Perfluorooctane Sulfonamide–Mediated Modulation of Hepatocellular Lipid Homeostasis and Oxidative Stress Responses in Atlantic Salmon Hepatocytes

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EFFECTS ON DEVELOPMENT, GROWTH RESPONSES AND THYROID-HORMONE SYSTEMS IN EYED-EGGS AND YOLK-SAC LARVAE OF ATLANTIC SALMON (*Salmo salar*) CONTINUOUSLY EXPOSED TO 3,3′,4,4′-TETRACHLOROBIPHENYL (PCB-77)

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Thyroid hormones (triiodothyronine, T3; and thyroxine, T4) play significant roles in development, metamorphosis, metabolism, homeostasis, cellular proliferation, and differentiation, for which the effects are mediated through thyroid hormone receptors (TRα and TRβ). Similarly, the insulin-like growth factor (IGF) is involved in growth and development through regulation of somatic growth. This study was designed to examine the effects of the dioxin-like 3,3′,4,4′-tetrachlorobiphenyl (PCB-77) on responses related to growth and thyroid hormone system in eyed eggs and yolk-sac larvae of Atlantic salmon. Salmon eggs were continuously exposed to two waterborne concentrations of PCB-77 (1 or 10 ng/L) over a period of 50 d covering hatching and through yolk-sac absorption stages. Sampling was performed regularly throughout the exposure period and at different time intervals. Gene expression patterns were performed on whole-body homogenate at age 500, 548, 632, 674, and 716 dd (dd: day degrees) using quantitative polymerase chain reaction (PCR). Total T3 (TT3) and total T4 (TT4) were measured using radioimmunoassay (RIA). Data showed that 10 ng PCB-77 increased dioiodinase 2 (Dio2) at 500 dd and both PCB-77 concentrations decreased dio2 expression at 548 dd. PCB-77 elevated cellular TT3 at 500 dd and was lowered at 548 dd only at 10 ng. Otherwise, time-related reduction was not affected by PCB-77 exposure as observed for the rest of the exposure period. For TT4, 1 ng PCB-77 produced a rise at 500 dd, and an apparent concentration decrease at 548 dd, before a total inhibition at 632 dd. The IGF-1 and IGF-1R were variably affected by PCB-77. For IGF-2, PCB-77 produced a concentration-dependent increase at 548 dd, and thereafter an elevation (1 ng) and fall (10 ng) at 632 dd. TRβ mRNA demonstrated PCB-77 related increases during the exposure period, and this effect returned to control levels at 716 dd. For TRα, a rise was noted only after exposure to 10 ng PCB-77 at 500 dd. Overall, the present study demonstrates some possible growth and developmental consequences following exposure to PCB-77 during early life stages of Atlantic salmon.

Thyroid hormones (TH) including triiodothyronine (T3) and thyroxine (T4) play important roles in metabolism, growth, and differentiation in many tissues and organs (Power et al., 2001; Brent, 2000). Thyroid hormones are bioavailable to the embryo via maternal deposition in the egg yolk, prior to active embryonic thyroid gland (Power et al., 2001; Brent, 2000). T4 is synthesized in the thyroid gland and bioactivated to T3 by deiodinases (dio1 and dio2) in peripheral tissues (Gereben et al., 2008). Thus, T4 is the main secretory product of the thyroid gland in all vertebrates and is recognized as a long-lived active molecule (Gereben et al., 2008). Deiodinase catalyzes the first step of

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TH action by conferring biological activity on T4, a metabolic pathway that removes one iodine residue from the T4 molecule to produce T3, the short-lived, most active form of TH (Sagar et al., 2008). T3 modulates gene expression in virtually every cell through ligand-dependent transcription factors; the thyroid hormone receptors (TR) are TRα and TRβ. Homeostasis of the TH system is maintained through the hypothalamus–pituitary–thyroid (HPT) axis involving the thyroid-stimulating hormone (TSH), T4, and T3 (Carr and Patino, 2011). Similar to the thyroid hormone system, the insulin-like growth factor (IGF) is involved in growth and development of vertebrates through the regulation of somatic growth (Moriyama et al., 2000; Mendez et al., 2001; Wood et al., 2005). The specific biological roles of TH and the IGF system render these specifically susceptible to organic pollutants (Mortensen and Arukwe, 2006; Shi et al., 2009; Kortner et al., 2009).

Polychlorinated biphenyls (PCB) are persistent, bioaccumulating, toxic organic pollutants (Andersson et al., 2001). In mammalian and avian species, the effects of PCB on the thyroid status were reported demonstrating that PCB may increase metabolism and excretion of TH or reduce circulating T4 concentrations (Sonstegard and Leatherland, 1979; Brouwer, 1991; Borga et al., 2005). Particularly, the coplanar 3,3′,4,4′-tetrachlorobiphenyl (PCB-77) is a dioxin-like congener, with a relatively high toxicity compared to most PCB. PCB-77 has structural similarities with T4 and was found to interfere with thyroid hormone homeostasis (Roelens et al., 2005) by decreasing T4 levels (Bastomsky et al., 1976), but not always producing symptoms of hypothyroidism. PCB-77 may act as an agonist or antagonist to thyroid hormone receptors (TR) (McKinney and Waller, 1998).

