Stony Brook University

Measures of Immune System Status in Young-of-the-Year Winter Flounder (*Pseudopleuronectes americanus*) from Long Island Coastal Bays

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Abstract of the Thesis

Measures of Immune System Status in Young-of-the-Year Winter Flounder (*Pseudopleuronectes americanus*) from Long Island Coastal Bays

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The immune status of YOY winter flounder was evaluated in fish collected from six areas around Long Island, including Jamaica Bay, Little Neck Bay, Manhasset Bay, Oyster Bay, Port Jefferson, and Shinnecock Bay; sites geographically distributed from west to east, representing a large gradient in urbanization, and thus likely contaminant inputs from the water shed. Fish were collected from June to October, and expression of pleurocidin, a gene coding for an antimicrobial peptide found in skin mucus, as well as antimicrobial activity of skin mucus assessed in individual fish from each site. Cytochrome P4501A (CYP1A) expression was also measured as an indicator of contaminant exposure in these fish. Gene expression was quantified using qRT-PCR, and antimicrobial activity was assessed using a growth assay with *Vibrio anguillarum* and

V. parahaemolyticus, two common marine bacteria known to affect winter flounder. Pleurocidin expression was ten times higher in fin tissue than in liver tissue, was highly variable between individual fish, and demonstrated no clear site specific differences associated with degree of urbanization of the watershed. Expression seemed to be related in part to fish size: a positive correlation between expression and total length of fish was observed, and fish in the largest size class (>125 mm TL) demonstrating significantly elevated expression as compared to fish in the smaller size class levels indicating that immune competency increases with age. Antimicrobial activity was also highly variable, showing no large site specific differences, and no significant correlation to pleurocidin expression. No differences in CYP1A expression were observed. These data suggest that exposure to aromatic hydrocarbon contaminants is fairly wide-spread throughout the study area and that any difference in pleurocidin expression in YOY winter flounder observed are due to other factors. The lack of correlation between pleurocidin expression and antimicrobial activity indicates that other antimicrobial peptides may be involved or that other factors are influencing antimicrobial activity. This is to my knowledge the first reports quantitatively evaluating pleurocidin expression in YOY winter flounder from an urban area. Further work is needed to characterize factors controlling pleurocidin expression, as well as other indicators of immune response in young fish.

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INTRODUCTION

Fish populations inhabiting urban and industrialized estuaries are often exposed to high levels of complex mixtures of anthropogenic contaminants from early stages of development through adulthood (Sorrentino et al., 2005). Such contaminants include both organic and inorganic chemicals such as polychlorinated biphenyls (PCBs) dioxins, mercury and other heavy metals, pesticides such as DDT, polycyclic aromatic hydrocarbons (PAHs), and an expanding suite of emerging contaminants that have not yet been well characterized (Stenberg et al., 2004; EPA, 2006). Active sources of these contaminants are industrial discharges, surface runoff, leaks and spills, landfills, sewage effluent, inputs from combined sewer overflows (CSOs), and atmospheric depositions. Despite improvement in water quality due to implementation of secondary sewage treatment and point source pollution control mandated by the Clean Water Act, certain areas of western Long Island Sound and parts of the New York/New Jersey harbor such as Jamaica Bay are still in very poor condition.

Contaminants can have many deleterious effects on fish – these include reproductive impairment (Sumpter et al., 2005); impaired growth and development, (Black et al., 1988), genetic damage (Brown et al., 1996) and immune suppression (Zelikoff, 1993; White et al., 1994). Disease occurrence in fish and fish populations is believed to be influenced by the interaction of three variables: the quality of the environment, differential susceptibility of individuals to the pathogen as a result of genetic predisposition or the physiological health of individual members of the host population, and the presence and virulence of the pathogen (Snieszko, 1973). Modification of any of these factors can disrupt the interaction between host

and pathogen, which may consequently alter the susceptibility of fish populations to disease (Arkoosh et al., 1998).

The immune system protects fish from serious diseases caused by external bacteria, parasites and viruses. In winter flounder, like other teleosts, a gradual increase in immune competence occurs during early growth with innate immunity developing first, followed by cell-mediated and then humoral immunity (Ewing, 1980). Innate immunity includes defense mechanisms that protect an organism against infection, which is independent of prior exposure to any particular microorganism. Innate immunity is more significant in fish, since it provides an ever-present protection as opposed to adaptive or acquired immune response which takes longer and develops later than in mammals (Alexander and Ingram, 1992; Ellis, 2001). Humoral components involved in innate immunity identified from fish include lysozyme, complement, lectins and antimicrobial peptides such as pleurocidin (Ellis, 1981).

Vertebrates and other organisms have the ability to fight infections by synthesizing diverse groups of antimicrobial peptides along with a highly specific cell-mediated immune system (Oren and Shai, 1998). Antimicrobial peptides are known to be among the earliest developed molecular effectors of innate immunity and play an important role in the first line of defense in many species (Boman, 1995). In teleosts, natural antimicrobial compounds have been isolated from the skin of the winter flounder (*Pleuronectes americanus*) (Cole et al., 1997), cat fish (*Parasilurus asotus*) (Park et al., 1998), carp (*Cyprinus carpio*) (LeMaitre, 1996) and rainbow trout (*Oncorhynchus mykiss*) (Smith, 2000). Pleurocidin is a linear, cationic alpha-helical antimicrobial peptide obtained from skin mucous secretions of winter flounder (Cole et al., 1997) and shows high amino acid sequence homology with two other antimicrobial peptides;

dermaseptin from the skin of the arboreal frog (*Phyllomedusa sauvagii)* and ceratotoxin from the Mediterranean fruit fly (*Ceratitis capitata*) (Jia et al., 2000).

Pleurocidin has been shown to exert microbial inhibition against a large range of both gram-negative and gram-positive bacteria from diverse aquatic species, thus exhibits broad-spectrum antibacterial action (Cole et al., 1997). Although the exact mechanism by which antimicrobial peptides kill bacteria is yet to be determined, studies have shown that peptide-lipid interaction, rather than receptor mediated recognition, leading to loss of membrane integrity, plays a role in their activity (Oren and Shai, 1998; Brogden, 2005). Patrzykat et al. (2002) found that the lowest concentration of pleurocidin with antimicrobial activity could cross the bacterial cell wall and inhibit RNA synthesis prior to affecting membrane permeability. Pellegrini et al. (2000) also determined that cationic peptides inhibit DNA and RNA synthesis prior to altering membrane permeability in *E. coli*. These studies indicate that pleurocidin's mechanisms of action may include an intracellular target as well as membrane level effects.

In this study, young-of-the-year (YOY) winter flounder, *Pseudopleuronectes americanus*, are used to assess immune status in fish from urbanized environments around Long Island, NY. While adult winter flounder spawn during the autumn and winter months within coastal bays and estuaries and return to the ocean following reproduction (Klein-MacPhee, 2002), juveniles stay in the coastal estuaries and bays for one or two years before joining the adult population in the ocean (Klein-MacPhee, 1978). Juvenile fish are resident in sediment that may contain contaminants and also feed on other sediment-dwelling organisms that have accumulated toxicants from the sediment reservoir during important periods of their life history, namely early development and growth (Ferguson et al., 2001). These life history traits make YOY winter flounder an ideal model for investigating the condition of fish inhabiting coastal embayments.

The first objective of this study was to assess immune capacity of wild populations of YOY winter flounder from Long Island coastal bays. Pleurocidin gene expression was used as a potential biomarker for innate immunity in these fish, and skin mucus antimicrobial activity measured as a functional measure of immune response. Antimicrobial activity against two common marine pathogens was assessed; *Vibrio anguillarum* and *Vibrio parahaemolyticus*.

A serological evaluation of winter flounder and European flounder (*Platychthys flesus*) from clean and polluted waters of the New York bight found that the greatest proportion of raised titers was against *Vibrio* species (Robohm et al., 1979). Disease due to vibriosis, *V. anguillarum* in particular, has been a cause for global concern after it was found to be responsible for 67.8% of bacterial disease in gilt-head seabream (*Sparus aurata*) in Spain during 1990 – 1996 (Balebona et al., 1998) and found to associated with major problems in seabass (*Lates calcarifer*) in Singapore, Thailand and Australia (Azad et al., 2004).

V. anguillarum is an opportunistic pathogen, and its virulence mechanisms in systemic infections in fish have been shown to involve production of a siderophore, as well as extra-cellular products with haemolytic, proteolytic and cytotoxic effects (Krovacek et al., 1987). Olsson et al. (1996) studied the *in vitro* effects of *V. anguillrum* and turbot (*Scophthalmus maximus*) intestinal mucus and determined that chemotaxis and penetration of mucus in the intestine may be important virulence factors for *V. anguillarum*. The capability to breach an intact mucus layer would allow the pathogen to get closer to the epithelium, increasing the likelihood of an infection. Subsequently, this bacterium was found to cause disease and mortality, and has been isolated from the skin and muscle lesions in winter flounder on many occasions (Levine et al., 1972).

V. parahaemolyticus was also chosen in this study as a ubiquitous organism to marine environments, and consistently found in inshore sediments and invertebrates (Baross and Liston, 1970). Given that juvenile winter flounder are resident in sediment and feed on invertebrates, *V. parahaemolyticus* should be a bacterium these fish are commonly exposed to, but there are no reports of pathogenicity of this bacterium in this host species. Therefore, it provides for a rather different relationship with the host from *V. anguillarum*, but still relatively closely related to *V. anguillarum*.

The second objective of this study was to determine contaminant exposure in these same YOY winter flounder, to evaluate potential interactions or correlations with immunological status.

It was not possible within the scope of this study to measure contaminant levels in the tissues of fish collected for this study. Even if we had the funds for this analysis, there was insufficient tissue for routine contaminant analysis. As a biomarker for contaminant exposure we chose to measure expression of cytochrome P4501A (CYP1A). A large suite of organic contaminants are known to stimulate the expression of CYP1A (Monosson and Stegeman, 1991). This gene is highly inducible by PAHs, PCBs, furans and dioxins and the mechanism of its induction has been evaluated to teleosts (Cao et al., 2000). Since some of the compounds that induce CYP1A are rapidly metabolized and excreted from the body, in some instances it can be a better measure of exposure and potential effect than either tissue or environmental residues.

