Coastal Environmental and Ecosystem Issues of the East China Sea,
Eds., A. Ishimatsu and H.-J. Lie, pp. 205–213.

Estrogenic Activity in Estuaries by Measuring Serum Vitellogenin Concentration of Japanese Male Common Goby in Northwestern Part of Kyushu

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Abstract—Immersion and field surveys were conducted in the northwestern part of Kyushu, to investigate the estrogenic activity in male Japanese common goby. Serum vitellogenin (Vg) concentration and total estrogenic activity in male common goby, based on microanalysis of natural estrogens (E_1, E_2, E_3) and estrogenic compounds (NP, BPA, OP), were measured. The immersion survey in Ariake Sound suggested the possibility of the progress of environmental estrogen pollution. Field surveys in Ohmura Bay and Ariake Sound also detected estrogenic activity. An extremely high ratio of Vg-positive males were recorded in Ohmuta River Estuary, and total estrogenic activity in environmental water in this site were also higher than that recorded in other sites. The main causative substance of the estrogenic activity was not alkylphenols, but natural estrogens (E_2), probably due to a high population density and a low sewage diffusion rate.

Keywords: environmental estrogen, vitellogenin, biomarker, common goby, alkylphenols

1. INTRODUCTION

Endocrine-Disrupting Chemicals (EDCs)

Large influxes of natural and synthetic chemicals, derived from domestic, industrial and agricultural discharge, are released into the aquatic environment. It is widely recognized that several of these chemicals have hormone-like potency; these have been termed endocrine-disrupting chemicals (EDCs). Environmental estrogens have come to be seen as the most influential EDCs. Environmental estrogens include both natural substances (17β-estradiol (E_2), estrone (E_1), estriol (E_3), phytoestrogens, etc.) and synthetic substances (ethynylestradiol (EE_2), alkylphenols, etc.). These chemicals can be detected in the aquatic environment, and they have the potential to perturb

reproductive functions in teleosts through the disruption of the endocrine system (Sumpter, 1995; Tyler et al., 1998). Abnormal intersexuality has been reported in roach (*Rutilus rutilus*) and gudgeon (*Gobio gobio*) (Jobling et al., 1998; Van Aerle et al., 2001). Intensive studies using roach revealed the widespread and frequent occurrence of males with intersex gonads, the male gonad containing various stages of both male and female germ cells (Jobling et al., 1998). In addition, it was reported that the occurrence of sexual abnormalities was closely related to the elevation of serum vitellogenin (Vg) concentration.

Vitellogenin (Vg)

Vitellogenin (Vg) is the precursor of egg yolk glycophospholipoprotein in oviparous animals, including fishes (see review Specker and Sullivan, 1994). Vg is synthesized under the strong control of endogenous estrogen in the liver of sexually matured females, and is taken in by oocytes through the blood stream. Although Vg is a female-specific protein, its synthesis can be easily induced in males by the exposure of exogenous estrogens. Therefore, the existence of Vg in male blood has been proposed as a good and sensitive biomarker for the survey of environmental estrogens (Sumpter and Jobling, 1995; Kime et al., 1999). Until now, the survey of environmental estrogens as pollutants has only been reported in the coastal areas of Japan in adult males of several species including the marble sole (*Pleuronectes yokohamae*) (Hashimoto et al., 2000), the common goby (*Acanthogobius flavimanus*) (Ohkubo et al., 2003a), and the grey mullet (*Mugil cephalus*) (Aoki et al., 2010). In every report, serum or plasma Vg concentrations was found to be high in EDC contaminated areas. However, there is little information regarding the relationship between actual concentrations of estrogenic compounds in environmental waters and Vg concentrations in serum of male marine fish.

Common Goby

Sewage and industrial wastewaters enter rivers and coastal waters, including estuaries. In estuaries especially, many chemicals that are discharged into river valleys, including environmental estrogens, are inevitably accumulated. The Japanese common goby (*Acanthogobius flavimanus*) is distributed throughout Japan, mainly inhabiting estuaries and are a typical non-migratory species (Dotu and Mito, 1955). They are therefore directly influenced by the ambient levels of environmental estrogens in their habitats. Gobies are also easy to catch by fishing and easily held in the laboratory. In addition, a goby Vg assay system has been developed using a specific polyclonal antibody (Ohkubo et al., 2003b). Common gobies also inhabit the northwestern part of Kyushu, Japan. Ohmura Bay and Ariake Sound are enclosed water areas. Typically in enclosed waters, various types of pollutants are found and prevail for a long period of time. In the present survey using common goby as a test fish, the levels of environmental estrogens were evaluated by both biological (measurement of male serum Vg concentration) and chemical (microanalysis of natural estrogens and estrogenic compounds in environment water) analyses mainly in enclosed waters in the northwestern part of Kyushu.