The biological effects of T3 and T4 are mediated through TR proteins, TRα and TRβ (Power et al., 2001). Thyroid hormone receptors function as hormone-inducible transcription factors that regulate the expression of target genes and are members of the hormone and orphan nuclear receptor superfamily. TRα and TRβ genes have been characterized in many vertebrates including several teleost species such as Japanese flounder (*Paralichthys olivaceus*), zebrafish (*Danio rerio*), and Atlantic salmon (*Salmo salar*) (Power et al., 2001). Thus, thyroid-disrupting chemicals may target any of the biological processes within the TH system, including the IGF pathways, in a chemical-specific manner (Ishihara et al., 2003a). Several environmental contaminants with endocrine-disrupting effects interfere with TH homeostasis by binding to transport proteins such as transthyretin (TTR) (Ishihara et al., 2003b). PCB and their hydroxylated metabolites (OH-PCB), dibenzo-*p*-dioxins, and dibenzofurans were shown to interact strongly with mammalian TTR, inducing a rise in plasma clearance rates of TH, and resulting in hypothyroxinemia in rats, seals, and humans (Brouwer et al., 1999). Data suggested that other chemicals may interact in a potent manner with transport proteins, and when compared with studies examining chemical effects on steroid hormone receptors, only a few investigations evaluated simultanously transcriptional modulation of TR and changes in growth and cellular TH levels mediated by endocrine-disrupting chemicals (EDC) during early development in fish (Crump et al., 2002).

Recently, Olufsen and Arukwe (2012) demonstrated that PCB-77 produced developmental effects related to angiogenesis and osteogenesis and disruption of vascular system development as evidenced by cardiac edema, anemia, and arrhythmia during early life stages of salmon. Because TH plays an integral role during organismal metamorphosis and interference by EDC on the TH system appears to be mediated, in part, by competition for binding to transport proteins (Ishihara et al., 2003a), it remains to be clarified whether EDC modulate transcriptional patterns of TR and their dependent genes during early development of fish embryo. The present study was designed with the objective of investigating the potential effects of environmentally relevant concentrations of PCB-77 on biological responses related to development, growth systems, and TH systems in eyed eggs and yolk-sac

larvae of Atlantic salmon. It was postulated that exposure of Atlantic salmon embryo/larvae to dioxin-like PCB-77 might produce changes in transcriptional, physiological, and endocrine responses indicative of potential adverse health and developmental effects in fish.

MATERIALS AND METHODS

Chemicals and Reagents

3,3′,4,4′-Tetrachlorobiphenyl (PCB-77) was purchased from Fluka Chemica-Biochemika (Buchs, Switzerland). iScript cDNA synthesis kit, iTAQ SYBR Green Supermix with ROX, and deoxynucleotide triphosphates (dNTPs) were purchased from Bio-Rad Laboratories (Hercules, CA). Trizol reagent for ribonucleic acid (RNA) purification was purchased from Invitrogen Corporation (Carlsbad, CA).

Animals, Exposure, and Sampling

Atlantic salmon (*Salmo salar*) eggs were purchased from AquaGen AS (Rearing Center located at Tingvoll). The eggs were fertilized and disinfected with Buffodine and thereafter treated with formalin (Rach et al., 1997) to remove possible fungus prior to delivery. Disinfection was performed both during incubation and before packaging, and formalin treatment was performed when eggs reached the eyed stage. The development of salmon embryo is dependent of water temperature (Gorodilov, 1996). Accumulated temperature units (number of days multiplied by degrees Celsius: dd) were therefore used to describe the developmental stage/age of the embryo/larvae since this is the best estimator of effect responses (Gorodilov, 1996). Upon delivery, eggs were 404 dd and kept at an average temperature of 5.8°C. On arrival, eggs were transferred to 14-L plastic tanks with fresh tap water and kept in a refrigerator at 6°C in the dark. The tanks were aerated by "bubbling stones" providing water circulation and air. During egg rearing, it is normal to utilize a flow-through system to maintain fresh water, but it was decided to manually change half the volume of tank water each week to reduce PCB-77 waste, while the nominal PCB-77 concentration was maintained by applying the appropriate 50% concentration. Tanks were placed vertically in the refrigerator and their position was altered weekly. Eggs were continuously exposed to waterborne 1 and 10 ng PCB-77/L. PCB-77 was dissolved in ethanol (carrier vehicle) and applied directly to water. Control group received the carrier vehicle only, and concentration of ethanol never exceeded 0.001% of total exposure tank volume.

In total, 1800 eggs were divided (600 each/group) into three exposure groups and kept in the dark to acclimatize. Exposure was initiated at 416 dd, when eggs were still unhatched. The hatching day was at 500 dd for all groups and defined as the time when 50% of all eggs had hatched (all groups hatched within 42 h), and hatched eggs at this time were used further in the experiment. The experiment lasted for a total of 51 d from the start of exposure, corresponding to larval age spanning from 404 to 716 dd. Twenty larvae were collected from each exposure group and distributed for use in different assays at each sampling time, and 10 larvae from each exposure group were used for growth measurement, which was performed 12 times during the experimental period, and mortality was recorded at each sampling day. Between 416 and 590 dd, sampling was performed once a week, while sampling was performed twice a week between 590 and 716 dd. This was done to capture significant developmental changes during the larval period. After preliminary screening and due to the overwhelming sample materials, selected and representative groups of these samples were analyzed and are presented herein.

