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# The plasticizer bisphenol A affects somatic and sexual development, but differently in pipid, hylid and bufonid anurans☆

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ABSTRACT

Due to their terrestrial habitats and aquatic reproduction, many amphibians are both very vulnerable and highly suitable bioindicators. The plasticizer bisphenol A (BPA) is one of the most produced chemical substances worldwide, and knowledge on its impacts on humans and animals is mounting. BPA is used for the industrial production of polycarbonate plastics and epoxy resins and found in a multitude of consumer products. Studies on BPA have involved mammals, fish and the fully aquatic anuran model *Xenopus laevis*. However, our knowledge about the sexual development of non-model, often semi-terrestrial anuran amphibians remains poor. Using a recently developed experimental design, we simultaneously applied BPA to two non-model species (*Hyla arborea*, Hylidae; *Bufo viridis*, Bufonidae) and the model *X. laevis* (Pipidae), compared their genetic and phenotypic sex for detection of sex reversals, and studied sexual development, focusing on anatomical and histological features of gonads. We compared three concentrations of BPA (0.023, 2.28 and 228 μg/L) to control groups in a high-standard flow-through-system, and tested whether conclusions, drawn from the model species, can be extrapolated to non-model anurans. In contrast to previous studies on fish and *Xenopus*, often involving dosages much higher than most environmental pollution data, we show that BPA causes neither the development of mixed sex nor of sex-reversed individuals (few, seemingly BPA-independent sex reversals) in all focal species. However, environmentally relevant concentrations, as low as 0.023 μg/L, were sufficient to provoke species-specific anatomically and histologically detectable impairments of gonads, and affected morphological traits of metamorphs. As the intensity of these effects differed between the three species, our data imply that BPA diversely affects amphibians with different evolutionary history, sex determination systems and larval ecologies. These results highlight the role of amphibians as a sensitive group that is responsive to environmental pollution.



## 1. Introduction

Natural or synthetic endocrine disrupting compounds (EDCs) are increasingly detected in the aquatic environment. These substances act as agonists or antagonists of hormones, hormone receptors and their pathways, and thus interfere with the endocrine system of animals (Aris et al., 2014; Bhandari et al., 2015; Vandenberg et al., 2009). In the last decades, bisphenol A (BPA) has received enormous attention from scientists, politicians and the public. With more than two million tons per year it is one of the most produced chemicals worldwide (Burridge, 2003), and evidence that BPA affects humans and animals is mounting (Bhandari et al., 2015; Braun et al., 2011, 2009; Canesi and Fabbri, 2015). BPA is a synthetic organic compound used for the manufacturing of polycarbonate plastics and epoxy resins and is thus found in a multitude of consumer products, such as thermal receipts, water pipes, sport and medical equipment, toys and electronics (Bhandari et al., 2015; Kang et al., 2007; Vandenberg et al., 2007). Additionally, it is used for internal coatings of tin cans, food containers and plastic

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bottles, from where it leaches into foods and beverages (Kang and Kondo, 2002; Le et al., 2008; Wong et al., 2005). Although often termed a “xenoestrogenic” compound, once incorporated, BPA is able to bind several hormone receptors (e.g. estrogen, androgen, thyroid hormone receptors in brain, liver and gonads) and thus impairs the normal endocrine pathways (Flint et al., 2012; Vandenberg et al., 2013; Watson et al., 2011). In fact, BPA has been detected in a variety of human tissues and fluids (Vandenberg et al., 2009, 2007; vom Saal et al., 2012). In mice it has been shown to advance female puberty (Howdeshell et al., 1999) and causes abnormalities of reproductive organs (Gupta, 2000; Markey et al., 2005, 2001; Vandenberg et al., 2008; Vom Saal et al., 1998). In 2015, the European Food Safety Authority (EFSA) conducted a reassessment of BPA and lowered the safe level of incorporation from 50 μg/kg bodyweight/day to 4 μg/kg. Nevertheless, EFSA stated that BPA “poses no health risk to consumers of any age group (including unborn children, infants and adolescents) at current exposure levels” (www.efsa.europa.eu). However, through the increasing introduction of man-made plastic waste as well as industrial and municipal wastewater, BPA is found ubiquitously in surface and ground waters with increasing concentrations (Crain et al., 2007; Flint et al., 2012). Aquatic BPA has been detected at levels of ≤0.27 μg/L in Germany, ≤ 0.32 μg/L in the United States, ≤ 21 μg/L in the Netherlands, and up to ≤740 μg/L in Japan (Belfroid et al., 2002; Bolz et al., 2001; Kawagoshi et al., 2003; Kolpin et al., 2002; Kuch and Ballschmiter, 2001; Rudel et al., 1998). Although BPA seems to degrade quickly (half life time 3–24 h; Gallard et al., 2004; Stahlhut et al., 2009), because of continuous input, its presence in the environment remains high. When observing effects of EDCs in the limnic environment, fish and amphibians are the most endangered vertebrates (Caldwell et al., 2012, 2008). Due to their highly permeable skin and, in most species, aquatic larval phase, dissolved chemicals can easily enter the body of amphibians and impair their somatic and sexual development by interference with the organisms' hormonal control. In addition, most amphibians are terrestrial as adults, and are thus also potential recipients of EDCs found in the terrestrial environment (Bhandari et al., 2015; Kloas, 2002; Orton and Tyler, 2014). Several studies testing effects of BPA on the somatic development of amphibians have been conducted and multiple negative outcomes were observed. Most of these experiments investigated the African clawed frog *Xenopus laevis*, however, at very high, environmentally unrealistic doses. In this model species, treatments reaching from 4560 to 11,400 μg/L BPA resulted in mostly complex effects, including suppression of organogenesis, scoliosis (crooked vertebrate column), malformations of abdomen and the head (including eyes), and shortened the body length (Imaoka et al., 2007; Iwamuro et al., 2003; Oka et al., 2003; Sone et al., 2004). A delayed metamorphosis of clawed frog tadpoles was observed at a concentration of 2280 μg/L BPA (Iwamuro et al., 2003). Other negative effects on the somatic development, namely delayed metamorphosis, reduced body size and microcephaly with mouth malformations, were also observed in two distantly related (more than 200 million years divergence) ranid and bufonid non-model species: at 2280 μg/L BPA in the ranid frog *Rana chensinensis* (Canesi and Fabbri, 2015) and between 1250 and 40,000 μg/L in the bufonid toad *Rhinella arenarum* (Wolkowicz et al., 2014). In two studies investigating the same endpoints in the model *X. laevis* at lower concentrations (0.83–497 μg/L), no effects on growth, somatic development or metamorphosis were observed (Levy et al., 2004; Pickford et al., 2003). In contrast, in another model species from the same systematic family (Pipidae), *Xenopus tropicalis*, exposure to only 2.3 μg/L BPA (Kashiwagi et al., 2008) inhibited metamorphosis. This was also observed in the ranid *Rana rugosa* (at 228 μg/L; Goto et al., 2006). Due to BPA's estrogenic potency (Levy et al., 2004; Matthews et al., 2001), it has been in the focus of studies on sexual development, but mostly in fish. For anuran amphibians, only very few studies focused on the sexual development. In male amphibians, BPA, by binding to estrogen receptors in the liver, induces the synthesis of vitellogenin, the egg yolk precursor protein (Bhandari et al., 2015; Canesi and Fabbri, 2015; Gye and Kim, 2005; Kloas et al., 1999). Other feminization effects have been determined via skewed sex ratios. In *X. laevis*, treatment with 22.8 μg/L BPA resulted in sex ratios with approximately 62%–70% females (Kloas et al., 1999; Levy et al., 2004), but no feminization occurred at higher or lower doses (228 or 2.28 μg/L). In contrast, in a concentration range from 0.83 to 497 μg/L, sex ratios of *X. laevis* appeared unaffected (Pickford et al., 2003).

