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AROCLOR 1254 EXPOSURE REDUCES DISEASE RESISTANCE AND INNATE IMMUNE RESPONSES IN FASTED ARCTIC CHARR

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Abstract—To examine the immunological impacts of polychlorinated biphenyls (PCBs) in an environmentally relevant way, we orally contaminated Arctic charr (*Salvelinus alpinus*) with Aroclor 1254. After contamination, fish were either fed (0 and 100 mg Aroclor 1254 kg^{-1} fish wt) or fasted (0, 1, 10, and 100 mg kg^{-1}) to mimic cycles of feeding–fasting experienced by Arctic animals. After four months, PCB concentrations in muscle were the same in fasted and fed fish; however, PCBs in kidneys of fed fish were 33 to 50% of those in fasted fish. Arctic charr were exposed to *Aeromonas salmonicida*, the bacteria responsible for furunculosis, by cohabitation with infected conspecifics. Fasted fish had a significant trend toward lower survival with higher dose of PCBs—from 68% in controls to 48% in treatment involving 100 mg kg^{-1}. Independent of PCB contamination, fed fish had the lowest survival; we attribute this to stress associated with establishing and maintaining feeding hierarchies. A significant decrease in the activity of lysozyme was observed in skin mucus, as was hemagglutination ability of a putative rhamnose lectin in fasted, but not in fed, PCB-treated fish. These results demonstrate the immunosuppressive effects of PCBs on Arctic charr, and they illustrate the importance of considering environmentally relevant nutritional status in ecotoxicological studies.

Keywords—Arctic charr Polychlorinated biphenyl Disease Immune

INTRODUCTION

Aquatic pollution is an increasing problem worldwide, and even in remote Arctic areas, high levels of pollutants have been detected. Of particular concern are the polychlorinated biphenyls (PCBs), which are found at alarmingly high concentrations in the Arctic ecosystem [1]. For example, 5 mg PCB kg^{-1} body wet weight have been reported in Arctic charr (*Salvelinus alpinus*) from Bear Island (situated in the northeastern Atlantic, at 74°N latitude)—the highest PCB levels detected so far in Arctic fish [2]. Although levels of persistent organic pollutants (POPs) in many Arctic animals may exceed the thresholds associated with immune suppression and reproductive disorders reported in laboratory animals, knowledge is still lacking about possible threats posed by POPs in Arctic ecosystems [1].

Understanding the impacts of contaminants in the Arctic requires consideration of the physiological adaptations of animals living in this extreme environment. Many animals in the Arctic exhibit marked seasonal cycles of feeding and fasting. For example, during the summer feeding migration to seawater, anadromous Arctic charr may double their body weight and increase their body lipid stores several-fold [3]. During the subsequent overwintering in freshwater, however, food intake is minimal, and on return to the sea the following summer, the lipid stores may be totally depleted [3]. It appears that lipid mobilization associated with fasting may affect the fish's sensitivity to deposited POPs. In Arctic charr, emaciation resulted in a redistribution of the lipophilic pollutant octachlorostyrene from main lipid-storing tissues (e.g., muscle) to vital organs, including liver, kidney, and brain [4]. Furthermore, emaciation potentiated the PCB-induced hepatic P4501A enzyme activity in Arctic charr [5]. We have previously shown that PCB (Aroclor 1254) reduced resting levels of the stress-hormone cortisol in Arctic charr. Also, the cortisol response to a handling disturbance was compromised in this species; food deprivation further suppressed the hormonal and hyperglycemic responses to stress [6]. Nutritional status may, therefore, be an important determinant of contaminant impact on other physiological systems, such as the immune system, of Arctic animals, but few studies have addressed this possibility.

Contaminants, such as PCBs, can have direct effects on the fish immune system by suppressing the ability of cells to proliferate and produce specific antibodies [7], promoting apoptosis and necrosis [8], or by impairing immune memory [7]. Some studies have linked dysfunctions of the adaptive immune system (i.e., specific antibody production) to reduced disease resistance [7]. However, the innate immune responses associated with epithelium—the first line of defense—may be more important than adaptive immunity for insuring bacterial resistance in fish [9,10]. Jørgensen et al. [5] reported that Arctic charr fed PCBs had a higher incidence of fin erosion than did control fish, suggesting a possible PCB effect on innate immunity. Two innate immune factors of importance in resistance to bacterial pathogens are lysozymes, which are antimicrobial, hydrolytic enzymes in plasma and tissues of most fish [11,12], and lectins, which are pattern-recognition receptors that bind to specific carbohydrate moieties that are part of bacterial cell walls [13,14]. Blood lysozyme activity has been observed to decrease in rainbow trout (*Oncorhynchus mykiss*) exposed to polluted water [15] and has been identified as a factor in the resistance of Atlantic salmon (*Salmo salar*) to the bacterial pathogen *Aeromonas salmonicida*, the causative agent of furunculosis [16]. Lectins have also been implicated in the ability

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Table 1. Timelines of experiments

	Year 2000	Year 2001
Establish fed and fasted groups	Feburary 21	March 1
Contaminate fish with Aroclor 1254	March 3	March 14
Transfer fish for disease challenge	June 6	—
Disease challenge begins	June 13	—
Physiological sampling	July 10 and 11	July 14
Disease challenge ends	July 31	—

of Atlantic salmon to resist bacterial infections [17]. Thus, PCBs impact resistance to bacterial pathogens and adaptive immune responses in salmonids, and resistance to bacterial pathogens has been linked to innate immune responses. However, to our knowledge, the interaction between PCB contamination, disease resistance, and innate immunity has not been explored.