Previous studies of CYP1A inducibility and expression in fish have mainly relied on techniques that measure wither levels of expression protein, such as Western blotting, ELISA (enzyme-linked immunosorbant assay), or enzyme activity, such as AHH (arylhydrocarbon

hydroxylase activity) (Collier et al., 1992a), EROD (7-ethoxyresorufin O-deethylase) activity (Rees at al., 2003) or Northern blotting to measure up-regulation of CYP1A RNA. New methods in biotechnology have been developed in order to quantitatively detect small perturbations in gene expression in organisms under stress. These can be especially useful to study larval/juvenile animals in which only small amounts of tissue are available, and/or in organisms where antibodies/assays are not readily available to quantify post-translational mechanisms. A large number of studies have demonstrated up-regulation of CYP1A genes in response to contaminant exposure in fish species using quantitative real time polymerase chain reaction (qRT-PCR) and microarray analysis (Williams et al., 2003; Sheader et al., 2006).

Straub used suppression subtractive hybridization (SSH) and PCR to identify genes differentially expressed in adult winter flounder from a reference site and a polluted estuary (Straub et al., 2004). While SSH-PCR can be crucial in identifying genetic biomarkers (such as pleurocidin and CYP1A) from upregulated or downregulated libraries, this method is more qualitative. In this case, the use of more sensitive quantitative techniques such qRT-PCR is warranted. QRT-PCR is a highly sensitive technique with a wide range of amplification that allows quantification of specific target genes. In order to conduct our study the expression of CYP1A and pleurocidin were used as biomarkers and quantified in order to evaluate environmental stress in YOY winter flounder.

MATERIALS AND METHODS

Site Selection

YOY winter flounder were collected from six coastal embayments around Long Island, NY, USA: Jamaica Bay, Little Neck Bay, Manhasset Bay, Oyster Bay, Port Jefferson Harbor and Shinnecock Bay. Sites were chosen to represent a gradient in watershed population density, as well as varying amounts of sewage influent and general contaminant loadings.

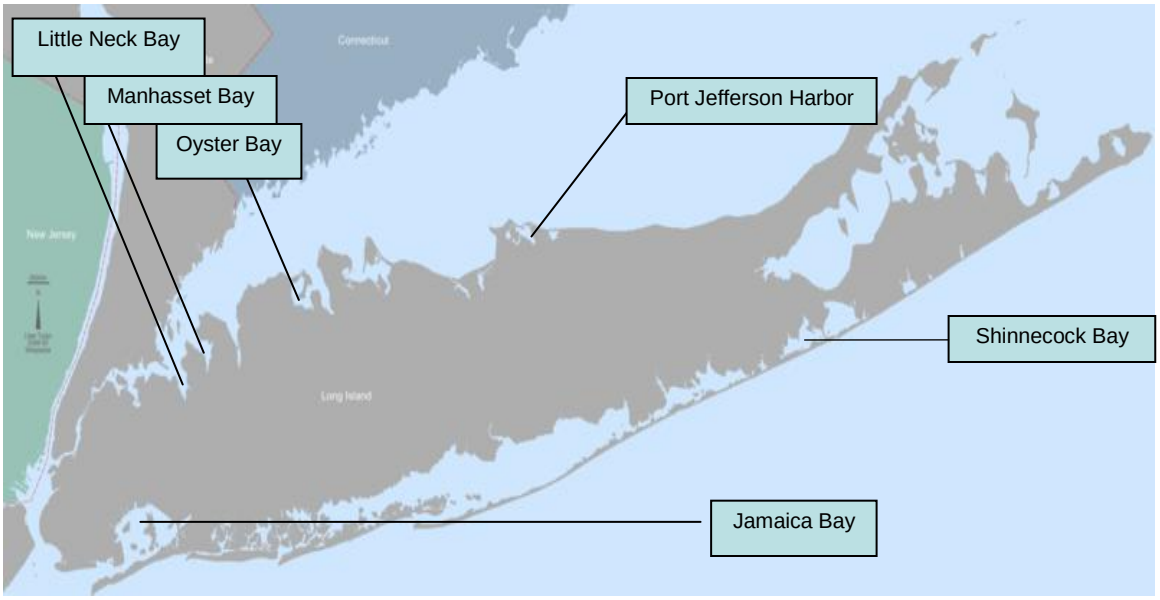

Figure 1. Map of Long Island, NY showing sampling sites.

Fish collection and maintenance

YOY winter flounder were collected in Jamaica Bay, Oyster Bay, Little Neck Bay and Manhasset Bay in the summer and fall of 2008 and 2009 using a 40 foot beach seine net. Winter flounder were collected from these embayments as part of the New York State Department of Environmental Conservation's fishery survey. Sampling was done in Port Jefferson Harbor in the

fall of 2008, the spring of 2009 and in Shinnecock Bay in August 2009 using a 1m beam trawl. Upon capture, fish were immediately flash frozen between two blocks of dry ice and transported to the laboratory where they were stored at -80 ^{o}C until processed.

Fish and tissue processing

Total length and total weight was measured on each fish, and then a mucus sample was taken from the right dorsal side of the winter flounder by gently scraping the skin on the dorsal side of the fish with sterile a scalpel from the pectoral fin to the end of the caudal fin. The mucus sample was added to 1 ml of sterile phosphate buffered saline 1X pH 7.2 (PBS) buffer and left on ice. The mucus in PBS buffer was mixed by vortexing for a few seconds and then centrifuged at 1,000 x *g* for 2 minutes to separate scales from the mucus. Supernatant containing the mucus was carefully removed using a sterile 3 ml syringe and 18G needle and then sterilized by passing through a 25 µm syringe filter into a clean 2 ml tube.

A 1 cm^2 clipping of the posterior dorsal fin was taken and added to 750 µl Trizol® LS Reagent in a screw cap tube containing 3 - 1 mm glass beads. The liver was carefully removed and its weight was recorded. Between 20 to 80 mg of liver tissue was placed in 750 µl Trizol ® LS reagent. Fin tissue was homogenized using a Mini Bead Beater (Cole-Parmer, Vernon Hills. IL) at 3,600 RPM for 15 seconds and the liver tissue was homogenized using a hand held cordless pellet homogenizer and sterile pestle (Sigma Aldrich, St. Louis, MO). Homogenized tissues in Trizol ® LS Reagent were kept on ice for RNA extraction as described below.

Antimicrobial assay

Measurement of antimicrobial activity of the mucus was based upon a turbidimetric assay from Noga et al. (1994), where inhibition of *Escherichia coli* D31 (*E. coli* D31: Monner 1971) treated with plasma was measured after 36 hours of incubation. *Vibrio anguillarum* and *Vibrio parahaemolyticus* were obtained from the culture collection at Yale University. 2 ml of sterile marine broth was inoculated with 100 µl of each bacteria and allowed to grow overnight at 37 ^{o}C and 200 rpm. Following incubation, the bacteria were centrifuged at 300 x *g* for 5 minutes at room temperature and the pellets rinsed with 1 ml sterile PBS. The wash was repeated twice more and the resulting bacterial suspension was diluted to a 50:50 mixture with PBS buffer to achieve an optical density (OD) of 0.100 at 560 nm prior to use in the assay.

Growth inhibition was calculated from the difference in growth between tube incubated with and without mucus after correcting appropriate controls using the following equation:

$$\frac{\text{(bacteria control – media) – (bacteria and mucus – mucus)}}{\text{(bacteria control- media)} \times 100}$$

Where media = PBS + media only; bacteria control = bacterium + PBS + media; mucus = mucus + media + PBS; and bacteria and mucus (the treatment) = mucus + bacterium + PBS + media. Antimicrobial activity was normalized to the protein concentration of the mucus solution determined using the Micro BCA Protein Assay Reagent protocol (Thermo Fisher Scientific, Pittsburgh, PA) using bovine serum albumin as a standard.

RNA extraction and cDNA synthesis

Total RNA was extracted from liver and fin tissues using Trizol® LS Reagent following the manufacturer's instructions (Invitrogen, Carlsbad, CA). RNA quantity and quality was determined using the NanoDrop-1000 Spectrophotometer (v3.2.1, Thermo Fisher Scientific, Pittsburgh, PA). Samples were then stored at -80^{o}C until cDNA synthesis. cDNA was synthesized using the Superscript^{TM} III First-Strand Synthesis SuperMix for qRT-PCR Universal Kit (Invitrogen, Carlsbad, CA) using 1.5 µg of total RNA from each sample diluted in DEPC-treated water in each reaction. Following first strand synthesis, cDNA samples were stored at -20^{o}C.

Primer design

Primer sequences were based on winter flounder sequences for pleurocidin (GenBank AF184223). CYP1A (GenBank X73631) was obtained from GenBank and BLAST searches for contiguous sequences using another flatfish species (i.e. *Pleuronectes platessa*) and ten other teleost species. Primers were designed using Primer3 software and purchased from Integrated DNA Technologies (IDT, Coralville, IA) Stock primers were diluted to 100 µM with DEPC-treated water and stored at -80 ^{o}C.

Table 1 Primer sequences, annealing temp, product sizes and PCR efficiencies of genes

Gene	Forward Primer	Reverse primer	Annealing temp (^{o}C)	Product Size (bp)	E%
CYP1A	AATCTGCAGGGTTTCCACTG	CCAATGTGATCTGCGGTATG	58	123	98 – 101%
Pleurocidin	CCTGCTTATCGCCAAGGTAA	CCATCTTCGTCCTCATGGTT	58.7	124	98 – 101%

PCR, cloning and standard curve construction

Polymerase Chain Reaction (PCR) was used to amplify products for both genes and production of a single product confirmed using agarose gel electrophoresis. Once good quality PCR products were obtained, they were purified (Wizard ® SV Gel and PCR Clean-Up System (Promega, Madison, WI). In the case of pleurocidin, the product was cloned into TA-cloning vectors (TOPO TA Cloning Kit for Sequencing (Invitrogen, Carlsbad, CA). PCR screening was done to analyze plasmids for inserts. Plasmid DNA was isolated using Purelink^{TM} HQ Mini Plasmid Purification Kit (Invitrogen, Carlsbad, CA) and submitted for sequencing. Once sequencing confirmed the cloning of the desired gene, the concentration of plasmid DNA was determined using the NanoDrop-1000 Spectrophotometer and used as a standard for qPCR. Ten-fold serial dilutions (6) were carried out using 1 ng/µl of plasmid standard as the starting concentration.

