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Changes in Steroid Hormones Levels of Olive Flounder, *Paralichthys olivaceus* Exposed to Phenanthrene

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Abstract - Phenanthrene, one of Polycyclic aromatic hydrocarbons with three aromatic rings, is a ubiquitous contaminant in the environment. Phenanthrene has been identified in ambient air, drinking water and sediment. We examined the effect of phenanthrene on steroid hormones level of olive flounder, *Paralichthys olivaceus*. Plasma testosterone level was increased significantly in fish exposed to phenanthrene($\geq$1.0 μM) at 4th week. However, there was no significant changes of estradiol-17β concentration in fish exposed to phenanthrene. The physiological variation in phenanthrene exposed fish was a dramatic increase in plasma cortisol level. It is concluded that chronic exposure of phenanthrene can induce increase of plasma testosterone levels and elevate the plasma cortisol level in flounder, *Paralichthys olivaceus*.

Key words : Blood, Steroid hormone, *Paralichthys olivaceus*, Phenanthrene

INTRODUCTION

The decrease of reproductive capability in aquatic organisms may be considered as one of a number of damaging effects of persistent pollutants released by man. A number of xenobiotics with widespread distribution in the environment have been reported to have endocrine activity which may affect reproduction and thus may threaten the existence of susceptible species (Peterson *et al.* 1993; White *et al.* 1994). The possibility that aquatic pollutants present in the environment may mimic hormones, causing deleterious physiological effects to aquatic biota, has been given considerable attention. Polycyclic aromatic hydrocarbons (PAHs) comprise of hundreds of structures and are carcinogenic and genotoxic in animal species.

Some PAHs have also been found to have an adverse effect on the process through which maturing oocytes in the ovary of fish accumulate yolk (e.g. vitellogenesis) (Nicolas 1999). The endocrine system plays a central role in fish stress mechanisms. Therefore, alterations in specific hormonal functions and consequent biochemical effects may be constituted important stress biomarkers. Convincing evidence has been reported in the freshwater and marine environment that a number of naturally occurring and synthetic chemicals lead to effects on wildlife (Harries *et al.* 1997; Jobling *et al.* 1998). Recent reviews suggest that PAHs can have a significant anti-oestrogenic effect by binding to the arylhydrocarbon (Ah) -receptor (Navas and Segner 2000), or a mild oestrogenic effect by binding to the oestrogen receptor (Nicolas 1999).

PAHs are major pollutants of the aquatic environ-

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ments (Meador *et al.* 1995). Nevertheless, only a few studies have focused on the disruptive potential of PAHs over the endocrine regulation of fish reproduction (Singh 1989; Thomas 1990). Moreover, the effects of chronic exposures to a single PAH, phenanthrene, on change in steroid hormones of fish are unknown. Therefore, in this study we examined the effect of phenanthrene, a single PAH, on plasma levels of steroid hormone in flounder, *Paralichthys olivaceus*.

MATERIALS AND METHODS

Experimental animals

Juvenile olive flounder (*Paralichthys olivaceus*), 51± 4.3 g mean body weight, were obtained from a commercial dealer, transferred to a maintenance tank and kept at 20°C. Fish were acclimated to the laboratory condition for several weeks prior to experiment. A 12 h light/dark cycle was used to minimize any influence on any physiological disturbance.

Exposure conditions

Test chamber (glass aquaria, 120 L capacity) were filled with 80 L of sea water. Water characteristics, measured by the method described in APHA (1995), were as follows: pH, 8.03±0.4; temperature, 20±1°C; salinity, 31.8±0.7 and dissolved oxygen 7.5-7.8 mg L^{-1}. Phenanthrene was initially dissolved in ethanol (Sigma Chemical, St. Louis, MO) to obtain initial stock solution. Prior to introduction of fish to test aquaria, solutions of phenanthrene (>96% purity, Sigma Chemical, St. Louis, MO) working solution were mixed with filtered seawater to attain a nominal concentration of 0.5, 1.0 and 2.0 μM. The working solutions were stirred 8-16 h prior to usage. In each test chamber, a group of ten fish was exposed for a period of 2 and 4 weeks under semi-static conditions with airstones to maintain dissolved oxygen levels greater than 75% saturation. The phenanthrene dose was renewed every second day along with the seawater.