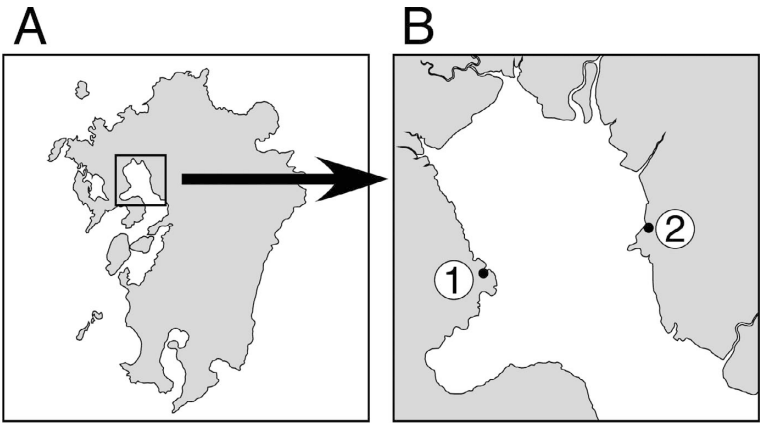

Fig. 1. Location of the immersion survey using Japanese common goby. A: Whole map of Kyusyu, B: Location of immersion survey in Ariake Sound. 1:Takesaki Fishing Port, 2: Suwa River Estuary.

2. IMMERSION SURVEY USING COMMON GOBY

As a preliminary test, an immersion survey was conducted using the common goby in Ariake Sound. Common gobies were caught by rod-and-line in the estuary of Taira River, Nagasaki, from November to December in 2003. Ohkubo et al. (2003a) reported that there were no Vg-positive male gobies captured in Taira River Estuary; this area was regarded as a clean site. Fish were transported to the Institute for East China Sea Research (Nagasaki University), and were kept in tanks with clean running seawater for two weeks to recover from fishing and transfer stress. They were fed an appropriate quantity of live lugworm daily.

After being kept in the laboratory tanks, fish were transferred to two sites in Ariake Sound for the immersion survey (Fig. 1). Ariake Sound has a large tidal range (max. 6 m), therefore, sites were carefully chosen, sufficient depth was available for cage immersion: such as within fishing ports. Within Ariake Sound, two sites were chosen; a clean site (Site 1 in Saga Prefecture) Takesaki Fishing Port and a contaminated site (Site 2 in Fukuoka Prefecture) Suwa River Estuary in Ohmuta city. Site 1 in particular was chosen as the population of Takesaki was very small. Conversely, Ohmuta city has a larger population, as well as industrial sources of EDCs, such as a sewage plant and chemical plant located near Suwa River Estuary. Cages were immersed at 50 m offshore from Suwa River Estuary to maintain a sufficient water depth.

Fish were allowed to adjust to the environmental water temperature at the caging site, with 15 individuals placed in one cage and held for 2 weeks. In this survey the immersion cage used for the goby was a modified trap cage (approx. W600 mm × D450 mm × H300) used previously for capturing conger eel (Fig. 2.). Fish were anesthetized with 2-phenoxyethanol and the standard body length (SL), body weight

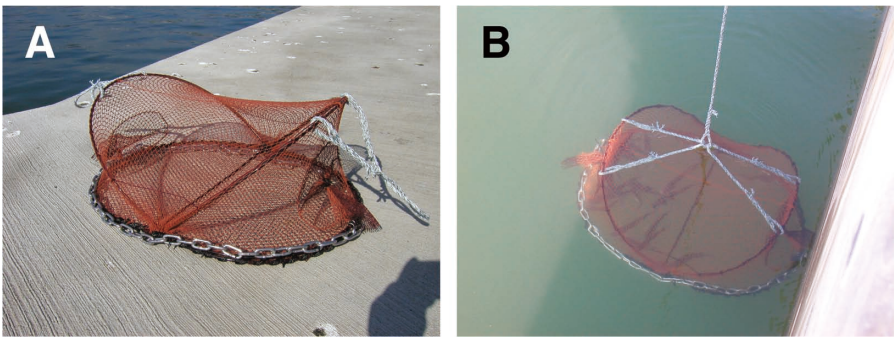

Fig. 2. Photographs of the cage used for immersion survey. A: an external form of cage, B: suspended and immersed cage in the survey site.