Physical handling of larvae was necessary when sampling; however, it is important to minimize stress to animals. This is because (1) stress affects result output and (2) it is important to minimize discomfort when working with live animals. Therefore, all sampling procedures were performed as rapidly and carefully as possible without compromising the experiment. All animals were anesthetized using tricaine

methane sulfonate (MS222) (500 ng/L) before further processing. Samples collected for gene expression analysis were transferred directly to Trizol reagent, homogenized, and then snap-frozen in liquid nitrogen and stored at –80°C until further processing.

Measurement of Growth

A general observation of visual traits like color, hemorrhage, heart rate, and length was performed using a dissecting light stereomicroscope without anesthetic. These procedures were initiated after hatching and performed each week at developmental age 548, 590, 608, 674, and 716 dd. The dissecting stereomicroscope was equipped with a measuring ocular lens, which is easily calibrated using a ruler. Ratio (lens ocular value:metric value) is employed to calculate length of larvae in millimeters from the anterior-most part of the head to the tip of the tail, and digital images were collected using a Nikon digital camera (Nikon, Inc., Melville, NY). At this age, larvae are pigmented on the head and along the back, but were predominantly transparent, making it easy to observe the heart. Larvae were then transferred to preweighed tin-foil caps and dried in a heat cabinet. A tin-foil cap containing larvae was weighed to obtain dry weight in milligrams using a microweight to ensure accuracy.

Quantitative (Real-Time) PCR

Total RNA was purified from whole-body tissues homogenized in Trizol reagent according to the manufacturer's protocol. Total cDNA for the real-time PCR reactions was generated from 1 μg total RNA using poly-T primers as described by the manufacturer (Bio-Rad). Quantitative (real-time) polymerase chain reaction (PCR) was used for evaluating gene expression profiles. The expression of individual gene targets was analyzed using the Mx3000P real-time PCR system (Stratagene, La Jolla, CA). Each 25-μl DNA amplification reaction contained 12.5 μl of iTAQ SYBR Green Supermix with ROX (Bio-Rad), 1 μl cDNA, and 200 n*M* of each forward and reverse primers. The 3-step real-time PCR program included an enzyme activation step at 95°C (3 min) and 40 cycles of 95°C (30 s), 57–60°C for 10 s, depending on the primers used (see Table 1), and 72°C (20 s). Cycle threshold (Ct) values obtained were converted into mRNA copy number using standard plots of Ct versus log copy number. The standard plots were generated for each target sequence using known amounts of plasmid containing the amplicon of interest. Data obtained are given as percent of control ± standard error of mean (SEM: $n = 5$).

Radioimmunoassay (RIA)

The cellular levels of thyroid hormones (total thyroxine, TT4, and total triiodothyronine, TT3) in postmitochondrial supernatant (PMS) from homogenized whole-body tissues were determined by radioimmunoassay (RIA). Assay kits for TH were purchased from Siemens Medical Solution Diagnostic (Coat-A-Count, Los Angeles, CA) and performed according to the manufacturer's protocol. A gamma-scintillation counter (Cobra

TABLE 1. Primer pair sequences, accession numbers, amplicon size and annealing temperature conditions for genes of interest used for real-time PCR

Target gene	Primer sequence		Amplicon size (nucleotides)	Annealing temperature (°C)	GenBank accession number
	Forward	Reverse			
TRα	CGCATCTTTGATTTGGG	GGGGAATGTTGTGCTTGC	194	57	AF146775
TRβ	GGAAACATGAGGCCATGC	ACACGCTACGTTGGGTT	176	57	AF302251
IGF-1	ACTGTGCCCCTGTCAAGTCT	TTTGTCTTGTCTGGGTGCTG	150	60	EF432852
IGF-1R	AGAGACGTGTGTGGCCATTA	CACCACGTGATGACAGTTGA	112	60	DQ358691.1
IGF-2	ATTGCGCTGGCACTTACTCT	CTCCACGATACCACGGTTCT	171	60	EF432854
Dio2	TTTATCTTGACGGCCCAGAC	TCTCAACTCCTGCCACACAC	159	60	AF312396

Autogamma Counting System, model 5003, Packard Instrument, Dowers Grove, IL) was used to register the radioactivity levels of the samples.

Statistical Analysis

Statistical analysis was performed with GraphPad Prism, version 5.00 (GraphPad Software, Inc., 2007). Significant differences between control and exposure groups were performed using one-way analysis of variance (ANOVA) after testing for normality and variance homogeneity. Statistical differences between exposure groups and their respective controls were analyzed using the Dunnet's and Tukey–Kramer tests. The level of statistical significance was set at $p < .05$.

RESULT

PCB-77 did not produce any significant exposure-related mortality during the study period. A representative number of larvae ($n = 10$) was sampled from each exposure group (including control) and measured for length and weight growth and at each sampling time. Larval dry weight did not show any marked exposure-related differences at any sampling time, and when larvae within the same exposure group of different ages were compared no time-dependent alterations were observed (data not shown). In contrast, larvae exposed to 10 ng PCB-77 were significanly longer than control at 716 dd (Figure 1).

