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Determining the optimal developmental stages of *Xenopus laevis* for initiating exposures to chemicals for sensitively detecting their feminizing effects on gonadal differentiation

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ABSTRACT

Xenopus laevis is an important model for detecting feminizing effects of endocrine disrupting chemicals (EDCs) on amphibians because its genetic males can be induced to phenotypic females by estrogenic chemicals. It is crucial that chemical exposures begin at sensitive developmental stages for gonadal sex-reversal in *X. laevis*. To determine the optimal stages for initiating exposures, we investigated gonadal sex-reversal induced by low concentrations of 17α-ethinylestradiol (EE2) when exposures were initiated at different stages (3/4, 45/46, 48 and 50) until stage 58. We found that 0.1 nM EE2 resulted in 85%, 86%, 43%, and 19% intersex, whereas 1 nM EE2 caused 77%, 81%, 17%, and 8% phenotypic females, when genetic male tadpoles were exposed from stages 3/4, 45/46, 48 and 50, respectively. The data show the sensitivity of *X. laevis* gonads to EE2 at stages 45/46 is similar with that at stages 3/4, but the sensitivity decreases at stage 48 and stage 50, displaying a developmental stage-dependent manner. In another experiment using the offspring of another pair of frogs, we confirmed high sensitivity of *X. laevis* gonads at stages 45/46 to low concentrations of EE2. Considering that stages 45/46 tadpoles are easier to manipulate and have higher survival rates than earlier embryos, we propose that stages 45/46 are the optimal stages for initiating exposure for detecting feminizing effects of EDCs on gonadal differentiation in *X. laevis*. The developmental stages for initiating exposures we determined will guarantee the high sensitivity for detecting feminizing effects of EDCs with low estrogenic activities on gonadal differentiation in *X. laevis*. Also, our study suggests that gonadal differentiation in *X. laevis* possibly begins at stages 45/46, but not at later stages.

1. Introduction

In the past several decades, the presence of endocrine disrupting compounds (EDCs) that have the ability to interfere with the normal functions of the endocrine systems of humans and wildlife in the environment has aroused extensive attention (Beronius and Vandenberg, 2016; Colborn et al., 1993; Roig et al., 2013). Many EDCs, including some of hormone pharmaceuticals, personal care products, growth promoters in animal agriculture, pesticides, etc. are constantly discharged into water (Gore et al., 2015; Hotchkiss et al., 2008; Locatelli et al., 2016). The adverse influences of EDCs on the reproductive systems of organisms in water, such as fish and amphibians, have received special attention (McRobb et al., 2014; Sumpter and Johnson, 2008). Due to a semi-aquatic life cycle, aquatic reproduction and a highly permeable skin, amphibians like fish are especially vulnerable to EDCs (Bernanke and Köhler, 2009; Egea-Serrano et al., 2012). Some amphibian species can be feminized by EDCs via mimicking estrogens, altering estrogen levels or other pathways, such as 17α-ethinylestradiol (EE2), polychlorinated biphenyls (PCBs), atrazine, bisphenol A (BPA), dibutyl phthalate (DBP) (Mosconi et al., 2002; Gyllenhammar et al., 2009; Orton and Tyler, 2015). In addition to these laboratory data, wild investigations have provided evidences for apparent correlations between estrogenic EDCs and reproductive abnormalities in wild amphibians (Lambert et al., 2015; McDaniel et al., 2008). Thus, estrogenic EDCs have become one of suspicious contributors to global amphibian declines in recent years (Collins, 2010; Hayes et al., 2010a).

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Table 1
Previous data concerning gonadal feminization in *Xenopus laevis* following exposure to some endocrine disrupting chemicals in the literature.

