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Cumulative Effects of Natural and Anthropogenic Stress on Immune Function and Disease Resistance in Juvenile Chinook Salmon

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Abstract.—Previous studies have shown that juvenile chinook salmon *Oncorhynchus tshawytscha* exposed in the field or the laboratory to polychlorinated biphenyls (PCBs), an anthropogenic stressor, are immunosuppressed. It is not known whether simultaneous exposure to natural stressors can increase this immunosuppression. To examine the effects of natural and anthropogenic stressors on immune function, we infected juvenile chinook salmon with metacercariae of the trematode *Nanophyetus salmincola* by exposing the fish to infected freshwater snails *Juga plicifera*. Infected (>300 metacercariae per fish) and noninfected salmon were then injected with either the commercial PCB mixture Aroclor 1254 or an acetone–emulphor carrier. B cell function was examined by in vitro hemolytic plaque-forming cell (PFC) assay. *Nanophyetus salmincola* infection resulted in significantly lower anterior kidney primary PFCs and lower splenic secondary PFCs. The combination of *N. salmincola* infection and Aroclor 1254 exposure caused a lower anterior kidney primary PFC response than did either stressor alone. The immune function of juvenile chinook salmon was also measured by challenging them with the marine bacterium *Listonella anguillarum* (formerly known as *Vibrio anguillarum*). Fish infected with *N. salmincola* had higher mortalities than noninfected fish when challenged with *L. anguillarum*. These experiments demonstrated that *N. salmincola* infection in juvenile chinook salmon can impair immune function and disease resistance. The findings also show that in combination these natural and anthropogenic stressors can have a greater negative effect on salmon health than either stressor alone.

Environmental stressors can influence host–parasite interactions and cause disease outbreaks in fish (Wedemeyer 1970; Snieszko 1974; Pickering 1993). However, a parasitic infection can also be a stressor. The cestode *Eubothrium salvelini* compromises the ability of yearling sockeye salmon *Oncorhynchus nerka* to adapt to saltwater (Boyce and Clarke 1983), as does the gill

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parasite *Ichthyobodo necator* in chum salmon *O. keta* (Urawa 1993). Parasitic infections can also increase the susceptibility of fish to the toxic effects of heavy metals, such as cadmium chloride (Pascoe and Cram 1977), zinc (Boyce and Yamada 1977), and petroleum hydrocarbons (Moles 1980).

A single stressor can be debilitating if it is sufficient in magnitude or duration. Similarly, multiple stressors, even at low magnitudes, can be debilitating due to the cumulative effects of stress. Juvenile anadromous salmon encounter a cascade of natural and anthropogenic stressors, both acute and chronic, during their first year of life. The combined effects of these stressors may ultimately result in impaired growth, reproduction, and survival, ultimately affecting populations.

Studies of multiple-stressor effects on juvenile salmon could lead to better estimates of natural mortality. A number of studies have examined the effects of infection by *Renibacterium salmoninarum* (the causative agent of bacterial kidney disease) in juvenile salmon in combination with various acute to moderate stressors such as hypoxia, agitation (Mesa et al. 2000), smoltification (Moles 1997; Mesa et al. 1999), and dissolved gas supersaturation (Weiland et al. 1999). Also, Harrahy (2001) examined the effects of multiple acute stressors, such as handling and elevated acclimation temperature, on juvenile salmon. Although these studies provide important information on some cumulative effects, they demonstrate the need to address the potential cumulative effects of additional stressors on juvenile salmon.

Polychlorinated biphenyls (PCBs) are highly persistent contaminants that are resistant to degradation, have strong lipophilic properties, and can be bioaccumulated by teleosts (reviewed by Safe 1990; Wang 1998). McCain et al. (1990) reported that juvenile chinook salmon *O. tshawytscha* from an urban estuary in Washington State are exposed to both PCBs and polycyclic aromatic hydrocarbons (PAHs). Arkoosh et al. (1991) found that the B cell response in juvenile chinook salmon from the same urban estuary was lower than that of cohorts from a nonurban estuary and those from the hatcheries that released into those systems. Immunosuppression of B cell function and increased susceptibility to infections resulting from exposure to PCBs and PAHs were later confirmed by exposing chinook salmon smolts in the laboratory to commercial forms of these contaminants (Arkoosh et al. 1994, 2001). The commercial PCB mixture Aroclor 1254 has been used to demonstrate the immunotoxic effects of PCBs in salmon (Arkoosh et al. 1994) and other teleosts (Thuvander and Carlstein 1991; Sweet et al. 1998).

The trematode *Nanophyetus salmincola* is a parasite that uses salmonids as a second intermediate host, remaining in them throughout the life of the fish until ultimately maturing in piscivorous birds and mammals (Bennington and Pratt 1960; Millemann and Knapp 1970a). The distribution of the parasite extends from northern California to the Olympic Peninsula of Washington State (Simms et al. 1931). Cercariae of the parasite penetrate juvenile salmon in freshwater after emerging from their snail host. The metacercariae of *N. salmincola* can encyst in gills, muscle, and the organs but predominate in the posterior kidney (reviewed in Millemann and Knapp 1970b). *Nanophyetus salmincola* can be lethal to salmon fry as a result of tissue destruction caused by the penetration and migration of the cercariae (Baldwin et al. 1967).