Transcript levels for TRα and TRβ were analyzed at different developmental stages following PCB-77 exposure (Figure 2). During the exposure periods (between 500 and 674 dd), TRβ showed significant increases in expression pattern after PCB-77 exposure compared with control (Figure 2A). However, no marked differences were observed between 1 and 10 ng/L groups. At 716 dd, TRβ mRNA, in all exposure groups, returned to control levels (Figure 2A). For TRα, significant elevation in transcript expression was noted at 500 dd in larvae exposed to 10 ng PCB-77 compared to control (Figure 2B). Otherwise, no other marked exposure-related changes were found.

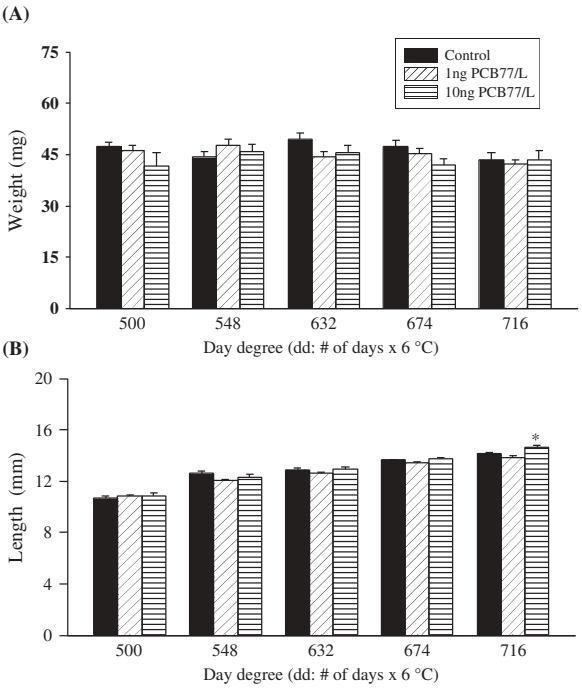

FIGURE 1. Effects of PCB-77 on somatic growth, measured as (A) length (mm) and (B) dry weight (mg). Samples were collected at five developmental stages, noted as day degrees (dd), after exposure. Data are provided as mean ($n = 6$) ± standard error of the mean (SEM). Asterisk denotes means that are significantly different from control at the same age ($p < 0.05$), analyzed using the Tukey–Kramer test.

Larval IGF-1 mRNA expression displayed significant rise compared to control, at 500 and 674 dd in larvae exposed to 10 ng PCB-77 (Figure 3A). At 716 dd, 1 ng PCB-77 produced significant increase compared with control in IGF-1 mRNA (Figure 3A). For IGF-2, a significant concentration-dependent elevation was noted at 548 dd. While this rise was maintained at 632 dd by 1 ng PCB-77, with 10 ng PCB-77 there was significant decrease in IGF-2 mRNA levels (Figure 3B). At 674 dd, a significant reduction in IGF-2 mRNA was observed in larvae exposed to 10 ng PCB-77 and no marked changes were found at 716 dd, although the expression levels showed a trend towards reduction (Figure 3B). The IGF-1R displayed an apparent concentration-dependent fall at 548 and 632 dd, and these effects were reversed at 674 dd, showing

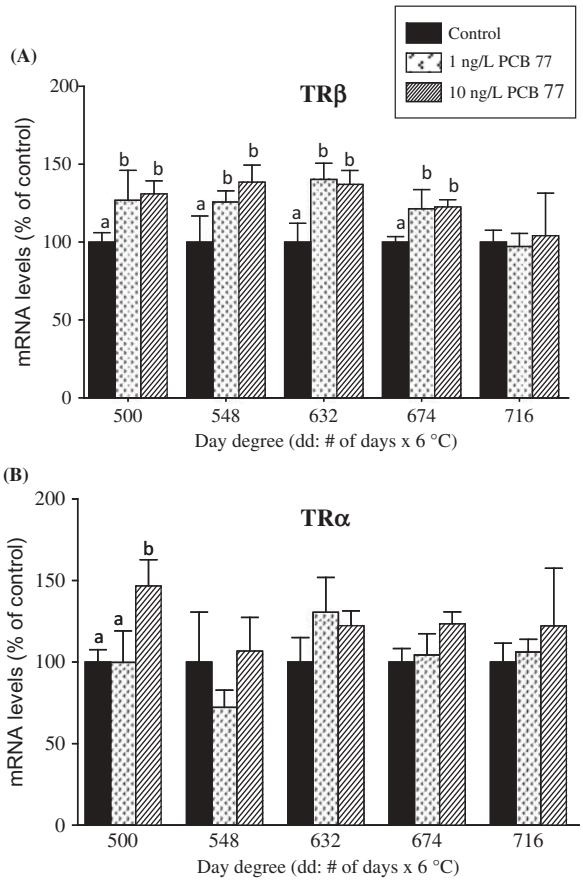

FIGURE 2. Thyroid receptor β (TRβ: A) and α (TRα: B) mRNAs of salmon larvae continuously exposed to PCB-77 and sampled at five developmental stages, noted as day degrees (dd), after exposure. Messenger RNA (mRNA) levels were quantified using quantitative real-time (RT)-PCR with specific primer pairs. Data are presented as mean percent of control ($n = 6$) ± standard error of the mean (SEM). Different letters denote means that are significantly different within the same developmental age ($p < .05$), analyzed using the Tukey–Kramer test.

an apparent concentration-dependent increase (Figure 3C). At 716 dd, 1 ng PCB-77 produced a significant rise in IGF-1R compared with control or 10 ng PCB-77 (Figure 3C).

