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Adverse effects of tributyltin on reproduction of Japanese medaka, *Oryzias latipes*

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Abstract — Tributyltin (TBT) is a typical endocrine disruptor that can induce imposex in mollusks and interfere with the reproduction in bivalves and fish. Our group has been studied the toxic effects of TBT using Japanese medaka (*Oryzias latipes*) as a model organism, and evaluated the effects on reproduction and behavior of medaka exposed to TBT. A series of our studies has demonstrated that TBT suppresses mating behavior in male and depresses fertility. We also observed that TBT exposure causes abnormalities in embryonic development, such as eye deformity, decreased hatchability and swim-up failure, but does not affect sexual development in the progenies from TBT-exposed fish. Furthermore, TBT alters not only the mating behavior but also other general, non-sexual, behaviors. Since behavioral abnormalities may influence individual survivability or populational structure, we believe that it is necessary to evaluate the effects of environmental pollutants on animal behaviors. In addition, the developmental toxicity of TBT was enhanced by co-exposure to PCBs, even when their exposure levels were below the no-observed-effect concentrations of the individual chemicals. Mixture toxicity of environmental contaminants is an emerging issue. It is quite important to emphasize and evaluate mixture toxicities of contaminants at environmentally relevant levels.

Key words: tributyltin, Japanese medaka, reproductive toxicity, behaviors, mixture toxicity

Introduction

The number of anthropogenic chemicals in the environment has dramatically increased with industrial development. Tributyltin (TBT) has been used as an antifoulant in marine environments, and it is a globally ubiquitous contaminant (Yamada et al. 1997, Sudaryanto et al. 2002, Ueno et al. 2004a). Organotin levels in the environment show declining trends in the past few decades (Harino et al. 1999), but these compounds have still been detected in a wide variety of aquatic organisms, even in deep-sea organisms (Takahashi et al. 1997, Harino et al. 2005). Additionally, several studies have revealed that considerably high organotin concentrations have been detected in estuaries and harbors, which are so-called "hot spots" (Leung et al. 2006, Smith et al. 2006). Thus, environmental contamination by organotin compounds is still ongoing, and their effects on marine organisms are of concern.

Tributyltin is known to be typical endocrine disruptor that can induce imposex in mollusks (Gibbs and Bryan 1986, Horiguchi et al. 1997). Horiguchi et al. (2006) suggested that imposex caused by TBT and/or triphenyltin is a leading factor for the decline of ivory shell (*Babylonia japonica*) populations in Japan. In addition, maternal exposure to TBT clearly resulted in developmental toxicity in the progeny of pearl oysters (*Pinctada fucata martensii*; Inoue et al. 2004) and Manila clams (*Ruditapes philippinarum*; Inoue et al. 2006). Thus, TBT severely affects reproduction in aquatic organisms, which may lead to population declines in highly affected species.

Recently, it has been demonstrated that the brain also can be a target organ of TBT. Omura et al. (2004) have demonstrated that a relatively high concentration of TBT is detected in the brain of exposed rats compared to other tissues. In addition, relatively high concentrations of TBT were detected in the brains of several fish species collected from Osaka Bay, Japan (Harino et al. 2000) and of Japanese whiting (*Sillago japonica*) following 67-day dietary administration of TBT (Shimasaki, unpublished data). Waterborne exposure to TBT led to accumulation of the compound or its metabolites in the diencephalon of rainbow trout (*Oncorhynchus mykiss*; Rouleau et al. 2003). In terms of neurotoxic effects, acute exposure to TBT reduced the levels of dopamine, norepinephrine, and serotonin in the whole brain of rats (Elsabbagh et al. 2002). Tsunoda et al. (2004) also reported that subacute exposure to TBT altered dopamine metabolism in the midbrain of mice. Thus, TBT accumulates in the brain and may act as a neurotoxicant, which could alter animal behavior.