Quantitative real-time PCR (qRT-PCR)

Gene expression was measured using RT-PCR. All reactions were run using a real-time thermal cycler (Eppendorf, Westbury, NY) using 1.5 µg cDNA within a reaction volume of 25 µl following the SYBR® GreenER^{TM} Two-Step RT-PCR Universal Kit (Invitrogen, Carlsbad, CA) protocol. All samples and standards were run in duplicate and two samples were run in every assay to account for inter-assay variability. Duplicates with greater than 30% fluorescence difference were re-run on another plate. The PCR cycling program started with a 10 minute denaturation at 95 ^{o}C, followed by 40 cycles of; 15 seconds at 95 ^{o}C and 30 seconds at 58 ^{o}C

(annealing and extension). Single product amplification was confirmed for all runs by melt curve analysis (increase in temp of 0.25 ^{o}C every 15 seconds at 60 ^{o}C. Since housekeeping gene products for elongation factor β-actin and could not be verified using gel electrophoresis, data were analyzed as 1/Ct normalized by RNA concentration.

Statistical analysis

Data were analyzed by one-way analysis of variance (ANOVA) followed by pair-wise multiple comparisons to discern differences between sites if the sample population me the criteria for using parametric statistics. In some cases, data were transformed to approximate a normal distribution before analysis. If the criteria for parametric statistics could not be met, the Kruskal-Wallis test followed by Dunn's method for pair-wise multiple comparisons was used to examine differences between sites. SigmaStat 3.1 software (Jandel Scientific Corp, San Rafael, CA) was used to perform statistical tests.

RESULTS

Pleurocidin Expression

Pleurocidin expression in the fin and liver expressed as $1/C_T$ normalized to total RNA for all fish collected are shown in Figure 2. Expression is significantly higher in the fin ($P = <0.001$) being approximately 10 times higher than seen in the liver (one $1/C_T$ value difference is equivalent to a ten fold difference in gene expression). Regression of fin versus the liver for all samples (Fig. 2B) shows a statistically significant positive correlation between pleurocidin expression in these two tissues ($P = 0.0433$, $r = 0{,}260$).

Expression in the fin of fish collected from the different sites is shown in Figure 3A. Median pleurocidin expression ($1/C_T$ per ug RNA) ranges from a high of 0.413 at Port Jefferson to a low of 0.33 at Manhasset, differing by a factor of 1.2 which corresponds to an approximately order of magnitude variation in median expression between sites overall. Expression in fish from Manhasset was significantly lower than that observed at all other sites. Fish from Oyster Bay had the next lowest median expression level, being significantly lower than expression observed in fish from Port Jefferson Harbor. Pleurocidin expression in fish from Jamaica Bay, Little Neck Bay and Shinnecock Bay was intermediate.

Median pleurocidin expression in fish from each site varied only between 0.031 and 0.0286, although median expression in Manhasset was still lowest, it was only significantly different than expression observed in Shinnecock and Little Neck Bay fish (Figure 3B).

Figure 2.

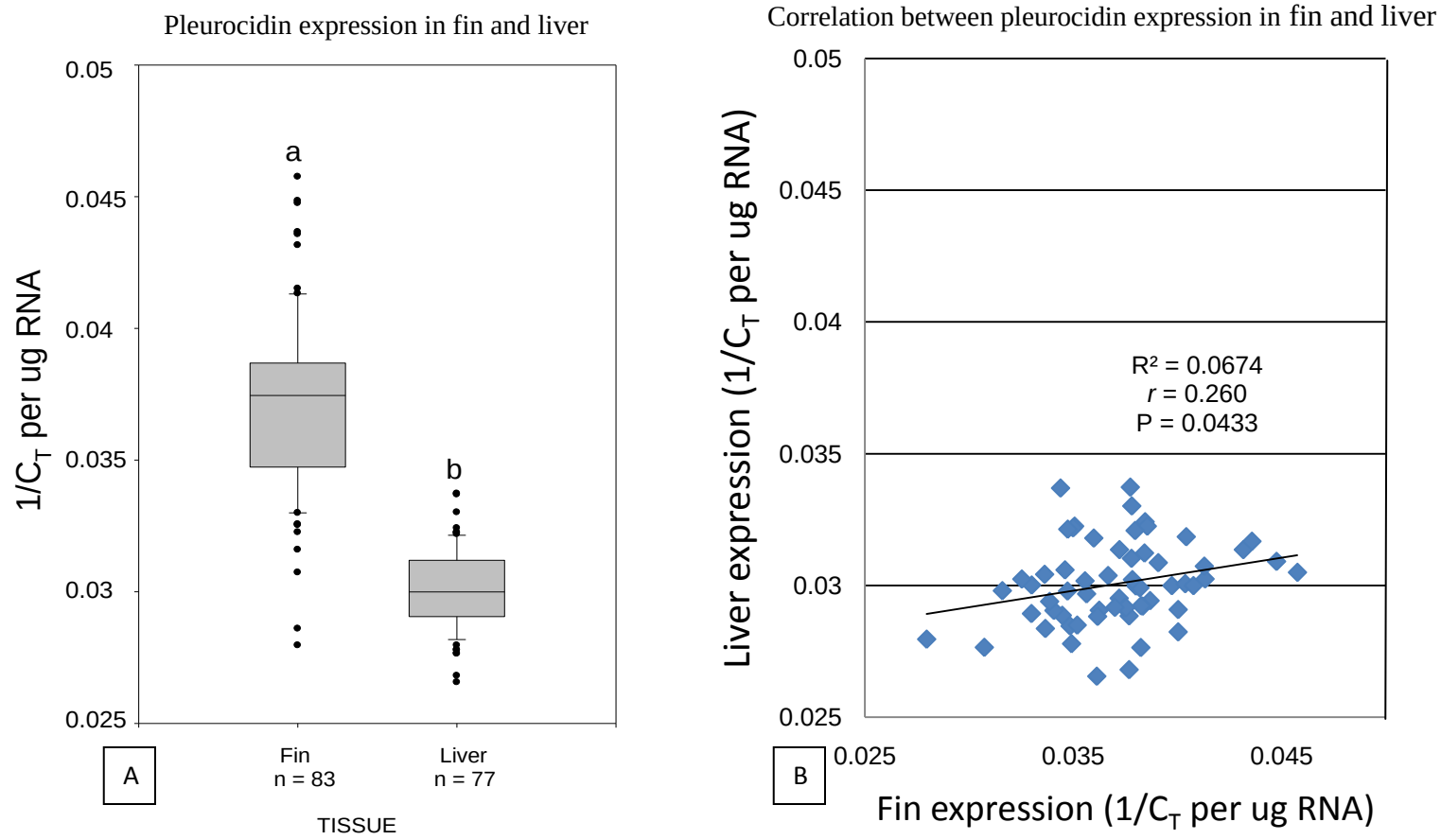

Figure 2A. Pleurocidin expression in fin and liver tissues ($1/C_T$ per ug RNA) from *Pseudopleuronectes americanus* analyzed by Real Time qPCR. N = number of samples analyzed. Data were analyzed by Mann-Whitney Rank Sum Test (P = <0.001). The ends of the boxes define the 25th and 75th percentiles, with a line at the median and error bars defining the 10th and 90th percentiles. Outliers are represented by dots. Figure 2B. Data were analyzed by Pearson-Product Moment Correlation

Figure 3

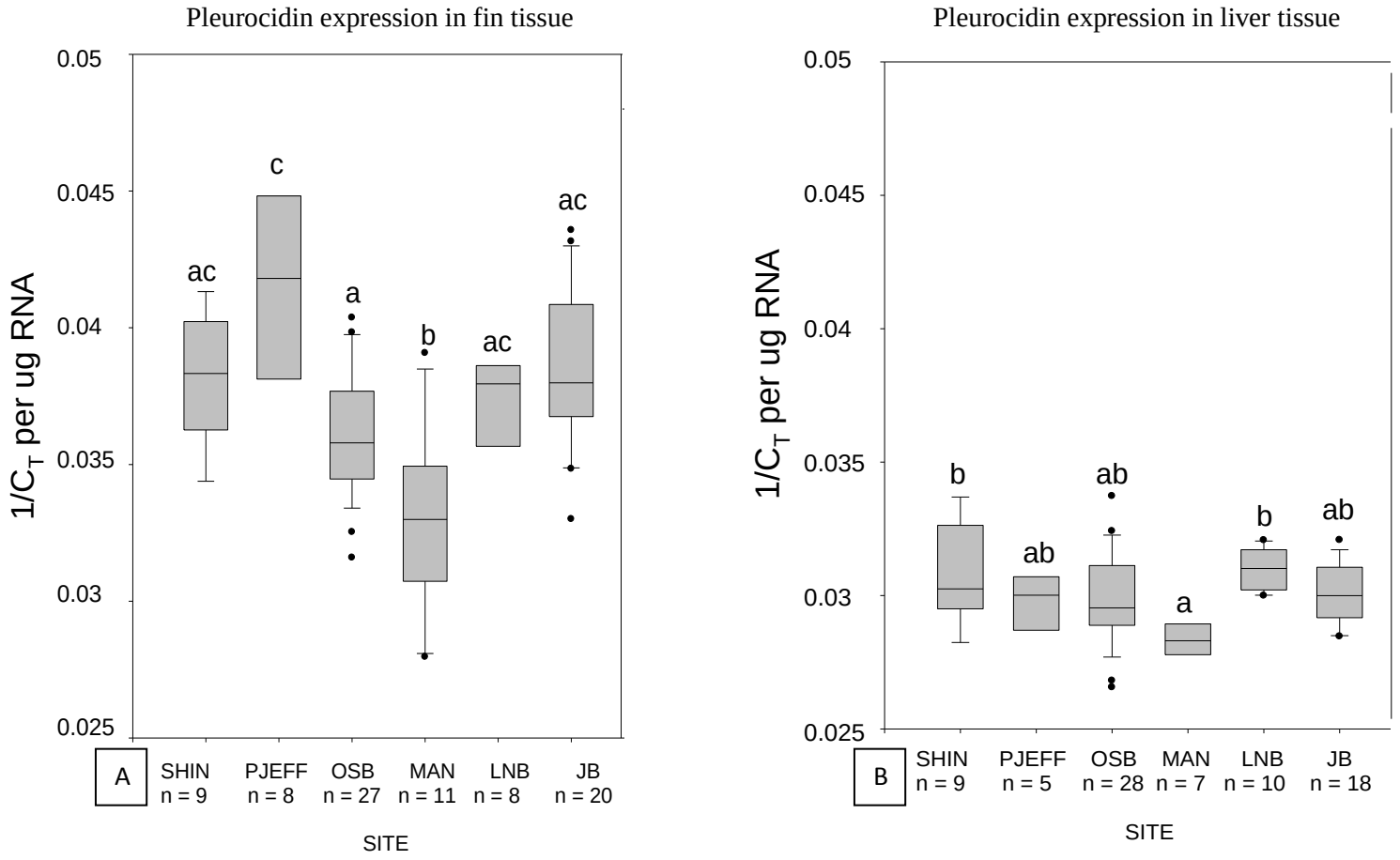

Figure 3A and B. SHIN = Shinnecock Bay, PJEFF = Port Jefferson Bay, OSB = Oyster Bay, MAN = Manhasset Bay, LNB = Little Neck Bay, JB = Jamaica Bay. N = number of individual fish analyzed. Differences between treatment groups were assessed using the Holm-Sidak method for pleurocidin fin expression, and the Dunn's method for pleurocidin liver expression. The ends of the boxes define the 25th and 75th percentiles, with a line at the median and error bars defining the 10th and 90th percentiles. Outliers are represented by dots.