The objective of this study was to examine the combined effects of an anthropogenic stressor (PCBs) and a natural stressor (infection with the trematode *N. salmincola*) on immune function and disease resistance in juvenile chinook salmon.

Methods

Parasites.—Freshwater snails *Juga plicifera*, the first intermediate host of *N. salmincola*, were collected from Big Elk Creek and the Siletz River, Oregon, in June and July of 1997. To screen for parasitic infections, the snails were placed in individual 2-mm petri dishes with a piece of lettuce and dechlorinated city water at 28°C. The covered petri dishes were left overnight at room temperature and checked the following morning for released cercariae with a dissection microscope at 100× magnification. Snails releasing only cercariae of *N. salmincola* were maintained in the laboratory in rectangular 0.37-m^3 flow-through tanks with dechlorinated freshwater and fed lettuce weekly.

Fish.—Juvenile fall chinook salmon were raised by the U.S. Fish and Wildlife Service, Abernathy Fish Technology Center, Longview, Washington. Fish were transported in July 1997 in an aerated 0.93-m^3 transport tank to the fish disease laboratory at the Hatfield Marine Science Center in Newport, Oregon. Following transport, fish were maintained in flow-through dechlorinated freshwater and allowed to acclimate for 3 weeks prior to any further handling. Fish were fed 3% of their body weight daily with BioDiet Grower pelletized feed (Bio-Oregon, Inc., Warrenton, Oregon).

Experimental infection with Nanophyetus salmincola.—Fish (mean weight, 9.6 g; mean length, 100 mm) were exposed to infected snails in rectangular 0.37-m^3 flow-through tanks covered with screens and black plastic in which slits had been cut for air exchange. Dechlorinated freshwater with a flow rate of 2 L/min was maintained at 9–11°C by means of a standard aquarium heater. Fish used in the immune function assays were exposed to 120 infected snails in one of these tanks for 3 weeks. The mean intensity of metacercariae in the posterior kidney from a sample of seven exposed fish was 430 (range, 354–543). Fish used in the disease challenge experiments were exposed to infected snails in two of four identical tanks. Each exposure tank held 180–200 fish and 60 infected snails, while tanks with 180–200 fish and no snails were used as controls. Fish to be used in the disease challenge were removed from the tanks of infected snails after 6 weeks of cohabitation. The mean number of metacercariae in the posterior kidney from a sample of seven of these fish was 394 (range, 257–504). Fish were transferred to 100-L circular tanks and acclimated to full-strength seawater over 7 d. These fish remained in seawater (10–11°C) for an additional 3 weeks prior to further treatment.

Contaminant exposure.—Three weeks after transfer to full-strength seawater, the infected and noninfected juvenile chinook salmon were transferred to 330-L tanks. After a minimum of 7 d in the new tanks, fish were anesthetized with 1.5 mg/mL metomidate and injected intraperitoneally with either the commercial PCB mixture Aroclor 1254 (AccuStandard, Inc., New Haven, Connecticut) in a 1:1 acetone–emulphor solution or with the acetone–emulphor carrier alone (control). The PCB dose (54 mg/kg wet weight) was 20% of the dose previously determined by Arkoosh et al. (1994) to be lethal to 50% of their test specimens within 96 h.

Water treatment.—All incoming seawater was sand-filtered and UV treated. Before the effluent was released into Yaquina Bay, Oregon, it was treated with charcoal to remove organic contaminants and chlorine to prevent the introduction of pathogens into the estuary.

Immune function analysis.—Ten days after injection with the contaminant or carrier, the fish were immunized by intraperitoneal injection with 100 mg trinitrophenyl-keyhole limpet hemocyanin (TNP-KLH) emulsified (50:50) in Freund's Complete Adjuvant (FCA; Sigma Chemical Co., St. Louis, Missouri). Control fish were injected with a mixture of phosphate-buffered saline (PBS) and FCA. Eight groups of fish resulted from these treatments: (1) fish that were infected with *N. salmincola*, injected with Aroclor 1254, and immunized; (2) fish that were infected with *N. salmincola*, injected with Aroclor 1254, and not immunized; (3) fish that were infected with *N. salmincola*, injected with the acetone–emulphor carrier, and immunized; (4) fish that were infected with *N. salmincola*, injected with the acetone–emulphor carrier, and not immunized; and (5)–(8) fish that were subjected to the above four treatments but not exposed to *N. salmincola*.

