Trophic Transfer of Contaminants and Mechanisms of Behavioral Effects on Juvenile Bluefish, *Pomatomus saltatrix* from the Hackensack River

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Introduction

Behavior is essential to a species' survival in their environment. Subtle changes in behavior, whether food acquisition, activity, predator avoidance, or reproduction due to toxic effects of contaminants can have severe implications for survival. We have previously shown in lab experiments that young-of-the-year (YOY) bluefish (*Pomatomus saltatrix*) from Tuckerton, NJ (a relatively clean estuary) accumulate very high levels of PCBs, mercury, and chlorinated pesticides when fed prey from the contaminated Hackensack River, NJ. Feeding on the contaminated prey resulted in a significant reduction in appetite, swimming activity, and growth (Candelmo 2007). Field-collected YOY bluefish and prey fish from the Hackensack River were also found to have significantly elevated concentrations of PCBs, pesticides and mercury compared to those from Tuckerton. Length and weight of HR field-caught bluefish were also significantly less than TK field-caught bluefish and the percentage of YOY HR bluefish caught with food in the gut was low (27%) compared to YOY bluefish reported from other regions (80%), suggesting reduced feeding also occurs in the wild population. Alterations of bluefish behavior and growth from exposure to contaminants may have detrimental effects on condition, migratory competence and overwinter survival, and ultimately recruitment success.

Possible underlying causes of the behavior and growth deficiencies in the fish include altered thyroid hormone status and/or neurotransmitters. PCB's and methylmercury are known endocrine disrupting chemicals in fish and mammals. Xenobiotics may alter hormonal status a number a ways: altered synthesis, via binding to receptor sites, altering transport of the hormones in the blood, or altering enzymes, such as deiodinases that metabolize the thyroid hormones (Zhou et al. 1999a, Weis et al 2001). Alterations in the thyroid system could have severe implications on the behavior, growth, and life cycle of

the organism. Endocrine disruption, particularly thyroid dysfunction, from contaminant exposure may be an underlying mechanism for the observed behavioral and growth effects (Zhou et al., 1999a). Neurobehavioral development is contingent on proper thyroid hormone synthesis. It is possible that the exposure to PCBs and other contaminants may result in alterations in the function of the pituitary-thyroid axis, causing alterations in synthesis of thyroid hormones. The system often responds to decreased thyroid hormones by the pituitary secreting increased levels of TSH, which results in the enlargement, budding, and irregularity of the thyroid gland, commonly known as a goiter. These alterations may result in hypertrophied thyroid follicles, or the fish equivalent of a "goiter." Mummichogs from the contaminated Piles Creek near Newark Bay NJ were found to have irregularly shaped and greatly expanded thyroid follicles or goiters compared to those from the reference site, Tuckerton in southern New Jersey (Zhou et al. 1999a). These fish also had impaired prey capture and reduced growth.

The synthesis, storage, or release of neurotransmitters, their receptor sites, reuptake mechanisms or postsynaptic action have also been shown to be affected directly by exposure to toxicants including metals and organic pollutants. PCBs have been shown to alter levels of neurotransmitters, particularly dopamine, which is associated with altered behavior in fish (Fingerman and Russell 1980, Shain and Seegal 1991). Exposure to mercury has been linked with alterations of behavior and levels of dopamine, serotonin and it metabolites in fish (Smith et al. 1995, Tsai 1995, Zhou et al. 1999b). The effects of xenobiotics and influence on different neurotoxic mechanisms are dependent on the particular toxicant, exposure time, dosage and species (Zhou et al. 1999b). Neurotransmitters such as norepinephrine (NE), serotonin (5-HT) and dopamine (DA) and its metabolite L-DOPA are have been shown to be associated with various behaviors including locomotion, condition responses and feeding (Grippo 2003, Weis et al. 2001). There is evidence that neurotoxic changes lead to behavioral changes.

PCBs and methylmercury may have additive or interactive adverse effects on nervous system function. A mixture of methylmercury and PCBs was found to reduce dopamine levels in rat brains more than either chemical did alone (Bemis and Seegal 1999). Low concentrations of these xenobiotics may

not present sublethal behavioral or physiological changes on their own, but could cause detrimental alterations when combined together.

The purpose of this study was to investigate physiological and biochemical changes from contaminant exposure that may be mechanisms underlying the changes observed in the YOY bluefish behavior (reduced activity and feeding) and growth. Comparisons of the physiology of the laboratory bluefish and the field-caught bluefish from Tuckerton and Hackensack River will provide insight into the health of the YOY bluefish from the individual estuaries.

