then processed by standard paraffin embedding. Tissue sections were affixed to microscope slides and stained with hemotoxylin and eosin.

Spleen sampling and preservation.—Individual spleen samples aseptically dissected from fish were placed directly into autoclaved 1.5-ml microcentrifuge tubes containing 350 µl of Buffer RLT (Qiagen, Valencia, CA, USA) with beta-mercaptoethanol. These spleen samples were kept on ice and subsequently frozen at -80º C within hours. Between each sampled fish, instruments were wiped, dipped in 70% ethanol, and flamed. Instruments used to dissect fish were changed frequently and always between replicate groups of fish, as were the anesthetic baths.

RNA/cDNA preparation.—Total RNA was extracted from spleen samples using RNeasy Minikit (Qiagen) following the manufacturer's instructions. Total RNA was frozen at -80º C for later synthesis into cDNA using the QuantiTect Reverse Transcription kit with DNase treatment (Qiagen), following the manufacturer's instructions.

Primer design.—Published primers for Mx-1 and Vig-8 in rainbow trout (Purcell et al. 2004) were used for amplifying sequences in Chinook salmon, due to the close taxonomic relationship of those species. To validate these primers in Chinook salmon, conventional PCR was performed using Platinum Taq DNA Polymerase (Invitrogen, Carlsbad, CA, USA) and a MJ Research PTC-200 thermocycler (Bio-Rad, Hercules, CA, USA) under the following conditions: 95º C for 3 min, then 40 cycles of 95º C for 30 s, 55º C for 1 min, 70º C for 1 min with a final extension step of 70º C for 10 min. Amplified products were separated by electrophoresis in 2% agarose gels and visualized by ethidium bromide staining. Resulting amplicons were single bands of correct size and were purified using QiaQuick PCR purification kit (Qiagen) and submitted for sequencing. Sequences were evaluated using computer applications from MacDNASIS (Mirai-Bio., Alameda, CA, USA), Amplify 1.2 (University of Wisconsin Genetics, WI, USA) and NCBI GenBank BLAST to verify correct amplicon and primer binding. Validated sequences were used for designing Mx-1 and Vig-8 primers for Chinook salmon. The primer sequences are: Mx-1 forward primer: 5'-GGG TAG CTG TCA AGG GTC AA-3', Mx-1 reverse primer: 5'-GTC CAC CTC TTG TGC CAT CT-3', Vig-8 forward primer: 5'-AGA AGC TCC ATT TGC CAA GA-3', and Vig-8 reverse primer: 5'-TTC ATT ATT TTC TTA ATG GTT TTC TGA-3'. These primers and resulting amplicons were validated with conventional PCR, sequencing, and software applications as described above with the exception that the annealing temperature was increased to 60° C. Degenerate primers for the ribosomal subunit S9 housekeeping gene were used for designing Chinook salmon S9 primers as described above with the exception that the PCR reactions were 94° C for 3 min, then 40 cycles of 94° C for 1 min 30 sec, 55° C for 30 sec and 70° C for 1 min.. Sequences for Chinook-specific S9 primers are: S9 forward primer: 5'-GAC AGC CAG AAG CAC ATT GA-3', and S9 reverse primer: 5'-TGG CGT TCT TTC TCT TGA CA-3'.

Real-time PCR.—Quantitative analysis of Mx-1, Vig-8 and S9 transcripts were completed with the ABI 7300 Real Time PCR system (Applied Biosystems, Foster City, CA, USA) using Quantitect SYBR Green PCR Master Mix (Qiagen) plus 0.5 units of

uracil DNA glycosylase (Invitrogen). All primer pairs were run in separate wells and in duplicate for all tissue samples with water as a control. Because the comparative C_T method ($\Delta\Delta C_T$ method) was used for quantifying gene transcription, primer efficiency curves were conducted using 10-fold serial dilutions of cDNA to assure equal priming ability of all primer pairs (Leutenegger et al. 1999). The Chinook salmon ribosomal subunit S9 housekeeping gene was used to normalize transcription of the immune genes by correcting for variation in reverse transcriptase efficiency, template quantity, or both. Transcription amounts were calibrated using the weakest transcription signal of one individual fish for Mx-1 or Vig-8 and all data was expressed as fold-increase transcription relative to this fish. Amplicons synthesized with real time PCR were verified by analysis of dissociation curves and by sequencing multiple wells for each primer set in duplicate using methods described above for conventional PCR. Infrequently, samples were excluded from comparative analysis wherever duplicate real time PCR reactions yielded a difference in cycle threshold crossing greater than 1.5 for either housekeeping or immune genes. This was true for no more than 1 fish in any given group.

Statistical analysis.—Statistical analysis was performed using Number Crunching Statistical Software (NCSS, Kaysville, UT, USA). Differences in mortality, mean day to death, serum osmolality and Mx-1 and Vig-8 transcription among all treatment groups were evaluated with GLM ANOVA with *P*-values ≤ 0.05 considered statistically significant.

