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Reproductive Inhibition and Transgenerational Toxicity of Triphenyltin on Medaka (Oryzias latipes) at Environmentally Relevant Levels

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An increasing number of studies have reported unexpectedly high body burdens of triphenyltin (TPT) in wild fishes around the world. To assess the effects of TPT on fish, we exposed pairs of medaka (Oryzias latipes) to different levels of TPT for 5 weeks, and the reproduction responses and transgenerational effects were studied. The results demonstrated that TPT exposure markedly suppressed the spawning frequency, spawned egg number, egg quality and gonad development, and induced teratogenesis, such as hemorrhaging, eye defects, morphological malformation, and conjoined twins, less hatchability, and swim-up failure in the F1 generation, thereby resulting in a significant decrease in the capacity to produce viable offspring (p < 0.01). The residual TPT levels in the exposure fish are in the range of 6.52 ± 0.56 to 5595 ± 1016 ng of TPT/g of wet weight, similar to those reported in wild fish around the world, indicating TPT contamination in the real world would have a significant adverse effect on the health of fish population. Down-regulation of vitellogenin (VTG) genes in the female of the TPT exposure groups was recognized as a cause for the decreased fecundity. Expressions of VEGFs and PAX6 associated with vascular or ocular development, respectively, were measured in hemorrhaging and eye defects embryos and showed good correlations with response outcomes.

Introduction

Two triorganotin compounds, triphenyltin (TPT) and tributyltin (TBT) have contaminated the aquatic environment globally for their worldwide uses as fungicides and pesticides in agriculture and as ingredient of antifouling paints for ship hulls, fishnets, etc. (1, 2). In previous studies, much attention has been paid to the pollution of TBT due to the facts that TBT dominated over TPT in usage amount in the ingredient of antifouling paints and the inducement of imposex in female of gastropods by TBT at environmentally relevant levels (1, 3, 4). Although the uses of organotin antifouling paints have been prohibited by countries and the International Maritime Organization, TPT compounds are continuing to be widely used as contact fungicide to treat crops. Recently, an increasing number of studies have reported unexpectedly high levels of TPT in predatory fishes around the world (5–12), because TPT can be easily biomagnified through the aquatic food web (7, 9, 12). Therefore, the effects of the bioaccumulated TPT on fish are of great concern.

Studies have demonstrated that TPT exposure inhibits cytochrome P450 aromatase (13), the key enzyme catalyzing the conversion of testosterone to estrogen (14), and significantly suppresses the levels of plasma vitellogenin (VTG) (15), the precursor of yolk protein, which is essential ingredient for vitellogenesis in oocyte development normally induced by 17β-estradiol (E2) (16), in fish. And the experiment based on mammal cells revealed that TPT could inhibit 11β-hydroxysteroid dehydrogenase type 2 (11β-HSD2) and 17β-hydroxysteroid dehydrogenase (17β-HSD) (17, 18). In teleost, 11β-HSD2 plays an important role in conversion of 11β-hydroxytestosterone (11β-OHT) to 11-ketotestosterone (11-KT), a major androgen in fish (19), and 17β-HSD is the enzyme catalyzing the conversion of estrone (E1) to E2 and interconversion between androstenedione and testosterone (20, 21). Considering androgens and estrogens play critical roles in gonad development and gametogenesis, the effects of TPT on the gonad development and reproduction of fish may not be negligible. In addition, TPT can be highly accumulated in eggs via the maternal transfer (22), and early life stages of fish are known to be very vulnerable to TPT as exemplified by the lethal effect in fathead minnow (Pimephales promelas) larvae (96 h LC50s of 7.1 μg/L) (23) and teratogenic potential for inducing abnormal eye development and spinal deformation in European minnows (Phoxinus phoxinus) and zebrafish (Danio rerio) (24, 25). Thus, it is necessary to investigate the effects of maternally transferred TPT on embryonic and larval stages for better understanding the effects of TPT on fish development in the real world.

In this paper, we investigated the effects of TPT on fish reproduction and offspring development at environmentally related concentration by exposing adult Japanese medaka (Oryzias latipes) to TPT. Expression of genes involved in several biological processes was also analyzed to better understand the toxicities and to explore the underlying mechanism.

