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Contaminations by Endocrine Disrupting Chemicals in Coastal Waters of the East China Sea

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Abstract—There are many chemicals that have estrogen like activity in natural envrionment, and are well known as endocrine disrupting chemicals (EDCs). These chemicals disrupt the endocrine system of wildlife and induce the abnormality of reproductive phenomena. Recently, many scientists reported that EDCs in the aquatic environment interfere with the reproductive process of wild fish. However, there are few information about that in Asian countries. We conducted the survey of contamination by environmental estrogens (EEs) of coastal areas in Korea, People's Republic of China, and Japan using the wild grey mullet. The grey mullet were collected from five sites (Ansan, Jeju, Yeosu, Tongyeong, and Busan) in Korea, three sites (Zhonshan, Luchaogang, and Pudong) in P.R. China, and three sites (Nagasaki, Omuta, and Fukuoka) in Japan. Contamination by EEs was determined by measuring vitellogenin (VTG) levels in serum and identifying gonadal abnormalities histologically (i.e., testis-ova). In four sites in Korea (Ansan, Yeosu, Tongyeong, and Busan), one site (Luchaogang) in P.R. China, and two sites in Japan (Nagasaki and Fukuoka), serum VTG in immature and male grey mullet was detected at levels greater than 1.0 μg/mL, which is considered to be an abnormal level. Testis-ova was detected in many sites of all countries, except for Yeosu and Jeju Island in Korea. These results suggest that the coastal areas of East China Sea are contaminated with EDCs.

Keywords: endocrine disrupting chemicals, environmental estrogen, grey mullet, vitellogenin, testis-ova, environmental pollution

1. INTRODUCTION

Endocrine disrupting chemicals (EDCs)

Endocrine disrupting chemicals (EDCs), which are exogenous natural substances (e.g. phytoestrogens) or man-made chemical compounds (e.g., synthetic steroids, alkylpheonols, phthalates, organochlorine pesticides, etc.), are well known to have similar properties to estrogenic hormone of wild animals including fish (Sumpter, 1995). These EDCs, which are called environmental estrogen (EEs), can be found in runoff water, domestic and industrial wastes that flow into rivers, lakes, and costal areas (Desbrow et al., 1998; Belfroid et al., 1999; Kolpin et al., 2002). The aquatic environment is the ultimate sink for the majority of these chemicals. Consequently, aquatic animals including fish are often exposed to these chemical compounds. Therefore, the impact of domestic and industrial sewage effluents on the endocrine system in fish has been demonstrated by many scientists. The EEs in the sewage effluents induce a feminization in wild male fish due to disruption of the endocrine system, and interfere with the reproductive process of wild fish by mimicking the action of an endogenous hormone (Jobling and Tyler, 2003; Robinson et al., 2003). In wild animals, it is know that the EEs also have effects on induction of precursor protein synthesis and gene expression of yolk protein (vitellognenin, VTG) in male (Jobling et al 1998; Tarrant et al., 2008), alterations of sex steroid synthesis (Folmar et al., 1996), and impediment of gonadal development and reproductive phenomena (Jobing et al., 1998). As described above, the EEs have a broad range of impact. However, the mechanism of EEs action in wild animals has not been clearly understood yet.

