Coastal Marine Science 32(1): 57–66, 2008

Genes differentially expressed in testis of a marine fish, the mummichog (*Fundulus heteroclitus*) after exposure to tributyltin oxide

Kazuhiko MOCHIDA*, Katsutoshi ITO, Toshimitsu ONDUKA, Akira KAKUNO and Kazunori FUJII

Fisheries Research Agency, National Research Institute of Fisheries and Environment of Inland Sea, Maruishi 2–17–5, Hatsukaichi, Hiroshima 739–0452, Japan
** E-mail: kmochida@fra.affrc.go.jp*

Received 2 October 2007; Accepted 26 December 2007

Abstract — There is still concern about the effects of organotin compounds (OTs) on marine organisms, and especially on their reproductive systems. We investigated the toxicity of tributyltin oxide (TBTO) on spermatogenesis in a marine fish, the mummichog, *Fundulus heteroclitus*. The primary purpose of this study is to find biomarkers for evaluation of the effects of TBTO on fish spermatogenesis. We used cDNA subtraction and differential display methods to clone genes differentially expressed in the mummichog testis by TBTO exposure. Six downregulated genes have been identified to date. A GenBank homology search revealed that these genes were homologous to the arginase II gene, a testicular type of creatine kinase gene, two different types of dynein heavy-chain genes, a mitochondrial carrier gene, and a functionally unidentified gene. Real-time PCR revealed that exposure of the fish to 4 μg/L of TBTO significantly reduced expression of at least three of the six genes: those encoding creatine kinase, a dynein heavy chain, and the functionally unidentified gene in the testis. In the 4 μg/L exposure group, significant reduction in spermatogenic cell proliferation and significant increase in numbers of apoptotic germ cells in the testis were observed. These genes can therefore be used as biomarkers for evaluating the effects of TBTO on fish spermatogenesis.

Key words: apoptosis, bromodeoxyuridine, cDNA subtraction, differential display, real-time PCR, teleost

Introduction

Organotin compounds (OTs) has been used worldwide as biocides in antifouling paints. Severe toxicity of OTs to marine organisms such as Pacific oysters, marine gastropods, and mud snails has been reported in many countries, including Japan (Fent 1996, Horiguchi et al. 2000). Because of these toxicological impacts, the use of OTs has been strictly regulated by many countries for the last two decades. Monitoring studies have revealed that although concentrations in the water have been declining, OTs have remained in the environment at concentrations that may have sublethal effects such as immunotoxicity on fish and mammals (Boyer 1989, Schwaiger et al. 1992, Rice et al. 1995, Takahashi et al. 1999). Hence, there is still concern about the effects of OTs on marine organisms.

OTs such as tributyltin (TBT) have several toxic effects on fish, including neurotoxicity, behavioral changes, immunotoxicity, and endocrine disruption (Triebskorn et al. 1994, Matthiessen and Gibbs 1998, O'Halloran et al. 1998, McAllister and Kime 2003, Nakanishi et al. 2006). Indeed, TBT can induce masculinization in the Japanese flounder, *Paralichthys olivaceus*, and expression of the P450 aromatase gene is also decreased in this fish (Shimasaki et al. 2003). Irreversible sperm damage has also been induced by TBT in the zebra fish, *Danio rerio* (McAllister and Kime 2003). In addition, Shimizu and Kimura (1987) observed that long-term exposure to tributyltin oxide (TBTO) resulted in marked depression of the gonad somatic index (GSI) in male goby (*Chasmichthys dolichognathus*). Our previous study also showed that TBTO exposure induced severe histological damage in the testis of mummichog, *Fundulus heteroclitus* (Mochida et al. 2007). Thus, it is obvious that TBT has reproductive toxicity in fish. In order to maintain wild populations of fish, we need to clarify the adverse effects of TBT, especially in regard to reproduction.

However, as far as we know, few studies have addressed the molecular mechanism by which TBT might affect fish gametogenesis. Here, we focused on elucidating the effect of TBT on spermatogenesis. First, we used cDNA subtraction and differential display methods in order to detect differential gene expression in the mummichog testes, both control and TBTO-exposed fish. To date, we have identified six differen-

tially expressed genes in the testis of the TBTO-exposed fish. Subsequently, we have produced recombinant proteins coded by four of the six genes, and developed antisera in rabbit to analyze characteristics of the gene expression. In addition, we examined germ cell proliferation by the incorporation ratio of bromodeoxyuridine (BrdU) and frequency of the apoptotic germ cell appearance in the testis after TBTO exposure. We discuss the possibility that quantification of these spermatogenesis-related genes is useful for evaluating the toxicity of TBTO to fish spermatogenesis.

Materials and Methods

Animals

The mummichog (Arasaki strain, Shimizu 1997) has been bred for several years in our laboratory. They were maintained in 1-kL indoor tanks at the National Research Institute of Fisheries and Environment of the Inland Sea at 20 to 25°C under a natural photoperiod and were fed an appropriate commercial diet (C-2000, Kyowa Hakko, Tokyo, Japan) once a day (1% of body weight). Male fish weighing 8 to 15 g with secondary sexual characteristics were used.

Tributyltin treatment

TBTO was purchased from Aldrich (Milwaukee, WI, USA). A stock solution of TBTO was prepared by dilution with a mixture of acetone–HCO-40 (polyoxyethylene hydrogenated castor oil, Nikko Chemicals, Tokyo, Japan) (1 : 1) at 2000 mg/L. To make test substance solutions, the stock solutions were further diluted with tap water in rectangular glass aquaria, and the concentration of TBTO was adjusted to 320 μg/L. As a solvent control solution, the acetone–HCO-40 mixture was diluted with tap water at a concentration of 1.3 mL/L.

The test apparatus was a continuous flow-through system of glass vessels with a minimum of other materials (Teflon tubing and adhesives). The tanks used for the exposure tests were rectangular aquaria with a working volume of 60 L. The test substance solutions and the solvent control solution were fed by a pump (STD-08S, KNF Lab, Trenton, NJ, USA) directly into the exposure tanks at flow rates of 2, 4, 7, or 10 mL/min for TBTO and 0.5 mL/min for the solvent control to adjust the nominal concentrations described below. In the exposure tanks, the test solutions and the solvent control solution were diluted by seawater that had been filtered through sand and activated carbon and then fed from a header tank (flow rate of 320 mL/min). For the ordinary control, seawater alone was supplied to the tank at the same flow rate. At least 10 tank volumes of the test solutions were supplied to each exposure tank per day.