Blood samples

At the end of each period (at 2nd and 4th week) fish were anesthetized in buffered 3-aminobenzoic acid ethyl ester methanesulfonate (Sigma Chemical, St. Louis, MO) and blood samples were taken from caudal vein using heparinized syringes. Anesthesia, measurement and blood withdrawal generally took less than 3 min/fish with minimum disturbance. Plasma was collected after centrifugation 3,000 g for 5 min at 4°C (MIKRO 22R, Hettich, Germany), divided into 200 μl aliquots and then stored at −80°C until analyzed.

Radioimmunoassay (RIA)

Plasma cortisol, estradiol-17β (E_2) and testosterone (T) levels were measured by radioimmunoassay (RIA), in the Fisheries Bioscience Information Center (NFRDI), according to the methods of Lou *et al.* (1984). Rabbit anti-Cortisol-3-CMO-BSA, Rabbit anti-E_2-6-CMO-BSA and Rabbit anti-T-3(E)-CMO-BSA sera were purchased from Cosmo-Bio Co. Ltd. (Tokyo, Japan). Nonradioactive steroids to be used as standards were purchased from Steraloids Inc. (Wilton, NH, USA). Radiolabeled steroids ([1, 2, 6, 7-^{3}H]-Cortisol, [2, 4, 6, 7-^{3}H]-E_2 and [1, 2, 6, 7-^{3}H]-T were purchased from Amersham Life Science (England).

The sensitivities of the assay were 22.5 pg ml^{-1}, 12.5 pg ml^{-1} and 10 pg ml^{-1} for cortisol, E_2 and T, respectively. The intra- and inter-assay coefficients of variations at the 50% binding were 2.8 (n = 5) and 8.1% (n = 6) for cortisol, 3.4 (n = 5) and 11.5% (n = 6) for E_2, 2.3 (n = 5) and 12.5% (n = 6) for T, respectively. The cortisol antibody cross-reacted with 11-deoxycortisol (16.3%), cortisone (2.9%) and corticosterone (3.3%). Cross-reactivities of other steroids in the cortisol assay were less than 0.01%. The E_2 antibody cross-reacted with estron (0.5%), estriol (0.9%) and T (0.01%). All other steroids tested showed less than 0.01%. The T antibody cross-reacted with dihydrotestosterone (2.7%), androsten-3, 17-dione (0.5%), 11-ketotestosterone (0.5%) and androstenedione (0.35%). All other steroids tested showed less than 0.001%.

Statistical analysis

Statistical analyses were performed using SPSS/PC+ statistical package. Significant differences between groups were determined using the Student's *t*-test for

two groups. Significance level was established at $P < 0.05$.

RESULTS AND DISCUSSION

The flounder (*Paralichthys olivaceus*) is a pleuronectiform species living in the sediment of coastal and estuarine areas, and it is particularly vulnerable to sediment-associated chemical pollution as a bottom-feeding fish. These ecological characteristics together with the relative ease of sampling on the field and the observed favorable adaptation to laboratory conditions makes flounder a good sentinel species to monitor pollution by PAHs (Monteiro *et al.* 2000).

In this study, plasma testosterone levels were significantly elevated in fish exposed to 1.0 μM and 2.0 μM phenanthrene, respectively, with a peak increase of 356 % over control fish occurring at the 2.0 μM concentration at 4th weeks (Fig. 1, B). These result can be explained when testosterone production was stimulated by activation of the cyclic adenosine monophosphate (cAMP)/protein kinase A (PKA) signal transduction pathway provides several sites where PAHs act, resulting in significant potentiations in testosterone production (Evanson and van Der Kraak. 2001).