2. VITELLOGENIN: BIOMARKER FOR SURVEY OF EDCS

It is known that VTG is good biomarker for understanding the EDCs contaminations in wild animals (Sumpter and Jobling, 1995; Kime et al., 1999). VTG is precursor protein of yolk in oviparous animals, and is usually produced under endogenous estrogen stimulation in the liver of mature females, released into the blood, and taken in by growing oocytes (Fig. 1). As described above, ovarian estrogen has an important role in the regulation of vitellogenin synthesis and vitellogenesis in many teleosts. On the other hand, immature females and all males have no or low concentrations of VTG in the blood. However, VTG is induced by exposed to exogenous EEs in immature and male. Thus, we can know the existence or nonexistence of EEs in environmental waters by detection of VTG levels in immature and male animals. To evaluate the presence of EEs, methods to measure VTG have been developed for various species of fish (Nishi et al., 2002; Ohkubo et al., 2003a). VTG was made available for the survey of EDCs using the male flounder (*Pleuronectes yokohamae*) (Hashimoto et al., 2000), the male common goby (*Acanthogobius flavimanus*) (Ohkubo et al., 2003b), and the male grey mullet (*Mugil cephalus*) (Aoki et al., 2010) in the coastal waters and estuarine areas of Japan. In the survey, these fishes showed high levels of VTG in their serum or plasma. The male flounder (*Platichthys flesus*) lived in estuarine areas of the UK has been also found with elevated concentrations of VTG in their serum (Allen et al., 1999). Since it is

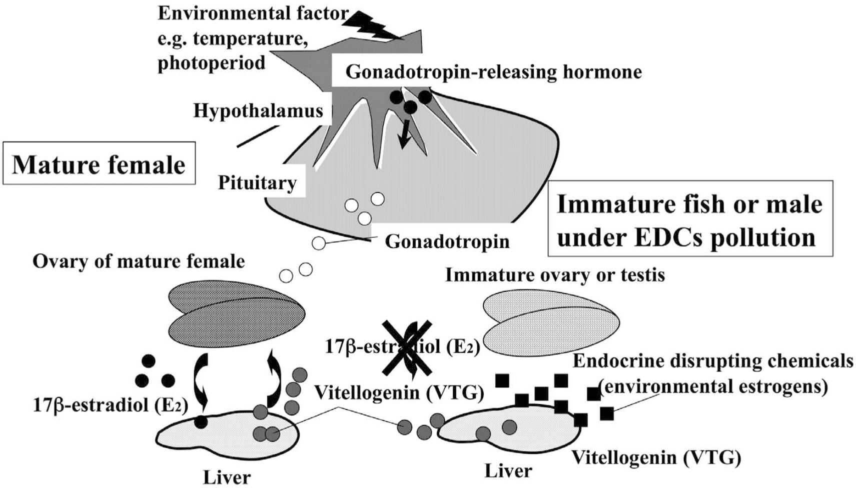

Fig. 1. Illustration of the mechanism of vitellogenin synthesis by 17β-estradiol and environmental estrogens in fish.

considered that EEs in the environment are responsible for raised serum VTG levels, these results suggest that these waters are polluted by EEs.

3. TESTIS-OVA: GONADAL ABNORMALITY

EEs also induce abnormalities of gonadal development (e.g., testis-ova and lack of germ cells). In many teleost species, the sex is fixed except for sex-changing fish species. Abnormal gonad such as testis-ova does not usually occur in sex-fixed fish species in natural conditions. However, sex determining mechanism is particularly susceptible to the effects of environmental factors and sex steroids. Then, the individuals having sex different from the genetically-determined sex appear in wildlife frequently. Especially, natural estrogen and estrogenic substances such as nonylphenol and bisphenol A can induce the production of abnormal gonad in fish (Kang et al., 2002, 2003). Therefore, histological analysis is also useful in investigating the effects of EEs at a cellular level. The analysis is also an important method to determine the adverse effects of EDCs exposure in fish.

Testis-ova are most popular abnormal morphological change in fish exposed by estrogenic chemicals. It is well known that testis-ova are induced by these chemicals in the fish that is sex determination phase, which occur during fly and larval stage. However, it is very difficult to induce the testis-ova in adult fish. In a previous study, testis-ova was observed in male flounders and male konoshiro gizzard shads (*Konosirus punctatus*) in Tokyo bay (Hashimoto et al., 2000, 2001; Cho et al., 2003) and suggested exposure to EEs in early stage of life cycle. Although testis-ova are observed in European countries (Allen et al., 1999), there is no report about histological abnormality of gonad in Asian countries except for Japan.