Materials and Methods

Animals and TPT-Cl Exposure. Adult Japanese medaka (Orange-Red strain), 5 months old, with body weight 650 mg/body length 32 mm, were selected from brood stock maintained for several years at our laboratory. Triphenyltin chloride (TPT-Cl) was purchased from Wako (Osaka, Japan). Chemical-stock solutions were prepared in dimethyl sulfoxide (DMSO) and the ratio of chemical-stock solution/water was 0.005% (v/v). Twelve breeding pairs of the fish were used in each of the 1.6, 8, 40, 200, and 1000 ng/L TPT-Cl exposure group or vehicle control (0.005% DMSO), with a final volume of 10 L in glass tanks. A flow-through system with a 4-fold volume of water flowing through every 24 h was used, and the concentrations of the water were kept to the designed exposure doses. The water used in the experiment was activated carbon treated with hardness 81.1 ± 0.3 mg/L calcium carbonate, pH 7.9 ± 0.1, dissolved oxygen 7.8 ± 0.3 mg/L, un-ionized ammonia 0.012–0.020 mg/L, temperature 25 ± 1 °C. The fish were kept under a constant 16:8 h light:dark photoperiod and fed with live brine shrimp (Artemia salina) twice a day. Whole exposure was continued for 5 weeks without mortality. After the exposure, the fish were sampled and the gonad, brain, and liver were isolated from each fish. Half of the gonad was fixed in 10% neutral buffered formalin for histological analysis, and liver, brain, and the

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TABLE 1. Relative Expressions of Significantly Regulated Genes in Liver, Gonad, and Brain of Medaka (exposure: 5 weeks; 200 ng/L TPT-Cl).

<table>
<thead>
<tr>
<th>tissue</th>
<th>gene symbol</th>
<th>sex</th>
<th>fold changed*</th>
</tr>
</thead>
<tbody>
<tr>
<td>liver</td>
<td>UGT2A3</td>
<td>female</td>
<td>2.87 ± 0.49b</td>
</tr>
<tr>
<td></td>
<td></td>
<td>male</td>
<td>2.36 ± 0.54b</td>
</tr>
<tr>
<td></td>
<td>CYP1A</td>
<td>female</td>
<td>2.62 ± 1.58b</td>
</tr>
<tr>
<td></td>
<td></td>
<td>male</td>
<td>2.15 ± 0.87b</td>
</tr>
<tr>
<td></td>
<td>CYP2A1</td>
<td>male</td>
<td>2.39 ± 0.57b</td>
</tr>
<tr>
<td></td>
<td></td>
<td>female</td>
<td>2.91 ± 1.08b</td>
</tr>
<tr>
<td>gonad</td>
<td>17/βHSD1</td>
<td>male</td>
<td>2.35 ± 0.57b</td>
</tr>
<tr>
<td></td>
<td>CYP19A</td>
<td>female</td>
<td>2.88 ± 0.38b</td>
</tr>
<tr>
<td></td>
<td></td>
<td>male</td>
<td>5.58 ± 1.32b</td>
</tr>
<tr>
<td>brain</td>
<td>CYP19B</td>
<td>female</td>
<td>3.97 ± 0.63b</td>
</tr>
<tr>
<td></td>
<td></td>
<td>male</td>
<td>1.65 ± 0.49</td>
</tr>
</tbody>
</table>

* The change folds were counted against control. Data are presented as means ± standard deviation (n = 8). b p < 0.05 compared with control.

FIGURE 1. Decreases of fecundity caused by TPT-Cl exposure. (A) Spawning frequency; (B) spawned egg number per female/day; (C) fertilization success. Data are presented as means ± standard deviation. * indicates p < 0.05; **, p < 0.01.

left-half of the gonad was frozen in liquid nitrogen till RNA isolation. And the remains (excluding head, gill, and gut) of the fish were frozen at −20 °C for TPT analysis.

Evaluation of Reproductive Success. During the last week of the exposure, eggs from the females were carefully collected daily a few hours after oviposition and gently separated. Spawning frequency and mean number of spawned eggs per spawning female were calculated. The collected eggs of each female were separately cultured in a glass plate using carbon-treated water. Fertilized eggs were identified under a microscope before late morula (Stage 9) and fertilization success was estimated by calculating the number of fertilized eggs relative to that of spawned. In addition, total protein content of egg was determined with a bicinchoninic acid (BCA) protein assay kit (Novagen, Rockford, IL) for ten randomly selected eggs of each group per day during the fourth week of the exposure.