In the present study, our objectives were to investigate the impact of PCB contamination on disease resistance and innate immune responses in Arctic charr and to determine if those effects are modulated by the nutritional status of the animal. The rationale for the present study stems from the fact that most examinations of PCB contamination have been performed with fed fish, which is not representative of the seasonal cycles of fattening and emaciation that are experienced by high-latitude, anadromous Arctic charr. Here, we contaminated the fish with an environmentally realistic PCB mixture (Aroclor 1254) when they were feeding (absorptive state), and then we fasted (or fed) the fish for four months before we investigated the resultant impact. We examined the effects of this long-term PCB treatment on the ability of the fish to resist *A. salmonicida*; activity of lysozyme in plasma, skin, and nares mucus; and levels of pattern recognition by a putative plasma lectin.

MATERIALS AND METHODS

Fish and experimental conditions

A total of 360 Arctic charr (age, 2.5 years) were used in the 2000 experiment (Table 1). The fish were fourth-generation offspring from anadromous Arctic charr collected at Svalbard (79°N latitude) in 1990. The fish were hatched at Kårvik Research Station (Tromsø, Norway) in 1998, and the experiment was conducted at the station from February through July 2000. Before the experiment, the fish were held under natural water temperatures and light conditions and were fed in excess according to normal rearing procedures at the research station.

On February 21 and 22, 2000, the fish (mean wt, 234 g) were individually tagged (Floy FTF-69 fingerling tags; Floy Tag, Seattle, WA, USA) and randomly sorted into six groups. They were then transferred to six centrally drained, circular, 500-L tanks that were supplied with freshwater maintained at approximately 0.5°C. During the two weeks following establishment of the groups, water temperature was gradually elevated to 7.0 ± 0.5°C, and photoperiod was adjusted to 12:12-h light:dark. These conditions were maintained until sampling and until the start of the disease challenge experiment. The tanks were supplied with freshwater in an amount sufficient to keep oxygen saturation at greater than 90%.

The 2001 experiment was performed with 2.5-year old, fifth-generation offspring of the same stock of anadromous Arctic charr from Svalbard (Table 1). On March 1, 2001, a total of 60 fish (mean wt, 240 g) were randomly sorted from a larger group of fish into four 300-L, centrally drained tanks. Water temperature was gradually elevated from 0.5°C (natural temperature) to 7°C within 14 d, and this temperature was maintained throughout the rest of the experiment. Light conditions were again maintained at a 12:12-h light:dark photoperiod, and fish were deprived of food for the experimental period.

Feeding, contamination, and sampling

From February 22, 2000, onward, the fish in four groups were deprived of food (hereafter termed fasted [FD]), whereas those in two other groups continued to receive food (designated Fed). The latter groups were fed to satiation with commercial pelleted dry feed (Skretting, Stavanger, Norway) 5 d a week before and after contamination with PCBs. The contamination and feeding regime followed here has been published previously [6]. Briefly, one of the four FD groups was held as an uncontaminated, vehicle-treated control (FD-C), whereas the fish in the other three groups were given one of three doses of the technical PCB mixture Aroclor 1254 (Promochem AB, Ulricehamn, Sweden). The doses were 1, 10, and 100 mg (FD-1, FD-10, and FD-100, respectively) of Aroclor 1254 kg^{-1} fish. Of the two groups of fed fish, one was held as an uncontaminated, vehicle-treated control, and the other was given 100 mg Aroclor 1254 kg^{-1} fish. Administration of PCB was performed (on March 3, 2000) by force-feeding lightly anesthetized fish (40 ppm of benzocaine) with gelatin capsules containing Aroclor 1254 dissolved in fish oil. The control fish were given capsules containing only fish oil. The calculation of individual PCB doses was based on the fish weights recorded on February 21 and 22, 2000. On July 10, 2000, 10 fish from each treatment that were not used in the disease challenge were sampled to determine their innate immune responses. On July 11, 2000, 10 prestress control fish from each of the six treatments were sampled in an experiment to determine the effects of PCB on the stress response of Arctic charr [6]; innate immune responses were also measured in these fish.

In 2001, Arctic charr were treated identically as described above, with the exception that the two groups of fed fish were not included. These groups of fish were contaminated on March 14, 2001, with 0, 1, 10, or 100 mg Aroclor 1254 kg^{-1} fish, and they were sampled on July 14, 2001. In the absence of a fed control group, we also sampled 10 fish from the stock tank of fed fish.

All 10 fish in each sample were rapidly netted and killed within 1 min with a lethal dose of benzocaine (300 ppm). Blood samples were collected from the caudal vein using lithium-heparinized vacutainers (Becton Dickinson, Franklin Lakes, NJ, USA). These blood samples were kept on ice until centrifuged (6,000 *g*, 10 min, 4.0°C) to separate plasma, which was stored frozen (−70°C) until used in assays. Mucus from the skin and nares was collected as described by Schrock et al. [12]. Briefly, a 1.0-μl plastic inoculation loop was rubbed on the skin or inserted into the nares of the fish to collect mucus. The mucus was immediately extracted in 50 μl of 0.04 M phosphate buffer at pH 6.2 and stored frozen (−70°C).