4. MULLETS: TARGET SPECIES FOR SURVEY OF EDCS

The target fish for survey of EDCs pollutants should have the following four characteristics: 1) to allow assessment of effects by EEs, the fish should be able to live in both clean and contaminated waters; 2) the fish should be sensitive to the environmental contaminants; 3) the fish should be widely distributed; and 4) the fish should be caught easily. In previous field surveys, the flounder, the common goby, and the konoshiro gizzard shad have been used as the target fish. These species are applicable to the characteristics for target fish basically and are available for the survey of local area. However, in extensive international survey on EDCs pollutants, we involve the introduction of excellent model species. Grey mullet (*Mugil cephalus*) is widely distributed from estuarine to oceanic waters. This fish species mainly eats detritus and diatoms with sediment (Boglione et al., 2006, Cardona, 2000). The grey mullet easily acquires chemical compounds compared to other fish in the environment. In addition, the fish is distributed in tropical and temperate waters in the world. This species is convenient for worldwide studies of EDCs contaminations, and is an excellent as a target fish for EDCs pollutant. In this survey, we also collected and used red lip mullet (*Chelon haematocheilus*) in Chinese coastal waters.

5. CONTAMINATIONS BY EDCS IN JAPANESE COASTAL WATERS

We monitored the contamination by EEs in Japanese coastal waters using wild grey mullet. The mullet were collected at Nagasaki Harbor, mouth of the Omuta River, and Hakata Harbor (Fukuoka city) in Japan. These sampling locations are shown in Fig. 2. Sampling was conducted between October 2003 and November 2008. A description of the sampling sites follows: Nagasaki Harbor is long and narrow bay and has many large and small shipyards. The sampling site was located near the small creek contaminated with domestic wastewater at the bottom of the long bay. The population of Nagasaki City is approximately 430 thousand. The mouth of Omuta River is an artificial small river contaminated with domestic wastewater and industrial effluent. In this area, there are some chemical factories and residential district around the river. The population of Omuta City is approximately 130 thousand. Hakata Harbor is the main port of Fukuoka City with a population of about 1.4 million. The sampling site in this area is adjacent to the sewage treatment works and oil terminal.

Wild grey mullet were captured by casting net or fishing (Fig. 3), and were anesthetized with phenoxyethanol (0.05%). Blood samples were taken from the caudal blood vessel with a syringe, and were centrifuged at 1500g for 10 min to separate the serum. The serum samples were stored at –80°C until VTG analysis. The gonad was removed from the body cavity and a small piece of the gonad was preserved in Bouin's solution for about 24 hours and subsequently kept in 70% ethanol until processing for histological examination.

Serum VTG concentration of the grey mullet was determined by enzyme-linked immunosorbent assay (ELISA) or single radial immunodiffusion (SRID).

ELISA: Lipovitellin (Lv) purification and its antibody were performed according to the method of Ohkubo et al. (2006). Specific antiserum to Lv was raised in a rabbit.

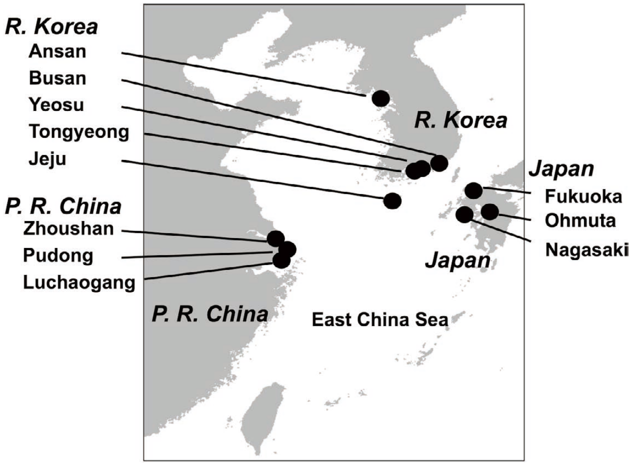

Fig. 2. Sampling sites of the grey mullet in Japan, Korea, and China.