Antimicrobial activity of mucus

Antimicrobial activity (% inhibition normalized to mg protein) is shown in Figure 4. This measure of immune response was also highly variable, particularly for *V. anguillarum* where values from individual samples ranged from 20% inhibition to almost 20% stimulation in growth. Despite this, antimicrobial inhibition was significantly higher in *V. anguillarum* than in *V. parahaemolyticus* ($P = <0.001$).

V. anguillarum was inhibited by the mucus from fish at all sites, with the median level of inhibition ranging from 0.0543% to 3.97% (Figure 5A). Variability between activity in individual fish was high (CV between 50 to 350%). Median values were highest at Oyster Bay and Jamaica Bay, and lowest at Shinnecock Bay, with activity in Port Jefferson Harbor, Manhasset Bay and Little Neck Bay being intermediate.

Site specific variation in antimicrobial activity against *V. parahaemolyticus* was smaller with values of inhibition being slightly lower than observed for *V. anguillarum,* ranging from 1.217 to -0.288 (Figure 5B). No significant differences were observed between sites in this case.

CYP1A Expression

CYP1A was measured in the liver of all fish processed. However, at two sites, Jamaica Bay and Port Jefferson Harbor, CYP1A expression in fin tissue was also measured. Data for expression in both tissues are shown in Figure 6. Expression in the liver was significantly higher ($P <0.001$) than that observed in the fin with an approximate 10 fold difference in expression.

Figure 4

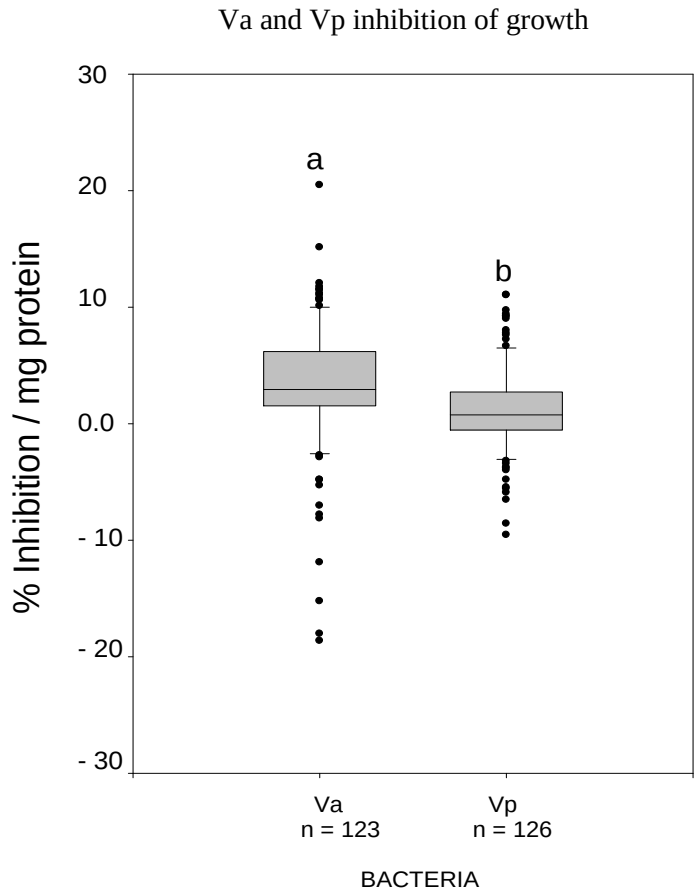

Figure 4. N = number of individual samples analyzed. Va = *Vibrio anguillarum*, Vp = *Vibrio parahaemolyticus*. Data were analyzed by Mann-Whitney Rank Sum Test (P = <0.001). The ends of the boxes define the 25th and 75th percentiles, with a line at the median and error bars defining the 10th and 90th percentiles. Outliers are represented by dots.

Figure 5

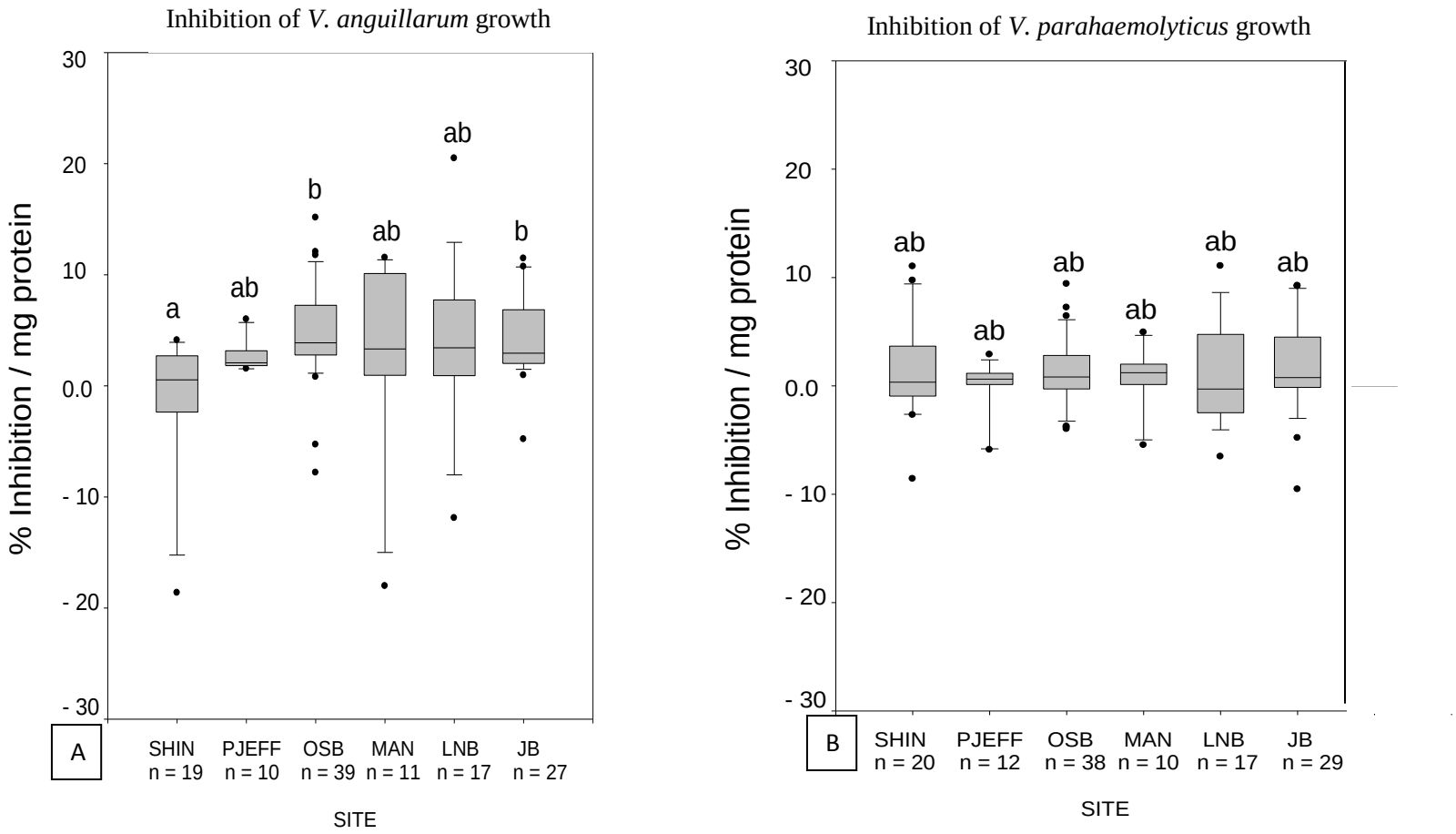

Figure 5A and B. Differences between sites were evaluated using Dunn's method. SHIN = Shinnecock Bay, PJEFF = Port Jefferson Bay, OSB = Oyster Bay, MAN = Manhasset Bay, LNB = Little Neck Bay, JB = Jamaica Bay. N = number of individual fish analyzed. The ends of the boxes define the 25th and 75th percentiles, with a line at the median and error bars defining the 10th and 90th percentiles. Outliers are represented by dots.

Figure 6

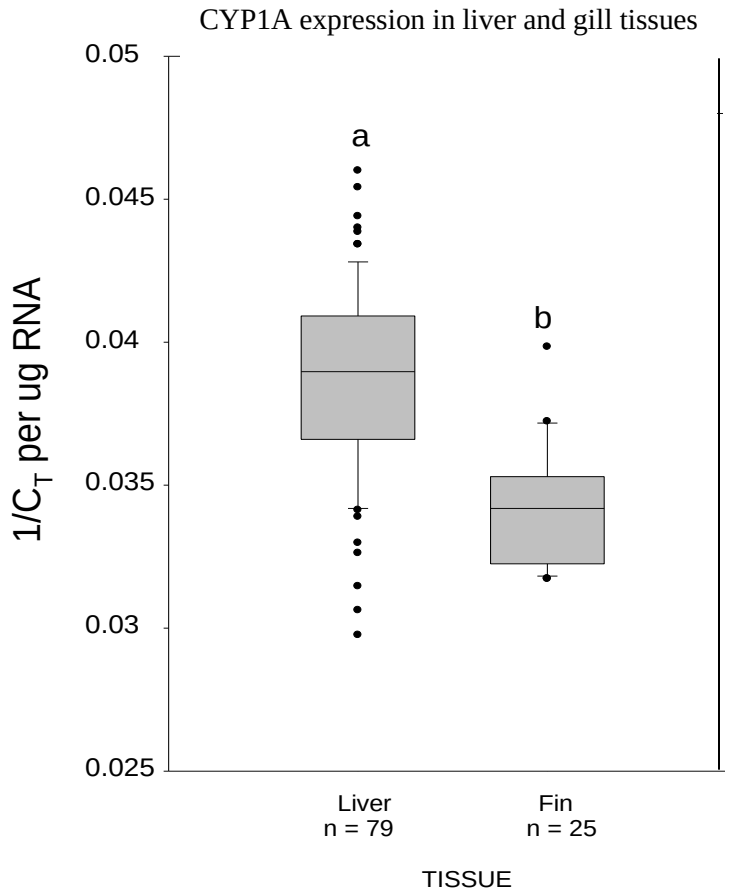

Figure 6. Data were analyzed by Mann-Whitney Rank Sum Test (P = <0.001). N = number of individual fish analyzed. The ends of the boxes define the 25th and 75th percentiles, with a line at the median and error bars defining the 10th and 90th percentiles. Outliers are represented by dots.