Disease challenge

On June 6, 2000, 40 previously tagged fish were randomly selected from each experimental group and transferred to the challenge facility at the disease laboratory at Kårvik Research Station. The fish were placed together in a 2,000-L circular

tank supplied with freshwater in a sufficient amount to keep the oxygen saturation at greater than 90%. The water temperature was kept at 7.0 ± 0.5°C, and the day length was kept equivalent to the normal day length for the summer season in Tromsø (24-h constant light). The fish were not fed during the disease experiment.

On June 13, 2000 (day 0), the experimental fish were challenged by the addition of 26 cohabitant Arctic charr (from the same stock of fish as the experimental fish), each of which received an intraperitoneal injection of 100 μl of phosphate-buffered saline containing 8×10^2 colony-forming units of *A. salmonicida*. The bacterium was a freshly reisolated strain of *A. salmonicida* subsp. *salmonicida* (LFI 4017) originally isolated from Atlantic salmon in 1989. The bacteria had been grown overnight at room temperature in brain heart infusion broth (BHI; Difco, Detroit, MI, USA) without shaking. The bacteria were washed once, resuspended, diluted in phosphate-buffered saline, and injected into the cohabitants. Mortality of cohabitants and experimental fish was recorded every 24 h. The cause of mortality of the experimental fish was confirmed in the first group of mortalities by culturing (BHI agar supplied with 0.1 g of Coomassie Brilliant Blue G; Sigma-Aldrich Norway, Oslo, Norway) bacteria sampled from their head kidneys. The remaining mortalities were confirmed by examination for clinical signs specific to furunculosis.

Lysozyme

Plasma and mucus were assayed for lysozyme activity using the microplate turbidimetric method with *Micrococcus lysodeikticus* as the target for lysozyme and hen egg-white lysozyme (HEWL) as standard [12]. All the lysozyme data are expressed relative to the HEWL standard curve. The HEWL likely is not the same as Arctic charr (or other fish) lysozyme, because optimal assay conditions are not the same for the two lysozymes [12]. For this reason, the Arctic charr lysozyme data are expressed in terms of units of HEWL activity per milliliter of serum or mucus (μg ml^{-1}) as opposed to molar concentration of lysozyme.

Lectin

Differences in plasma lectin were assessed using a hemagglutination assay similar to that described by Fock et al. [18]. Briefly, 25 μl of plasma were serially diluted twofold in wells of a 96-well, conical-bottomed microtiter plate (Corning, Corning, NY, USA) using Tris-buffer (0.01 M Tris, 0.77 M NaCl, with or without 0.01 M $CaCl_2$, pH. 7.4 overall buffer). We then added 25 μl of red blood cells (RBCs; 1% v/v in Tris buffer) from one of several species (rabbit, horse, sheep, chicken, or guinea pig) and incubated the covered plates at 4°C. Negative-control wells contained 25 μl of RBCs and 25 μl of Tris buffer. We found no differences in hemagglutination titers when plates were examined from 4 to 24 h after the assays were set up. To determine the carbohydrate specificity of the putative Arctic charr lectin, we conducted inhibition assays with *N*-acetyl-galactosamine, *N*-acetyl-glucosamine, D(+) galactose, D(+) glucose, D(+) mannose, L(−) mannose, D(−) arabinose, L(−) rhamnose, fructose, and lactose, which were serially diluted twofold beginning with a stock concentration of 800 mM. Each well of the 96-well plate contained 25 μl of carbohydrate, 25 μl of serum (25% in Tris-buffer), and 50 μl of rabbit RBCs (1% in Tris buffer). Positive-control wells contained plasma, RBCs, and 25 μl of Tris buffer in place of carbohydrate; negative-control wells contained RBCs and 50 μl of Tris buffer in place of plasma and carbohydrate. We identified the lowest concentration of carbohydrate to inhibit hemagglutination.

Bacterial agglutination by lectin was determined using the same strain and culture methods of *A. salmonicida* as in the disease challenge. Before use, the bacteria were heat-killed (70°C, 30 min) and resuspended to an optical density of 0.25 as measured spectrophotometrically at 630 nm. Bacterial agglutination titers were assessed similar to the hemagglutination with serial dilutions of Arctic charr plasma (25 μl) and bacterial suspension (25 μl). The plasma–bacteria mixtures were examined microscopically to determine the lowest titer at which bacterial agglutination occurred.

PCB analyses

To determine body burden of Aroclor 1254 in the treatment groups from the year 2000, a piece of the muscle from which the skin had been removed (~1 cm wide in cross-section) was taken immediately anterior to the dorsal fin and frozen for later PCB analyses (see below). Organ burdens were measured in the kidneys from which approximately 10 g were removed and frozen for later analyses. The tissues from the 10 fish sampled from each group were divided into three pooled samples representing three, three, and four fish. The pooled samples were homogenized and extracted with acetone followed by hexane: acetone (3:1). After clean-up, PCB was determined using a high-resolution gas chromatograph (Model 5890 Series II; Fision Instruments, Milano, Italy) equipped with a splitless injector, electron-capture detector, and a high-performance HP-5 capillary column (length, 50 m; inner diameter, 0.20 μm; film thickness, 0.11 μm; Hewlett-Packard, Folsom, CA, USA). Nitrogen was used as carrier gas (42 ml min^{-1}). The injector and detector temperatures were 280 and 320°C, respectively. The temperature program was 60°C (held for 1 min), a ramp of 15°C min^{-1} to 160°C (held for 0 min), and a ramp of 1.5°C min^{-1} to 270°C (held for 20 min). Calculations were done on the basis of external (PCB 53) and internal standards with which the chromatograph had been calibrated. The analyzed congeners (International Union of Pure and Applied Chemistry numbers), including the percentage of recovery, were as follows: PCB 28, 50%; PCB 31, 51%; PCB 52, 67%; PCB 99, 54%; PCB 101, 50%; PCB 105, 69%; PCB 118, 53%; PCB 138, 63%; PCB 153, 57%; PCB 156, 80%; and PCB 180, 72%. The PCB data were adjusted in accordance with the recovery values.