For detection of genes differentially expressed in testis, mummichog were exposed to TBTO at 0, 7, or 10 μg/L which is about half of the 96-h LC0 value reported by Bushong et al. (1988) for 2 weeks. In the experiment to analyze the effective range of TBTO concentration on fish spermatogenesis, fish were exposed to TBTO at 0, 2, or 4 μg/L or to the acetone–HCO-40 mixture as the solvent control at 5 μl/L for 2 weeks. At least five individuals were used in treatment. The experimental fish were fed the abovementioned commercial diet once a day (2% of body weight) and were maintained under a 14 : 10-h light : dark photoperiod. At the end of the exposure period, the fish were euthanized, and their total length, body weight, and gonad weight were measured before further tissue preparation.

Water-quality parameters during the exposure test were as follows: water temperature, 19.4±0.3°C; dissolved oxygen, 7.5±0.1 mg/L; oxygen saturation, 96.2±0.6%; pH, 7.6±0.0; and salinity, 29.8±0.1 psu.

Chemical analysis

In the TBTO exposure test, water samples were taken once a week to determine the actual concentrations of TBTO in each experimental group. On day 3 and 10, 200-mL samples were taken from the 2- and 4-μg/L tanks and solvent control tanks. The concentrations of TBTO in these samples were then analyzed in accordance with the method described previously (Mochida et al. 2007) by capillary gas chromatography with mass spectrometric detection on a gas chromatograph (model 5890 Series II, Hewlett-Packard, Palo Alto, CA, USA).

Detection of proliferating germ cells and apoptotic germ cells

Proliferation of germ cells was assayed by the immunohistochemical detection of 5-bromo-2′-deoxyuridine (BrdU) incorporated into replicating DNA. Before sampling, fish were injected intraperitoneally twice, 12 h apart, with a mixture of BrdU (Sigma, St Louis, MO, USA) and 5-fluoro-2′-deoxyuridine (Sigma) (10 : 1, respectively, by weight) at 25 μg/g body weight after the mixture was adjusted to 1.25 mg/mL by dissolution in an adequate volume of 10 mM phosphate-buffered saline (PBS) [pH 7.2 to 7.4] .

At the time of sampling, fish were euthanized and the testes were freshly removed. One part of the testis was fixed with Bouin's solution for 4 to 5 h at room temperature. Another part of the testis was prepared for total RNA purification as described below. The tissues were washed with PBS for 2 days (four or more changes) at 4°C, and then embedded in paraffin (melting point, 54°C). The tissues were sectioned at a thickness of 5 μm and then rinsed in 0.3% hydrogen peroxide in methanol for 20 min to eliminate endogenous peroxidase activity. After being washed with PBS, the sections were treated with 10% normal goat serum in PBS to prevent non-specific binding of antibodies. They were then incubated overnight at 4°C with an anti-BrdU mouse monoclonal anti-

body (Sigma) at a dilution of 1 : 200. Subsequently, the sections were incubated at room temperature for 3 h with biotinylated goat anti-mouse IgG (Dako, Glostrup, Denmark) at a dilution of 1 : 400 and then for 30 min with streptavidin–biotin–horseradish peroxidase (Dako). After each section was washed with 50 mM Tris-HCl (pH 7.6) (TB), 3.3′-diaminobenzidine (0.1 mg/mL) in TB containing 0.03% H_2O_2 was applied to visualize the peroxidase reaction products. To examine proliferation, the percentage of cysts containing germ cells labeled with the anti-BrdU mouse monoclonal antibody among 50 randomly selected cysts was calculated for each individual.

Another section was used for the detection of apoptotic germ cells. Apoptotic germ cells were visualized by ApopTag Plus Peroxidase *In Situ* Apopotosis Detection Kit (Chemicon International, Temecula, CA, USA) in accordance with the manufacturer's instructions. The frequency of the apoptotic germ cell occurrence was determined by counting the cells in five randomly selected fields, each 200 μm^2 in area, for each experimental group.

Detection of differentially expressed genes

For total RNA isolation, the testes were removed and immediately immersed in RNAlater (Ambion, Austin, TX, USA). These samples were stored at −20°C until use.

Genes differentially expressed in the testes of the TBTO-treated fish were detected by subtraction of cDNA with a PCR-Select cDNA Subtraction Kit (BD Biosciences, Palo Alto, CA, USA) or a Delta Differential Display Kit (BD Biosciences) in accordance with the manufacturers' instructions. Briefly, total RNA from the testis of mummichog exposed to TBTO at 0, 7, or 10 μg/L for 2 weeks was prepared by using ISOGEN (Nippon-Gene, Tokyo, Japan). Poly(A)+RNA was isolated from total RNA by using Oligo(dT)-latex beads (Oligotex dT30, Takara, Otsu, Japan). The isolated 1 μg of poly(A)+RNA from the testes of fish treated with 0 μg/L (controls), 7, or 10 μg/L TBTO was converted into cDNA with an oligo(dT) primer using AMV reverse transcriptase or MMLV reverse transcriptase for the cDNA subtraction or the differential display, respectively, according to the above mentioned manufacturers' instructions. The synthesized cDNA was then amplified by PCR. One part of the cDNA was labeled with digoxigenin (DIG) by using DIG-HIGH Prime (Roche, Bazel, Switzerland) and used for cDNA cloning as a probe.

cDNA cloning and sequencing

A library of cDNA derived from the mummichog testis was constructed in λZAPII phage vector (Stratagene Cloning Systems, Cedar Creek, TX, USA) in accordance with the manufacturer's instructions. A total of 10^5 plaques were screened with the subtracted probe. Clones that were positive after two rounds of screening were subcloned into pBluescriptII SK-plasmid by *in vivo* excision. In cases where the 5′-terminus of the clone was truncated, 5′-rapid amplification of cDNA ends (5′-RACE) was performed with a 5′-RACE System for Rapid Amplification of cDNA Ends (Gibco BRL, Gaithersburg, MD, USA). Both strands of the insert cDNA were sequenced by the primer-walking method with a DYEnamic ET Terminator Cycle Sequencing kit (Amersham Biosciences, Buckinghamshire, UK) and an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). Sequence analysis and comparison were performed with DNASIS software (Hitachi, Tokyo, Japan). Homology searching against the amino acid sequence deduced from the obtained cDNAs was done with a FASTA Sequence Similarity Search protein query (Genome Net, Kyoto, Japan).

Preparation of antiserum to recombinant proteins

The pCold I DNA vector (Takara) inserted into the partial frame of the cloned cDNA was constructed as follows. A PCR-amplified fragment corresponding to the partial protein-coding region of the abovementioned cloned cDNA was subcloned into pCR II vector (Invitrogen, Carlsbad, CA, USA). The primer pairs used for each cDNA are shown in Table 1. The pCR II was digested with the appropriate restriction enzymes. The digested fragment was inserted into the corresponding sites of pCold I vector, which could produce a fusion protein with six histidine (6×His) residues in *Escherichia coli*.

