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Accumulation and Pharmacokinetics of Estrogenic Chemicals in the Pre- and Post-hatch Embryos of the Frog *Rana rugosa*

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Abstract. *Amphibian eggs spawned in water are exposed immediately to various chemicals present in their water. The present study aimed to investigate the accumulation and pharmacokinetics of 17α-ethynylestradiol (EE_2), bisphenol A (BPA), and nonylphenol (NP), as well as 17β-estradiol (E_2), in the pre-hatch and post-hatch embryos of the frog Rana rugosa. Fertilized eggs were exposed to chemicals at a final concentration of 500 nM in breeding water for two days, then the embryos with jelly coats were reared in fresh-breeding water without supplementation of the xenoestrogens for six more days. All exogenous chemicals were concentrated in the embryo body at two days after fertilization, whereas their concentrations in the jelly coat were the same as those in the breeding water. The bioconcentration factors for E_2, EE_2, BPA, and NP were 217.9, 170.2, 382.3, and 289.1, respectively, suggesting that the estrogenic chemicals were concentrated in the embryo body through the jelly coat.*

*Bis*phenol A (BPA) and *p*-nonylphenol (NP), as well as 17α-ethynylestradiol (EE_2), have long been known as potential estrogenic endocrine-disrupting chemicals (EDCs). BPA has been used for polycarbonate plastics as a raw material of epoxy resin and in the inner coating of metal food cans. NP is a degradation product of alkylphenol polyethoxylates, of which large amounts have been used as non-ionic surfactants. EE_2 is a component of the oral contraceptive pill. Several studies have shown potent estrogenic activity of EE_2 (1, 2) and comparably weak estrogenic activities of BPA and NP (1-3). Many studies demonstrated their contamination in the aquatic environment and adverse effects on the reproduction of aquatic animals. BPA (4-6), NP (7-12) and EE_2 (13-17) have been detected in sewage effluent and river water all over the world. NP and BPA bioaccumulate in fish living in polluted water (5, 10). Apart from other abnormalities, intersex gonad formation and an elevated level of vitellogenin are common in the male roach (*Rutilus rutilus*) (18) and rainbow trout (*Oncorhynchus mykiss*) (19) grown near sewage-effluent discharge areas in the UK. Feminized male fish with higher serum vitellogenin levels or abnormal gonads have also been captured in the fields of the USA (20-22) and Japan (23, 24).

In contrast to fish, considerably little information has been made available on field observations of amphibians with abnormal gonads (25), *e.g.* gonadal deformities induced by atrazine (26, 27). However, laboratory reports using amphibians highlight the adverse effects of BPA, NP, and EE_2 on endogenous endocrine conditions and the development of gonads in tadpoles of *Xenopus laevis* (28). Exposure of tadpoles of *Silurana* (*Xenopus*) *tropicalis* to EE_2 induced sex reversal from male into female (29). Other exposure experiments using amphibian tadpoles under laboratory conditions have shown that NP and BPA elevate the levels of serum vitellogenin (30) and of its mRNA (31), which probably results in disordered sexual differentiation of the gonad (28, 31, 32).

In addition to these laboratory experiments using tadpoles, EDCs have been found to induce abnormal development of pre-hatch embryos, *i.e.* abnormalities of body shape and neural cell differentiation (33-36). NP ethoxylate induces growth and developmental abnormalities of pre-hatch embryos of *X. laevis* and two other Australian frog species, *Litoria adelaidensis* and *Crinia insignifera*, in the frog embryo teratogenesis assay-*Xenopus* (FETAX) assay (37). BPA exerted toxicity and delayed or arrested development,

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reduced body size, and led to persistent yolk plug and microcephaly in early-stage embryos of the common South American toad *Rhinella arenarum* (38). Most female amphibians spawn eggs in water and the eggs are thus exposed immediately and directly to the surrounding contaminants. Most EDCs with estrogenic activity are lipophilic chemicals. Since amphibian eggs have a large amount of lipid-enriched yolk, and detoxification systems are not established in pre-hatch embryos, it is important to investigate accumulation and pharmacokinetics of estrogenic chemicals in early-stage embryos during development.

