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Combined effect of tributyltin and benzo[a] pyrene on the levels of sex hormone and vitellogenin in female *Sebastiscus marmoratus*

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Abstract: Tributyltin(TBT), an organometal used as an antifouling biocide, has been reported to induce masculinization of fish. Benzo [a]pyrene (BaP), a widespread carcinogenic polycyclic aromatic hydrocarbon, has been reported that its microsomal metabolites can produce an estrogenic response when tested *in vitro*. This study was therefore designed to examine the potential *in vivo* influence of TBT, BaP and their mixture on sex hormone levels in serum of *Sebastiscus marmoratus*, which were given 2 separate intraperitoneally (ip) injections(a single injection every 7 d) of TBT(0.5, 1, 5 and 10 mg/kg), BaP(0.5, 1, 5 and 10 mg/kg), or both in combination(0.5, 1, 5 and 10 mg/kg); control fish received olive oil vehicle only. Six days after the 2nd injection, serum samples were collected and analyzed for sex hormone levels and alkali labile protein phosphorus (ALPP), which is related to the yolk precursor protein vitellogenin. The pollutants at all doses significantly reduced serum testosterone, estradiol and ALPP content after 2 injections compared with the corresponding controls. The reduction of the estradiol levels should be response for the decrease of the vitellogenin levels. The results in the present study suggested that aromatase seems not the major target acted by TBT and BaP in fish. This study demonstrated that TBT or BaP exposure both inhibit the reproductive potential in female *Sebastiscus marmoratus*. Combined effect of TBT and BaP on the serum testosterone, estradiol and ALPP was not antagonism from the anticipation.
Keywords: tributyltin; benzo[a]pyrene; estosterone; 17β-estradiol; vitellogenin; *Sebastiscus marmoratus*

Introduction

Organotin compounds, particularly tributyltin (TBT) are widely used as biocides in a variety of consumer and industrial products. Among them, antifouling paints are the most important contributors of organotin compounds to the aquatic environment, where they are known to cause deleterious effects to non-target organisms(Alzieu, 1991). Besides the acute toxicity of TBT, some studies show that TBT has embryotoxicity (Marin *et al.*, 2000), genotoxicity(Jha *et al.*, 2000) and can produce endocrine disruptive effects. Potential reproductive impairment has been reported at a concentration of TBT in water as low as 1 ng/L as Sn, viz. the induction of imposex in the dogwhelk(*Nucella lapillus*)(Gibbs *et al.*, 1988). It was reported that TBT potentially induced masculinization, to the extent of complete sex reversal, of genetically female Japanese flounder (*Paralichthys olivaceus*)(Shimasaki *et al.*, 2003). However, limited information concerning the mechanism of action of organotin compounds in fish is available.

Polycyclic aromatic hydrocarbons (PAHs) are present worldwide due to anthropogenic activity. PAHs have been demonstrated to be mutagenic and carcinogenic precursors (McElroy *et al.*, 1991; Maccubin, 1994), as well as to have a deleterious effect on the vitellogenesis of fish from feral populations as well as in laboratory experiments (Nicolas, 1999). The reported effects include reduction in circulating hormones and plasma vitellogenin, estrogenic and antiestrogenic effects, retardation of oocyte maturation and reduction of reproductive success (Nicolas, 1999). Benzo[a]pyrene (BaP), a representative PAH, was found to have no direct estrogenic effect (Thomas and Smith, 1993). However, microsomal metabolites of BaP did produce an estrogenic response when tested *in vitro* (Bulger *et al.*, 1985).

TBT and BaP are widespread pollutants that occur simultaneously in many aquatic environments under both dissolved and particulate forms. Because animals inhabiting polluted areas are exposed to mixtures of chemicals, additive, synergistic, or antagonistic effects of endocrine-disrupting chemicals are possible, Padrós *et al.* (2003) reported combined effect of TBT and BaP on a suite of biomarkers, but there are not any previous study reporting the combined effect of the mixture of TBT and BaP on the level of sex hormone and vitellogenin in fish. The result in previous study showed that the level of BaP in surface sediment was 0.13—0.69 μg/g (Heit *et al.*, 1981). Many studies show sediment concentrations of TBT in the hundreds of ng/g to low μg/g levels(Wade *et al.*, 1990; Langston and Burt, 1991; Dowson *et al.*, 1993; Espourteille *et al.*, 1993; Krone *et al.*, 1996; Fent, 1996). There would be an equal level between TBT and BaP in realistic marine environment. So in the present study the concentration ratio of the mixture of TBT and BaP was designed to 1 ∶ 1. The selection

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of the test species was based on its availability, commercial importance (fisheries and aquaculture), and distribution throughout the coastal waters of China.