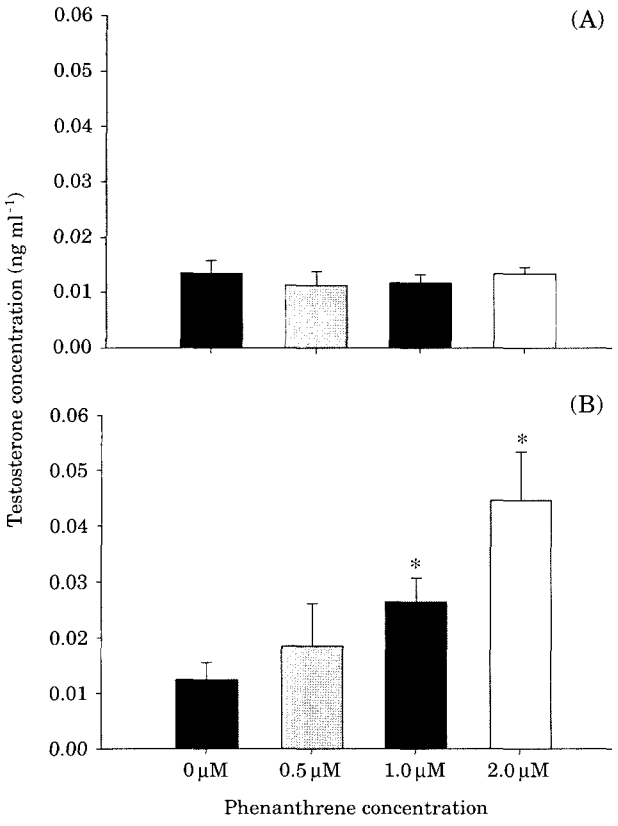

Fig. 1. Changes of plasma testosterone level in *Paralichthys olivaceus* exposed to various concentrations of phenanthrene for 4 weeks (2nd week, A; 4th week, B). Each column represents the mean ± S.E. (n = 4). *Significantly different from control ($P < 0.05$).

The numerous studies that include plasma estradiol-17β (E_2) concentrations as a variable have demonstrated with few exceptions the reduction of circulating E_2 in the presence of organic contaminants both in the field (Johnson *et al.* 1993; Casillas *et al.* 1991), and in the laboratory exposures (Snowberger and Stegeman 1987; Thomas and Budiantara 1995). The understanding of the processes by which organic contaminants affect estradiol levels is not clear. It has been established that the drop in plasma estradiol in the presence of PAHs, when it occurs, is associated with an increase of the excretion of E_2 metabolites in the bile (Hansson and Rafter 1983; Johnson *et al.* 1993). The mechanism of this enhanced excretion of E_2 metabolites remains under debate. Hansson and Rafter (1983) and Sivarajah *et al.* (1978) claimed that the induction of phase I (oxidative) and:or II (conjugative) detoxifying enzyme activities associated with PAH exposure increases the catabolism and excretion of estradiol into the bile (as steroids are a substrate for some forms of P450 enzymatic activities). However, during vitellogenesis the thecal cell layer, under the influence of gonadotropins, secretes the aromatizable androgen testosterone which is then converted to E_2 by aromatase in the granulosa cell layer. The ability of the follicle cell layers to secrete E_2 depends upon the activity of the enzyme aromatase, which in turn varies with the developmental stage of the follicle (Young *et al.* 1983). However, in this study, plasma estradiol concentration did not differ significantly between fish from each treatment group (Fig, 2, $P > 0.05$).

In this study, the exposure of the flounders to phenanthrene revealed plasma cortisol concentration was stable, in particular for 2 week exposure (Fig. 3, A), whereas plasma cortisol level markedly affected by the phenanthrene exposure at 4th week (Fig. 3, B). As seen in Fig. 3 (B), plasma cortisol levels were increased (by

