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A Reproductive Toxicology Study of Phenanthrene in Medaka (*Oryzias latipes*)

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Abstract Environmental contaminants, such as polycyclic aromatic hydrocarbons (PAHs), can disrupt the endocrine system and affect reproductive function of humans and wildlife. In this study, we exposed Japanese medaka (*Oryzias latipes*) to water-borne or food-spiked phenanthrene, an ubiquitous PAH, and investigated the chronic effects of the chemical on medaka reproduction. The results showed that phenanthrene significantly prolonged the time to hatch for embryos. Other parameters such as fecundity and fertility, organ-somatic ratios, hepatic vitellogenin production, and the histology of testes and ovaries were not different between the control and phenanthrene-treated groups. This suggests that the time to hatch in embryos might be a more sensitive biomarker for environmental contaminants.

Environmental contaminants could disrupt the endocrine system and affect the reproductive function of humans and animals (reviewed by Caserta et al. 2008; Dickerson and Gore 2007; Edwards et al. 2006; Kang et al. 2007; Rasier et al. 2006; Soin and Smagghe 2007). Polycyclic aromatic hydrocarbons (PAHs) are among the most abundant contaminants to which people and wildlife are commonly exposed. There has been evidence showing that the egg yolk precursor protein vitellogenin (VTG) levels in rainbow trout were decreased 15-fold by creosote, a product mainly composed of PAHs (Sherry et al. 2006). Patel et al. (2006) have shown that ovarian aromatase activity in killifish was suppressed by benzo(*a*)pyrene. Benzo(*a*)pyrene also lowered the number of offspring and destroyed primordial oocytes in mice (Mattison et al. 1980).

In addition, PAHs can raise testosterone production in goldfish and rainbow trout by promoting testicular steroidogenesis (Evanson and Van Der Kraak 2001), suppress spermatogenesis in clams (Frouin et al. 2007) and rats (Takeda et al. 2004), and inhibit sex steroid synthesis in female teleosts (Monteiro et al. 2000a, b; Seruto et al. 2005). The chemicals might act as antiandrogens for the human androgen receptor (Chang and Liao 1987; Vinggaard et al. 2000) or as estrogen through an estrogen receptor (Hilscherova et al. 2002; Tsai et al. 2004). Furthermore, bacteria in the human colon have been found to transform PAHs into estrogenic metabolites (Van de Wiele et al. 2005).

Polycyclic aromatic hydrocarbons are contaminants frequently found in aquatic environments around the world. In Taiwan, the total PAHs concentrations in the sediments from the south ranged from 8 to 356 ng/g in a river (Doong and Lin 2004) and from 98 to 3382 ng/g in coastal areas (Fang et al. 2003). Three- and four-ring PAHs, including phenanthrene, anthracene, and pyrene, were the dominant species in the sediment samples. The major sources of these chemicals were the neighboring factories and illegal dumping of used motor oil.

Toxicological studies on PAHs are abundant, but PAH mixtures or field sediments containing multiple PAHs and other contaminants are often used (Barron et al. 2004a; Reynolds et al. 2003; Seruto et al. 2005). As each PAH could have distinct and specific targets in organisms

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(Incardona et al. 2004, 2006), investigating action of common PAHs individually will help us better understand the interaction within a compound mixture. Because phenanthrene is one of the most abundant PAHs in urban environments (Hwang and Wade 2008; Lin et al. 2002), we chose to use it as the focus of this study.

Phenanthrene is present in fumes from vehicles, coal and agricultural burning, and cooking and cigarette smoking. It has only three aromatic fused rings and is considered less toxic to fish and mammalian systems (Barron et al. 2004b) but more water soluble and, thus, more bioavailable (reviewed by Hylland 2006).

Studies on the effects of phenanthrene on the reproduction of aquatic animals are scant. It has been known to lower egg production and hatching success rates in a marine calanoid copepod (Bellas and Thor 2007) and to reduce reproductive output in the enchytraeids (Sverdrup et al. 2002).

In this study, we investigated the long-term effects of phenanthrene on reproduction, using Japanese medaka (*Oryzias latipes*) as the experimental animal. The fish is ideal for toxicological testing because of its relatively short life cycle, easy maintenance, and sensitivity to environmental contaminants.

