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Toxic effects of estradiol E_2 on development in the European tree frog (*Hyla arborea*)

Karim Ghali[1,2], Julien Leuenberger[1], Michel Ansermet[2], Nicolas Perrin[1] and Christophe Dufresnes[1,*]

Abstract. Oestrogenic hormones are a major environmental threat to aquatic wildlife. Here we report on chronic toxic effects of larval exposure to the naturally excreted oestrogen 17β-estradiol (E_2), in the European tree frog (*Hyla arborea*), by means of an experimental setting and long-term monitoring. Larval survival was significantly lower in treated tanks when compared to controls, as more treated larvae died during metamorphosis. Morphometric data suggested an impact on froglet phenotypes, with treated individuals being heavier (but not different in size) than control ones. Survival during juvenile growth was also significantly lower for exposed frogs, which after two and a half years of monitoring furthermore seemed to have developed slower. In addition to the well-documented impact on sexual differentiation and mating behaviour, the general toxicity of human-released oestrogens likely contributes to global amphibian declines, particularly for tree frogs which are threatened in many countries.

Keywords. amphibian, endocrine disruptors, estradiol-17β, oestrogens, sex hormones, xenobiotics

Introduction

Natural and synthetic estrogenic steroids are a major environmental issue. Oestrogens are naturally produced by humans and massively used in drugs and medication (e.g. birth control pills, hormone replacement therapies). As they are not fully metabolized when excreted through urine, these molecules remain active long after their transfer to the environment via wastewater systems (Braun et al., 2003). Consequently, they are present in effluent, surface, and even drinking waters (Ternes et al., 1999; Kuch and Ballschmiter, 2001), and can display high estrogenic activities even at extremely low concentrations (Jobling et al., 1998). Especially aquatic wildlife is therefore affected by oestrogens, which have been shown to impact mating behaviour and interfere with sexual differentiation (Freedberg et al., 2006; Lahnsteiner et al., 2006; Hu et al., 2008; Partridge et al., 2010; Saaristo et al., 2010; Hoffman and Kloas 2012; Bhandari et al., 2014). In amphibians, oestrogenic and other endocrine disruptive compounds (EDCs, e.g. pesticides) are assumed to contribute to the worldwide decline of populations via their feminization (Carey and Bryant, 1995; Hayes et al., 2002, 2006b). This effect was well demonstrated for artificial oestrogen derivatives, such as used for human contraception (17α-ethynylestradiol, EE_2; Bhandari et al., 2014; Tamschick et al., 2016). In parallel, the human endogenous sex hormone 17β-estradiol (E_2) is also known to impact aquatic vertebrates (e.g. Friedman et al., 2004). E_2 is naturally released by men and women and is also widely used for pharmaceutical purposes (> 500 kg/year sold in the past decade in the US; Laurenson et al., 2014), accumulating in sewage effluent and surface waters up to concentrations sufficient to induce sex-reversal in sensitive fishes (Routledge et al., 1998). Importantly, the sensitivity of amphibians to environmental oestrogens seems to greatly differ between species (Tamschick et al., 2016) and experimental observations are needed to understand species-specific responses.

The European tree frog *Hyla arborea* (Linnaeus, 1758) is one of the most widespread amphibian species of the Western Palearctic, distributed from the Balkan Peninsula to North-western France, but it is declining

[1] Department of Ecology & Evolution, Biophore Building, University of Lausanne, CH-1015, Lausanne, Switzerland
[2] Vivarium de Lausanne, Chemin de Boissonnet 82, 1010 Lausanne, Switzerland
* Corresponding author. E-mail: Christophe.Dufresnes@unil.ch

in many regions (Dufresnes and Perrin, 2015). Like in other amphibians, xenobiotic compounds, notably pesticides, are assumed to significantly contribute to these declines; tree frogs are particularly threatened in industrial and densely populated countries (Dufresnes and Perrin, 2015), where pollution by pesticides and other chemicals is a major environmental concern. Accordingly, recent experimental data suggested an effect of the contraceptive EE_2 on sex-differentiation in this species (Tamschick et al., 2016). Here we take advantage of an experiment involving artificial exposure of European tree frog tadpoles to the human natural oestrogen (E_2), followed by long-term husbandry and monitoring. We analysed survival and morphometric data, and report toxic effects on development in this threatened species.