To our knowledge, the present study is the first on non-model anurans from the Hylidae and Bufonidae on sexual development under BPA-exposure. Both taxa are widespread in southeastern and Central (*Bufo viridis, Hyla arborea*) or Northwestern Europe (*H. arborea*). The species complexes of tree frogs and green toads show an Eurasian distribution, where their breeding ponds, specifically shallow river-side standing waters and ponds in flooding areas of rivers and streams as well as pools and ponds (Schneider and Grosse, 2009; Stöck et al., 2009) are potentially BPA-polluted from various sources (e.g. Careghini et al., 2015; Papaevangelou et al., 2016), including urban wastewaters as generally known sinks for EDCs including BPA (cf. Lambert et al., 2015; Lambert and Skelly, 2016). Indeed, BPA is also frequently detectable in European and Asian water bodies (Bhandari et al., 2015; Crain et al., 2007; Duong et al., 2010) and therefore highly relevant for both species complexes. We aimed at testing whether previous results obtained in the model *X. laevis* (Pipidae) are applicable to the European non-model anurans *Hyla arborea* (European tree frog) and *Bufo viridis* (European green toad), or if we find susceptibility differences between these anuran lineages (cf. Tamschick et al., 2016). The Pipidae diverged from the Neobatrachia, including Bufonidae and Hylidae, more than 206 million years ago (www.timetree.org), and there are notable differences in their ecotoxicologically relevant genome composition (e.g. Helbing, 2012), feeding behavior and ecology (Avila and Frye, 1978; Degani, 1986; Wilbur, 1980; Schneider and Grosse, 2009; Stöck et al., 2009; www.amphibiaweb.org). While *X. laevis* tadpoles are filter-feeders on suspended algae, *H. arborea* uses water surface particles (or rasps organic material from plants or stones), and *B. viridis* mostly consumes benthic algae and detritus. These ecological differences might cause variance in EDC-exposure, especially between ground-feeding and filter-feeding larvae. Importantly, these species also differ in their sex determination systems with either male heterogamety (XX/XY) in *H. arborea* (Berset-Brändli et al., 2006; Stöck et al., 2011a, 2011b) and *B. viridis* (Stöck et al., 2011a; Tamschick et al., 2015) or female heterogamety (ZZ/ZW) in *X. laevis* (Chang and Witschi, 1956; Yoshimoto et al., 2008).

Using a high-standard flow-through-system under identical experimental conditions, we simultaneously exposed larvae of *H. arborea*, *B. viridis* and the well-investigated but deeply diverged model species *X. laevis* to an environmentally relevant concentration range of BPA. Our study combines genetic sexing and histology to detect potentially feminizing effects and impairments of sexual and somatic development in metamorphs.

## 2. Material and methods

### 2.1. Study animals

*Xenopus laevis* tadpoles were obtained from the breeding stock at the Leibniz-Institute of Freshwater Ecology and Inland Fisheries (IGB). Induction of spawning and rearing of tadpoles followed Lorenz et al. (2011). Parts of clutches of *H. arborea* and *B. viridis* were