Data analyses

All continuous data are presented as the mean ± standard error. The PCB data are the sum of all congeners in the tissue pools and are presented as the mean and range for each treatment and tissue. The survival data in the disease challenge experiment were tested for differences between groups by performing a log-rank test for trend combined with a chi-square test for significance. Differences in plasma and mucous lysozyme data were tested using the Kruskal–Wallis nonparametric test followed by Dunn's multiple-comparison test, with $p < 0.05$ as the level of significance. Plasma lectin data are discontinuous, so we expressed the data as the $\log_2$ of the greatest dilution that resulted in agglutination and also analyzed these data using the nonparametric tests. Analyses were accomplished in GraphPad Prism Version 3.00 for Windows (GraphPad Software, San Diego, CA, USA).

Table 2. Mean and range of polychlorinated biphenyls (ng g wet wt^{-1}) in the muscle and kidney of fasted (FD) and fed Arctic charr orally contaminated with 0 (controls [C]), 1, 10, or 100 mg Aroclor 1254 kg fish^{-1} [a]

	Muscle		Kidney	
Treatment	Mean	Range	Mean	Range
FD-C	14	14–15	19	16–23
FD-1	129	92–157	339	241–473
FD-10	1,073	941–1,309	2,032	1,438–2,588
FD-100	8,366	5,847–10,390	18,103	16,401–21,209
Fed-C	12	10–14	22	20–24
Fed-100	8,074	7,204–9,214	8,094	5,604–10,009

[a] Sample sizes = 3 (from tissue pools of 4, 3, and 3 fish).

RESULTS

Weight and PCBs

Between the time of sorting in February 2000 (mean wt, 234.0 g) and subsequent sampling in July 2000, the FD fish lost weight (180.5 g), whereas the fed fish gained weight (358.0 g), independent of Aroclor 1254 treatment. No significant differences in fish weights were observed between PCB treatments within each of the FD and fed groups. The total PCB concentrations in muscles and kidneys increased with increasing Aroclor 1254 dose (Table 2). Among fasted fish, kidney PCB concentrations (per unit wt) were approximately twice as high in the muscles, regardless of Aroclor 1254 dose. Kidney PCB concentrations were also approximately twice as high in fasted than in fed fish when both groups were contaminated with 100 mg kg^{-1} Aroclor 1254, whereas muscle PCB concentrations were similar in these two groups (Table 2).

Disease challenge

Fish exposed to *A. salmonicida* started to die on day 18, and the experiment was terminated after 48 d (5 d after the last fish died) (Fig. 1). A total of six, five, three, two, four, and four fish in the FD-C, FD-1, FD-10, FD-100, Fed-C, and Fed-100 groups, respectively, lost their tags and were consequently excluded from the experiment. All cohabitant fish (injected with *A. salmonicida*) died between days 8 and 11. A decrease in survival was observed with increasing PCB dose among the FD fish, ranging from 68% in the FD-C group to 48% in the FD-100 group (Fig. 1). Although no significant differences were found between the survival curves when these were compared pairwise ($p = 0.176$, chi-square test), a significant trend ($p = 0.026$, log-rank test) toward a lower disease resistance with increasing PCB dose was noticed among the FD fish. The lowest survival was in the two Fed groups (45 and 39% in the Fed-C and Fed-100 groups, respectively). The survival curves were not significantly influenced by PCB among the Fed fish (Fig. 1). The weights of the experimental fish that died during the challenge were 207.1 ± 13.8 ($n = 11$), 196.1 ± 10.3 ($n = 14$), 202.3 ± 13.2 ($n = 18$), 208.1 ± 6.9 ($n = 20$), 282.2 ± 14.9 ($n = 20$), and 287.1 ± 12.7 g ($n = 22$) in the FD-C, FD-1, FD-10, FD-100, Fed-C, and Fed-100 groups, respectively, and did not differ among the FD groups nor among the Fed groups. The presence of *A. salmonicida* was confirmed in the first 24 mortalities from the treatment groups, and all remaining mortalities had clinical signs of furunculosis. Although they were not part of the experimental design for the disease challenge, no mortalities were observed in cohorts of the same treatment groups used for the stress-response experiment [6].

