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Nakayama, Kei
Center for Marine Environmental Studies, Ehmie University

Oshima, Yuji
Laboratoy of Drainage and Water Environment, Division of Refional Environmenta Science, Department of Bioproduction Environmental Sciences, Faculty of Agriculture, Kyushu University

Hiramatsu, Kazuaki
Laboratory of Fisheries Environmental Science, Division of Marine Biological Chemistry, Department of Bioresource and Bioenvironmental Sciences, Faculty of Agriculture, Kyushu University;

Honjo, Tsuneo
Laboratory of Fisheries Environmental Science, Division of Marine Biological Chemistry, Department of Bioresource and Bioenvironmental Sciences, Faculty of Agriculture, Kyushu University;

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Alteration of General Behavior of Male Medaka, *Oryzias latipes*, Exposed to Tributyltin and/or Polychlorinated Biphenyls

Kei NAKAYAMA[1], Yuji OSHIMA*, Kazuaki HIRAMATSU[2], and Tsuneo HONJO

Laboratory of Fisheries Environmental Science, Division of Marine Biological Chemistry,
Department of Bioresource and Bioenvironmental Sciences, Faculty of Agriculture,
Kyushu University, Fukuoka 812–8581, Japan
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We examined the general behavior of male Japanese medaka, *Oryzias latipes*, after exposed to tributyltin (TBT), polychlorinated biphenyls (PCBs), or a mixture of these chemicals at a concentration of $1 \mu g/g$ body weight daily for 3 weeks. We analyzed swimming velocity as an indicator of acute toxicity and counted the frequencies of straight swimming and swimming in circles. We also calculated the entropy of the positions of the fish within the experimental chamber. Neither TBT nor PCBs nor their mixture affected the swimming velocity, suggesting that there were no acute toxic effects. However, PCBs exposure increased the frequencies of both straight and circular swimming, suggesting hyperactivity. TBT increased the positional entropy value. Both TBT and PCBs affect the general behavior of mature male medaka, although exposure to the 2 chemicals together has no significant additive behavioral effect.

INTRODUCTION

Tributyltin (TBT) and polychlorinated biphenyls (PCBs) have been widely distributed in sediments, fish, and mammals (Kannan and Falandysz, 1997; Nakata *et al.*, 1997; Senthilkumar *et al.*, 1999; Schlenk *et al.*, 2002), despite regulation or prohibition of their usage. In addition, both TBT and PCBs can be detected in the liver of squid collected from the same area (Yamada *et al.*, 1997). Therefore, the effects of mixtures of TBT and PCBs on marine organisms should be considered along with the effects of individual chemicals.

Recent studies have noted that TBT and PCBs lead to abnormal physiological responses in fish and have adverse effects on their reproductive system (Monosson *et al.*, 1994; Kim and Cooper, 1999; Nirmala *et al.*, 1999). In our previous study, TBT reduced fertilization success in mating pairs of medaka (Nakayama *et al.*, in press).

Chemicals are also known to have the potential to alter the behavior of organisms. Behaviors, such as feeding, avoiding from predators, and mating directly influence the survival of individuals and future population structures. Weis *et al.* (2001, 2003) reported that poor feeding ability was observed in adult and larval mummichog, *Fundulus heteroclitus*, collected from a site contaminated with PCBs and heavy metals. TBT affects anti–predatory behavior in three–spine stickleback, *Gasterosteus aculeatus* L

[1] Center for Marine Environmental Studies, Ehime University
[2] Laboratory of Drainage and Water Environment, Division of Regional Environment Science, Department of Bioproduction Environmental Sciences, Faculty of Agriculture, Kyushu University
* Corresponding author (E–mail: yoshima@agr.kyushu–u.ac.jp)

(Wibe *et al.*, 2001). Moreover, TBT suppressed sexual behavior in male medaka (Nakayama *et al.*, in press). Therefore, behavior might be a good endpoint in the evaluation of the effects of these chemicals.

