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<td>Aoki, Jun-ya; Nagae, Masaki; Takao, Yuji; Hara, Akihiko; Lee, Young-Don; Yeo, In-Kyu; Lim, Bong-Soo; Park, Chang-Beom; Soyano, Kiyoshi</td>
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</table>

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Survey of contamination of estrogenic chemicals in Japanese and Korean coastal waters using the wild grey mullet (*Mugil cephalus*)

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Abstract

We monitored the contamination by environmental estrogens (EEs) of coastal areas in Korea and Japan using the wild grey mullet. The grey mullet were collected from Ansan, Jeju, Yeosu, Tongyeong, and Busan in Korea and 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) 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. Although, testis-ova were observed in some individuals collected in Ansan, Tongyeong, and Busan in Korea and Omuta in Japan, there was no correlation between individuals with testis-ova and individuals with abnormal levels of VTG. Furthermore, in Japan, serum VTG levels of fish collected from Nagasaki and Fukuoka was also greater than 1.0 μg/ml. Although individuals with testis-ova were found in Omuta, these fish expressed normal levels of serum VTG. Our results suggest that the grey mullet living in these coastal areas are influenced by EEs in the environment. Furthermore, it appears that the production of VTG and the occurrence of testis-ova are caused by different mechanisms.

Keywords: fish; estrogen activity; endocrine disrupting chemicals; vitellogenin; testis-ova; East Asia
1 Introduction

There are many chemicals that have the potential to disrupt the endocrine systems of wildlife (Colborn et. al., 1993), and are known as endocrine disrupting chemicals (EDCs). It is known that most of these chemicals in the environment have estrogenic activity (Sumpter, 1995), such as natural and synthetic estrogens, alkylphenols, and organochlorines. These chemicals, environmental estrogens (EEs), can be found in run off water, domestic and industrial wastes that flow into rivers, lakes, and coastal areas. The aquatic environment is the ultimate sink for the majority of synthetic and natural chemicals. Therefore, aquatic organisms are often exposed to EEs.

To evaluate the presence of EEs, methods to measure vitellogenin (VTG), which is the female specific yolk protein precursor in oviparous animals, have been developed for various species of fish (Nishi et al., 2002, Ohkubo et al., 2003a). VTG is synthesized in the liver, released into the blood, and taken in by growing oocytes (Sumpter and Jobling, 1995). It is usually produced under estrogen stimulation in mature females, while immature females and all males have no or low concentrations of VTG in the blood (Sumpter and Jobling, 1995). However, VTG production in immature and mature males can be induced by exposed to EEs. Thus, VTG is a useful biomarker for the estimation of EEs exposure in fish (Sumpter and Jobling, 1995, Kime et al., 1999). In Japanese estuarine and coastal areas, the male flounder (*Pleuronectes yokohamae*) (Hashimoto et al., 2000) and the male common goby
(Acanthogobius flavimanus) (Ohkubo et al., 2003b) showed high levels of VTG in their serum. In estuarine areas of the UK, the male flounder (Platichthys flesus) has been also found with elevated concentrations of VTG in their serum (Allen et al., 1999). Since it is considered that EEs in the environment are responsible for raised serum VTG levels, these results suggest that these waters are polluted by EEs.

Abnormalities in gonadal development (e.g., testis-ova and lack of germ cells) are also thought to be caused by EEs. Specifically, testis-ova does not usually occur in fish species where the sex is fixed except for sex-changing fish species. Weakly estrogenic chemicals such as nonylphenol and bisphenol A can induce the production of testis-ova in male medaka (Kang et al., 2002, Kang et al., 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. However, there are few examples of field surveys in coastal areas using these biomarkers for EEs exposure. For example, 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, Hashimoto et al., 2001) and suggested exposure to EEs.

In previous field surveys, the flounder, the common goby, and the konoshiro gizzard shad have been used as the target fish for the investigation of EDCs pollutants. However, it remains to be determined whether these fish are appropriate for the general investigation of EDCs pollutants. The target fish 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. Grey mullet (*Mugil cephalus*) is widely distributed from estuarine to oceanic waters. This fish species consumes a variety of prey distributed from the water surface to bottom mud, however, mainly eats detritus and diatoms with sediment as an energy source (Boglione et al., 2006, Cardona, 2000). Therefore, the grey mullet easily acquires chemical compounds compared to other fish in the environment. Moreover, the grey mullet is found worldwide inhabiting tropical and temperate waters. Therefore, this species is convenient for worldwide studies of EDCs contaminations, and is an excellent sentinel for EDCs research fish as a target fish for EDCs pollutant. For these reasons, we believe that the grey mullet offers a number of advantages for field surveys.

