Polybrominated diphenyl ethers (PBDEs) are added to plastics, polyurethane foam, paints, and synthetic fabrics as a flame retardant. Recently, concerns have arisen about possible health impacts of PBDE exposure because studies have revealed rising PBDE levels in the tissues of humans and wildlife (Hites 2004; Law et al. 2003). The chemical structure of PBDEs resembles that of polychlorinated biphenyls (PCBs), and PBDEs may act similarly as disruptors of the hypothalamic-pituitary-thyroid axis (Birnbaum and Staskal 2004; Boas et al. 2006; McDonald 2002). In studies with rats, mice, and fish, in vivo PBDE exposure reduced plasma levels of the thyroid hormone, thyrotropin (TH) thyroxine (T4) (Hallgren et al. 2004; Boas et al. 2006; McDonald 2002). Supporting this idea, rats with reduced T4 after exposure to the PBDE mixture Bromkal 70-SDE also showed elevated hepatic uridine-5-diphosphoglucuronosyltransferase (UDP-GT) activity, suggesting that the decline in T4 may result in part from increased biliary excretion of conjugated TH (Skarman et al. 2005). Alternatively, some hydroxylated PBDEs bind the TH transport protein transthyretin with sufficient affinity to displace T₄ (Hamers et al. 2006; Meer et al. 2000). The mechanism by which PBDEs depress circulating T₄ remains unclear, and it is also not known whether PBDE exposure impacts TH-regulated pathways in the brain or peripheral tissues. PBDEs appear to have only weak binding affinity for TH receptors (TRα) (Marsh et al. 1998), yet Schriks and co-workers (2007) found recently that one T₃-like hydroxylated PBDE, 4′-hydroxy-2,4,6-tribromodiphenyl ether, increases T₃-induced TRα activation in reporter-gene assays, whereas PBDE 209 inhibits T₃ activation of both TRα and TRβ. These new findings suggest that PBDE exposure may affect TH-regulated pathways in target tissues.

In the vertebrate brain, THs play key roles in regulating neural development and functioning (Koibuchi and Chin 2000; König and Neto 2002). THs influence neurogenesis by mediating the proliferation of neural progenitor cells, influencing dendritic and synapse formation, and regulating myelination (Gould and Butcher 1989; Porterfield and Hendrich 1993). In rodents, neurogenesis in the brain is induced by T₃ (Uchida et al. 2005), and THs stimulate stem cell proliferation and neuronal differentiation in the olfactory system of mammals and fish (Lema and Nevitt 2004; Paternostro and Meisami 1994). Nevertheless, it remains unclear whether exposure to PBDEs impacts TH-mediated neural development (Porterfield 2000).

In the present study, we used teleost fish as an animal model for investigating the influence of PBDEs on neurogenesis and brain development. Teleost fish show cell proliferation and neural differentiation in the brain throughout adult life (Adolf et al. 2006; Lema et al. 2005; Zupanc et al. 2005), and the extent of neurogenesis in the adult fish brain greatly exceeds that in mammals (Cayre et al. 2002). Embryonic exposure of zebraspike (Danio rerio) to PBDE-47 has been found to lead to cardiac and morphologic defects that appear to be caused by a primary effect on neural function (Lema et al. 2007). Whether such neural and behavioral impacts of PBDE exposure result from disruption of TH signaling during brain development, however, has yet to be examined.

We tested the hypothesis that dietary exposure to PBDEs affects TH-regulated gene
transcripts in target tissues in the adult fathead minnow (*Pimephales promelas*). The fathead minnow is a teleost model for assessing the toxic and endocrine-disrupting effects of chemical pollutants (Ankley and Villeneuve 2006), and oral exposure to the PBDE con-
gen 2.2',4',4''-tetrabromodiphenyl ether (PBDE-47) impaired reproductive activity in this species (Muirhead et al. 2006). We exposed adult minnows to a dietary source of PBDE-47, typically the most concentrated PBDE congener in humans and wildlife (Hites 2004; Schecter et al. 2005). We then examined the effects of PBDE-47 exposure on plasma T4 and T3 status and mRNA levels for thyrotropin β-subunit (TSHβ) and glycoprote-
in hormone α-subunit (GPHα) in the pitu-
ity. We also examined how PBDE-47 affected key target tissues for THs by quantifying.
transcripts for the autoinduced TRα and TRβ genes in the brain and liver, and by quantifying brain mRNA for basic transcription element-binding protein (BTEB), a TH-
responsive transcription factor that regulates neural differentiation (Cayrou et al. 2002; Denver et al. 1999).