We have previously investigated and assessed impacts of chemicals and hormones on amphibians (36, 39-42). During the course of work using fertilized amphibian eggs, we were curious to learn whether the jelly coat of the egg accumulates chemicals. In the present study, therefore, pre-hatch embryos of *Rana rugosa*, a native Japanese frog, were exposed to E_2, EE_2, BPA and NP, then the levels of chemicals in the jelly coat and embryo were analyzed separately.

Materials and Methods

Artificial insemination. Rana rugosa were collected in Higashi-Hiroshima, Japan, and kept for generations in our laboratory. Since estrogen treatment induces male-to-female sex reversal (data not shown), it is very useful for investigating the impact of estrogenic chemicals on this frog species. Acetone-dried pituitaries were used to induce artificial ovulation of female *R. rugosa*, and were prepared as follows: fresh female pituitaries of adult *R. nigromaculata* were collected in acetone. After collection, pituitaries were transferred twice to fresh acetone at 30-min intervals, and then dried in a fresh glass vial under light, and stored in a desiccator until use. Two acetone-dried pituitaries were crushed in a mortar, and suspended in 0.5 ml of Ringer's solution immediately before injection. Single intra-peritoneal injection of pituitary suspension along with 200 IU human chorionic gonadotropin (HCG) (Novartis Animal Health Co., Tokyo, Japan) (dissolved in 0.2 ml of ringer's solution) was given to female *R. rugosa*. The activated female frog was kept at 22˚C in a dark environment for 16 h, following which unfertilized eggs were pressed out onto a glass slide. Meanwhile, the testes were dissected from male frogs and homogenized in 1.0 ml of Ringer's solution to prepare sperm suspension. The unfertilized eggs were artificially inseminated with the sperm suspension on the glass slide. Developmental stages of embryos were determined according to the table by Shumway (43).

Chemical treatments and sample collection. E_2, EE_2, BPA, and NP, purchased from Sigma Chemical (St. Louis, MO, USA) were dissolved in dimethylsulfoxide (DMSO) at a concentration of 1 mM as stock solutions. After 20 min of artificial insemination, the fertilized eggs on the glass slides were transferred and reared in 50 ml of de-chlorinated water supplemented, singly, with 500 nM of E_2, EE_2, BPA, and NP (E_2, 0.136 ppm; EE_2, 0.148 ppm; NP, 0.110 ppm; BPA, 0.114 ppm) for two days. DMSO was used as a vehicle control. The embryos were then reared in fresh breeding water devoid of chemicals for six days. The breeding water was changed every 24 h. Twenty embryos were pooled in one tube at 2, 4, 6, and 8 days after fertilization (DAF). Because the embryos hatched between 2 and 4 DAF, the jelly coat was removed from twenty embryos at 2 DAF with fine forceps, and pooled in a glass tube. Ten milliliters of water from each treatment group was collected in a glass bottle at 2, 4, 6, and 8 DAF. Each estrogenic chemical were also added to the breeding water in the absence of the embryos as a reference experiment; the test water was also collected from the breeding water one day later. All these samples were weighed and stored at –30˚C until gas chromatography/ mass spectroscopy (GC/MS) analysis was conducted (44).

Measurement of estrogenic chemicals with GC/MS. Concentrations of the supplemented chemicals in the embryos, jelly coats, and breeding water were measured using a high-performance liquid chromatography (HPLC) method, explained in detail as follows.

Experimental reagents. N,O-*Bis*(trimethylsilyl)trifluoroacetamide (BSTFA) (Sigma) was used as a reagent for silylation. Analytical-grade reagents (Kanto Chemical Co., Tokyo, Japan) were used, unless stated otherwise. E_2-d_3, BPA-d_{16}, and NP-d_4 (*p-n*-nonylphenol-d_4) were used as internal standards for GC/MS analysis.

