Marine Pollution Bulletin xxx (xxxx) xxx–xxx

Contents lists available at ScienceDirect

Marine Pollution Bulletin

journal homepage: www.elsevier.com/locate/marpolbul

Effects of polychlorinated biphenyls on metamorphosis of a marine fish Japanese flounder (*Paralichthys olivaceus*) in relation to thyroid disruption

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ARTICLE INFO

Keywords:
Flatfish metamorphosis
Morphological abnormalities
Delayed effects
Thyroid hormones
Polychlorinated biphenyls

ABSTRACT

This study examined the influence of environmental concentrations of Aroclor 1254 (10, 100, and 1000 ng/L) on metamorphosis of *Paralichthys olivaceus*, and analyzed the mechanisms in relation to thyroid disruption. Results showed that 100 and 1000 ng/L Aroclor 1254 delayed metamorphosis and that 1000 ng/L Aroclor 1254 caused abnormal morphology. Thyroxine and triiodothyronine levels in the control group were significantly elevated at metamorphic climax, but treatment with 100 and 1000 ng/L delayed the increase in thyroid hormones (THs) and retarded metamorphic processes. In larvae exposed to 1000 ng/L Aroclor 1254, TH levels at metamorphic climax were significantly lower than those of the control group at the same metamorphic stage. We suggest that the effects of Aroclor 1254 on larval metamorphosis can be explained by disruption of thyroid homeostasis. These findings provide a new perspective and biological model for thyroid-disrupting chemicals (TDCs) screening and investigating interference of thyroid function by TDCs.

1. Introduction

Thyroid-disrupting chemicals (TDCs) such as planar halogenated aromatic hydrocarbons, polycyclic aromatic hydrocarbons (PAHs), heavy metals, and steroids have the potential to affect thyroid status (Brown et al., 2004; Rolland, 2000). Administration of ammonium perchlorate, sodium arsenate, potassium-perchlorate and polychlorinated biphenyls (PCBs) to fish influences the morphology of the thyroid gland (Crane et al., 2005; Liu et al., 2008; Schmidt et al., 2012) and can have positive or negative effects on levels of circulating thyroid hormones (THs) (Coimbra et al., 2005; LeRoy et al., 2006; Schnitzler et al., 2011). PCBs are frequently detected in aquatic environments. For example, the PCB content of surface water in the Minjiang River Estuary and Bohai Bay, China, average 985 and 210 ng/L, respectively (Wang et al., 2007; Zhang et al., 2003). The total concentration of PCBs ranged from 2.33 to 44 μg/kg in marine sediments in Barcelona, Spain (Castells et al., 2008), and from 10 to 899 μg/kg in surface sediments of Naples Harbor, Italy (Sprovieri et al., 2007). It has been reported that PCBs can alter thyroid histopathology, combine transthyretin (TTR), deiodinase activity, and metabolism of THs, thereby interfering with TH levels in fish (Adams et al., 2000; Brown et al., 2004; Coimbra et al., 2005; Ishihara et al., 2003; Klaassen and Hood, 2001; Schnitzler et al., 2011).

Several studies in fish have highlighted the important role of TH during flatfish metamorphosis (Inui and Miwa, 1985; Miwa and Inui, 1987; Power et al., 2001; Shao et al., 2017). Exogenous THs, administered by simple immersion protocols to gravid females, larvae, or pre-metamorphic juveniles, can enhance maturation of oocytes, improve larval survival, synchronize metamorphosis, and produce uniform cohorts in flatfish (Gavlik et al., 2002; Schreiber and Specker, 1998; Solbakken et al., 1999). Japanese flounder (*Paralichthys olivaceus*) were treated with exogenous thiourea (TU), an inhibitor of thyroxine (T_4) synthesis, significantly reduced their T_4 levels and retarded the metamorphosis (Okada et al., 2005). Thus, TDCs that alter larval TH levels may affect larval metamorphosis. It has been confirmed that TDCs can retard metamorphosis and increase rates of offspring deformity in amphibians. Croteau et al. (2009) found that the estrogenic chemical 4-tert-octylphenol caused a significant delay in development starting from Gosner stage 29, by observing that fewer *Rana pipiens* tadpoles developed past this stage when they had lowered levels of deiodinase type 2 or increased mRNA levels of deiodinase type 3. Balch et al. (2006) found significant inhibition of tail resorption, delayed metamorphosis, and effects on skin pigmentation in *Xenopus laevis* exposed to polybrominated diphenyl ethers. Exposure of eggs or