Observation of Embryonic or Larval Development. All the fertilized eggs of females in one group each day were collected together and cultured in a glass plate using carbon-treated water without TPT exposure for studying the transgenerational toxicity. A microscope was used to observe the embryonic and larval development. Embryos were observed each day till hatch or death. And larvae were observed from hatching to 10 days posthatch (dph). The embryonic or larval malformations were sorted and recorded, and their occurrence frequencies were determined in all the groups. Hatching success and swim-up success were calculated by hatched fries/fertilized eggs and swim-up fries (10 days posthatch, dph)/hatched fries, respectively.

Histological Examination. The gonad samples were fixed in the 10% neutral buffered formalin for more than 24 h, and then dehydrated in a graded series of ethanol solutions, embedded in paraffin blocks according to standard methods. Sections were cut at 5–10 µm and stained with hematoxylin and eosin. Slides were examined by light microscopy for routine histology and morphometrics, and histological measurements were taken using an ocular micrometer.

Quantitative Real-Time RT-PCR Assay. RNA preparation, first-strand cDNA synthesis and quantitative real-time PCR assay were performed mainly according to the methods in our previous paper (26), and the details were provided in Supporting Information. On the basis of the symptoms observed in the exposed fish and F1 generation, as well as knowledge from previous studies, 103 genes involved in sex differentiation and gametogenesis, steroids metabolism and steroidogenesis, signal transduction, retinoid synthesis and metabolism, and embryonic development were analyzed to explore the underlying mechanism of the observed toxicities (Table S1 in the Supporting Information). All the primer sequences were showed in Table S2 (Supporting Information). Ribosomal protein L7, a housekeeping gene, was used as the internal control (26), and relative expression was evaluated by the 2−∆∆Ct method provided by Applied Biosystems (Foster City, CA, USA).

TABLE 2. Differential Expression of Genes (with 2 or More Fold Change) in Abnormal Development Embryos from TPT-Cl Exposure Groups

<table>
<thead>
<tr>
<th>gene symbol</th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
<th>E</th>
<th>F</th>
<th>G</th>
<th>H</th>
</tr>
</thead>
<tbody>
<tr>
<td>VEGFa</td>
<td>-3.37</td>
<td>-5.89</td>
<td>-2.65</td>
<td>-3.32</td>
<td>-2.98</td>
<td>-1.66</td>
<td>-1.77</td>
<td>-1.19</td>
</tr>
<tr>
<td>VEGFc</td>
<td>-3.22</td>
<td>-3.86</td>
<td>-3.51</td>
<td>-3.01</td>
<td>-3.09</td>
<td>-1.89</td>
<td>-1.11</td>
<td>-1.10</td>
</tr>
<tr>
<td>PAX6</td>
<td>-1.32</td>
<td>-7.87</td>
<td>-3.98</td>
<td>-1.56</td>
<td>-2.65</td>
<td>-2.45</td>
<td>-4.78</td>
<td>-2.13</td>
</tr>
</tbody>
</table>

* The change folds were counted against normal embryos from control. A, embryo that was retarded in development and with hemorrhaging (3 dpf); B, eyeless embryo with hemorrhaging (3 dpf); C, small eye embryo with hemorrhaging (3 dpf); D, hemorrhaging embryo (6 dpf); E, embryos that were deformed, hemorrhaging, and with small eye (6 dpf); F, single eye embryo (6 dpf); G, eyeless embryo (9 dpf); H, single eye embryo (9 dpf).
the exposure. The extraction and analysis of these chemicals were mainly according to the methods described in previous papers (9, 27), and the details are provided in the Supporting Information.

Statistical analyses. The statistical program SPSS (version 11.5; Chicago, IL) was used to collate and analyze all the collected data. Differences were evaluated by ANOVA followed by Tukey’s test.

