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THYROIDAL STATUS OF MUMMICHOGS (*FUNDULUS HETEROCLITUS*) FROM A POLLUTED VERSUS A REFERENCE HABITAT

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Abstract—Previous studies have demonstrated that mummichogs (*Fundulus heteroclitus*) collected from Piles Creek (PC), New Jersey, USA (a site polluted with heavy metals and organic materials), were sluggish and showed poorer prey capture and predator avoidance than those from a reference site (Tuckerton [TK], NJ, USA). We hypothesized that the behavioral dysfunction of the PC fish might be associated with thyroid impairment due to toxic exposures. In this preliminary study, we compared thyroid histology and thyroid hormones in the two populations and experimentally determined whether a polluted or an unpolluted environment could alter levels of thyroid hormones. In comparison to TK, PC fish had larger thyroid follicles and greater follicle epithelial cell heights and also contained significantly higher plasma thyroxine (T4) levels. However, no significant differences were found in levels of either plasma or tissue triiodothyronine (T3). The TK fish raised in a simulated PC environment contained higher plasma T4 and lower plasma T3 levels than those sampled from the field. The PC fish raised in clean water showed a reduction in both plasma T4 and T3 levels in comparison to field-sampled fish. In either case, no significant difference was found in tissue T3 content. The contaminants in PC may cause the differences in thyroid structure and function, which may be partly responsible for the behavioral differences between fish from the polluted PC and from the reference TK.

Keywords—Thyroid Pollution Fish *Fundulus heteroclitus* Endocrine disruptors

INTRODUCTION

The mummichog, *Fundulus heteroclitus*, is one of the most abundant euryhaline fish distributed along the East Coast of North America and can be readily found in salt marshes such as Piles Creek (PC) and Tuckerton (TK) in New Jersey, USA. Both PC and TK provide similar habitat for mummichogs. However, TK is an uncontaminated nonindustrialized area, whereas PC is a highly polluted site surrounded by many industrial plants and a major highway in Linden, New Jersey, USA, about 170 km north of TK. The sediments of PC contain elevated levels of various heavy metals and organic materials [1–4] (Table 1), some of which (e.g., mercury, lead, PCBs, and DDTs) are considered endocrine disruptors [5–7]. The mummichogs from PC show significantly higher body burdens of many contaminants than fish from nonpolluted reference sites [8,9].

Biological differences have been found between the TK and PC populations [10]. Previous studies have found that adult mummichogs collected from PC showed lower spontaneous activity [11], were poorer at catching grass shrimp (a principal prey species), and were more vulnerable to predation by blue crabs (a main predator) than those from TK [9]. The poor behavioral performance in PC adults was correlated with the elevated mercury body burden and lowered levels of baseline brain serotonin [12], a neurotransmitter involved in locomotion [13]. The polluted environment in PC is associated with the neurobehavioral impairments of the resident mummichogs, which may contribute in part to their previously noted poorer growth, condition, and shorter life span than their conspecifics from reference sites [14].

Environmental toxicants may exert adverse influences on thyroid function [15], which in turn may affect development, growth, and reproduction. Thyroid hormones, which regulate metabolism and growth, may directly affect CNS or sensory organs and influence the CNS response to external stimuli. Exogenous thyroid hormone has been reported to alter locomotor activity of fish [16,17]. Because the level of thyroid activity is considered to be related to the level of locomotor activity, we hypothesized that thyroid dysfunction resulting from chronic toxic exposure may contribute to the poor behavioral performance in PC fish. This preliminary research had two phases. In the first phase, both histological and radiochemical criteria were used to evaluate and compare thyroidal status of adult mummichogs sampled from PC and TK. In the second phase, we aimed to ascertain whether a polluted or an unpolluted environment could alter levels of thyroid hormones in mummichogs.