Behaviors such as schooling, feeding, anti-predatory behavior and sexual behavior may directly influence the survival of individuals and reproduction at the population level, and alteration or disruption of these behaviors could cause

'ecological death' (Scott and Sloman, 2004). Many contaminants are known to affect behavior in aquatic organisms. Poor feeding ability was observed in adult and larval mummichogs (*Fundulus heteroclitus*) collected from a site contaminated with polychlorinated biphenyls (PCBs) and heavy metals (Weis et al. 2001, 2003). The suppression of sexual behavior was also reported in Japanese medaka (*Oryzias latipes*) exposed to 17β-estradiol (E2; Oshima et al. 2003) and to octylphenol (OP; Gray et al. 1999). Scott and Sloman (2004) and Jones and Reynolds (1997) reviewed the effects of environmental pollutants on fish behavior and reproductive behavior, respectively. TBT has been reported to affect swimming activity and orientation of rainbow trout (Triebskorn et al. 1994) and thornfish (*Terapon jarbua* Forsskål; Wang and Huang 1998). It has also been reported that TBT affects antipredatory behavior in three-spine stickleback (*Gasterosteus aculeatus* L; Wibe et al. 2001). These behavioral abnormalities may lead to decreased survivability and reproductive ability.

TBT by itself has severe effects on sexual differentiation or maturation, reproduction, and behavior in aquatic organisms, but organisms inhabiting polluted areas are exposed to complex mixtures of chemicals, not single chemicals. Indeed, it has been reported that skipjack tuna (*Katsuwonus pelamis*) were contaminated with not only butyltin compounds (Ueno et al. 2004a), but also dioxin-like compounds, PCBs and other organochlorines, and brominated flame retardants (Ueno et al. 2003, 2004b, 2005, 2006). Yamada et al. (1997) also have reported that both TBT and PCBs were detectable in the livers of squid collected from the same area (TBT: up to 243 ng g^{-1}, PCBs: up to 280 ng g^{-1}). Therefore, the assessment of mixture toxicity of environmental contaminants is one of the most important and relevant issues in ecotoxicology.

Mixture toxicities of environmental contaminants have been studied using both *in vitro* and *in vivo* testing systems. *In vitro* yeast estrogen screening (YES) assays have demonstrated that mixtures of weak estrogenic chemicals produce significant effects, even when the individual chemicals are combined at below their no-observed-effect concentrations (NOECs; Silva et al. 2002). *In vivo* testing and whole effluent toxicity (WET) testing have been used to elucidate the toxicities of mixtures of environmental contaminants [U.S. Environmental Protection Agency (http://www.epa.gov/waterscience/WET/)] (Markle et al. 2000, Smolders et al. 2002). Kim and Cooper (1998) have also reported that 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) and 3,3′,4,4′,5-pentachlorobiphenyl (PCB 126) additively induce toxic effects in Japanese medaka embryos. Furthermore, mixed exposures to 25 or 50 different organic chemicals below their NOECs additively affect growth or mobility, respectively, of *Daphnia magna* (Hermens et al. 1985, Deneer et al. 1988). Thus, mixtures of chemicals may have more toxic effects compared to single chemicals. Most recent publications, however, only focus on effects of single chemicals; mixture toxicity is less frequently studied, making such research necessary.

In this article, we review a series of our studies evaluating toxicities of TBT using Japanese medaka as a model species (Nakayama et al. 2004a, 2004b, 2005b, 2007, Hano et al. 2007). We observed behavior, reproduction and early-stage development of medaka exposed to TBT. Mixture toxicities of TBT and PCBs were also analyzed, since PCBs are also widely distributed in the environment (Tanabe 2002, Monirith et al. 2003, Ueno et al. 2003, 2004a), and they also show reproductive toxicity in some fish species (Monosson et al. 1994, Örn et al. 1998). The experimental conditions of each test are summarized in Table 1.

Swimming Behavior

We assessed the effects of TBT and PCBs on swimming behavior of male medaka using the procedures described by Oshima et al. (2003) and Nakayama et al. (2004a). Fish were administered 1 μg TBT/g body weight (b.w.)/day, 1 μg PCBs/g b.w./day, or 1 μg TBT+1 μg PCBs/g b.w./day in the diet for three weeks (Table 1). After the exposure period, each treated male was put in another 2.5 L glass chamber, and its behavior while it swam freely was recorded with a CCD video camera for 30 min at 23°C. The first 15 min in the chamber was used as the acclimation period, and the next 15 min was the analysis period. Recorded behavior was analyzed with a two-dimensional Image Tracking Analyzer that traced the swimming trajectory of the medaka every 1/3-s. Mean swimming velocity and position of the fish were measured by the trajectory data. Behaviors were categorized into three types, and we defined the behaviors as resting, swimming in a straight line, and swimming in circles (along the wall of the chamber). The frequencies of these behaviors were counted. One person analyzed the data to minimize artifacts.