Materials and Methods

Thyroid Histology

Eleven YOY bluefish were collected using a bottom trawl net and hook and line in late September 2007 from both sites, the Hackensack River (HR-field) and Great Bay Tuckerton, NJ (TK-field) estuaries prior to emigration. The ventral portion of the head was immediately removed and preserved in a 10% formalin solution and then transferred to 70% ethanol for preservation. They were then then decalcified, dehydrated, embedded in paraffin, and sliced sagittally near the midline, mounted on slides and stained with hematoxylin and eosin. Thyroid follicles were located and their area and epithelial cell heights measured. Follicle morphology (area of follicle and epithelial cell height) was measured using a light microscope and UTHSCSA Image Tool version 3.0 software. The ten largest thyroid follicles were measured for each fish. The area was recorded and the cell height was measured in four spots around the cell equally spaced around the circumference of the follicle. The cell height for each follicle was averaged from these four measurements. Average follicle area, greatest follicle area, and average cell height was compared between the field caught bluefish from the two study sites.

In addition, thyroids from the bluefish from the previous laboratory experiments in which TK fish were fed prey fish from TK or HR were also analyzed. These fish had been sacrificed and stored in a -80˚ C freezer. The heads of ten HR-fed and ten TK-fed were dissected while still frozen and placed in a

solution of 10% formalin to defrost and then transferred to a 70% ethanol solution and processed the same as above.

Neurotransmitters

To determine neurotransmitter levels, 15 fish from both study sites HR and TK were collected, brains were removed in the field and put on dry ice and then stored in a -80˚ C freezer. In addition the ten HR-fed and TK-fed lab fish were stored frozen -80˚ C until the brain was dissected immediately prior to analysis. Each brain was placed in 0.5 ml 0.1 N perchloric acid, sonicated and centrifuged for 8 min at 1000×*g*. Supernatants were stored at −80 °C until analyzed by HPLC-EC to determine concentrations of dopamine (DA) and its metabolites, 3,4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA), serotonin (5-hydroxytryptamine (5-HT)) and its main metabolite, 5-hydroxy-3-indoleacetic acid (HIAA), and norepinephrine (NE). DOPAC:DA ratios and HVA:DA ratios, an index for dopaminergic activity, as well as HIAA:5HT ratios, an index for serotonergic activity, were also calculated for each measurement (Thiruchelvam et al., 2000; Zhou et al., 1999b). Detailed methods for HPLC-EC analysis followed the procedure and instrumentation described by Thiruchelvam et al. (2000). The pellets were later digested in 1ml of 0.5 N NaOH for measurements of protein concentration using Bio-Rad (Hercules, CA) assay reagents.

The concentration of each neutrotransmitter measured was compared between the Tuckerton field-caught fish and Hackensack field-caught fish and then between the Tuckerton lab fish and the Hackensack lab fish.

Statistical Analysis

All statistical analyses were performed on Minitab13.1 software program. The differences between the groups of bluefish were tested for significance using a one way ANOVA

for each variable. The level of significance was set at P<0.05. Pairwise comparison on certain variables was performed using Fisher's Least Significant Difference (LSD).

Results

Thyroid Histology Analysis

HR-Field and TK-Field

Thyroid follicles of HR field caught bluefish were noticeably enlarged and irregular, and often the colloid was partially depleted (Figure 1).

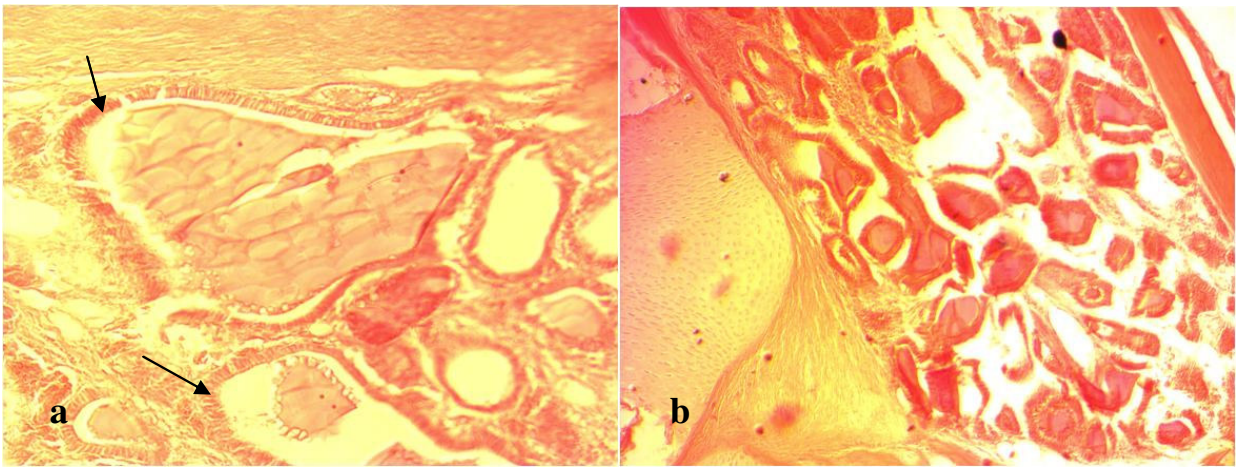

Figure 1 Sagittal section of thyroid tissue and follicles of HR-field **(a)** and TK-Field **(b)**. Picture taken at same magnification of 20x. Arrows point to enlarged follicles.