Determination of the concentrations in test water. Ten-milliliter water samples were mixed with 0.2 ml of 1 M HCl, 0.3 g of NaCl, and 1 μg of internal standards. The hormones were extracted twice with 2 ml of dichloromethane by shaking for 30 min. Anhydrous sodium sulfate was used for dehydration of dichloromethane. The solution was then concentrated to approximately 0.5 ml by high-purity nitrogen gas. Then 200 μl of BSTFA and 100 μl of pyridine were added to the solution for the silylation. The resultant sample was sealed and left to stand at 80˚C for 30 min to allow for the reaction to proceed. Trimethylsilylated E_2 (E_2-TMS), EE_2 (EE_2-TMS), BPA (BPA-TMS) and NP (NP-TMS) were analyzed by GC/MS. The concentration of each chemical was determined by calculation of the ratio of the peak area of the target chemical to that of the internal standard.

Determination of the concentrations in embryo and jelly. The stocked embryos and jellies were enriched with 1 μg of the internal standards before homogenization in 5 ml of methanol. After centrifugation, the supernatant was collected. The extraction was repeated once. Hexane was added to remove non-polar substances. The resultant solution was mixed with 5 ml of dichloromethane, 25 ml of water, 500 μl of 1 M HCl, and 1 g of NaCl. The dichloromethane phase was collected and the extraction was repeated. The solution was then dehydrated and concentrated using N_2 gas. The extracts were cleaned by hexane using a silica-gel column. E_2, EE_2, and BPA were eluted through the column with a solution of acetone:hexane (30%:70%). NP was eluted with dichloromethane. The eluted solution was concentrated and silylated as mentioned above. Trimethylsilylated E_2 (E_2-TMS), EE_2 (EE_2-TMS), BPA (BPA-TMS) and NP (NP-TMS) were analyzed by GC/MS. The concentration of each chemical was determined by calculating from the ratio of the peak area of the target chemical to that of the internal standard.

GC/MS analysis. Analysis was conducted on a CP-3800 GC system (Agilent Technologies, Santa Clara, CA, USA) equipped with an ion trap MS detector (Saturn 2000, Agilent Technologies) and a capillary column (DB-5ms; length, 30 m; film thickness, 0.25 μm; diameter, 0.25 mm; Agilent Technologies), in splitless mode (10 psi, split ratio=30), using helium as the carrier gas. The injection volume

Table I. *Concentrations and bioconcentration factors (BCFs) for estrogenic chemicals in the embryo and jelly coat of Rana rugosa.*

Treatment	Sample	Concentration (ppm)	BCF
E_2	Embryo (3)[a]	53.726±10.571	217.9±42.9[b]
	Jelly (3)	0.916±0.080	3.7±0.3
	Breeding water (3)	0.247±0.020	
EE_2	Embryo (3)	5.470±1.894	170.2±58.9
	Jelly (3)	0.106±0.011	3.3±0.4
	Breeding water (3)	0.032±0.010	
BPA	Embryo (3)	13.754±11.340	382.3±315.2
	Jelly (3)	0.047±0.029	1.3±0.7
	Breeding water (3)	0.036±0.010	
NP	Embryo (3)	26.820±13.597	289.1±146.6
	Jelly (3)	3.495±0.677	37.7±7.3
	Breeding water (3)	0.093±0.025	

[a]No. of samples pooled; [b]mean±SE.

was 1 μl. The split vent was opened 1 min after starting the analysis. Injection port and ion trap temperatures were set at 300 and 250˚C, respectively. Column temperature was initially maintained at 35˚C for 1 min and then increased to 250˚C at a rate of 10˚C/min. After a 5-min pause at 250˚C, the temperature was increased again to 300˚C at a rate of 20˚C/min and kept constant for another 5 min. MS analysis was carried out using electron ionization (EI) auto mode with an MS scan speed of 1.2 scan/s and an MS detector voltage of 1450 V.

The quantitative ions were E_2-TMS at m/z=416 and 417, E_2-d_3-TMS at m/z=420, EE_2-TMS at m/z=426 and 425, BPA-TMS at m/z=357 and 358, BPA-d_{16}-TMS at m/z=368 and 369, NP-TMS at m/z=193, 207, 221 and 235, and NP-d_4-TMS at m/z=183 and 184. The amount of each chemical was calculated from the ratio of the peak area of the target chemical to that of the internal standard. E_2-d_3 was used as an internal standard for EE_2.

Calculation of bioconcentration factor (BCF). BCF values were calculated according to the following formula: BCF=C/Cw, where C is the concentration of the chemical in the embryo or jelly and Cw, the true concentration of the chemical dissolved in the breeding water without embryos.