Results

Mortality.—Dead fish were detected among groups of Chinook salmon exposed to both IHNV and to esfenvalerate by 68 h post virus exposure. Cumulative mortality among replicate groups ranged from 14.3% to 28.6% (Table 1). Mortality among fish exposed only to IHNV began 9 dpe and dead fish were detected subsequently up to day 21 when the experiment was terminated with an average mortality of 17.8 % (Table 1). There was

Treatment Group	Sampled at 68 h?	Replicate	Cumulative Mortality (%)	Mean Time to Death (days)
Control	no	A	0	NA
	no	B	0	NA
	yes	C	0	NA
	yes	D	0	NA
IHNV	no	A	21.4	14.6
	no	B	28.6	15.5
	yes	C	14.3	17.5
	yes	D	7.1	16.0
Es	no	A	0	NA
	no	B	0	NA
	yes	C	0	NA
	yes	D	0	NA
IHNV+Es	no	A	21.4	3.0
	no	B	14.3	3.0
	yes	C	28.6	3.0
	yes	D	21.4	3.0

Table 1.—Mean time to death (days) of juvenile Chinook salmon exposed to infectious hematopoietic necrosis virus (IHNV) only, esfenvalerate (Es) only, Es and IHNV concurrently, or to controls. Each replicate initially contained 14 fish, while 10 fish were removed from designated replicate groups for sampling at 68 hours post-virus exposure. Mortality on day 3 was recorded prior to sampling procedure and no other fish died that day. Groups that had no mortality have no mean time to death (NA).

nomortality among any other groups of fish, including unexposed control groups at any time during the study. Total cumulative mortality was not significantly different between groups of fish exposed to IHNV only, or to esfenvaletate and IHNV, though both these groups had higher mortality ($F_{3,16} = 17.49$; $P < 0.001$) than control groups or groups exposed to esfenvalerate alone. Mean day-to-death was lower ($F_{1,4} = 915.06$; $P<0.001$) in groups of fish exposed to both esfenvalerate and IHNV (3.0 d) as compared to groups exposed to IHNV alone (15.9 d).

Osmolality.—Fish sampled from the stock tank (time zero) had the highest serum osmolality with a mean of 88.0 mM/kg, the greatest value when compared to all treatment groups, including controls. The control groups had the next highest serum osmolality ($\bar{x}$ = 79.5 mM/kg), which was greater than groups exposed to esfenvalerate or IHNV alone. The remaining treatment groups (esfenvalerate only, IHNV only, and esfenvalerate/IHNV) had mean serum osmolalities of 70.1, 71.0 and 78.0 mM/kg, respectively (Table 2).

Treatment Group	Mean Serum Osmolality (mMoles/kg)
Stock tank	88.0
Control	79.2
IHNV	70.5
Es	69.5
Es + IHNV	76.9

Table 2.—Mean serum osmolality of juvenile Chinook salmon from stock tank and from replicate groups exposed to, infectious hematopoietic necrosis virus (IHNV) only, esfenvalerate (Es) only or to Es and IHNV concurrently. All fish were sampled at 68 h post-virus exposure except stock tank fish, which were sampled before any fish were allocated for replicate groups; control fish were not exposed to either virus or esfenvalerate.

Histopathology.—No obvious microscopic pathological changes were observed in the gill and anterior kidney in any experimental or control groups sampled at 68 h post virus exposure. Separation of the epithelial from supporting pillar cells was noted, but this occurred inconsistently and among all groups suggesting this was an artifact of the sampling procedures.

Mx-1 and Vig-8 transcription.—All replicate groups exposed to IHNV had elevated levels of Mx-1 transcripts, but only groups exposed to IHNV alone were significantly higher ($F_{4,43} = 6.69$; $P < 0.001$) than all other treatment groups (Table 3). Though transcription of Mx-1 was elevated in groups exposed to esfenvalerate and IHNV concurrently, it was not significantly greater than any other treatment group (Table 3). Similar to Mx-1, all groups exposed to IHNV had elevated levels of Vig-8, but only groups exposed to IHNV alone were significantly higher ($F_{4,42} = 4.15$; $P < 0.007$) than all other treatment groups including groups exposed to IHNV and esfenvalerate (Table 3). Vig-8 transcripts were elevated in esfenvalerate-only groups relative to stock tank fish, but not significantly (Table 3).

Table 3.—Mean-fold transcription of Mx-1 and Vig-8 (±*SE*) in juvenile Chinook salmon from stock and control tanks, and in replicate test groups: esfenvalerate (Es) only, infectious hematopoietic necrosis virus (IHNV) only, or IHNV and Es concurrently. Significantly different ($P < 0.05$) groups are designated with an asterisk.

	Treatment group				
Gene	Stock Tank	Control	Es only	IHNV only	Es + IHNV
Mx-1	20.2 ± 8.2	4.9 ± 0.8	5.0 ± 1.9	2,898.8 ± 1,018.4*	861.9 ± 183.9
Vig-8	10.0 ± 3.6	8.4 ± 2.7	79.4 ± 33.4	3,751.6 ± 1,675.6*	519.1 ± 233.7

Discussion

As demonstrated in prior studies, replicate groups of juvenile Chinook salmon exposed to both IHNV and to sub-lethal levels of esfenvalerate experienced an unusual and significant early mortality event not seen in any other treatment group (Clifford et al. 2005). Initial investigations of the causes of this early mortality in the current study suggest a potential role for an inhibition in the transcription of important anti-viral gene transcription in fish exposed to IHNV and esfenvalerate, compared to fish exposed to the virus or the pesticide alone.

