Quantitative Polymerase Chain Reaction for Transforming Growth Factor-β Applied to a Field Study of Fish Health in Chesapeake Bay Tributaries

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Fish morbidity and mortality events in Chesapeake Bay tributaries have aroused concern over the health of this important aquatic ecosystem. We applied a recently described method for quantifying mRNA of an immunosuppressive cytokine, transforming growth factor-β (TGF-β), by reverse transcription quantitative-competitive polymerase chain reaction to a field study of fish health in the Chesapeake Basin, and compared the results to those of a traditional cellular immunoassay macrophage bactericidal activity. We selected the white perch (*Morone americana*) as the sentinel fish species because of its abundance at all of the collection sites. White perch were sampled from Chesapeake Bay tributaries in June, August, and October 1998. Splenic mononuclear cell TGF-β mRNA levels increased and anterior kidney macrophage bactericidal activity decreased, particularly in eastern shore tributaries, from June to August and October. The results of the two assays correlated inversely (Kendall's τ b = -0.600; p = 0.0102). The results indicated both temporal and spatial modulation of white perch immune systems in the Chesapeake Basin, and demonstrated the utility of quantitative PCR for TGF-β as a molecular biomarker for field assessment of teleost fish immune status. ***Key words*: Chesapeake Bay, field study, macrophage bactericidal activity, *Morone americana*, quantitative PCR, transforming growth factor-β, white perch. *Environ Health Perspect* 108:447–452 (2000). [Online 28 March 2000]**

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Marine and freshwater models for environmental health research are receiving increasing attention (*1*), and fish immunology is one focus of this attention (*2*). The assessment of fish immune status in response to environmental conditions has by necessity been dominated by assays not requiring species-specific or taxon-specific reagents. Examples include lymphoid organ weight and histology, hematology, serum protein and protein fraction quantification, disease incidence or response to pathogen challenge, lymphocyte blastogenesis, and macrophage and granulocyte functional assays measuring chemotaxis, phagocytosis, respiratory burst, and bactericidal activity (*2–5*). Recent studies in fish have focused on the detection of cytokines, proteins that regulate cellular and immune function. Evidence for the presence of specific cytokines in fish was indirect (*6,7*) until the cloning and sequencing of transforming growth factor (TGF)-β (*8,9*).

TGF-βs are cytokines with diverse functions that affect cell growth and differentiation, extracellular matrix regulation, wound healing, and immune function (*10–14*). TGF-β immunoregulatory properties are primarily suppressive. Immune functions down-regulated by TGF-β include the following: major histocompatibility complex and Fc receptor expression, some cytokine production, thymocyte proliferation, T- and B-cell proliferation, IgG and IgM production, IL-2 receptor expression, cytotoxic T-cell generation and function, lymphokine-activated killer and natural killer cell activation and function, macrophage activation, macrophage respiratory burst activity, neutrophil adhesion to endothelium, and hematopoiesis (*13*). Three isoforms of TGF-β (-β_1, -β_2, and -β_3) are expressed by mammals. In addition, one (-β_4) has been identified from the chicken, and one (-β_5) from the African clawed frog, *Xenopus laevis*. TGF-β_4 and -β_5 are considered homologues of mammalian TGF-β_1 (*15*). TGF-β_1 is the major TGF-β isoform within the context of the mammalian immune system (*10*).

Coding sequences for a TGF-β from hybrid striped bass [*Morone saxatilis* × *M. chrysops*, Genbank (*16*) accession no. AF140363 (*9*)] and rainbow trout [*Oncorhynchus mykiss*, Genbank (*16*) accession no. AJ007836 (*8,17*)] grouping with *Xenopus* TGF-β_5, chicken TGF-β_4, and mammalian TGF-β_1, were recently reported. Homology with TGF-β_1 suggests that the fish TGF-β isolates may also be important in regulating immune responses in these species. Evidence for the conserved nature of TGF-β activity in fish is provided by both biologic cross-reactivity and antigenic cross-reactivity. Bovine TGF-β_1 enhanced respiratory burst activity of resting rainbow trout macrophages at low doses. At higher doses, however, bovine TGF-β_1 inhibited activated rainbow trout macrophages and countered the effects of activating signals on resting macrophages (*18*). Chicken antiporcine TGF-β_1 antibodies enhanced the ability of rainbow trout leukocyte-derived supernatants to stimulate respiratory burst activity of anterior kidney macrophages (*19*).

The ability to measure TGF-β mRNA production in fish could provide a means to detect dysfunctional responses to stressors in the aquatic environment before the development of overt infectious disease, developmental abnormalities, or neoplasia. Harms et al. (*20*) used a quantitative polymerase chain reaction (PCR) technique to measure TGF-β mRNA from lymphoid cells of teleost fish (*9*) in a controlled setting to demonstrate an inverse relationship between anterior kidney macrophage bactericidal activity and splenic mononuclear cell TGF-β mRNA levels in hybrid striped bass treated with a known immunomodulator. The application of quantitative PCR for TGF-β to a field study of fish health would help validate its use as an immunologic marker in more complex settings. The opportunity for field application of the technique was presented by recent fish mortality and ulcerative skin lesion events in Chesapeake Bay tributaries.