Cell culture and plaque-forming cell (PFC) assay.—To measure the in vitro primary and secondary PFC response to TNP, spleen cells and anterior kidney cells were removed from fish 9 weeks after injection with either TNP-KLH or PBS. Fish were euthanatized by a lethal dose (200 mg/L) of tricaine methanesulfonate (MS-222; Sigma) in a container with 4 L of seawater and were weighed and measured (fork length) for determination of condition factor. Fish were bled by removal of the caudal peduncle to remove circulating red blood cells. The spleen and anterior kidney from each fish were removed using sterile techniques. Each organ was placed into 2 mL of chilled RPMI-1640 cell medium (Gibco Laboratories, Grand Island, New York) and maintained on ice. The organs were homogenized gently with sterile 1-mL tuberculin syringes to obtain single-cell suspensions. These cell suspensions were maintained on ice until all large particulate matter had settled to the bottom of the tubes. The cell suspensions were then transferred to new centrifuge tubes and washed twice at 4°C in complete RPMI-1640 (Kaattari et al. 1986). Leukocytes were counted with a hemocytometer and viability determined by trypan blue exclusion. Cells were adjusted to a final concentration of 2×10^7 cells/mL. In vitro PFC assays followed the procedures of Kaattari et al. (1986). In brief, 50 μL of the cell suspension was dispensed in triplicate with different concentrations (0.4, 4.0, or 40.0 μg/mL) of the T-cell-independent antigen TNP-lipopolysaccharide (TNP-LPS) in complete RPMI-1640 medium or in medium without TNP-LPS in flat-bottomed 96-well tissue culture plates. These tissue culture plates were placed in a CO_2 (10%) incubator at 17°C for 13 d. Cells were fed on alternate days with 10 μL of a nutritional supplement (Kaattari et al. 1986).

On the 13th day of incubation, a modified Jerne plaque assay (Cunningham and Szenberg 1968) was performed to measure the primary and sec-

ondary PFC responses of spleen and anterior kidney cells. The 96-well plates were centrifuged, and the supernatant was removed from each well. Cells were suspended with a 1:50 dilution of coho salmon *O. kisutch* sera, as a source of complement, and sheep red blood cells conjugated to TNP (Henry 1980). This cell suspension was then incubated in Cunningham slides at 17°C in an open-atmosphere incubator for 2–3 h. Plaques were counted under a dissection microscope at 100× magnification immediately following incubation.

Disease challenge with Listonella anguillarum (formerly known as *Vibrio anguillarum*).—A frozen 2-mL aliquot of *L. anguillarum* (strain 1575) was thawed, diluted with 500 mL of trypticase soy broth supplemented with 0.5% NaCl, and placed on an environmental shaker. Dilutions of the bacterial culture were made following 18 h of incubation at 20°C. The peak of the exponential growth of the bacterial culture was determined as an optical density between 1.7 and 1.9 at 525 nm (Arkoosh et al. 1998).

Fish were separated into replicate groups of 16–19 and exposed to 1×10^5 bacteria/mL in 4 L of aerated seawater for 1 h in 7-L buckets. The sample sizes of the exposure groups in this experiment were unequal due to the lower survival of fish infected with *N. salmincola* during the infection and smoltification periods (the differential survival was not significant). Control groups of fish were also placed in 4 L of aerated seawater without *L. anguillarum*. Following bacterial challenge, fish were placed in 100-L tanks with flow-through, sand-filtered, and UV-sterilized pathogen-free seawater. Fish were not fed on the day of or immediately following bacterial exposure. Tanks were checked and mortalities removed twice daily.

Statistical analyses.—A three-factor analysis of variance (ANOVA) using parasite (two levels), contaminant (two levels), and immunization (two levels) was performed on square-root-transformed PFC data to measure the significance between main factor effects. The square root transformation was used because the raw data were not normally distributed and the group variances were proportional to the means (Zar 1999). Data from the two organs (spleen and kidney) were analyzed separately. The different in vitro TNP-LPS antigen concentration levels (0.4, 4.0, and 40.0 μg/mL) were first included within the ANOVAs as a main effect. Antigen concentration was a significant factor only for splenic PFCs of unimmunized fish (data not shown), and within this ANOVA there was no interaction effect between antigen concentration and the other factors. For this reason, antigen concentration was not included as a factor in subsequent ANOVAs. Fisher's protected-least-significant-difference (PLSD) test was used to determine the significance between treatment groups. Results were deemed to be statistically significant at $P < 0.05$.

Generalized linear modeling was used to test for statistical significance between treatments in the *L. anguillarum* challenge experiments (Arkoosh et al. 1998). We assumed that the number of survivors and mortalities followed a binomial distribution (Hilden and Hirvi 1987; Kerr and Meador 1996). The analyses were performed with the GLIMStat computer application (Beath 1995). The logistic generalized linear model was used for these analyses; for this model, the error structure was binomial and the linear predictor was related to the expected value of the datum by the logit link function.

Measuring the effects of combined stressors.—We used models with additive, multiplicative, and simple comparative effects to qualitatively examine the potential cumulative effects of parasitic infection and contaminant exposure on immune function. These models are described in detail by Folt et al. (1999). Reductions in the mean PFC responses in fish exposed to stressors singly and in combination were calculated as the percent differences from the control responses. The predicted value for the simple-comparative-effects model was the percentage response of the single stressor that induced the lowest effect (or greatest suppression) relative to its control. For the additive-effects model, the predicted value was the sum of the observed percentage reductions from the individual stressors. For the multiplicative-effects model, the predicted value was the product of the observed percentage reductions from the individual stressors. The observed values were then compared with the predicted values to determine which model best fit the observed data.