Chemicals	Stages for initiating exposure	Source of *Xenopus*	End exposure	Concentrations	Feminizing effects	References
E2	stages1-10	Xenopus Express	stage 66	1, 10, and 100 μg/L (3.67, 36.7, 367 nM)	66, 99, 100% females	Hu et al. (2008)
	within 24 h of fertilization	Xenopus Express	stage 66	1 μg/L E2 (3.67 nM)	70% females	Sharma and Patino (2010)
	stage 40	Department of Inland Fisheries	stage 66	10 and 100 nM	>75% or near 100% females	Bogi et al. (2002)
	stages 42/43	Ecology and Inland Fisheries	stage 66	10 and 100 nM	75%-80% females	Levy et al. (2004)
	stages 45/46	Xenopus I	stage 66	0.2, 1.5, 6.0 μg/L (0.73, 5.5, 22 nM)	88, 95, and 98% females	Lutz et al. (2008)
	just after hatching or stage 51	Jagiellonian University	stage 49, 52, 54, 58, 61, 66	100 μg/L (367 nM)	94% and 86% females in just after hatching and stage 51 group	Piprek et al. (2012)
	stages 46/47	Chinese Academy Sciences	stage 66	100 μg/L (376 nM)	80% females	Qin et al. (2003)
	stage 47/48	Xenopus I	stage 66	0.2, 1.5 μg/L (0.73, 5.51 nM)	70% and 92% females	Lutz et al. (2008)
	48 h posthatch	Xenopus Express	stage 66	100 μg/L (376 nM)	67% females	Carr et al. (2003)
	8 dpf	Xenopus I	stage 66	0.2, 1.5, 6.0 μg/L (0.73, 5.51, 22 nM)	86%, 92% and 98% females	Wolf et al. (2010)
	72 h posthatch	Xenopus Express	2–3 months post-metamorphosis	100 μg/L (376 nM)	8% genetic male reversed to female	Coady et al. (2005)
	Stages 49/50	Gunma University	stage 57	0.1, 1, 10, and 20 nM	0, 97, 100 and 100% ovaries in males	Oka et al. (2006)
EE2	stages 42–44	Freshwater Ecology and Inland Fisheries	stage 66	50, 500, 5000 ng/L (0.16, 1.6, 16 nM)	31.3%, 76.5% and 100% sex-reversal in genetic males (ZZ)	Tamschick et al. (2016)
	fertilized eggs	Boreal Laboratories	89 d	0.09, 0.84, 8.81 μg/L (0.29, 2.7, or 28 nM)	0, 7, and 17% females in genetic males	Tompsett et al. (2012)
BPA	stages 38–40	University of Karlsruhe	12 weeks	10 and 100 nM	65% females	Kloas et al. (1999)
	stages 42/43	Institute of Freshwater Ecology and Inland Fisheries	stage 66	10 and 100 nM	69% and 65% females	Levy et al. (2004)
	stages 43/45	Jagiellonian University	stage 66	0.83, 2.1, 9.5, 23.8, 100, 497 μg/L	no observable effect	Pickford et al. (2003)
atrazine	hatching	Tecalote Creek	3 years	2.5 μg/L	10% feminized in genetic ZZ males	Hayes et al. (2010b)
	stage 48 (4dpf)	University of California	stage 66	0.01–200 μg/L	20% multiple gonads	Hayes et al. (2002)
	48 h posthatch	Xenopus Express	stage 66	25 μg/L	4.7% intersex gonads	Carr et al. (2003)
	stage 46–47	no information	120 d	100 μg/L	sperms were reduced to 17.51%.	Chen et al. (2015)
	stage 47	no information	stage 62	200 or 400 μg/L	no differences in sex ratio	Zaya et al. (2011)
	8dpf	Xenopus I	stage 66	0.01–100 μg/L	no significant changes	Kloas et al. (2009a)
	stage 48	Xenopus I	stage 66	25 μg/L	no affecting sex differentiation	Kloas et al. (2009b)
	72 h posthatch	Xenopus Express	2–3 months post-metamorphosis	10, or 25 μg/L	2.7% and 2.6% mixed sex	Coady et al. (2005)
	stage 49	Yamamura frog store	stage 66	10 and 100 μg/L	62% and 72% females	Oka et al. (2008)
PCB3 and PCB5	stages 46/47	Chinese Academy Sciences	stage 66	5–80 μg/L	12%-17% abnormal testes	Qin et al. (2003)

E2: 17β-estradiol; EE2: 17α-ethynylestradiol; BPA: bisphenol A; PCB: polychlorinated biphenyl.

Although many amphibian species have been demonstrated to be sensitive to estrogenic EDCs (Hogan et al., 2008; Ohtani et al., 2001), *Xenopus laevis* is the most used model species for detecting feminizing effects of EDCs on amphibians (Kloas, 2002; Oka et al., 2006). For example, some EDCs with weak estrogenic activities, such as BPA and PCBs, were reported to cause significant intersexes in *X. laevis* (Kloas et al., 1999; Levy et al., 2004; Qin et al., 2003). Furthermore, estrogenic pharmaceuticals were demonstrated to result in complete male-to-female reversal in *X. laevis*; for instance, Tamschick et al. (2016) reported that 50, 500, 5000 ng/L EE2 caused 31.3%, 76.5% and 100% phenotypic females in genetic males, respectively. The data have enough demonstrated high sensitivity of *X. laevis* gonadal differentiation to estrogenic EDCs. In the literature, however, there are inconsistent results concerning feminizing effects of EDCs on *X. laevis*. For example, Hayes et al. (2002, 2010b) reported that atrazine induced gonadal feminization of *X. laevis*, but other investigators cannot repeat the findings of Hayes (Kloas et al., 2009a,b). Even, Lutz et al. (2008) reported that the same concentration (0.2 μg/L) of estradiol (E2) resulted in different female percentages (88.4% and 70.5%) of *X. laevis* in two independent experiments. The inconsistent results suggest that certain key factor(s) that determine the outcome of weak estrogenic EDCs or low concentration of estrogens may not have been noticed.

As we know, the developmental stages for initiating exposure are crucial for sex reversal in amphibians (Hu et al., 2008; Phuge and Gramapurohit, 2014). Villalpando and Merchant-Larios (1990) reported that exposures of *X. laevis* to 100 μg/L estradiol benzoate (EB) beginning at stages 44–50 produced all phenotypic females, whereas exposures beginning at stages 51–54 resulted in intersexes. According to Villalpando and Merchant-Larios' findings,