Regression analysis indicated that expression in fin was not significantly correlated with expression in liver ($P > 0.05$ data not shown).

Median CYP1A expression in the liver of fish collected from different sites ranged only from 0.0399 to 0.0375 with no significant differences noted between sites and high variability (Figure 7A). There were also no significant differences between CYP1A expression in fin tissue observed between Port Jefferson Harbor and Jamaica Bay (Figure 7B), the only two sites where CYP1A expression in fin was evaluated.

Gene Expression as a Function of Size

The fish examined in this study ranged in size from 48 to 200 mm total length and were collected over the period from April to October in each of two years, 2008 and 2009. Visual inspection of all the data indicated that gene expression appeared to increase with size, and thus age of the fish. Unfortunately, most of the fish were collected during the early summer period when YOY flounder are abundant, so there were many more small ($n = 76$) fish than large fish ($n = 7$). Larger fish were obtained at only two sites; Port Jefferson Harbor and Jamaica Bay. As there seemed to be a break between fish less than 100 mm in length and those of greater than 125 mm in length, we split the data into 2 categories based on these size classes, and analyzed the data separately. Pleurocidin expression in the fin showed a significant difference between these two size groups. Comparing data at all site with expression being approximately 10 fold higher in the larger fish ($P = <0.001$) (Fig. 8A). Elevated expression in larger fish was not observed for CYP1A expression in liver (Figure 8B), where no significant differences between the size groupings was observed ($P = 0.299$). Furthermore, when pleurocidin fin expression was

regressed against the size of the fish, a significant correlation was observed (Figure 9), $P = 2.64 \times 10^{-9}$, $r = 0.356$. Regression of size versus CYP1A expression revealed no statistically significant relationship (data not shown).

Figure 7

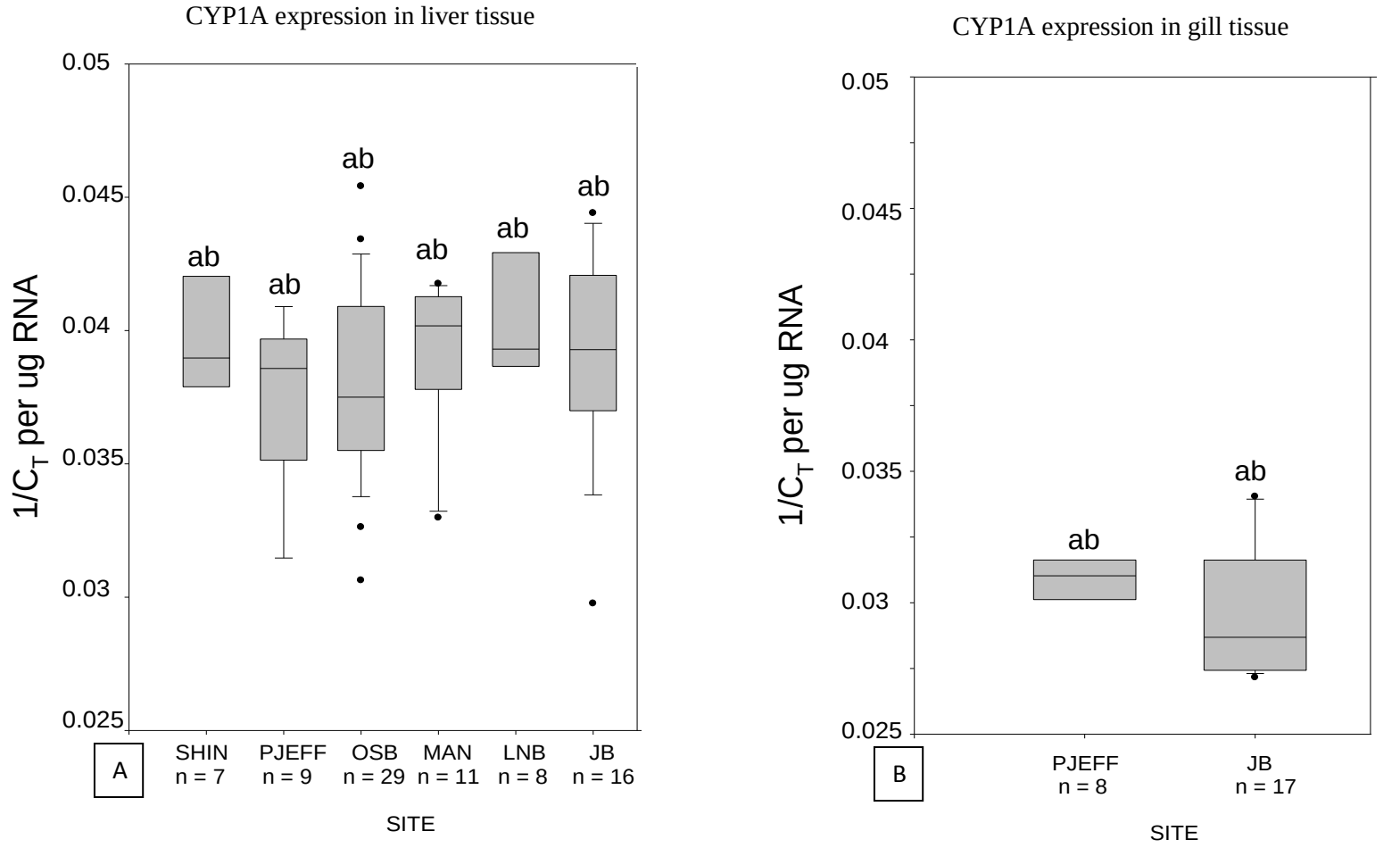

Figure 7A and B. Data for CYP1A liver expression were analyzed by One Way ANOVA (P = 0.481). SHIN = Shinnecock Bay, PJEFF = Port Jefferson Bay, OSB = Oyster Bay, MAN = Manhasset Bay, LNB = Little Neck Bay, JB = Jamaica Bay. N = number of individual fish analyzed. Data for CYP1A fin expression were analyzed by t-test (P = 0.167). The ends of the boxes define the 25th and 75th percentiles, with a line at the median and error bars defining the 10th and 90th percentiles. Outliers are represented by dots.

Figure 8

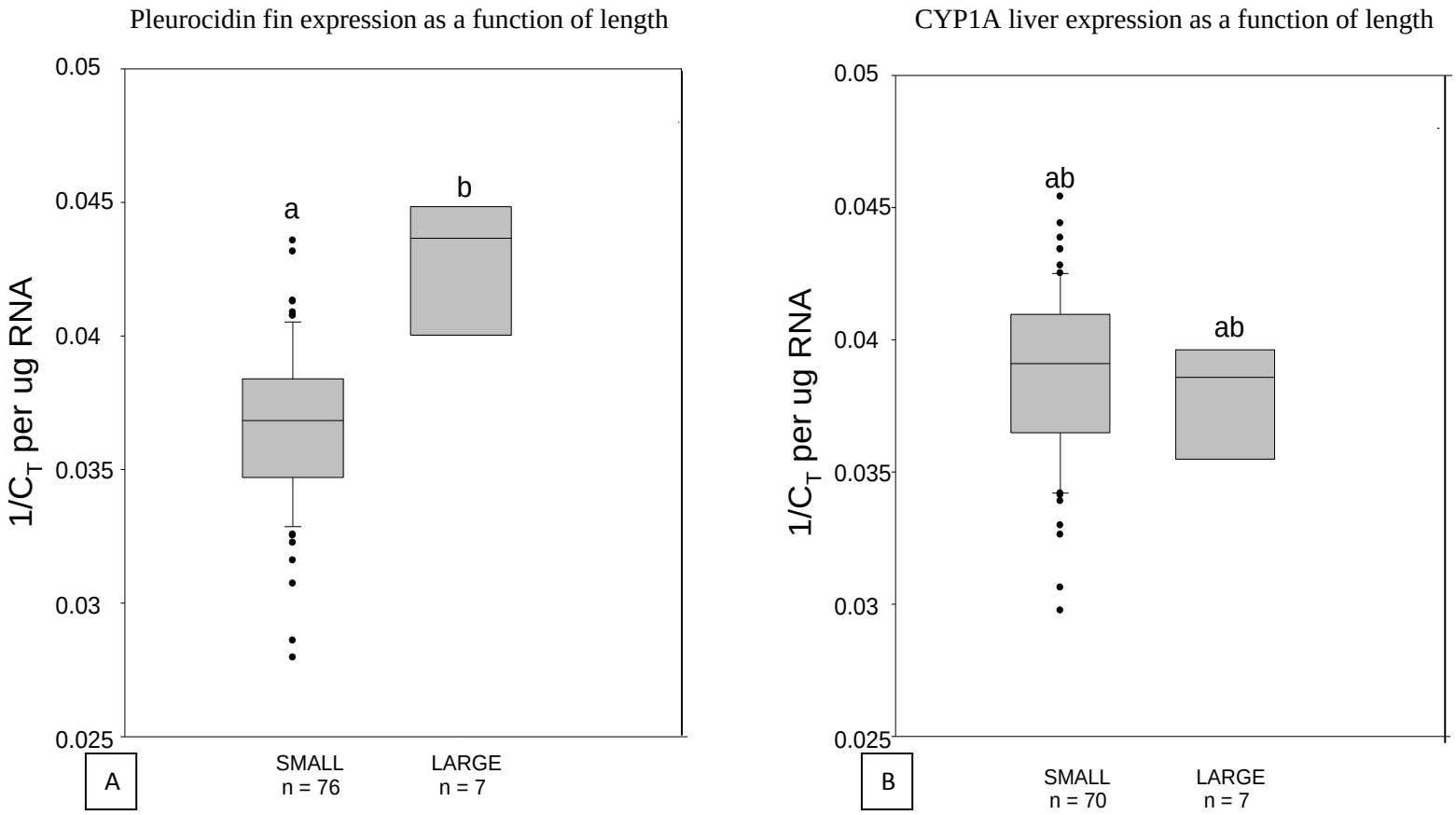

Figure 8A and B. N = number of individual fish analyzed. Data were analyzed by the t-test (P = <0.001), (P = 0.299) for pleurocidin and CYP1A expression respectively. Small = <125mm. Data for CYP1A fin expression were analyzed by t-test (P = 0.167). The ends of the boxes define the 25th and 75th percentiles, with a line at the median and error bars defining the 10th and 90th percentiles. Outliers are represented by dots.