Preliminary results in our lab demonstrated the amount of biomass per aquaria and the tendency of pyrethroids to adsorb to organic materials greatly affect the outcome of concurrent esfenvalerate and IHNV exposure (Clifford, CDFW, unpublished data). A nominal concentration of 0.00322 µg esfenvalerate per g of fish resulted in 0% mortality, but when increased to 0.00812 µg esfenvalerate per g of fish (by reducing amount of fish per replicate), the early mortality event was observed. Given the hydrophobic nature of pyrethroids, these values will most likely vary under different experimental conditions regarding the composition and surface area of tanks, the amount of suspended materials in water, methods of aeration, and any other surfaces in the experimental vessels. For example, toxic levels of a pyrethroid insecticide resulted in 100% mortality of rainbow trout by 24 h post exposure, but when water containing 15 mg/L suspended solids was used with the same pyrethroid concentrations there was 0% mortality observed (Hill 1985). Carefully accounting for these factors and using replicate groups, we have repeatedly witnessed the synergistic mortality event using different ages, weights and strains of fish, and different strains and concentrations of IHNV.

Investigating the potential causes of the early mortality following exposure of Chinook salmon to both esfenvalerate and IHNV did not indicate that disruption in osmoregulation was a contributing factor. Both pyrethroids and IHNV are reported to disrupt gill-ion regulation (Kumaraguru and Beamish 1981, Kumaraguru et al. 1982, Bradbury et al. 1987, Symonik et al. 1989, Congleton 1996), but in our trials no significant differences in blood osmolality were detected at 68 h between any of the experimental or control groups. This suggests at the sampling time (68 h) osmotic imbalances are unlikely to underlie the early synergistic mortality episodes that have been observed.

Apart from their primary neurotoxic effects, pyrethroids are reported to disrupt non-neural mechanisms, including gill and renal function, leading to lethal toxicity to fish species (Bradbury et al. 1987). Histopathological studies of gill surfaces in rainbow trout exposed to pyrethroids, including esfenvalerate, reported epithelial separation and necrosis, mucous cell hyperplasia, clubbing and fusion of secondary lamellae, and damage consistent with gill irritation (Kumaraguru et al. 1982, Bradbury et al. 1987). These results were reported whether pyrethroids were administered directly by aqueous exposure or indirectly through dietary intake. Histopathological examinations of fish in our studies did not reveal any obvious cellular changes to gill or anterior kidney. The absence of microscopic changes in our investigation compared to prior studies may be due to differences in experimental design. Kumaraguru et al. (1982) exposed fish to higher pyrethroid concentrations (0.09–35.0 µg/L) and exposed fish for 20–40 d before taking tissue samples. Bradbury et al. (1987) investigated acute lethal toxicity and exposed fish to very high concentrations of pyrethroids ($\bar{x}$ = 412 ± 50 µg/L) and performed extensive surgical procedures (spinal transection, catheritization, copper electrodes). The use of much lower concentrations of pyrethroid and the shorter exposure periods in our study are likely explanations for the lack of histopathological changes in the gill and anterior kidney in our study.

Exposure to IHNV is known to result in the transcription of many early, non-specific anti-viral cytokines including Mx-1 and Vig-8 (Purcell et al. 2004, 2006). While we observed this in all IHNV-exposed groups, concurrent esfenvalerate exposure resulted in significantly lower Mx-1 and Vig-8 transcription, suggesting an immune suppression or inhibition effect (Table 3). Eder et al. (2004) reported that exposure of juvenile Chinook salmon to 0.08 µg/L esfenvalerate led to a slight but significant decrease in Mx protein transcription to below basal levels, a trend consistent with our results (Table 3). Clifford et al. (2005) first reported the observed lethal synergy of juvenile Chinook salmon exposed to IHNV and esfenvalerate, an event occurring in the absence of detectable increases in virus replication above that of fish exposed only to IHNV. In our study, fish exposed to both IHNV and esfenvalerate demonstrated significant decrease in the transcription of type-1 interferon-regulated early non-specific anti-viral genes (Mx-1 and Vig-8), which may indicate a less adequate immune response to IHNV.

In the trials presented here, concurrent esfenvalerate and IHNV exposure resulted in a significant and rapid synergistic mortality event that may be linked to the disruption of the innate, early anti-viral response of juvenile Chinook salmon. Additional environmental stressors including water quality parameters (e.g., temperature) and co-contaminant or pathogen exposures would likely result in even greater losses of juvenile salmonid fish. Further studies of the observed lethal synergism are needed to define potential mechanisms involved. These include more sensitive molecular detection methods for virus (Purcell et al. 2006) and more comprehensive gene transcription analyses (von Schalburg et al. 2005, Purcell et al. 2006). Lastly, our study supports the importance and difficulty of taking into account the many variables required to establish lawfully acceptable concentrations of pesticides in aquatic environments.

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