To analyze positional dispersion patterns we calculated the entropy of each male. As shown in Figure 1, a side image of the chamber (20×15 cm) was separated into a grid of 20 small rectangles, and the frequencies at which the medaka stayed in each grid area were counted. The counts were then transformed into probability data. The probability that a male was present in a certain area was represented by p_{ij} (i=horizontal grid coordinates 1, 2, 3, 4, 5; j=vertical grid coordinates 1, 2, 3, 4). From the probability, entropy (H) was calculated by the formula shown in Figure 1.

The swimming velocity of male medaka treated with TBT and/or PCBs for three weeks was affected by neither TBT nor PCBs, nor a mixture of both (Table 2). Swimming velocity has been used as an indicator in some acute toxicity

Table 1. Experimental conditions in each exposure test.

Test	Medaka strain	Sex	Stage	Exposed chemical(s)	Temperature (°C)	Photoperiod (light : dark; hr)	Diet
General behavior	Orange-red	Male	Mature	TBT and/or PCBs (33 μg each/g diet)	23	14 : 10	Commercial diet[a)]
Reproduction and mating behavior	Orange-red	Male	Mature	TBT and/or PCBs (33 μg each/g diet)	23	14 : 10	Commercial diet[a)]
Reproduction and early stage development	FLF-II	Male, Mature[d)]	Female[d)]	TBT and/or PCBs (1, 5 or 25 μg TBT/g diet plus 25 μg PCBs/g diet)	25	16 : 8	Freeze-dried brine shrimp flake[b)] *Artemia nauplii*[c)]
Nanoinjection	Orange-red	—	Embryo	TBT (0.16, 0.80, 3.96, 19.2, 82.1 ng TBT/egg)	25–27	—	—

TBT: tri-*n*-butyltin oxide (>95% pure; Tokyo Kasei Kogyo, Tokyo, Japan).
PCBs: polychlorinated biphenyls (PCB-48, equivalent to Kanechlor-400; Tokyo Kasei Kogyo, Tokyo, Japan).
FLF-II: female leucophore free-II
[a)] Particle size,<0.3 mm; purchased from Kyorin, Himeji, Japan.
[b)] TetraDelica®, Tetra Japan, Tokyo, Japan.
[c)] <24 hr after hatching.
[d)] Parental fish.

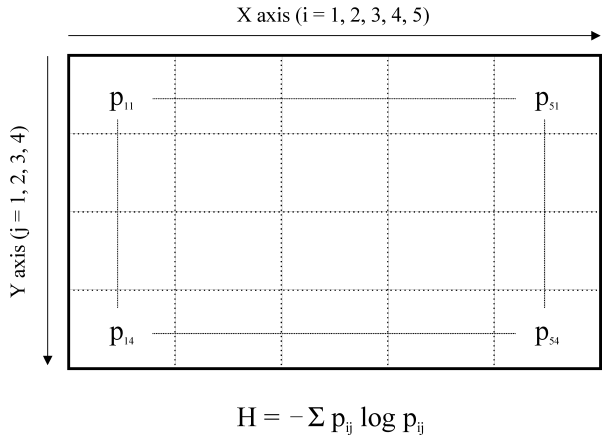

Fig. 1. Calculation of entropy values. Fish behavior was recorded from the side of a glass chamber. The side view of chamber was separated into a 5×4 grid. The probability that the fish would be present in each area was presented as p_{ij}. From the probability values, entropy was calculated by the formula shown in the figure.

tests; this result demonstrated that TBT or PCBs did not exert acute toxic effects on male medaka. Fish exposed to considerable concentrations of toxicants show reduced swimming behavior (Ososkov and Weis 1996, Grillitsch et al. 1999). Gerhardt et al. (2006) detected decreased swimming velocity in medaka exposed to some toxicants, such as fenitrothion, benthiocarb or diazinon. Therefore, we concluded that exposure to TBT and PCBs at the level set in this study does not have any acute toxic effects in male medaka.

