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# Effects of the environmental endocrine disrupting compound benzo[a] pyrene on thyroidal status of abu mullet (*Liza abu*) during short-term exposure

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ABSTRACT

Benzo[a]Pyrene (BaP) is a ubiquitous polycyclic aromatic hydrocarbon (PAH) that has been shown to disrupt the metabolism of thyroid hormone. Then, the present investigation aimed to study the effects of BaP on thyroid function in *Liza abu*. Fish were injected with 2, 10 and 25 mg/kg-bw of BaP. Samples were taken from blood, thyroid and muscle tissues at days 1, 2, 4, 7, and 14. Blood was evaluated for changes in the plasma levels of TSH, T3 and T4. Also, BaP bioaccumulation in the fish muscle was measured. Thyroid tissues were processed for routine histology. BaP concentration in the muscle of treated fish reached a maximum level after 4 days. Exposure of fish to BaP resulted in a significant decrease in T3 and T4 plasma level and increase in TSH concentration up to day 4. Also some pathological alterations were observed in BaP-exposed fish such as hemorrhage and increased number of large follicles with squamous epithelium. In conclusion, according to the results of the present investigation, short term exposure to sublethal concentrations of BaP significantly affected thyroid function in fish. The results revealed BaP ability to alter thyroid function.

## 1. Introduction

Thyroid follicular cells synthesize and secrete the thyroid hormones after activation of the hypothalamo-pituitary-thyroid (HPT) axis. The hypothalamus induces the pituitary gland to secrete thyroid-stimulating hormone (TSH) which activates synthesis of thyroxine (T4; 3,5,3,5-tetraiodo-L-thyronine) and triiodothyronine (T3; 3,5,3_-triiodo-L-thyroinine) in the thyroid gland. The level of T4 is generally higher than T3 in the blood circulation. T4 acts as a prohormone that can be converted into T3 by 5-iodothyronine deiodinases in target tissues [1]. Thyroid hormones are essential for regulating development, growth, morphogenesis, basal metabolism, reproduction, osmoregulatory properties, and behavior in fishes [1].

Different environmental contaminants disturb the thyroid system at many levels. Polycyclic aromatic hydrocarbons (PAHs) have two or more fused aromatic rings. They are classified as a group of organic pollutants [2] that arise from anthropogenic activities, such as vehicle exhausts, oil shipping and refineries. These organic chemicals presented in the priority pollutant list of the United States Environmental Protection Agency because of their mutagenic, carcinogenic and immunosuppressive properties [3]. These compounds have adverse effects on development and function of the thyroid gland in mammals [2]; however, there are few studies on the effects of PAHs on thyroid development or function in fish. 3-Methylcholanthrene is a carcinogen PAHs which adversely affected thyroid function in rat [4]. Teles et al. [5] reported that naphthoflavone, an aryl hydrocarbon receptor prototype ligand, significantly decreased plasma T4 levels, whereas TSH, T3 and plasma cortisol remained constant in adult *Anguilla anguilla*. Also as it seems, many PAH compounds are able to bind to transthyretin (TTR), in vitro [6]. According to Sun et al. [7], 1- naphthol and 2-naphthol, two hydroxylated PAHs, inhibit the TRβ1-mediated transcription in vitro. Totally, PAHs decrease the circulating and tissue levels of thyroid hormones through at least three mechanisms: 1. PAHs may directly interfere with thyroid gland function and then change the structure of thyroid gland that lead to disruption of hormone synthesis [8]. 2. PAHs can target the metabolism of thyroid hormone. They may

*Abbreviations:* BaP, benzo[a]pyrene; EDC, endocrine disrupting chemical; PAH, polycyclic aromatic hydrocarbon; PCB, polychlorinated biphenyl; ROPME, Regional Organization for the Protection of the Marine Environment; RSA, ROPME sea area; T3, triiodothyronine (3,5,3-triiodo-L-thyroinine); T4, thyroxine (3,5,3,5-tetraiodo-L-thyronine); TSH, thyroid-stimulating hormone

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affect 5′-iodothyronine deiodinases (enzymes that convert T4 to T3 in target tissues). 3. PAHs may attach to thyroid hormone binding proteins in the blood stream [8].