The pCold I was introduced into the *E. coli* BL21 strain. A colony of the transformants was inoculated into LB medium (10 g/L bactotrypton, 5 g/L yeast extract, 10 g/L NaCl) and grown at 37°C until the OD600 reached to 0.4 to 0.5. Fusion protein production was induced by incubation at 15°C for 30 min, then 1 mM isopropyl-β-D-thiogalactopyranoside was added to the medium, which was further incubated for 24 h at 15°C. Cells were pelleted by centrifugation at 4°C and resuspended in 50 mM NaH_2PO_4, 8 M urea, 300 mM NaCl, 20 mM imidazole (pH 8.0), containing 1 mM phenylmethylsulfonyl fluoride. The suspension was subjected to sonication (Vibra Cell, Sonics, Newtown, CT, USA) and centrifuged again at 10000×g for 10 min. From the final supernatant, 6×His-tagged protein was purified with Histrap (Amersham Biosciences) in accordance with the manufacturer's instructions. The purified 6×His-tagged protein was dissolved in 50 mM NaH_2PO_4, 2 M urea, 2 mM imidazole (pH 8.0) containing 1 mM phenylmethylsulfonyl fluoride (PMSF).

Specific antiserum to the 6×His-tagged protein was obtained from a rabbit immunized with a 2 mL solution containing 500 μg of the purified 6×His-tagged protein mixed with an equal volume of Freund's complete adjuvant. The rabbit received four subcutaneous injections at a 2-week interval.

Table 1. Predicted proteins encoded by genes differentially expressed in the testis of the mummichog, *Fundulus heteroclitus*, after exposure to TBTO, and primer pairs used in recombinant protein production

Predicted protein	Method	Size of open reading frame (bp)	Predicted molecular weight of protein (kD)	GenBank accession number	Primer pair
Arginase II	DD	1047	37.6	AB290198*	F: 5′-GCCGCATATGGACGTGAAGTTCCCGAGG-3′ R: 5′-GCCGCTCGAGCAGAGACGACGCAATGAC-3′
Creatine kinase	cDNA subtr.	1161	43.3	AB290197*	
DD-4 (unknown)	DD	1059	39.9	AB365518	F: 5′-CCGGATCCCATATGGCCTCTGTTGCAGCA-3′ R: 5′-CCGGATCCCTCGAGCACCAGCGCGCCGCT-3′
Dynein heavy chain I	cDNA subtr.	12360	467.6		F: 5′-GCCGCATATGATTAAGAAAGCACTGAC-3′ R: 5′-GCCGCTCGAGTTAGTCAAGATGTTTCCCTGT-3′
Dynein heavy chain II	DD	11979	454.5		F: 5′-GCCGCATATGGAGCGTGAATCCAAGGA-3′ R: 5′-GCCGCTCGAGTTACTGTCCCAGGTTTAAGGC-3′
Mitochondrial carrier	DD	1425	53.1	AB365519	

DD, differential display; cDNA subtr., cDNA subtraction.; F, forward primer; R, reverse primer. *, published in Mochida et al., 2007

Immunohistochemistry

Serial paraffin sections of mummichog testis were prepared in accordance with the method described above. The sections were first rinsed in 0.3% hydrogen peroxide in methanol for 30 min to eliminate endogenous peroxidase activity. After being washed with PBS, the sections were treated with Target Retrieval Solution (Dako) for 1 h at 70°C. The sections were blocked with 10% normal goat serum in PBS to prevent nonspecific binding of antibodies, and they were then treated overnight at 4°C with rabbit antiserum to the recombinant proteins obtained by the method described above. Subsequently, the sections were incubated at room temperature for 3 h with biotinylated goat anti-rabbit IgG (Dako) at a dilution of 1 : 200 and with streptavidin-biotin complex HRP (Dako) for 30 min. After the sections had been washed with 50 mM TB, 3,3′-diaminobenzidine (0.1 mg/mL) in TB containing 0.03% H_2O_2 was applied to visualize the localization of the antigen. To confirm specificity, preimmune serum was substituted for the antiserum.

Electrophoresis and western blot analysis

Milt freshly stripped from male mummichog was centrifuged at 10000×*g* for 15 min at 4°C. The precipitated spermatozoa were washed twice with PBS and then used for further analysis. Fish were euthanized and the testes freshly removed and then homogenized with a polytron homogenizer in 50 mM Tris-HCl (pH 8.0), containing 1 mM ethylenediamine tetraacetic acid, 1 mM PMSF, and 0.25 M sucrose. The homogenate was centrifuged at 1000×*g* for 20 min at 4°C, and then at 12000×*g* for 20 min at 4°C. The pellet from this second was then centrifuged by high-speed centrifugation, and the resulting supernatant was further centrifuged at 100000×*g* for 1 h at 4°C with a Himac CP80α ultracentrifuge (rotor, RP55T; Hitachi, Tokyo, Japan) to separate the microsomal fraction and the cytosolic fraction.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis was performed by a method described previously (Laemmli, 1970). Samples containing 80 μg protein, as determined by Lowry's method, were pretreated with 2% SDS or with 0.1% SDS and then applied to a precast 5% to 20% gradient polyacrylamide gel (ATTO, Tokyo, Japan). After electrophoresis, the gels were stained with Coomassie Brilliant Blue R-250 (Bio-Rad Laboratories, Hercules, CA, USA). Molecular weights were estimated with a protein molecular weight marker (Sigma Marker Wide Range; Sigma, or HiMark Unstained HMW protein standard, Invitrogen).

Unstained gels were transferred to a polyvinylidene difluoride membrane (Immobilon-P; Millipore, Bedford, MA, USA) by a method described previously (Towbin et al., 1979). Before immunostaining, the membrane was incubated for 30 min with 5% skim milk in Tris-buffered saline (TBS; 20 mM Tris-HCl [pH 7.5], containing 500 mM NaCl) to block non-specific protein binding sites. The membrane was then incubated overnight at 4°C with rabbit antiserum to the 6×His-tagged proteins obtained by the method described above. This was followed by treatment for 1 h at room temperature with alkaline-phosphatase–conjugated goat anti-rabbit IgG (Sigma) at a dilution of 1 : 10000 in TBS. After washing the membrane with TBS, nitro blue tetrazolium (NBT, Promega, Madison, WI, USA) and 5-bromo-4-chloro-3-indolyl-phosphate (BCIP, Promega) in 100 mM Tris-HCl (pH 9.5), 100 mM NaCl, and 50 mM $MgCl_2$ (TNM) was applied to visualize the alkaline phosphatase reaction products. The reaction mixture was composed of 330 μg of NBT and 165 μg of BCIP in every 1 ml of TNM.