The significance of model outcomes comparing predicted and observed effects on PFCs were assessed using Mann–Whitney *U*-tests to measure differences between individual- and combined-stressor treatments. To determine whether the combined effects were additive, we randomly paired the differences (control minus treatment) from individual stressors to obtain the mean difference and variance that would be expected from the summation of the separate effects of *N. salmincola* infection alone and Aroclor 1254 treatment alone. These differences were summed and the means

TABLE 1.—Total number of leukocytes isolated from the anterior kidney and spleen of juvenile chinook salmon exposed to various combinations of the stressors *Nanophyetus salmincola* and the commercial polychlorinated biphenyl Aroclor 1254 followed by immunization with phosphate-buffered saline (PBS) or trinitrophenyl-keyhole limpet hemocyanin (TNP-KLH). Values are means ± SEs, with sample sizes in parentheses. The abbreviation AE stands for the acetone–emulphor control. Asterisks indicate significant ($P \leq 0.05$) differences between fish exposed to *N. salmincola* and those not exposed.

			N. salmincola	
Organ	Exposure	Immunization	Not infected	Infected
Kidney	AE	PBS	220 ± 49 (8)	364 ± 34* (9)
	Aroclor 1254	PBS	202 ± 24 (8)	270 ± 37 (9)
	AE	TNP-KLH	225 ± 30 (8)	323 ± 33* (11)
	Aroclor 1254	TNP-KLH	265 ± 48 (8)	320 ± 28 (11)
Spleen	AE	PBS	330 ± 56 (8)	339 ± 53 (9)
	Aroclor 1254	PBS	323 ± 54 (8)	355 ± 57 (9)
	AE	TNP-KLH	335 ± 63 (8)	378 ± 34 (11)
	Aroclor 1254	TNP-KLH	289 ± 67 (8)	302 ± 34 (11)

compared with those obtained from the fish subjected to both stressors in combination. The effects of combined stressors were considered additive if they were not statistically different from the summed differences of individual stressors (Hay et al. 1994).

Results

Condition Factor

There were no significant differences in weight or condition factor (weight/length^3) between any of the treatment groups used in either the disease challenge experiments or the immunocompetence assays (data not shown).

Total Number of Leukocytes

There was a significant treatment effect on the number of leukocytes isolated from the anterior kidneys (ANOVA: $P = 0.02$; Table 1). The total mean number of leukocytes isolated from the anterior kidneys (measured by hemocytometer counts prior to in vitro stimulation) of *N. salmincola*-infected fish (both immunized and not immunized) was significantly higher (Fisher's PLSD test: $P = 0.01$ and 0.05, respectively) than that of noninfected cohorts, suggesting that an immune response had been activated in these fish. Significant differences were not observed between infected and noninfected fish also exposed to the contaminant Aroclor 1254. There were no significant differences between any of the treatments in the number of leukocytes isolated from spleens (Table 1).

TABLE 2.—Results of a three-way ANOVA to identify differences in the primary and secondary plaque-forming cell responses of leukocytes isolated from the spleen of juvenile chinook salmon that were infected with the parasite *N. salmincola,* exposed to the contaminant Aroclor 1254, or both and then immunized with PBS or TNP-KLH. The ANOVA was performed on square-root-transformed data.

Source	df	Mean square	*F*	*P*
Parasite (P)	1	93.55	7.04	0.01
Contaminant (C)	1	235.42	17.71	<0.0001
Immunization (I)	1	417.09	31.38	<0.0001
P × C	1	42.06	8.16	0.07
P × I	1	11.91	0.89	0.34
C × I	1	49.07	3.69	0.05
P × C × I	1	12.73	0.96	0.33
Residual	239	13.29		

Plaque-Forming Cell Response of Splenic Lymphocytes

We examined the effects of the parasite and the contaminant on juvenile chinook salmon B cell function by measuring their ability to produce primary (initial) and secondary (memory) PFC responses in vitro to the T-cell-independent antigen TNP-LPS. Infection with the parasite *N. salmincola,* injection with Aroclor 1254, and immunization with TNP-KLH each had significant effects on the number of splenic PFCs (Table 2). Aroclor 1254 had a significant negative effect on the primary PFC response in the spleen (Figure 1a). The splenic primary PFC response was significantly lower (Fisher's PLSD; $P < 0.0001$) in the fish that received Aroclor 1254 alone and in the fish exposed to both *N. salmincola* infection and Aroclor 1254 than in the noninfected, acetone–emulphor controls (Figure 1a). The lower numbers of splenic PFCs observed in the *N. salmincola* -infected, nonimmunized fish were not significantly different from the PFCs of control fish ($P = 0.19$).