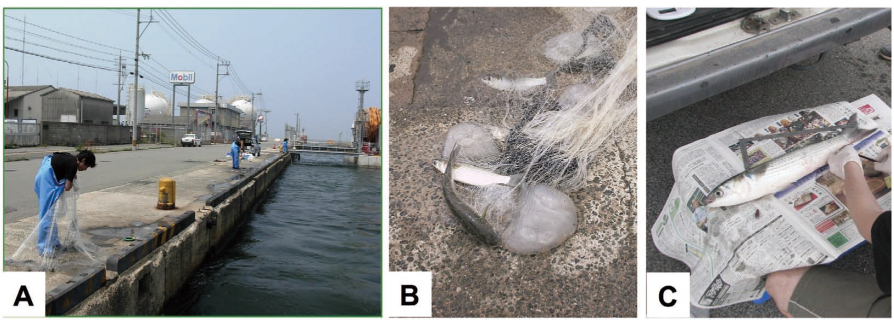

Fig. 3. Photomicrographs of sampling site and the grey mullet. A: Sampling site of Hakata Harbor (Fukuoka city); B: fish sampling using casting net; C: the grey mullet.

IgG was purified with ammonium sulfate precipitation and DE52 (Watman International Ltd. Kent, UK) ion-exchange chromatography. In this assay, wells of 96-well microtiter plates (SUMITOMO BAKELITE CO., LTD., Japan) were coated with the primary antibody in 0.05 M carbonate buffer (pH 9.6) and incubated overnight at 4°C. Non-specific sites of wells were blocked with 1% bovine serum albumin (SIGMA-ALDRICH, Inc., USA) and 5% skim milk (Yukizirushi, Japan) in 0.05 M carbonate buffer for 1 hour at room temperature. Then each well received an aliquot of the VTG standard solutions or serum sample diluted ten folds with 0.5% BSA in 0.05 M PBS for 1.5 h at room temperature. The secondary antibody labeled biotin in 0.5% BSA-0.05 M PBS was added, and incubated for 1.5 h at room temperature. After that, streptavidin-horse radish peroxidase (DakoCytomation, Denmark) conjugated solution in 0.5% BSA in PBS was added and incubated for 1 h at room temperature. For color development, enzyme substrate solution (0.2 M citric acid buffer containing H_2O_2 and *o*-phenylenediamine (SIGMA-ALDRICH CO., USA)) was added to each well and incubated for 40 min at room temperature. The reaction was stopped by the addition of 6 N H_2SO_4. The absorbance of each well was measured at 492 nm using microtiter plate reader (Multiskan, ThermoLabsystems, Finland) and analyzed by DeltaSOFT 3 (BioMetallics Inc., USA) software.

SRID: SRID assay was conducted by the grey mullet VTG kit purchased from COSMO BIO CO. LTD. (Tokyo, Japan). Ten μL of serum sample diluted with the dilution buffer (i.e., to 50% original concentration) was injected into a sample hole of the SRID plate containing an antibody against grey mullet VTG. The plate was incubated at 37°C for 48 h. After the reaction, the diameter of a precipitin ring was measured with a caliper, and converted to VTG level by referring to the conversion table in the kit.

Fixed gonad were dehydrated in an ethanol and butanol series, embedded in paraffin, and sectioned serially at 5 μm intervals. These sections were stained with hematoxylin-eosin for observation under a light microscope.