Real-time quantitative PCR

Total RNA was prepared as described above from the testes of fish exposed to various concentrations of TBTO for 2 weeks. After treatment with DNase I (Takara), the isolated

Table 2. Primer pairs and probes used in real-time quantitative PCR measurement

cDNA	Primer pair	Labeled hybridization probes
Arginase II*	F: 5′-GCTAAACCATTCCTCTCTTCA-3′	FITC (3′ end): 5′-CCCAGGTTCTTTAGAATGTGGTGCTCTCCG-3′
	R: 5′-AGATGGTCAAGAGCAACTTC-3′	LCRed640 (5′ end): 5′-GGTCAACGTCCCGCAGTCCAATGTACAC-3′
Creatine kinase*	F: 5′-GCACAACGACAACAAGACC-3′	FITC (3′ end): 5′-ACGAGGAGGACCACCTGCGTGTGATT-3′
	R: 5′-AGAACGTAACCCAGATGTC-3′	LCRed640 (5′ end): 5′-CCATGCAGAAAGGCGGCAACATGAAG-3′
DD-4	F: 5′-GTTGTACGAGACCAAATCCTTC-3′	FITC (3′ end): 5′-AGGCTCTGTTCCTGGAGGCGGTCAAACT-3′
	R: 5′-GATGACCTTGATGCCCTTGT-3′	LCRed640 (5′ end): 5′-CAAGTCGGACCTGGAGCACTTCATGCT-3′
Dynein heavy chain I	F: 5′-GAGACAGGTCGGCTGACAGA-3′	FITC (3′ end): 5′-CGCAGGGTCTTGTAGACAGGACAGAGGTAGAT-3′
	R: 5′-GGCGGTCACGTAATTGTTGG-3′	LCRed640 (5′ end): 5′-CACAGAAGTTGGAGGTCTACGGTTGGCTTTT-3′

F, forward primer; R, reverse primer; FITC, fluorescein isothiocyanate; LCRed640, LightCycler Red 640. *, published in Mochida et al., 2007

total RNA was converted into cDNA with an oligo(dT) primer and AMV reverse transcriptase by a First Strand cDNA Synthesis kit for RT-PCR (AMV) (Roche). Synthesized cDNA was stored at −30°C until use. Standard samples were prepared as follows: first, PCR was performed with the above mentioned mummichog testis cDNA by using the primer pairs described in Table 2. Then the PCR product (~200 bp) was subcloned into the pT7Blue-2 T-Vector (Novagen, Madison, WI, USA), and the plasmid was used as a standard sample after determination of the gene concentration. A series of increasing concentrations of the standard sample (10^3 to 10^8 copies/μL) was used to construct a standard curve.

Real-time PCR for quantitative detection of the genes was performed on LightCycler (Roche) with the hybridization probes listed in Table 2. Hybridization probes were synthesized on the basis of the open reading frame sequences of the genes. Mummichog testis cDNA or the standard plasmid was added to a master mix of Light Cycler FastStart DNA Master Hybridization Probes (Roche) with each primer pair and hybridization probe. PCR amplification was performed in LightCycler (Roche). All quantitative PCR was performed in duplicate for each cDNA sample. The relative expression of target genes was calculated by comparison with those of the standard plasmid. Specificity of the amplified products was confirmed by dissociation curve analysis.

Statistics

Because the data distributions were skewed, the expression levels of the genes were log transformed for further statistical analysis. Data were analyzed by one-way analysis of variance followed by Dunnett's multiple comparison tests. All statistical analyses were performed with the Microsoft Excel add-on software MacTOUKEI ver. 1.5 (Esumi, Tokyo, Japan). Differences were regarded as significant at $P<0.05$.

Results and Discussion

Genes differentially expressed in testis after exposure to TBTO

Six differentially expressed genes in the testis of the TBTO-treated fish were identified by the cDNA subtraction method and differential display method (Table 1).

The gene encoding arginase, mArg, was identified as a downregulated gene in the testis by differential display method. The cDNA sequence of mArg has been reported in a previous paper (Mochida et al. 2007). A GenBank homology search revealed that mArg showed a high level of similarity to the arginase II gene reported in other vertebrates. Arginase exists in two forms and distributes in a wide range of tissues. A cytosolic form, arginase I, is highly expressed in the liver and is responsible for the hydrolysis of arginine to urea and ornithine (Cederbaum et al. 2004). Arginase II is thought to be more widely expressed and involved in the biosynthesis of polyamines, ornithine, proline, and glutamate. It is highly expressed in the prostate, and probably plays an important role in the function of human sperm (Elgün et al. 2000; Cederbaum et al. 2004). Western blot analysis of subcellular fractions of the testis by using antiserum to recombinant mArg revealed that mArg protein at approximately 37 kD was especially abundant in the cytosol of the testis but was almost none in the spermatozoa (Fig. 1). Other immune-positive signals, at approximately 62 kD and 87 kD, were observed in the other subcellular fractions of the testis, but not in the sperm (Fig. 1). The molecular weights of these proteins suggest that they are not dimers or trimers of mArg protein, but perhaps hetero-dimeric or hetero-timeric proteins formed with mArg protein. Further study is needed to clarify the molecular structures of these proteins. Immunohistochemistry using antiserum to the recombinant mArg demonstrated that the protein existed in spermatocytes and spermatids, and in somatic cells such as Sertoli cells and interstitial cells of the mummichog testis (Fig. 2A and B), although its exact localization on the testicular somatic cells was not clear yet. Because no immune-positive signal was observed on spermatogonia and

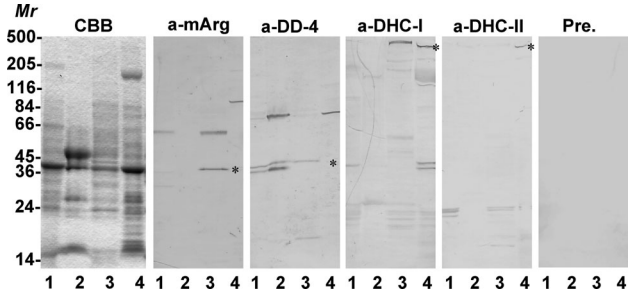

Fig. 1. Western blot analysis of several differentially expressed genes proteins in subcellular fractions of mummichog testis and sperm. The left-most panel is a 5% to 20% polyacrylamide gel stained with Coomassie Brilliant Blue (CBB). Other panels show western blots probed with polyclonal rabbit antiserum to recombinant mArg (a-rec. mArg), DD-4 (a-rec. DD-4), DHC-I (a-rec. DHC-I), DHC-II (a-rec. DHC-II), and preimmune serum as a negative control (Pre.) Asterisks in each panel indicate positions of intact form of each protein. Numbers at the left are molecular weight standards (x 10^{-3}), below; 1, high speed centrifugation pellet of testis; 2, sperm; 3, cytosolic fraction of testis; and 4, microsomal fraction of testis.

spermatozoa, mArg protein is not expressed in these germ cells. This result supports the fact that mArg protein was not detected in sperm by western blot analysis.