The frequencies of swimming straight and swimming in circles were significantly increased by the administration of PCBs ($p<0.001$ for both behaviors; Fig. 2), while TBT alone did not affect the frequency of each behavior, and no additive effect of TBT and PCB co-exposure was detected. These data indicated that PCBs increased the frequencies of changes in behavioral patterns in male medaka, suggesting hyperactive behavior. It is now known that PCBs are neurotoxic and affect behaviors such as motor activity, response acquisition, and spatial discrimination in mammals. Holene et al. (1998) reported that 2,2′,4,4′,5,5′-hexachlorobiphenyl (PCB 153) induced attention deficit hyperactivity disorder in rats, a finding similar to ours in fish.

Table 2. Swimming velocities and entropy values of male medaka in control and treatment groups ($n=12$).

Treatment	Swimming velocity (mm/s)	Entropy value of positions
Control	3.2±1.8	1.00±0.03
TBT	3.0±1.3	1.11±0.03*
PCBs	3.8±1.9	1.05±0.05
TBT+PCBs	2.9±0.9	1.09±0.03*

Data are presented as mean±standard deviation.
*: Significantly different from control ($p<0.05$; two-way analysis of variance).

Entropy value was significantly increased by TBT administration ($p=0.043$; Table 2). The value in the PCB treatment group was slightly higher than in the controls, although the difference was not statistically significant. In this study, medaka in the control group preferred a certain area of the chamber, especially near the chamber walls, and they spent most of the testing time around this area. This behavior may indicate a kind of positional preference in medaka. The relatively low entropy values in the control might be reflective of

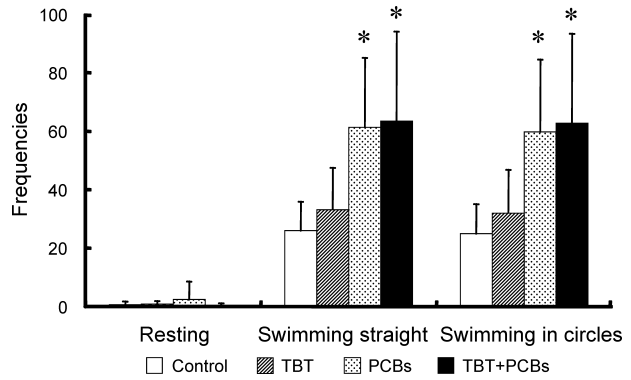

Fig. 2. Frequencies of resting, swimming straight and swimming in circles in male medaka exposed to tributyltin (TBT) and/or polychlorinated biephenyls (PCBs). Data are presented as the mean±standard deviation (n=12). *: PCB-exposure significantly increased the frequency; $p<0.001$; two-way analysis of variance.

this preference. The significant increase in entropy in TBT-exposed medaka indicated a loss of their preference for a specific swimming area. We propose that entropy values are applied to evaluate the orderliness of behavior in fish and other animals, especially in species with territories.

Swimming behaviors of medaka were clearly altered by TBT exposure. Previous studies have reported that TBT affects swimming activity and orientation of rainbow trout (Triebskorn et al. 1994) and thornfish (Wang and Huang 1998). It has also been reported that TBT affects anti-predatory behavior in three-spine stickleback (Wibe et al. 2001). These effects on fish behaviors might be due to neurotoxic effects of TBT. TBT can be detected in fish brain (Harino et al. 2000, Shimasaki, unpublished data), and axonal transport of TBT was also observed in rainbow trout (Rouleau et al. 2003). Therefore, we hypothesize that a potential mechanism for behavioral abnormalities caused by TBT or PCBs might involve brain monoamines, since both compounds alter the concentration of monoamines in the brain (Khan and Thomas 1996, 2001, Elsabbagh et al. 2002, Tsunoda et al. 2004). We also found that administration of TBT decreased dopamine, norepinephrine and serotonin concentrations in the brain of medaka (Nakayama et al. 2007). The concentration of monoamines in fish brains may directly influence their behavior. Serotonin suppresses aggressive behavior (Adams et al. 1996), whereas dopamine stimulates it (Munro 1986, Tiersch and Griffith 1988). In addition, exposure to tryptophan, a serotonin precursor, stimulates serotonergic neurons and increases locomotor activity in medaka (Koutoku et al. 2003). Furthermore, brain monoamines are closely related to the neuroendocrine system, which is responsible for reproduction (Billard et al. 1984; Khan and Thomas 1992; Saligaut et al. 1998). Therefore, imbalance of brain monoamines could play a key role in the reproductive impairment and behavioral disorders we reported here and elsewhere (Nakayama et al. 2004a, 2004b, 2005a, 2005b).