MATERIALS AND METHODS

Thyroidal status of field-sampled fish

Sample collection. Thyroid activity in teleost fish is influenced by environmental factors, including the lunar cycle, day length (photoperiod), and temperature (seasonal changes) [18]. To minimize variations in the hormonal response to environmental factors and capture stress, adult mummichogs (60–90 mm standard length, about 2–3 years old) were sampled by Gees minnow traps from PC and TK between 1200 and 1600 h on 2 consecutive days (for each batch sampled, 1 d at each site), mainly in May 1997. Fish were anesthetized with 3-aminobenzoic acid ethyl ester (Sigma Chemical, St. Louis,

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Table 1. Examples of sediment contaminants (mean ± standard error or mean values) in Tuckerton, NJ, USA (TK) and Piles Creeks, NJ, USA (PC) sample sites

Sediment contaminant	Sample site	
	TK	PC[a]
Mercury (μg/g)[b]	0.15 ± 0.06	16.41 ± 3.08*
Lead (μg/g)[c]	13.68 ± 0.14	55.42 ± 0.02*
Copper (μg/g)[c]	7.16 ± 0.04	1,895 ± 178*
Zinc (μg/g)[c]	32.89 ± 0.14	1,407 ± 0.02*
Chromium (μg/g)[c]	13.53 ± 0.04	88.75 ± 6.60*
Cadmium (μg/g)[b]	1.90 ± 0.67	7.05 ± 1.45*
PCBs (ng/g)[d]	11.9	285*
PAHs (ng/g)[d]	1,190	8,690*
DDTs (ng/g)[d]	2.45	138*

[a] Asterisk indicates significant differences in sediment contaminants between TK and PC.
[b] [2]
[c] [3]
[d] Estimated from nearby sites by Environmental Monitoring and Assessment Program [4].

MO, USA) right after capture, fixed immediately in 10% Formalin, and then transferred to 70% ethanol for preservation (for thyroid histology study). Other fish were maintained in aquaria with 15-ppt synthetic seawater (Instant Ocean Salt, Marine Enterprises, Baltimore, MD, USA) under room temperature and were fed artificial flakes. After 3 to 4 d in the laboratory, they were sacrificed. To evaluate the thyroidal status, both histology (follicle morphology) and thyroid hormones (thyroxine [T4] and triiodothyronine [T3]) were examined.

Thyroid histology. Fish heads were cut off, decalcified, dehydrated, embedded in paraffin, sliced sagittally at 5 μm, and stained with hematoxylin and eosin. The thyroid tissue, composed of follicles (epithelial cells, vacuoles, and colloid; Fig. 1), was scattered around the ventral aorta and branchial arteries near the gills and tongue in the lower jaw region. Follicle morphology (area of follicle and epithelial cell height) was measured using Neurolucida 2.1® software (MicroBrightField, Colchester, VT, USA) with a Microsys computer connected to a camera (Hitachi, Tokyo, Japan) and a light microscope (Olympus BH2, Olympus, Tokyo, Japan). Because the size of follicles varied considerably, the 10 largest follicles per fish were chosen for measurements. Along the perimeter of each chosen follicle, five epithelial cell heights were measured in 8 TK and 15 PC fish. In addition, colloid density was visually inspected.

Plasma thyroid hormones. Blood was taken between 1400 and 1600 h right after fish were anesthetized with 3-aminobenzoic acid ethyl ester. The caudal peduncle was severed, and blood was collected from the caudal vasculature with a heparinized microhematocrit capillary tube and centrifuged at 1,400 rpm at room temperature for 10 min. Blood cells were then discarded. Supernatants (plasma samples) were then kept in microcentrifuge vials and stored at −70°C until analysis. Whenever necessary, plasma samples were pooled (from two to three fish) to get enough volume for measurements. Plasma T4 (in duplicates of 20 μl) and plasma T3 (in triplicates of 10 μl) concentrations were analyzed with a double antibody radioimmunoassay modified from John-Alder [19]. The detection limits were 2.5 pg for both T4 and T3. Blood was taken from 80 TK and 80 PC fish for T4 measures and from 37 TK and 38 PC fish for T3 analysis.

Muscle tissue T3. Preparation of muscle tissue T3 extract was done following procedures slightly modified from Brown et al. [20]. Tissues were rinsed in 0.74% sodium chloride, homogenized in potassium sodium phosphate solution with propylthiouracil, and extracted with methanolic ammonia. The dried T3 extract was reconstituted with 300-μl assay buffer (1.5 mg 8-anilino-1-naphthalene sulfonic acid per milliliter of bovine gamma-globulin barbital buffer). After the mixture was centrifuged, the aqueous layer was assayed via double antibody radioimmunoassy as described previously. Thirty-microliter samples were measured in triplicate. A 75% extraction efficiency was reported for a similar method [21] and our trial analysis. The measured tissue T3 levels, reported as ng/g tissue wet weight, were not corrected for extraction efficiency.