Atlantic menhaden (*Brevoortia tyrannus*) mortalities and ulcerative skin lesions with a predilection for the anal area occurred during August and September 1997 in Maryland tributaries of the Chesapeake Bay, including the Pocomoke River, Pocomoke

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Sound, Chicamacomico River, and Kings Creek, a tributary of the Manokin River (*21,22*). Menhaden lesions were characterized by necrotizing dermatitis and myositis with severe chronic granulomatous inflammation containing aseptate fungal hyphae (*23,24*). Similar ulcerative mycosis lesions were originally described in menhaden from North Carolina in the early 1980s (*25*). More recently these lesions and mortality events of menhaden have been associated with blooms of the toxic dinoflagellate, *Pfiesteria piscicida* (*26,27*). Although acute dermonecrotic lesions of fish have been induced in laboratory studies of *P. piscicida* (*28*), the histologic appearance of chronicity suggests caution in linking the skin lesions of wild menhaden to the acute effects of the dinoflagellate toxin (*23,24*).

The morbidity and mortality exhibited by Pocomoke-area menhaden, in addition to the increased prevalence of lesions in other fish species, provided the impetus for a broad-based study of fish health in Chesapeake Bay tributaries. The study was conducted by the U.S. Geological Survey's National Fish Health Research Laboratory (Kearneysville, WV) and the Maryland Department of Natural Resources (Stevensville, MD). However, menhaden were not sufficiently abundant at all of the sites scheduled for monitoring. Therefore, the white perch (*Morone americana*) was used as the sentinel fish species because of its abundance at all of the collection sites. In addition, methods for TGF-β reverse transcription quantitative-competitive PCR (RT-qcPCR) have been validated for white perch (*9*), and its position in the middle to upper fraction of the water column makes it less likely to be impacted by toxins accumulated in the sediment.

Our objectives were to apply the newly-described method for measuring TGF-β mRNA production in teleost fish (*9*) to a field study of fish health, and to relate constitutive TGF-β transcription levels to a standard cellular immune function test, macrophage bactericidal activity, under complex field conditions.

Materials and Methods

Fish and collection sites. In the summer and fall of 1998, we collected adult white perch from the lower Pocomoke [37°58′ N, 75°38′ W (all coordinates ± 1′); collected on 16 June, 13 August, and 5 October], the Wicomico (38°15′ N, 75°48′ W; collected on 17 June, 4 August, and 14 October), and the Choptank (38°39′ N, 75°58′ W; collected on 24 June, 3 August, and 7 October) Rivers on the Eastern Shore of the Chesapeake Bay. Perch were also collected from the Back River (39°15′ N, 76°27′ W; collected on 10 June, 12 August, and 8 October) on the western shore of the bay. We timed the collections to span the periods before and after dates when menhaden mortalities were recorded from the Pocomoke the previous August.

The Pocomoke and Wicomico Rivers pass through rural areas of intense poultry rearing and are subject to high levels of nutrient run-off (*21*). There were menhaden mortalities in the Pocomoke in August 1997. The Choptank River also drains a rural area but is further removed from the areas of reported fish mortality. However, *Pfiesteria* sp. was identified from sediments of Jenkins Creek of the Choptank River in 1992 (*29*). The Back River runs through an industrialized area of Baltimore, Maryland, and was thought likely to contain fish with altered immune function. We recorded air temperature, water depth, surface and bottom water temperature, dissolved oxygen, pH, and salinity at each sample site.

We collected fish by 4.9-m semiballoon otter trawl in 10-min tows starting between 0715 and 0918 hr. Twenty-four white perch > 170 mm total length from each site were collected and transported 15–30 min in a live well to shore for processing. Nonquantitative observations of skin lesions were made before the release of menhaden captured incidentally.

Fish processing. White perch were euthanized with an overdose of tricaine methanesulfonate. We recorded the weight and total length and noted the presence of external lesions. Fish were bled from the caudal vein for related hematology studies, and to reduce peripheral blood mononuclear cell content of anterior kidneys and spleens. We determined packed cell volume (PCV) and plasma total solids by refractometry on site. Spleens and anterior kidneys were aseptically harvested for TGF-β mRNA quantification and cellular immunoassays. Sex was determined by internal examination.

TGF-β RT-qcPCR (spleen). We placed spleens from 10 white perch in sterile transport medium [RPMI 1640 plus 10% heat-inactivated fetal bovine serum (FBS), 100 U/mL penicillin, 100 µg/mL streptomycin, 2 mM EDTA; subsequently referred to as complete RPMI] at 4°C. The perch were transported on wet ice by overnight courier to the laboratory, where they were processed for TGF-β mRNA quantification.

We prepared mononuclear cells for RT-qcPCR as previously described (*9*). Spleens were minced finely, forced through a fine wire mesh, and resuspended in complete RPMI. Spleen homogenates were centrifuged on two-step Percoll gradients (specific gravity 1.053 and 1.066 g/mL in 0.15 M saline) at 400*g* for 5 min, then 800*g* for 20 min at 4°C. Mononuclear cells were harvested from the 1.053/1.066 g/mL interface. Cells were washed twice in complete RPMI. Viable cell counts were performed with cells suspended in 0.2% trypan blue, and differential counts were performed on cytospin preparations stained with LeukoStat (Fisher Scientific, Pittsburgh, PA). Cell viability typically exceeded 95%.

Total RNA was isolated by the guanidine thiocyanate method (Tri Reagent; Molecular Research Center, Cincinnati, OH). The RNA pellet was resuspended in sterile diethyl pyrocarbonate-treated water at a concentration of 5×10^4 cell equivalents/µL. Messenger RNA was reverse transcribed (Superscript II RT; Gibco-BRL, Gaithersburg, MD) to cDNA with oligo dT15 priming of 3×10^6 cell equivalents of total RNA. We stored samples of cDNA at -20°C until their use. Negative RT controls were run in parallel.