The creatine kinase gene (mCK) which is downregulated in the testis of the mummichog after exposure to TBTO has been also identified (Mochida et al. 2007). Creatine kinase (CK) is an enzyme participating in an energy shuttle that utilizes phosphocreatine to transfer the energy from ATP generated by the mitochondrion in the sperm midpiece to axonemal dynein (Tombes and Shapiro 1985). Besides the mCK gene characteristics shown in Table 1, as has been previously reproted (Mochida et al. 2007), the protein encoded by the mCK gene has a unique amino terminal sequence. Garber et al. (1990) cloned a unique type of CK with 14 amino-terminal residues from rainbow trout testis that are unlike any CK sequence previously reported. They revealed by northern blot analysis that CK transcripts are expressed almost exclusively in the testis, and they identified it as a testicular type of CK. The characteristics of the mCK sequence are similar to those of the CK sequence (Garber et al. 1990). In addition, northern blot analysis using a cRNA probe synthesized on the basis of the cDNA sequence of the amino terminal region revealed that the transcript of the mCK gene was expressed specifically in the testis (Mochida et al. 2007). Thus, the mCK gene was also confirmed as a testicular type of CK gene in this study. As has been previously demonstrated by *in situ* hybridization that mCK mRNA was expressed in the spermatocytes and spermatids before spermiogenesis in the testis (Mochida et al. 2007).

A GenBank homology search revealed that the DD-4 gene showed similarity to the genes encoding uncharacterized proteins obtained in several mammalian species. The function of the putative protein is unpredictable, and the gene is tentatively called Differential Display (DD)-4. Western blot analysis using antiserum to recombinant DD-4 protein revealed that this protein were abundant in the the spermatozoa (Fig. 1), which characterized by three protein bands at approximately 33 kD, 38 kD, and 75 kD. Because of the molecular weight of DD-4 protein predicted from the cDNA sequence (i.e. 39.9 kD), the 38-kD band and 75-kD band correspond to an intact form and a dimeric form of the DD-4 protein. It is likely that the 33-kD protein band is the degradation product of the DD-4 protein. Immunohistochemical study using anti-recombinant DD-4 protein also demonstrated that the signal was detected especially on the spermatids (Fig. 2D): a weak signal was also observed on the spermatozoa. The DD-4 protein may have a specific function in spermiogenesis and/or spermatozoa.

In this study, the genes encoding two forms of axonemal dynein heavy chain (DHC) have been found downregulated by TBTO exposure. One of the genes was cloned by the cDNA subtraction method and the other was cloned by the differential display method. The clone from the cDNA subtraction is tentatively named as DHC-I and the other one from the differential display as DHC-II. Dynein is a motor protein involved in axonemal beating in the sperm tail (Gibbons 1981). Western blot analysis demonstrated that anti-DHC-I and anti-DHC-II antisera gave signals at approximately 500 kD, and the molecular weight of DHC-I protein was slightly greater than that of DHC-II protein (Fig. 1 and Table 1). The molecular weight of DHC-I protein in the cytosol was slightly higher than that in the microsomal fraction (Fig. 1). Although the reason for this remains unknown, the cytosolic form of DHC-I may have some kind of modification. In addition, immunohistochemical study using anti-DHC-I antiserum and anti-DHC-II antiserum showed that DHC-I protein was localized in the tail region of the spermatids and DHC-II protein was localized in the head region (Fig. 2E and F, respectively). Weak signals in the spermatozoa were also observed using the two antisera (data not shown). Although a homology search indicated that both DHC-I and DHC-II are of the axonemal dynein type, but the possibility cannot be denied that DHC-II belongs to the cytoplasmic dynein type identified in mammals (Vallee and Shpetner 1990). Further study is needed to clarify the exact localization of these two forms of dynein. DHC-I protein is thought to play a role in flagellar beating of spermatozoa as a motor protein and also in spermiogenesis, including in the sperm maturation process. The amount of DHC protein in the testis may become a biomarker for evaluation of the TBT toxicity to the number and quality of spermatozoa.

So far only sequence information on the sixth gene, the mitochondrial carrier protein gene has been sequenced. A homology search revealed that this gene encodes a protein belonging to the mitochondrial solute carrier family, which