Dio2 transcript was first increased in an apparent concentration-dependent manner at 500 dd, and thereafter at 548 dd showed a concentration-dependent decrease (albeit not significant; Figure 4A). Otherwise, no marked changes were noted for the remaining exposure periods (Figure 4A). Cellular levels of total T3 (TT3) and T4 (TT4) were also measured using RIA, demonstrating that TT3 was first elevated by PCB-77 concentraions at 500 dd and then significantly lowered by 10 ng PCB-77 at 548 dd (Figure 4B). Otherwise, no other marked changes were observed, including after the recovery period. For TT4, 1 ng PCB-77 produced a significant increase at 500 dd, and thereafter an apparent concentration-dependent decrease was observed at 548 dd (Figure 4C). At 632 dd, PCB-77 produced complete inhibition of TT4, and after the recovery period, a PCB-77-mediated significant decrease was observed compared with the control (Figure 4C).

DISCUSSION

In a recent study using the same experimental material from the present study, data showed that PCB-77 produced developmental effects related to angiogenesis and osteogenesis and disruption of vascular system development as evidenced by cardiac edema, anemia, and arrhythmia during early life stages of salmon (Olufsen and Arukwe, 2012). Results in this investigation demonstrated that environmentally relevant concentrations of PCB-77 produced time- or age- and concentration-specific modulations on biological responses related to development, growth systems, and TH systems in eyed eggs and yolk-sac larvae of Atlantic salmon. Given that chemical disruption of the TH system may occur at several steps in the thyroid cascade, from synthesis and regulation to metabolism, and because TH regulate many metabolic and physiological processes, the effects of organic contaminants and their hydroxylated (OH) metabolites may exert deleterious consequences for wildlife species including Atlantic salmon. Cellular TT4 and TT3 levels, together with transcript patterns for TRα, TRβ, Iod2, IGF-1, IGF-2, and IGF-1R, were analyzed at different developmental stages during continuous exposure to PCB-77, showing that these responses were variably affected by PCB-77. Overall, the present study demonstrates some possible growth and developmental consequences arising from exposure to PCB-77 during early life stages of Atlantic salmon. However, while these effects appeared