Liver somatic index. Because liver is a major site of T3 production, liver somatic index was determined to see whether a size difference in liver existed between the two populations. Total body weight of each individual fish was measured prior to sacrifice. After blood was collected, fish liver (free of gallbladder) was taken and liver wet weight measured. Liver somatic index of each individual was calculated as liver wet weight over body weight. A total of 57 TK fish and 68 PC fish were sampled for liver somatic index.

Effects of environment on thyroidal status

Adult mummichogs (60–90 mm S.L.) were sampled by Gees minnow traps from PC and TK in May 1998. After capture, fish were maintained in aerated experimental tanks at 22°C with a 14:10 h LD (light:dark) photoperiod. Water quality (water temperature, pH, dissolved oxygen, and total ammonia) was monitored weekly.

To determine effects of environment on thyroidal status, we performed reciprocal experiments from May to June 1998 and compared results with those in experiment 1. To see whether a polluted environment could alter levels of thyroid hormones, 40 TK fish, 10 in each tank, were raised for 1 month in four 56.8-L tanks with simulated PC environment (10 cm in depth of PC sediments and about 40 L of PC water). The sediments were randomly sampled from three sites of PC. The water was not changed but was refilled with freshly sampled PC water when evaporated. The TK fish in each tank were fed with 60 PC grass shrimp (*Palaemonetes pugio,* 50–70 mm standard length) and 10 chopped PC yearling mummichogs once a week.

To see whether an unpolluted environment could alter levels of thyroid hormones, 40 PC fish (10 in each tank) were raised in four 56.8-L tanks with 40 L of 15-ppt synthetic saltwater for one month. The water in each tank was changed once a week to remove contaminants possibly depurated from the PC fish. The PC fish were fed artificial flakes on a daily basis and 10 chopped TK yearlings once a week.

After one month in the experimental tanks, all live fish were removed, and their blood was taken for plasma thyroid hormones following the procedure as previously described.

Data analysis

All statistical analyses were performed on SAS/STAT® 6.11 (SAS Institute, Cary, NC, USA). If not normally distributed, data were log10-transformed before statistical tests [22]. Means and standard errors are presented in the text and graphs. To take nested effects of follicles and follicle epithelial cells into consideration, generalized linear model procedure was applied for nested analysis of variance (ANOVA), using nested models.



Fig 1 Sagittal section of representative thyroid tissue from TK (A) and PC (B) adult mummichogs. Magnification = 40×. Epithelial cell (c), vacuole (d), colloid (e), gill (f), branchial artery (g). The arrows indicate irregularly shaped follicle and partially depleted colloid.

The relationship between follicle size and epithelial cell heights for the two populations was examined by analysis of covariance (ANCOVA) using multiple regression models followed by comparison of least square means on transformed data. To compare the T3, T4, and liver somatic index between two groups, a t test (for equal variance) or an approximate t test (for unequal variance) was used. Significance level (α) was set to 0.05.

RESULTS

Field-sampled fish

Thyroid histology In general, larger follicles were found in PC than TK fish (Fig. 1A and B). The PC fish also had irregularly shaped follicles and partially depleted colloid (Fig. 1B), neither of which was found in TK fish (Fig. 1A). Nested

Table 2. Nested effects of follicles and epithelial cells on follicle size (size) and cell height (height), followed by ANCOVA results[a]

Source of variance		Dependent variable[b]			
		Follicle size (size)		Cell height (height)	
Nested ANOVA	df	*F*	*P* > *F*	*F*	*P* > *F*
Site	1	3,571.52	0.0001**	1,056 96	0.0001**
Fish	21	112.65	0.0001**	19.01	0.0002**
Follicle (fish)	206	15.18	0 0003**	2.57	0.1445
Cell (fish × follicle)	916	0 00	1.0000	0.34	0.9872
ANCOVA	df			*F*	*P* > *F*
Model	3			577.17	0.0001**
Site	1			4.36	0.0371*
Size	1			212.02	0.0001**
Size × site	1			20.00	0.0001**

[a] ANCOVA, GLM model. log (height) = log (size) site log (size) × site.
[b] * indicates significance at the 0.05 level; ** indicates significance at the 0.001 level.