Histological observation. Embryos were fixed in Bouin's solution overnight, embedded in paraffin, and sectioned at 7-μm thickness. The sections were stained with haematoxylin, and observed under a light microscope.

Statistical analysis. Significance of differences in the values of chemical concentrations and BCFs between the embryo, jelly coat, and breeding water were analysed using one-way ANOVA, followed by Tukey-Kramer test.

Results

Firstly, to clarify whether estrogenic chemicals in water accumulate in pre-hatch embryos through jelly coats, we reared newly fertilized eggs in the presence of E_2, EE_2, BPA or NP for two days and measured their concentrations in the embryo, jelly coat, and breeding water. At 2 DAF, the fertilized eggs, developed to the tail bud stage, were covered with a jelly coat (Figure 1a). BCF values for E_2, EE_2, BPA and NP in the embryo and jelly coat are shown in Table I. All four estrogenic chemicals were accumulated in the embryo at concentrations ranging from 5.470-53.726 ppm. The highest mean concentration among the four estrogenic chemicals in the embryo (53.726 ppm) was found in the E_2 treatment. Although insignificant, the mean BCF value for E_2 in the embryos (217.9) was lower than those of BPA (382.3) and NP (289.1). On the other hand, BCFs of the jelly coat ranged from 1.3 to 37.7. The mean BCF for each chemical in the embryos was 7.7- to 294.1-fold higher than that of the respective jelly coats.

Next, we investigated the pharmacokinetics of the estrogenic chemicals accumulated in the embryos by morphological and histological analysis (Figure 1). The majority of the internal organs were under-differentiated at the tail bud stage (2 DAF), and the abdominal region was occupied with many cells containing yolk (Figure 1b) (45). Embryos hatched between 2-4 DAF, were developed to stage 21 (Figure 1c) at 4 DAF. Although various organs, such as the liver, heart, and pronephros, were differentiated at stage 21, the abdominal region was still occupied by many cells containing yolk (Figure 1d). Thereafter, the embryos were developed to stages 24 and 25 at 6 and 8 DAF, respectively (Figure 1e and 1g). The yolk-containing cells greatly diminished in the abdominal region of the embryos at 6 DAF (Figure 1f), and the intestine was differentiated in the same region of the embryos at 8 DAF (Figure 1h).

After two days of exposure, concentrations of different estrogenic chemicals were analyzed at different time points (Figure 2). E_2 concentrations in the embryos decreased slightly between 2 and 4 DAF, but a drastic reduction was observed between 4 and 6 DAF and concentrations remained