In our previous studies, the serum VTG level of the grey mullet living in clean water was lower than 1 μg/mL (Hara et al., 2001; Soyano et al., 2001). Thus, we considered that less than 1 μg/mL was the cut off point for normal levels of VTG. Therefore, the ratio of the fish detected higher than normal levels of VTG (1 μg/mL) were indicated in each sampling sites (Table 1). VTG levels of some fish were detected at levels greater than 1.0 μg/mL in Nagasaki, Omuta, and Fukuoka. Especially, a lot of individuals with abnormal levels of VTG were confirmed in the fish collected from Hakata Harbor in Fukuoka city. The high value was approximately 3 μg/mL in Hakata Harbor which has an oil terminal and large sewage treatment works. It is known that the abnormal VTG levels in the flounder collected in the UK were induced by EEs found in industrial and sewage treatment effluent (Lye et al., 1997; Allen et al., 1999). In addition, the highest value of VTG were detected in grey mullet and Japanese common goby collected from Osaka and Tokyo Bay which are industrial metropolitan area (Hara et al., 2001; Soyano et al., 2001; Ohkubo et al., 2003b). It appears that high value of VTG synthesis is induced by the domestic wasters and industrial effluent. These results suggest that the coastal areas of Kyusyu were likely contaminated with EEs.

In this experiment, appearance of fish with abnormal VTG levels was different with season in all sampling sites. The male flounder in UK indicated high VTG levels in mid-winter than those of spring and summer seasons (Kleinkauf et al., 2004). This species migrates from estuary to outside for spawning, and after that the fish come again into estuary areas in late summer. The flounder fed and came into contact with EEs in the estuary during winter season. The grey mullet also migrate to spawning grounds in autumn and early winter. However, the migration for spawning is only observed in mature fish.. In this observation, we don't need to think about this

Table 1. Number of the individuals with abnormal levels of VTG (>1 μg/mL) collected from Japanese coastal waters.

Sampling sites	Dates	Number of individuals	Number of individuals with	
			Abnormal VTG levels	Testis-ova
Nagasaki	Nov. 2003	26	0	0
	Jul. 2005	27	1	0
	Nov. 2005	15	0	0
	Aug. 2006	18	2	1
	Dec. 2006	25	0	0
	Jul. 2007	20	0	1
	Nov. 2007	20	0	0
	Jul. 2008	20	0	3
	Nov. 2008	20	0	1
Omuta	Oct. 2003	18	0	2
	Aug. 2005	15	0	0
	Nov. 2005	19	0	2
	Jul. 2006	12	1	5
	Nov. 2006	25	0	3
	Jul. 2007	20	7	1
	Nov. 2007	20	2	3
	Jul. 2008	20	0	0
	Nov. 2008	20	0	0
Fukuoka	Jun. 2005	20	19	0
	Oct. 2005	10	0	0
	Jun. 2006	10	0	0
	Jul. 2007	20	7	—
	Nov. 2007	25	5	3
	Jun. 2008	10	0	0
	Nov. 2008	13	5	0

possibility, because we collected immature fish for experiment in coastal areas. Consequently, we deemed that seasonal changes in VTG levels are influenced by seasonal changes in environmental EEs.

Photographs of histological analysis of gonads are shown in Fig. 4. Testis-ova (the presence of oocytes in the testis) were observed in some individuals collected from Nagasaki, Omuta, and Fukuoka (Table 1). The oocytes of the perinucleolus stage that are the characteristic of the testis-ova were scattered in the testis. In particular, testis-ova were observed in many individuals collected from the estuarine area of Omuta River. The number of individuals having testis-ova was approximately 9% of the total fish examined in Omuta. The sampling site is located at the head of the Ariake Sound, and there are some chemical factories and residential area which has a population of approximately 130 thousand. The high VTG levels were detected in the mudskipper (*Pheriophthalmus modestus*) and Japanese common goby