The sampling sites for the survey of EDCs pollution have been centered in Europe, United States of America, and Japan. However, the evaluation of EDCs pollution is not fully investigated in East Asian areas where industrialization is advancing. Survey of EEs pollution in this area is certainly needed in the future. Thus, this study describes the first monitoring of EDCs carried out in coastal areas of Korea and Japan using wild grey mullet.

2 Materials and methods
2.1 Fish collection and sampling sites

Wild grey mullet were collected from Ansan, Jeju, Yeosu, Tongyeong, and Busan in Korea and at Nagasaki Harbor, mouth of the Omuta River, and Hakata Harbor (Fukuoka city) in Japan. These sampling locations are shown in Fig. 1. Sampling was conducted between October 2003 and November 2005. A description of the sampling sites follows: Ansan is a rural residential town and has many paddle fields. This city is 30 km away from Seoul and the population of this area is approximately 730 thousand. Jeju is a South Korean resort. The population of the island is approximately 560 thousand. Jeju Island is famous for flounder aquaculture and cultivation of orange. Yeosu has a petrochemical industry and a prosperous fisheries industry with a population of approximately 300 thousand. Tongyeong is known for oyster cultivation and has a population of approximately 140 thousand. Busan is the second largest city in Korea and the population is approximately 3.7 million, and has a deep harbor and large container handling port. Nagasaki Harbor is long and narrow bay and has many large and small shipyards. The collection site in this area was located 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. There are some chemical factories and residential areas around this river. The population of Omuta City is approximately 130 thousand. Hakata Harbor was the main port of Fukuoka City with a population of about 1.4 million.
The collection site in Hakata Harbor is adjacent to the sewage treatment works and oil terminal.

2.2 Blood and gonad sampling

Wild grey mullet were captured by casting net or fishing, and were anesthetized with phenoxyethanol (0.05 %). After total length (mm) and body weight (g) were measured, blood samples were taken from the caudal blood vessel with a syringe. These samples 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.

2.3 Measurement of VTG levels in serum

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

SRID: SRID 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 hours. 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.

ELISA: Purification of lipovitellin (Lv) and its antibody were performed as described by Ohkubo et al. (2006). Specific antiserum to Lv was raised in a rabbit. IgG was purified with ammonium sulfate precipitation and DE52 (Watman International Ltd. Kent, UK) ion-exchange chromatography. It is known that the grey mullet has VTG protein of three forms (A, B, C) (Amano et al., 2007). The used antibody in our experiment indicated a positive reaction to complete type of VTG-A and -B, which is the main VTG protein induced by estrogens. 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 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 H2O2 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$_2$SO$_4$. 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.

2.4 Histological analysis

The preserved gonadal samples were dehydrated in an ethanol and butanol series and embedded in paraffin. Sections 5 μm thick were stained with hematoxylin-eosin and mounted on a microscope slide.

2.5 Statistics

Statistical analyses were performed using StatView for Mac (version 5.0) (SAS Institute Inc. USA). The result on total length of the grey mullet collected is expressed as mean ± S.D. The normality of the distribution of the data was evaluated by the Bartlett test. Since the data was not normally distributed, the non-parametric Kruskal-Wallis test was utilized followed the Games-Howell test. $p < 0.05$ was considered statistically significant.
3 Results

3.1 Body size of the collected fish

Number of samples, total length, and body weight of the grey mullet collected from Korean and Japanese coastal waters are shown in Table 1. Total length of the collected fish was divided into three groups, statistically ($p<0.05$). Statistical analysis of body weight data has not been conducted, because body weight varies depending on food consumption.

3.2 Serum VTG levels

VTG level in serum was measured in immature and male grey mullet (Fig. 2). The range of values of serum VTG levels were 0.1-1.75 µg/ml in Ansan, 0.1-0.21 µg/ml in Jeju, 0.29-56.8 µg/ml in Yeosu, 0.1-4.01 µg/ml in Tongyeong, and 0.1-5.69 µg/ml in Busan in Korea, and 0.1-1.35 µg/ml in Nagasaki, 0.1-0.57 µg/ml in Omuta, and 0.1-3.36 µg/ml in Fukuoka. Thus, the variation in the value of the serum VTG was large. In four sites in Korea (Ansan, Yeosu, Tongyeong, and Busan) and in the two sites in Japan (Nagasaki and Fukuoka), serum VTG levels of some fish were detected at levels greater than 1.0 µg/ml.