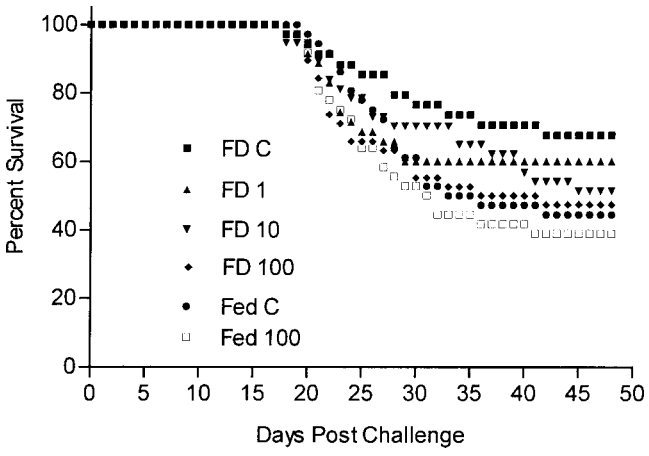

Fig. 1. Survival of Arctic charr that were orally contaminated in March 2000 with 0 (control [C]), 1, 10, or 100 mg Aroclor 1254 kg^{-1} fish and either fasted (FD) or fed (Fed). In June 2000, 40 fish from each group were exposed to cohabitants infected with *Aeromonas salmonicida* on day 0; first mortalities occurred on day 18 after exposure as shown. Survival did not differ between groups (chi-square test); however, log-rank test indicated a significant trend ($p = 0.026$) for decreasing survival with increasing Aroclor 1254 in the fasted groups.

Lysozyme

Lysozyme activity did not differ in plasma or nares mucus from Arctic charr in the FD groups (Fig. 2); however, plasma lysozyme activity was significantly lower in Fed fish treated with 100 mg Aroclor 1254 kg^{-1} as compared to all other groups. Lysozyme in the skin mucus of FD-C fish was significantly higher than in all other groups except for FD-100 (Fig. 2).

Lectin

The putative Arctic charr plasma lectin(s) hemagglutinated RBCs from various species, with or without $CaCl_2$; however, rabbit RBCs had the highest binding titer ($\log_2$ titer, 8) and bound equally well in either buffer. Rabbit RBC hemagglutination was most easily inhibited by L(−) rhamnose (minimum concentration to inhibit, 0.024 mM), followed by L(−) mannose (0.780 mM), D(+) galactose (3.125 mM), and to a lesser extent, other sugars (50 to >200 mM). Thus, the putative lectin on which we focused appears to be a Ca^{2+}-independent, L(−) rhamnose lectin. Hemagglutination titers to rabbit RBCs were significantly lower in plasma from FD fish treated with 100 mg Aroclor 1254 kg^{-1} as compared to FD controls in both years (Fig. 3); furthermore, hemagglutination titers in plasma from fish treated with 10 mg Aroclor 1254 kg^{-1} in the year 2001 were also significantly less than those in controls. No effect of the high Aroclor 1254 dose was observed on hemagglutination titers of fed fish. The ability of plasma to agglutinate *A. salmonicida* was relatively low and did not differ among any of the treatments or controls (Fig. 4).

DISCUSSION

In the present study, we have shown that PCBs ingested by Arctic charr and subsequently deposited in various organs over several months decreased resistance to the bacterial fish pathogen responsible for furunculosis (*A. salmonicida*) (Fig. 1). The tissue concentrations of PCBs in our experiments are ecologically relevant, because mean PCB concentration (~8.0