Epithelial cell height was found to be positively correlated with the area of the follicle when analyzing both the largest follicle per fish and the ten largest follicles per fish. Utilizing follicle area as a covariate, ANCOVA revealed that the epithelial cell height of the largest follicle of each fish was significantly greater in the HR fish than TK fish ($F = 180.90$; $p < 0.0001$) and that the average cell height of the 10 largest follicles per fish was significantly greater in the HR fish than TK fish ($F = 15.20$; $p = 0.001$). There was no difference found between the epithelial cell height of the largest follicles of HR bluefish and the mean of the cell

height of the 10 largest follicles of HR bluefish. This was also true for the TK bluefish (Figure 2a).

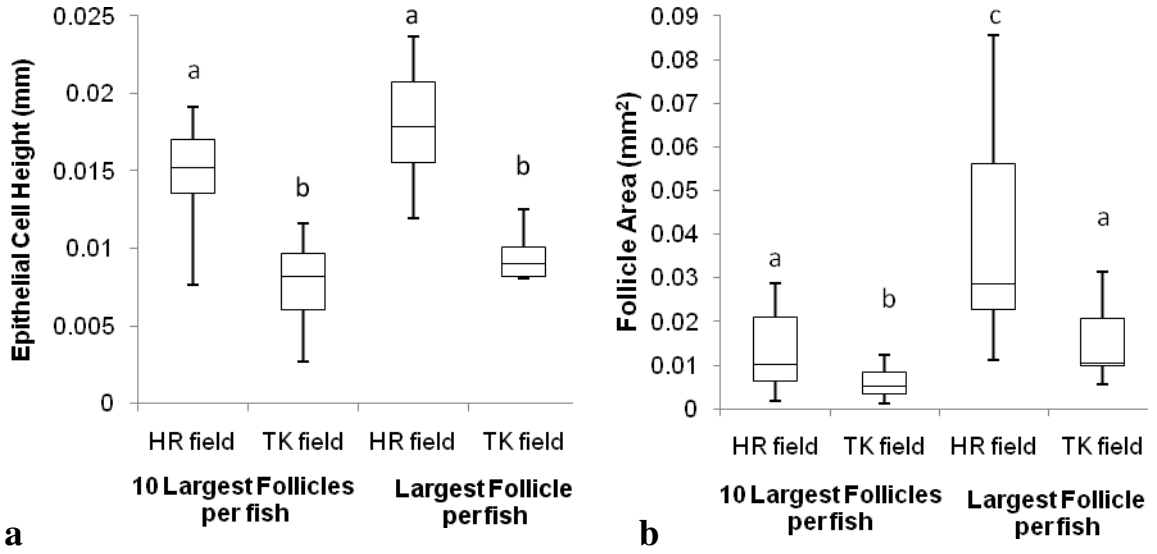

Figure 2.

a. Box plots of the mean epithelial cell height from the 10 largest thyroid follicles from 10 HR and TK bluefish and the mean epithelial cell height from the largest follicle for each fish. Four cells were measured and averaged per follicle. Plots with different letters are significantly different (ANOVA, $p < 0.05$; pairwise comparison with Fisher's protected least significant difference).

b. Box plots of the cross-sectional area of the ten largest thyroid follicles from 10 HR and TK fish and the area of the single largest thyroid follicle from 10 HR and TK bluefish. Plots with different letters are significantly different (ANOVA, $p < 0.05$; pairwise comparison with Fisher's protected least significant difference).

The mean area of the largest HR follicle was significantly larger than mean area of the largest TK follicle per bluefish ($F=10.70$; $p=0.004$). The mean area of the ten largest follicles was significantly larger in HR than in TK fish ($F=38.32$; $p<0.0001$). Significant differences were also found in pairwise comparison of the ten largest and largest follicle area per fish from TK and HR ($F = 30.38$; $p < 0.0001$) (Figure 2b). The mean of the largest TK-fed bluefish was not significantly different than the mean of the ten largest HR-fed bluefish.

Laboratory HR and TK fed Bluefish

The mean area of the largest HR-fed bluefish follicle was significantly larger than mean area of the largest TK-fed bluefish follicle per bluefish (F=5.47; p=0.031). The mean area of the ten largest follicles was significantly larger in HR-fed than in TK-fed fish (F=29.82; p<0.0001). Significant differences were also found in pairwise comparison of the ten largest and largest follicle area per fish from TK and HR (F = 35.43; $p < 0.0001$) (Figure 3). The mean of the largest TK-fed bluefish was not significantly different than the mean of the ten largest HR-fed bluefish.