3.3 Histological analysis
Photographs of histological analysis of gonads are shown in Fig. 3. Histological abnormalities such as the presence of oocytes in the testis (testis-ova) were observed in some individuals collected from Ansan, Tongyeong, and Busan in Korea and from Omuta in Japan. The oocytes of the perinucleolus stage and vitellogenesis that are the characteristic of the testis-ova were scattered in the testis. The number of individuals having testis-ova was approximately 10% of the total fish examined (Table 2).

4 Discussions

VTG was detected in the serum of immature and male fish collected from all sampling sites, and the variation in the values of the serum VTG was large. Although the difference in the total length of the fish between sampling sites was large (Table 1), there was no correlation between total length and serum VTG level. Thus, we evaluated the value of serum VTG level irrespective of fish size. However, Scott et al. (2006a) described that there was a positive relationship between VTG level and BW in male cod (*Gadus morhua*). As one of the reason in elevated VTG of large male cod, they showed the possibility that large cod was accumulating EEs through the food chain. They pointed out that a change in food species during the life cycle of the cod, from benthic invertebrates to mainly other fishes may be the cause. However, in the grey mullet, it is thought that the food chain has no influence,
because the species mainly consumes detritus and diatoms with sediment. We should give more thought to changes in food type during the life cycle and investigate and estimate the influence of EDCs, depending on the target species.

The highest value in each sampling site in Korea was 1.75 μg/ml in Ansan which is a rural residential town and has a population of approximately 730 thousand, 0.21 μg/ml in Jeju which is a resort area and has a population of approximately 560 thousand, 56.8 μg/ml in Yeosu which has a petrochemical industry and prosperous fisheries industry with a population of approximately 300 thousand, 4.01 μg/ml in Tongyeong which has an oyster cultivation industry and has a population is approximately 140 thousand, and 5.69 μg/ml in Busan which is the second largest city in Korea and has a population of approximately 3.7 million. In Japan, the highest value from each sampling site was 1.35 μg/ml in Nagasaki which has many shipyards with a population of approximately 430 thousand, 0.57 μg/ml in Omuta which has some chemical factories, residential areas, and a population of approximately 130 thousand, and 3.36 μg/ml in Fukuoka which has an oil terminal and a population of approximately 1.4 million. 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 levels of the grey mullet in Ansan, Yeosu, Tongyeong, Busan, Nagasaki, and Fukuoka were greater than the normal value. These results indicate that EEs in environmental water induced VTG expression of the collected fish.
In previous studies, high levels of VTG were detected in the serum of the grey mullet collected from Osaka (highest value: 3500 μg/ml) and Tokyo Bays (highest value: 37.9 μg/ml) (Hara et al., 2001, Soyano et al., 2001). Additionally, the highest values of VTG, which is the complete type of VTG (Vg-530), were 6.035 and 2.780 μg/ml in Japanese common goby from Osaka and Tokyo Bay, respectively, (Ohkubo et al., 2003b) and 2.2 μg/ml in the flounder from Tokyo Bay (Hashimoto et al., 2000). These sites are in an industrial metropolitan area, and it is believed that VTG production is induced by the effluent of industrial and domestic waste. The flounder collected in the UK had also high levels of VTG (Allen et al., 1999, Lye et al., 1997), assuming that the abnormal VTG levels were induced by EEs found in industrial and sewage treatment effluent. In our study, higher than normal levels of VTG (> 1 μg/ml) were detected in the grey mullet collected from 4 sites in Korea and 2 sites in Japan. Although, these levels are lower than that of the fish collected from Osaka Bay, the fish were influenced by the environmental EEs. These sites were also near industrial and densely populated areas, which were likely contaminated with EEs.