Results

Reproductive Inhibition. During the fifth week of the TPT-Cl exposure period, the spawning frequency, i.e., the fraction of females spawning per day, and the number of eggs for each spawning female per day significantly decreased in the 8, 40, 200, and 1000 ng/L TPT-Cl groups, compared with that of the control (panels A and B in Figure 1), whereas fertilization success was not significantly affected by TPT exposure with the exception of a transient decrease in the 200 ng/L group (Figure 1C). The eggs spawned from the TPT-Cl exposure groups were found to be colorless as shown in Figure 2A, and the protein content in the newly spawned eggs was significantly (*p < 0.05) decreased in all the TPT-Cl exposure groups (Figure 2B). Histological examination presented retarded-development ovaries in the fish which never spawned during the last week of exposure from in the TPT-Cl exposure groups. As shown in Figure 2C, a decrease of mature oocytes and an increase of preovulatory atretic follicles were observed in the retarded-development ovaries.

To clarify the potential mechanism for egg colorlessness, reduction of egg protein, preovulatory atretic follicle, and even the decreases of spawning frequency and number of spawned eggs, we studied expressions of vitellogenin (VTG) genes (VTG-1 and VTG-2) in the liver. And dose-dependent transcriptional inhibitions of both VTG-1 and VTG-2 were found in female medaka exposed to TPT-Cl (Figure 2D). Because the transcription of VTG is dependent on estrogen activation in fish, the genes for enzymes involved in biosynthesis and metabolism of estrogens were determined in control and the 200 ng/L TPT-Cl exposure group. Table 1 shows the relative expressions of significantly (*p < 0.05) regulated genes in the liver, gonad and brain of medaka in the 200 ng/L TPT-Cl exposure group. Expressions of 17β-hydroxysteroid dehydrogenase type 1 (17β-HSD1) and CYP19A in the ovary and CYP19B in the brains of females were

| TABLE 3. Residue Levels (ng/g wet weight) of TPT and its Metabolites (DPT and MPT) in Fish of Control and TPT-Cl Exposure Groups* |
|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| sex             | control         | 1.6             | 8               | 40              | 200             | 1000            |
| TPT female      | ND              | 6.52 ± 0.56     | 28.9 ± 5.73     | 141 ± 9.18      | 720 ± 113       | 4919.86 ± 571   |
| male            | ND              | 8.39 ± 2.22     | 36.6 ± 8.12     | 215 ± 29.8      | 949 ± 102       | 5595 ± 1016     |
| DPT female      | ND              | ND              | ND              | 10.43 ± 0.83    | 75.1 ± 11.3     | 370 ± 68.4      |
| male            | ND              | ND              | ND              | 17.13 ± 1.76    | 92.9 ± 16.3     | 455 ± 59.0      |
| MPT female      | ND              | ND              | ND              | ND              | 5.15 ± 2.57     | 13.4 ± 1.74     |
| mMale           | ND              | ND              | ND              | ND              | 4.56 ± 1.16     | 21.8 ± 6.53     |

* Data are presented as means ± standard deviation (n = 8).
significantly suppressed ($p < 0.05$), and those of CYP1A, CYP2A1 and UDP glucuronosyltransferase 2A3 (UGT2A3) involved in metabolism of estrogen were significantly increased ($p < 0.05$) in the liver. In addition, the 11\textbeta-HSD2 was up-regulated 1.67 ($0.72$ folds in male and $1.31$ ($0.56$ folds in female of the 200 ng/L TPT-Cl exposure group, but no significant difference from that of control was found ($p > 0.05$).

**Transgenerational Toxicity.** Several kinds of developmental abnormalities have been observed in embryo and larvae of the F1 generation from TPT-Cl exposure groups. Hemorrhaging occurred in spots of the vascular network was observed in embryos from the TPT-Cl exposure groups (Figure 3 A, C, and D). Generally, the hemorrhaging embryos also exhibited retarded development. And the frequency of hemorrhaging embryos significantly increased with increasing TPT-Cl exposure concentrations (Figure 3M). Abnormal ocular development as indicated by eyelessness (single eye or no eyes), small eyes and abnormally shaped eyes in embryos and larvae were observed in the TPT-Cl exposure groups (Figure 3A, E–I). Abnormal ocular development occurred in all exposure groups at embryonic stage, and larvae with eye defects were found in the 8 ng/L or higher dose groups (Figure 3N). Morphological abnormalities, including deformed embryo (Figure 3E), lordosis larvae with retarded yolk sac resorption (Figure 3J) and larvae with curled bodies or tails (Figure 3K), were observed in embryo and larvae from the TPT-Cl exposure groups. The frequency of the larval morphological deformation was analyzed and found to be dose-dependent (Figure 3O). In addition, several cases of conjoined-twin (images C and L in Figure 3) were observed in the embryos or larvae from TPT-Cl exposure groups. Since many embryos with severe developmental defects died before hatching and most of the abnormal larvae could not be fed and died in several days post hatch, hatching success (Figure 3A) and swim-up success (Figure 3B) were both significantly decreased in all the TPT-Cl exposure groups ($p < 0.05$). As an overall result, the mean number of surviving swim-up larvae per female each day was found to be significantly decreased in all the TPT-Cl exposure groups ($p < 0.01$), as shown in Figure 3C.