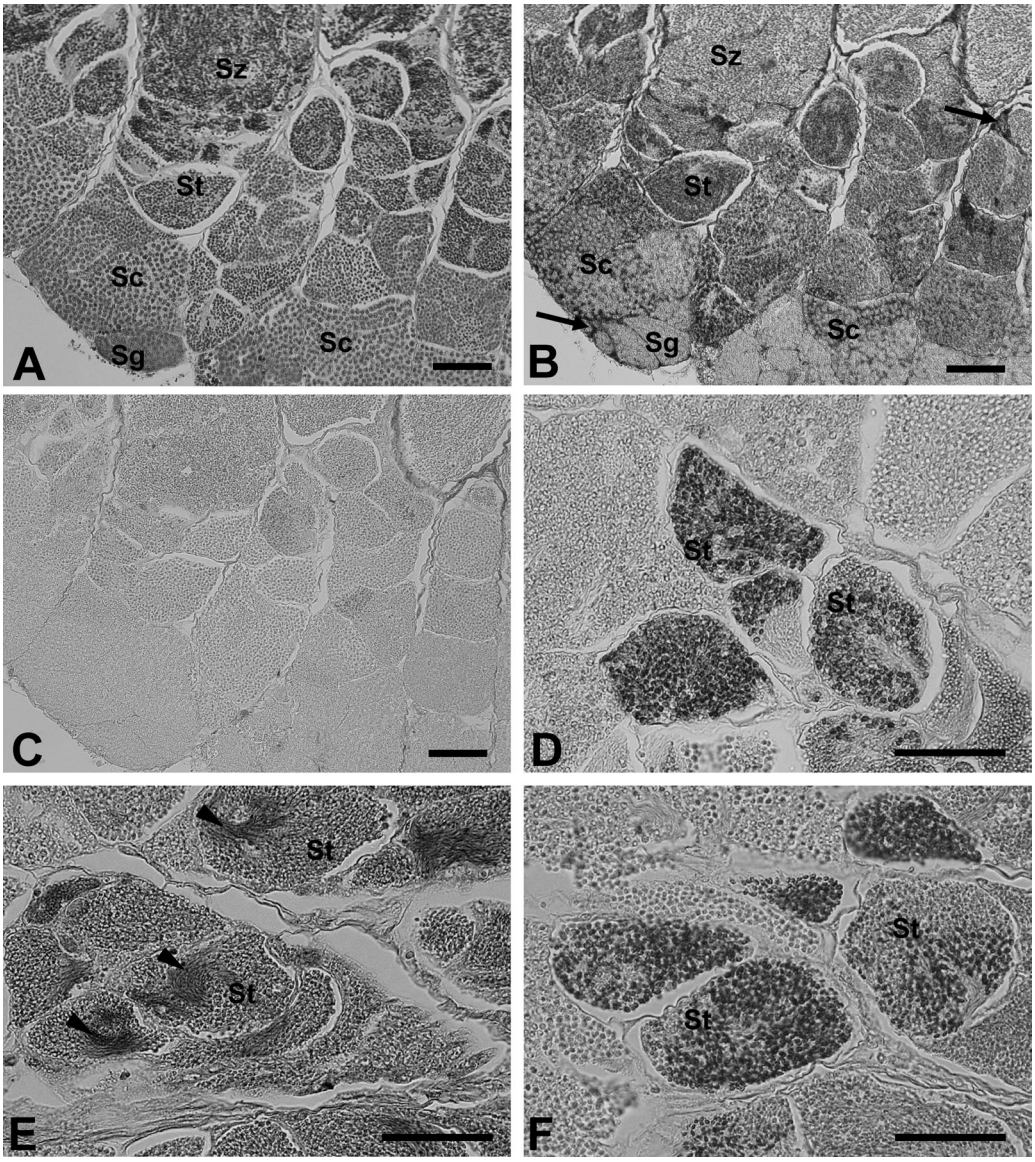

Fig. 2. Localization of the mArg protein, DD-4 protein, DHC-I protein, and DHC-II protein in testis of mummichog. Semi-adjacent sections of testis stained with hematoxylin-eosin (A) and immunostained with anti-rec. mArg protein (B), preimmune serum as a negative control (C), anti-rec. DD-4 (D), anti-rec. DHC I (E), and anti-rec. DHC II (F). Sg, spermatogonia; Sc, spermatocytes; St, spermatid; Sz, spermatozoa. Arrows in B indicate positive signals on testicular somatic cells such as Sertoli cells and interstitial cells. Arrowheads in E indicate tail region of spermatids. Bars, 50 μm.

supports a variety of transport activities across the mitochondrial inner membrane (Kuan and Saier Jr. 1993). Several studies have demonstrated that this protein family plays an indispensable role in spermatogenesis and sperm maturation in the epididymis of mammals (Griffin et al. 2005, Sipilä et al. 2006, Brower et al. 2007). One family of proteins encoded by these genes is the adenosine nucleotide translocases (Ant), which facilitate the transport of ADP and ATP across the inner mitochondrial membrane and play an important role in cellular energy metabolism (Brower et al. 2007). A novel member of the *Ant* family in mice, *Ant4*, is selectively expressed in testicular germ cells, and in *Ant4*-deficient mice a significant decrease in testicular size is observed owing to a severe reduction of spermatocytes, accompanied by apoptosis (Brower et al. 2007). Although the specific function of the mitochondrial solute carrier protein, the gene for which was recognized downregulated by TBTO exposure in the present study, remains unknown, it is likely that the protein plays a crucial role in spermatogenesis.

Germ cell proliferation activity and frequency of the apoptotic germ cell appearance after exposure to TBTO

The average TBTO concentrations in the test solutions were not detected (control and solvent control), 2.1 (nominal concentration, 2 μg/L), and 3.9 (nominal concentration, 4 μg/L).

Because depression of the proliferation of spermato-

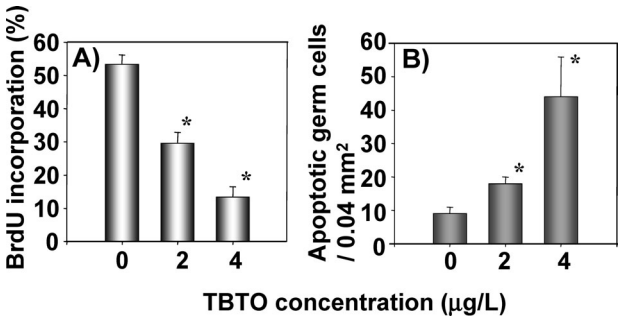

Fig. 3. Changes in germ cell proliferation A) and frequency of appearance of apoptotic germ cells B) in mummichog testis after exposure to various concentrations of TBTO for 2 weeks. Asterisks indicate that values are significantly different from control value.

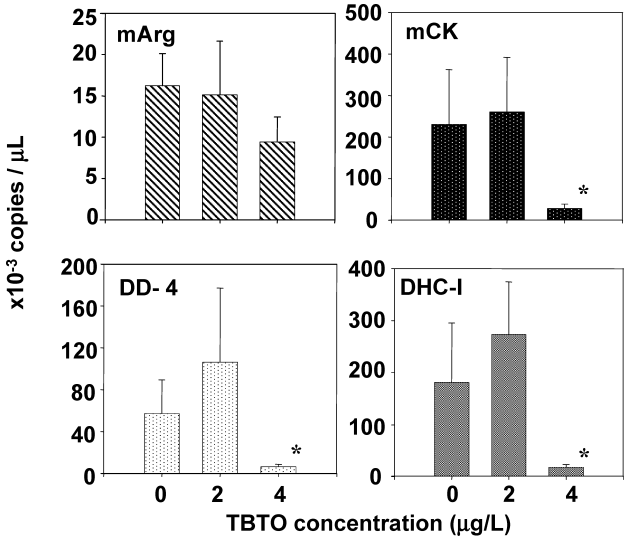

Fig. 4. Expression of genes encoding arginase II (mArg), creatine kinase (mCK), DD-4, and dynein heavy chain I (DHC-I) in testis of mummichog after exposure to various concentrations of TBTO for 2 weeks; quantified by real-time PCR assay. Asterisks indicate that the values are significantly different from that of the control (0 μg/L).

genic cells and the occurrence of apoptotic germ cells is directly correlated with a reduction in the numbers of spermatozoa, these cell biological analyses are excellent markers for the evaluation of toxic effects on reproduction. After exposure of fish to TBTO, proliferation of germ cells was monitored by BrdU incorporation (Fig. 3A). BrdU incorporation declined in a concentration-dependent manner. No significant difference was observed between the control group and the solvent control group (data not shown). This result indicates that the solvent alone had no effect on germ cell proliferation. In the 2-μg/L and 4-μg/L treatment groups, the values were significantly lower than in the control group (0 μg/L) ($P<0.05$). Immunopositive cysts usually contained spermatocytes and spermatids. No difference between spermatocytes and spermatids was observed in terms of inhibition of BrdU incorporation by exposure to TBTO. The frequency of apoptotic germ cell occurrence increased in a concentration-dependent manner (Fig. 3B). Apoptosis was usually seen in spermatocytes, and apoptotic spermatogonia were also observed in some cases. In the 2-μg/L and 4-μg/L treatments, the values were significantly higher than in the control group (0 μg/L) ($P<0.05$).