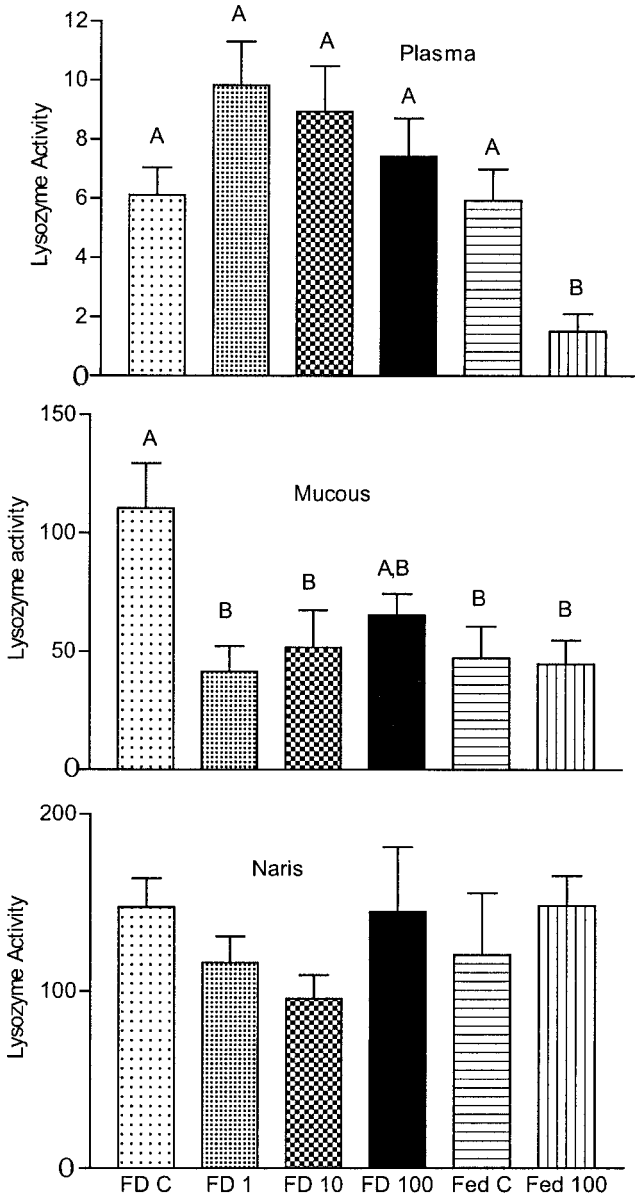

Fig. 2. Lysozyme activity (as hen egg-white lysozyme activity per ml of plasma or mucus [μg ml^{-1}]) in Arctic charr plasma, skin mucus, and nares four months after fish were orally contaminated with 0 (control [C]), 1, 10, or 100 mg Aroclor 1254 kg^{-1} fish and either fasted (FD) or fed (Fed). Groups without letters in common differ significantly from each other ($p < 0.05$, Kruskal–Wallis nonparametric ranked sums test and Dunn's pairwise comparisons). Data are presented as the mean + standard error ($n = 20$).

mg kg^{-1} wet wt) in the muscle of Arctic charr treated with a high dose was equivalent to that reported in charr at Bear Island, Norway (5 mg PCB kg^{-1} body wet wt) [2]. Moreover, the mean concentration in kidneys (339 ng g^{-1} wet wt) of the low-dose treatment charr was the same as that in the livers (300 ng g^{-1} wet wt) of emigrating juvenile Chinook salmon (*O. tshawytscha*) in an urban waterway near Seattle (WA, USA) [7]. We were also able to confirm PCB exposure based on hepatic P4501A activity (M.M. Vijayan, E.H. Jorgensen, A.G. Maule, unpublished data), which was the same as that reported previously for similarly treated Arctic charr [5].

Several researchers have demonstrated that PCB contamination decreases resistance of salmonid fishes to viral [19] and bacterial pathogens [7]. Others, however, have reported that

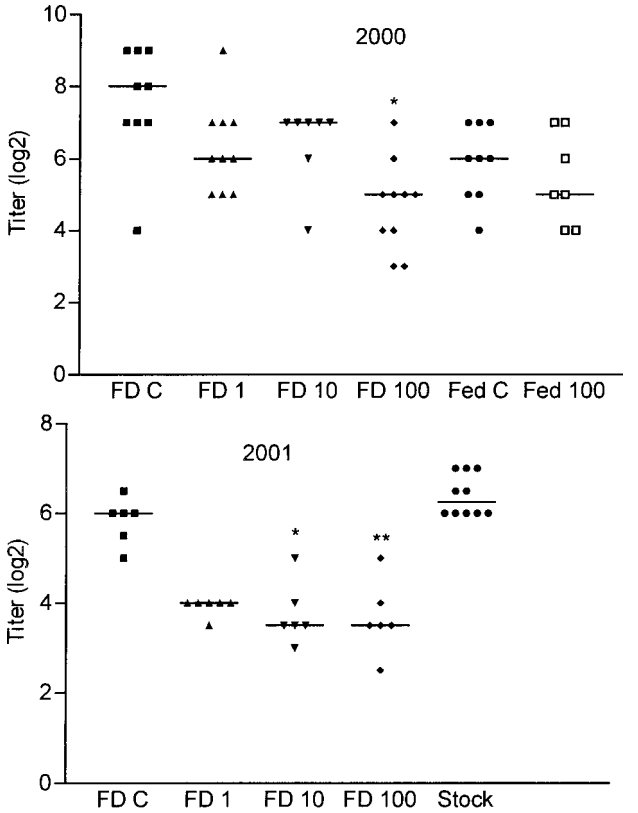

Fig. 3. Plasma lectin titers in Arctic charr four months after fish were orally contaminated with 0 (control [C]), 1, 10, or 100 mg Aroclor 1254 kg^{-1} fish and either fasted (FD) or fed (Fed). In 2001, nontreated controls (Stock) were used rather than fed treatment fish. Asterisks indicate groups that differ significantly from fasted controls (FD C) at $p < 0.05$ (one asterisk) or $p < 0.01$ (two asterisks) based on the Kruskal–Wallis nonparametric ranked sums test and Dunn's pairwise comparisons. Data are for individual fish, and horizontal lines represent median values.

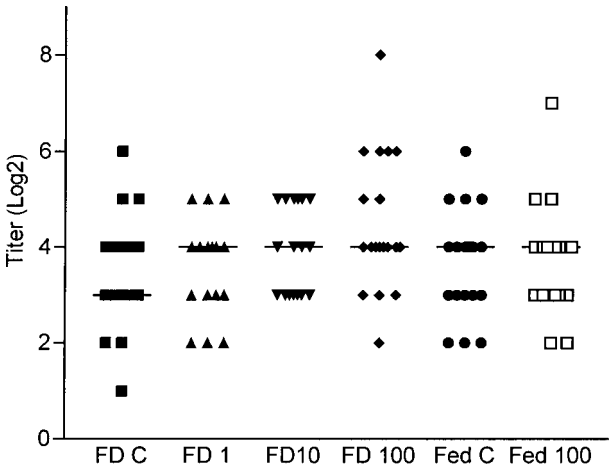

Fig. 4. Agglutination of *Aeromonas salmonicida* by plasma from Arctic charr four months after fish were orally contaminated with 0 (control [C]), 1, 10, or 100 mg Aroclor 1254 kg^{-1} fish and either fasted (FD) or fed (Fed). None of the groups differ significantly based on the Kruskal–Wallis nonparametric ranked sums test and Dunn's pairwise comparisons. Data are for individual fish, and horizontal lines represent median values.