We performed RT-qcPCR based on a previously described procedure (*30*) that was further developed using primers and competitive fragments specific for teleost fish TGF-β and β-actin (*9*). Each competitive PCR contained 2.5×10^4 cell equivalents of cDNA as prepared above. The final reaction volume of the competitive PCR was 25 µL, and was reached by adding 20 µL of a PCR master mix to 5 µL of each competitive fragment dilution (1.5×10^8 copies down to 6 copies) in a Thermowell 96-well thin-wall polycarbonate plate (Costar, Acton, MA) on ice. The final reaction concentrations were 1 × PCR buffer [10 mM Tris-HCl (pH 8.3), 1.5 mM $MgCl_2$, 50 mM KCl], 250 µM each dNTP, 0.375 µM each primer, 30 µU/µL Taq DNA polymerase. Reactions were cycled on a PTC-100 thermocycler (MJ Research, Inc., Watertown, MA) at 94°C for 1 min, (94°C, 30 sec denature; 55°C, 1.5 min anneal; 72°C, 2 min extend) × 35 cycles, with a final extension at 72°C for 7 min.

PCR products were separated in 2% agarose gels in TAE, stained with ethidium bromide, and photographed on a 300-nm ultraviolet transilluminator. The images were digitized and analyzed with the Alphaimager 2000 Documentation and Analysis System (Alpha Innotech Co., San Leandro, CA). We measured fluorescence of target and competitor bands in each lane; these were expressed as area under the curve. We compensated for fluorescence differences due to molecular weight differences by the formula:

$$CFR = \frac{TF(\text{area})}{CF(\text{area})} \times \frac{CS(\text{bp})}{TS(\text{bp})}$$

where CFR = the corrected fluorescence ratio, TF = the target fluorescence, CS = competitor size, CF = competitor fluorescence, and TS = target size. The $\log_{10}$ of the CFR was plotted against the $\log_{10}$ of the number of copies of

competitor in the sample, and the point of molecular equivalence (the point at which the copy number of target cDNA equals the copy number of competitor DNA) is the *x*-intercept. Finally, to control for sample-to-sample variation in RNA isolation, reverse transcription, amplification, and gel loading during quantification, we normalized the TGF-β results to those of the housekeeping gene, β-actin.

Macrophage bactericidal assay (anterior kidney). We isolated mononuclear cells from the anterior kidney using a modification of the procedures described by Sharp et al. (*31*). Cells were kept cold during processing except where noted. Aseptically harvested anterior kidney tissues from individual white perch were homogenized in 10 ml of L-15 medium supplemented with 2% FBS, 100 U/mL penicillin, 100 µg/mL streptomycin, and 10 U/mL sodium heparin (L-15/2% FBS). Homogenates were produced at the sample sites and transported back to the laboratory on ice. Transportation time ranged from 3 to 5 hr.

Homogenized anterior kidney tissues were pelleted by centrifugation at 4°C for 10 min at 500*g*. Tissues were washed by resuspension in 5 mL of L-15/2% FBS followed by centrifugation. Washed cell suspensions were resuspended in 6 mL of L-15/2% FBS and layered on a single step Percoll gradient [density 1.047 g/mL in Hank's balanced salt solution (HBSS) without phenol red]. Cell suspensions on Percoll were centrifuged at 4°C for 20 min at 400*g* and then leukocytes were removed from the media/Percoll interface. Leukocytes were pelleted at 500*g*, washed as described previously, and resuspended in 3 ml L-15/2% for counting. We determined the number of viable leukocytes isolated from each fish by trypan blue exclusion (0.1% trypan blue in L-15/2% FBS) and the cells were pelleted at 500*g* as described previously. Leukocytes were resuspended at 2×10^7 viable cells/mL in L-15, supplemented with 0.1% FBS, 100 U/mL penicillin, and 100 µg/mL streptomycin (L-15/0.1% FBS). We loaded leukocytes in L-15/0.1% FBS into 96-well tissue-culture plates at 100 µL/well and incubated them for 2 hr in a container of humidified air at 20°C. After incubation, we removed the L-15/0.1% FBS containing nonadherent cells and replaced it with 100 µL/well of L-15/5% FBS at 20°C. We evaluated the adherent cells for macrophage bactericidal activity.

We determined bactericidal activity using a modification of the assay described by Graham et al. (*32*). The temperature of all solutions was adjusted to 20°C before use. Forty-eight-hour cultures of *Yersinia ruckeri* (Hagerman strain; National Fish Health Research Laboratory no. 11.40) were washed 3 times in HBSS and the suspension density was adjusted to 0.15 OD600 in HBSS.