We assessed fish behavior not only by direct observation but also by image analysis to minimize artifacts. In addition, we introduced the concept of entropy as a parameter to analyze the disorder of the fish's position within the observation chamber. Entropy is defined as a numerical measure of the uncertainty of an outcome. We hoped that application of entropy would enable us to evaluate changes in the dispersion patterns of fish inside the aquarium.

Our objective was therefore to evaluate the effects of TBT and PCBs, individually or in mixtures, on the general, nonsexual, behavior of Japanese medaka.

MATERIALS AND METHODS

Special chemicals

Tri–n–butyltin oxide and PCBs (PCB–48; equivalent to Kanechlor–400) were purchased from Tokyo Kasei Kogyo, Tokyo, Japan.

Fish

Approximately 400 mature Japanese medaka (ca. 11 months after hatching, body weight ca. 0.3 g) were purchased from a fish farm in Nagasu, Kumamoto Prefecture, Japan. The fish were acclimated for 1 month in indoor glass aquaria equipped with aerating filter systems and induced to spawn under a summer photoperiod (14 hr light/10 hr dark) at 23 °C. The fish were fed a commercial diet (Medakanoesa, Kyorin, Himeji, Japan) at 30 mg/g body weight (b.w.). During the acclimation period, two–thirds of the aquarium water was replaced with aerated tap water every other day.

Exposure conditions

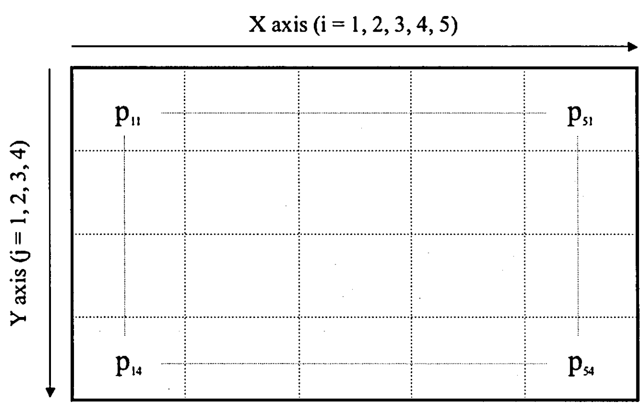
For the exposures test, fish were divided into 4 groups consisting of 12 pairs. Each pair of medaka was placed in a 2.5–L glass chamber. All fish were induced to spawn under the summer photoperiod (14 hr light/10 hr dark) at 23 °C. Half of the water in each chamber was replaced with aerated tap water everyday. For 3 weeks, the fish pairs were fed 1 of 3 diets, containing TBT (1 μg/g b.w. per day), PCBs (1 μg/g b.w. per day), or TBT+PCBs (1 μg/g b.w. of each chemical per day), or a pollutant–free diet as a control. No adverse morphological appearance or mortality was observed in the fish during the exposure period.

Behavior test

We assessed the general behavior of the males by the procedure described by Oshima *et al.* (2003). After the administration period, each treated male was put in a 2.5–L glass chamber. Its behavior while it swam freely was recorded with a CCD video camera (model IK–642, Toshiba, Tokyo, Japan) 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 2–dimension Image Tracking Analyzer that traced the swimming trajectory of the medaka in every 1/3 sec. Mean swimming velocity and position of the fish were measured by the trajectory data. Behaviors was categorized into 3 types: resting, swimming in a straight line, and swimming in circles (along the wall of the aquarium). The frequencies of these behaviors were counted. One person analyzed the data to minimize the artificial effect.

Calculation of entropy

To analyze positional dispersion patterns we calculated the entropy for each male. A side image of the chamber (20×15 cm) was separated into 20 small grid areas (Fig. 1), and the frequencies with 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.



$$H = -\Sigma\, p_{ij} \log p_{ij}$$

Fig. 1. Calculation of entropy values. Fish behavior was recorded from the side of an aquarium. The side view of aquarium was separated into 5 (horizontal) × 4 (vertical) grid areas. 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 this figure.

