

SYNERGISTIC EFFECTS OF ESFENVALERATE AND INFECTIOUS HEMATOPOIETIC NECROSIS VIRUS ON JUVENILE CHINOOK SALMON MORTALITY

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Abstract—Sublethal concentrations of pollutants may compromise fish, resulting in increased susceptibility to endemic pathogens. To test this hypothesis, juvenile chinook salmon (*Oncorhynchus tshawytscha*) were exposed to sublethal levels of esfenvalerate or chlorpyrifos either alone or concurrently with infectious hematopoietic necrosis virus (IHNV). Three trials were performed with fish exposed to concentrations of IHNV between 0.8×10^2 and 2.7×10^6 plaque-forming units/ml and to 5.0 $\mu\text{g/L}$ of chlorpyrifos or 0.1 $\mu\text{g/L}$ of esfenvalerate. The presence and concentration of IHNV in dead fish were assayed by virus isolation and plaque assay techniques, respectively. Among groups exposed to both esfenvalerate and IHNV, 83% experienced highly significant ($p < 0.001$) mortality, ranging from 20 to 90% at 3 d post-virus exposure, and cumulatively died from 2.4 to 7.7 d sooner than fish exposed to IHNV alone. This trend was not seen in any other treatment group. Virus assays of dead fish indicate a lethal synergism of esfenvalerate and IHNV. Chlorpyrifos had no observed effect on total mortality or IHNV susceptibility. The present results suggest that accepted levels of pollutants may be seemingly nonlethal to fish but, in fact, be acting synergistically with endemic pathogens to compromise survivorship of wild fish populations through immunologic or physiologic disruption.

Keywords—Salmon Esfenvalerate Infectious hematopoietic necrosis virus Pollution Synergy

INTRODUCTION

Fish concurrently exposed to sublethal concentrations of toxins and microbial pathogens may undergo higher mortality than fish exposed to pathogens alone [1]. The unprecedented worldwide decline of wild fisheries has prompted the study of specific pollutants and their impacts on fish health, including that of Pacific salmon [2,3]. Sublethal levels of toxins can disrupt the immune system and, thus, render fish more susceptible to disease [3–5], but studies that establish correlations between specific chemical pollutants and disease impacts on wild fish populations are needed [4].

Declines of anadromous salmonid populations have been attributed to many factors, including construction and operation of dams, loss of marshlands and estuaries, river-water allocation for cities and farms, overfishing, and pollution [6]. The delta region of California, USA, formed by the confluence of the San Joaquin and Sacramento rivers, is one area where fish populations have declined severely because of anthropogenic activities [7]. All natural chinook salmon (*Oncorhynchus tshawytscha*) populations have declined in California, some even to extinction [6], and are now provided access to only 20% of their historical spawning and rearing habitat in the Sacramento–San Joaquin watershed [8]. Even with population declines [8], annual chinook salmon commercial and recreational fishing continues to contribute \$43 million to California's economy (<http://www.dfg.ca.gov/mrd/fforum2004.html#salmon>), and substantial numbers of chinook salmon from the streams of California's Central Valley are caught in Oregon and Washington, USA, and in British Columbia, Canada [8]. Many toxicological and pathological studies of salmonid fish use rainbow trout (*O. mykiss*); however, because

chinook salmon represent a separate and equally important species, studies of these fish are necessary.

The loss of habitat as well as the exposure to agricultural and urban pollutants may be key factors affecting the performance and success of chinook salmon in California. A compromise of the immune system because of environmental pollution has been associated with an increased severity of disease in salmonids [3,9] and other fish species, as indicated by studies in the North Sea, where a greater prevalence of the viral disease lymphocystis was found among flounder (*Platichthys flesus*) from polluted compared to those from nonpolluted waters [4,10,11]. Many studies of fish disease and pollution, however, lack definitive data regarding the actual chemicals responsible or the concentrations of these pollutants [4]. In experimental challenge exposures with the marine bacterial pathogen *Vibrio (Listonella) anguillarum*, juvenile chinook salmon from a contaminated estuary were more susceptible to the pathogen than were salmon from a reference hatchery [3]. Similarly, juvenile rainbow trout exposed to sublethal levels of copper followed by waterborne challenge with infectious hematopoietic necrosis virus (IHNV) were twice as likely to die from IHNV infection compared to control trout exposed to the virus alone [12].