ANOVA (Table 2) indicated significant differences in follicle size and cell heights among individual fish ($p < 0.0002$) as well as significant differences in follicle size among follicles from the same fish ($p = 0.0003$). No significant differences were found in cell heights among follicles from the same fish ($p = 0.1445$). Follicle cell heights within the same follicle did not differ from each other ($p = 0\ 9872$). The PC fish had larger average thyroid follicle area (PC: 19,144 ± 1,070, ranging from 376 to 23,7053. vs TK: 2,834 ± 171, ranging from 419 to 15,535 μm², $p = 0.0001$) and greater epithelial cell heights (PC: 11.42 ± 0.16, ranging from 3.04 to 31.33, vs TK: 5 48 ± 0.07, ranging from 1.84 to 10.07 μm, $p = 0.0001$) than TK fish (Fig. 2) Follicle cell height was positively related with follicle size (area) in a linear fashion ($p = 0.0001$), but elevations of the regression lines for the two populations were significantly different ($p = 0.0001$) (Table 2, ANCOVA). When cell heights were compared in equal sized follicles, analysis of least square means showed a significant difference in cell heights of equal-size follicles (PC: 2.24 ± 0.01 vs TK: 1.79 ± 0.03, $p = 0.0001$)

Thyroid hormones. Females and males within each population showed comparable levels of plasma T4 and T3. In comparison to TK, the PC fish contained significantly higher levels of plasma T4 (PC: 13.92 ± 1.38 vs TK: 8.64 ± 0.72 ng/ml, $p = 0\ 0103$) when sexes were separated or pooled (Fig. 3a). No significant difference in plasma T3 levels was found between TK and PC fish (Fig. 3b). Similarly, no statistically significant difference in tissue T3 levels was found between

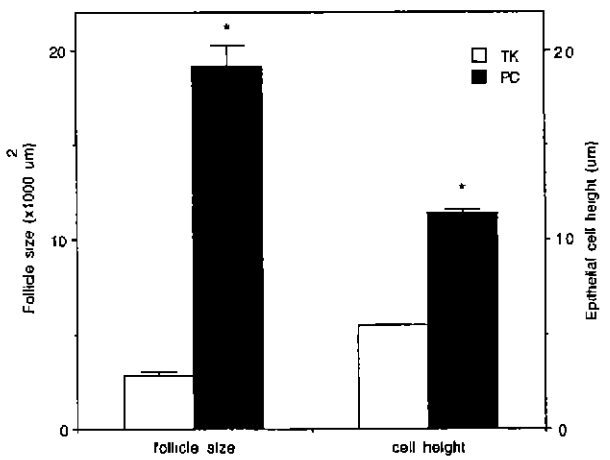

Fig. 2. Follicle size and epithelial cell height (mean ± SE) from field-sampled TK and PC mummichogs Five epithelial cell heights per follicle out of 10 follicles per fish were measured. * denotes significant difference between the populations ($p < 0.0001$).