The bioavailability of PAHs in the aquatic environment is dependent on their sorption mechanisms, which involve particle properties and nonaqueous-phase liquids (reviewed by Luthy et al. 1997). Each of these components might also vary with organic matters and oxide coatings on clay particles. Although the hydrophobic nature makes PAHs readily adsorb to particulate matters and eventually accumulate in sediments, the distribution of PAHs in sediment might be a function of the local environment. This makes the interpretation of an experiment with sediment-bound PAH difficult. In order to simplify the exposure conditions, we used waterborne phenanthrene to simulate the habitat immediately adjacent to the contaminated sediments for embryos. We then used food-spiked phenanthrene (Experiment A) or water-borne phenanthrene (Experiment B) for juvenile and adult fish. This allowed us to investigate how exposure routes affect the action of phenanthrene on fish. Several parameters were used to assess the effects of phenanthrene: embryo hatchability, time to hatch, fecundity (number of eggs), fertility (percent fertilized), gonadal histology, organ-somatic ratios, and the egg yolk protein VTG expression.

Materials and Methods

Experimental Animals

Sexually mature Japanese medaka was generously donated by Dr. Chien-Min Chen at Chia Nan University of Pharmacy and Science (Tainan, Taiwan). The fish were maintained at 28°C under a constant 14-h light:10-h dark photoperiod in glass tanks filled with flow-through filtered water (pH 7.5–7.8). They were fed four times daily with freshly hatched brine shrimp (<24 h after hatching). All procedures were carried out in accordance with the "Guidelines for Animal Experimentation" of Chang Jung Christian University.

Test Solutions and Food Preparations

Phenanthrene crystals (purity >99.5%; from Sigma-Aldrich, St. Louis, MO) were dissolved in ethanol to make a 1-mg/mL stock solution, which was stored in an amber glass bottle at 4°C. To make the test solutions, the stock solution was diluted to desired concentrations with filtered water. The amount of ethanol added was the same in all groups, with a final concentration less than 0.1%. The control solution was prepared by adding the same amount of ethanol to filtered water.

To prepare phenanthrene-spiked food, diluted solutions of various concentrations from the stock were added to fish food flakes (TetraMin, Melle, Germany). The designated concentrations were 0, 5, 10, 25, and 50 ppm of phenanthrene in the food flakes. The doses were chosen based on our previous analyses on three-ring PAHs in the low-density fraction of sediment samples collected from contaminated creeks in Taiwan (0.1–24 ppm; Chen 2003) and New Jersey (26.5–715 ppm; Horng 1998). The preparations were stored in tubes lined with Teflon to prevent the adsorption of phenanthrene on the inside of the tubes. The tubes were lidded and placed on a roller at 4°C for 2 days to allow time for the phenanthrene and the food to mix evenly. The lids of the tubes were opened periodically to release ethanol vapor. On the third day, the tubes were centrifuged, the water was removed, and the food pellets were freeze-dried.

Care was taken so that all phenanthrene solutions and spiked food were prevented from exposure to light, as phenanthrene can be degraded by sunlight (Valerio et al. 1984).

Experimental Design

Experiment A: Exposure Through Water and Food

Fertilized eggs from the broodstock were pooled and randomly divided into five groups ($n = 40$ each). They were placed in glass dishes containing 0%, 25%, 50%, 75%, and 100% phenanthrene-saturated filtered water. The concentration of 100% saturated phenanthrene in water is reported to be 1.28 ppm (Meador et al. 1995). The LC_{50} for medaka is unknown; for rainbow trout, the published figures range from 0.2 ppb to 3.2 ppm (PAN Pesticide Database 2008).

The eggs were placed in an incubator maintained at 26 °C. Test solutions were changed and the embryos observed daily until hatching. Juveniles were then transferred to fish tanks and fed twice daily with fish food spiked with 0, 5, 10, 25, and 50 ppm (the concentrations later measured to be 0, 11.8, 19.2, 32.2, 55.0 ppm, respectively) of phenanthrene, respectively, for 60 days. Afterward, four pairs of medaka from each treatment group were randomly chosen and fed with the same phenanthrene-spiked food as earlier. The eggs spawned from each group were collected and counted consecutively for 30 days; the numbers of unfertilized eggs were also determined. The fish were then sacrificed and measured for body weight and length. The liver, brain, and gonads were removed and weighed to obtain the organ-somatic ratios. The livers were also processed and analyzed for the VTG content, whereas the gonads were processed for the histological examination.