Table 1. Characteristics of the male Japanese common goby using caging survey.

Caging site	SL (cm) Mean ± SEM	BW (g) Mean ± SEM	GW (g) Mean ± SEM	Number of male fish
1) Takesaki	14.3 ± 0.6	40.2 ± 4.8	0.038 ± 0.005	12
2) Suwa	14.1 ± 0.4	37.6 ± 3.4	0.035 ± 0.004	8

SL: Standard length, BW: Body weight, GW: Gonad (testis) weight

(BW) and gonad (testis) weight (GW) were measured (Table 1). Blood samples were taken from the dorsal vessel with a syringe and transferred to tubes, allowed to clot at 4°C and centrifuged at 5,000 g for 5 min to separate the serum. The sera were collected and stored at –30°C until further analysis. Serum concentrations of Vg (Vg-530) were analyzed by Japanese common goby Vg specific enzyme-linked immunosorbent assay (ELISA) developed by Ohkubo et al. (2003b). For the Vg 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. Wells were blocked with 1% bovine serum albumin (BSA) and 1% skimmed milk in 0.05 M carbonate buffer for 1 hour at room temperature. Each well received an aliquot of the VTG standard solutions or serum sample diluted fifty-folds with 0.05 M PBS–0.5% BSA for 1.5 h at room temperature. The secondary antibody labeled with biotin in 0.05 M PBS–0.5 % BSA was added and incubated for 1.5 h at room temperature. Streptavidin-horse radish peroxidase (DakoCytomation, Denmark) conjugated solution in 0.05M PBS–0.5% BSA was subsequently added and incubated for 1 h at room temperature. Enzyme substrate solution (TMB+ Substrate-Chromogen, DakoCytomation, Denmark) was added to each well and incubated for 20 min at 15°C to promote colorimetric development,

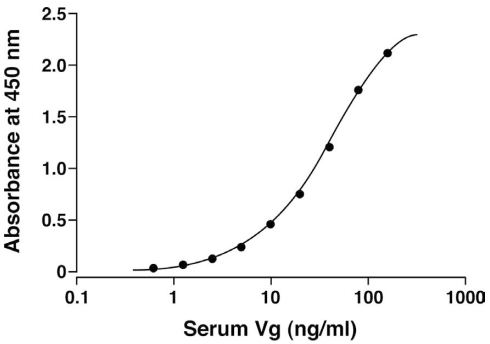

Fig. 3. A typical standard curve of Japanese common goby Vg ELISA.

where the reaction was terminated by the addition of 6 N H_2SO_4; absorbance was measured at 450 nm using a microtiter plate reader and analyzed by Assayzap software. A typical standard curve of this assay is represented in Fig. 3. The lower limit of quantification was determined as 2 ng/mL (serum Vg concentrations of <100 ng/mL were therefore considered Vg non-detectable (Vg-negative), >100 ng/mL were considered Vg-positive).

Characteristics of the male common goby from the immersion survey are listed in Table 1 (standard length, body weight, gonad (testis) weight). There were no significant differences in the chosen parameters between the two sites. Serum Vg concentrations of each male individual at the two sites are shown in Fig. 4. In Takesaki Fishing Port, both the ratio of Vg-positive male and the serum Vg concentration in Vg-positive male were low. In contrast, an obvious increase of serum Vg levels was observed in Suwa River Estuary. A statistically significant difference in serum Vg levels can be reported between the two immersion survey sites ($p < 0.05$, Mann-Whitney's *U*-tests), suggesting that the concentration of environmental estrogens in Suwa River was markedly higher than that found in Takesaki.

3. FIELD SURVEY

Immersion survey results described above suggest that the pollution of environmental estrogens has progressed also in the northwestern part of Kyushu, including Ariake Sound. However, there is no direct evidence for the causative agents to induce Vg synthesis in male common goby. In the field survey, the serum Vg concentration in field-collected male goby were quantified, and were compared with the concentration of environmental estrogens (natural estrogens and alkylphenols) in the water of the sampling sites to evaluate the estrogenic activity in the northwestern part of Kyushu.