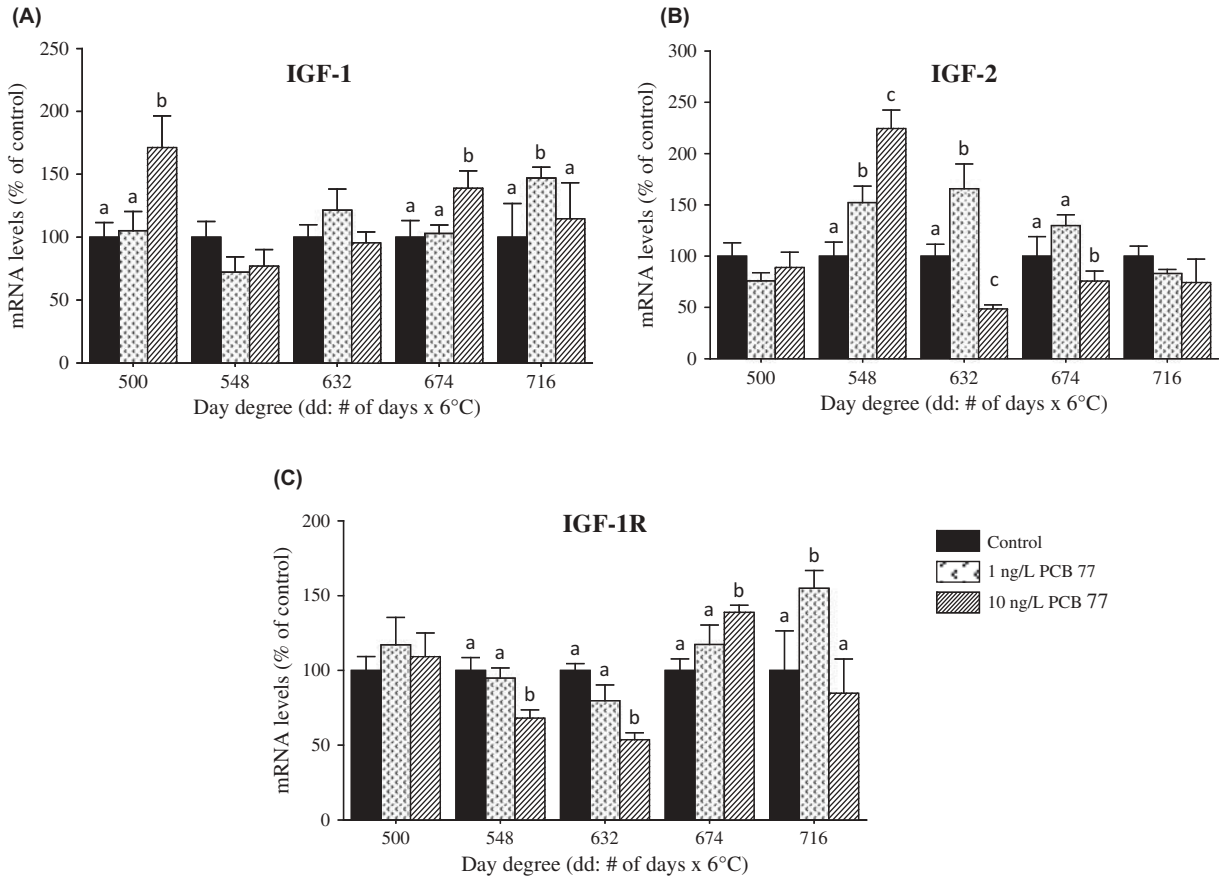

FIGURE 3. Effects of PCB-77 on insulin-like growth factor 1 (IGF-1) (A), IGF-2 (B), and IGF-1R (C) mRNAs of salmon larvae. Samples were collected at five developmental stages, noted as day degrees (dd), after exposure. Messenger RNA (mRNA) levels were quantified using quantitative RT-PCR with specific primer pairs. Data are given as mean percent of control ($n = 6$) ± standard error of the mean (SEM). Different letters denote means that are significantly different within the same developmental age ($p < .05$), analyzed using the Tukey–Kramer test.

minimal, most likely these findings may be attributed to use of whole organ homogenate and may represent serious biological or physiological consequences for developing embryos.

The biological actions of TH are dependent on several processes that include blood transport proteins (Ishihara et al., 2003a), levels of TR in target tissues, and activating and inactivating enzymes (outer and inner ring deiodinases: dio1 and dio2) (Orozco et al., 2002) and elements that upregulate or downregulate upstream gene responses for growth, development, and reproduction (Lazar, 1993). As a result, the entire TH system is susceptible to chemical insult (Brown et al., 2004a). In this study, PCB-77 produced changes in transcript levels for TRα and TRβ at different developmental stages. However, while these effects were transient, occurring only at 500 dd for TRα, they were continuous for TRβ, but these effects were not concentration dependent. An integral mechanism of TH action involves hormone binding to TRα and TRβ, resulting in tissue-specific activation/repression of gene transcription (Yamano and Miwa, 1998). When our finding are considered, there are several aspects of our data that are comparable with previously findings showing that dioxins, furans, dioxin-like PCB, and PCB mixtures produced effects on the TH system of fish. Cheek et al. (1999) found that examination of hydroxylated PCB (OH-PCB), DDT, DDT metabolites, and several other OC herbicides when used in