Abbreviations: TDCs, thyroid-disrupting chemicals; PAHs, polycyclic aromatic hydrocarbons; PCBs, polychlorinated biphenyls; THs, thyroid hormones; TTR, transthyretin; TU, thiourea; T_4, thyroxine; TCDD, tetrachlorodibenzo-p-dioxin; PCB 126, 3, 3′, 4, 4′, 5-pentachlorobiphenyl; T_3, triiodothyronine; MS222, tricaine methane sulfonate; PBS, phosphate-buffered saline; ELISA, enzyme linked immunosorbent assay; ANOVA, analysis of variance; PTU, propylthiouracil; TSH, thyroid-stimulating hormone

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http://dx.doi.org/10.1016/j.marpolbul.2017.04.033
Received 9 January 2017; Received in revised form 14 April 2017; Accepted 15 April 2017

tadpoles of *Bufo boreas*, *R. pipiens*, *and Rana clamitans* to 73 ng/g 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) caused minor morphological abnormalities (Jung and Walker, 1997). In contrast, no changes in T_4 levels or obvious inhibition of metamorphosis was observed in *X. laevis* and *Rana temporaria* exposed to 200 mg/kg Clophen A50 (Gutleb et al., 2000). Accordingly, the amphibian metamorphosis assay has been adopted for testing of TDCs in Tier 1 testing of the endocrine screening program developed by the United States Environmental Protection Agency (USEPA) (USEPA, 2009); however, it appeared that exposure to chemicals at doses greater than the actual environmental contamination can affect amphibians metamorphosis (Miyata and Ose, 2012). Indeed, early life stage exposure to 3, 3′, 4, 4′, 5-pentachlorobiphenyl (PCB 126) at 1, 3, and 10 ng/L was reported to cause delayed development in common sole (*Solea solea*) (Foekema et al., 2008). Soffientino et al. (2010) also found 15 ng/g of PCB 126 delayed metamorphic progress and resulted in abnormal gastric gland morphology in larval summer flounder (*Paralichthys dentatus*). Regarding the definitive evidence that TH plays a major role in initiating metamorphosis in flounder larvae (Inui and Miwa, 1985; Inui et al., 1994), we hypothesized that the thyroid-mediated metamorphosis in this flatfish model would be more suitable for detecting potential TDCs in the aquatic environment.

Metamorphosis is a crucial developmental phase in flatfish species and the transformation from symmetric pelagic larva to asymmetric benthic juveniles most conspicuously involves eye migration and craniofacial remodeling (Klaren et al., 2008). Defects in metamorphosis of flatfish, such as migration of the wrong eye, arrest of metamorphosis, and various pigmentation flaws could result in maldevelopment of juveniles and adults (Bisbal and Bengston, 1993; Ellis et al., 1997; Pittman et al., 1998). Japanese flounder (*P. olivaceus*) inhabits coastal waters of Asia, has significant economic potential, and is cultured at a commercial scale. Furthermore, *P. olivaceus* is the first teleost species in which a role for TH in metamorphosis has been demonstrated (Inui and Miwa, 1985), and a recent study also demonstrated the molecular basis of metamorphosis in this flatfish (Shao et al., 2017). Here, we used premetamorphic *P. olivaceus* larvae in an experimental examination of the influence of environmental concentrations of Aroclor 1254 on metamorphosis (Castells et al., 2008; Wang et al., 2007; Zhang et al., 2003). Since PCBs exposure has been shown to exert thyroid-disrupting properties in teleosts by influencing TH levels (Coimbra et al., 2005; LeRoy et al., 2006; Schnitzler et al., 2011) and cause developmental delay in larval flatfish (Foekema et al., 2008; Soffientino et al., 2010), changes in whole-body T_4 and triiodothyronine (T_3) levels during metamorphosis were determined in *P. olivaceus* larvae exposed to Aroclor 1254 to analyze the mechanisms behind abnormal metamorphosis. This study provided a new understanding for study of thyroid interference by TDCs in fish and suggested a potential teleost model for testing of TDCs in aquatic environments.