We also examined the splenic PFC response to

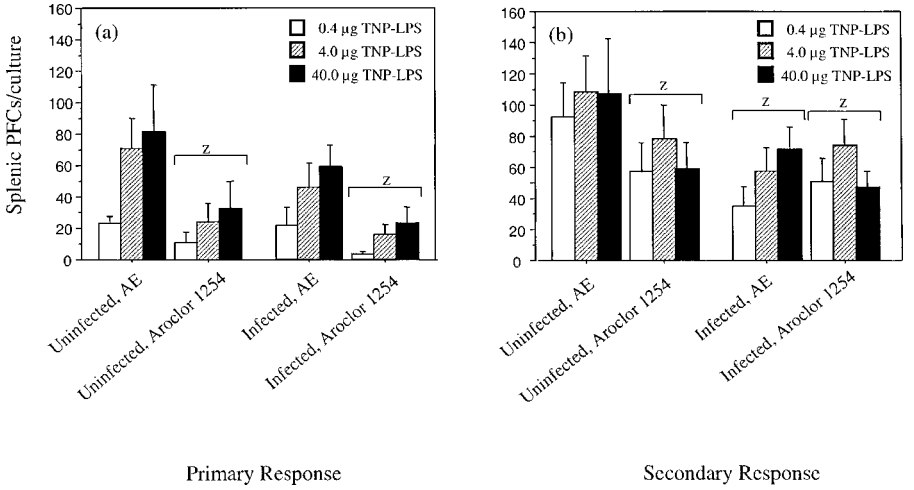

FIGURE 1.—Mean number of in vitro splenic plaque-forming cells (PFCs) per culture from juvenile chinook salmon that were (1) infected with *Nanophyetus salmincola*, (2) injected with the polychlorinated biphenyl Aroclor 1254, or (3) exposed to both stressors. Control fish were not exposed to the parasite but were injected with an acetone–emulphor (AE) carrier. Panel **(a)** shows the results for fish that were injected with Freund's Complete Adjuvant alone to measure a primary PFC response; panel **(b)** shows the results for fish that were injected with trinitrophenyl (TNP)-keyhole limpet hemocyanin in Freund's Complete Adjuvant to measure a secondary PFC response. Cells were incubated in vitro with three concentrations (0.4, 4.0, or 40.0 µg) of TNP-lipopolysaccharide (TNP-LPS). Whiskers indicate SEs. In panel (a) the lowercase *z* denotes statistically significant ($P < 0.0001$) differences between fish exposed to Aroclor 1254 and both infected and uninfected controls; in panel (b) it denotes statistically significant ($P < 0.02$) differences from uninfected controls.

TNP-LPS in vitro in fish immunized with TNP-KLH (Figure 1b) to measure effects on the secondary B cell response. Immunized healthy fish should mount an elevated secondary immune response to TNP-conjugated sheep red blood cells following incubation in vitro with TNP-LPS. Immunization with TNP-KLH had a significant effect (ANOVA: $P < 0.0001$; Table 2) and increased the number of splenic PFCs in immunized fish (Figure 1a, b). This increase was most apparent between nonimmunized and immunized control fish at the 0.4-µg/mL concentration of TNP-LPS (Figure 1a, b). There was a significant interaction effect in the ANOVA between the contaminant and immunization ($P < 0.05$; Table 2) that was consistent with the change in the trend of PFC responses to different concentrations of TNP-LPS in groups of immunized fish exposed to Aroclor 1254 (Figure 1b). All three groups of immunized and stressed fish (*N. salmincola* infection alone, contaminant exposure alone, *N. salmincola* infection plus contaminant exposure) had significantly (Fisher's PLSD test; $P < 0.02$) lower numbers of secondary PFCs than immunized control fish (Figure 1b).

TABLE 3.—Results of a three-way ANOVA to identify differences in the primary and secondary plaque-forming cell responses of leukocytes isolated from the anterior kidney of juvenile chinook salmon. See Table 2 for additional details.

Source	df	Mean square	*F*	*P*
Parasite (P)	1	111.38	6.20	0.01
Contaminant (C)	1	90.33	5.03	0.03
Immunization (I)	1	546.79	30.42	<0.001
P × C	1	6.68	0.37	0.54
P × I	1	7.74	0.43	0.51
C × I	1	8.67	0.48	0.48
P × C × I	1	16.18	0.90	0.34
Residual	245	17.97		

Plaque-Forming Cell Response of Anterior Kidney Lymphocytes

Both infection with *N. salmincola* and injection with Aroclor 1254 had significant effects on the anterior kidney PFC response (ANOVA: $P = 0.01$ and 0.03 respectively; Table 3). Each stressor alone caused small reductions in the anterior kidney primary PFC response, but they were not significantly different from the responses in control fish (Fisher's PLSD: $P = 0.07$; Figure 2a). In contrast, fish exposed to the two stressors combined had significantly lower primary PFC responses than control fish ($P = 0.0002$; Figure 2a).