Experiment B: Exposure Through Water Only

Fertilized eggs from the broodstock were pooled and randomly divided into triplicate of two groups ($n = 20$ each). One group was placed in glass dishes containing the control solution; the other was in test solution containing phenanthrene-saturated water (theoretically 1.28 ppm; later measured to be 1.40 ± 0.20 ppm). They were maintained and observed as in Experiment A.

After hatching, juveniles were transferred to fish tanks, subjected to control or phenanthrene treatments, and reared for 60 days. The tanks were prepared as follows: Phenanthrene crystals were placed in a sponge-capped 15-mL glass tube, and the tube was placed on the bottom of the tanks. An air stone was inserted into the tube to promote water circulation and phenanthrene solubility. This design attempted to minimize the loss of phenanthrene through photodegradation and eliminated the necessity of changing tank water daily. Control tanks were prepared with the same setup without phenanthrene. The phenanthrene concentration was later measured to be 0.55 ± 0.07 ppm.

At 2 months of age, four pairs of mature medaka from each treatment group were randomly chosen and subjected to the same control or phenanthrene treatments. The eggs spawned from each group were collected and counted consecutively for 30 days. Afterward, the fish were sacrificed and processed as Experiment A.

General and Histological Examination

The fish ($n = 3$–6 each group) were anesthetized in 0.06% MS222 (Sigma-Aldrich, St. Louis, MO), and the liver, brain, and gonads were removed and weighed to obtain the organ-somatic ratios. Some of the gonads were processed with standard methods for paraffin embedding. Briefly, the tissues were fixed in 4% neutral-buffered formalin for 16 h, then dehydrated sequentially through 70%, 80%, 90%, 95%, and 100% alcohols for 1 h each, cleared in xylene twice for 1 h each, infiltrated and embedded with paraffin at 60 °C, and sectioned to 5-μm thickness. The sections were then stained serially with hematoxylin and eosin, dehydrated, mounted, and, finally, observed under the light microscope.

Western Blot Analysis of VTG

The liver ($n = 3$–5 each group) from adult medaka was dissected and homogenized with TES buffer (5 mM Tris-HCl, pH 6.8; 1 mM EDTA, pH 8.0; 0.5 M sucrose). Total protein in each sample was determined with Bradford reagent (Sigma-Aldrich, St. Louis, MO), separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE), and transferred onto nitrocellulose membranes (Hybond-ECL; Amersham). The membranes were blocked with 5% dry milk dissolved in Tris-buffered saline containing 50 mM Tris-HCl, pH 7.6, 0.9% NaCl, and 0.1% Tween-20 (TBST). The VTG and actin expressions were detected by incubation with anti-fish VTG (Biosense, Bergen, Norway) and anti-actin (Chemicon) primary antibodies, and then peroxidase-conjugated anti-mouse secondary antibody (Chemicon). Both antibodies were diluted 1:2000. The bound peroxidase was visualized by reacting with chemiluminescent substrate (ECL; Amersham) and then detected and measured with UVP AutoChemi Image System (UVP, Upland, CA).

Determination of Phenanthrene Concentrations

Saturated phenanthrene concentrations in the test solutions were determined by liquid–liquid extraction with *n*-hexane, followed by characterization of the extract by a gas chromatography/flame ionization detector (GC/FID; USEPA 2006). Phenanthrene concentrations in spiked food were determined by microwave extraction with acetone/*n*-hexane (50%/50%, v/v) solvent (US EPA SW 846 Method 3546 Microwave Extraction), followed by characterization of the extract by GC/FID. Because phenanthrene was the only peak present in the chromatograms of the extracts of both the stock solution and spiked food, but not in the analytical blank, further GC/MS (mass spectrometry) confirmation and internal standards were deemed unnecessary.