1 Materials and methods

1.1 Chemicals

Tributyltin chloride was obtained from Fluka AG, Switzerland, with a purity of greater than 97%. BaP was obtained from Sigma Chemical Co.(St Louis, MO, USA). All other chemicals were of analytical grades and were obtained from commercial sources.

1.2 Experimental species and estimation of LD_{50}

Female cuvier(*Sebastiscus marmoratus*) weighing 25—50 g, were captured from a pristine coast in Xiamen city, Fujian Province, China. Before the exposure experiment, the fish were acclimated in tanks containing 60 L of aerated sand-filtered seawater, under flow-through conditions with natural photoperiod for 7 d.

Short-term static toxicity tests were performed. Median lethal doses LD_{50} were estimated by the Bliss method (1938). The 95% confidence limits (UCL, LCL) of the LD_{50} values were determined according to Sokal and Rohlf (1995). A series of degressive doses with a ratio of 1.2, from 48, 40, 33.33, 27.78 to 23.15 mgTBT/kg body weight, were employed for intraperitoneally(ip) injection. A single ip injection of BaP at 200 mg/kg bw had no effect upon the fish survival for up to 7 d post-injection.

1.3 Exposure conditions

Ten female fish per dose group received two separate ip injection (7 d apart) of TBT (0.5, 1, 5 and 10 mg/kg), BaP (0.5, 1, 5 and 10 mg/kg) or both in combination (0.5, 1, 5 and 10 mg/kg); control fish received an equal volume of the olive oil (injection volume in each case 1 ml/kg). Injections were repeated every 7 d. The fish were fed with fresh clam *Meretrix meretrix* flesh for 2 h to satiation before replacing the water, the clam was collected from a pristine coast and previously maintained in aerated marine water for up to 7 d. This process was repeated every other day until the third day before sampling. The water temperature was maintained at 14 ±2℃ and salinity 22—24.

1.4 Sample collection

Six female fishes were randomly sampled from each treatment group for 6 d after the 2nd injection. Sampling was between 8:00 a.m. and 11:00 a.m. in order to minimize diurnal variability. 1 ml of blood was collected from the heart using heparinized syringes. Blood samples were left at 25℃ and allowed to clot. The clotted blood was then centrifuged at 1000 ×g for 20 min. The supernatant fraction was collected as serum and stored at -80℃ until they were analyzed. The fish of 10 mg TBT/kg group was dead before sampled.

1.5 Sex hormone analysis

Radioimmunoassay of 17β-estradiol and testosterone was done in serum of fish using a commercial radio-immunoassay (RIA) kit (Furui Biological Engineering Co, Beijing, China.), following the instruction of the manufacturer.

1.6 Alkali labile protein phosphorus (ALPP) estimation

Vitellogenin was measured as alkali labile protein phosphorus following the method of Deeley *et al.* (1975) as modified by Dasmahapatra *et al.* (1983). Free phosphates of vitellogenin were estimated by the method of Chen *et al.*(1956).

1.7 Data processing

Values were presented as mean ± *SD* of untransformed data in the figures. The data were processed by Student-Newman-Keuls multiple comparison test and $P<0.05$ was accepted as significant. Two-way ANOVA analysis using SPSS 10.0 software was employed to determine whether there is an interaction between TBT and BaP.

2 Results

2.1 LD_{50} of TBT or BaP to the fish

In this study, exposure of the fish to levels of BaP as high as 200 mg/kg bw for up to 7 d post-exposure had no effect upon the fish survival and no noticeable effects (compared to the vehicle control) upon behavior. This observation coincides with data obtained in fish studies that demonstrated BaP not to be acutely toxic(Carlson *et al.*, 2002).

The (24-h) LD_{50} value for TBT on *Sebastiscus marmoratus* was 43.02 mg/kg. The 95% confidence limits (UCL, LCL) of the (24-h) LD_{50} values were 42.97—43.07 mg/kg. The fish exposed to 10 mg TBT/kg were all dead at the day 6 after the 2nd injection.