Infectious hematopoietic necrosis virus is the most important viral pathogen found among Pacific salmon and rainbow trout in the western United States and is the cause of significant losses of juveniles in hatcheries and trout farms [13,14]. The virus multiplies in the skin, gills [15], and/or gastrointestinal tract, eventually reaching the hematopoietic tissues of the kidney and spleen [13,16]. Young fish die because of anemia, kidney failure [17], or severe electrolyte and fluid imbalances [18,19]. Rarely have population-scale losses been reported as a result of IHNV infection [20], but significant juvenile mortality at a smaller scale likely can occur and go unrecognized

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in salmon migrating from freshwater to the marine environment, particularly when various stressors, such as environmental pollutants, compromise the immune response. Because juvenile salmon in the Sacramento and San Joaquin rivers can encounter pesticides during early life stages in freshwater, it is suspected that the combined negative impacts of pollutants and IHNV can reduce survivorship.

The pesticides chlorpyrifos (Cp) and esfenvalerate (Es) represent two of the more important groups of insecticides (organophosphates and pyrethroids, respectively) known to be present in the Sacramento–San Joaquin watershed and estuary [21,22]. For example, Cp was the primary toxicant in 87% of samples collected from the delta [22], and pyrethroid insecticides were detected in 75% of sediment samples collected from small creeks and irrigation canals in California's Central Valley [23]. Whereas organophosphates, such as Cp, generally are not acutely toxic to fish at environmentally realistic concentrations, the pyrethroid Es can be lethal to fish at concentrations in the nanomole-per-liter range [24]. Use of this pesticide is increasing in agriculture, in forest-spray applications, and for household purposes [24]. Esfenvalerate reaches waterways either by agricultural runoff, drift, or direct administration to bodies of water [24]. Storm-water runoff from Es-treated orchards in California was highly toxic to fathead minnows (*Pimephales promelas*) [25]. In the present experimental trials, the effects of concurrent exposures of chinook salmon to sublethal levels of Es or Cp and multiple doses of IHNV were examined.

MATERIALS AND METHODS

Fish

Three trials were conducted. Fish for trials 1 and 2 were from one group of fall-run chinook salmon obtained as fertilized eggs from the Nimbus Hatchery (Rancho Cordova, CA, USA), operated by the California Department of Fish and Game (CDFG). Eggs were transferred to the University of California Fish Health Containment Laboratory (Davis, CA, USA), where fry and juveniles were maintained throughout the experimental period. Juvenile fish in trial 3 were winter-run chinook salmon (four weeks posthatch) from a captive-breeding program at the Bodega Marine Laboratory (University of California–Davis, Bodega Bay, CA, USA) and obtained with a permit from the U.S. Fish and Wildlife Service and the CDFG. All fish were maintained in 8 to 10°C, single-pass well water, and fish used in all trials were acclimated to 12°C for use in experiments. Within the normal range of wild juvenile salmon habitat, 12°C is a temperature at which both IHNV and the pesticides examined in the present study demonstrate effects on fish [26,27]. The age and mean weight of fish in trials 1, 2, and 3 were six weeks and 0.38 g, 11 weeks and 0.42 g, and four weeks and 0.40 g, respectively.

Juvenile chinook salmon were randomly selected from 12°C stock tanks and placed in glass aquaria, each holding 16 L of well water supplied with aeration. This provided a static-system environment for the addition of known concentrations of both pesticide and IHNV. Two replicate groups of 30 fish were used for each treatment group. Aquaria were partially submerged in flow-through, chilling water baths to maintain a water temperature of 12°C. Approximately 75% of the water volume to each aquarium was replaced every 24 h. Dissolved oxygen, pH, and ammonia were checked daily. During pesticide exposures, fish in all treatment groups were fed only 1 h before water changes to minimize pesticide binding to or-

ganic material in the tanks. At all other times, fish were fed daily (~1.5–2.5% of body wt per day). Any uneaten food or fecal matter was removed by siphon during water changes. Following pesticide and virus exposures, all groups were transferred from 16-L static-system aquaria to 15-L flow-through tanks continuously receiving 12°C well water and supplemental aeration. Fish were held in the 15-L flow-through tanks for the remainder of experiments, and water temperature and appearance of fish were monitored one or more times per day.