Microwave extraction was performed in CEM MARSX with a temperature-control program, ramped at 11.5°C/min to 115°C and held for 15 min. The extract was volume-reduced to about 2 mL using a rotary evaporator (Brinkmann, Westbury, NY; R110 Rotavapor) under a slight

vacuum (–50 kPa) at 35°C. It was then quantitatively transferred to a 15-mL amber vial, further reduced in volume to about 0.5 mL under a high-purity nitrogen stream, and sealed with a Teflon-lined screw cap until GC analysis.

Phenanthrene was separated and quantified with an Agilent GC6890 series GC equipped with a FID. An aliquot of extract was injected and separated by a Hewlett-Packard HP-5 column (30 m × 0.53 mm × 1.5 μm). The carrier gas was high-purity nitrogen. The GC oven program had a starting temperature of 40°C with a hold time of 1 min. The oven was ramped at 30°C/min to 170°C, then 4°C/min to 240 °C, and, finally, at 12°C/min to 300°C, where it was held for 10 min. Chromatographic peaks of phenanthrene were identified by comparing retention times with those of phenanthrene standards and then quantified by calibration curves developed in advance using known concentrations of standards.

Statistical Analysis

The Kolmogorov–Smirnov one-sample test for normality and the Levene test for the homogeneity of variance were first conducted to examine the validity of applying analyses of variance (ANOVAs) on our datasets. If the datasets failed to meet the assumptions of ANOVA, nonparametric methods were applied. Kruskal–Wallis ANOVA by ranks was used to test the effect of phenanthrene treatment on embryo hatching time as well as various organs and somatic parameters. Significance was considered when $p < 0.05$. Statistical grouping of different treatments was further computed by the post hoc comparisons of mean ranks, if the treatment effect proved significant.

Results

Concentrations of Phenanthrene in Test Solution, Spiked Food, and Tank Water

The concentration of phenanthrene in phenanthrene-saturated test solution used for incubating embryos was analyzed to be 1.40 ± 0.20 ppm, close to the 1.28 ppm published figure (Meador et al. 1995). The concentrations of phenanthrene in the spiked food in Experiment A were determined to be 0, 11.8, 19.2, 32.2, and 55.0 ppm for the five groups, which were intended to be 0, 5, 10, 25, and 50 ppm, respectively. Analysis of phenanthrene concentration in the treatment tanks of Experiment B for juveniles and adults, however, showed that the actual concentration was 0.55 ± 0.07 ppm. In the control tanks, phenanthrene was not detectable. We will use these measured concentrations for the figures and the rest of the text.

Embryo Hatchability and Time to Hatch

Experiment A

Without exposure to phenanthrene, the time to hatch for embryos ranged widely (Table 1 and Fig. 1a), and averaged 13.2 ± 2.0 days. Embryos exposed to various concentrations of phenanthrene also had a wide range in the time to hatch. Those exposed to 1.40 ± 0.20 ppm phenanthrene took a significantly longer time to hatch, averaging 15.1 ± 3.0 days ($p = 0.006$). For the hatchability of embryos, indicated by the percent hatch (Table 1), the group exposed to the highest dose (1.40 ± 0.20 ppm) of phenanthrene was the lowest among the groups.

Table 1 Percent hatchability and time to hatch for embryos exposed to 0%, 25%, 50%, 75%, and 100% water-saturated phenanthrene

Designated % saturation of phenanthrene	% Hatchability	Time to hatch (days)		
		Mean ± SEM	Minimum	Maximum
Experiment A ($n = 40$)				
0	97.5	13.2 ± 2.0	11	18
25	90.0	14.1 ± 2.6	10	22
50	92.5	14.6 ± 2.4	12	22
75	95.0	14.3 ± 2.8	11	22
100[a]	85.0	15.1 ± 3.0*	11	26
Experiment B ($n = 20$ in triplicate)				
0	93.3 ± 5.4	14.2 ± 0.7	12	29
100[a]	91.7 ± 2.0	15.3 ± 1.9*	12	27

[a] Chemical analysis showed that the concentration of phenanthrene was 1.40 ± 0.20 ppm

* Significant difference from control ($p < 0.05$)