Reproduction and Mating Behavior

The effects of TBT on reproduction were tested using paired medaka exposed to TBT, PCBs, or TBT plus PCBs for three weeks (Nakayama et al. 2004b). The concentrations of chemicals were the same as those used in the swimming behavior test. During the third week of the three-week exposure period, the number of spawned and fertilized eggs was counted. After that, the sexual behavior of the males was assessed using the procedures described by Oshima et al. (2003) and Nakayama et al. (2004b). For the test, chemically naive females from the same brood stock of medaka used in the exposure test were transferred to a 2.5 L glass chamber two days before the assay. Then each male medaka was placed in a new 2.5 L chamber; after five minutes, an untreated female that had been injected intramuscularly with prostaglandin F2α was introduced into the chamber. We recorded the sexual behavior of the males for one hour with a CCD video camera, and then counted the frequencies of each behavior. Sexual behaviors were classified as: following (male swims behind female), dancing (male swims in a circle in front of female), crossing (male crosses its cloaca with that of female), and mating (male releases its sperm; Ono and Uematsu, 1957). One person analyzed all data to minimize artifacts.

During week 3 of the exposure period, fertility in mating pairs of medaka was significantly decreased by TBT exposure, while fecundity was unaffected. In addition, the profile of fertility frequencies showed a bimodal distribution, comprising low-fertility (0–10%) and high-fertility (90–100%) peaks (Nakayama et al. 2004b). The daily fertility of each mating pair in the TBT+PCBs group fluctuated dramatically during week 3. Reproductive toxicities of TBT have been studied in some aquatic organisms. Manning et al. (1999) assessed the toxicity of TBT to sheepshead minnow (*Cyprinodon variegatus*) using a life-cycle test and found obvious reduction of viable eggs compared to the control group, although the difference was not statistically significant because of a small number of replicates. Shimasaki et al. (2006) reported that 30-day exposure to TBT significantly decreased floating egg rate (equivalent to fertility) and the number of viable larvae of Japanese whiting. These results support our observation that TBT depresses fertility in exposed fish, but not fecundity.

Exposure to TBT or a mixture of TBT and PCBs significantly suppressed the sexual behavior of male medaka. The frequency of dancing was suppressed significantly in the male fish treated with TBT (p=0.020; Fig. 5), and the number of males performing sexual behaviors was decreased in both the TBT and TBT+PCBs groups (Table 3). In the same groups, as mentioned above, the fertility data showed a bimodal distribution. The results of the reproduction test and

Table 3. Sexual behavior of male medaka treated with tributyltin (TBT) and/or polychlorinated biphenyls (PCBs).

Treatment	Number of male medaka that performed (n=12)			
	Following	Dancing	Crossing	Mating
Control	12	9	6	6
TBT	8	5	3	3
PCBs	12	10	7	6
TBT+PCBs	5**	3*	2	2

Asterisks represents significant differences compared to control values (**: p<0.01, *: p<0.05; Fisher's exact test).

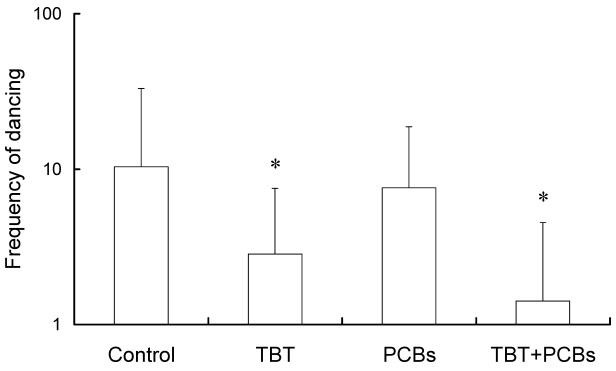

Fig. 3. Frequency of dancing by male medaka exposed to tributyltin (TBT) and/or polychlorinated biephenyls (PCBs). Data are presented as the mean±standard deviation (n=12). *: TBT exposure significantly reduced the frequency; p=0.020; two-way analysis of variance (Nakayama et al. 2004b).

mating behavior test suggest that male medaka exposed to TBT or to mixture of TBT and PCBs can induce egg spawning in females; however, they sometimes fail to release their sperm after the females spawn. Male medaka normally release their sperm after female spawn their eggs (Ono and Uematsu 1957). This observation is consistent with our findings that exposure to TBT depressed fertility, but fecundity was unaffected (Nakayama et al. 2004b). In addition, the testes of male medaka exposed to TBT and/or PCBs lacked noteworthy abnormalities. Therefore, it is likely that impairment of sexual behavior directly impedes reproduction in medaka.