Pesticide treatments

Fish transferred to 16-L static-system aquaria were held for 24 h, after which the first water change took place. On completing the first water change, the first pesticide and control treatments were administered to aquaria. Water changes and pesticide dosing were conducted four times at 24-h intervals to complete a 96-h pesticide exposure time. Methanol served as the solvent for all pesticides in our studies and for the control nonpesticide groups (10 ml per aquarium per dosage). Esfenvalerate was examined in all trials; Cp was used only in trials 1 and 2. Solid, crystalline Es (purity, 98%; ChemService, West Chester, PA, USA) was diluted in methanol to make a stock solution of 160.0 µg/L. Ten milliliters of this stock solution were added to the 16-L static-system aquaria for a final nominal concentration of 0.1 µg/L. Because 100% of Es was assumed to break down or adsorb to glass after 24 h, 10.0 ml of stock solution were added after each subsequent water change to keep concentrations as close to 0.1 µg/L as possible. In trials 2 and 3, water samples from Es groups were taken on the fourth day of pesticide dosing immediately after administration and again 24 h later (just before water change) to test the accuracy of Es concentrations in aquaria. These water samples were analyzed by the CDFG Fish and Wildlife Water Pollution Control Laboratory using gas chromatography and dual electron-capture detectors, with positive samples confirmed using gas chromatography–mass spectrometry (see *Results*).

Colorless, crystalline, solid Cp (purity, 99.5%; ChemService) was diluted in methanol to make a stock solution of 8,000 µg/L. Ten milliliters of this stock solution were added to the 16-L static-system aquaria for a final nominal concentration of 5.0 µg/L. Based on previous experiments, 75% of Cp in fish aquaria water was lost over a 24-h period. Considering that 25% of the dosed water was left in the aquaria during water changes, 9.4 ml of stock solution were added to the 16-L static-system aquaria after each subsequent water change to keep concentrations near 5.0 µg/L. In trial 2, water samples were taken from the Cp groups immediately after dosing on the fourth day of administration and again 24 h later to test for the accuracy of Cp concentrations in aquaria. As with Es, these samples were analyzed by the CDFG Wildlife Water Pollution Control Laboratory. On completing 96 h of pesticide exposures, water in all 16-L static-system aquaria was changed daily for an additional 2 d, allowing fish to remain in clean and pesticide-free water (to meet requirements of laboratory flow-through water discharge). All groups of fish were then transferred to the 15-L flow-through aquaria receiving 12°C well water supplied with aeration for the remainder of the experiments.

Virus and cell culture

The chinook salmon embryo 214 (CHSE-214) cell line [28] was used to propagate the IHNV isolates in the present study.

Determinations of virus concentrations were obtained by plaque-forming cell assay (plaque assays) using the epithelioma papulosum cyprini (EPC) cell line incubated at 15°C for 5 d [29]. Both cell lines were grown in minimal essential media (MEM) supplemented with 7.5% (v/v) fetal bovine serum, 50 IU/ml of penicillin, 50 µg/ml of streptomycin, and 2 mM L-glutamine [30]. The amount of fetal bovine serum added to the growth medium was reduced to 2% (v/v; MEM-2) for virus propagation and during plaque assays. The IHNV used in trials 1 and 2 (Nimbus lot 122, eighth passage) was isolated from adult fall-run chinook salmon at the CDFG Nimbus hatchery in January 1974. The IHNV isolate used in trial 3 (Winter 2001-114, third passage) was isolated from adult winter-run chinook salmon in the upper Sacramento River by the U.S. Fish and Wildlife Service (Anderson, CA, USA) in July 2001. The IHNV isolates in all trials were maintained as frozen stocks at -80°C. Virus used for fish exposures in trial 3 was concentrated by ultracentrifugation at 30,000 g for 1 h at 10°C. The virus pellet was resuspended in 50 ml of 4°C, double-distilled water and kept on ice for approximately 2 h before fish exposures took place. Before virus exposures, a small aliquot of the virus suspension was removed for determinations of the IHNV concentration by plaque assay.