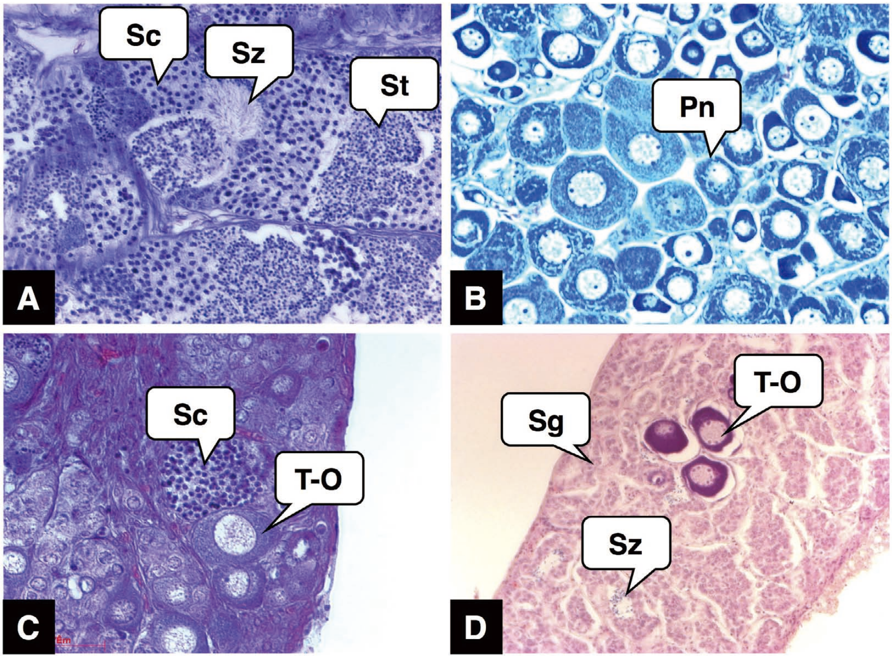

Fig. 4. Photomicrographs of the gonad of the collected grey mullet. A: Testis of mature male, B: ovary of immature female, C and D: testis having oocytes, Sg: Spermatogonium, Sc: Spermatocyte, St: Spermatid, Sz: Spermatozoa, Pn: Perinucleolus stage oocyte, T-O: Testis-Ova.

collected from same site of Omuta (Soyano and Okamatsu, 2003; Nagae et al., unpublished data). It seems that the both species were influenced by EEs containing sediment, because the life style such as food habit and nest of both species of goby are deeply linked the bottom sediment of the habitat. Consequently, it appears that the testis-ova in the grey mullet were also induced by EEs in the sediment.

6. CONTAMINATIONS BY EDCS IN KOREAN COASTAL WATERS

We monitored the contamination by EEs in Korean coastal waters using wild grey mullet. The mullet were collected from Ansan, Jeju, Yeosu, Tongyeong, and Busan. These sampling locations are shown in Fig. 2. Sampling was conducted between November 2003 and May 2007. A description of the sampling sites follows: Ansan is new industrial district which is 30 km away from Seoul, and the population of this area is approximately 730 thousand. Jeju is a South Korean resort island located in the East China Sea. The population of the island is approximately 560 thousand. Yeosu is known a city of petrochemical and fisheries industry and has a population of approximately 300 thousand. Tongyeong is rural residential town and is known for oyster cultivation. The city has a population of approximately 140

Talbe 2. Number of the individuals with abnormal levels of VTG (>1 μg/mL) collected from Korean coastal waters.

Sampling sites	Dates	Number of individuals	Number of individuals with	
			Abnormal VTG levels	Testis-ova
Jeju	Nov. 2003	25	0	0
Tongyeong	Dec. 2003	19	2	3
Ansan	Nov. 2004	18	2	2
Yeosu	Nov. 2003	13	4	0
	May 2007	20	0	0
Busan	Dec. 2003	27	2	2
	Sep. 2006	19	—	0
	May 2007	20	0	0

thousand. Busan is the second largest city in Korea and has a deep harbor and large container handling port. The population is approximately 3.7 million.

Wild grey mullet were captured by casting net or fishing. Blood samples were taken from the caudal blood vessel with a syringe for VTG analysis. Serum VTG concentration of the grey mullet was determined by enzyme-linked immunosorbent assay (ELISA) or single radial immunodiffusion (SRID). The gonad was removed from the body cavity and a small piece of the gonad was preserved in Bouin's solution for histological observation.