Figure 9

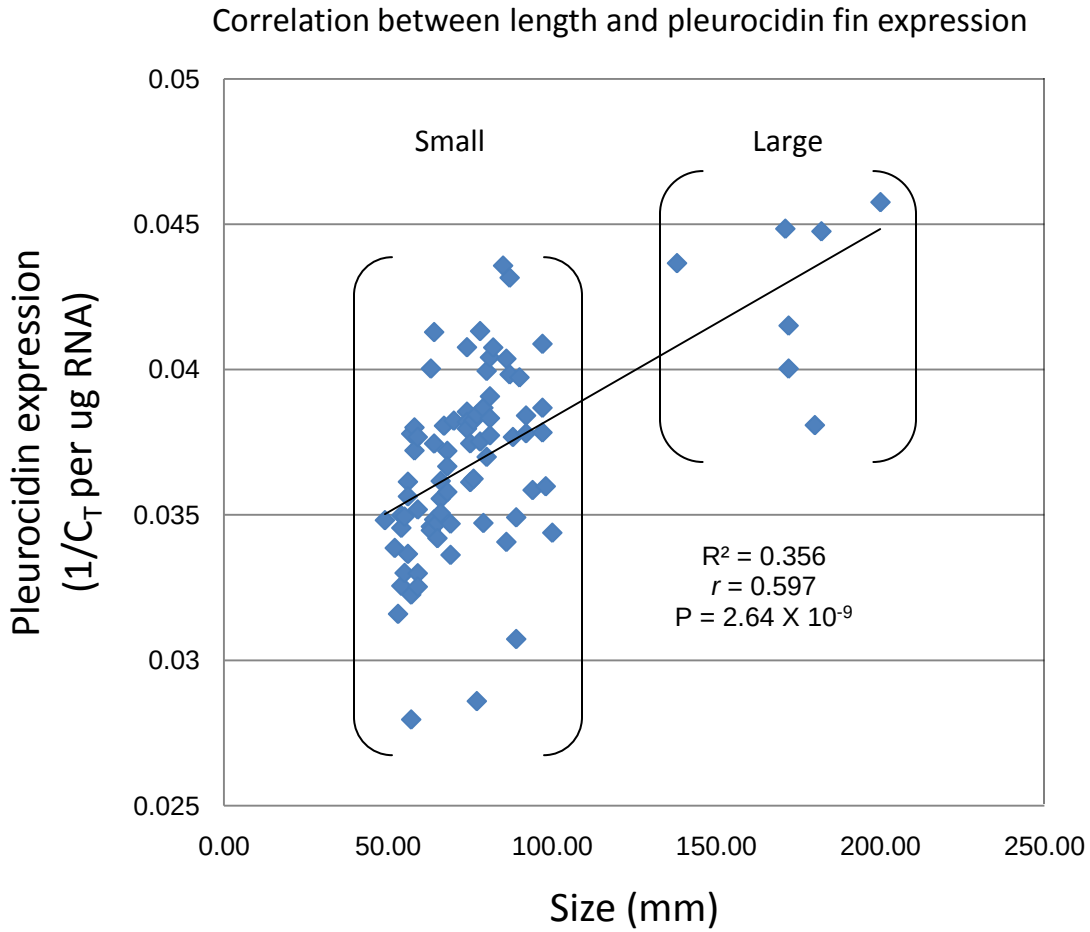

Figure 9. Data were analyzed by Pearson-Product Moment Correlation

DISCUSSION

Interactions between the host, pathogen and environment are believed to be the key factors controlling the development of infection into overt clinical disease. In fish, genetic predisposition, exposure history and physiological fitness of individuals within the population are similar to that of terrestrial host species, however the pathogens and environment are quite different. As in other systems, presence and virulence of the pathogen can drive infection towards disease, but the nature of the host immersion in a medium containing potentially high pathogen densities and/or contaminant concentrations is unique within aquatic systems. In this study, we were specifically concerned with the immune capacity of wild populations of YOY winter flounder from Long Island coastal bays and sought to determine whether contaminant exposure in these same YOY winter flounder would influence immunological status.

In fish, innate immunity provides a pre-existing and system of protection as opposed to adaptive or acquired immune response which takes longer and develops later (Alexander and Ingram, 1992; Ellis, 2001). Humoral components involved in innate immunity identified from fish include lysozyme, complement, lectins and antimicrobial peptides (Ellis, 1981). One of the most widespread antimicrobial peptides in fish are the piscidins, which display broad-spectrum activity against viruses, fungi, and parasites. Pleurocidin is an antimicrobial peptide identified in winter flounder (Cole et al. 1997) has been isolated, cultured and synthetically produced for use as an antimicrobial agent (Douglas et al., 2001; Patrzykat et al., 2003; Corrales et al., 2009).

We targeted YOY winter flounder liver and skin tissues for the expression analysis of pleurocidin in fish collected from six bays around Long Island and found that expression was

variable across locations, and expression in the skin was significantly higher than that in the liver. Previously, pleurocidin has been localized from within skin and intestinal tissues in winter flounder. Using transmission election microscopy and immune gold staining, Cole et al. (2000) revealed that the preferential location of pleurocidin in winter flounder was near the mucin granule membranes in the skin and within the globlet cells obtained from the proximal portion of the small intestine. However, pleurocidin was not shown to be present in sections of the heart, striated muscle, gills, stomach, liver and spleen. Douglas et al. (2001) substantiated this by finding pleurocidin transcripts in both skin and intestine tissues using Northern blot and RT-PCR analyses. Our study, agrees with findings from these previous reports finding pleurocidin expression to be approximately ten times higher in the skin than the liver (Fig. 2A). The localization of pleurocidin in skin mucus cells and the goblet cells of the small intestine of the winter flounder supports pleurocidin playing an important role in mucosal immunity since the epidermis, like the gut, is an interface between organism and environment.

Although our sampling pool was predominantly comprised of smaller (<125 mm TL) and younger individuals, we did have larger and older individuals at two sites; Port Jefferson Harbor and Jamaica Bay. Assessment of these two sites showed that there was a significant difference in pleurocidin expression as a function size between groups and a significant correlation between expression and length in individual fish (Figs 8A and 9). Age dependent pleurocidin expression has been previously observed by Douglas et al. (2001) who reported pleurocidin transcripts in winter flounder showed a progressive increase from early larval stages, thirteen days post hatch, towards adulthood.

The presence of other antibacterial proteins during early life stages has also been reported in fish. Seppola et al. (2009) found transcripts and activity of lysozyme in unfertilized Atlantic

cod (*Gadus morhua*) eggs. The transcriptional onset of other antibacterial genes (hepcidin and pentraxin) occurred during the gastrula period. After hatching, and at the time of first feeding the transcript level of cathelicidin increased in larvae. The authors suggested that this may represent an adaptation to improve egg quality and increase offspring viability by preventing bacterial invasion of eggs and embryos and prepares the larvae for increased pathogen exposure after leaving the protection of the egg. Antibacterial activity has also been observed in eggs from coho salmon (*Oncorhynchus kisutch*) (Yousif et al., 1991) and zebrafish (*Danio rerio*) (Wang et al., 2008). This ability to have a functional component of innate immunity present during the period of early development may be crucial to survival since ingestion of exogenous food is expected to introduce larvae and juveniles to a diverse range of viruses and bacteria.

Although pleurocidin expression was quantified in individuals from six sites around Long Island with variable population density and presumed contaminant loadings along a west to east gradient, no special gradient in pleurocidin expression was observed. Mean expression in fin differed by a factor of 10 between the highest (Port Jefferson) and the lowest (Manhasset) site, but individual variability was high, and the elevated expression levels in Port Jefferson population were likely influenced by the greater number of larger fish at this site. If fish from the larger size classes (>125 mm TL) were removed from the analysis, expression levels in Port Jefferson fish were only significantly elevated over the fish from Manhasset (data not shown) where pleurocidin expression was significantly less than all other sites evaluated. No reports on antimicrobial peptide expression in the skin and its relationship with contaminant exposures have been published, but there have been studies on hepcidin (an antimicrobial peptide and iron-regulating hormone) and the effects of two known environmental contaminants, the hormone, 17β estradiol, and the polycyclic aromatic hydrocarbon, benzo[a]pyrene. Robertson et al. (2009)

found that the induction of hepcidin-2 by the bacteria (*Edwardsiella ictaluri*) in 1 to 2 yrs old largemouth bass (*Micropterus dolomieu*) was abolished by 17β estradiol. On the other hand, Wang et al. (2009) reported that hepcidin transcripts in juvenile black porgy (*Acanthopagrus schlegelii* B.) were significantly induced by exposure from 1.0 ug/l benzo [a] pyrene after 4 hrs of continuous exposure. While this study assessed winter flounder that are most likely chronically exposed to contaminants, these studies done under acute conditions show that antimicrobial peptides can either be inhibited or induced by different compounds. The pleurocidin expression patterns observed here suggest that fish are responding to unknown factors in the environment, not directly linked to degree of urbanization.