Virus exposures

Virus was added to the 16-L static-system aquaria during the daily water change. This took place after 24 h of pesticide exposure. Virus groups in trial 1 were exposed to either a low dose (0.8×10^2 plaque-forming units [PFU]/ml) or high dose (8.2×10^3 PFU/ml); control groups received 4 ml of MEM-2 (same volume as IHNV high dose groups but without virus). Virus groups in trial 2 were exposed to either a low dose (1.2×10^5 PFU/ml) or a high dose (1.4×10^6 PFU/ml); control groups received 45 ml of MEM-2. Virus groups in trial 3 were exposed to either a low dose (6.2×10^4 PFU/ml) or a high dose (2.7×10^6 PFU/ml); control groups received 175 ml of double-distilled water. Groups in all trials were exposed at the specified virus concentrations for 1.3 h, after which the static-system aquaria were replenished to 16 L with freshwater and the toxicant or methanol control dosages resumed as described.

Fish were examined twice daily in both the static and flow-through system phases, and dead fish were removed, individually bagged, placed on ice, and either processed immediately for virus analysis or frozen at -80°C. Depending on size, either whole fish or fish with the head and tail removed were tested for the presence of IHNV using plaque assay techniques [31]. Virus concentrations in fish tissues were estimated by plaque assays run in duplicate with controls.

Statistical analysis

Statistical analysis of percentage cumulative mortality was conducted using analysis of variance with NCSS (Kaysville, UT, USA) and SAS (Cary, NC, USA) statistical software. Mean time to death was analyzed using NCSS software [32].

RESULTS

Trial 1

At 3 d postexposure (DPE), chinook salmon exposed to both Es and IHNV at the low or high virus dose experienced

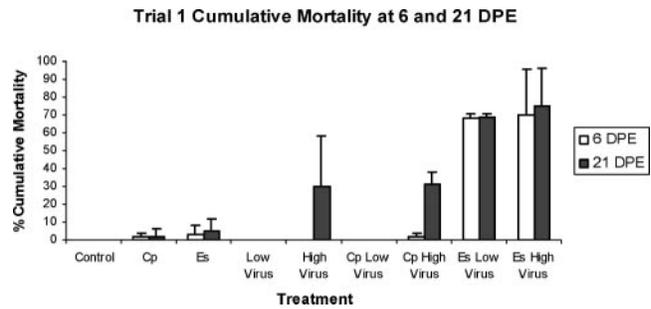


Fig. 1. Cumulative mortality among juvenile chinook salmon exposed to the pesticides chlorpyrifos (Cp) or esfenvalerate (Es) at 6 and 21 d following exposure to infectious hematopoietic necrosis virus at a low or high dose (0.8×10^2 and 8.2×10^3 plaque-forming units/ml, respectively) in trial 1. Error bars represent the standard deviation between replicates. DPE = days postexposure.

68.0 and 70.0% mortality, respectively. At this point, no mortality was observed in any other treatment group, with the exception of one dead fish in the Cp-with-high-virus-dose group and one in the Es-only group (1.6% mortality each). Cumulative mortality at 6 DPE was significantly ($p < 0.001$) higher in the Es-with-low-virus-dose group and the Es-with-high-virus-dose group than in all other groups (Fig. 1). At 21 DPE, cumulative mortality in the Es-with-high-virus-dose and Es-with-low-virus-dose groups was significantly higher ($p < 0.05$) than in all other groups but not significantly different ($p < 0.05$) from each other (Fig. 1). No significant ($p < 0.05$) differences were found in mortality of virus-dose-only groups compared to Cp-with-IHNV groups at 6 or 21 DPE (Fig. 1). Mean time to death was lowest in all groups exposed to Es and IHNV than in all other groups (Table 1). Fish in the Es-with-high-virus-dose group died 7.7 d sooner than groups exposed to high virus dose alone (Table 2). At 21 DPE, mean cumulative mortality in the Es-with-low-virus-dose group was 68.5%, whereas no fish died in groups exposed to low virus dose alone (Fig. 1). Of fish that died on 3 DPE, virus was isolated from only one fish in the Es-with-low-virus-dose group and from only four fish in the Es-with-high-virus-dose group. Virus was consistently isolated from IHNV-exposed fish