**Expressions of several genes involved in vascular, ocular, and skeletal development were evaluated in the abnormal development embryos.** Table 2 shows the differential expressions of genes with 2 or more folds change in abnormal development embryos from TPT-Cl exposure groups. Transcripts of vascular endothelial growth factors (VEGFa and VEGFc) in hemorrhaging embryos at 3 dpf (days postfertilization) and 6 dpf were down-regulated. Transcription levels of the paired box gene 6 (PAX6) decreased in eyeless, small eye, and abnormally shaped eye embryos at 3–9 dpf.

**Residue TPT Levels in Fish and Eggs.** The accumulated body burdens of TPT and its metabolites, diphenyltin (DPT) and monophenyltin (MPT), were measured in both sexes of the fish in this study (Table 3). TPT was detected in all exposure groups and its level was much higher than those of DPT and MPT. The residue levels of TPT were higher in males than in females in each group, which indicated the
maternal transfer of TPT as found in a previous study (22). In fact, TPT was detected in eggs collected from the medaka after 4 week exposure, and the concentrations were 1.02, 4.64, 21.2, 117, and 876 ng/g of ww, corresponding with 1.6, 8, 40, 200, and 1000 ng/L TPT-CI groups, respectively. And the DPT was only detected in eggs from the 200 and 1000 ng/L TPT-CI groups with the concentrations of 2.38 and 16.1 ng/L TPT-CI groups with the concentrations of 2.38 and 16.1 ng/g of ww, respectively. No MPT was detected in the egg samples. The bioconcentration factor (BCF) of TPT was estimated to be 5038 – 6161 in male and 3882 – 5418 in female in the TPT-CI exposure groups, which were similar to those reported in Pagrus major (3100 – 3300) and Rudarius erodecaes (4100) exposed to TPT-CI for 8 weeks in laboratory (28) and the Empirical BCFs in Western Scheldt fish species (12). The accumulated body burden of TPT in this study covered a broad range of TPT levels (~4216 ± 186 ng/g of wet weight) in wild fish reported in recent years as reviewed in Table S3 (Supporting Information).

Discussion

This study demonstrated that TPT-CI exposure caused decrease of fecundity, including the lower spawning frequency (Figure 1A) and the reduced egg number (Figure 1B), poor quality of eggs as exemplified by the colorlessness of eggs (Figure 2A) and the decline of egg protein (Figure 2B), retarded development of ovary (Figure 2C), and down-regulation of VTG-1 and VTG-2 (Figure 2D) in medaka. As a histological finding, retarded development of ovary was found to be an important cause for the decreased fecundity. At molecular level, the down-regulations of VTG were consistent with the decrease of plasma VTG level reported in medaka after TPT exposure (15). Since VTG is essential material for vitellogenesis, oocyte maturation and yolk biosynthesis in fish (14, 16), the down-regulations of VTG are suspected to be a cause for the reduced egg quality and the retarded development of ovary. And on the basis of the linkage of alterations in plasma VTG levels to adverse effects in fecundity illustrated in female fathead minnows (Pimephales promelas) by 21 day laboratory toxicity tests with aromatase inhibitor fadrozole (29), the down-regulations of VTG genes in the female of the TPT exposure groups should be recognized as an important reason for the decreased fecundity. Considering that TPT inhibits the activity of cytochrome P450 aromatase (2, 13) and transcription of VTGs is strongly dependent on the presence of estrogen in fish (30), the down-regulated VTGs are regarded as the consequence of lower estrogen levels, which was also supported by the evidence of down-regulation of CYP19A and 17β-HSD1 genes in gonads and CYP19B in the brain, and up-regulation of CYP1A, CYP2A1 and UGT2A3 in the liver of female medaka exposed to TPT-CI (Table 1). Inhibitions of both CYP19A and CYP19B expression have been reported in brain and gonad of developing fugu (Takifugu rubripes) exposed to an aromatase inhibitor (fadrozoie) for a long-term (from the ‘first feeding’ to the 100th day after hatching) (31), whereas no effect on CYP19 transcripts abundance was found after a short-term exposure (7 days) of zebrafish (Danio rerio) female to androstradienedione, an aromatase inhibitor (32). The potential reason for such different phenomena is that the long-term deprivation of E2 due to aromatization inhibition may lead to a decreasing transcript abundance of the CYP19 genes.