Methods

Tadpoles of *H. arborea* were exposed to three different concentrations of E_2 via food intake (fish flakes, administrated throughout the larval stage; 10, 100 and 500 µg/g of flakes) while their development was monitored from hatching to sexual maturity. To this end, food (flakes) was treated by baths of absolute ethanol with solubilized E_2, until complete evaporation; control food received E_2-free ethanol baths. Similar administration techniques are used in fish (Lee et al., 2009). The experimental design comprised four replicate tanks for each concentration (12 treated tanks in total) and five control replicates. At the beginning of the experiment (spring 2013), each tank (50×30×30cm) included 40 newly-hatched tadpoles of the same clutch. Clutches were obtained the same night, by capturing mating pairs in a local Swiss population (46.502507°, 6.419579°; details in Dufresnes et al., 2011). In total, four different clutches were sampled and divided between treatments and controls, allowing testing for potential family effects. The experiment was conducted in a homogenous room, with stable room temperature (~20°C) and artificial light (12:12 hours cycle). Tadpoles were fed *ad libitum*, exclusively with flakes.

Larval development was monitored by counting tadpoles every 7 days after hatching. At metamorphosis, individuals were measured (snout-ventral length, SVL), weighted, and transferred into terrariums for husbandry (120×40×50cm, 5 individuals per terrarium). For logistic reasons, froglets from the three treated groups were pooled together. This seems anyway appropriate because no significant difference was observed among treated groups at larval and metamorphosis stages (see Results). Diet (*ad libitum*) included fruit flies and crickets. Juveniles were counted on a regular basis (every ~9 days) during the first four months of development. Then, in November 2013, individuals were set for hibernation by adapting the light cycle (to 6:18 hours) and progressively lowering the temperature down to 4°C. The next spring (April 2014), the frogs were waken from hibernation and raised in outdoor cages (40×40×120cm) until fall 2015 (except for a second indoor hibernation during winter 2014-2015). These better mimicked environmental conditions but regular censing was not possible. Prior to their third winter (fall 2015), the surviving individuals were measured (SVL) and weighted. All statistical analyses were performed in R (R Development Core Team, 2008).

To understand whether the effects of E_2 exposure on survival were sex-specific, we sexed individuals that died at metamorphosis by genotyping three sex-linked microsatellite markers (WHA5-22, WHA5-201 and *Ha*-H108). These loci feature fixed diagnostic X and Y-specific alleles in the population of origin (Dufresnes et al., 2014), allowing unambiguous genetic sexing. DNA was extracted from tissues (stored in absolute ethanol) using the Qiagen Robotic workstation and markers were amplified and genotyped following the methods by Dufresnes et al. (2014; multiple PCR Mix E). It was unfortunately not possible to infer the rates of sex-reversal because (i) gonads are usually still undifferentiated at metamorphosis in *Hyla,* and (ii) most older individuals (with more developed gonads) suffered post-mortem decomposition when discovered (including DNA degradation), preventing phenotypic or genetic sexing.

Results

Larval development lasted 60-70 days, with high initial mortality in all tanks (~50% within the first two weeks) when tadpoles are extremely small (< 10mm) and vulnerable. The proportion of larvae succeeding metamorphosis was significantly higher between the control (23.5%) compared to treated groups (15%) ($P = 0.024$, χ^2 test, logistic regression; Figure 1A), but did not differ among the three treated groups ($P = 0.45$), and without family effects (between control and treated: $P = 0.49$; among treated: $P = 0.74$). Control individuals showed overall significantly higher survival at metamorphosis (control: 69%; treated: 51%; $P = 0.0046$), and the tadpole stage (control: 34%; treated: 30%; $P = 0.36$). The duration of larval development, which depends on the average density of tadpoles in