Table 1. Results from three separate trials showing mean time to death among groups of juvenile chinook salmon exposed to the pesticides chlorpyrifos (Cp) or esfenvalerate (Es) and infectious hematopoietic necrosis virus at a low^a or high^b dose

Treatment group	Mean time to death (d)		
	Trial 1	Trial 2	Trial 3
Control	None	None	12.5
Cp	8.5	10	NA ^c
Es	6.3	5.6	3
Low virus	None	10.2	9.9
High virus	11.1	9.4	8.9
Cp low virus	None	11	NA
Cp high virus	10.5	10.5	NA
Es low virus	3	4.9	5.3
Es high virus	3.4	10.6	6.5

^a Low doses in trials 1, 2, and 3 were 0.8×10^2 , 1.2×10^5 , and 6.2×10^4 plaque-forming units/ml, respectively.

^b High doses in trials 1, 2, and 3 were 8.2×10^3 , 1.4×10^6 , and 2.6×10^6 plaque-forming units/ml, respectively. Each exposure group consisted of two replicate groups of 30 juvenile chinook salmon.

^c NA = not applicable.

Table 2. Virus isolation and concentrations (titer^a) observed in the tissues of juvenile chinook salmon that died at 2 or 3 d post-virus exposure (DPE) in trial 3^a

DPE	Treatment group	IHNV detected	Titer (PFU/g tissue)
2	Es low virus	-	NA ^b
2	Es high virus	+	1.4×10^4
3	Es only	-	NA
3	Es only	-	NA
3	Es only	-	NA
3	Es only	-	NA
3	Es low virus	+	2×10^3
3	Es low virus	+	4×10^3
3	Es low virus	-	NA
3	Es low virus	-	NA
3	Es low virus	-	NA
3	Es low virus	-	NA
3	Es low virus	-	NA
3	Es low virus	-	NA
3	Es low virus	-	NA
3	Es low virus	-	NA
3	Es low virus	-	NA
3	Es high virus	-	NA
3	Es high virus	+	4×10^3
3	Es high virus	+	2.8×10^5
3	Es high virus	+	1×10^4
3	Es high virus	+	1×10^4
3	Es high virus	+	4×10^3
3	Es high virus	+	2×10^3
3	Es high virus	+	1×10^3

^a The titer of virus is given in plaque-forming units (PFU) per gram of tissue if infectious hematopoietic necrosis virus (IHNV) was detected. Fish were exposed to esfenvalerate (Es) alone or together with IHNV at a low or high virus dose. Low and high virus dose was 6.2×10^4 and 2.6×10^6 PFU/ml, respectively.

^b NA = not applicable.

that died beyond 6 DPE but never from fish in control groups not exposed to the virus.

Trial 2

Chinook salmon exposed to Es with low virus dose experienced 56.6% mortality at 3 DPE. This mortality event did not occur in any other group (Fig. 2), including the Es-with-high-virus-dose group. Cumulative mortality at 6 DPE was significantly higher ($p < 0.001$) for chinook salmon exposed to Es with low virus dose than in all other treatment groups (Fig. 2). Total cumulative mortality was determined at 21 DPE and was significantly greater ($p < 0.05$) in the Es-with-low-virus-dose group than in fish exposed to the low virus dose alone (Fig. 2). Cumulative mortality for chinook salmon in the

Trial 2 Cumulative Mortality at 6 and 21 DPE

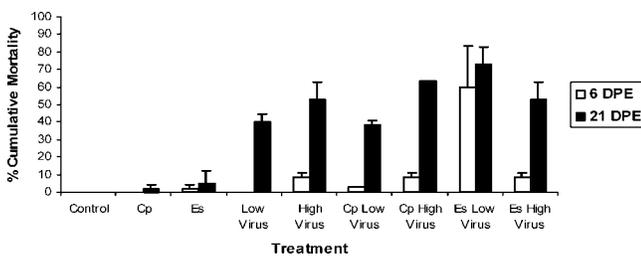


Fig. 2. Cumulative mortality among juvenile chinook salmon exposed to the pesticides chlorpyrifos (Cp) or esfenvalerate (Es) at 6 and 21 d following exposures to infectious hematopoietic necrosis virus at a low or high dose (1.2×10^5 and 1.4×10^6 plaque-forming units/ml, respectively) in trial 2. Error bars represent the standard deviation between replicates. DPE = days postexposure.