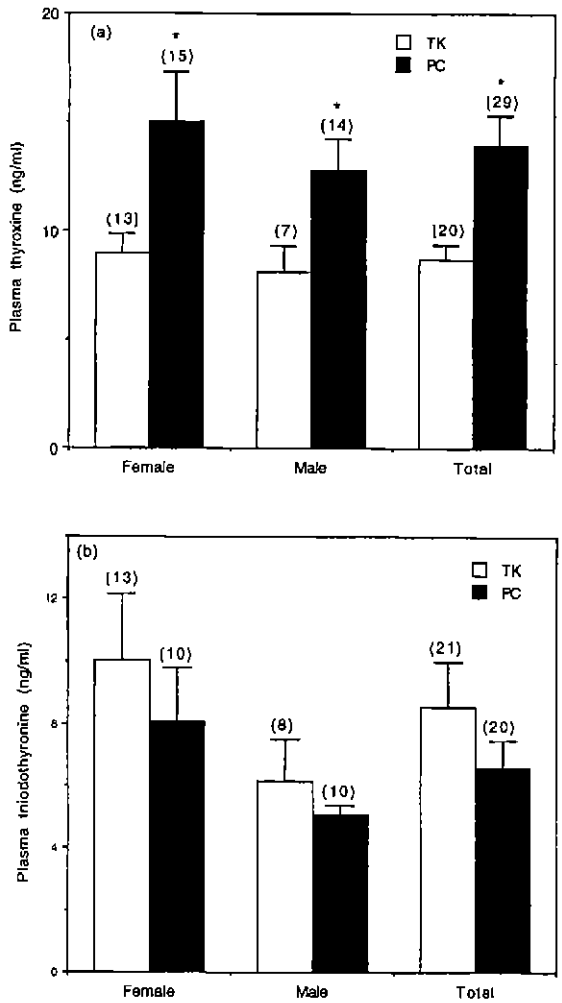

Fig. 3. Plasma thyroxine (a) and plasma triiodothyronine levels (b) in field-sampled TK and PC mummichogs. Values are mean ± SE. Numbers in parentheses are sample size for each assay. * denotes significant difference between the populations ($p < 0.05$).

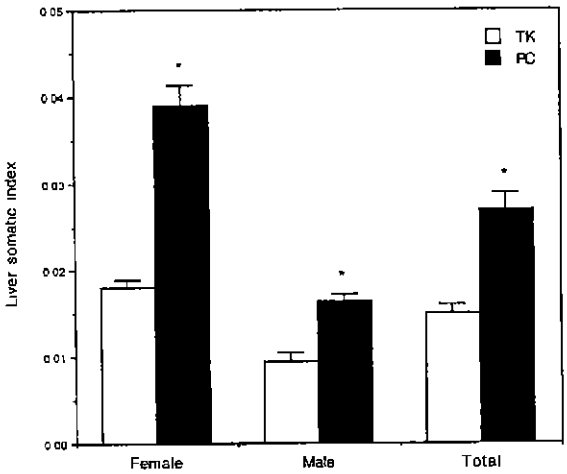

Fig. 4. Liver somatic index (mean ± SE) of field-sampled TK and PC mummichogs * denotes significant difference between the populations ($p < 0.05$).

PC (0.52 ± 0.04 ng/g) and TK fish (0.83 ± 0.22 ng/g) (p = 0.9470).

Liver somatic index. Both female and male PC fish exhibited a greater liver somatic index than TK fish of the same sex (Fig 4). The result was based on 33 female TK, 24 male TK, 37 female PC, and 31 male PC fish. For both populations, a sex difference was found in liver somatic index (p = 0.0001) Females generally had larger liver than males. When pooled, PC fish also showed significantly greater liver somatic index in comparison to TK (PC: 0.027 ± 0.002 vs TK: 0.015 ± 0.001, p = 0.0001).

Reciprocal environment experiment

The TK fish raised in simulated PC environment showed a significant increase in plasma T4 level but a decrease in plasma T3 level (Fig. 5a). Interestingly, the plasma T4 level was much higher than that of field-sampled PC fish (TKP: 34.14 ± 9.88 ng/ml vs PC: 13.92 ± 1.38 ng/ml, p = 0.0057). However, the plasma T3 level was lower than that of field-sampled PC fish (TKP: 4.13 ± 1.38 ng/ml vs PC: 6.56 ± 0.92 ng/ml, p = 0.0421).

When the PC fish were raised in clean water, both plasma T4 and T3 levels decreased in comparison to field-sampled PC fish (Fig. 5b). However, the plasma T4 level dropped to 5.26 ± 1.42 ng/ml, which was even less than that found in the field-sampled TK fish (8.64 ± 0.72 ng/ml, p = 0.0118). Similarly, the plasma T3 level dropped to 1 70 ± 0.21 ng/ml, which was also considerably less than that in the TK fish (8.54 ± 1 45 ng/ml, p = 0.0000).

In both cases, plasma T3 levels decreased whenever the environment was changed (Fig 5a and b). In contrast, tissue T3 levels were not affected. Neither of the two cases showed significant alterations in T3 concentrations at the tissue level.