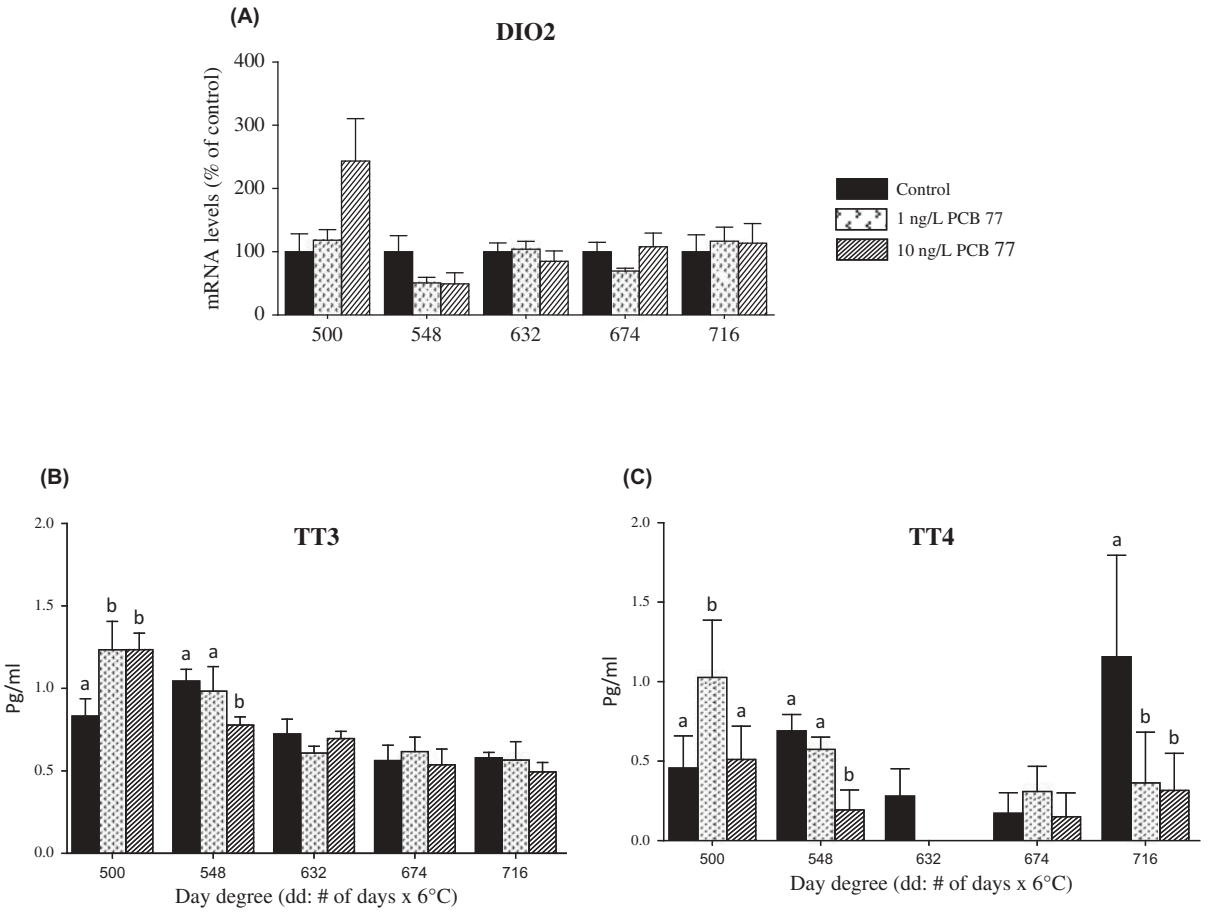

FIGURE 4. Deiodinase-2 (dio2) (A) mRNA, total triiodothyronine (TT3) (B) and total thyroxine (TT4) (C) in salmon larvae continuously exposed to PCB-77 and sampled at five developmental stages after exposure, noted as day degrees (dd). Messenger RNA (mRNA) levels were quantified using quantitative RT-PCR with specific primer pairs. Data are given as mean percent of control ($n = 6$) ± standard error of the mean (SEM). Different letters denote means that are significantly different within the same developmental age ($p < .05$), analyzed using the Tukey–Kramer test.

a competitive inhibition experiments showed that only the OH-PCB competed for binding to TR.

In Arctic grayling (*Thymallus arcticus*) exposed to single dietary doses of PCB-77 at 10, 100, and 1000 mg/kg body weight, plasma TH levels and/or thyroid histology were not markedly affected after 30 or 90 d (Palace et al., 2001). Elsewhere, male or immature female American plaice (*Hippoglossoides platessoides*) exposed once to 5–500 mg/kg of either PCB-77 or PCB-126 demonstrated elevation in increased plasma T4 levels and liver T4ORD activity, and decreases or no change in plasma T3 levels after PCB-77 exposure; and none of the PCB congener markedly affected brain deiodination activity (Adams et al., 2000). Further, in vitro exposure of PCB-77, PCB-126, or OH-PCB metabolites did not produce marked effects on hepatic microsomal deiodination activities of plaice or compete with T4 for binding to plasma proteins (Adams et al., 2000), thus prompting the suggestion that PCB-induced changes in deiodination may be compensatory responses to disrupting effects that might otherwise have reduced plasma T3 levels (Adams et al., 2000; Brown et al., 2004b). This is further supported by the fascinating new role of the deiodinase enzymes that was unveiled recently, showing that the activating deiodinase (dio1) and inactivating deiodinase (dio2) may locally increase or decrease TH

signaling in a tissue- and temporal-specific fashion, independent of changes in TH serum concentrations (Gereben et al., 2008). In addition, sublethal exposure of pre-metamorphic summer flounder (*Paralichthys dentatus*) to dioxin-like PCB126 at 20.9 mg/kg produced accelerated metamorphosis (Soffientino et al., 2002). A reduction in plasma TT4 levels, without corresponding changes in either TT4 or plasma TT3 levels, was observed when European flounder (*P. flesus*) were injected intraperitoneally (ip) with 100 mg of TCDD/kg (Besselink et al., 1997). In lake trout (*Salvelinus namaycush*), a chronic ip exposure to PCB-126 temporarily elevated plasma T4 level and clearance, probably as a result of enhanced hepatic T4 glucuronidation (Brown et al., 2004b).

In the present study, changes in transcriptional levels of TSH were not measured. Previously, Mortensen and Arukwe (2006) found that TSHβ mRNA levels were inhibited by T4 and DDE, when given alone and in combination, in the salmon brain. In addition, DDE also induced TSHβ mRNA in salmon liver. Changes in the production of plasma TSH levels are negatively regulated by T3 and T4 through a feedback mechanism to stimulate the production of TH. The typical pattern of physiological effects of TH modulators demonstrates negative correlation between increased plasma concentration of TSH and reduced T3 and/or T4 (O'Connor et al., 1999). Yamada et al. (2004) reported that DDE exposure of rats to 100 mg/kg/d significantly decreased serum T4 but not T3 or TSH. These studies were relevant to the present study, since the quantified gene expression thyroid pathways are indicative of potential modulation of TSH production. When the current findings are viewed together with our recent paper (Olufsen and Arukwe, 2012) illustrating that PCB-77 produced developmental effects related to angiogenesis and osteogenesis and disruption of vascular system development, a realistic overview of the effects of dioxin-like compounds on early life stages of fish emerges. Although several endpoints of thyroid function were altered by PCB-77 exposure, the sensitivities of these effects were largely dependent on developmental stage or sampling time and probably reflected physiological function of developmental modes in salmon embryo/larvae. The action of TH depends on the transport proteins in the blood (Ishihara et al., 2003b; Yamauchi et al., 2002), the nuclear thyroid receptors in the target tissues (Cheek et al., 1999), the activating and inactivating enzymes (outer and inner ring deiodinases dio1 and dio2) (Orozco et al., 1997), and the upstream response elements to upregulate or downregulate genes for growth, development, and reproduction (Power et al., 2001), making the entire system susceptible to chemical insult (Brown et al., 2004b). Herein, PCB-77 produced changes in TRβ mRNA expression throughout the sampling period, while the effect on TRα mRNA was significantly restricted to the early development stage (500 dd). These findings support the understanding that major mechanism of TH action involves hormone binding to nuclear TRα and TRβ, resulting in tissue-specific activation/repression and developmental stage-specific expression of gene transcription (Yamano and Miwa, 1998).