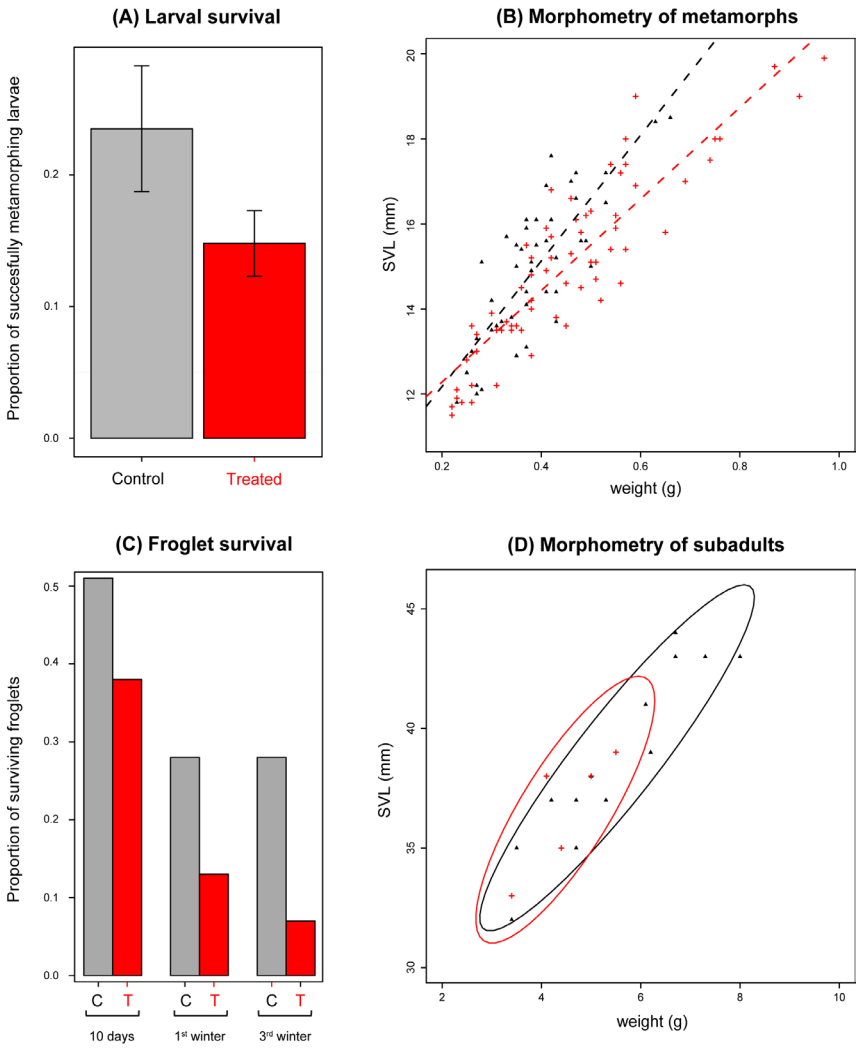

Figure 1. Effects of E_2 exposure on tree frog (*Hyla arborea*) development. Black/grey: controls; red: E_2 exposed (treated). **(A)** Overall larval survival. Error bars show standard error (between tanks of each group). **(B)** Morphometry of live metamorphs (snout-ventral length (SVL) and weight), with trend lines (linear regressions). **(C)** Juvenile survival at three different time points after metamorphosis (ten days, first winter (four months), third winter (28 months). **(D)** Morphometry of control (n = 13) and E_2-exposed (n = 5) sub adults; ellipses show 80% confidence levels.

tanks (negative relationship, linear regression, $R^2 = 0.41$, $P = 0.003$), did not covary with treatments (between control and treated, $P = 0.42$; among treated, $P = 0.66$; ANCOVAs) nor with families ($P = 0.54$).

The SVL and weight (both log-transformed) at metamorphosis were expectedly highly-correlated (linear regression, $R^2 = 0.75$, $P < 0.001$; n = 117); moreover, this relationship significantly covaried with treatment (control *vs* treated; $P < 0.001$, ANCOVA): E_2 exposure influenced the morphometrics of froglets, which were heavier compared to control individuals for the same SVL (weight: $P = 0.024$, SVL: $P = 0.70$, ANOVAs; Figure 1B). No significant difference was detected among the three treated groups ($P = 0.16$), neither among families ($P = 0.21$), nor depending on tadpole density during larval growth ($P = 0.35$).