DISCUSSION

Thyroid histology revealed that the PC fish had greater epithelial cell heights and larger follicles than TK fish. Because PC fish also showed partially depleted colloid, the larger follicle did not necessarily indicate more stored T4. Irregularity of follicle outline, increased epithelial cell heights, and decreased colloid density observed in PC fish are typically seen when the thyroid gland undergoes stimulation [23] and are

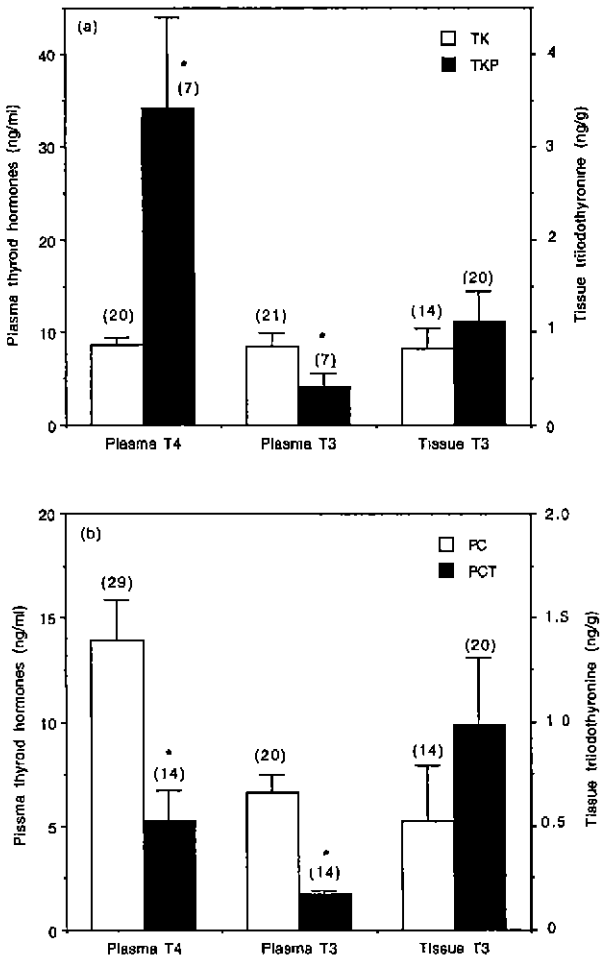

Fig. 5. Plasma and tissue thyroid hormone levels in (a) TK mummichogs sampled from the field (TK) or raised in simulated PC environment (TKP) and (b) PC mummichogs sampled from the field (PC) or raised in clean water (PCT) Values are mean ± SE. Numbers in parentheses are sample size for each assay * denotes significant difference between the populations ($p < 0.05$).

similar to the goiter described by Leatherland [15] in Great Lakes fish. Leatherland et al. [24] found that the goiter tissue in coho salmon (*Oncorhynchus kisutch*) from the polluted Great Lakes had small follicles, composed of masses of tall epithelial cells, containing little or no colloid material. These were called microfollicular goiter or afollicular goiter. In addition, other forms of thyroid lesions, such as simple hyperplasia, trabeculate goiter, colloid goiter, and papillary goiter, were recognized in the same species from the Great Lakes [15]. In contrast, PC fish displayed enlarged or irregularly shaped follicles proportional to epithelial cell height, which contained partially depleted colloid. However, PC fish also showed elevated plasma T4, which typically would not be seen in goitrous fish. After failing to induce goiter experimentally with contaminants, Leatherland [15] suggested that waterborne environmental goitrogens (possibly by-products of the microflora of the lake system) rather than environmental toxicants (heavy metals and aromatic halogenated hydrocarbons) were responsible for the prevalence of thyroid gland hyperplasia or goiter in coho salmon from the polluted Great Lakes. However, heavy metals such as lead and cadmium have been found to induce goiters in rats [25]. Because the PC habitat differs from

TK in levels of contaminants, we suspect that the differences in thyroid histology between the populations are due to the contaminants in PC.

Goitrogens usually decrease thyroid hormone synthesis, thereby increasing the activity of the hypothalamus-pituitary-thyroid axis and inducing goiters without necessarily elevating thyroid hormone synthesis [26]. However, the sluggish PC fish demonstrated higher levels of plasma T4, the primary secreted form [23]. The two populations showed comparable levels of T3, the biologically active form in fish [23], in both plasma and tissue compartments. Exposure of TK mummichogs to contaminated PC sediments for 1 month resulted in increased plasma T4. This finding is consistent with what was found in the two populations and supports the hypothesis that the contaminants in PC may be responsible for the differences in thyroidal status between fish from the polluted PC and reference TK. On the other hand, the contaminated PC mummichogs showed a reduction in plasma T4 as a result of living in an unpolluted environment, which was again in agreement with what was found in the two populations. The recovery of plasma T4 level may be related to a decreased body burden of pollutants in the fish; presumably depuration occurred. In another comparison study, Hontela et al. [27] reported lower plasma T4 and comparable plasma T3 in yellow perch (*Perca flavescens*) from the contaminated St. Lawrence River than in perch from a reference site. Yet, when fish are exposed to a single chemical, the direction of changes in the thyroid hormones varies with species and chemicals [5–7,28] and exposure time [29]. Adult *Fundulus* at the PC site are exposed over time to a mixture of heavy metals and organic materials, which may act either alone or together in a synergistic or antagonistic manner. The mixture may complicate responses of the thyroid of PC mummichogs.