Immunization with TNP-KLH had a significant

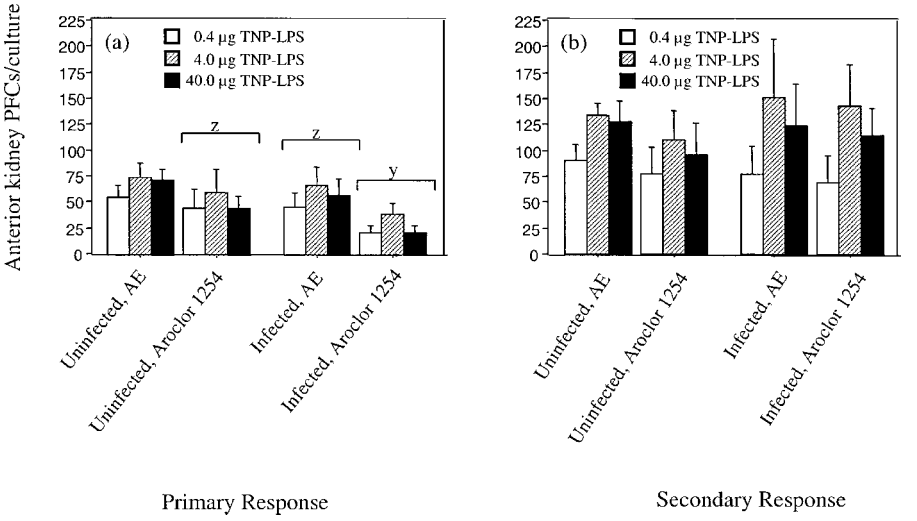

FIGURE 2.—Mean number of in vitro plaque-forming cells (PFCs) per culture from the anterior kidneys of juvenile chinook salmon that were (1) infected with *N. salmincola*, (2) injected with Aroclor 1254, or (3) exposed to both stressors. The lowercase *z* denotes nonsignificant ($P = 0.07$) differences from uninfected controls and the lowercase *y* statistically significant differences from both controls ($P = 0.0002$) and other stressed fish ($P < 0.05$). See Figure 1 for other details.

effect (ANOVA: $P < 0.001$; Table 3) on the PFC response of anterior kidney leukocytes. Anterior kidney leukocytes from control fish immunized with TNP-KLH were able to mount an enhanced secondary antibody response (Fisher's PLSD test; $P < 0.05$) relative to that of their nonimmunized cohorts (Figures 2a, b). Juvenile chinook salmon injected with Aroclor 1254 appeared to have a lower secondary anterior kidney PFC response than the acetone–emulphor controls; however, this difference was not statistically significant (Figure 2b). *Nanophyetus salmincola* infection, alone or in combination with Aroclor 1254, did not significantly reduce the number of secondary anterior kidney PFCs.

Combined Effects

To evaluate the effects of the combined parasitic infection and contaminant treatment, we measured the PFC responses of splenic and anterior kidney leukocytes in fish exposed to both stressors and compared these responses with those in fish exposed to the individual stressors. No statistical interaction effects between the parasitic infection and the contaminant were determined by the three-way ANOVA for splenic or anterior kidney leukocytes (Tables 2, 3). Thus, the effect of each factor was statistically independent of the presence of the other factor.

Examination of the primary PFC responses of both the anterior kidney and splenic leukocytes of nonimmunized fish suggests that the parasite and contaminant stressors had cumulative effects (Figures 1a, 2a). The primary PFC response of anterior kidney leukocytes from infected and contaminant-injected, nonimmunized fish was significantly lower (Fisher's PLSD test; $P < 0.05$) than the response measured following either individual treatment (Figure 2a). The primary PFC response of splenic leukocytes from fish exposed to the combined stressors was also lower than that from fish exposed to individual stressors (Figure 1a); however, this difference was not significant. The secondary PFC response of splenic or anterior kidney leukocytes of immunized fish given the combined parasite and contaminant treatment was not different from that of immunized fish exposed to either stressor alone (Figures 1b, 2b).

To further characterize the biological impact of these multiple stressors on the B cell response, we evaluated their combined effects by qualitatively comparing the observed PFC responses in the simple- and multiple-stressor treatments with those predicted by three different models, namely, single-comparative-effects, multiplicative-effects, and additive-effects models (Table 4). The primary PFC response of anterior kidney leukocytes in fish exposed to the combined stressors (40.5% of the control response) was lower than the responses predicted by any of the models but not statistically

TABLE 4.—Effects of multiple stressors predicted by three different models (simple comparative, additive, and multiplicative) and the observed effects of single and multiple stressors on the primary and secondary plaque-forming-cell responses of splenic and anterior kidney leukocytes of juvenile chinook salmon. Predicted and observed effects are expressed as percentages of unstressed control fish. The stressors, to which fish were exposed both individually and jointly, are the parasite *N. salmincola* and the contaminant Aroclor 1254. In each case, the prediction of the simple-comparative-effects (SCE) model is the percentage response of an individual stressor that induced the lowest effect (or greatest suppression); the prediction of the additive-effects (AE) model is the sum of the effects of the individual stressors within the treatment; and the prediction of the multiplicative-effects (ME) model is the product of the effects of the individual stressors within the treatment.

Organ	Immune response	Stressor	Model predictions			Observed effects
			SCE	AE	ME	
Kidney	Primary	Parasite				74.4
		Contaminant				85.3
		Contaminant + parasite	74.4	59.7	63.5	40.5
	Secondary	Parasite				100.0
		Contaminant				80.3
		Contaminant + parasite	80.3	89.3	80.3	91.7
Spleen	Primary	Parasite				72.6
		Contaminant				38.7
		Contaminant + parasite	38.7	11.3	28.1	24.5
	Secondary	Parasite				53.4
		Contaminant				62.8
		Contaminant + parasite	53.4	16.2	33.5	55.4

different from the prediction by the additive model (59.7%; Table 4). The splenic primary PFC response of fish exposed to both stressors (24.5% of the control response) was lower than those predicted by the simple-comparative-effects model (38.7%) and the multiplicative-effects model (28.1%) but higher than that predicted by the additive-effects model (11.3%; Table 4). These results suggest a multiplicative effect between the two stressors on the primary splenic PFC response because the observed response was not significantly different from that predicted by the multiplicative-effects model.