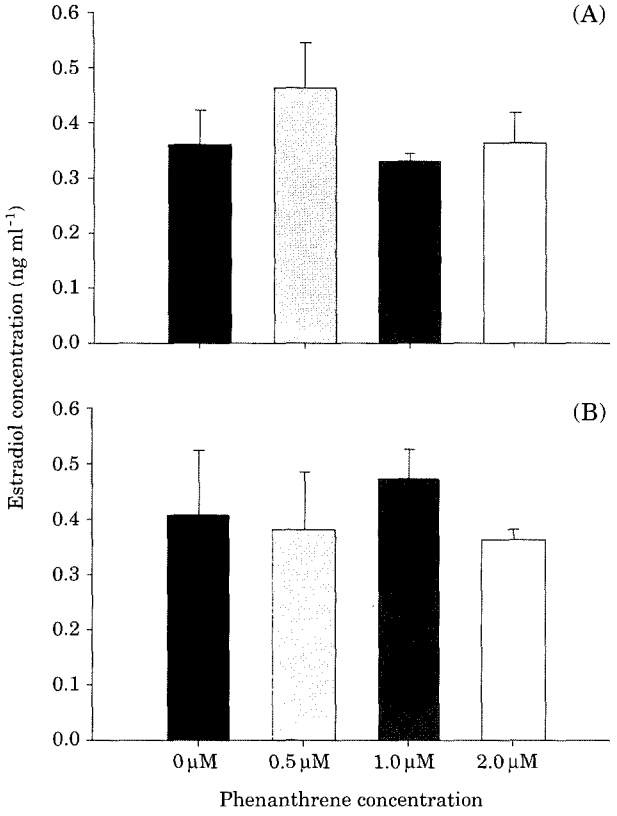

Fig. 2. Changes of plasma estradiol-17β level in *Paralichthys olivaceus* exposed to various concentrations of phenanthrene for 4 weeks (2nd week, A; 4th week, B). Each column represents the mean±S.E. (n = 4).

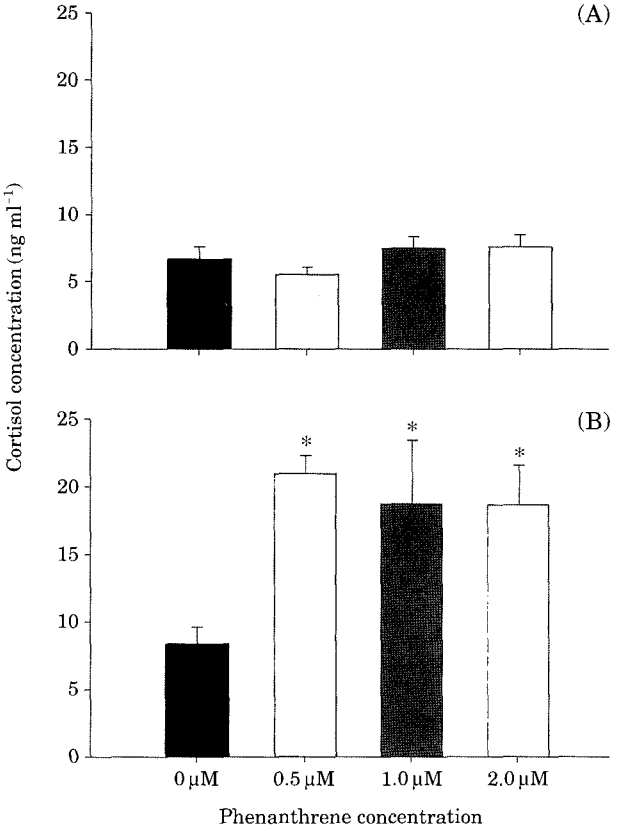

Fig. 3. Changes of plasma cortisol level in *Paralichthys olivaceus* exposed to various concentrations of phenanthrene for 4 weeks (2nd week, A; 4th week, B). Each column represents the mean±S.E. (n = 4). *Significantly different from control ($P<0.05$).

2.2-2.5 fold) in all treatment flounders at 4th week ($P<0.01$). Cortisol is the main steroid hormone produced in the interrenal tissue of teleost fish, the tissue homologous to the mammalian adrenal (Lacroix and Hontela 2001), has a major role in the physiological response to stressors. Therefore, exposures to xenobiotics alter interrenal function and cortisol secretion (Hontela 1997). Previous investigations demonstrated that acute exposures to heavy metals (Hontela *et al.* 1996), components of pulp and paper effluents (Kennedy *et al.* 1995) as well as capture and handling (Vijayan *et al.* 1997), elevate plasma cortisol. Changes in plasma cortisol concentrations (Santos and Pacheco 1996), were recognized as general stress biological indicators in fish. The current data corroborate the conclusions of Hontela *et al.* (1995), who stated that the impaired ability to elevate blood cortisol may constitute a biomarker of toxic stress in health assessment. In summary, significant increase of plasma testosterone and cortisol concentrations have been detected in phenanthrene exposed olive flounder. This result suggests that endocrine system might be affected by phenanthrene exposure. Further study should be made to evaluate effect of phenanthrene on aquatic orgasnisms.

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