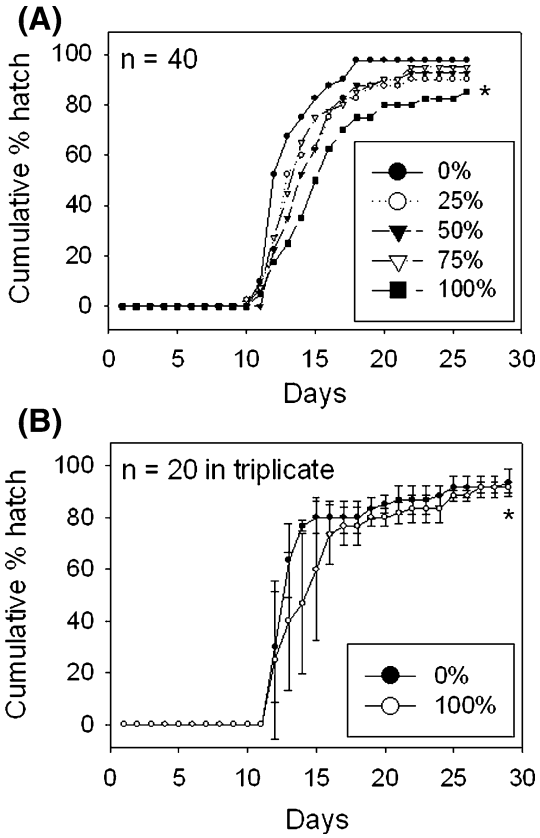

Fig. 1 Cumulative percent hatch of embryos from Experiment A (**a**) and Experiment B (**b**). * Significant difference from the control at $p < 0.05$

Experiment B

The time to hatch for embryos again ranged widely from 12 to 29 days (Table 1). The phenanthrene-treated group also had a prolonged embryonic development period, compared to that of the control group (15.3 ± 1.9 vs. 14.2 ± 0.7 days, $p = 0.04$).

Fecundity and Fertility

The eggs spawned by the adult fish were collected and counted for 30 consecutive days. The result in Experiment A showed that the total numbers of eggs collected from each group ranged from 1295 to 1872 (Table 2). The daily averages of eggs spawned from phenanthrene-treated groups were not significantly different from that of the control, except the dose 11.8-ppm group ($p = 0.01$). The daily percentages of unfertilized eggs of all groups were also not significantly different.

The result from Experiment B showed that the total number of eggs collected from the triplicate control group was 1152 ± 74 (Table 2) and that of the phenanthrene-treated group was 1015 ± 164. Both were not significantly different ($p = 0.55$). The daily averages of spawned eggs and percentage of unfertilized eggs were also not significantly different ($p = 0.07$ and 0.24, respectively). These results demonstrated that fecundity and fertility were not significantly different between the two groups.

Table 2 Total numbers and daily averages of eggs spawned during the 30-day period as well as the daily percentage of unfertilized eggs

Dose (ppm)	Total	Daily average	Daily % unfertilized
Experiment A			
0	1458	49 ± 3	5.3 ± 0.8
11.8	1872	62 ± 5*	7.7 ± 1.1
19.2	1295	43 ± 4	7.8 ± 1.4
32.2	1570	52 ± 3	9.6 ± 1.8
55.0	1591	53 ± 4	8.3 ± 3.0
Experiment B			
0	1152 ± 74	40 ± 3	14.0 ± 1.5
0.55	1015 ± 164	34 ± 3	17.1 ± 2.2

Note: Total numbers of eggs spawned in Experiment B were averages from the triplicate groups. Data were expressed as mean ± standard error

* Significantly different from the control, $p = 0.01$

Organ-Somatic Ratios

The body weight and length of the fish in both experiments were not significantly different between the control and phenanthrene-treated groups (data not shown). The male body weight in the control group averaged 0.49 ± 0.03 g, with body length 3.65 ± 0.14 cm; the female in the control group averaged 0.52 ± 0.09 g, with body length 3.69 ± 0.15 cm. The organ-somatic ratios (% body weight) for the liver, brain, and gonads are shown in Fig. 2a and b. Except for the liver-somatic ratio of 19.2 ppm for the male group in Experiment A ($p = 0.02$), no other significant difference was observed among all groups of both males and females.