Trial 3 Cumulative Mortality at 6 and 21 DPE

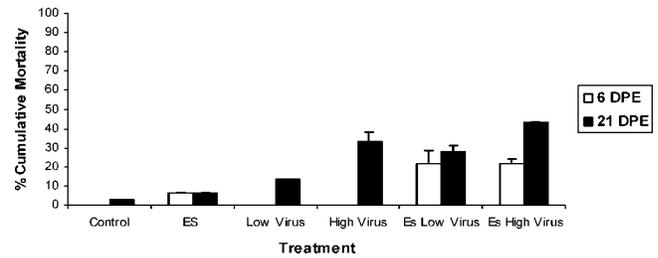


Fig. 3. Cumulative mortality among juvenile chinook salmon exposed to the pesticide esfenvalerate (Es) at 6 and 21 d following exposures to infectious hematopoietic necrosis virus at a low or high dose (6.2×10^4 and 2.6×10^6 plaque-forming units/ml, respectively) in trial 3. Error bars represent the standard deviation between replicates. DPE = days postexposure.

Es-with-high-virus-dose and the Cp-with-high-virus-dose groups were both higher than in groups exposed to the high virus dose alone, but the difference was not statistically significant at $p < 0.05$ (Fig. 2). Chinook salmon in the Es-with-low-virus-dose groups died an average of 5.3 d sooner than fish exposed to the low virus dose alone (Table 1). Of the fish that died at 3 DPE, virus was isolated from 7 of 16 fish tested. Virus was consistently isolated from IHNV-exposed fish that died at 4 DPE but never from fish in control groups not exposed to the virus. A composite water sample taken from Cp-treated tanks immediately after pesticide administration on the fourth day of pesticide treatment contained $3.750 \mu\text{g/L}$ of Cp (target concentration, $5.0 \mu\text{g/L}$), whereas a composite water sample taken 24 h later contained $1.100 \mu\text{g/L}$ Cp. A composite water sample taken from Es-treated tanks immediately after pesticide administration on the fourth day of pesticide treatment contained $0.079 \mu\text{g/L}$ of Es (target concentration, $0.1 \mu\text{g/L}$). Water samples also were taken 24 h later from one replicate tank of the Es-with-low-virus-dose treatment and from one replicate tank of the Es-with-high-virus-dose treatment. The Es-with-low-virus-dose group contained $0.075 \mu\text{g/L}$ of Es, and the Es-with-high-virus-dose group contained $0.038 \mu\text{g/L}$ of Es.

Trial 3

At 3 DPE, chinook salmon exposed to Es with low virus dose and ES with high virus dose experienced 20.0 and 21.6% mortality, respectively. This mortality event was not seen in any other treatment group (Fig. 3). Cumulative mortality at 6 DPE was significantly higher ($p < 0.001$) in the Es-with-low-virus-dose and Es-with-high-virus-dose groups than in all other groups, but these two groups were not significantly different from each other (Fig. 3). Total mortality was determined at 21 DPE (Fig. 3), and although the two Es-with-IHNV groups experienced higher mortality than groups exposed to virus alone, they were not significantly different at $p < 0.05$. However, chinook salmon exposed to both Es and IHNV at the high or low virus dose died sooner (2.4 and 4.6 d, respectively) than fish exposed to virus only (Table 1). Mean time to death was lowest in the Es-only group, although this group only experienced 6.7% total mortality (Table 1 and Fig. 3). Of fish that died at 2 or 3 DPE, virus was isolated from 30.0% in the Es-with-low-virus-dose group and from 89.0% in the Es-with-high-virus-dose group. Concentrations of virus isolated from these dead fish ranged from 1.0×10^3 to 2.8×10^5 PFU/g tissue (Table 2). Virus was consistently isolated from IHNV-exposed fish that died after 5 DPE but never from fish in control

groups not exposed to the virus. A composite water sample taken from Es-treated tanks immediately after pesticide administration on the fourth day of pesticide treatment contained 0.080 $\mu\text{g/L}$ of Es (target concentration, 0.1 $\mu\text{g/L}$). A composite water sample taken 24 h later from tanks treated with Es and IHNV contained 0.040 $\mu\text{g/L}$ of Es.