Benzo[a]Pyrene (BaP) is an ubiquitous PAH that could be considered as one of the most mutagenic and carcinogenic pollutants. It has been shown to be a CYPlA inducer. BaP resulted from incomplete combustion of fossil fuels and identified in ambient air, surface water, drinking water, and wastewater, and in char-broiled foods (I.A.R.C. 1983). BaP is primarily released to the air and removed from the atmosphere by photochemical oxidation and dry deposition on land or water. The main route of excretion is hepatobiliary followed by bowel elimination. BaP has evoked much interest due to its carcinogenic properties [8]. Existence of detergents or liquid hydrocarbons in the water bodies, simplify BaP solution in the sewage and surface water leading to its infiltration to the plant and animal cells [9]. BaP entrance to the organism, could priming a chain of oxidative processes mediated by the mixed-function oxygenase (MFO) system (phase I). By-products of the first phase may affected by conjugative enzymes (phase II), which will make them more readily excretable. BaP would cause immune changes in fish such as those observed in mammals.

The objective of the present study was to evaluate the effects of BaP on the tissue structure of thyroid gland and plasma levels of T3, T4 and TSH in the abu mullet (*L. abu*) under laboratory conditions. *L. abu* was selected due to its commercial importance in the ROPME (Regional Organization for the Protection of the Marine Environment) Sea Area. BaP was used in the present study because of its endocrine disruptive properties.

## 2. Material and methods

Benzo [a] pyrene was obtained from Aldrich (USA), with a purity of greater than 97%. All other chemicals were of analytical grade and were obtained from Merck (Germany). Hanks balanced salt solution (HBSS) was bought from Sigma (USA).

The range finding tests were required to conduct before running the experiment, to determine the adequate concentrations. Based on the primary range finding experiments, a 14 day BaP LC50 of 48 mg/kg was determined and then high nominal concentration and two low nominal concentrations were set at 25 mg/kg and 2 and 10 mg/kg, respectively. BaP solutions were injected with nominal amounts of 2, 10 and 25 mg/kg-bw in 1 mL coconut oil into the peritoneal cavity.

500 *L. abu* (150 ± 7.9gr mean body weight and 15.8 ± 0.2 cm mean body length) were collected from Bahrakan Creek located at the north west of the Persian Gulf during October 2011. To study the effects of BaP, 300 *L. abu* were randomly placed in fifteen 300 L tanks containing 200L running, UV-treated, aerated seawater and 20 *L. abu* were maintained in each tank. Firstly, fish were adapted to the experimental condition for 10 days. The tanks were then divided into five experimental groups, with each group run in triplicate: (1) control, (2) solvent (tested only with coconut oil), (3) low concentration of BaP (2 mg/kg-bw), (4) medium concentration of BaP (10 mg/kg-bw), and (5) high concentration of BaP (25 mg/kg-bw). Fish were captured with a hand-held net and were injected intraperitoneally (IP) with BaP (2, 10 and 25 mg/kg-bw) in I ml coconut oil. The control fish received no injection or handling. The fish were kept under the experimental conditions for 2 weeks. 70% of the water in the tanks was renewed each day. Fish were not fed during the experiment [10]. During the experiment the chemical characteristics of water were as follows: Water temperature 26 ± 1 °C, environmental temperature 29 ± 1 °C, pH 8.8 ± 0.1 and salinity 50 ± 1 ppt.