Embryonic or larval abnormalities including hemorrhaging, abnormal ocular development, and morphological deformation which were occurred in the F1 generation, are similar to those reported in fish by in ovo exposure of TPT or TBT in previous studies (24, 25, 33), but conjoined twins (images C and L in Figure 3) were reported for the first time in our observation, indicating that even worse effects would be induced by the maternally transferred TPT. The hemorrhaging occurred in spots throughout the vascular network, suggesting it would be caused by impairment of blood vessel development. Because VEGFs are crucial for angiogenesis and play a pivotal role during embryo vasculogenesis (34, 35), the down-regulation of VEGFa and VEGFc (Table 2) observed in this study would be a cause of the hemorrhaging symptom. Nishikawa et al. (2004) reported that TPT can bind the retinoid X receptors (RXRs) with even higher affinity than 9-cis retinoic acid (9-cis RA), the natural ligand of RXRs (36). And RXRs are reported to be involved in suppressing VEGF expression after binding RA (37). The hemorrhaging caused by TPT, was therefore considered to be through the RXR signal pathway.

In the embryos with eye defects (including eyelessness and small eyes), Pax6 was obviously down-regulated. Pax6 is a key regulator of eye development, which conserves in teleosts and mammals (38). Pax6 knockout mice show significant defects in eye development (39). Heterozygous mutations of Pax6 result in the human eye defects (40), and homozgyous Pax6 mutations result in the absence of eyes (41). Pax6 is mediated by RAR/RXR complexes (42, 43), and therefore down-regulation of Pax6 should be the underlying action of the abnormal ocular development induced by TPT through the RXR signal pathway.

In our study, as an overall consequence, the capacity to produce viable offspring significantly (p < 0.01) reduced in all the TPT exposure groups (Figure 4C), through inhibiting
spawning frequency, number of pawed eggs and inducing embryonic or larval death and abnormality in the F1 generation, and therefore would lead to population decline. Miller et al. (29) demonstrated that decreases of VTG concentration of female fathead minnow, caused by chemicals (including aromatase inhibitor), would lead to population declines, and exemplified that a 25% decrease in VTG concentration in females from baseline values would exhibit a 34.6% projected decrease in size after two years of exposure and reach an equilibrium population size that was only 30.2% of the preexposed population (29). It should be noted that the residual TPT concentrations in the TPT exposure groups (Table 3) were similar or even lower than those reported in some marine fishes around the world (see Table S3 in the Supporting Information). This indicated that the reduced capacity to produce viable offspring would be occurred in wild fish. Moreover, unprecedented population declines and species extinction of marine fish, in particular predatory fish, have occurred in the past few decades (44, 45). According to Reynolds et al. (46), “by 2001, based on data from 98 North Atlantic and northeast Pacific populations, marine fish had declined by a median 65% in breeding biomass from known historic levels; 28 populations had declined by more than 80%. Most of these declines would be sufficient to warrant a status of threatened with extinction under international threat criteria” (46). Thus, while overfishing is commonly recognized as the primary cause, the TPT contamination might be another notable factor affecting the health of marine fish population.

Acknowledgments

Financial support from the National Natural Science Foundation of China (40632009, 20777002) and the National Basic Research Program of China (2007CB407304) is gratefully acknowledged.

Supporting Information Available

Additional information, including the methods for gene expression analysis and TPT analysis, the categories and the primer sequences of 105 genes studied in this paper, and the reviewed TPT levels in wild fishes worldwide reported in recent years (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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