2. Materials and methods

2.1. Chemicals

Aroclor 1254 (CAS 11097-69-1) was purchased from AccuStandard Inc. Tricaine methane sulfonate (MS222) was obtained from Sigma-Aldrich Co. Ethanol (analytically pure), Na_2HPO_4, KH_2PO_4, NaCl, and KCl were from Sinopharm Chemical Reagent Co. Aroclor 1254 stock concentrate (1 mg/mL) was prepared by dissolving 50 mg Aroclor 1254 in 50 mL ethanol.

2.2. Fish maintenance and exposure

Fertilized eggs (approximately 2000) of *P. olivaceus* were provided by the Huanghai Sea-farming Company, Haiyang, China, on May 3, 2013. The eggs were obtained from a spawning tank containing several males and females and the fertilization rate was 80%. Eggs were stocked in a 240-L tank with noncirculating sand-filtered natural seawater at 15 °C. Hatching (50% hatched) occurred after 2 days. Newly hatched larvae (3 days post-hatching, dph) were randomly assigned to four 240-L tanks, a solvent control (0.0001% ethanol) group, and three treatment groups (n = 500 larvae per treatment), containing 200 L of sand-filtered natural seawater with a salinity of 33 ± 1‰ and pH 8.0 ± 0.1 at ambient temperature (16.8 ± 1.6 °C). Only one replicate was performed for each condition. A 24-h light photoperiod (24 hL/0 hD) was maintained to stimulate larval feeding, and promote survival, growth, and development of *P. olivaceus* larvae. Larval *P. olivaceus* were exposed to Aroclor 1254 (CAS 11097-69-1, AccuStandard Inc., NH, USA) at 0 (solvent control), 10, 100, and 1000 ng/L. The larvae were fed fatty acid-enriched rotifers, *Brachionus rotundiformis*, at a prey density of 5–10 individuals/mL; newly hatched brine shrimp, *Artemia nauplii*, were also provided from 17 dph. Fish were fed twice a day during the experiment. Microalgae (*Nannochloropsis gaditana*, 3×10^5 cells/mL) were also added to the rearing tanks from the first feeding. Chang et al. (1965) reported that flounder larvae metamorphosis began after 17 dph. The exposure duration was designed to 41 dph, covered pre-metamorphosis, pro-metamorphosis, metamorphic climax, and post-metamorphosis stages. During exposure, 50% of each test solution was changed once per day, and the appropriate amount of seawater and stock solution was added to maintain the desired chemical concentrations.

2.3. Sampling procedures

To characterize metamorphic progress, larvae (n = 16–25) were randomly removed from the four tanks by scooping with a small strainer at the time points indicated in Fig. 3. It was not necessary to anaesthetize the larvae for this procedure. Experimental animals were handled carefully during this transfer in order to minimize stress and avoid any injury to the larvae.

To determine whole-body total TH concentrations, 18-, 22-, 24-, 28-, 30-, 37-, and 41-dph larvae were randomly selected from each experimental group and anesthetized with 500 ng/L of MS-222. Subsequently, larvae were rinsed with distilled wate and adhered water was removed with a tissue. Because the larvae were too small to collect blood from, larvae from each group in each metamorphic stage were pooled to obtain a total sample wet weight of 0.04–0.2 g (n = 4). Samples were frozen in liquid nitrogen and stored at − 80 °C until further analysis.

2.4. Assessing metamorphic progress

The metamorphic stage of each larva was determined according to the scheme presented in Table 1, which was based on a scheme developed by (Minami, 1982). Frequency distributions were constructed from which the maximal frequency (%) of each developmental stage and the larval age (dph) at which each stage was observed were derived.

At the end of the experiment (41 days), total body length (L_T) and body weight (W_T) of the larvae (n = 9) were measured.

2.5. Thyroid hormone analysis

Briefly, larval samples were homogenized in 500 μL phosphate-buffered saline (PBS; 10 mM phosphate buffer, pH 7.3, containing 140 mM NaCl) by using an Ultra-TurraxT8 basic homogenizer (IKA, Staufen, Germany). After centrifugation for 20 min at 3000 rpm, the supernatant was collected. Whole-larvae total T_4 and total T_3 concentrations were measured with enzyme linked immunosorbent assay (ELISA) Kits (Bogoo, Shanghai, China) according to the manufacturer's instructions. Each sample was measured twice. Calibration samples were prepared in the same PBS as the test samples. A logit-log plot produced linear calibration curves from which total TH levels in the