Common goby were caught by rod-and-line in the estuary at five sites (site 1: Kawatana River, site 2: Ohe River, site 3: Nagayo River, site 4: Urakami River, site 5: Ohmuta River) in 2007 and 2008 (Fig. 5). Fish were anesthetized, and SL, BW

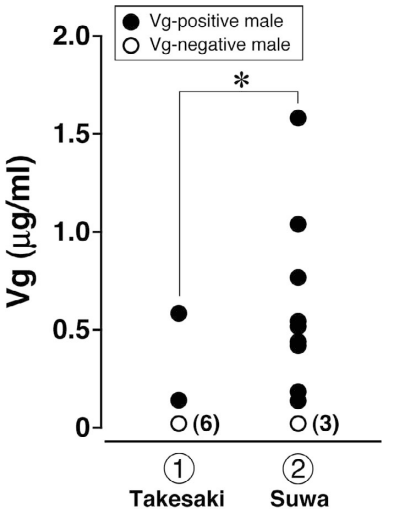

Fig. 4. Scatter diagram of male serum Vg concentration in immersion survey. Vg concentration of 100 ng/mL or less was considered Vg non-detectable (Vg-negative, open circle), 100 ng/mL or over was considered Vg-positive (closed circle). Asterisk (*) indicates significant difference ($p < 0.05$, Mann-Whitney's *U*-tests).

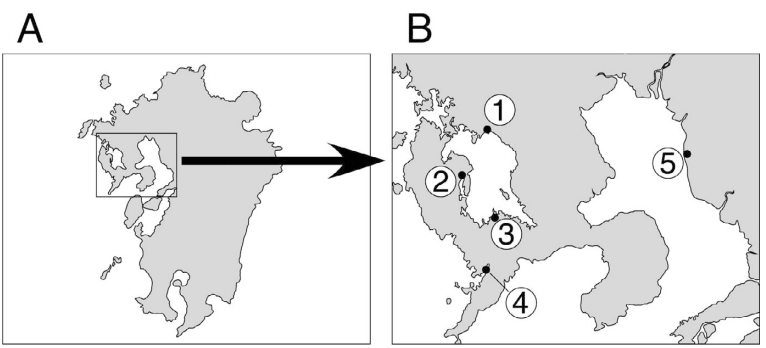

Fig. 5. Location of the field survey of Japanese common goby. A: Whole map, B: Location of field survey in Ohmura Bay and Ariake Sound. 1: Kawatana River Estuary, 2: Ohe River Estuary, 3: Nagayo River Estuary, 4: Urakami River Estuary, 5: Ohmuta River Estuary.

and GW were measured. Then, sera were collected and used for Vg quantification as described above. The water samples were also collected at each sampling site. Water samples were collected in clean shaded glass bottles with ascorbic acid (1 g/L) to prevent oxidative degradation of target substances. In the laboratory, target chemicals

(natural estrogen (E_1, E_2 and E_3), synthetic estrogen (EE_2) and alkylphenols (Nonylphenol (NP), Bisphenol-A (BPA) and Octylphenol (OP)) were extracted by the combination of solid and liquid phase extraction. Concentrations of natural estrogens, EE_2 and alkylphenols in environmental water were quantified by HPLC/MS/MS, and by GC/MS/MS, respectively. Total estrogenic activity (E_2-equivalent, ng/L) was calculated using the conversion rate reported by Nishihara et al. (2000). The conversion rate of each chemical was determined as: E_1 0.16, E_2 1, E_3 0.0027, NP 0.00033, BPA 0.000042, OP 0.0021.

The ratios of Vg-positive males varied from 0 to 88% at all sites (Table 2). In Kawatana River, no Vg-positive males were observed. Relatively high ratios of Vg positive males (more than 10%) were detected at Nagayo River, Urakami River and Ohmura River Estuary. In Ohmuta River, the highest ratio of Vg-positive males was detected (88%), and the mean Vg concentrations were also higher than 1,000 ng/mL. Concentrations of natural estrogens and alkylphenols in environmental water at each site were also quantified (Table 2). EE_2 and OP were not detected at any sampling sites and there was little difference between the concentrations of alkylphenols at the five sites. In Kawatana River, neither E_1 nor E_2 was detected. The extremely high level of E_2 was observed in Ohmuta River estuary (4.1 ng/L). Total estrogenic activity (E_2-equivalent) was calculated using these data, the highest concentration was 4.6 ng/L in Ohmuta River. Comparatively high levels of total estrogenic activity were detected in Ohe and Nagayo River Estuaries. In Kawatana River, the lowest activity (0.4 ng/L) was observed among sampling sites. As listed in Table 2, E_2 was found to be the main causative compound of total estrogenic

Table 2. Field sampling data.