**Materials and Methods**

**Animals and housing.** Adult fathead minnows (*Pimephales promelas*) were obtained from Environmental Consulting & Testing (Superior, WI). Minnows were maintained at the Battelle Pacific Northwest Division in Sequim, Washington, under a 16 hr:8 hr light:dark photoperiod with water quality parameters of 24–26°C, 6.6–7.4 mg/L dis-
solved oxygen, and 8.1–8.3 pH for the dura-
tion of the experiment. All animals were treated humanely and with regard for allevia-
tion of suffering, in accordance with guide-
lines of the Battelle Institutional Animal Care and Use Committee.

**Bioencapsulation of PBDE-47 in Artemia shrimp.** We obtained PBDE-47 from ChemServices (> 99% purity; West Chester, PA). A stock solution of PBDE-47 was prepared by dissolving 10.0 mg/mL in hexane. One milliliter of stock solution was added to a 1-L Erlenmeyer flask, the hexane evaporated, and approximately 15,000 adult brine shrimp (*Artemia franciscana*) added and incubated overnight to bioencapsulate the PBDE-47 (Muirhead et al. 2006). Before dosing minnows with the bioencapsulated brine shrimp, aliquots of the *Artemia* were assayed for PBDE-47 concentration using gas chromatography.

**PBDE-47 exposures.** We placed minnows in 38-L aquaria with one adult male and one adult female per aquarium. Each aquarium contained a 10.2-cm diameter clay pot that was split longitudinally to provide spawning substrate. Before beginning PBDE-47 expo-
sure, the breeding pairs for all treatments (n = 9–11 pairs per treatment) were fed clean, frozen *Artemia* (San Francisco Bay Brand, Newark, CA) diluted 1:1 with sterile filtered seawater (0.5 g wet weight/mL) *ad libitum* twice daily for 7 days, during which time we checked the spawning sub-
strate every morning to confirm that each pair was reproducitively active.

Following this 7-day period, minnows were fed PBDE-47 bioencapsulated *Artemia* (1 mL) twice daily for 21 days. Minnow pairs were given PBDE-47 either as a low dose (2.38 ± 0.63 µg PBDE-47/pair/day) or a high dose (12.30 ± 3.61 µg PBDE-47/pair/day). We selected these doses based on previous PBDE exposures with this species (Muirhead et al. 2006). A third, control group of minnows continued to be fed *Artemia* not bio-
encapsulated with PBDE-47. We monitored spawning activity daily for the duration of PBDE-47 exposure, as described above.

After 21 days of PBDE-47 exposure, minnows were euthanized with tricaine methane-
sulfonate (Argent Chemical, Redmond, WA), and body mass (grams) and fork length (milli-
imeters) were measured. Plasma was collected, and the pituitary gland, brain, and liver were dissected and frozen rapidly in liquid nitrogen, although the liver was first weighed to determine liver somatic index (LSI). We also dissected one gonad and immersed it in Bouin’s fixative for histologic analysis. After removal of the digestive tract, the remaining carcass of each animal was frozen to quantify body burdens of PBDE-47. All tissues were stored at –80°C.

T4 and T3 radioimmunoassays. Plasma concentrations of T4 and T3 were measured by radioimmunoassay as described previously (Dickhoff et al. 1982) using anti-L-T4 (1:4,000) or anti-L-T3 antiserum (1:10,000) (Accurate Chemical & Scientific Corp., Westbury, NY) and 125I-labeled T4 or T3 (Perkin-Elmer, Waltham, MA). The intra-
assay coefficient of variation was 4.1% for the T4 assay and 5.4% for the T3 assay. All sam-
ples were run in single assays. Given the small body size of fathead minnows, only the larger male sex provided sufficient plasma to quanti-
fy both T4 and T3 from the same individual.