We obtained very interesting results in the present study. In the fish collected from Nagasaki and Fukuoka, VTG levels in the present study were lower than those in 1999 and 2000 (Soyano et al., 2001). Similar changes in VTG levels was observed in the UK. VTG levels of the male flounder of the Mersey Estuary in the UK decreased for six years (Scott et al., 2006b). It is difficult to prove this phenomenon through evidence. However, it is obvious that the decrease of VTG levels were caused by the decrease of the amount of EEs in
environment. We presume that decrease in EEs is by the improvement of the performance and ability of sewage treatment. Therefore, in future studies, it is important that field surveys of EEs contamination be carried out in consideration of the ability of the sewage treatment facility (amount of drainage containing EEs) and the characteristic of the target fish (body size, feed, migration, etc.).

When mature female fish have vitellogenic oocytes during the normal spawning season, they also display high level of VTG in serum. This fact indicates that serum VTG level in females change with season. On the other hand, VTG level of immature and male fish shows no seasonality (Unpublished data). However, in our survey, the values differed with season within Nagasaki and Fukuoka in 2005. VTG levels of the fish collected from Nagasaki in July 2005 and Fukuoka in June 2005 tended to be higher than those of Nagasaki in November 2005 and Fukuoka in October 2005, respectively. If the quantity of EEs in water and/or sediment varies with season, VTG levels in the grey mullet may also change under the influence of these environmental EEs. The male flounder (*Platichthys flesus*) in the Mersey of the UK had high levels of VTG in mid-winter but not in late spring and summer (Kleinkauf et al., 2004). They considered that the reason for the seasonal difference in VTG levels in males could be because most of the flounder enter the Mersey Estuary in the late summer. The flounder fed and came into contact with EEs in the estuary, their VTG levels gradually built up and peaked just before they were ready to migrate back to the sea. Although the grey mullet also migrate to spawning grounds during spawning season in
autumn and early winter, the migration is only observed in mature size fish. The fish collected in our survey is immature and stay in the same area around estuary and coastal area. Consequently, in the grey mullet, a discussion of the reason for seasonal changes in VTG should be done without any regard for the influence of spawning migration. Thus, we conceived that seasonal changes in VTG levels is influenced by seasonal changes in environmental EEs.

Although abnormal gonads were not found in fish collected from Jeju and Yeosu in Korea and Nagasaki and Fukuoka in Japan, testis-ova were found at Ansan, Tongyeong, and Busan in Korea and at Omuta in Japan. The grey mullet is a gonochoristic species and therefore testis-ova should not be present (Chang et al., 1995). It has been experimentally established that testis-ova can be caused by EEs (Kang et al., 2002, Kang et al., 2003). Therefore, it is likely that the testis-ova found in our study were attributed to EEs. The flounder and the konoshiro gizzard shad in Tokyo Bay and the grey mullet in Tokyo, Osaka, and Fukuoka also had testis-ova (Hara et al., 2001, Soyano et al., 2001, Hashimoto et al., 2000, Hashimoto et al., 2001). Moreover, the flounder collected in the UK had also testis-ova (Allen et al., 1999). These results suggest that EEs, which can induce the testis-ova, exist in coastal areas world wide.

Analysis of chemicals has been carried out in many locations (Auriol et al., 2006). In the UK, natural and synthetic steroidal estrogens were detected in the drain of the sewage works (Desbrow et al., 1998). In New Zealand, natural and the synthetic estrogens were also
observed in animal wastes and sewage treatment plant effluents (Sarmah et al., 2006). In Japan, natural and synthetic substances having estrogenic activity were found in the Tokyo metropolitan area (Isobe et al., 2001, Isobe et al., 2003). Unfortunately, we do not have data of chemical analysis of the sampling sites. We need to investigate the chemical composition of water and mud in sampling sites to elucidate the mechanism which testis-ova is formed.

There was no correlation between high level of serum VTG and the appearance of testis-ova in this study. It is assumed that the mechanisms of induction of the two abnormalities are different. The expression of VTG is induced immediately under all circumstances, on the other hand, the appearance of the testis-ova may be caused by EEs exposure during the critical period of sexual differentiation. We suggest that the two biomarkers show the following: Abnormal levels of serum VTG level indicate individuals were exposed to EEs in the present or the very near past. The appearance of testis-ova indicates that the individuals were exposed to EEs in the past, especially, during the sexual differentiation period. In this study, the grey mullet might be exposed to EEs during various periods in the life cycle. To understand whether the reproductive function in the grey mullet is adversely affected by EEs in Japanese and Korean coastal areas, further field surveys and exposure experiments are needed.