sublethal PCB exposure increased resistance of rainbow trout exposed to the bacterial pathogens *A. hydrophila* [20] and *Yersinia ruckeri* [21]. The variability in these studies most likely results from differing modes of exposure to the contaminant (ingestion, injection, or flush exposure) and the pathogen (injection, flush exposure, or cohabitation), different compositions of congeners in the PCB mixtures, and perhaps, differences in the pathogen used. We believe that PCB contamination by ingestion, and pathogen exposure by cohabitation, most closely mimic conditions in the wild, where PCBs biomagnify up trophic levels and most pathogens are transmitted horizontally. In the present study, ingested PCBs and nutritional status modified the innate immune responses and reduced disease resistance in a cohabitant disease challenge. Although the differences in survival between groups were not significant, a significant trend of decreasing survival was observed with increasing PCB treatment in the fasted groups, with percentage survivals ranking as follows: FD-100 $<$ FD-10 $<$ FD-1 $<$ FD-C (Fig. 1).

We were surprised that fish in the fed groups (Fed-C and Fed-100) had the lowest survival (Fig. 1), independent of whether they were contaminated with PCBs. Mortality from disease, or unspecified causes, was higher in starved compared to fed juvenile striped bass (*Morone saxatilis*) [22] and juvenile channel catfish (*Ictalurus punctatus*) [23]. Okwoche and Lovell [24] reported, however, that two-year old channel catfish that were starved for six months over the winter survived at an approximately eightfold higher rate than fish that were fed continuously before being challenged with a bacterial pathogen (*Edwardsiella ictaluri*). In that same study, starved less than 1-year-old catfish died at approximately twice the rate of fed fish, suggesting that a suite of physiological factors (e.g., development, maturity, bioenergetics) must influence survival of starved fish. The similarity of our results with an anadromous cold-water species as well as the results with a warm freshwater species [24] suggest that fish may have evolved to maintain a high level of disease resistance during times of naturally low food availability (i.e., when water temperatures drop in winter). One way that providing food in a laboratory experiment might decrease disease resistance is by increasing stress associated with establishing and maintaining dominance hierarchies and competition for food. Compared to dominant fish, subordinate fish in a hierarchy are stressed, as indicated by elevated plasma cortisol [25] or brain serotonergic activity [26], and have reduced innate immune responses [27]. Indeed, it has been reported that brain serotonergic activity increased in subordinate Arctic charr [26] and in stressed Arctic charr but was not affected by starvation [28]. The fed fish in our disease challenge were taken from experimental tanks in which they had been fed for four months; during this time, they presumably established hierarchies and experienced the resultant social stress. Such chronic stress in these fish would result in immune suppression and reduced disease resistance [29] compared to those that had been held without food before the disease challenge experiment. Somewhat contradictorily, we reported previously that our fasted control fish had significantly higher resting plasma cortisol levels than did fed control fish [6]. However, after a handling stress, plasma cortisol in the fed control fish was significantly higher than that in fish from all the fasted groups, independent of PCB contamination [6]. We speculate that another scenario leading to increased mortality in fed fish is that when all the fish were combined, the fish that were previously fed were more stressed (i.e., had higher cortisol levels) than the fasted fish, because the fed fish tried to reestablish hierarchies in anticipation of being fed. Stress-induced elevated cortisol then reduced the disease resistance [29] of fed fish.

In the present study, we also demonstrated that PCBs can modulate innate immune responses in Arctic charr. We monitored factors of the innate immune system (i.e., lysozyme and a putative lectin) because others have already shown PCB impacts on primary and secondary adaptive immune responses in laboratory studies [30]. Rice et al. [31] also demonstrated the value of assessing innate immune function in fish collected from a stream contaminated with PCBs and heavy metals. Furthermore, several others [17,32] identified plasma or serum constituents that correlated with salmonid resistance to *A. salmonicida*, the pathogen used in our disease challenge. Lysozymes are hydrolytic, antimicrobial peptides secreted by cells of the immune system and found in tissues and fluids throughout most vertebrates. As pattern-recognition molecules, lysozymes lyse the peptidoglycan polymer in bacterial cell walls. Our lysozyme data do not show a clear correlation with disease resistance (Fig. 2). However, this is not surprising, because reports of both positive and negative correlations between disease resistance and blood lysozyme activity have appeared. Balfry et al. [33] compared two strains of coho salmon and found that the strain with high plasma lysozyme also had higher survival when challenged with *V. anguillarum*. Grinde [11] also demonstrated that rainbow trout lysozymes could lyse several fish pathogens in vitro. On the contrary, others [34] reported that lysozyme in rainbow trout and Atlantic salmon were correlated negatively with resistance to several pathogens, including *A. salmonicida*. In the present study, plasma lysozyme activity in Arctic charr that were fed and received 100 mg Aroclor 1254 kg^{-1} body weight was significantly lower than that in all other groups, including fed controls (Fig. 2). This suggests that neither PCBs nor feeding (as opposed to starvation) affected plasma lysozyme activity independently but, rather, that the combination suppressed this variable. Previous reports concerning the effects of dietary deficiencies on innate immune variables suggest that the opposite effect (i.e., immunosuppression of starved fish) would be expected [35], indicating the possibility that starvation of anadromous Arctic charr over the winter is the norm to which they have adapted. As pointed out by Winberg et al. [28], both stress and starvation increase brain serotonergic activity (a measure of stress response) in mammals, but starvation does not affect brain serotonergic activity in Arctic charr.