For that reason, we assayed T3 in males only.

**Cloning of cDNA for BTEB.** We first identified and sequenced the cDNA for BTEB from the brain of fathead minnow using primers designed for zebrafish BTEB (GenBank accession no. AJ979399 (National Center for Biotechnology Information 2008)). First strand cDNA was amplified in a 50-µL polymerase chain reaction (PCR) containing 2 µg of total RNA from the brain under the thermal profile: 94°C for 2 min, followed by 30 cycles of 94°C for 30 sec, 50°C for 30 sec, and 72°C for 90 sec, and ending with 72°C for 10 min. The cDNA was purified and sequenced to provide a 151-bp partial sequence, which was used to design primers (see Supplemental Material, Table 1 (available online at http://www.ehponline.org/members/2008/11570/suppl.pdf)) to obtain the full length BTEB sequence (SMART RACE cDNA Amplification Kit; BD Biosciences, Palo Alto, CA). The full-length cDNA sequence for fathead minnow BTEB is available online [GenBank accession no. EF432310 (National Center for Biotechnology Information 2008)].

**Real-time quantitative reverse-transcribed PCR assays.** We extracted total RNA from the pituitary gland using the MiniTriz RNeasy Kit (Qiagen, Inc., Valencia, CA) and from the brain and liver using Tri-Reagent (Molecular Research Center, Cincinnati, OH). Extracted RNA was quantified (NanoDrop Technologies, Wilmington, DE) and diluted to 15 ng/µL. Total RNA was reverse-transcribed (RT) in 15-µL reactions containing 3.0 µL 5x buffer and 1.5 µL dithiothreitol (DTT); Invitrogen, Carlsbad, CA), 0.75 µL deoxyribonucleotide triphosphate (dNTP) and 0.255 µL random hexamer (Promega, Madison, WI), 0.3 µL RNase inhibitor (20 U/µL; Applied Biosystems, Inc., Foster City, CA), 0.1875 µL Superscript II reverse transcriptase (Invitrogen), 6.075 µL ddH2O (nuclease-free water; Sigma, St. Louis, MO), and 3.0 µL of total RNA template (15 ng/µL) under a profile of 25°C for 10 min, 48°C for 60 min, and 95°C for 5 min.

Primers and probes for real-time quantitative RT-PCR assays were designed for *TSHβ* (GenBank accession no. DQ778789) (Lema et al. 2008), *GPHα* (DQ256072), *TRα* (DQ74645), *TRβ* (AY533142) and *BTEB* (EF432310) from fathead minnow using Primer Express software (ABI). All primers and probes were synthesized by Integrated DNA Technologies (Coralville, IA) [see Supplemental Material, Table 2 (available online at http://www.ehponline.org/members/2008/11570/suppl.pdf)].

Quantitative RT-PCR reactions (25 µL) contained 12.5 µL Master Mix (ABI Universal MasterMix Reagent), 0.5 µL forward primer, 0.5 µL reverse primer, 8.0 µL nuclease-free H2O, and 3.0 µL of reverse-
transcribed cDNA template. Reactions were run on an ABI 7700 Sequence Detector under a profile of 50°C for 2 min, 95°C for 10 min, and then 40–45 cycles of 95°C for 15 sec and 60°C for 1 min. All samples for each gene were run on a single 96-well plate. For each gene, we tested for DNA contamination by analyzing a total RNA sample that was not reverse-transcribed, and each run included duplicate samples lacking cDNA template. We used serial dilutions of total RNA from the experiment as a standard curve. Standard curve samples were run in triplicate, but sam-
ples themselves were not duplicated. We also quantified expression for *18S* (Universal 18S;
ABI) as a potential normalizing gene. In the pituitary gland and liver, 18S transcript expression was affected by PBDE-47 treatment, and total RNA yield from the pituitary was insufficient to screen additional housekeeper genes. Therefore, instead of normalizing the genes of interest to 18S, pituitary and liver transcripts were expressed relative to the total yield of RNA. Relative gene transcript levels were then made within staging classes to identify which stages were altered by PBDE exposure. To examine how PBDE-47 affected spawning frequency, we used an analysis of covariance model with treatment, baseline spawning frequency as a covariate, and treatment × baseline spawning frequency as factors.