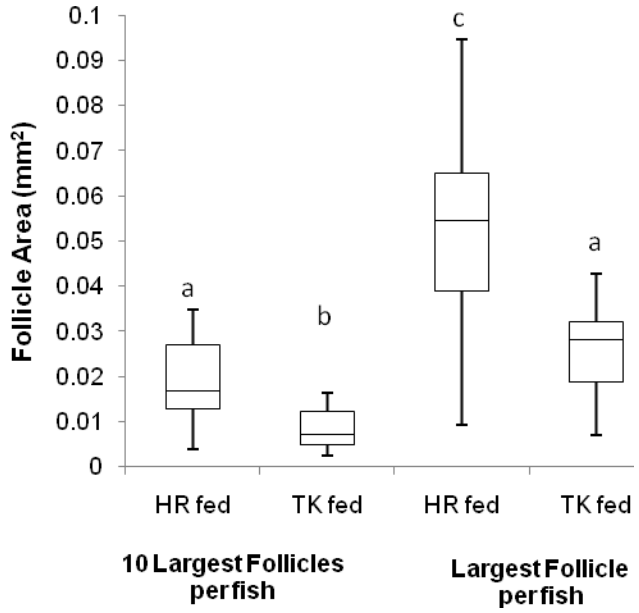

Figure 3. Box plots of the cross-sectional area of the ten largest thyroid follicles from 10 HR and TK fed bluefish and the area of the single largest thyroid follicle from 10 HR and TK bluefish. Plots with different letters are significantly different (ANOVA, $p < 0.05$; pairwise comparison with Fisher's protected least significant difference).

Neurotransmitters

HR-Field and TK-Field

The mean concentration of protein (mg/L) was not significantly different between HR and TK brains ($p = 0.161$). However, the mean concentration of the DA metabolites DOPAC and HVA were significantly lower in the HR fish than in the TK fish ($p < 0.0001$). The concentrations of DA, 5-HT and its metabolite HIAA were not significantly different (p = 0.163, 0.507 and 0.188, respectively) between the fish groups although they were lower in the HR fish (Figure 4 a & b). NE concentrations were not significantly different, but there was a trend that HR was greater than TK (p = 0.091) (Figure 4a).

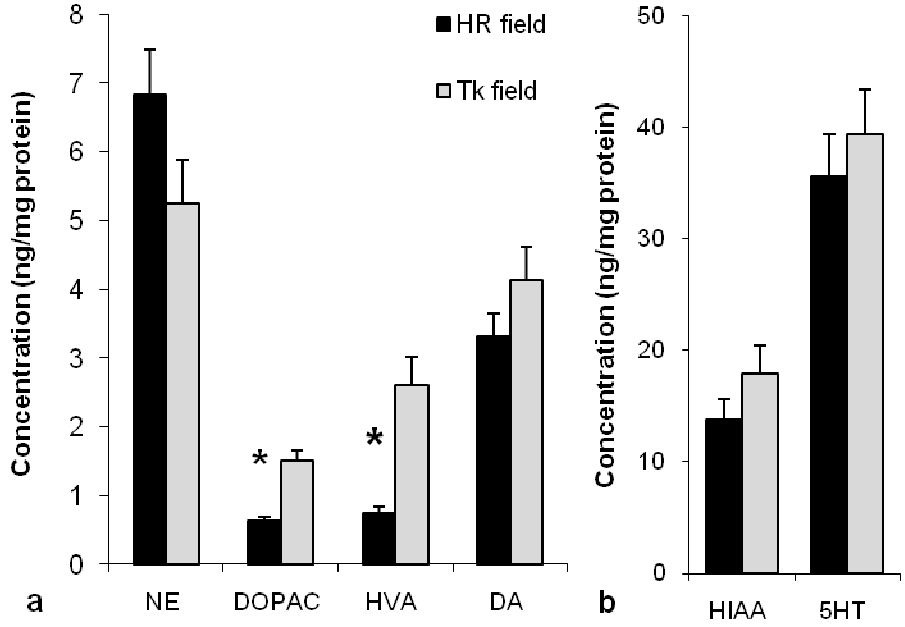

Figure 4. a. HR-field and TK field bluefish neurotransmitter concentration levels of dopamine (DA), its metabolites, 3,4-dihydroxyphenylacetic acid (DOPAC) and homovanillicacid (HVA), and and norepinephrine (NE).
b. HR-field and TK field bluefish neurotransmitter concentration levels of serotonin (5-HT), its main metabolite, 5-hydroxy-3-indoleacetic acid (5-HIAA)
Significance level p<0.05. Significance difference found between TK & HR is labeled with an asterisk *.

The dopaminergic activity levels (ratios of DOPAC:DA and HVA: DA) of HR fish were significantly lower than that of TK fish for both metabolites DOPAC and HVA ($p = 0.002$ and

0.008, respectively). However, there was no difference in serotoninergic activity, HIAA:5HT (p = 0.121) (Figure 5).