After 36 hr incubation in a humidified container at 20°C, the L-15/5% was removed from the wells of macrophage plates and replaced with 100 µL/well of unsupplemented L-15. The L-15 was then replaced with an equal volume of L-15/5% without antibiotics and *Y. ruckeri* suspension was added at 25 µL/well. Plates were incubated for 4 hr at 20°C in a humidified container. After incubation, we removed the media from the wells and replaced it with 50 µL/well of 0.2% Tween 20 in tissue-culture grade water. The resulting well contents were serially diluted ($\log_{10}$) in tryptic soy broth. Dilutions were plated as 10 µL drops on tryptic soy agar plates and incubated for 24 hr at room temperature. We determined the number of colony-forming units (CFUs) for each well at the 10^{-4} or 10^{-5} dilution. Bactericidal activity was expressed as % CFU reduction = (1 - CFU treated/CFU control) × 100, where CFU treated = the replicate mean number of CFUs in wells containing adherent leukocytes ($n = 3$) and CFU control = the replicate mean number of CFUs from wells with media only ($n = 6$). The number of CFU treated was replicated on a per-fish (cell source) basis. The number of CFU control was replicated on a per-plate basis (i.e., all of the CFU treated mean values from a given plate were compared to a single CFU control mean value).

Statistical analyses. We used nonparametric statistical tests for comparisons due to nonnormal distribution of TGF-β transcription and macrophage bactericidal activity data from some sample sites (Shapiro-Wilk test; JMP statistical software, SAS Institute, Inc., Cary, NC). Values for splenic mononuclear cell TGF-β transcription, anterior kidney macrophage bactericidal activity, and fish length and weight were grouped according to sample (month and locality) and analyzed by the Kruskal-Wallis test against the null hypothesis that sample medians did not differ (*33*). We performed Dunn's multiple comparison test after significant Kruskal-Wallis tests to detect which pairs of sample medians differed (*33*). We used Kendall's τ-b to test for correlation of variables according to samplings (JMP). We performed least-squares linear regression (JMP) using sample medians to illustrate the inverse association between TGF-β transcription and macrophage bactericidal activity. Sex distribution was compared between samplings by χ^2. Significance was set at $p < 0.05$ in all cases.

Results

White perch length (overall mean ± SD = 197 ± 17.7 mm) and weight (112 ± 30.8 g) did not differ significantly between sample sites and months (Kruskal-Wallis, $p > 0.05$). There was a predominance of females in the sample population (65.5%, range 37.5–90.1% females), but sex distribution was not significantly different between samplings (χ^2, $p > 0.05$).

Menhaden with deep ulcerative skin lesions were collected incidentally from the Pocomoke and Wicomico Rivers in August and the Wicomico and Choptank Rivers in October, although no mass mortalities were reported in the summer of 1998. External lesions of white perch were minor, mostly consistent with capture and transport, and included foci of erythematous skin and reddened or frayed fins in 23% (range 0–60%) of fish sampled for TGF-β determination (Table 1). Mean PCVs ranged from 29.1 to 49.3%, and plasma total solids from 4.3–6.1 g/dL (Table 1). Physical measurement ranges for air temperature, surface water temperature, surface dissolved oxygen, surface pH, and surface salinity were 18–31.8°C, 18–28.1°C, 4.7–8.1 mg/L, 7.1–8.8, and 0.0–9.9 g/L, respectively; bottom-water temperature, bottom dissolved oxygen, bottom pH, bottom salinity, and bottom sample depths were 18.0–27.8°C, 2.4–6.9 mg/L, 7.1–8.8, 0.0–9.9 g/L, and 1.2–9.0 m, respectively (Table 2).

Splenic mononuclear cell TGF-β:β-actin ratios varied significantly by collection site and month (Kruskal-Wallis test, $p < 0.0001$; Figure 1). Although TGF-β transcription was greatest initially from Back River fish in June, over the course of the sampling period the levels remained relatively constant while they increased in fish from the Eastern Shore tributaries. The increase was greatest for fish from the Wicomico River.

Anterior kidney macrophage bactericidal activity also varied significantly by collection site and month (Kruskal-Wallis test, *p*

Table 1. Skin lesions, PCV, and plasma total solids in white perch of Chesapeake Bay tributaries in June, August, and October 1998.[a]

Month/river	Skin lesions (%)	PCV (%) ± SD	Plasma total solids (g/dL) ± SD
June			
Back	20	39.2 ± 5.6	6.1 ± 0.5
Choptank	60	29.9 ± 6.3	5.0 ± 1.2
Pocomoke	40	30.1 ± 6.1	4.4 ± 0.9
Wicomico	0	40.0 ± 5.7	5.1 ± 0.9
August			
Back	30	32.3 ± 6.2	4.7 ± 0.7
Choptank	36	37.5 ± 4.7	5.3 ± 0.8
Pocomoke	10	34.4 ± 4.9	4.8 ± 0.5
Wicomico	0	35.5 ± 3.2	4.5 ± 0.6
October			
Back	10	49.3 ± 7.3	5.3 ± 0.8
Choptank	20	39.1 ± 4.4	5.0 ± 1.2
Pocomoke	20	45.5 ± 8.5	5.4 ± 1.2
Wicomico	30	35.5 ± 3.1	4.5 ± 0.6

[a] $n = 10$ at each sample site and time, except for the Choptank River in August ($n = 11$).

Table 2. Physical measures from Chesapeake Bay tributaries obtained during fish sampling in June, August, and October 1998.