**Results**

*Plasma thyroid hormones.* We observed decreased plasma T3 levels in both sexes after dietary PBDE-47 exposure (*p* = 0.002; Figure 1). Males had higher plasma T4 levels than females (*p* = 0.0447), but this sex difference was independent of PBDE exposure. Plasma T3 levels in males were unaffected by PBDE-47.

*Pituitary gene transcripts.* At the lower exposure dose, PBDE-47 elevated gene transcripts for TSHβ in the pituitary gland (Figure 2; *p* = 0.0043). At the higher PBDE-47 dose, however, pituitary mRNAs for GPHα were reduced in both males and females (*p* < 0.0001) without a change in transcript for TSHβ.

*TR and BTEB mRNAs in the brain.* Gene transcripts for TRα were elevated 37% in the brain of females (n = 0.002), but not males, exposed to the high PBDE-47 dose (Figure 3A). Transcript levels for TRα also differed between males and females (*p* = 0.0431). In both sexes, PBDE-47 exposure depressed brain TRβ mRNA levels at both PBDE dosing levels (Figure 3B; *p* = 0.001). There was no difference in brain TRβ transcript levels between sexes.

Dietary PBDE-47 exposure also altered mRNA abundance for the TH-regulated transcription factor BTEB, although this effect differed between sexes (Figure 3C; *p* = 0.029). In males, BTEB transcript was reduced in both the low and high PBDE-47 exposures. Females had lower levels of BTEB transcript than males (*p* = 0.0008), but expression in females was not affected by PBDE exposure.

*TR transcripts in the liver.* LSI was greater in females than in males and was elevated 38% in males exposed to the high dose of PBDE-47 (*p* = 0.009; see Supplemental Material, Table 3 [available online at http://www.ehponline.org/members/2008/11570/suppl.pdf]). Transcript levels for TRα and TRβ in the liver were not altered by PBDE-47 [see Supplemental Material, Figure 1 (available online at http://www.ehponline.org/members/2008/11570/suppl.pdf)]. Male minnows, however, had greater levels of TRβ mRNA in the liver than females (*p* < 0.0001). TRα mRNA levels did not vary between the sexes.

*Gonad staging and reproductive behavior.* Male minnows exposed to PBDE-47 had fewer mature spermatocytes and more primary spermatocytes and spermatids compared with control males (low dose vs. control: χ² = 17.78, *p* = 0.001; high dose vs. control: χ² = 57.22, *p* < 0.001; see Supplemental Material, Table 4 [available online at http://www.ehponline.org/members/2008/11570/suppl.pdf]).
Dietary PBDE-47 depressed plasma T4 in male minnows exposed to PBDE-47 had body burdens of PBDE-47 related to their dietary dose, but females had greater tissue concentrations of PBDE-47 than males. In the low-dose treatment, males had a body burden of 11.43 ± 1.24 µg PBDE-47/g carcass, whereas females had 20.07 ± 7.38 µg PBDE/g carcass. In the high-dose treatment, PBDE-47 levels were 64.62 ± 6.10 µg/g carcass in males and 107.60 ± 29.40 µg/g carcass in females. This sex difference in PBDE-47 body burdens corresponds to a previous study using similar dosing procedures with this species (Muirhead et al. 2006).