Impairment of sexual behavior after exposure to chemicals is known to decrease reproduction in fish. We previously reported that administration of E2 depressed reproduction and suppressed sexual behavior in medaka (Oshima et al. 2003), and Gray et al. (1999) documented impairment of reproduction and sexual behavior in medaka exposed to OP. Suppression of sexual behavior also occurs in other species exposed to E2 or OP (Bayley et al. 1999, Bjerselius et al. 2001). These results support our hypothesis that TBT suppressed sexual behavior and consequently impaired reproduction in medaka.

The potential mechanisms for the impairment of sexual behavior by TBT and/or PCBs observed in this study are unknown. There are, however, some possible targets of the chemicals in male medaka. Firstly, TBT and PCBs may act directly on the testis or indirectly through the hypothalamus and pituitary and suppress the production of androgens that are essential for the occurrence of the male sexual behavior (Kobayashi et al. 2000). Secondly, TBT and PCBs may directly affect the brain (Andersson et al. 1998, Bachour et al. 1998, Harino et al. 2000) and suppress the neural system that regulates sexual behavior (Koyama et al. 1984, Satou et al. 1984). It remains to be elucidated how TBT and PCBs act on the neural system and suppress sexual behavior. It is also important to clarify whether the behavior suppression can be attributed to neural toxicity or to an estrogenic effect of TBT and PCBs.

Early Stage Development

To assess early stage toxicities of TBT, we conducted two different types of exposure tests. In the first experiment, we collected embryos spawned by TBT- and/or PCB-exposed parent medaka, and cultured them in six-well plates in embryo-rearing medium, both TBT- and PCB-free (Nakayama et al. 2005a). In the second experiment, we injected TBT into embryos collected from unexposed parent medaka (Hano et al. 2007). Deformities, hatchability, developmental delay, and swim-up behavior were observed in both experiments. Genetic and phenotypic sexes of the fish at 60 days post hatch (dph) were also determined to clarify whether TBT causes sex reversal in Japanese medaka.

Larval swim-up and hatchability were significantly affected by both maternally transferred (Table 4) and injected TBT. These results also agreed with those we reported previously (Nirmala et al. 1999). Fent and Meier (1992) have reported that TBT induces high mortality in larval minnows (*Phoxinus phoxinus*) within a few days after hatching and severely alters skeletal muscle fibers. Such skeletal muscle abnormalities could explain the swim-up failure.

In contrast, exposure to PCBs alone had no effect on swim-up and hatchability (Table 4). Our results agree with those of a previous report (Foster and Berlin 1997). Some field studies have reported blue-sac disease (Symula et al. 1990) and swim-up syndrome (Mac et al. 1985) in lake trout fry (*Salvelinus namaycush*), although a cause-and-effect relationship between swim-up syndrome and body burden of PCBs still has not been established. However, thiamine deficiency might be a potential mechanism of early life mortality (Fitzsimons 1995), as a previous study showed a strong association between early life mortality and low thiamine levels in the eggs of lake trout from the Great Lakes (North America) and inland lakes (Fisher et al. 1996). Moreover, PCB exposure can reduce thiamine storage in rats (Yagi 1979), and

Table 4. Early-life effects in embryos spawned by parent medaka exposed to tributyltin (TBT) with (w/) or without (w/o) polychlorinated biphenyls (PCBs; n=6).

TBT concentration (μg/g-diet)	Swim-up failure (%)		Hatchability (%)		Abnormal eye (%)		Time to hatching (d)	
	w/o PCBs	w/ PCBs[b]	w/o PCBs	w/ PCBs[b]	w/o PCBs	w/ PCBs	w/o PCBs	w/ PCBs[b]
0	2.5±3.0	0.5±0.8	93.3±7.2	90.3±19.7	0	0	6.7±0.2	7.0±0.3[a]
1	1.1±1.7[a]	11.8±11.4[a]	97.1±2.4	79.5±17.8	0	6.4±7.5	6.8±0.3	6.9±0.4[a]
5	10.4±5.8[a]	9.2±4.7[a]	65.4±25.2[a]	85.3±6.7[a]	4.6±3.1	4.4±3.8	6.4±0.4	7.4±0.4[a]
25	5.1±4.0[a]	9.7±4.8[a]	77.0±14.5[a]	86.7±5.1[a]	9.1±14.0	2.3±3.2	6.6±0.3	7.3±0.3[a]