Juvenile mortality was high within the first ten days after metamorphosis, as expected during this critical period, but was higher for the treated (62%) than control group (49%), although the difference was not significant (P = 0.16, χ^2 test, logistic regression). Fourteen weeks after metamorphosis, prior to the first hibernation, 28% of control froglets had survived *vs* 13% of treated ones (P = 0.04). Two years and four months after metamorphosis, prior to the third winter, no more control frogs had died and only 7% of the treated metamorphs remained (P = 0.0024; Figure 1C). Morphometrics (SVL and weight, log-transformed) of these sub adult frogs were not significantly different between control and treated (weight: P = 0.17, SVL: P = 0.27, ANOVAs), nor their relationship (linear regression, R^2 = 0.83, P < 0.001; ANCOVA with treatment, P = 0.62), although control individuals (n = 13) were on average larger than treated ones (n = 5; Figure 1D).

Mortality at metamorphosis was not significantly sex-specific, neither for control (χ^2 = 0.69, P = 0.40, n = 26) nor any treated group (χ^2 = 0-0.82, P = 0.37-1.00, n = 12-27). More treated males (n = 36) than females (n = 25) died at metamorphosis when considering all treated individuals together, but the deviation from an even sex-ratio was not significant (χ^2 = 0.99, P = 0.32, n = 61).

Discussion

Although limited by sample sizes in later stages of the experiment, as well as by the exposure protocol (via food intake) which do not directly relate to natural conditions (contaminated environmental waters), our experimental study provides some evidence for toxic effects of oestrogen E_2 on tree frog development. Namely, these effects included higher mortality at metamorphosis and during juvenile/sub adult development, as well as modifications regarding froglet physiognomy. Mortality at metamorphosis may be more severe for males than females, but the effect was not significant from our data. All tanks including controls generally suffered inexplicably high mortality in early larval and juvenile stages, which may stem from the high sensitivity of tree frogs in the genetically impoverished Swiss populations (Dufresnes and Perrin 2015). As a consequence, toxic effects on the long term could not be assessed, due to the low sample size, along with the heterogeneity of outdoor raising conditions.

The proximate causes of the chronic toxicity of E_2 in tree frogs remain unknown. Various adverse symptoms following long-term oestrogen exposure have been reported in some laboratory mammals, including diseases and the development of tumours (Maier and Herman, 2001). Oestrogen metabolites are also known carcerogenics, causing DNA damage and cancerous mutations (Miller, 2003, Harvey et al., 2004), and are considered genotoxics (Hundal et al., 1997). In *Xenopus laevis*, exposure to natural and artificial oestrogens induced malformations of the head and abdomen, as well as suppressed organogenesis (Nishimura et al., 1997). While non-model amphibians have being less frequently studied, acute toxicity following E_2 exposure was reported in few species (e.g. toad embryos, Fridman et al., 2004), in line with our results on tree frogs. In contrast with most previous studies on this topic, our study had the advantage to document long-term consequences of larval oestrogen exposure. Yet, future experimental surveys with environmentally relevant hormonal concentrations will be necessary to better assess the impact of these xenobiotic chemicals on natural tree frog populations.

In addition, E_2 is also famous to interfere with amphibian sexual differentiation, causing feminization or gonadal malformation (e.g. Hayes et al., 2006a; Hu et al., 2008), which we unfortunately could not test in our study. Numerous surveys reported sex-reversals induced by the artificial derivative EE_2 in many organisms, including tree frogs, but this effect seems to be strongly species-dependent (Tamshick et al., 2016). Additional symptoms may also include disturbed mating behaviours (Bhandari et al., 2014), that we might be able to assess from our treated animals if they reach adulthood. Our sub adult tree frogs exposed to E_2 during the larval stage appear smaller than unexposed ones (Figure 1D), which might indicate slower development. Together with other environmental pollutants like pesticides (Hayes, 2002, 2006b; Brühl et al., 2013; Dufresnes and Perrin, 2015), our study emphasizes the strong impact of human-released chemicals on global amphibian declines.

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