5 fish per group were sampled at days 1, 2, 4, 7 and 14 following the injection. The fish were euthanized with 2- phenoxy ethanol (0.35 mL/l) and the blood samples were then collected from the caudal vein into heparinized syringes. The blood samples were centrifuged for 10 min and plasma was separated and frozen at −20 °C for further thyroid hormones analysis. Also, the tissue samples were taken from the fish muscle to measure BaP concentration in the fish muscle. Then the muscle samples were stored in aluminum foil at −20 °C. For histological study of thyroid gland, the fish jaws were cut to expose pharyngeal region, and all tissues between the gills were fixed in Bouin's solution for 72 h.

Extraction of BaP was carried out using Moopam [11] method. Samples of the fish muscles were moved to pre-washed glass jars to freeze-dry using freeze drier apparatus (Zirbus, Germany) for 4–5 days. Then the samples were weighed again to verify dry/wet ratio and powdered into a porcelain mortar. The BaP extraction procedure was performed using soxhlet apparatus (a soxhlet extractor). For hydrocarbon measurement, the extract was loaded onto a silica/alumina column. The silica and alumina were first soxhlet extracted with the mixture of *n*-hexane and di-chloromethane for 8 h and then activated at 200 °C within 4 h. They partially deactivated with 5% water. In the end, 1 g sodium sulfate was added to the surface of column to prevent disturbance of the top layer when decanting the solvent. Finally two fractions ($F_1$ and $F_2$) were separated. $F_2$ was concentrated to about 10–15 mL using rotary evaporator and then clean and dried $F_2$ was concentrated to about 1-1.5 mL under moderate nitrogen flow [11]. To analyze the BaP using High-Performance Liquid Chromatography (HPLC), samples were dried under a pure nitrogen flow, and 20 µL acetonitrile was added to the concentrated $F_2$. $F_2$ then was injected into the High-Performance Liquid Chromatography (HPLC) System (Knaeur, Germany) [11] outfitted with double piston pump(K-1001), UV detector (K-2600) and reverse phase column (25 cm length and 4.6 mm internal diagonal) (Eurospher 100-5$C_{18}$). The limit of detection of HPLC System was higher than 0.07 mg/kg-bw BaP.

The *L. abu* were dissected to expose the internal organs and the jaws were cut to expose pharyngeal region. All the pharyngeal tissues were fixed in Bouin's fixative for 72 h and then stored in 70% ethanol. Tissues were dehydrated through an ethanol series and embedded in paraffin. The samples were then sectioned at 5–6 µm and were stained with hematoxylin and eosin (H&E). The height of the thyroid epithelium and diagonal of thyroid follicles were measured in a total of 15 follicles per fish. Measurements were made at four points within each follicle at 90◦ to one another.

The concentration of thyroid hormones (T3 and T4) was measured in the plasma samples by radioimmunoassays (RIAs) method using commercial T3 and T4 RIA kits (Immunotech, Beckman Culture Company, France), as previously described by Morgado et al. [12]. TSH was also measured using radioimmunoassay method according to Forest et al. [13].

Results are reported as mean ± SE. Analysis was carried out using One-way ANOVA with SPSS 16.0 software. The data were processed by post hoc test and $P \leq 0.05$ was accepted as statistically significant.

## 3. Results

No significant difference was observed in all studied parameters between the control and solvent control groups.

BaP concentration in the muscle of control groups were undetectable across all sampling days and then considered 0 (Fig. 1). BaP concentration was also undetectable in all treatments on the first sampling day. BaP accumulation in the muscle samples from all treatments during the experiment is presented in Fig. 1. BaP concentration in the muscle of BaP- treated fish reached a maximum level after 4 days (0.36-14.37 mg/kg-bw). It then decreased until the end of the experiment. There were significant differences in BaP accumulation between treatments in both sampling days (days 4 and 7) ($P < 0.05$; Fig. 1).

According to the results, the thyroid gland of *L. abu* is not encapsulated. It consisted of follicles distributed all over the pharyngeal region along the dorsal surface of ventral aorta and bronchial arteries. The wall of spherical thyroid follicles in the control groups was lined with one layer of cuboidal epithelial cell that surrounded a central lumen full of colloid fluid. Interstitial connective tissue existed among