Month/river	Air temp (°C)	Water temp (°C) surface/bottom	pH surface/bottom	Dissolved oxygen (mg/L)	Salinity (g/L) surface/bottom	Depth (m)
June						
Back	31.0	28.1/27.8	6.5/6.1	8.0/8.1	0.2/0.2	1.2
Choptank	30.8	22.1/22.1	6.2/6.1	7.1/7.1	0.0/0.0	3.3
Pocomoke	29.2	22.8/22.6	5.3/5.1	7.2/7.2	2.4/2.4	4.5
Wicomico	29.9	24.7/24.6	6.8/6.9	7.3/7.3	5.5/6.8	3.1
August						
Back	31.2	27.8/27.6	8.1/5.3	8.4/8.4	2.1/3.0	2.3
Choptank	29.6	26.1/26.1	5.5/2.4	7.3/7.3	4.3/4.4	7.4
Pocomoke	31.8	27.8/27.8	4.7/4.7	7.3/7.3	8.4/9.0	3.9
Wicomico	29.4	26.1/26.1	5.1/5.1	7.3/7.3	5.4/5.5	2.5
October						
Back	18.0	18.0/18.0	6.4/6.4	8.8/8.8	5.4/5.4	5.0
Choptank	19.0	19.0/19.0	6.8/6.8	7.6/7.6	8.1/8.1	7.0
Pocomoke	21.1	20.0/20.0	5.7/5.7	7.4/7.4	7.5/7.5	6.0
Wicomico	18.0	18.3/18.3	6.8/6.8	7.8/7.8	9.9/9.9	9.0

< 0.0001), but with a pattern inverse to that of the TGF-β results (Figure 2). Similar to the TGF-β results, bactericidal activity from Back River fish varied least as compared to fish from the other sites throughout the sampling period. The general decline in bactericidal activity observed from June to October was greatest for the Eastern Shore tributary fish as compared to fish from the Back River.

Sample medians for splenic mononuclear cell TGF-β:β-actin ratios correlated inversely with anterior kidney macrophage bactericidal activity (Kendall's τ b = -0.600, p = 0.0102). This inverse correlation is illustrated by least-squares linear regression (Figure 3). There were no significant correlations of splenic mononuclear cell TGF-β mRNA levels with sex, weight, length, presence of skin lesions, PCV, plasma total solids, air temperature, surface or bottom temperature, dissolved oxygen, or pH. There was a positive association of TGF-β mRNA levels with both surface and bottom salinity (Kendall's τ b = 0.504 and 0.546, p = 0.0233 and 0.0136 for surface and bottom, respectively). Anterior kidney macrophage bactericidal activity correlated significantly (inversely) only with splenic mononuclear cell TGF-β mRNA levels.

Discussion

This report marks the first time that quantitative measurement of specific cytokine production has been used in a field assessment of fish health. There were significant differences in splenic mononuclear cell TGF-β:β-actin ratios based on sample time and location. Constitutive TGF-β production generally increased from June to August and October, primarily in the Chesapeake Bay Eastern Shore tributaries. Based on its primarily immunosuppressive effects, elevated TGF-β production could be interpreted as an indicator of immunosuppression. However, TGF-β has some proinflammatory effects, including promotion of macrophage and neutrophil chemotaxis (*13*). Whether TGF-β is pro- or antiinflammatory depends on its concentration, the state of differentiation of target cells, and the concentration of other proinflammatory compounds (*11*). Therefore, TGF-β:β-actin ratios should be interpreted in the context of other health and immune function indicators. As compared to splenic mononuclear cell TGF-β transcription, anterior kidney macrophage bactericidal activity declined from June to August and October, and bactericidal activity correlated inversely with TGF-β mRNA levels. The inverse relationship between TGF-β production and macrophage bactericidal activity was expected based on the known effects of TGF-β on macrophage activation and respiratory burst (*13*). This field-based finding of inverse correlation between the two assays also concurred with a laboratory based study of hybrid striped bass in response to administration of a known immunomodulator, triamcinolone (*20*). The agreement between the quantitative PCR for TGF-β and the functional cellular immunoassay, conducted in two separate laboratories, indicates the occurrence of immunomodulation consistent with immunosuppression both spatially and temporally in white perch of the Chesapeake Basin. This immunomodulation took place in the absence of the widespread fish mortalities noted the previous year, and may indicate a seasonal and site predisposition to disease outbreaks under appropriate adverse conditions.

The immunomodulation detected (lower bactericidal activity and higher TGF-β mRNA) coincided with the qualitative observations of deep ulcerative lesions in

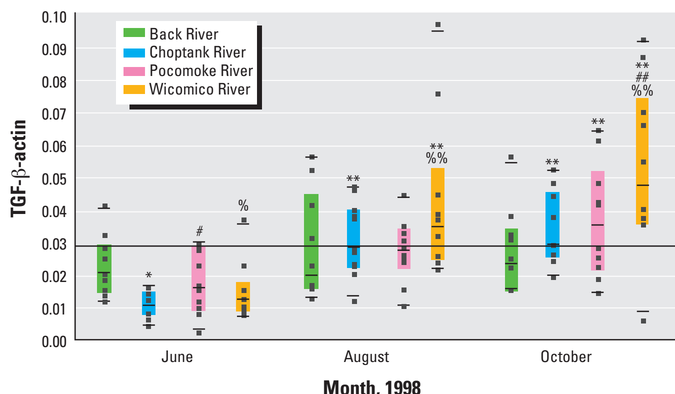

Figure 1. TGF-β:β-actin ratios varied significantly by month and river (Kruskal-Wallis test, p < 0.0001; n = 10 for all, except August in the Choptank River, where n = 11). Quantile boxes show nonparametric measures of dispersion: the 10th, 25th, 50th (median), 75th, and 90th quantiles; the horizontal line indicates the total response sample mean. Sample pairs that differed significantly from each other are indicated by single versus double shared symbols (Dunn's multiple comparison test, α < 0.05); all other sample pairs did not differ significantly.