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Figure captions

Fig. 1. Sampling sites of the grey mullet in Korea and Japan.
a - e show sites in Korea, and f - h show sites in Japan.
a: Ansan, b: Jeju, c: Yeosu, d: Tongyeong, e: Busan, f: Nagasaki, g: Omuta, h: Fukuoka

Fig. 2. Serum VTG levels of collected immature or male grey mullet.

Fig. 3 Photomicrographs of the gonad of the collected grey mullet.
A: gonad of immature fish, B: testis of mature male, C: ovary of mature female, D, E, and F: testis having oocytes, Sg: Spermatogonium, Sc: Spermatocyte, St: Spermatotid, Sp: Sperm, Yo: Yolk stage oocyte, Pn: Perinucleous stage oocyte, T-O: Testis-Ova Scale bar: 50 μm
Fig 1. Aoki et al.
Serum VTG level (µg/ml)

Fig. 2 Aoki et al.
Fig. 3 Aoki et al.
Table 1. Number of individuals, total length (TL), and body weight (BW) of the grey mullet collected from coastal area in Japan and Korea.

<table>
<thead>
<tr>
<th>Sampling sites and dates</th>
<th>Number of individuals</th>
<th>TL (mm) ± SD</th>
<th>BW (g) ± SD</th>
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<tr>
<td>Ansan Nov. 2004</td>
<td>18</td>
<td>445.3 ± 52.8(^a)</td>
<td>780.9 ± 329.6</td>
</tr>
<tr>
<td>Jeju Nov. 2003</td>
<td>25</td>
<td>214.7 ± 38.6(^b)</td>
<td>91.0 ± 55.7</td>
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<tr>
<td>Yeosu Nov. 2003</td>
<td>13</td>
<td>467.7 ± 55.9(^a)</td>
<td>911.5 ± 576.7</td>
</tr>
<tr>
<td>Tongyeong Dec. 2003</td>
<td>19</td>
<td>426.4 ± 21.2(^a)</td>
<td>701.7 ± 108.2</td>
</tr>
<tr>
<td>Busan Dec. 2003</td>
<td>27</td>
<td>486.2 ± 29.3(^a)</td>
<td>836.5 ± 138.8</td>
</tr>
<tr>
<td>Nagasaki Nov. 2003</td>
<td>26</td>
<td>227.9 ± 21.5(^b)</td>
<td>114.7 ± 32.5</td>
</tr>
<tr>
<td>Nagasaki Jul. 2005</td>
<td>27</td>
<td>208.2 ± 42.7(^b)</td>
<td>92.9 ± 72.9</td>
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<tr>
<td>Nagasaki Nov. 2005</td>
<td>15</td>
<td>222.9 ± 30.4(^b)</td>
<td>101.2 ± 44.2</td>
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<tr>
<td>Omuta Oct. 2003</td>
<td>18</td>
<td>211.7 ± 55.3(^b)</td>
<td>105.7 ± 102.8</td>
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<tr>
<td>Omuta Aug. 2005</td>
<td>15</td>
<td>202.8 ± 80.6(^b)</td>
<td>123.0 ± 130.5</td>
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<tr>
<td>Omuta Nov. 2005</td>
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<td>232.4 ± 25.5(^b)</td>
<td>119.7 ± 36.7</td>
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<tr>
<td>Fukuoka Jun. 2005</td>
<td>20</td>
<td>62.3 ± 5.4(^c)</td>
<td>2.6 ± 0.7</td>
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<td>Fukuoka Oct. 2005</td>
<td>10</td>
<td>229.0 ± 67.2(^b)</td>
<td>143.4 ± 129.9</td>
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Results are shown as mean and standard deviation. \(^a\), \(^b\) and \(^c\) groups indicate significant difference (\(p < 0.05\)), respectively.
Table 2. Number of the individuals with testis-ova by observation of histological analysis of the gonad

<table>
<thead>
<tr>
<th>Sampling sites and dates</th>
<th>Number of individuals</th>
<th>Number of individuals with testis-ova</th>
<th>Appearance ratio of testis-ova (%)</th>
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<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Tongyeong Dec. 2003</td>
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<td>15.8</td>
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<tr>
<td>Busan Dec. 2003</td>
<td>27</td>
<td>2</td>
<td>7.4</td>
</tr>
<tr>
<td>Nagasaki Nov. 2003</td>
<td>26</td>
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<td>0</td>
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Table 2 Aoki et al.