Control fish received 0 μg TBT/g-diet without PCBs.
The concentration of PCBs in the diet was 25 μg/g.
Data are presented as mean±standard deviation.
[a]: Significantly different from control (two-way analysis of variance; $p<0.05$).
[b]: Significant interaction of TBT and PCBs detected (two-way analysis of variance; $p<0.05$).

administration of thiamine reduced early mortality syndrome in lake trout (Fitzsimons et al. 2001). Therefore, it is hypothesized that low levels of thiamine might be caused by PCB exposure and that this thiamine deficiency could enhance the incidence of swim-up failure induced by TBT.

Abnormal eye development, as indicated by eyelessness or small eyes, was observed in embryos spawned by parent fish exposed to 5 or 25 μg TBT/g in the diet (Table 4). These effects were also caused by nanoinjection of TBT (Hano et al. 2007). Weis et al. (1987) reported that TBT induced optical abnormalities in mummichog larvae, and Fent and Meier (1992) also have observed histological abnormalities of the eye in minnow larvae exposed to TBT. Thus, abnormal eye development is a typical effect of TBT exposure on early stage fish.

The effects of TBT on sexual development were also observed. Followed by *in ovo* exposure to TBT, hatched larvae were kept until 60 dph. Their phenotypic sex completely agreed with their genetic sex (Hano et al. 2007). We also assessed the effects of maternally transferred TBT on sexual development in the fish used in our previous report (Table 5; Nakayama et al. 2005a). Sex reversal was not induced by maternally transferred TBT, which agreed with the data from the nanoinjection test. Furthermore, the male : female sex ratio was close to 1 : 1 in both tests, which means that TBT exposure did not result in a male- or female-biased population in medaka in the present study. In contrast, Shimasaki et al. (2003) have reported that TBT has the potential to induce masculinization—to the extent of complete sex reversal—of genetically female Japanese flounder (*Paralichthys olivaceus*). In addition, early stage exposure to TBT results in a male-biased population of zebrafish (*Danio rerio*) and causes severe sperm damage (McAllister and Kime 2003). These studies suggest that TBT may disrupt sexual differentiation or maturation of fish. However, neither injected TBT nor maternally-transferred TBT affected sexual development in Japanese medaka. Therefore, the effects of TBT on sexual development may depend on fish species, since sex determination and sexual development processes are quite different among species.

Table 5. Sex ratio of progeny medaka spawned by tributyltin (TBT)- and/or polychlorinated biphenyl (PCB)-exposed parental fish (90 days post hatch; n=48 per group)[a].

TBT conc (μg/g-diet)	Parent diet without PCBs		Parent diet with PCBs[b]	
	Male	Female	Male	Female
0	28	20	25	23
1	29	19	25	23
5	27	21	24	24
25	20	28	26	22

[a]: Control fish received the 0 μg TBT/g diet without PCBs.
[b]: The concentration of PCBs was 25 μg/g in the parents' diet.

We found that treatment with TBT alone at the lowest dose had no significant effect in on development (Table 4). However, the same concentration of TBT severely affected embryological development when mixed with PCBs. Even if individual chemicals are below their NOECs, mixtures of the same chemicals may have toxic effects.

Summary

Our studies have demonstrated that TBT severely affects reproduction of Japanese medaka. TBT suppressed mating behavior in male and depressed fertility, but not fecundity. Exposure of embryos to TBT resulted in developmental toxicity, such as abnormal eye formation, decreased hatchability, and swim-up failure, but did not affect sexual development. In addition, TBT clearly alters both general (non sexual) and mating behaviors in medaka. We propose that behavior should be monitored to evaluate the effects of environmental contaminants, because it may directly influence the survival of individuals and affect population structure. Furthermore, the developmental toxicity of TBT was enhanced by co-ex-

posure to PCBs, even when their exposure levels were below the NOECs of the individual chemicals. Since organisms inhabiting polluted areas are exposed to complex mixture of chemicals, it is quite important to monitor mixture toxicities of contaminants at environmentally relevant levels.

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