These results obviously indicate that TBTO causes a reduction in the number of germ cells and an induction of apoptosis. However, the toxicological mechanism for the two processes remains unknown. In terms of apoptosis, it is intriguing to discuss the relationship between arginine metabolism and apoptosis induction. Nitric oxide (NO), which is a messenger molecule synthesized from arginine by NO synthase (NOS), plays important roles in vascular regulation, immune response, neurotransmission, and other systems (Wu and Morris Jr. 1998, Mori 2007). However, excessive amounts of NO cause apoptosis through endoplasmic reticulum stress (Mori 2007). Arginase (Arg) can downregulate the production of NO by decreasing intracellular arginine concentration (Mori and Gotoh 2000). In macrophages, NOS and Arg activities are regulated interchangeably by cytokines, and this may help the efficient production of NO (Mori and Gotoh 2000). Incidentally, considerable correlations have been established between Arg activity in the seminal plasma and sperm dysfunction (Elgün et al. 2000). In addition, it has been suggested that NO plays a role in the modulation of spermatogenesis, sperm motility, and sperm maturation (Perera et al. 1996). Although it remains unclear whether Arg and NOS are co-expressed in testicular germ cells, it would be useful to elucidate the toxic effect of TBT on the activities of the two enzymes.

Quantification of several genes in the testis after exposure to TBTO

After exposure to TBTO for 2 weeks, the levels of the genes encoding mArg, mCK, DD-4, and DHC-I in the testis were measured by quantitative real-time PCR assay. The levels of expression of all genes tested were unchanged at 2 μg/L, and the levels were significantly lower than in the controls at 4 μg/L ($P<0.05$) except for the gene encoding mArg (Fig. 4). There was tendency for the expression level of the mArg gene to decrease at 4 μg/L, but the difference in levels from the control was not statistically significant. Incidentally, the levels of the genes encoding DHC-II and mitochondrial carrier in the testis are under investigation.

In light of the fact that the DD-4 and DHC-I proteins exist mainly in spermatozoa, the genes encoding them are thought to be expressed in the earlier stages of sperm generation, such as in the spermatocytes and spermatids. In addition, we have already demonstrated by *in situ* hybridization that the mCK gene is expressed in spermatocytes and spermatids (Mochida et al. 2007). Real-time quantitative PCR

demonstrated that the expression levels of the mCK, DD-4, and DHC-I genes were decreased after exposure to TBTO. This result is coincident with our previous histological observations that germ cells such as spermatids and spermatozoa at the late stage are the first cells to be impaired by TBTO toxicity (Mochida et al. 2007). The inhibition mechanism of the gene expression has not been clarified, yet. Analysis of the promoter regions of these genes may explain whether the transcription activity of these genes is directly or indirectly inhibited by TBTO.

Expression of the mArg gene was not greatly affected by the TBTO exposure (Fig. 4). The differences in expression pattern among genes may be due to differences in their localization site. The immunohistochemical results suggest that the mArg gene is localized in the testicular somatic cells as well as the germ cells such as spermatocytes and spermatids, whereas the other genes such as the mCK, the DD-4, and the DHC-I gene are localized especially in the spermatids, which are among the first cells to be impaired by TBTO toxicity (Mochida et al. 2007). Considering the characteristic of the mArg gene expression being not greatly influenced by TBTO exposure, it is advantageous to make use of the gene as an internal control gene in real-time quantitative PCR to identify target genes by toxicity of TBTO exposure more specifically. We have already applied the mArg gene as an internal control gene and demonstrated that the mCK gene was one of specific targets by toxicity of TBTO (Mochida et al. 2007).

Thus, real-time quantitative PCR revealed that exposure to TBTO induced a significant reduction in the testicular gene expression, with the excepetion of the mArg gene. In the 4-μg/L TBTO treatment, this reduction was coincident with that of BrdU incorporation into the spermatogenic cells and also with the appearance of apoptotic germ cells. Thus these genes can be used as biomarkers for TBTO toxicity effects on fish spermatogenesis.

Surveillance from 1989 to 1990 in some coastal areas of Japan showed that concentrations of TBT in the water ranged from undetectable to 94 ng/L (Harino et al. 1998, Takahashi et al. 1999, Inoue et al. 2002). The sensitivity of real-time quantitative PCR using the genes may not be environmentally relevant for the evaluation of TBTO toxicity in fish spermatogenesis, because significant reduction in the expression of the genes was observed at TBTO level of 4-μg/L. McAllister and Kime (2003) reported that the result of TBT exposure at an environmentally relevant level (10 ng/L) on zebrafish from hatching to 70 days examined sperm quality in adults 3–5 months after cessation of exposure, sperm motility was significantly lower than that of the control group and all sperm lacked flagella. Although the authors did not mention histological alteration in testis and depression of some spermatogenesis-related genes, it is possible that these changes occurred in the zebrafish testis. In addition, TBT is highly concentrated in fish tissue (Yamada et al. 1994). With regard to the effect of long-term exposure of the environmentally relevant concentrations of TBT on spermatogenesis, further study should be conducted on various fish species. Toxic effect of long-term exposure of the ng/L-level of TBTO on mummichog spermatogenesis is now under investigation in our laboratory.

There are likely many other genes that are differentially expressed in the testis of TBTO-treated mummichog. Analysis of the unidentified genes will be necessary to understand the mechanism of TBTO toxicity to fish spermatogenesis. In addition, some of those genes would be useful as biomarker at lower level of xenobiotics, including TBT. Further analysis of genes differentially expressed in the testis after exposure to TBTO is still remained for future studies.

Acknowledgement

We are grateful to Dr. Satoshi Arima and Ms. Chiaki Hiramoto of the National Research Institute of Fisheries and Environment of Inland Sea, Fisheries Research Agency, for their kind assistance. This work was supported in part by a Grant-in-aid (Hazardous Chemicals) from the Ministry of Agriculture, Forestry, and Fisheries of Japan (HC-07-2232-1).

Literature cited

Boyer, I. J. 1989. Toxicity of dibutyltin, tributyltin and other organotin compounds to humans and to experimental animals. Toxicology 55: 253–298.

Brower, J. V., Rodic, N., Seki, T., Jorgese, M., Fliess, N., Yachnis, A. T., McCarrey, J. R., Paul Oh, S. and Terada, N. 2007. Evolutionarily conserved mammalian adenine nucleotide translocase 4 is essential for spermatogenesis. J. Biol. Chem. 282: 29658–29666.

Bushong, S. J., Hall Jr., L. W., Hall, W. S., Johnson, W. E. and Herman, R. L. 1988. Acute toxicity of tributyltin to selected Chesapeake Bay fish and invertebrates. Wat. Res. 22: 1027–1032.

Cederbaum, S. D., Yu, H., Grody, W. W., Kern, R. M., Yoo, P. and Iyer, R. K. 2004. Arginases I and II: do their functions overelap? Mol. Genet. Metab. 81: S38–S44.

Elgün, S., Kaçmaz, M., Sen, I. and Durak, I. 2000. Seminal arginase activity in infertility. Urol. Res. 28: 20–23.