Histology of Gonads and Hepatic VTG Expression

The histology of testes and ovaries of phenanthrene-treated groups were not different from those of the controls in both experiments and no ovo-testis was found (Fig. 3). Western blot analysis (Fig. 4) also showed that phenanthrene did not induce the hepatic VTG expression in male fish; neither did it alter the levels of VTG in the females.

Discussion

In both Experiment A and Experiment B, phenanthrene prolonged the embryonic development and lengthened the average time to hatch by 1–2 days. Whether this effect was

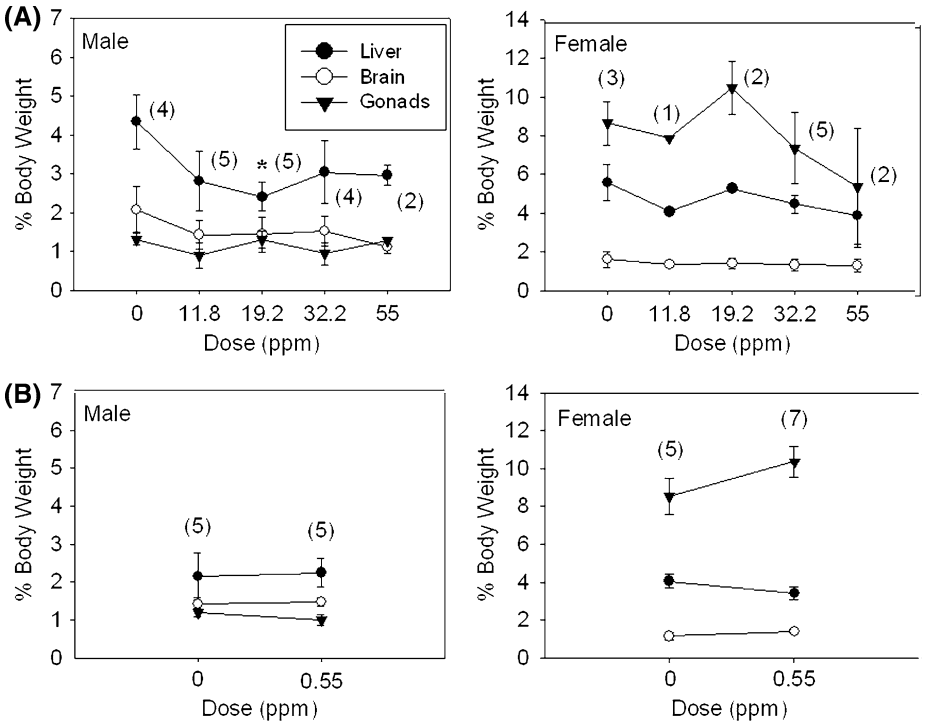

Fig. 2 Organ-somatic ratios (expressed as % body weight) of the liver, brain, and gonads in Experiment A (**a**) and Experiment B (**b**). The numbers in parenthesis represent the numbers of individuals in each group. * Significant difference from the control at $p < 0.05$