Antimicrobial activity against 2 *Vibrio* species was evaluated using YOY winter flounder mucus. *V. anguillarum* has been found to be responsible for disease in many fish globally as well as the causative agent for numerous winter flounder disease outbreaks (Levine et al., 1972; Robohm et al., 1979). While there are there are no reports of pathogenicity of *V. parahaemolyticus* on winter flounder, this bacterium is considered an opportunistic pathogen found ubiquitously in inshore sediments and invertebrates (Baross and Liston, 1970) which are habitats and food sources of winter flounder. Although activity against both pathogens was observed, it was more consistent against *V. anguillarum.* The ability of pleurocidin amide to protect against *V. anguillarum* in Coho salmon *in vivo* was demonstrated by Jia et al. (2000) suggesting a potential use of pleurocidin as a therapeutic agent in aquaculture to replace conventional antibiotics. Shen et al. (2010) have described antimicrobial peptide resistance mechanisms by *V. parahaemolyticus* through effective repair of damaged membranes and prevention of cellular penetration of antimicrobial peptides. To understand this, further characterization of the isolates of *V. anguillarum* and *V. parahaemolyticus* is needed. In any

case, data indicate that YOY winter flounder have some ability to protect themselves from common marine pathogens via mucus antimicrobial peptides.

Antimicrobial activity also did not demonstrate clear environmental patterns. Activity in fish from only one site was significantly different from others for *V. anguillarum*, and no significant differences between sites were observed for *V. parahaemolyticus*. There also appeared to be no correspondence between antimicrobial activity and pleurocidin expression and no significant correlation was observed between these two responses in individual fish. These data indicate that the antimicrobial activities measured here may be mediated at least in part by antimicrobial peptides other than pleurocidin.

CYP1A expression in liver was measured to provide an indication of contaminant exposure in these fish. Somewhat surprisingly, no site specific differences in expression were observed indicating that contaminant exposure did not vary greatly between the sites examined. An earlier study by our lab on adult winter flounder reported significant elevation of CYP1A protein levels and EROD activity in liver tissue from Jamaica Bay as compared with Shinnecock Bay fish (Mena et al., 2006). However, earlier studies of CYP1A activity and expression in adult winter flounder from around New England indicate that induced levels are common along the entire northeast coast. Monosson and Stegeman (1994) reported EROD activity and CYP1A protein levels were correlated with PCB tissue content, finding elevated levels at two sites in Long Island Sound, Hempstead Bay (an area evaluated in this study) and Niantic (an area further east in Long Island Sound on the CT shore). Only remote areas of coastal Maine and Georges Bank showed significantly lower levels of induction. Similar CYP1A enzyme activities were observed by Collier et al. (1998) who examined the induction of hepatic aryl hydrocarbon hydroxylase (AHH) in adult non-spawning winter flounder in the northeast from 1991 to 1994.

They reported that there were trends towards increasing AHH activities over time in fish from the Raritan Bay/Long Island Sound area, and generally increasing trends for sites near Massachusetts (especially Boston Harbor) and Rhode Island. A decreasing trend was noted in fish from Great Bay, New Jersey, and there appeared to be decreasing activities in fish from the nearshore waters of Maine. Over the period of study elevated levels of AHH activity were seen in winter flounder from both the western and eastern portions of Long Island Sound. Contaminant levels commensurate with areas of the Northeast coast known to be significantly impacted by contaminants such as Boston and New Bedford Harbors and Raritan Bay.

The only available data on sediment contaminant levels at the sites examined in this study come from the Environmental Protection Agency's National Coastal Assessment a single sediment sample is collected randomly within an area on an annual basis to provide unbiased estimates of regional condition. Table 2 shows PCB and PAH (ng/gdw) sediment concentrations in samples collected from 2000 to 2005. Although mean levels averaged over the six year period are indeed higher in the urbanized sites to the west, high variability between individual measurements was observed at all sites evaluated, with coefficients of variation (CV) ranging from 52 to 188% and concentration in sites that are less urban (Shinnecock Bay) matching or at times exceeding those that are more urbanized (Jamaica Bay).

However, levels in inducing agents in sediment may not adequately reflect levels of bioavailable contaminants fish actually are responding to. Recent data indicates highly variable levels of PCBs in Jamaica Bay (Lauenstein and Kimbrough 2007), one of our study sites where differences of more than a factor of 10 were observed over a relatively small area (e.g.

http://www.bnl.gov/wrdadcon/publications/image/jamaica-bay-pcb.jpg). Nacci et al. (2010) comparing the sensitivity of populations of the Atlantic killifish (*Fundulus heteroclitus*) from 24 locations around the east coast from Virginia to Massachusetts to CYP1A induction after exposure to PCB126 found tissue PCB levels to be a better indicator of exposure than sediment contaminant levels. Furthermore, the sensitivity of early life stage fish from Jamaica Bay (a location investigated in this study) compared more closely to less contaminated sites along the coast, further indicating that fish from this site may not be chronically exposed to extremely high levels of inducing chemicals.

Genetic variability among populations may in part explain the variability seen in gene expression observed in individual fish from each site. Early work suggested that there were discrete spawning stocks of winter flounder within Long Island Sound (Berry et al., 1965), But more recent work examining genetic variability among winter flounder larvae collected from presumed spawning groups within the Niantic River and Thames River, CT in eastern Long Island Sound, Crivello et al. (2004) observed substantial genetic differences among the reputed source populations over relatively small spatial scales (<5km). However, similar results have been reported for fish in Narragansett Bay. In a similar genetic analysis Buckley et al. (2008) found that there were 16 distinct populations of winter flounder larvae. Juveniles collected from within Narragansett Bay were found to arise from adjacent winter flounder larval populations (>99%), suggesting that YOY juveniles do not move far geographically from natal nurseries. The high genetic variability within small geographic areas and limited movement of juveniles away from spawning grounds and could also account for the variability observed in individuals within each site in this study.

Table 2. Background data on PCB and PAH sediment contaminant levels at collection sites from EPA's National Coastal Assessment from 2000 to 2005

	Data all years PCBs (ng/gdw)					
	SHIN	**PJEFF**	**MAN**	**OSB**	**LNB**	**JB**
	21.40	2.10	69.20	32.10	7.60	1.00
	5.00	4.40	97.60	41.30	78.40	39.00
	11.20	14.50	82.20	17.30	262.30	12.80
	17.60	15.90	19.30	11.80	51.00	70.60
			30.30	1.70	59.40	167.10
				9.00		6.80
				5.70		34.60
						61.80
Mean	**13.80**	**9.23**	**59.72**	**16.99**	**91.74**	**49.21**
Max	21.40	15.90	97.60	41.30	262.30	167.10
Min	**5.00**	**2.10**	**19.30**	**1.70**	**7.60**	**1.00**
SD	7.22	6.99	33.65	14.56	98.81	53.83
CV	**52.32**	**75.73**	**56.35**	**85.70**	**107.71**	**109.37**

	Data all years PAHs (ng/gdw)					
	SHIN	**PJEFF**	**MAN**	**OSB**	**LNB**	**JB**
	324	70	1712	676	1052	23
	533	148	242	688	2448	7.6
	28.4	11.4	171	66.9	764	3.7
	84.3	69.9	61.8	2.9	778	133
		78.4	134	64.2	3116	318
				967		8.9
				413		954
						3076
Mean	**242.43**	**75.54**	**464.16**	**411.14**	**1631.60**	**565.53**
Max	533.00	148.00	1712.00	967.00	3116.00	3076.00
Min	**28.40**	**11.40**	**61.80**	**2.90**	**764.00**	**3.70**
SD	232.31	48.57	700.59	378.88	1082.50	1064.55
CV	**95.83**	**64.29**	**150.94**	**92.15**	**66.35**	**188.24**

Table 2. SHIN = Shinnecock Bay, PJEFF = Port Jefferson Bay, OSB = Oyster Bay, MAN = Manhasset Bay, LNB = Little Neck Bay, JB = Jamaica Bay. Represents available data from 2000 to 2005 at each site.

CONCLUSIONS AND SIGNIFICANCE

This study demonstrates that evaluating pleurocidin expression in fin tissue may be an effective tool in determining innate immunity in juvenile fish. Data indicate that YOY winter flounder have some ability to protect themselves from common marine pathogens via mucus antimicrobial activity. No differences in CYP1A expression were observed. These data suggest that exposure to aromatic hydrocarbon contaminants is fairly wide-spread throughout the study area and that any difference in pleurocidin expression in YOY winter flounder observed are due to other factors. The lack of correlation between pleurocidin expression and antimicrobial activity indicates that other antimicrobial peptides may be involved or that other factors are influencing antimicrobial activity. This is to my knowledge the first reports quantitatively evaluating pleurocidin expression in YOY winter flounder from an urban area.

Contaminant exposure, from either the water column or sediments, in known to be a significant cause of acute and in some cases chronic stress to fish physiology. However, while these effects are well studied with respect to resulting growth and/or reproductive effects (i.e. endocrine disruption) on wild populations, the effects on immune responses are often overlooked. Furthermore, in the case of Long Island Sound winter flounder, in which depressed populations are not associated with reduced recruitment, identification of factors affecting survival and morbidity of juveniles are important in determining their significance towards the lack of population recovery. Further work is needed to characterize factors controlling pleurocidin expression, as well as other indicators of immune response in young fish.

Results of this study suggest that larger sample sizes should have been used in conjunction with a comparison between life stages from all sites may provide greater insight into the differences between individual sites/populations. Given that many other proteins are present in mucus, future work could also include the purification method adapted by Cole et al. (1997) to provide a more specific measurement of pleurocidin antimicrobial activity. Also, a pathogen/stimulation challenge should be conducted to see if pleurocidin can be induced under immunological priming and whether a correlation exists with survival and expression of immune parameters.

Although qRT-PCR has been reported to be more sensitive than other techniques (i.e. EROD activity, immunohistochemistry, ELISA and Western blotting) in determining CYP1A induction in fish (Rees and Li, 2004), CYP1A activity or protein levels as well as gene expression should be done in parallel to see if these are more responsive measures. Since data suggest that contaminants do not vary in sites around Long Island, fish from George's Bank and/or Northern Maine should be used as references and compared with those from Long Island.

Finally, a total gene expression analysis between sites should be assessed to look for up and down regulated genes and gene function for more sensitive biomarkers. With the advent of next-generation sequencing and RNA-sequence technologies, whole genome and transcriptome analysis could provide a series of sensitive biomarkers to assess population dynamics and individual fitness to build on this and the SSH work by Straub et al. (2004). If funds are available, RNA-seq or microarray analysis, with the potential use of European flounder (*Platichthys flesus*) chip, should be applied since they have the potential to provide informative data on the degree of toxicant exposure and disease vulnerability in juvenile winter flounder in an urban estuary through an understanding of the functional pathways (Williams et al., 2008).