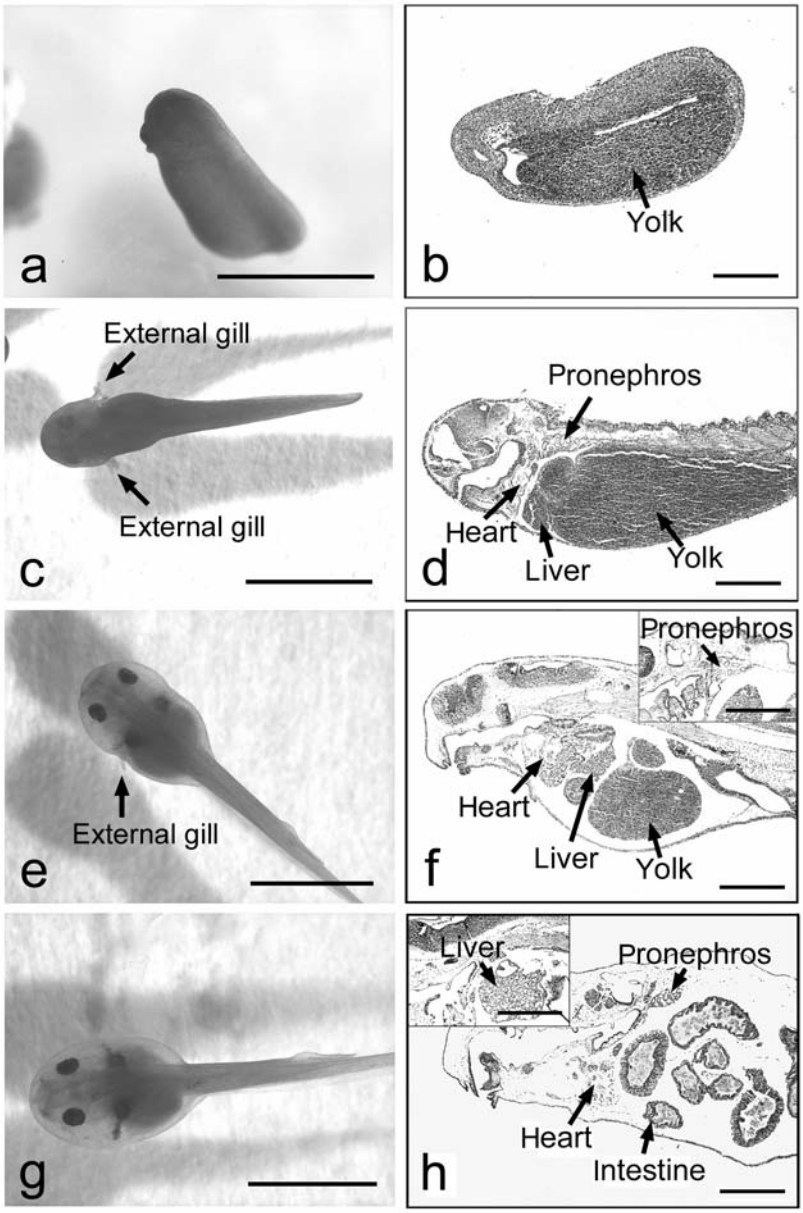

Figure 1. *External appearances (a, c, e, g) and histological features (b, d, f, h) of the early embryos of Rana rugosa. Fertilized eggs were developed to the neurula (a, b) at two days after fertilization (DAF) and the embryos at stages 21 (c, d), 24 (e, f), and 25 (g, h) of Shumway (43) at 4, 6, and 8 DAF, respectively. At 2 DAF, the chemical treatments were terminated. Hatching occurred at 3 DAF. Two external gills can be seen at both sides of the embryo at stage 21 (c), and thereafter only one gill disappears in the embryo at stage 24 (e). A mass of cells containing yolk can be seen in the abdominal region of the neurula (b), and thereafter this diminished in the embryos during development (d, f, h). Organs such as the pronephros, heart, liver, and intestine, are differentiated in the embryos during development (d, f, h). Scale bar=2 mm.*

low, to 8 DAF (Figure 2a). On the other hand, concentrations of EE_2, BPA and NP in the embryos decreased gradually from 2 to 8 DAF (Figure 2b-d), except for BPA groups, where no reduction was observed between 2 and 4 DAF (Figure 2c). The estrogenic chemicals in breeding water collected at 3, 4, 6, and 8 DAF were not detected by the assay system (data not shown).

Discussion

The present study showed that the embryo body of fertilized eggs and pre-hatch embryos of *R. rugosa* accumulated xenoestrogens (EE_2, BPA and NP) and native estrogen (E_2) through the jelly coat with similar BCFs. Since the BCFs for the estrogenic chemicals in the jelly coat of the pre-hatch