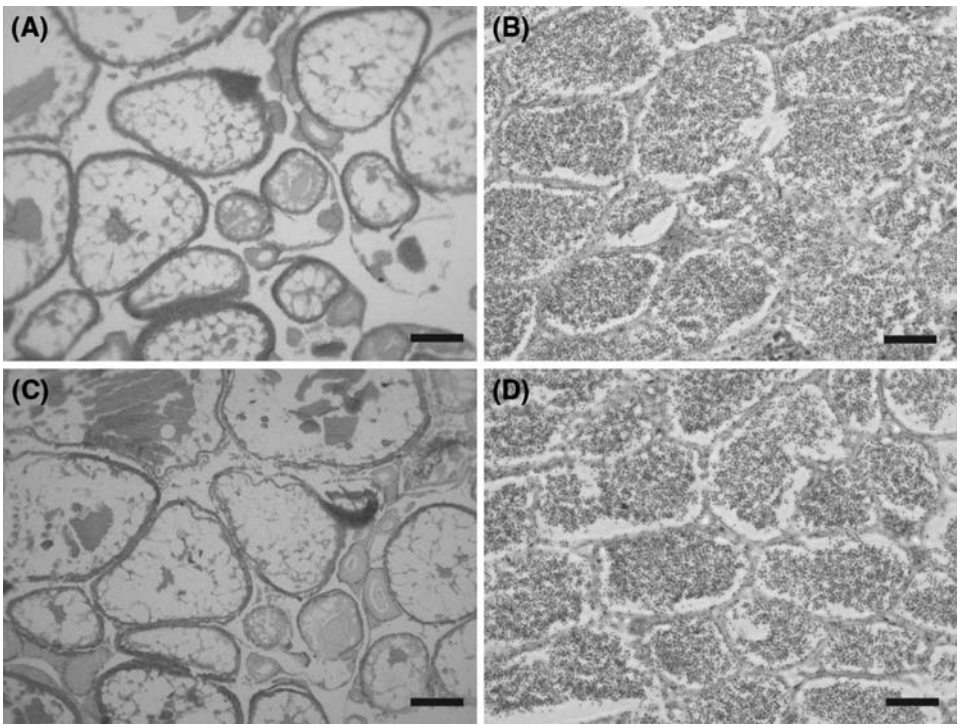

Fig. 3 Longitudinal sections of the ovaries (**a, c**) and testes (**b, d**) from individuals of the control group (**a, b**) or individuals exposed to 55.0 ppm phenanthrene through food (**c, d**) in Experiment A. The sections were 5 μm thick; the bar in (**a**) and (**c**) represents 200 μm; the bar in (**b**) and (**d**) represents 50 μm

biologically relevant is unknown. However, one could argue that an immobile medaka embryo on the bottom of a creek or attached to a plant might be more vulnerable to predators than a small, yet agile, larva.

No significant difference was found for other parameters examined. In other words, the chemical at its solubility limit did not affect the hatching success of embryos nor did it influence the animals' fecundity and fertility. This result was consistent with the histological examination of the testes and ovaries; both gonads appeared normal and no ovo-testis was found. Results from the organ-somatic ratios of the liver, brain, and gonads were also similar and there

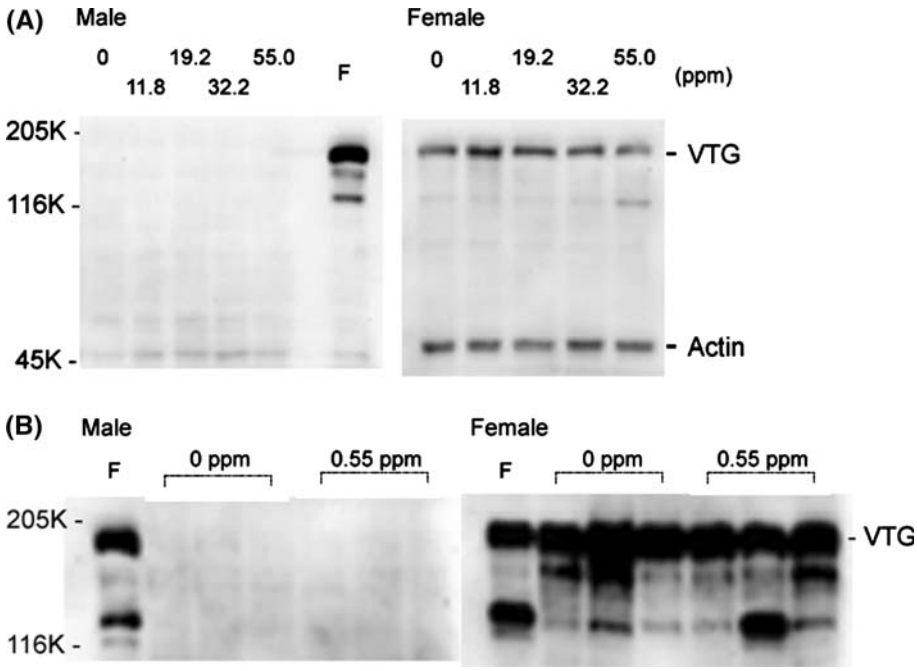

Fig. 4 Western blot analysis on the levels of the egg yolk precursor protein VTG in the liver of adult males and females. Fifty micrograms of total proteins were loaded onto the gels. F: female liver homogenate serving as a positive control. Immunoblotting for actin, serving as a loading control, is shown in (**a**)

was no change in the levels of hepatic VTG expression in both male and female fish. In addition, exposure routes through food or water appeared to cause no significant difference in medaka reproductive function.