Lysozyme activity in mucus from the skin of Fed-100 fish was also significantly lower than that in the fasted controls, as was that in all other groups except the FD-100 group (Fig. 2). Thus, it appears that interaction between nutrition and PCB contamination also affected activity of skin mucous lysozyme; however, no additivity or dose relation was observed in the effects. The lack of a PCB dose–related reduction in mucous lysozyme activity in the present study may indicate the involvement of other immune factors in the reduced disease resistance. It has been shown that skin mucous lysozyme acts synergistically with other antimicrobial peptides as a first line of defense for salmonids [36]. Similar to a study of lysozyme in Pacific salmon [12], the highest lysozyme activity we found was in mucus from the nares of Arctic charr; however, no effect of PCBs or nutrition was observed on nares lysozyme activity (Fig. 2).

Lectins are pattern-recognition receptors that recognize mo-

lecular patterns specific to, and invariant among, microbes. Interest in pattern-recognition receptors as innate factors of vertebrate immune systems has grown, because these molecules have similar functions throughout the plant and animal kingdoms and appear to be evolutionary and functional links between the adaptive (e.g., production of antigen-specific antibody) and innate immune responses [14]. Phylogenetically, adaptive immunity arose with teleost fishes—that is, more primitive plants and animals have only innate immunity—so it seems to be reasonable that salmonids would still rely significantly on innate immunity for protection against microbial pathogens. Lectins in fish (and other vertebrates) are preliminarily defined by which species of RBCs they agglutinate, their carbohydrate specificity, and whether they are Ca^{2+}-dependent. The plasma lectin we monitored in Arctic charr was Ca^{2+}-independent and hemagglutinated rabbit RBCs; this hemagglutination was most easily inhibited by L(−) rhamnose. These variables matched one of two lectins (designated WCL1) identified in white-spotted charr (*S. leucomaenis*), the amino acid sequence of which has 91% similarity to steelhead lectin (STL1), which originates in the liver and is found in the blood as well as the immune organs and ovaries [37,38].

In the present study, hemagglutination by the putative lectin was reduced significantly in fasted Arctic charr given mid or high doses of Aroclor 1254 (Fig. 3). Several researchers have reported that lectins in rainbow trout and Atlantic salmon bound *A. salmonicida* [13,32] or enhanced immune responses [17]; however, these were Ca^{2+}-dependent, mannose-binding molecules. Plasma from Arctic charr agglutinated *A. salmonicida* (Fig. 4). However, the binding was relatively low, and no indication was observed that PCBs affected that binding. It is important to note that all six of the RBC species we tested were agglutinated to some degree by the Arctic charr plasma, which suggests the presence of other agglutinins (putative lectins) that we did not characterize. We chose instead to focus on the RBCs with maximum hemagglutination. It is possible that a Ca^{2+}-dependent, mannose-binding lectin is more important in conferring resistance to *A. salmonicida* than the Ca^{2+}-independent, L(-)rhamnose lectin we identified. Indeed, Bricknell et al. [39] reported that rhamnose is a major component of *A. salmonicida* cell wall extracts, which have very little mannose—the dominate sugar in the *A. salmonicida* culture supernatants (i.e., extracellular product). Moreover, passive immunization of Atlantic salmon with antisera to the cell wall extracts (i.e., high in rhamnose) afforded no protection when fish were challenged with the bacteria, whereas antisera to the extracellular products (i.e., high in mannose) increased survival significantly [39]. Other acute-phase proteins, such as serum amyloid-A (SAA), may also be important in resistance to *A. salmonicida*. The SAA mRNA was not detectable in livers of Arctic charr before challenge (intramuscular injection) with *A. salmonicida*, was first detected 48 h after injection, and was 40-fold higher at 120 h after injection [40]. Our experimental design (i.e., determining innate immune responses before pathogen challenge) did not allow us to consider effects of PCBs on SAA.

CONCLUSION

In summary, the present study has shown that dietary contamination with PCBs and subsequent long-term starvation—mimicking natural feeding–fasting cycles of animals in the Arctic—can reduce disease resistance of Arctic charr. Determining the role of starvation in disease resistance is clouded by possible stress responses of fish that were fed before being moved into the disease challenge tank. Other studies on the effects of establishing social hierarchies, stress, and starvation in Arctic charr [26,28] support our view that long-term starvation is the norm for anadromous Arctic charr and that feeding the fish contributed to high mortalities in those groups. Innate immune responses (skin mucus lysozyme and plasma lectin) decreased significantly in PCB-treated fish; however, additional studies are needed to determine if these are mechanisms by which PCBs impair disease resistance. It is important to note that disease resistance in Arctic charr seemed to be decreased even by very low PCB burdens in fasted fish, whereas a very high dose of Aroclor 1254 in fed fish had no effect relative to fed controls. This observation supports previous findings that long-term fasting and emaciation potentiate the sensitivity of fish to persistent, lipophilic pollutants, such as PCB [4–6]. Hence, lipid status seems to be an important determinant for dose–response relations and must be considered in ecotoxicological studies of fish.

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