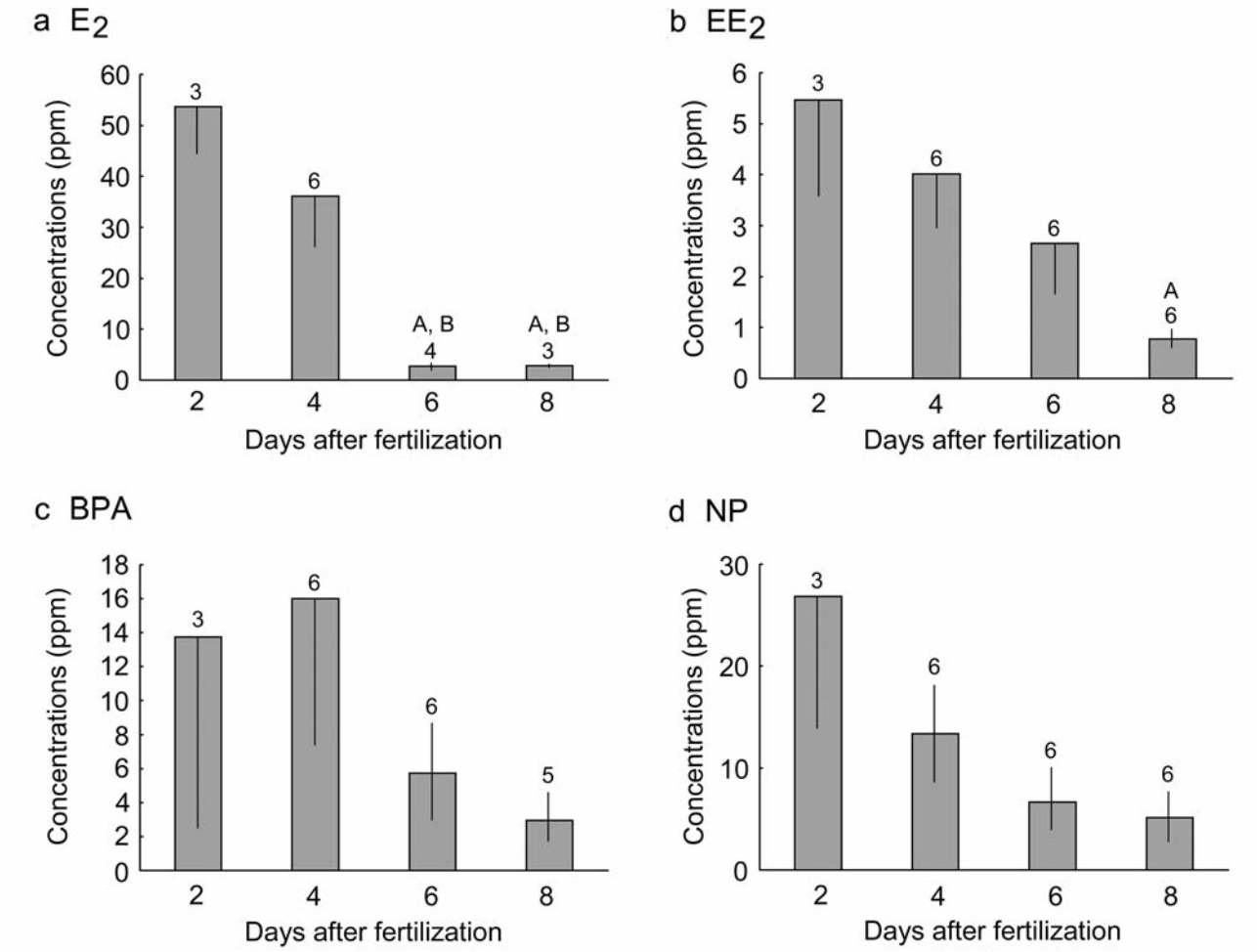

Figure 2. *Concentrations of accumulated exogenous native estrogen and xenoestrogens in the embryo and jelly coat of Rana rugosa during development. The pre-cleaved embryos were exposed to 17β-estradiol (E_2; a), 17α-ethynylestradiol (EE_2; b), bisphenol A (BPA; c), and nonylphenol (NP; d) from 20 min after fertilization for two days. After two days, embryos were transferred to breeding water without estrogenic chemicals and reared for an additional six days. The breeding water was exchanged every day. The embryos were collected at 2, 4, 6, and 8 days after fertilization (DAF). Concentrations of the chemicals in the embryos were measured using a HPLC method. Numbers of samples examined are indicated above each column. Each column and the vertical line represent the mean±SE. Significance of differences: A, $p<0.05$ vs. 2 DAF; B, $p<0.05$ vs. 4 DAF.*