Sampling site	(year)	Number of Vgpositive males/total males (ratio, %)	Total estrogenic activity (E_2-equivalent, ng/L)	Natural estrogens (ng/L)			Alkylphenols (ng/L)		
				E_1	E_2	E_3	NP	BPA	OP
1) Kawatana	(2007)	0/18 (0%)	0.40	ND	ND	109.8	300	20	ND
2) Ohe	(2008)	2/29 (7%)	1.86	0.7	1.5	71.9	200	20	ND
3) Nagayo	(2007)	5/48 (10%)	1.67	ND	1.4	35.6	400	20	ND
4) Urakami	(2008)	5/35 (16%)	0.23	1.7	ND	47.0	—	—	—
5) Ohmuta	(2008)	28/32 (88%)	4.60	0.5	4.1	157.7	ND	20	ND

ND: less than the lower limit of quantification; —: no data

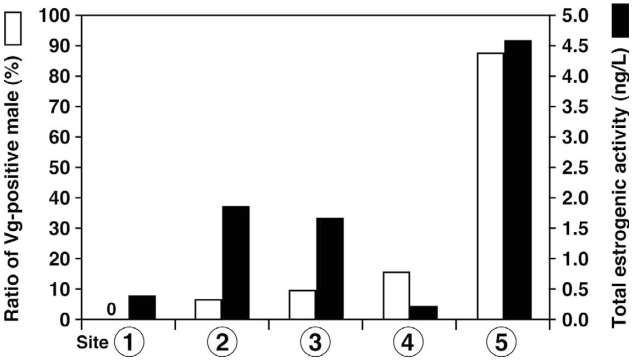

Fig. 6. The ratio of Vg-positive males to total males and total estrogenic activity (E_2-equivalent) in environmental waters in field sampling sites.

activity in the sampling sites of this survey.

A strong correlation was observed between the ratios of Vg-positive male and total estrogenic activity in each site (Fig.6). Total estrogenic activity in environmental water was also correlated with the population size and population density in each river valley (data not shown). Therefore, it was suggested that natural estrogens detected in this survey were derived from human activity. In addition, especially in Ohmuta River Estuary, both the ratios of Vg-positive male and total estrogenic activity were higher by far than those of other sites. The sewage diffusion rate in Ohmuta city is extremely low (about 33%) and may be one of the factors accountable for the concentration of natural estrogens. It will be also necessary for the detailed understanding to investigate the discharge route and the structural changes of natural estrogens.

The highest estrogenic activity found was 4.6 ng/L in Ohmuta River Estuary. It was revealed in an E_2 exposure laboratory test using common goby that the minimal E_2 concentration in water to induce serum Vg in male was 10 ng/L (Ohkubo et al., 2003b). Compared with this concentration, the highest concentration of estrogenic activity (4.6 ng/L, E_2-equivalent) detected in this survey was about 50%; however, Vg synthesis in male was markedly induced. Pajapakse et al. (2002) reported the combined additive effect of the xenoestrogens led to a dramatic enhancement of E_2 action, even when each single xenoestrogen was below its NOEL. Many xenoestrogens, including alkylphenols, also detected in this survey coexist with natural estrogens in the field, the synergistic effect of them may occur in wild animals.

4. CONCLUSIONS

From the results of both immersion and field survey using male common goby, it was strongly suggested that the pollution of environmental estrogens progressed in Ohmura Bay and Ariake Sound, highly closed water areas in the northwestern part of

Kyusyu. Additionally, the main causative substance of the estrogenic activity was not man-made chemicals, but natural estrogens (E_2), probably due to a high population density and a low sewage diffusion rate. However, it is still unknown the effects of environmental estrogens on the physiological functions, especially reproductive functions, in common goby of this water area. It will be also investigated by the morphological observation whether gonadal abnormalities (testis-ova, the lack of germ cell, etc.) are occurred.

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