VTG levels of some fish were detected at levels greater than 1.0 μg/mL in Ansan, Yeosu, Tongyeong, and Busan (Table 2). The high value was approximately 56 μg/mL in Yeosu which has a petrochemical industry. However, there was no fish having abnormal gonad, testis-ova in Yeosu. The testis-ova was detected in fish collected from Ansan, Tongyeong, and Busan. It appears that high value of VTG synthesis and appearance of testis-ova are induced by the domestic wasters and industrial effluent. These results suggest that the Korean coastal areas were contaminated with EEs. However, the place that is not polluted by EDCs exists in Koran coastal area, such as Jeju Island.

7. CONTAMINATIONS BY EDCS IN CHINESE COSTAL WATERS

We monitored the contamination by EEs in Chinese coastal waters using wild grey mullet. The mullets were collected from Zhonshan, Luchaogang, and Pudong in P.R. China. These sampling locations are shown in Fig. 3. Sampling was conducted between October 2007 and September 2008. These sampling sites are located near Shanghai. Shanghai is the largest city in P.R. China and the population is approximately 18.8 million, and has a vast industrial area and large harbor.

Wild mullets were captured by casting net or fishing. Blood samples were taken from the caudal blood vessel with a syringe for VTG analysis. Serum VTG concentration

Talbe 3. Number of the individuals with abnormal levels of VTG (>1 μg/mL) collected from Chinese coastal waters.

Sampling sites	Dates	Number of individuals	Number of individuals with	
			Abnormal VTG levels	Testis-ova
Zhoushan	Sep. 2007	12*	—	1
		3**	—	0
Luchaogang	Oct. 2007	19*	4	—
	Sep. 2008	21*	0	—
Pudong	Sep. 2008	7*	0	2

*Red lip mullet, **Grey mullet

of the mullets were determined by enzyme-linked immunosorbent assay (ELISA) or single radial immunodiffusion (SRID). The gonad was removed from the body cavity and a small piece of the gonad was preserved in Bouin's solution for histological observation.

VTG levels of some fish were detected at levels greater than 1.0 μg/mL in Luchaogang (Table 3). The testis-ova was detected in fish collected from Zhonshan and Pudong. It appears that high value of VTG synthesis and appearance of testis-ova are induced by the domestic wasters and industrial effluent. These results may suggest that the Chinese coastal areas were contaminated with EEs. However, our results are too little to draw a conclusion. We are continuing this survey in China now.

8. MECHANISM OF INDUCTION OF VITELLOGENIN SYNTHESIS AND TESTIS-OVA BY EES

Both VTG synthesis and testis-ova are induced by EEs. However, there was no correlation between high level of VTG and the appearance of testis-ova in fish collected from the coastal waters in Japan, Korea, and P.R. China. These results indicate that the mechanism of induction of VTG synthesis and testis-ova are different. The gene expression and protein synthesis of VTG are induced immediately by estrogen regardless of sex and degree of maturity. Moreover, serum VTG is maintained for approximately 2 months after estrogen exposure (Aoki et al., in preparation). However, testis-ova is not induced by estrogen treatment in mature fish. The appearance of the testis-ova may be only caused by EEs exposure during the critical period of sexual differentiation. Abnormal levels of serum VTG level indicate individuals were exposed to EEs in the present or the very near past (Aoki et al., 2010). On the other hand, the appearance of testis-ova indicates that the individuals were exposed to EEs in the past, especially, during the sexual differentiation period (Aoki et al., 2010). In our survey, the mullets might be exposed to EEs during various periods in the life cycle. However, we don't have enough information to understand the mechanisms of abnormality of gonadal development and physiological phenomena by EDCs exposure. We should do the exposure and in vitro experiment in addition to field surveys in coastal areas of Asian countries.

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