2.2 Effect of TBT, BaP and their mixture on testosterone level in the serum

The two chemicals and their mixture at all doses significantly reduced serum testosterone content compared with the corresponding controls(Fig.1). The levels of the serum testosterone in the fish exposed to TBT were reduced in dose-dependent manner. The serum testosterone contents were significantly reduced after exposed to the mixture, while the reduction extent of the testosterone level was decreased in dose-dependent manner as increasing of the dose of the mixture. Treatment with 5 mg TBT/kg, 5 mgBaP/kg and 0.5 mg(TBT+BaP)/kg resulted in the highest decrease of the testosterone level in each treatment by 53%, 57% and 53% respectively. There was not a significant difference between TBT+BaP groups and TBT or BaP groups according to two-way ANOVA analysis.

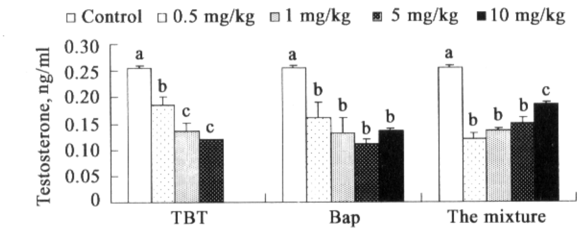

Fig.1 The serum testosterone content in female *Sebastiscus marmoratus* for 6 d after the 2nd injected with TBT, BaP and their mixture at 1 ∶ 1 concentration ratio(n=6)
Different superscript letters indicate there is a significant difference between the two groups

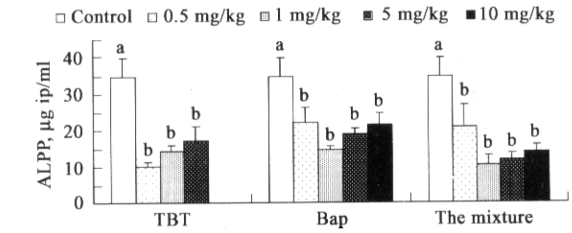

Fig.3 The serum alkali labile protein phosphorus (ALPP) content in female *Sebastiscus marmoratus* for 6 d after the 2nd injected with TBT, BaP and their mixture at 1 ∶ 1 concentration ratio(n=6)
Different superscript letters indicate there is a significant difference between the two groups

2.3 Effect of TBT, BaP and their mixture on 17β-estradiol level in the serum

The two chemicals and their mixture at all doses significantly reduced the serum 17β-estradiol content compared with the corresponding controls (Fig.2). Treatment with BaP alone significantly decreased the levels of the serum 17β-estradiol in dose-dependent manner. Treatment with 5 mgTBT/kg, 10 mgBaP/kg and 10 mg (TBT+BaP)/kg resulted in the highest decrease of 17β-estradiol level in each treatment by 38%, 42% and 56% respectively. There was not a significant difference between TBT+BaP groups and TBT or BaP groups according to two-way ANOVA analysis.

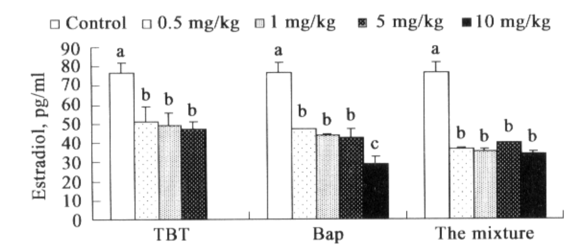

Fig.2 The serum 17β-estradiol content in female *Sebastiscus marmoratus* for 6 d after the 2nd injected with TBT, BaP and their mixture at 1 ∶ 1 concentration ratio(n=6)
Different superscript letters indicate there is a significant difference between the two groups

2.4 Effect of TBT, BaP and their mixture on the alkali labile protein phosphorus (ALPP) content of the serum

The two chemicals and their mixture at all doses significantly reduced serum ALPP content compared with the corresponding controls (Fig.3). Treatment with 0.5 mg TBT/kg, 1 mg BaP/kg and 1 mg (TBT+BaP)/kg resulted in the highest decrease of ALPP level in each treatment by 71%, 58% and 70% respectively. There was not a significant difference between TBT+BaP groups and TBT or BaP groups according to two-way ANOVA analysis.