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APPENDIX 1: ANTIMICROBIAL ASSAY

1. Into sterile 2 ml colorless Eppendorf tubes, mix 10 µl mucus + 10 µl bacterium + 30 µl PBS buffer.
2. Place 50 µl PBS buffer into a sterile 2 ml tube as the negative control and use a mixture of 10 µl bacterium + 40 µl PBS buffer as the positive control.
3. Use 10 µl mucus + 30 µl PBS buffer in a sterile 2 ml tube as the growth control.
4. Incubate samples at 28 ^{o}C for 30 minutes followed by the addition of 450 µl of ice cold, sterile marine broth.
5. Incubate assays involving *V. parahaemolyticus* and *V. anguillarum* at room temperature for 24 hours and48 hours respectively.
6. After incubation, pipette contents of each tube up and down 8 to 10 times with a sterile filter tip 100 µl pipette to resuspend the bacterial culture.
7. Add 100 µl of each sample to separate wells in a 96-well flat bottom plate (Microtest^{TM}, Franklin Lakes, NJ) in triplicate and measure the OD at 560 nm using the Wallac Plate Reader (PerkinElmer Life Sciences, Downers Grove, IL).

APPENDIX 2: RNA EXTRACTION

Use sterile filter pipette tips and RNAse/DNAse free microcentrifuge tubes. Wash down work surface with 70% ethanol before commencing and wear gloves and lab coat. Do all work with Trizol in fume hood.

1. Homogenize tissue samples in 0.75 ml Trizol ® LS Reagent.
2. Incubate the homogenized tissues at room temperature for 5 minutes.
3. Add 0.2 ml chloroform (Sigma Aldrich, St. Louis, MO) and shake vigorously for 15 seconds and incubate at room temperature for 10 minutes.
4. Centrifuge samples for 12,000 x *g* for 15 minutes at 4 ^{o}C.
5. Place samples on ice and carefully remove the upper aqueous phase to clean 1.5 ml tubes (colorless eppendorf). Be careful not to take any of the interphase.
6. Precipitate the RNA by adding 0.5 ml of isopropyl alcohol (Sigma Aldrich, St. Louis, MO) and incubate at room temperature for 10 minutes.
7. Centrifuge samples at 12,000 x g for 10 minutes at 4^{o}C and remove the supernatant without disturbing the RNA pellet.
8. Wash the pellet once with 1 ml of 75% ethanol (Sigma Aldrich, St. Louis, MO) and vortex for a few seconds.
9. Centrifuge at 7,500 x g for 5 minutes at 4^{o}C and discard the ethanol.
10. Air dry the RNA pellets for 30 minutes and dilute with 50 µl RNase-free water and place on ice.

APPENDIX 3: CDNA SYNTHESIS

1. Thaw RNA and reagents provided in the III First-Strand Synthesis SuperMix for qRT-PCR kit Universal (Invitrogen) and keep on ice.
2. 1.5 µg of Total RNA is required for cDNA synthesis with a total volume of 20 µl. Calculate the volume of RNA required for this reaction by the following equation: 1500/RNA concentration.
3. At most 8 µl and at least 1.5 µl of RNA can be used. If needed, calculate the volume of DEPC-treated water needed to make up a total volume of 8 µl.
4. Prepare a Master Mix by adding; (10 µl 2X Reaction Mix + 2 µl RT Enzyme Mix) x no. of samples.
5. Combine 12 µl of the Master Mix, the calculated volume of DEPC-treated water and RNA in a 0.2 ml tube and keep on ice.
6. Put tubes in the real-time thermal cycler (Eppendorf) programmed at 25 ^{o}C for 10 minutes, 50 ^{o}C for 30 minutes, 85 ^{o}C for 5 minutes. Maintain the reaction at 4 ^{o}C.
7. Add 1 ul of *E. Coli* RNase H to each tube and incubate at 37 ^{o}C for 20 minutes then place on ice.

APPENDIX 4: PCR

1. Thaw cDNA and reagents provided in the Platinum® *Taq* DNA Ploymerase kit (Invitrogen) on ice.
2. Make a 10 µM working solution of primers.
3. Prepare a master mix for 3 cDNA samples and a negative control with each primer set by adding; (20 µl 10X PCR Buffer, Minus Mg + 6 µl $MgCl_2$ + 16 µl dNTP mixture + 8 µl forward primer + 8 µl reverse primer + nuclease-free 143 µl H_2O + 1 µl Platinum® *Taq* DNA Ploymerase).
4. Dispense 48 µl into each of four 0.5 ml tubes and add 2 µl of cDNA template and 2 µl nuclease-free H_2O as the negative control. Mix well.
5. Place tubes in the Mastercycler (Eppendorf) programmed at 94 ^oC for 2 minutes, 35 x (94 ^oC for 30 seconds, annealing temperature for 30 seconds and 72 ^oC for 60 seconds), 72 ^oC for 10 minutes. Maintain the reaction at 4^oC.
6. Place tubes on ice and evaluate the PCR products by agarose gel electrophoresis using 1.5% agarose gel with ethidium bromide and 10 µl PCR product. Observe using UV transillumination.

APPENDIX 5: PCR PURIFICATION

1. Excise DNA fragment using a clean blade and place in a 1.5 ml microcentrifuge tube. Add 10 µl Membrane Binding Solution for every 10 mg of gel. Vortex the mixture and incubate at 50-65^{o}C for 10 minutes, vortexing every 5 minutes to dissolve the gel.
2. Place a SV Minicolumn into a Collection Tube and pipette the dissolved gel mixture onto the column and incubate for 1 minute at room temperature.
   Centrifuge at 16,000 x *g* for 1 minute and discard the flow through.
3. Replace the column into the Collection Tube and add 700 µl Membrane Wash Solution with ethanol to the column and centrifuge at 16,000 x *g* for 1 minute and discard the flow through.
4. Repeat the wash with 500 µl Membrane Wash Solution and centrifuge at 16,000 x *g* for 5 minutes. Discard the flow through and centrifuge for 1 minute to get rid of residual ethanol.
5. Place the column into a 1.5 ml microcentrifuge tube and add 50 µl nuclease-free water into the center of the column and incubate at room temperature for 1 minute. Centrifuge at 16,000 x *g* for 1 minute. The content of the microcentrifuge tube is the prepared purified DNA.

APPENDIX 6: TA CLONING AND PCR SCREENING

1. Gently mix 4 µl purified DNA + 1 µl Salt Solution + 1 µl TOPO® vector and incubate at room temperature for 5 minutes. Place mixture on ice.
2. Add 2 µl of the above mixture to 25 µl TOP10F' competent cells (do not pipette up and down) and incubate on ice for 30 minutes.
3. Heat shock the cells at 42 ^{o}C for 30 seconds and replace on ice.
4. Add 250 µl of room temperature S.O.C. medium, cap tightly and shake the tube horizontally (200 rpm) at 37 ^{o}C for 1 hour.
5. Spread 10 to 50 µl from tube unto each of 6 prewarmed selective plates and incubate overnight at 37 ^{o}C.
6. After cloning, pick 10 colonies for analysis and inoculate ½ of each colony into 1.5 ml LB broth containing ampicillin and grow overnight.
7. Prepare a PCR Master Mix using a gene-specific primer and a non-specific primer, T3 and T7, (TOPO TA Cloning® Kit for Sequencing; Invitrogen). Substitute cDNA with ½ of each colony left from step 6.
8. Evaluate PCR products by agarose gel electrophoresis. Once screening test is positive, proceed to plasmid DNA isolation and sequencing.

APPENDIX 7: PLASMID DNA ISOLATION AND SEQUENCING

1. Centrifuge culture at 1,500 x *g* for 15 minutes and discard the culture media. Resuspend the pellet in 240 µl Resuspension Solution prepared with RNAse A.
2. Add 240 µl of Lysis Buffer and gently mix by inverting the tube 4 to 8 times then incubate for 5 minutes at room temperature.
3. Add 340 µl of Neutralization/Binding Buffer and mixing by inverting as previously done. Using a tabletop centrifuge, spin for maximum speed for 10 minutes.
4. Place a Purelink^{TM} spin column into a collection tube and pipette the supernatant, from the previous step, into the spin column and centrifuge at 14,000 x *g* for 1 minute at room temperature. Discard the flow through and replace the spin column into the collection tube.
5. Add 650 µl Wash Buffer prepared with ethanol to the column and centrifuge at 14,000 x *g* for 1 minute at room temperature. Discard the flow through and replace the spin column into the collection tube.
6. Centrifuge the column at maximum speed for 1 to 3 minutes to get rid of residual wash buffer.
7. Place the spin column into a 1.7 ml elution tube and add 100 µl DEPC-treated water to the center of the spin column. Incubate at room temperature for minute and centrifuge at maximum speed for 1 minute.
8. The content of the elution tube is the prepared plasmid DNA.
9. Determine quantity of the plasmid DNA using the NanoDrop-1000 Spectrophotometer (v3.2.1, Thermo Fisher Scientific) and store at -20 ^{o}C.
10. Acquire a plasmid DNA concentration of 2 µl/ng. In 0.2 ml strip tubes, combine 1 µl plasmid DNA + 2 µl T3 or T7 primer + 5 µl DEPC-treated water and submit for sequencing.
11. Once sequences are confirmed, use prepared plasmid DNA as a standard for Real-time qPCR.

APPENDIX 8: QRT-PCR

1. Thaw cDNA and reagents provided in the SYBR® GreenER™ Two-Step qRT-PCR Universal Kit (Invitrogen) on ice.
2. Program mastercycler (Eppendorf) at optical cycling temperatures; 95°C for 10 min, 40 cycles of (95°C for 15 sec, 58°C for 30 sec), 95°C for 15 sec, 60°C for 15 sec, melt curve analysis for 20 min and terminate at 95°C for 15 sec.
3. Prepare 1:10 dilutions for standards by combining 2 µl plasmid DNA or PCR products with 18 µl DEPC-treated water. Prepare fresh standards each time and change pipette tips each time for an efficient curve.
4. Prepare a master mix (12.5 µl of SYBR greenER supermix + forward and reverse primers (i.e. 1 µl each, from 10 µM stock) + 9 µl DEPC water) = 23.5 µl per reaction. Make up one extra sample per 15 or so samples being made.
5. Dispense 23.5 µl into each well of a 96-well PCR plate addition of template.
6. Add 1.5 µl template and pipette 5-6 times to mix. Seal plate with sealing film and centrifuge plate at 300 x *g* for 3 minutes at 4 °C to remove air bubbles. If needed, keep on ice.
7. Enter the plate layout, place plate in preheated mastercycler and run the assay.