Statistical Analyses

All data were analyzed by the Levene's test to check homogeneity of variance across treatments. Swimming velocity, the frequencies of each swimming behavior, and entropy were analyzed by 2-way analysis of variance (2-way ANOVA). The frequencies of resting, swimming straight, and swimming in circles were logarithmically transformed before the analysis. All statistical analyses were performed by SPSS Advanced Models 11.0J (SPSS Japan Inc. Tokyo, Japan).

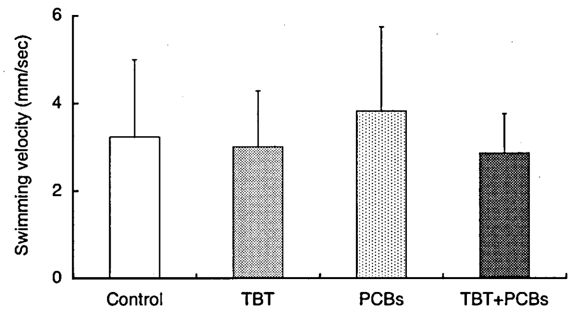


Fig. 2. Swimming velocities of male medaka in control and treatment groups. Data are presented as the mean ± standard deviation (N=12).

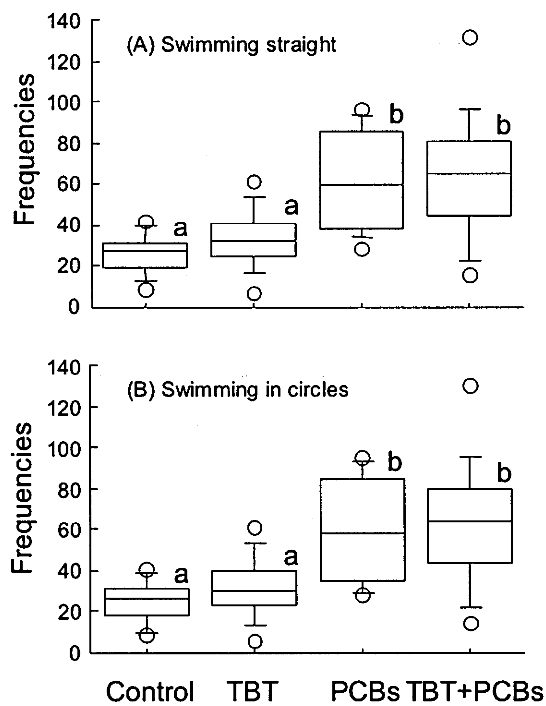


Fig. 3. Frequencies of swimming (A) in straight lines and (B) circles by male medaka in control and treatment groups. Box-whisker plots represent percentiles (bottom whisker=10th value, bottom of box=25th, horizontal line inside box=50th, top of box=75th, top whisker=90th. Open circles represent outlier values.). Pairs of boxes with the same letter do not differ significantly (2-way analysis of variance).

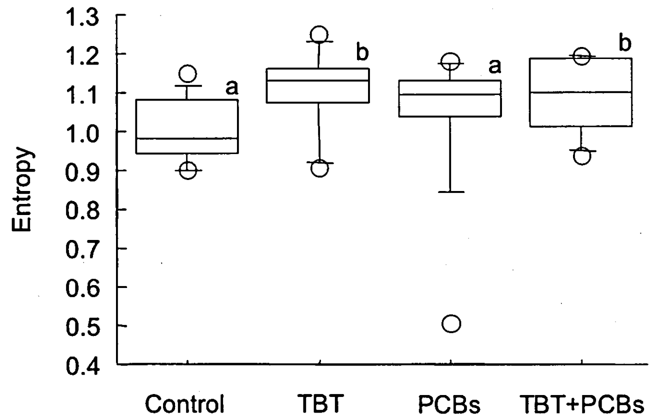


Fig. 4. Entropy values of positions of male medaka in control and treatment groups. Box–whisker plots represent percentiles (bottom whisker = 10 th value, bottom of box = 25 th, horizontal line inside box = 50 th, top of box = 75 th, top whisker = 90 th. Open circles represent outlier values.). Pairs of boxes with the same letter do not differ significantly (2–way analysis of variance).