**Discussion**

Dietary PBDE-47 depressed plasma T4 in male and female adult minnows. This reduction in T4 was associated with elevated transcript for TSHβ (low dose only) in the pituitary gland and changes in transcript expression for TR receptors at both exposure doses. The effects of PBDE-47 on TR receptor mRNA abundances were tissue specific. Transcripts for both TRα and TRβ in the liver were unaffected by PBDE-47. In the brain, however, PBDE exposure reduced levels of mRNA for TRβ in both sexes and elevated TRα mRNA in females. PBDE-47 also reduced brain mRNA levels of the TH-regulated transcription factor BTEB in male, but not female, minnows (Hallgren et al. 2001). As was observed in the current study, these effects on T4 generally correspond to a previous study using similar dosing protocols (Fernie et al. 2005; Meerts et al. 2000), but quantitative structural-activity binding models indicate only weak affinity of PBDE-47 to transthyretin (TRT), although the hydroxylated form of PBDE-47, 6-OH-PBDE-47, has greater affinity (Harju et al. 2007). An experimental study using recombinant sea bream TTR likewise found that PBDE-47 has lower affinity for teleost TTR than either TRα or TRβ, whereas the affinity of 6-OH-PBDE-47 is greater than that of the endogenous hormones (Morgado et al. 2007). Hydroxylated PBDEs such as 6-OH-PBDE-47 are produced metabolically from parent compounds (Mörick et al. 2003), and metabolic conversion of PBDE-47 may have occurred in the minnows. TTR, however, is generally not the dominant TH transport protein in fish, and binding of PBDEs to thyroxin-binding protein and serum albumin has yet to be examined.

Our data show that the PBDE-induced reduction in peripheral T4 is accompanied by changes in pituitary mRNAs for TSH. TSH is composed of an α- and β-subunit with each subunit synthesized separately. The GPH α-subunit of TSH is identical to that of follicle-stimulating hormone (FSH) and luteinizing hormone (LH), so it is TSH’s β-subunit that determines the hormone’s functional specificity. In the present study, TSHβ mRNA was elevated in both sexes by the low PBDE-47 dose, and transcript for GPHαβ was depressed in both sexes at the high dose. The elevation in TSHβ mRNA at the lower dose is consistent with reduced negative feedback on the pituitary from the decline in circulating T4. At the higher PBDE dose, however, alternative regulatory mechanisms or toxic effects may occur. Supporting this idea, the tests of PBDE-47–exposed males in the high dose had fewer spermatozoa. A similar decline in spermatozoa was observed in fathead minnows given an oral dose of 28.7 µg PBDE-47/pair/day (Muirhead et al. 2006), suggesting that PBDE exposures at high doses may impact gametogenesis and pituitary feedback from gonadal steroids.

Although the mechanism responsible for the PBDE-induced decline in circulating T4 cannot be discerned from alterations in pituitary mRNA levels alone, the reduced pituitary GPHαβ transcript may cause a reduction in bioactive TSH protein production and a decline in TH biosynthesis. To test whether PBDE-47 exposure affects TH-mediated gene transcripts in target tissues, we quantified mRNAs for TRα and TRβ in the brain and liver. TRs act as ligand-activated transcription factors by inducing or repressing the transcription of genes containing thyroid response elements (TREs). The genes for TRs themselves comprise TREs, so that transcripts for TRα and TRβ are autoinduced by T3 (Lema SC, Dickey JT, Schultz IR, Swanson P, unpublished data; Liu et al. 2000). This autoinduction means that TR transcripts are markers for assessing TH-induced activation of gene transcripts in target tissues (Opitz et al. 2006). In fish and other vertebrates, distinct expression patterns of the TRα and TRβ isoforms suggest that TRs have tissue-specific and developmental state-specific functions (Forrest et al. 1990; Yamano and Miwa 1998). Indeed, in neural development, the α and β receptors play distinct roles (Forrest et al. 2002). Studies with *in vitro* cell culture have shown that TRα regulates stem cell proliferation, whereas TRβ mediates differentiation of these newly proliferated cells into neurons (Jones et al. 2003; Lebel et al. 1994; Leroioualch et al. 1995).