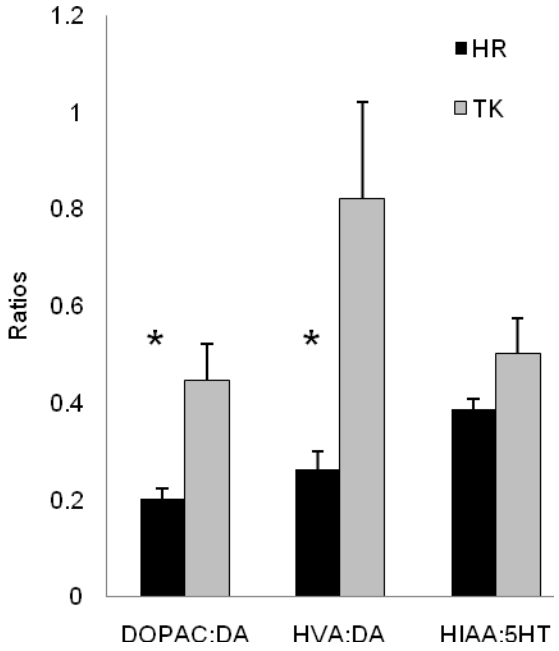

Figure 5. Dopaminergic activity levels (DOPAC:DA and HVA:DA) and the serotonergic activity levels (HIAA:5HT) of HR and TK field bluefish. Significance level $p<0.05$. Significance difference found between TK & HR is labeled with an asterisk *.

HR-fed and TK-fed lab fish

Comparison of the HR-fed and TK-fed laboratory experiment bluefish neurotransmitter concentrations revealed that all except the dopamine metabolite HVA were significantly higher in the HR-fed bluefish. The mean HVA concentration in the HR-fed bluefish was higher than that of TK-fed, but the p-value was 0.067. There were no differences found in metabolic activity (DOPAC:DA, HVA:DA and HIAA:5HT) for either dopamine or serotonin.

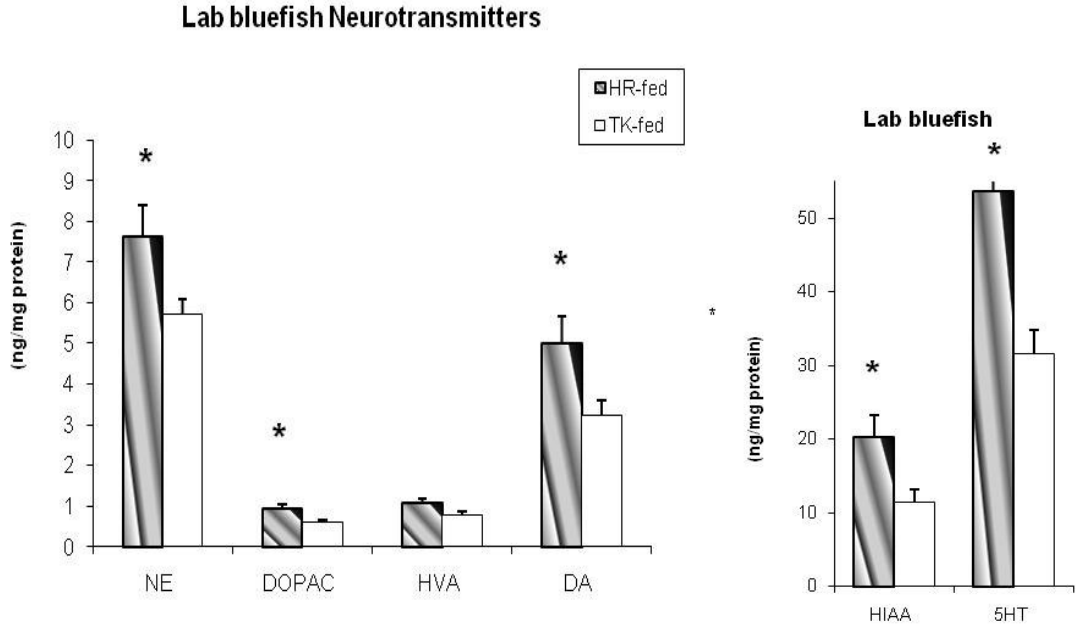

Figure 6. HR-fed and TK-fed lab bluefish neurotransmitter concentration levels. Abbreviations same as figure 3. Significance level $p<0.05$. Significance difference found between TK & HR is labeled with an asterisk *.