The TH signaling and IGF systems control biological processes involved in growth and development of vertebrates. In salmonids, IGF-1 is involved in the regulation of somatic growth and plays specific roles in the smoltification process (Beckman et al., 1998). In this study, data demonstrated that larval IGF-1 mRNA expression significantly increased at early development (500 and 674 dd) after exposure to 10 ng PCB-77. After the recovery period, 1 ng PCB-77 produced significant elevation. In addition, IGF-2 displayed a significant PCB-77 concentration-dependent rise at 548 and 632 dd at 1 ng PCB-77, and a decrease at 10 ng PCB-77. Furthermore, changes in IGF-1R mRNA were observed in larvae exposed to PCB-77, and these effects were apparently dependent on developmental stage or sampling time. Previously, Le Gac et al. (2001) reported that exposure of rainbow trout to the antiandrogenic fungicide prochloraz reduced IGF-1 response of male testicular cells, and at the same time increased the number of specific IGF-1 binding sites. In another study, pregnant Sprague-Dawley rats

injected on gestational day 16 and 18 with a PCB mixture (Aroclor 1221: A1221), a reconstituted PCB mixture representing those with highest human body burden (PCB 138, 153, 180) produced somatic and reproductive developmental effects (Dickerson et al., 2011). In female adult rats, androgen receptor, IGF-1, and TGFβ1 mRNA were significantly reduced in the preoptic area (POA) of exposed individuals (Dickerson et al., 2011). However, no marked or direct relationship was noted between changes in somatic growth (length and weight) and transcriptional changes in thyroid or IGF signaling pathways in this study. These discripancies may be explained by the fact that changes in transcript levels were measured in whole larval body homogenate and may therefore not represent real changes in gene expressions that regulate somatic growth at specific organs. However, previously Elonen et al. (1998) demonstrated that dioxins and dioxin-like compounds produced effects in an array of developmental disorders. These included an increase in rate of mortality that paralleled vascular dysfunction and edema in areas surrounding the yolk sac and heart, craniofacial malformations, and decreases in growth rate on early life stages of fish.

Arctic charr exposed to a high dose of Aroclor 1254 (A1254) displayed either a transient or a permanent reduction in plasma growth hormone, IGF-1, and T3 and T4 levels during the smoltification period (Jorgensen et al., 2004), and these hormonal changes paralleled the impaired hyposmoregulatory ability in May and June, as well as reduced growth rate and survival after transference to seawater (Jorgensen et al., 2004). In accordance with the present study, Mortensen and Arukwe (2006) previously found that DDE induced changes in deiodinase mRNA levels in kidney and liver of salmon, and combined exposure with T4 inhibited deiodinase expression in salmon brain, kidney, and liver. In addition, IGF-1R and TRα expressions were induced by DDE, and T4 in the brain, while combined exposure with both compounds did not markedly affect IGF-1R transcript levels (Mortensen and Arukwe, 2006). When all these studies are viewed together, it could be argued that these environmental chemicals share a common mode of action in affecting the transcriptional regulation of THR and IGF-1R dependent pathways, but with different developmental responses. Given that salmon parr go through an exponential growth phase during early development, the observed changes of IGF-signaling pathways in exposed fish are interesting and may represent significant developmental and physiological effects. However, further studies are needed in order to understand the mechanisms leading to changes at the molecular level and how these transcend to functional deficits.

In summary, disruption of thyroid hormone and growth factors status by halogenated organic chemicals, with associated developmental alteration, might occur at several steps, including synthesis, regulation, and metabolism. Since TH and growth factors regulate many developmental, metabolic, and physiological processes, our data represent a reliable indicator of PCB-77 effects on developing fish embryo/larvae. The present study showed that PCB-77 produced significant changes in TT3 levels at 500 dd (increased) and 548 dd (reduced), while TT4 levels were variably modulated throughout the entire experimental period. This supports previous findings that PCB-77 binds to TTR, resulting in changes in TT4 levels without consequent hypo- or hyperthyroidism. Dio2 exprssion paralleled TT3, supporting Dio2 as the main activator/deiodinating enzyme for converting T4 to T3. Transcription of TRα, TRβ, IGF-1, IGF-2, and IGF-1R were also modulated by PCB-77, and these effects were dependent on larval developmental stage or sampling time. In general, these findings indicate that the coordinated and integral roles of the TH and IGF systems, their changes between developmental stages, and susceptibility to chemical insults may represent overt developmental and physiological consequences during early development in fish species.

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