Based on the model predictions, the combination of both stressors did not cumulatively affect the secondary PFC response of either organ. The observed secondary PFC response of splenic leukocytes in fish exposed to both stressors (55.4% of the control response) was similar to that induced by the parasitic infection alone (53.4%; Table 4). The secondary PFC response produced by anterior kidney leukocytes was higher in fish exposed to both stressors (91.7% of the control response) but not significantly different than in the contaminant-alone treatment (80.3%).

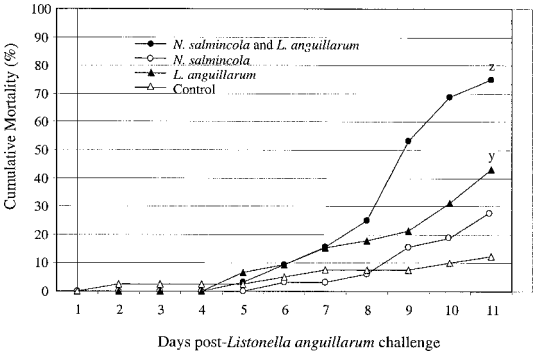

FIGURE 3.—Average cumulative mortalities of juvenile chinook salmon. Experimental treatment groups included fish infected with the parasite *N. salmincola* prior to *Listonella anguillarum* challenge, fish infected with *N. salmincola* but not challenged with *L. anguillarum*, uninfected fish challenged with *L. anguillarum*, and control fish. The lowercase *z* denotes a significant ($P < 0.05$) difference from all other treatments, the lowercase *y* denotes a significant ($P < 0.05$) difference from controls.

Disease Challenge

Infection with *N. salmincola* increased mortality following exposure to a secondary infection with the marine bacterium *L. anguillarum* (Figure 3). Mortalities in groups exposed to *L. anguillarum* began on day 5 postchallenge. Juvenile chinook salmon infected with *N. salmincola* and exposed to *L. anguillarum* had significantly ($P < 0.05$) higher cumulative mortalities (75%) than all other treatment groups by day 11 postchallenge. Fish exposed to *L. anguillarum* only had significantly greater cumulative mortalities (43%) than control fish (12.5%) but not than fish exposed to *N. salmincola* (28.0%).

Additional groups of infected and noninfected

fish were also injected with Aroclor 1254 and then challenged with *L. anguillarum*. Unfortunately, unexplained mortalities of control fish injected with Aroclor 1254 and not exposed to *L. anguillarum* prevent us from drawing conclusions from these combined treatments. Similar mortalities did not occur with the control fish not given Aroclor 1254 (12.5% on day 11, as reported above). Thus, we are unable to describe the combined effects of parasite and contaminant on a disease challenge at this time.

Discussion

We found the combination of a natural stressor (a parasitic infection) and an anthropogenic stressor (PCBs) to have a greater detrimental effect on juvenile salmon than either stressor alone. The in vitro primary PFC response of anterior kidney leukocytes was significantly lower in fish infected with *N. salmincola* and injected with Aroclor 1254 than in fish with the parasite or the contaminant alone. The primary PFC responses of splenic leukocytes were also lower in fish exposed to both stressors; however, the effect was not as great as that of the anterior kidney leukocytes. In contrast, we did not observe a combined effect of the stressors on the secondary B cell response in either the spleen or anterior kidney. These results suggest that when juvenile salmon experience these multiple stressors their ability to respond to an additional initial infection may be compromised.

We further described the type of biological interaction of the two stressors by using the additive-, multiplicative-, and simple-comparative-effects models. This approach was used by Folt et al. (1999) to qualitatively describe the type of synergism or antagonism among multiple environmental stressors on zooplankton reproduction and survivorship. The observed primary PFC responses of anterior kidney leukocytes indicated an additive effect between *N. salmincola* infection and Aroclor 1254 treatment when compared with the values predicted by the models. This additive effect on the primary PFC response of the anterior kidney suggests that the mechanisms of the two stressors are independent of each other and could act interchangeably in the same step of a multistep process (Rothman et al. 1980). The lack of a statistically significant interaction effect on anterior kidney PFC responses between the parasite and the contaminant in the ANOVA also supports the hypothesis that there are separate mechanisms of immune suppression. The combined effects of these two stressors on the primary PFC of splenic leukocytes indicated a multiplicative effect, a response that is more detrimental than the result caused by either stressor alone. Factors that are multiplicative can fall into a broad category and act at different steps in a biological process (Rothman et al. 1980). Either outcome (additive or multiplicative) results in greater risk than that from a single stressor.

Current understanding of the effects of multiple stressors on anadromous salmon is poor due to both the complexity of the problem and the limited number of studies addressing it. In addition, while some studies have found exacerbated effects with multiple stressors, others have not. For example, Harrahy (2001) examined the combined effects of elevated acclimation temperature and acute handling stress on the immune response of yearling spring chinook salmon. The combined acute stressors did not have an additive effect on plasma lysozyme concentrations, the number of anterior kidney leukocytes, or the number of antibody-producing cells. Barton et al. (1986) noted that juvenile chinook salmon infected with the virulent coldwater bacterium *Flexibacter psychrophilus* and suffering from chronic fin rot condition were less capable than healthy fish of coping with cumulative acute handling stresses. Results have been mixed when examining *R. salmoninarum* infection and additional stressors. Combinations of *R. salmoninarum* infection and seawater challenges or dissolved gas supersaturation led to increased mortality rates (Elliott et al. 1995; Moles 1997; Weiland et al. 1999), but multiple handling stressors following *R. salmoninarum* infection did not change the infection level or mortality rate (Mesa et al. 2000). These studies emphasize the variety of stressors to which juvenile salmon are subject and the need to examine a wide range of stressors to provide managers with improved understanding of the risks.