Lab and Field Bluefish

A pairwise comparison among the four groups using Fisher's (LSD) revealed significant differences. TK-field fish had significantly higher concentrations of the dopamine metabolites DOPAC and HVA than the other three groups. HR-fed lab fish contained significantly higher concentrations of serotonin. HR-fed fish also contained high concentrations of NE than TK-field fish and higher concentrations of dopamine than TK-fed lab fish and HR-field fish. (Figure 6)

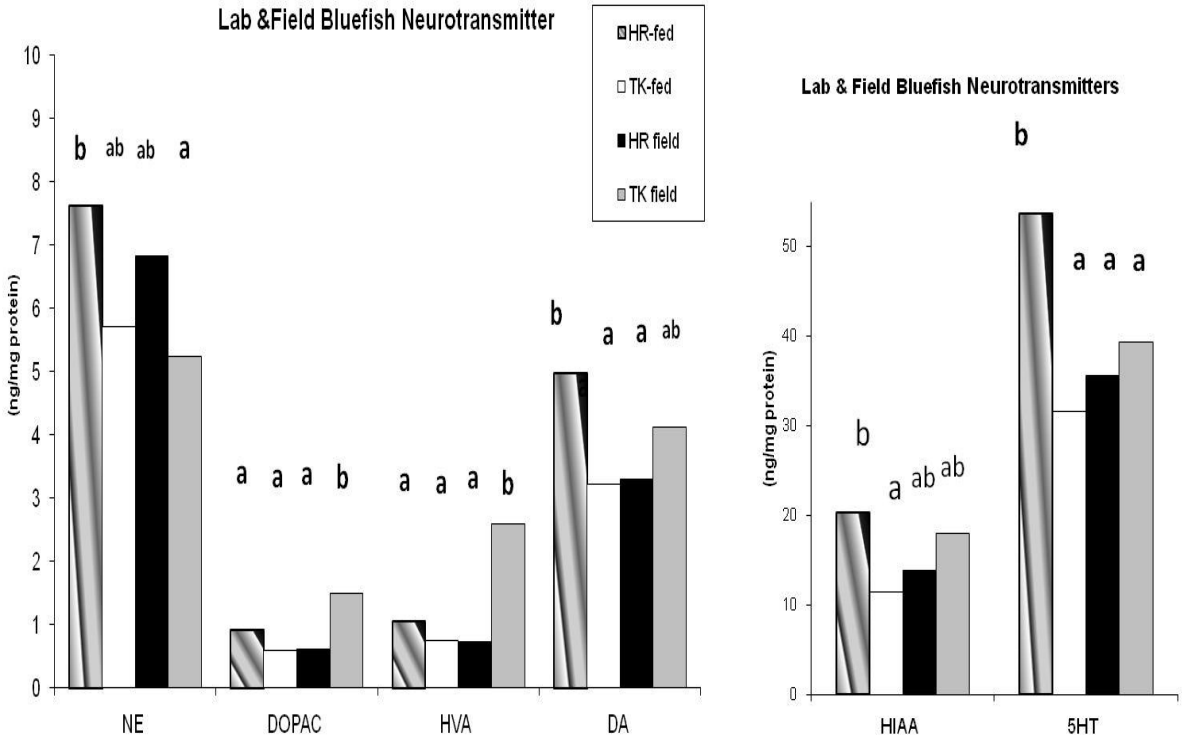

Figure 7. HR-fed and TK-fed lab bluefish neurotransmitter concentration levels. Abbreviations same as figure 3. A pairwise comparison with Fisher's protected least significant difference was performed on each neurotransmitter separately. The bars for each neurotransmitter with different letters are significantly different from one another (ANOVA, $P < 0.0001$)

In addition the TK-field fish also display significantly higher dopaminergic activity than all the other groups and higher serotonergic activity than the TK-fed lab fish. (Figure 7).