Fent, K. 1996. Ecotoxicology of organotin compounds. Crit. Rev. Toxicol. 26: 1–117.

Garber, A. T., Winkfein, R. J. and Dixon, G. H. 1990. A novel creatine kinase cDNA whose transcript shows enhanced testicular expression. Biochim. Biophys. Acta 1087: 256–258.

Gibbons, I. R. 1981. Cilia and flagella of eukaryotes. J. Cell Biol. 91: 107s–124s.

Griffin, K. P., Ward, D. T., Liu, W., Stewart, G., Morris, I. D. and Smith, C. P. 2005. Differential expression of divalent metal transporter DMT1 (Slc1 1a2) in the spermatogenic epithelium of the developing and adult rat testis. Am. J. Physiol. Cell Physiol. 288: C176–C184.

Harino, H., Fukushima, M., Kawai, S. and Megumi, K. 1998, Measurement of butyltin contamination of water and sediment in Osaka Bay, Japan. Appl. Organometal. Chem. 12: 819–825.

Horiguchi, T., Takiguchi, N., Cho, H. S., Kojima, M., Kaya, M., Shiraishi, H., Morita, M., Hirose, H. and Shimizu, M. 2000. Ovotestis and disturbed reproductive cycle in the giant abalone, *Haliotis madaka*: possible linkage with organotin contamination in a site of population decline. Mar. Environ. Res. 50: 223–229.

Inoue, S., Oshima, Y., Imada, N. and Honjo, T. 2002. Tributyltin pollution in coastal areas around northern Kyushu (in Japanese with English abstract). Jpn. J. Environ. Toxicol. 5: 43–50.

Kuan, J. and Saier, Jr., M. H. 1993. The mitochondrial carrier family of transport proteins: structural, functional, and evolutionary relationships. Crit. Rev. Biochem. Mol. Biol. 28: 209–233.

Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227: 680–685.

Matthiessen, P. and Gibbs, P. E. 1998. Critical appraisal of the evidence for tributyltin-mediated endocrine disruption in mollusks. Environ. Toxicol. Chem. 17: 37–43.

McAllister, B. G. and Kime, D. E. 2003. Early life exposure to environmental levels of the aromatase inhibitor tributyltin causes masculinisation and irreversible sperm damage in zebrafish (*Danio rerio*). Aquat. Toxicol. 65: 309–316.

Mochida, K., Ito, K., Kono, K., Onduka, T., Kakuno, A. and Fujii, K. 2007. Molecular and histological evaluation of tributyltin toxicity on spermatogenesis in a marine fish, the mummichog (*Fundulus heteroclitus*). Aquat. Toxicol. 83: 73–83.

Mori, M. 2007. Regulation of nitric oxide synthesis and apoptosis by arginase and arginine recycling. J. Nutr. 137: 1616S–1620S.

Mori, M. and Gotoh, T. 2000. Regulation of nitric oxide production by arginine metabolic enzymes. Biochem. Biophys. Res. Commun. 275: 715–719.

Nakanishi, T., Nishikawa, J. and Tanaka, K. 2006. Molecular targets of organotin compounds in endocrine disruption: do organotin compounds function as aromatase inhibitors in mammals? Environ. Sci. 13: 89–100.

O'Halloran, K., Ahokas, J. T. and Wright, P. F. A. 1998. Response of fish immune cells to in vitro organotin exposures. Aquat. Toxicol. 40: 141–156.

Perera, D. M. D., Katz, M., Heenbanda, S. R. and Marchant, S. 1996. Nitric oxide synthase inhibitor N^G-monomethyl-L-arginine preserves sperm motility after swim-up. Fertil. Steril. 66: 830–833.

Rice, C. D., Banes, M. M. and Ardelt, T. C. 1995. Immunotoxicity in channel catfish, *Ictalurus punctatus*, following acute exposure to tributyltin. Arch. Environ. Contam. Toxicol. 28: 464–470.

Schwaiger, J., Bucher, F., Ferling, H., Kalbfus, W. and Negele, R.-D. 1992. A prolonged toxicity study on the effects of sublethal concentrations of bis(tri-*n*-butyltin) oxide (TBTO): histopathological and histochemical findings in rainbow trout (*Oncorhynchus mykiss*). Aquat. Toxicol. 23: 31–48.

Shimasaki, Y., Kitano, T., Oshima, Y., Inoue, S., Imada, N. and Honjo, T. 2003. Tributyltin causes masculinization in fish. Environ. Toxicol. Chem. 22: 141–144.

Sipilä, P., Pujianto, D. A., Shariatmadari, R., Nikkilä, J., Lehtoranta, M., Huhtaniemi, I. T. and Poutanen, M. 2006. Differential endocrine regulation of genes enriched in initial segment and distal caput of the mouse epididymis as revealed by genome-wide expression profiling. Biol. Reprod. 75: 240–251.

Shimizu, A. 1997. Reproductive cycles in a reared strain of the mummichog, a daily spawner. J. Fish Biol. 51: 724–737.

Shimizu, A. and Kimura, S. 1987. Effect of bis(tributyltin) oxide on gonadal development of a salt-water goby, *Chasmichtys dolichognathus*: exposure during maturing period (in Japanese with English abstract). Bull. Tokai Reg. Fish. Res. Lab. 123: 45–49.

Takahashi, S., Tanabe, S., Takeuchi, I. and Miyazaki, N. 1999. Distribution and specific bioaccumulation of butyltin compounds in a marine ecosystem. Arch. Environ. Contam. Toxicol. 37: 50–61.

Tombes, R. M. and Shapiro, B. M. 1985. Metabolite channeling: a phosphorylcreatine shuttle to mediate high energy phosphate transport between sperm mitochondrion and tail. Cell 41: 325–334.

Towbin, H., Staehelin, T. and Gordon, J. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc. Natl. Acad. Sci. USA 76: 4350–4354.

Triebskorn, R., Köhler, H.-R., Flemming, J., Braunbeck, T., Negele, R.-D. and Rahmann, H. 1994. Evaluation of bis(tri-*n*-butyltin)oxide (TBTO) neurotoxicity in rainbow trout (*Oncorhynchus mykiss*). I. behaviour, weight increase, and tin content. Aquat. Toxicol. 30: 189–197.

Vallee, R. B. and Shpetner, H. S. 1990. Motor proteins of cytoplasmic microtubues. Annu. Rev. Biochem. 59: 909–932.

Wu, G.. and Morris Jr., S. M. 1998. Arginine metabolism: nitric oxide and beyond. Biochem. J. 336: 1–17.

Yamada, H., Tateishi, M. and Takayanagi, K. 1994. Bioaccumulation of organotin compounds in the red sea bream (*Pagrus major*) by tow uptake pathways: dietary uptake and direct uptake from water. Environ. Toxicol. Chem. 13: 1415–1422.