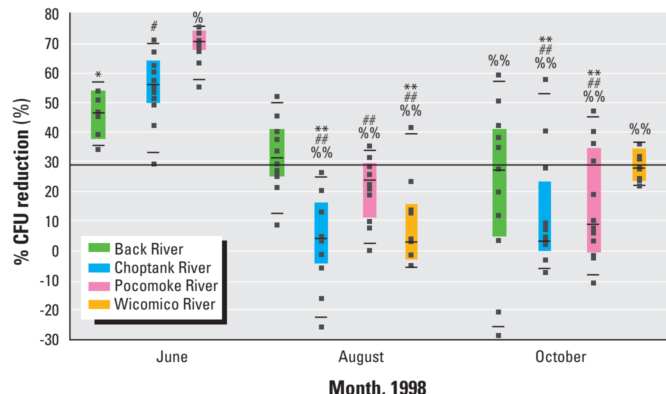

Figure 2. Macrophage bactericidal activity [CFU reduction (%)] varied significantly by month and river (Kruskal-Wallis test, p < 0.0001; n = 12 for all, except August in the Wicomico River, where n = 10). Quantile boxes show nonparametric measures of dispersion: the 10th, 25th, 50th (median), 75th, and 90th quantiles; the horizontal line indicates the total response sample mean. There were no bactericidal activity data for the Pocomoke River in June. Sample pairs that differed significantly from each other are indicated by single versus double shared symbols (Dunn's multiple comparison test, α < 0.05); all other sample pairs did not differ significantly.

menhaden collected in the same areas. The prevalence of skin and fin lesions in white perch did not correlate with TGF-β production or macrophage bactericidal activity, but the white perch lesions were minor as compared to observed and previously reported menhaden lesions, and may have represented acute effects secondary to capture and transport. Two indicators of general health, PCV and plasma total solids, also varied according to sample site and time (Table 1, statistical analyses not shown), but did not correlate with the more specific immunologic assessments of splenic mononuclear cell TGF-β production and anterior kidney macrophage bactericidal activity.

Production of TGF-β varies during development (*12*); therefore, it could vary according to age even in adult fish. In this study, the age of white perch was assessed indirectly by length and weight, which correlate with age in white perch (*34*). There were no significant length and weight differences between samplings, and TGF-β mRNA levels did not correlate with either length or weight, suggesting that age was not a factor in TGF-β differences. Sex and reproductive status could also affect TGF-β production because estrogens induce the production of both TGF-β_1 and TGF-β_2 in mammals (*35*). However, there were no significant differences in sex distribution between samplings, and there was no correlation between TGF-β:β-actin ratios and sex. White perch spawn from late March to May in the Chesapeake (*34*). Outdoor laboratory-reared white perch from the Roanoke River in North Carolina spawned in nearly the same time frame and had basal concentrations of testosterone and estradiol from June through October (*36*). Reproductive status and sex therefore most likely had no effect on the immunologic assays in this study.

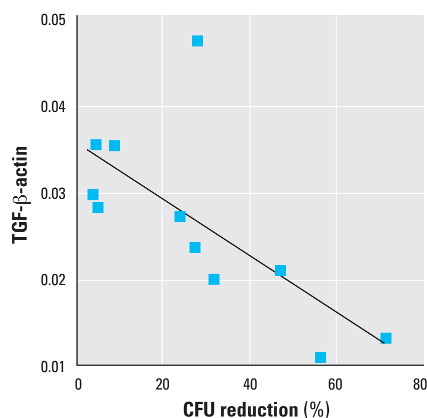

Figure 3. Least-squares linear regression of TGF-β:β-actin ratios versus macrophage bactericidal activity [CFU reduction (%)] sample medians to illustrate inverse correlation (Kendall's τ b = -0.600; *p* = 0.0102).

Of the physical conditions measured at the sample sites, none of the following correlated with TGF-β:β-actin ratios or macrophage bactericidal activity: air temperature, water temperature, pH, depth, and dissolved oxygen. Hypoxic conditions have been suggested as a possible factor in the development of menhaden ulcerative lesions (*27*), as well as other fish morbidity and mortality events in estuarine environments. Dangerously low dissolved oxygen (2.4 mg/L) was detected in a bottom-water sample from the Choptank River in August, although the surface water level was an acceptable 5.5 mg/L (*36*). The failure to detect associations between these physical conditions and the immune function indicators measured in this study may mean that parameters were within a range tolerable by white perch, or that adverse conditions were transient and not detected with the sample schedule that we used. The estuarine environment is mutable with the tides and with river flow rates, and any of the parameters we measured could vary considerably between sampling times. A pulsatile insult affecting immune function might no longer prevail at the time of sampling. Furthermore, freely moving fish collected during a field study might be recent immigrants to the sample location and may have been subject to different conditions before their capture.