DISCUSSION

In the three experimental trials described, concurrent exposure of juvenile chinook salmon to Es and IHNV induced significant and earlier onset of mortality than occurred from IHNV infection alone. The early death of fish in the Es-with-IHNV groups was an unexpected finding and suggests a synergistic effect between pathogen and toxicant. The mechanisms underlying this early mortality are unknown, but the effect was reproduced in three separate trials. No such effect was found on juvenile chinook salmon exposed to Cp with IHNV, which suggests that under the experimental conditions of the present study, these two toxicants interact differently with the host and the pathogen.

The early and significant mortality of chinook salmon exposed to Es and IHNV contrasts to the more normal time to death of 5 to 14 DPE when fish are exposed to IHNV alone at water temperatures of 12°C [13]. Results of virus isolations from fish experiencing early mortality in the Es-with-IHNV groups were inconsistent, and observed concentrations of virus were lower (mean, 3.3×10^4 PFU/g tissue) than those in fish exposed to virus alone that died at 5 DPE or later (mean, 4.1×10^5 PFU g/tissue). In trial 3, positive virus isolations were more frequent from dead fish at 3 DPE in the Es-with-high-virus-dose group than from those in the Es-with-low-virus-dose group (Table 2). This suggests that virus dose influences the ability to isolate virus from dead fish at these early time points. The inability to isolate virus consistently at the lower concentrations of virus indicates that the observed mortality may not be caused by the more rapid replication and spread of the virus in fish but are equally likely to be caused by separate effects of exposure to Es.

Esfenvalerate is a synthetic pyrethroid insecticide that disrupts nervous system function by interfering with sodium channels [33]. Symptoms associated with lethal administration of Es to fish also suggest effects on respiratory surfaces and renal system functions [24]. Rainbow trout experienced hyperplasia, increased mucus production, epithelial necrosis, epithelial separation, and fusion of gill lamellae when exposed to another synthetic pyrethroid, permethrin [34]. When exposed to fenvalerate, a related synthetic pyrethroid, rainbow trout experienced disrupted ion regulation, decreased heart rate, and increased oxygen consumption, cough rate, and ventilation volume [34,35]. The combined respiratory, metabolic, and renal disruption from Es exposure may have been sufficient predisposing factors to allow relatively low concentrations of IHNV to further compromise gill and renal functions, with the resultant mortality occurring without IHNV concentrations reaching those expected in fish dying solely of IHNV infection [13,19]. Although not assessed, the release of corticosteroids and catecholamines because of the combined stressors (Es and IHNV) may have resulted in increased metabolic rate, decreased osmotic and ionic stability, and decreased glycogen, lipid, and body protein reserves sufficient to induce metabolic exhaustion [36]. Decreased energy availability because of IHNV-infected fish refraining from taking food [13] could further worsen the toxicity of Es, because protein deficiency, in

addition to stressors, increases the toxic effects of pesticides [37]. Exposure to Es may have compromised early, nonspecific, antiviral immune mechanisms of fish in the present study, resulting in the unexpected early mortality. These immune mechanisms are important in resisting IHNV, as fish resist or succumb to the virus weeks before specific antibodies are detected [19, 38]. This idea is further supported by the observation that rainbow trout exposed to IHNV respond by expressing a suite of early, nonspecific immunogenes as early as 1 DPE [39].