embryo were not very high, it is conceivable that the jelly coat does not function as a barrier to the transport of estrogenic chemicals to fertilized eggs and pre-hatch amphibian embryos, as reported earlier, using cypermethrin (46) and isoproturon (47). But, it could not be excluded the possibility that the jelly coat functions to keep out the estrogenic compounds, probably through carriers/efflux pumps. Further experiments are needed to clarify this possibility. These low estrogenic chemicals are lipophilic and amphibian eggs contain large amounts of lipids in the form of yolk. Cells containing yolk were still observed in the abdominal region of the pre-hatch embryo, a stage when the chemical concentrations decreased slightly. This supports the notion of bioconcentration of estrogenic chemicals in the embryonic body, especially in cells containing yolk. Similarly to our findings, Honkanen *et al*. (48) also reported accumulation of BPA in both the yolk sac and embryonic body of landlocked salmon (*Salmo salar*) at pigmented-eye stages. Thus, it could be considered that the estrogenic chemicals accumulate preferentially in the yolk during development from the fertilized egg up to the pre-hatch embryo.

Concentrations of estrogenic chemicals accumulated in the embryo body decreased at 4-6 DAF. The reduction of chemicals in the embryo may be due to two reasons as follows: (i) a decrease in the number of cells containing yolk (as discussed above) and (ii) metabolism of the estrogenic chemicals by internal organs of the developing larvae.

Surprisingly, the estrogenic chemicals were not detected in the breeding water at 3 DAF, which suggests the strong possibility of their metabolism by the developing post-hatch embryo. Moreover, gradual reduction of the chemical concentration with development also supports the second explanation. In this regard, studies on metabolism of xenoestrogens have shown that mammalian hepatocytes glucuronidate exogenous BPA *in vitro* (49, 50). BPA, introduced orally into rats is absorbed by the intestine and glucuronidated exclusively in the liver, and the glucuronide of BPA was accumulated in the bile (51). In rats (52) and carp (53), intestines are also able to glucuronidate BPA. NP was found to be metabolized in hepatic microsomes in rats and humans (54). In the juvenile rainbow trout, the metabolites of exogenous NP were found predominantly in the pyloric caecum, intestine, liver, and bile several days after the injection of NP (55). Similarly in juvenile salmon, exogenous NP and its metabolites were found predominantly in the bile, viscera, fat, kidney, liver, and skin after dietary exposure (56). These studies indicate that inner organs play an important role in metabolizing xenoestrogens. Like mammals and fish, adult *R. temporaria* and *X. laevis* sulphurize and glucuronidate phenols into conjugate forms (57), and *X. laevis* tadpoles metabolize BPA (58) and tetrabromo*bis*phenol A (59). In the present study, histological observations showed that various organs such as the liver, intestine, and pronephros differentiated progressively in the developing embryos after hatching. Thus, it appears that the differentiated organs may metabolize the exogenous estrogenic chemicals accumulating in the pre-hatch embryos.

The half-life of BPA is several hours in adult rats (60), and half a day in juvenile rainbow trout (61) and adult zebrafish (62). The half-lives of isotope-labelled NP residues in carcass, viscera and muscle of the juvenile salmon are between 24 and 48 h (56). Tadpoles of *X. laevis* are capable of metabolizing almost all exogenous tetrabromo*bis*phenol A within 8 h (59). It took several days for possible metabolism of exogenous xenoestrogens in the amphibian embryos in the present study. Furthermore, concentrations of EE_2, BPA and NP decreased more slowly in the embryo body than that of E_2, which may indicate different efficacies of the metabolism of xenoestrogens and native estrogen. Toxic effects such as abnormalities in body shape (36) and melanocyte differentiation (34), and apoptosis of neural cells (35) are induced by exposure of *X. laevis* pre-hatch embryos to estrogenic chemicals. Sublethal toxicity is also observed in larvae of *Rhinella arenarum* at the end of embryonic development after 24 h exposure of BPA at the early-blastula stage (38). Thus, the present study together with these findings suggests that high accumulation and slow clearance of xenoestrogens in pre-hatch amphibian embryos probably induce abnormal development after hatching.

In conclusion, fertilized eggs and pre-hatch embryos of *R. rugosa* accumulated EE_2, BPA, NP and E_2 through the jelly coat with no significant differences of BCFs. Since BCFs for the estrogenic chemicals in the jelly coat of the pre-hatch embryo were very low, the jelly coat does not appear to function as a barrier for the transport of estrogenic chemicals to the fertilized eggs and pre-hatch amphibian embryos.

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