RESULTS

Figure 2 shows the swimming velocities of male medaka treated with TBT and/or PCBs for 3 weeks. Neither TBT, nor PCBs, nor a mixture of both decreased the swimming velocity. This result demonstrated that TBT or PCBs did not exert acute toxic effect on medaka.

The frequencies of swimming straight and swimming in circles were significantly increased by the administration of PCBs (Fig. 3; $p < 0.001$ for both behaviors). TBT alone did not affect the frequency of each behavior, and no additive effect of TBT and PCBs together was detected. The frequency of resting was not affected in any treatment group.

Entropy was significantly increased by TBT administration (Fig. 4; $p = 0.043$). Entropy in the PCBs treatment group was slightly higher than in the controls, although the difference was not statistically significant. No additive effect of TBT and PCBs was detected.

DISCUSSION

Swimming velocity has been used as an indicator in some acute toxicity tests. No acute toxicity in terms of swimming velocity was observed in medaka treated with TBT or PCBs. Fish exposed to considerable concentrations of toxicants show reduced swimming behavior (Ososkov and Weis, 1996; Grillitsch *et al.*, 1999). We have detected decreased swimming velocity in medaka exposed to some toxicants, such as cyanide and insecticide (unpublished data). Therefore, we could conclude that exposure to TBT and PCBs at 1 μg/g b.w. daily for 3 weeks does not have any acute toxic effects in male medaka.

Our PCBs–exposed males swam in straight and in circles twice as frequently as control fish, suggesting that PCBs induce 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'–hexachloro biphenyl, an isomer of PCBs, induced attention deficit hyperactivity disorder in rats, a finding similar to ours in fish.

Chronic exposure of TBT or PCBs affects general, non–sexual, behavior in fish. Wibe *et al.* (2001) reported that TBT affected anti–predatory behavior in three–spine stickleback. In other studies, larval and adult mummichog collected from the sites contaminated by PCBs and heavy metals showed poorer prey capture ability than those from cleaner reference site (Weis *et al.*, 2001, 2003). These results indicate that general behavior is a useful endpoint in chronic toxicity tests. Furthermore, TBT at the same concentration as used in our study suppresses sexual behavior in male medaka (Nakayama *et al.*, in press). Therefore, both sexual and nonsexual behavior should be used as behavioral biomarkers for evaluating the effects of contaminants at chronic levels.

Brain dysfunction is a potential mechanism behind the abnormal behavior seen upon exposure to TBT and PCBs, because both chemicals can accumulate in, and directly affect, the brain (Andersson *et al.*, 1998; Bachour *et al.*, 1998; Harino *et al.*, 2000). PCBs are known to alter the concentration of dopamine (DA), a neurotransmitter, in the brain of mammals (Seegal *et al.*, 1994) and fish (Khan & Thomas, 1996). In fish, 5–hydroxytryptamine (5–HT) suppresses aggressive behavior, in contrast, dopamine stimulates it (Munro *et al.*, 1986; Tiersch & Griffith, 1988; Adams *et al.*, 1996). Koutoku *et al.* have also reported that exposure to tryptophan (precursor of 5–HT) stimulates the serotonergic system in the brains of medaka and simultaneously increases locomotor activity. Therefore, TBT and/or PCBs could have altered the monoamine content in the brain of medaka and caused the abnormal behavior that we observed.

In this study, medaka in control group preferred a certain area of aquarium, especially near from wall of aquarium, and they spent most of testing time around the area. This may be a kind of preference for positioning. Relatively low entropy in control might be reflected their preference. Entropy was significantly increased in TBT–exposed medaka, which indicated loss of their preference for swimming area. We propose an application of entropy to evaluate an orderliness of behavior in fish and other animals.

In summary, neither TBT nor PCBs nor their combination at 1 μg/g b.w. daily for 3 weeks had acute toxic effects on mature male medaka in terms of swimming velocity. However, PCBs frequently altered swimming behavior to produce patterns suggestive of hyperactivity. Furthermore, TBT increased the entropy of fish's position. Therefore, both TBT and PCBs affect general, nonsexual, behavior of mature male medaka.

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