Salinity measurements did correlate with TGF-β:β-actin ratios, but an inverse association with macrophage bactericidal activity was not statistically significant. Osmotic shock alters the synthesis of specific proteins by hemocytes of the American oyster (*Crassostrea virginica*), an estuarine osmoconformer (*38*). Osmoregulatory mechanisms could account, in part, for altered TGF-β production by teleost fish responding to salinity changes in estuarine environments. Prolactin and cortisol both play a role in regulating electrolyte flux across the gills (*39*), and sequential prolactin and cortisol surges are important for smoltification of anadromous fish as they make the transition to seawater (*40*). In mammals, high blood prolactin concentrations correlate with TGF-β_2 levels in rat milk (*41*), and human T cells increase transcription of TGF-β_1 in response to the synthetic glucocorticoid dexamethasone (*42,43*). Osmoregulatory mechanisms could, therefore, affect the immune systems of estuarine fish as they move between waters of varying salinity. Alternatively, differences in salinity could be secondary to other conditions, such as river flow rates, and have no direct effect on TGF-β transcription. The interactions between salinity, osmoregulatory mechanisms, and TGF-β production of teleost fish will require investigation under controlled conditions to verify the field association reported here.

We did not assess other conditions that could affect fish immune function in Chesapeake Bay tributaries, such as nutrient load, toxic algae blooms, heavy metals, pesticides, and other toxins. Normal seasonal variations in immune function could play a role in the observed immunomodulation. Seasonal cues such as photoperiod and temperature, acting through the neuroendocrine system, have been proposed as responsible for multiple changes in immune function indicators in ectotherms (*44*). Irrespective of the proximate and ultimate causes, natural or anthropogenic, the results presented here indicate temporal and spatial immunomodulation in white perch of the Chesapeake Basin in a pattern consistent with previously observed fish morbidity and mortality events. The addition of a molecular-based assay for a specific cytokine, TGF-β, applicable to a wide range of teleost species occupying varied habitats promises to augment our ability to assess fish immune systems in response to experimental and natural conditions of interest.

References and Notes

1. Bonaventura C. NIEHS Workshop: Unique Marine/Freshwater Models for Environmental Health Research. Environ Health Perspect 107:89–92 (1999).
2. Stolen JS, Fletcher TC, eds. Modulators of Fish Immune Responses, Vol I: Models for Environmental Toxicology, Biomarkers, Immunostimulators. Fair Haven, NJ:SOS Publications, 1994.
3. Anderson DP. Environmental factors in fish health: immunological aspects. In: The Fish Immune System: Organism, Pathogen, and Environment (Iwama G, Nakanishi T, eds). San Diego, CA:Academic Press, 1996;289–310.
4. Dunier M, Siwicki AK. Effects of pesticides and other organic pollutants in the aquatic environment on immunity of fish: a review. Fish Shellfish Immunol 3:423–438 (1993).
5. Zelikoff JT, Enane NA, Bowser D, Squibb KS, Frenkel K. Development of fish peritoneal macrophages as a model for higher vertebrates in immunotoxicological studies. Fundam Applied Toxicol 16:576–589 (1991).
6. Secombes CJ. The phylogeny of cytokines. In: The Cytokine Handbook, 2nd ed (Thomson AW, ed). London:Academic Press, 1994;567–594.
7. Secombes CJ, Hardie LJ, Daniels G. Cytokines in fish: an update. Fish Shellfish Immunol 6:291–304 (1996).
8. Hardie LJ, Laing KJ, Daniels GD, Grabowski PS, Cunningham C, Secombes CJ. Isolation of the first piscine transforming growth factor β gene: analysis reveals tissue specific expression and a potential regulatory sequence in rainbow trout (*Oncorhynchus mykiss*). Cytokine 10:555–563 (1998).
9. Harms CA, Kennedy-Stoskopf S, Horne WA, Fuller FJ, Tompkins WAF. Cloning and sequencing hybrid striped bass (*Morone saxatilis* × *M. chrysops*) transforming growth factor-β (TGF-β), and development of a reverse transcription quantitative competitive polymerase chain reaction (RT-qcPCR) assay to measure TGF-β mRNA of teleost fish. Fish Shellfish Immunol 10:61–85 (2000).
10. Derynck R. Transforming growth factor-beta. In: The Cytokine Handbook. 2nd ed (Thomson AW, ed) London:Academic Press, 1994;319–342.
11. McCartney-Francis NL, Wahl SM. Transforming growth factor-β: a matter of life and death. J Leukoc Biol 55:401–409 (1994).
12. Roberts AB, Sporn MB. The transforming growth factor-βs. In: Peptide Growth Factors and Their Receptors I (Sporn MB, Roberts AB, eds). Berlin:Springer-Verlag, 1990;419–472.