The concentration of phenanthrene in the 100% saturated test solution was determined to be 1.40 ± 0.20 ppm. It might seem to be a dose too high for the subtle effect of delaying the time to hatch for embryos. However, Evans and Nipper (2008) have demonstrated that 30.6% of water-saturated phenanthrene can be lost in 1 h and 78.1% can be lost in 48 h. In addition, such loss is much greater with the presence of biomass, most likely due to bioaccumulation. Thus, even with a daily change of the test solutions, the actual exposure doses for embryos in this study might have been much lower. On the other hand, the concentration of phenanthrene in tanks for juveniles and adults in Experiment B was determined to be 0.55 ± 0.07 ppm, only 39% of the water-saturation level of 1.40 ppm; however, the design of our setup should have continuously replenished some of the lost phenanthrene. Unfortunately, we did not monitor the concentration of phenanthrene throughout the experiment.

Farwell et al. (2006) have demonstrated that a mixture of PAHs (up to 0.09 ppm) did not change the time to hatch in medaka embryos, whereas Rhodes et al. (2005) have reported that the time to hatch was slightly shortened by a much lower dose (0.01 ppm) and by a 0.2-ppm dose of phenanthrene, but not intermediate concentrations up to 0.1 ppm. Interestingly, in the latter study, the authors also found that the more toxic four-ringed dibenzothiophene (0.2 ppm) did delay the time to hatch by about 3 days. The inconsistencies from these studies and ours might simply reflect the complex nature of chemical toxicity: not only might each individual substance have distinct effects, but the concentrations of this substance or the interactions among various components within a mixture can also make a difference. Furthermore, the dose responses of an organism to either an individual or a mixture of PAHs might be nonmonotonic (Kohn and Melnick 2002) (*i.e.*, forming an inverted U-shaped curve). The biochemical mechanisms of these environmental contaminants remain largely unknown.

Overall, these studies have suggested that PAHs have an effect on embryonic development. The mechanism of such effect was unknown. McElroy et al. (2006) has reported that medaka embryos can rapidly accumulate PAHs and metabolize them to polar metabolites. These metabolites could influence the development of embryos (Mathew et al. 2008). In addition, evidence has shown that PAHs can disrupt ATP production in mitochondria (von Westerhagen 1988). Thus, energy deficiency might also contribute to the slow embryonic development.

Medaka embryos have been known to vary in their time to hatch, depending on temperature, light, dissolved oxygen levels, agitation (Farwell et al. 2006), and environmental contaminants. Ethanol (Oxendine et al. 2006) and an industrial effluent from a printing plant prolonged the time to hatch (Zha and Wang 2006), whereas thyroid hormone (Walpita et al. 2007) and bisphenol-A shortened the time to hatch (Ramakrishnan and Wayne 2008). This demonstrated that the time to hatch can be influenced by various factors and is probably a more sensitive biomarker for environmental variables than other end points, such as fecundity and fertility, organ-somatic ratios, histology of gonads, and hepatic VTG production.

In this study, there were some incidence of deformities in both Experiment A and Experiment B. Unfortunately, we did not record the numbers carefully. Deformities and reduced growth could lead to decreased numbers at maturity and reduced population size, thus affecting reproduction. PAHs have been known to cause deformities in fish (Debruyn et al. 2007; Incardona et al. 2004) and possibly in humans (Perera et al. 2006; Tang et al. 2008). Thus, it is important that the effects of PAHs be more intensively investigated.

Conclusion

We have demonstrated that phenanthrene has little effect on medaka reproduction, but it can slightly prolong the time to hatch for embryos. The result indicates that the time to hatch might be a more sensitive biomarker for detecting the effects of PAHs, at least in the case of phenanthrene. However, the biological significance of this, due to the small, yet statistically significant effect, is not clear. As the action and metabolism of PAHs are complex, more studies are needed to understand better the problems that are pervasive in the environment.

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