3 Discussion

The fish were fed an artificial diet containing tributyltin oxide (TBTO) at concentrations of 0.1 and 1.0 μg/g diet from 35 to 100 d after hatching. The ratio of sex-reversed males significantly increased to 25.7% of the flounder fed the 0.1 μg/g diet and to 31.1% of those fed the 1.0 μg/g diet compared with the control(2.2%)(Shimasaki *et al.*, 2003). As reported by Nakayama, the administration of TBT(1 μg/g body weight) decreased fertilization success in paired medaka (Nakayama *et al.*, 2004). However, there are few reports about the changes of sex hormones level by TBT in fish. The sex steroid hormones in fish play a key role in gonadal maturation, vitellogenesis and oocyte growth. Although certain pollutant in certain concentration would not do obvious harm to the embryo growth, hatch, it can result in physiological and biochemical response on the early stage of the reproductive system in molecular level (Singh, 1993; Thomas, 1990), thus it would influence the reproductive function of fish finally. In female fish, testosterone is the estradiol precursor, aromatase catalyses the conversion of testosterone to 17β-estradiol. TBT has been found to elevate testosterone levels in female gastropods (Spooner *et al.*, 1991). As discussed by Matthiessen and Gibbs (1998), the increase in testosterone titers resulted from TBT exposure is generally considered to be due to the direct inhibition of aromatase by TBT. The present study showed that TBT reduced the testosterone level in the ovary. This effect on ovarian testosterone level in the fish was different with that in gastropods. According to the aromatase inhibition hypothesis, TBT prevents the conversion of testosterone to 17β-estradiol. If the aromatase enzyme is the target of TBT, then a stoichiometric decrease in 17β-estradiol should occur commensurate with increases in testosterone (Gooding and LeBlanc, 2001). In the present study, the testosterone and 17β-estradiol levels in the ovary were both decreased by TBT treatment. These suggests that the effect of TBT on sex hormone levels in the fish might not be principally due to direct inhibition of aromatase, but possibly to an indirect effect such as downregulation. Animal studies have shown that TBT can lead to permanent brain damage (Sloviter *et al.*, 1986). The mechanism of TBT act on the fish's reproductive function is deserving of further study.

One of the roles of estradiol is to stimulate the liver to synthesize vitellogenin (MacKay and Lazier, 1993). Reduction of serum 17β-estradiol by environment pollutants like aromatic hydrocarbons, ketoconazole, polychlorinated biphenyls etc., with concomitant reproductive abnormalities have been documented (Feldman, 1986; Singh and Singh, 1987; Sivarajah *et al.*, 1978a, b; Anderson *et al.*, 1996a; Hutz *et al.*, 1999), although the exact reason is still under investigation. Thomas and Budiantara (1995) reported a decline in plasma concentrations of estradiol and testosterone after the exposure of Atlantic croaker (*Micropogonias undulatus*) to naphthalene and diesel oil. There are some reports that vitellogenin levels were reduced in fish exposed to environmental pollutants(Casillas *et al.*, 1991; Pereira *et al.*, 1992). The results of other laboratory exposures also showed a decrease of the level of vitellogenin in the serum of rainbow trout (*Oncorhynchus mykiss*) exposed to PAHs (Anderson *et al.*, 1996b). In the present study we observed that BaP and TBT reduced the serum testosterone and 17β-estradiol levels. The reduction of the 17β-estradiol levels should be response for the decrease of the vitellogenin levels.

Nakayama *et al.*(2004) administered TBT(1 μg /g body weight daily), PCBs(1 μg /g body weight daily), or both to medaka (*Oryzias latipes*) for 3 weeks, and assessed reproductive success during 3 weeks and the sexual behavior of male medaka after the exposure period. The results indicate that TBT, but not PCBs, affects sexual behavior and reproduction in medaka. However, there are not any previous study reporting the combined effect of the mixture of TBT and BaP on the level of serum testosterone, 17β-estradiol and vitellogenin in fish. As the inhibitor of aromatase, TBT was found to elevate testosterone levels in female gastropods and can causes imposex in these organisms (Spooner *et al.*, 1991). BaP was found to have estrogenic effect *in vitro* (Bulger *et al.*, 1985). It was speculated that the combined effect of TBT and BaP may be antagonism, but the results in the present study differ from the anticipation. The result in the present study would be conflict with the reporter that microsomal metabolites of BaP did produce an estrogenic response when tested *in vitro*(Bulger *et al.*, 1985). Recent results suggest that PAHs can have a significant antiestrogenic effect through an Ah-receptor mediated mechanism (Anderson *et al.*, 1996b), to which our results appear to support. Whether BaP has an androgenic effect on the fish *in vivo* is deserving of further study.

Finally, it should be emphasized that this short-term study was conducted using injection of high doses of pollutants and focused on combined effect. Therefore, a necessary next step will be to investigate how long the term of exposure to environmentally relevant concentrations of BaP and TBT affects reproductive potential of fish.

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