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 immunosassay 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 t 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] http://ehpnet1.niehs.nih.gov/docs/2000/108p447-452harms/abstract.html

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, and macrophage respiratory burst activity, neutrophil adhesion to endothelium, and hematopoiesis (13). Three isoforms of TGF-β (β1, β2, and β3) are expressed by mammals. In addition, one (β3) has been identified from the chicken, and one (β2) from the African clawed frog, Xenopus laevis. TGF-β1, TGF-β2, and TGF-β3 are considered homologues of mammalian TGF-β (15). TGF-β1 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 x M. chrysops, Genbank (16) accession no. AF140363 (9)] and rainbow trout [Oncorhynchus mykiss, Genbank (16) accession no. AF1007836 (8,17)] grouping with Xenopus TGF-βs, chicken TGF-βs, and mammalian TGF-βs, were recently reported. Homology with TGF-β1, suggests that the fish TGF-β isoforms 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-β1 enhanced respiratory burst activity of resting rainbow trout macrophages at low doses. At higher doses, however, bovine TGF-β1 inhibited activated rainbow trout macrophages and countered the effects of activating signals on resting macrophages (18). Chicken antiporcine TGF-β1 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 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...
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 asperate fungal hyphae (23,24). Similar ulcerative myositis 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 (22). 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 9-m semiballoon otter trawl in 10-30 m-nets 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 400g for 5 min, then 800g 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 × 106 cell equivalents/μL. Messenger RNA was reverse transcribed (Superscript II RT; Gibco-BRL, Gaithersburg, MD) to cDNA with oligo dT15 priming of 3 × 106 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 × 104 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 × 108 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 MgCl2, 50 mM KCl], 250 μM each dNTP, 0.375 μM each primer, 30 μL/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:

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

where CFR = the corrected fluorescence ratio, TF = the target fluorescence, CS = competitor size, CF = competitor fluorescence, and TS = target size. The log10 of the CFR was plotted against the log10 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 μg/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 500g. 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 400g and then leukocytes were removed from the media/Percoll interface. Leukocytes were pelleted at 500g, 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 500g as described previously. Leukocytes were resuspended at 2 × 10^6 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 non-adherent 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 trypsin soy broth. Dilutions were plated as 10 μL drops on trypsin 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 (Q^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--acin 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.^
| 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                     |

* 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) | pH surface/bottom | Dissolved oxygen (mg/L) | Salinity (g/L) | Depth (m) |
|-------------|---------------|-----------------|-------------------|-------------------------|---------------|-----------|
| June        |               |                 |                   |                         |               |           |
| Back        | 31.0          | 28.1/27.8       | 8.5/6.1           | 8.0/6.1                 | 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          | 22.8/22.8       | 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       | 5.5/4.2           | 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           | 8.9/8.8                 | 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 0.0156 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, trimcinolone (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...
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, and TGF-β2 in mammals (35). However, there were no significant differences in sex distribution between samplings, and there was no correlation between TGF-β-actin ratios and sex. White perch spawned 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.

Of the physical conditions measured at the sample sites, none of the following correlated with TGF-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-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. Progesterin and cortisol both play a role in regulating electrolyte flux across the gills (39), and sequential prolactin and cortisol surges are important for smolitization of anadromous fish as they make the transition to seawater (40). In mammals, high prolactin concentrations correlate with TGF-β levels in rat milk (41), and human T cells increase transcription of TGF-β, 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 ecotamers (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.

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