The reduced bioavailability of Es in trial 2 may have been responsible for the difference in survivability at 3 DPE in the Es-with-low-virus-dose group compared to the Es-with-high-virus-dose group. The principal difference between these two groups, other than virus concentration, was the amount of culture medium added to the tanks (4 ml in the low dose and 46 ml in the high dose), which contained not only residual cell debris from virus-infected cells but also 2% fetal bovine serum plus other essential nutrients for *in vitro* growth of fish cells. Because Es binds organic material [24], the high amount of organic material present in the virus culture medium likely bound the Es, thus reducing its bioavailability to the fish. In support of this hypothesis, studies reported by the National Research Council of Canada found fenvalerate to be 40-fold less toxic to channel catfish when premixed with suspended solids before administration to test water [24], and toxic levels of cypermethrin that resulted in 100% mortality in control tests resulted in 0% mortality when using aquaria water containing 15 mg/L of suspended solids [40]. In trial 2, the concentrations of Es in water samples taken 24 h after dosage from the Es-with-low-virus-dose and Es-with-high-virus-dose groups were 0.075 and 0.038 $\mu\text{g/L}$, respectively. These differences in Es concentration may explain, in part, the different mortality experienced by the two groups. In trial 3, the Es-with-IHNV groups received virus that was partially purified from the culture medium and, thus, should have had reduced amounts of organic material. In contrast to trial 2, the early and significant mortality in trial 3 was observed in both the Es with high-virus-dose and ES with low-virus-dose groups.

Several factors must be considered when evaluating the effects of both IHNV and Es on juvenile chinook salmon. Fish age is critical, because fish younger than six months of age are highly susceptible to the virus whereas older fish are generally more resistant [26]. Other key factors influencing mortality from IHNV infections include the water temperature, the strain of virus, and the species and genetic background of the salmonid host [26]. In contrast to IHNV, Es is reported to have equal lethality across fish species [24], but similar to IHNV, it is affected by water temperature [27]. Despite the many variables that affect Es- or IHNV-induced mortality, all three trials demonstrated accelerated and significant mortality in our laboratory when fish were exposed to both agents, despite using two different isolates of IHNV, three different ages of fish, and two different races of juvenile chinook salmon. Laboratory conditions (e.g., holding fish in aquaria, transferring fish from one aquarium to another, feeding) may influence the effects of the experimental parameters of the present study; therefore, studies of fish exposed to IHNV and the pesticides of the present study in the natural habitat should be conducted. Fish are often exposed to multiple pathogens concurrently, and polluted waters often contain more than one toxicant. For this reason, laboratory studies that limit experimental parameters,

although providing necessary data and minimizing other variables, seldom mimic exactly the natural aquatic environment.

In contrast to Es, the concurrent exposures of juvenile chinook salmon to IHNV and sublethal concentrations of Cp did not significantly ($p < 0.05$) affect total mortality or mean days to death in trials 1 and 2; therefore, this toxicant was not included in trial 3. It is possible, however, that higher concentrations of Cp would have an observable immunosuppressive or immunostimulatory effect on fish, as previously described [9]. Juvenile chinook salmon in the present study were exposed to sublethal levels of Cp for only 96 h, but it has been demonstrated that chinook salmon bioaccumulate toxic compounds during residence in polluted estuaries, which can result in increased susceptibility to endemic pathogens [3]. Chlorpyrifos has a propensity for leaving the aqueous phase and binding to organic material and accumulating in aquatic sediments because of its nonpolar nature [41]. However, despite a significant increase in expression of stress proteins, bioaccumulation in fathead minnow (*Aphanius iberus*) was not observed when the fish were exposed to Cp in a food-chain study [42].

In conclusion, the present study demonstrates that sublethal levels of Es operate by unknown mechanisms to significantly increase mortality following IHNV exposures of juvenile chinook salmon. Studies that examine the combined effects of Es and IHNV on immune gene transcription, respiratory epithelial functions, metabolic demand, serum electrolyte levels, and additional renal parameters may provide insights regarding the mechanisms involved in the mortality observed. The synergistic effect on mortality in chinook salmon in the present study might be observed using other pyrethroid insecticides, other fish species, or other fish pathogens. Lastly, the present study demonstrates the need to define acceptable concentrations of pollutants in the environment that consider potential synergisms between sublethal doses of toxicant and other environmental stressors, including endemic pathogens.

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