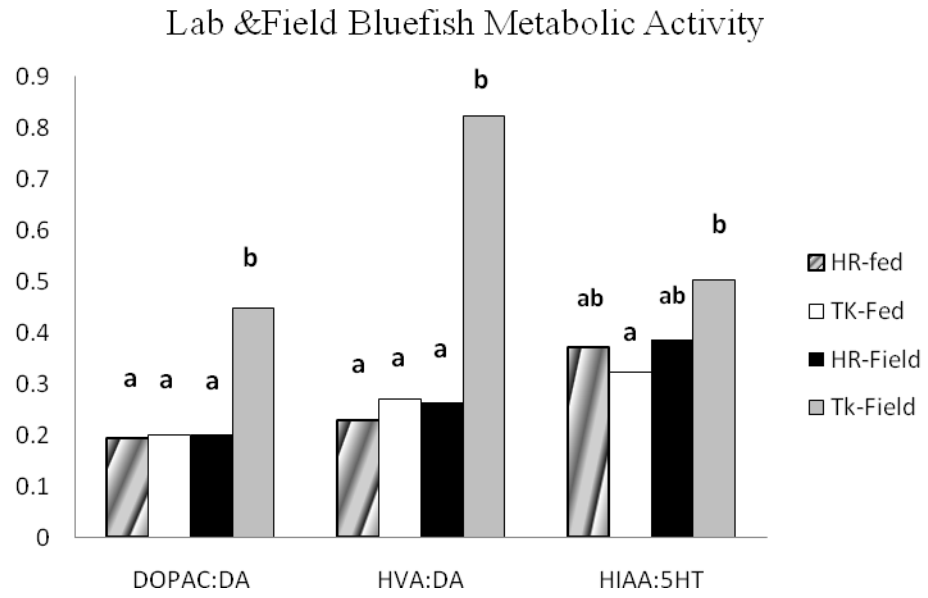

Figure 8. Dopaminergic activity levels (DOPAC:DA and HVA:DA) and the serotonergic activity levels (HIAA:5HT) of HR and TK field, HR-fed and TK-fed lab bluefish. A pairwise comparison with Fisher's protected least significant difference was performed on each neurotransmitter separately. The bars for each neurotransmitter with different letters are significantly different from one another (ANOVA, $P < 0.0001$)

Discussion

YOY bluefish residing in a contaminated estuary (HR) possessed enlarged, irregular thyroid follicles that were lined with epithelial cells of increased height compared to those from the relatively clean estuary (TK). The results of the thyroid histology analysis of the laboratory bluefish also reveal enlarged irregular follicles in the HR-fed bluefish compared to those fed TK prey. HR field caught were found to have altered DA metabolites and dopaminergic activity and HR-fed bluefish had significantly altered concentrations of neurotransmitters and their metabolites compared to their TK counterparts. These reduced irregular hormonal and neurotransmitter levels could have consequences for the behavior and ultimate fitness of these animals.

The results for the thyroid histology support the hypothesis that the contaminant exposure may be causing endocrine (thyroid) disruption in these fish, which could be a cause of the reduced activity and feeding and reduced growth. Behavior is heavily contingent on proper thyroid hormone synthesis. The presence of hypertrophied thyroid follicles or "goiters" in the HR-field caught fish may be an indication of the disruption to the function of the pituitary-thyroid axis, causing reduced synthesis of thyroid hormones, followed by increased secretion of TSH resulting in the enlarged and irregular thyroid follicles.

The HR-fed bluefish were found to have significantly reduced feeding, spontaneous swimming activity and growth in the laboratory (Candelmo et al. 2007). In addition, we have previously shown reduced feeding and size in HR field caught bluefish (Candelmo et al 2007). YOY bluefish from HR field and bluefish fed HR prey were also found to have elevated levels of PCBs, mercury and DDTs compared to TK field fish and TK-fed fish (Candelmo et al. 2007). These are known endocrine-disrupting chemicals in fish, mammals, and other organisms (Clotfelter et al., 2004; Baldigo et al., 2006). It is, therefore, plausible that a xenobiotic induced thyroid alteration is causing maladaptive behavior in these fish during their first few summer months in the estuary in which they are undergoing critical periods of rapid growth and development (Juanes and Conover, 1994). Delayed growth and altered behavior and migration as a result of a disrupted thyroid system may have serious implications for their overwinter migration preparation and success.

Dopaminergic activity levels (DOPAC:DA and HVA:DA) of the HR bluefish were reduced significantly. The dopamine metabolites, 3,4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA) were 60% and 73% less, respectively in the HR-field compared to the TK-field bluefish. These reduced concentrations and dopaminergic activity indicates that there is likely a disruption in the metabolism of dopamine. The concentration of dopamine was 20%

lower in the HR-field fish compared to the TK, although the difference was not significant, suggesting that there is a possible disruption in the synthesis or release of these chemicals as well. Decreased dopaminergic activity may be a response to a decreased release of dopamine as a means to retain what dopamine levels are available. NE was also observed to be 31% elevated in the HR-field bluefish although not significantly. Analysis of the whole brain instead of discrete regions may have obscured present brain region specific changes. More specific analysis may have resulted in significant differences in DA, 5-HT and NE between the HR field and TK field fish. Mummichogs from a polluted site (Piles Creek) had lower concentrations of 5-HT and its metabolite 5-HIAA in their medullas but not in their cerebellums (Smith et al. 1995). Future studies should analyze neurotransmitters in specific regions of the brain, as there is evidence that different regions are more sensitive to certain contaminants (Smith et al. 1995; Khan and Thomas, 2006; Lyng and Seegal, 2008).

The neurotransmitters concentrations in the HR-fed bluefish were also significantly altered compared to the TK-fed bluefish, however instead of a decrease in neurotransmitter concentrations as seen in the contaminated field fish, significantly elevated concentrations of all neurotransmitters except HVA (which was elevated but not quite significantly) were recovered in the HR-fed bluefish. There were no differences found in metabolic activity. DA and 5HT in the HR-fed fish appear to be getting metabolized at the same rate as in the TK-fed bluefish and therefore the increased concentrations of DA and 5HT are leading to increased concentrations of their metabolites, particularly DOPAC and HIAA. The serotonin system appears to be most affected with a 70% increase in 5HT and a 78% increase in HIAA in the HR-fed bluefish compared to the TK-fed bluefish. DA, DOPAC and HVA concentrations were 56%, 50% and

35% greater respectively in the HR-fed bluefish. NE was also significantly elevated in the exposed fish (33%) which similar is to the results of the HR field caught bluefish.