Because this is the initial study for the two populations, we do not have ample information to determine how the thyroidal status is impacted by pollutants. There are several possibilities.

Because T3 binds mainly to receptor sites and thyrogenic effects are mediated mainly by T3 [23], the comparable levels of both plasma and muscle T3 between PC and TK fish could suggest clinically euthyroidal status of PC fish.

It is also possible that less T3 binds to receptor sites of PC fish, which may be competitively blocked by pollutants. If the effectiveness of T3 binding were affected, the higher plasma T4 levels might result from elevated thyroid-stimulating hormone (TSH) due to an altered feedback mechanism in the hypothalamus or pituitary. Certain organic pollutants, such as PCBs and dioxins, which are also found in PC, can decrease or mimic the biological action of thyroid hormones [30]. If pollutants similarly bind to the thyroid hormone receptors in the nervous system and peripheral tissues, thus blocking the activity of thyroid hormones, sluggish behavior could result. However, for environmental chemicals that disrupt thyroid function, binding to the receptor has not been demonstrated. On the other hand, higher plasma T4 levels could also result from greater plasma binding.

Both PC fish and TK fish raised in simulated PC environment showed higher plasma T4 but lower T3 levels, suggesting altered activity of 5′-monodeiodinase, which catalyzes the conversion of T4 to T3 in peripheral tissues, such as liver and kidney [23]. Inhibition of 5′-monodeiodinase activity was documented in fish exposed to lead [31] and mammals exposed to cadmium [32,33]. Conversely, the comparable tissue T3 levels between the two populations may result from T3 autoregulation [23].

Because cortisol stimulates T4 to T3 conversion [34], disruption of renal cortisol secretion in PC fish may have impaired thyroid hormone conversion. Hypertrophy and hyperplasia of interrenal cells were noticed in brown trout (*Salmo trutta*) living in a cadmium- and zinc-contaminated environment [35]. Depressed cortisol secretion coupled with thyroid dysfunction was also reported in yellow perch from a site contaminated with organic materials and heavy metals [27]. A greater liver somatic index was observed in PC fish than TK fish. Because the liver is involved in detoxification and metabolism of xenobiotics, enlargement of the liver of PC fish may be an indication of stress due to living in the polluted environment. Because the liver is also a major site of T3 production, an enlarged liver may adversely affect thyroid hormone metabolism or T4 to T3 conversion.

On the other hand, a negative correlation may exist between levels of plasma T4 and spontaneous activity in mummichogs. Katz and Katz [17] found that exogenous T4 increased swimming speed in pearl danio *Brachydanio albolineatus* (Blyth). In contrast, yearling Atlantic salmon (*Salmo salar*) injected with T4 or T3 showed reduced swimming activity [16]. Elevated plasma T4 in adult PC fish may result in reduced spontaneous activity. However, exogenous T4 (60 ng/ml) did not induce hypoactivity in either TK or PC yearling mummichogs (unpublished data).

In summary, mummichogs from a contaminated environment (PC) showed stimulation of thyroid gland activity in comparison to fish from a cleaner environment (TK). Both elevated plasma T4 and histological activation of thyroid tissue were observed. Reciprocal experiments demonstrated that the simulated PC environment could elevate plasma T4 in TK fish, whereas an unpolluted environment could reduce both plasma T4 and T3 in PC fish. However, further investigation is required to determine specific mechanisms of thyrogenic responses in fish and the exact impacts of pollutants on thyroid mechanisms, whether at the levels of pituitary gland, deiodinase, metabolism, receptor sites, or a combination of the above. The differences in thyroidal status between the two populations may be in part responsible for their behavioral differences. We suggest that the contaminants in PC may impair thyroid function of PC fish and cause the differences in thyroxine levels and thyroid follicle morphology between the two sites.

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