13. Ruscetti FW, Palladino MA. Transforming growth factor-β and the immune system. Prog Growth Factor Res 3:159–175 (1991).
14. Sasaki H, Pollard RB, Schmitt D, Suzuki F. Transforming growth factor-β in the regulation of the immune response. Clin Immunol Immunopathol 65:1–9 (1992).
15. Burt DW, Law AS. Evolution of the transforming growth factor-beta superfamily. Prog Growth Factor Res 5:99–118 (1994).
16. National Center for Biotechnology Information, National Library of Medicine, National Institutes of Health. Genbank. Available: http://www.ncbi.nlm.nih.gov [cited 5 April 1999].
17. Daniels GD, Secombes CJ. Genomic organisation of rainbow trout, *Oncorhynchus mykiss* TGF-β. Dev Comp Immunol 23:139–147 (1999).
18. Jang SI, Hardie LJ, Secombes CJ. Effects of transforming growth factor-beta-1 on rainbow trout *Oncorhynchus mykiss* macrophage respiratory burst activity. Dev Comp Immunol 18:315–323 (1994).
19. Jang SI, Hardie LJ, Secombes CJ. Elevation of rainbow trout *Oncorhynchus mykiss* macrophage respiratory burst activity with macrophage-derived supernatants. J Leukoc Biol 57:943–947 (1995).
20. Harms CA, Ottinger CA, Kennedy-Stoskopf S. Correlation of TGF-β mRNA expression with cellular immunoassays in triamcinolone-treated captive hybrid striped bass (*Morone saxatilis* × *M. chrysops*). J Aquat Anim Health 12:9–17 (2000).
21. Hughes H, Fowler B, Frosh B, Guns RA, Baker WC, Nelson FW, Sommer A, Toll J, Bryce JC. Report of the Governor's Blue Ribbon Citizens Pfiesteria Action Commission. Annapolis, MD:Maryland Department of Natural Resources, 1997.
22. Macilwain C. Scientists close in on 'cell from hell' lurking in Chesapeake Bay. Nature 389:317–318 (1997).
23. Blazer VS, Vogelbein WK, Densmore CL, May EB, Lilley JH, Zwerner DE. Aphanomyces as a cause of ulcerative skin lesions of menhaden from Chesapeake Bay tributaries. J Aquat Anim Health 11:340–349 (1999).
24. Densmore CL, Blazer VS. Characterization of lesions of Atlantic menhaden from the Pocomoke River. In: Proceedings of the International Association for Aquatic Animal Medicine Conference, Vol. 29, 2–6 May 1998, San Diego, California. International Association for Aquatic Animal Medicine, 1998;129.
25. Noga EJ, Dykstra MJ. Oomycete fungi associated with ulcerative mycosis in menhaden, *Brevoortia tyrannus* (Latrobe). J Fish Dis 9:47–53 (1986).
26. Burkholder J M, Noga EJ, Hobbs CH, Glasgow HB, Smith SA. New 'phantom' dinoflagellate is the causative agent of major estuarine fish kills. Nature 358:407–410 (1992).
27. Noga EJ. Toxic algae, fish kills and fish disease. Fish Pathol 33:337–342 (1998).
28. Noga EJ, Khoo L, Stevens JB, Fan Z, Burkholder JM. Novel toxic dinoflagellate causes epidemic disease in estuarine fish. Mar Pollut Bull 32:219–224 (1996).
29. Lewitus AJ, Jesien RV, Kana TM, Burkholder JM, Glasgow HB, May E. Discovery of the "phantom" dinoflagellate in Chesapeake Bay. Estuaries 18:373–378 (1995).
30. Rottman JP, Tompkins WAF, Tompkins MB. A reverse transcription-quantitative competitive PCR (RT-QCPCR) technique to measure cytokine gene expression in domestic mammals. Vet Pathol 33:242–248 (1996).
31. Sharp GJE, Pike AW, Secombes CJ. Leucocyte migration in rainbow trout (*Oncorhynchus mykiss* [Walbaum]): Optimization of migration condition and responses to host and pathogen (*Diphyllobothrium dendrictium* [Nitzche]) derived chemoattractants. Dev Comp Immunol 15:295–305 (1991).
32. Graham S, Jeffries AH, Secombes CJ. A novel assay to detect macrophage bactericidal activity in fish: factors influencing the killing of *Aeromonas salmonicida*. J Fish Dis 11:389–396 (1988).
33. Hollander M, Wolfe DA. Nonparametric Statistical Methods. New York:John Wiley & Sons, 1973.
34. Mansueti RJ. Movements, reproduction, and mortality of the white perch, *Roccus americanus*, in the Patuxent estuary, Maryland. Chesapeake Sci 2:142–205 (1961).
35. Roberts AB, Sporn MB. Transforming growth factor β. In: Human Cytokines (Aggarwal BB, Gutterman JU, eds). Boston:Blackwell Scientific Publications, 1992;399–417.
36. Jackson LF, Sullivan CV. Reproduction of white perch: the annual gametogenic cycle. Trans Am Fish Soc 124:563–577 (1995).
37. Tucker CS. Water analysis. In: Fish Medicine (Stoskopf MK, ed). Philadelphia:Saunders, 1993;166–197.
38. Tirard CT, Grossfeld RM, Levine JF, Kennedy-Stoskopf S. Effect of osmotic shock on protein synthesis of oyster hemocytes in vitro. Comp Biochem Physiol 116A:43–49 (1996).
39. Stoskopf MK. Clinical physiology. In: Fish Medicine (Stoskopf MK, ed). Philadelphia:Saunders, 1993;48–57.
40. Smith LS. Anatomy and special physiology of salmonids. In: Fish Medicine (Stoskopf MK, ed). Philadelphia:Saunders, 1993;322–332.
41. Schneider SL, Gollnick SO, Grande C, Pazic JE, Tomasi TB. Differential regulation of TGF-β_2 by hormones in rat uterus and mammary gland. J Reprod Immunol 32:125–144 (1996).
42. Batuman OA, Ferrero A, Diaz A, Jimenez SA. Regulation of transforming growth factor-β_1 gene expression by glucocorticoids in normal human T lymphocytes. J Clin Invest 88:1574–1580 (1991).
43. Batuman OA, Ferrero A, Cupp C, Jimenez SA, Khalili K. Differential regulation of transforming growth factor-β_1 gene expression by glucocorticoids in human T and glial cells. J Immunol 155:4397–4405 (1995).
44. Zapata AG, Varas A, Torroba M. Seasonal variations in the immune system of lower vertebrates. Immunol Today 13:142–147 (1992).