Exposure to contaminants including mercury and PCBs has been shown to damage the nervous system and alter neurotransmitters (Bemis and Seegal, 1999; Zhou et al., 1999b; Weis et al., 2001; Grippo and Heath, 2003). However, in general, neurotoxicity of a contaminant and its impact on biogenic monoamines varies greatly depending on species, age, dosage and duration of exposure. The unpredictability of neurotransmitter response to contaminant exposure is further confirmed in the present study. HR field caught and HR-fed bluefish were exposed to multiple xenobiotics at varying degrees of concentrations and are likely experiencing multiple neurotoxic effects resulting in the different neurochemical alterations. Martiniuk et al. (accepted) found that contaminants including PCBs have the potential to modulate brain transcripts required for the synthesis of DA and other catecholamines and have different modes of action.

The TK field bluefish were found to have greater concentrations of the DA metabolites, HVA and DOPAC and increased dopaminergic and serotonic activity levels than the TK-fed, HR-fed and HR-field. The significant differences found between the TK-field and TK-fed lab fish indicate a possible response to the differences between residing in the laboratory and field. Water quality, diet, stress levels, activity levels and energy requirements may all be different in the field compared to the tank environment. Since the neurotransmitter concentrations found in the reference groups from the field and the lab are conflicting a more detailed discussion comparing the four groups (HR-fed, HR-field, TK-fed, TK-field) together would not be informative.

The results of the comparison of laboratory bluefish (HR-fed to TK-fed) neurotransmitter concentrations are conflicting with the concentration results found comparing the field-caught bluefish with the exception of NE. Concentrations of NE were found to be elevated in both the HR-fed compared to the TK-fed bluefish and the HR field-caught compared to the TK field-caught bluefish (although the field caught fish were not significantly different). HR field fish experienced reduced dopamine metabolic turnover. The HR lab fish on the other hand did not have altered DA or 5HT metabolic turnover, instead the metabolites were elevated because the DA and 5HT were elevated. As stated above, these differences in neurochemical alterations between exposed and reference fish in the field and lab may be due to a lab artifact. Or it is also possible that differences in chemical exposure and accumulation may be the cause of elevated neurotransmitters in the HR-fed fish and decreased concentrations in the HR-field fish. Overall the lab fish had significantly higher concentration of total PCBs and Hg than the HR-field bluefish. Exposure to specific PCB congers and other contaminants at varying concentrations may result in specific changes such as increases and decreases of certain neurotransmitter via various mechanisms.

The altered feeding behavior and activity levels observed in the YOY bluefish in the laboratory experiments (Candelmo et al. 2007) could be in part the result of altered neurotransmitter levels. Increases and decreases in levels of neurotransmitters including DA, 5-HT, and NE and their metabolites have been shown to have a variety of effects on behavior, although the studies interrelating changes in brain monoamines and behavior in fish, are limited and often conflicting.

Conclusion

The aim of this study was to investigate possible mechanisms from contaminant exposure underlying changes in behavior and growth previously observed in YOY bluefish (Candelmo et al., 2007). We found that fish from the contaminated estuary had abnormal thyroid follicles and reduced levels of dopamine metabolites, DOPAC and HVA as well as reduced dopaminergic activity. And bluefish fed HR contaminate prey for four months also possessed abnormal, enlarged thyroid follicles and had significantly elevated concentrations of neurotransmitters including, dopamine and its metabolite DOPAC, serotonin and its metabolite HIAA and norepinephrine. The present study, however, does not determine which toxicants are responsible for the neurological and thyroid impairments. Future work should examine the effects, mechanisms and pathways of disruption of specific PCB congeners and other contaminants. In addition, further research investigating whether the alterations are permanent would be beneficial. After emigration from the contaminated estuary, the fish will be exposed to lower levels of contaminants and their body burden may be reduced via depuration and growth dilution. If the thyroid and neurological impairments are reversible, then the fish may be able to recover. However, there is evidence that neurochemical changes may persist even after the contaminant has depurated (Seegal et al. 1997). It is also believed that in winter the fish undergo a period of reduced feeding and utilize stored lipids, in which case the lipophilic PCBs, pesticides and meHg that were deposited in the lipids during the summer may be subsequently redistributed to the brain and other sensitive organs (Boon and Duinker, 1985; Jorgensen et al., 1997; Jorgensen et al., 1999), and result in further disruptions and affect recruitment to the adult population.

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