Our study found suppressed immune function in salmon infected with *N. salmincola*, but we do not know the mechanisms underlying this immunosuppression. We also found that infection had different effects on the splenic and anterior kidney B cell responses, and although we do not have a definitive explanation for this, it is known that the PFCs from the two organs can respond differently to antigens and mitogens (Kaattari and Irwin 1985). Anterior kidney leukocytes respond much like immature immune cells from a primary lymphoid organ characterized by a homogeneous population of antibody-producing cells. In contrast, the spleen contains a more heterogeneous popu-

lation of antibody-producing cells, which is characteristic of a secondary lymphoid organ (Kaattari and Irwin 1985).

We also found that chinook salmon infected with *N. salmincola* had significantly greater cumulative mortality than noninfected salmon following a secondary infection with the marine bacterium *L. anguillarum*. Foott and coworkers (U.S. Fish and Wildlife Service, personal communication) also examined the effects of *N. salmincola* on fall chinook salmon exposed to secondary challenge by *L. anguillarum* and found less of an effect with a substantially lower dose of *L. anguillarum* and a shorter exposure time.

There has been little empirical evidence to date suggesting that *N. salmincola* has sublethal effects on the health of salmon smolts. Early experimental studies on the effects of *N. salmincola* infection were conducted on several salmon species, and high pathogenicity was observed in salmon fry exposed to high numbers of cercariae for 24 h (Baldwin et al. 1967). The lethal effects of the parasite were attributed to the penetration and migration of cercariae (Baldwin et al. 1967). Salmon fry survived exposure to the previously noted lethal numbers of cercariae when exposed over an extended period of time (Millemann and Knapp 1970b). A significant reduction in swimming ability was reported in coho salmon and steelhead *O. mykiss* (anadromous rainbow trout) 7–60 mm in length that were exposed to cercariae daily for 15 d but not 15 d postexposure, that is, after the encystment of the metacercariae (Butler and Millemann 1971). These results suggest that encysted metacercariae are not measurably detrimental.

A few studies, however, do suggest that established *N. salmincola* infections can negatively affect salmon. Negative effects on osmoregulatory control and saltwater survival have been observed in both laboratory (Foott and coworkers, personal communication) and field studies (T. Newcomb, National Marine Fisheries Service, personal communication). In addition, a study in the Chehalis River, Washington, by Schroder and Fresh (1992) found that coho salmon acquire high intensities of *N. salmincola* infection while emigrating through the lower areas of the river. They suggested that *N. salmincola* infections could have been contributing to the low coho salmon survival there. In spite of this and other reports of mortality caused by encysted metacercariae in fish (Lemly and Esch 1984), little concern has been expressed about potential detrimental effects once the metacercariae have encysted. Our data suggest that encysted metacercariae should not be deemed benign and thus ignored when considering the effects of *N. salmincola* on fish. Our finding of higher total numbers of anterior kidney leukocytes in infected fish than in noninfected ones suggests an active immune response against the encysted metacercariae and an ongoing interaction between parasite and host.

The immunotoxicity of PCBs in fish has been well documented (reviewed by Zelikoff et al. 2001; Rice and Arkoosh 2002). In rainbow trout, dietary Aroclor 1254 has been shown to suppress the antibody response and increase disease susceptibility (Cleland et al. 1988; Thuvander and Carlstein 1991). Arkoosh et al. (1994) have previously shown that injection of juvenile fall chinook salmon with Aroclor 1254 can suppress the primary and secondary PFC responses of both splenic and anterior kidney leukocytes. These earlier experiments provide the foundation for testing potential cumulative effects of anthropogenic (PCBs) and natural (a parasite) stressors on an important measure of juvenile salmon health, the immune response. This design was chosen to allow comparison of measurable immune responses. By quantifying the number of antibody-producing cells in fish exposed to both Aroclor 1254 (a known immunosuppressive agent) and the parasite relative to fish exposed to Aroclor 1254 alone, we were able to demonstrate cumulative effects on B cell function. Arkoosh et al. (2001) also showed that the same dose of Aroclor 1254 (54 mg/kg) could cause greater mortalities in juvenile salmon following challenge with *L. anguillarum*. We had planned to test for cumulative effects through disease challenge but were unable to draw conclusions on combined effects due to an unforeseen tank effect in a control tank of the PCB-exposed fish.

In conclusion, we found a cumulative effect on immune function in salmon exposed to both the parasite *N. salmincola* and the commercial PCB Aroclor 1254. Also, we determined that sublethal infection levels of *N. salmincola* may negatively affect juvenile chinook salmon. Although we do not know the precise mechanisms of immunodepression in *N. salmincola*-infected fish, clearly, salmon that are infected with this parasite are at higher risk than uninfected salmon when they encounter other stressors. Additional studies of this host–parasite relationship are warranted and should examine other measures of stress and immune responsiveness, the potential mechanisms of immunosuppression, and other combinations of stressors.

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