In the present study, found that dietary PBDE-47 exposure did not affect TR transcripts in the liver, but it decreased mRNA levels for TRβ by 15–22% in the brain of males and females at both doses and elevated TRα transcripts by 37% in females at the high dose. These changes in TR transcript expression may result from the PBDE-induced T4 decline or via interactions between PBDE-47 and TRs or their corepressors. It is important to note, however, that transcripts for both TRs are similarly autoinduced by T3 in the brain and liver in both sexes in adult fathead minnows (Lema SC, Dickey JT, Schultz IR, Swanson P, unpublished data). That PBDE-47 exposure altered...
TR transcripts in the brain only, and TRα in a sex-specific pattern, suggests that PBDE-47 or its metabolites act directly on TR gene transcription mechanisms in target tissues (see also Schirks et al. 2007). Moreover, this finding exemplifies how impacts of PBDEs on gene transcripts do not conform to the expectations predicted by general hypothyroidism and demonstrates that these PBDE-induced effects cannot be generalized across tissues or sexes. Although the mechanism for PBDE-47’s impacts on TR gene transcription remains unclear, recent evidence indicates that PBDEs 47, 99, and 209 interact with the mouse pregnane X receptor (PXR) and its human counterpart, the nuclear steroid and xenobiotic receptor (SXR) (Pacyniak et al. 2007). The SXR interacts with the corepressor SMRT (silencing mediator for retinoid and thyroid receptors) (Takeshita et al. 2002), and PBDE-induced impacts on SXR might contribute to changes in TR gene transcription.

Even though the mechanism by which PBDE-47 alters brain TR transcripts is unresolved, our results clearly provide new evidence that dietary intake of PBDEs may impact TH-mediated neural development. In support of this idea, PBDE-47 exposure reduced transcript levels for the TH-regulated gene BTEB in the male minnow brain by as much as 53%, but did not affect that of females. BTEB encodes a zinc-fingered transcription factor that binds GC-box domains to facilitate or inhibit TH-mediated gene transcription. In mammals, TH regulates specific to neurons (Denver et al. 1999), and in Xenopus, BTEB is responsive to TH in brain and other tissues (Furlow and Kanamori 2002; Hooper et al. 2002). Studies in which BTEB expression has been blocked or induced have revealed that BTEB mediates TH-induced neural differentiation and neurite branching via TH activation of TRβ (Cayrou et al. 2002; Denver et al. 1999). The TRβ1-induced BTEB protein also binds the promoter of the TRβ1 gene to regulate its autorepression by PTX (Bagamasbad et al. 2008).

Whether the PBDE-induced change in BTEB transcript observed here translates to altered neurogenesis is not clear. Cell proliferation and neural differentiation occur throughout the adult fish brain (Lema et al. 2005; Zapanc et al. 2005), and BTEB transcript is regulated by TH in the brain of adult fathead minnows (Lema SC, Dickey JT, Schultz IR, Swanson P, unpublished data). Still, it is not known if BTEB regulates neurogenesis during this leasto life stage as it does during mammalian development. There is, however, accumulating evidence in mammals that THs regulate adult neurogenesis (Desouza et al. 2005; Fernandez et al. 2004; Tekumalla et al. 2002). In adult rats, for instance, exogenous TH increases immunoreactivity for the cell proliferation marker Ki-67 in the subventricular zone of the brain (Giardino et al. 2000). Taken together, these studies suggest that THs may influence neurogenesis in adults as they do in embryonic and neonatal life.

In summary, our results provide evidence that oral PBDE-47 exposure affects the thyroid axis at several levels by depressing peripheral levels of T₃, altering pituitary transcripts for TSHβ and GPH, and changing brain mRNA levels for the TH-responsive genes Tα₁, TRβ, and BTEB. Taken together, these results provide evidence that oral intake of the brominated flame retardant PBDE-47 can impact TH-regulated gene transcription in the pituitary gland and brain, and they illustrate how PBDE-induced changes in TH-regulated transcripts do not conform to the effects predicted by general hypothyroidism. Given these findings, it becomes crucial to ask whether these changes in TH-mediated mRNA levels translate into health consequences for humans or wildlife (Birnbaum and Staskal 2004). Effects of polybrominated diphényl ethers (PBDEs) and polychlorinated biphenyls (PCBs) on the thyroid hormone and vitamin A levels in rats and mice. Arch Toxicol 75:200–208.

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