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p,p′-Dichlordiphenyldichloroethylene (*p,p′*-DDE) can elicit antiandrogenic and estrogenic modes of action in the amphibian *Xenopus laevis*

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HIGHLIGHTS

- *p,p′*-Dichlordiphenyldichloroethylene (DDE) is still environmentally relevant.
- Previous studies found that DDE acts as antiandrogen in amphibians.
- We show that DDE elicits estrogenic and antiandrogenic modes of action in *X. laevis*.
- We further demonstrate that DDE alters the reproductive behavior of male *X. laevis*.
- Disruption of such behavioral patterns might result in reduced reproductive success.

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ABSTRACT

p,p′-Dichlordiphenyldichloroethylene (DDE) is a metabolite of the insecticide dichlorodiphenyltrichloroethane (DDT), an organochloride which was massively used from its discovery in 1939 until the early 1970's. Due to the tremendous half-life of DDT and DDE, both substances are to date environmentally relevant. Furthermore, DDT is still employed in many African countries in the context of the WHO's antimalaria campaign. In amphibians, DDE was found to act as antiandrogenic endocrine disrupting chemical (EDC), whereas in other species DDE was found to act as an estrogen. To determine the mode of action (MOA) of DDE in adult male *Xenopus laevis*, we exposed adult male frogs to different concentrations of DDE, as well as to the estrogenic EDC ethinylestradiol (EE2) and the antiandrogenic fungicide vinclozolin (VIN) for four consecutive nights. We then analyzed the mate calling behavior, which was previously shown to be affected by (anti)androgenic and (anti)estrogenic EDC in a MOA-specific manner, in order to assess whether DDE exposure results in estrogen-specific or antiandrogen-specific alterations of the mate calling behavior. Our results demonstrate that DDE alters the reproductive behavior of male *X. laevis*. Lowered sexual arousal of exposed males was indicated by a decreased production of advertisement calls and higher amounts of calls that suggest a sexually unaroused state of the males. Our results further indicate that DDE can display both, estrogenic and antiandrogenic MOA, either of which can have adverse effects on reproductive physiology and behavior in *X. laevis*. The disruption of the affected mating behavior, which is crucial for a successful reproduction, might result in a reduced reproductive success of DDE exposed animals.

1. Introduction

p,p′-Dichlordiphenyldichloroethylene (DDE) is a metabolite of the insecticide dichlorodiphenyltrichloroethane (DDT), an organochloride which was massively used from its discovery in 1939 until the early 1970's, when various adverse impacts of this substance, such as an increased risk of liver cancer and non-Hodgkin lymphoma, were revealed [1,2]. Nevertheless, due to the tremendous half-life of DDT and DDE (animal and human tissue: >10 years; soil: up to 4 years; surface waters: up to 150 years; [3–7], both substances can still be found in the environment [8–12]. In addition, DDT is still used in many African countries as a way to control malaria in the frame of the WHO's antimalaria campaign [13–15].

DDE was shown to exhibit diverse actions on the endocrine system, most likely depending on the species and endpoints measured [16]. Kelce, Stone [17], for instance, provided evidence for ascribing an antiandrogenic mode of action (MOA) to DDE in mammals in vitro and in

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vivo. In reptiles, on the other hand, DDE can possess estrogenic MOA e.g. in alligators [18], while it did not exhibit any estrogenicity in various turtles, rodents and humans [16–21]. In amphibians, DDE was suggested to act as an antiandrogenic endocrine disrupting chemical (EDC), but specific results are rather inconclusive [22]. However, Arukwe and Jenssen [23] found clear evidence that DDE can also interfere with the thyroid system. These manifold actions of DDE make it difficult to assess environmental risks of this chemical using in vitro studies, since those studies are often limited and can only demonstrate specific mechanisms for particular receptor types (e.g. in humans or rodents). Therefore, in vivo studies using various species are the means of choice for examining the diverse effects of a DDE contamination in the frame of environmental risk assessment.

Previous studies demonstrated that DDE contamination can result in direct toxic effects in birds [24–26] but also, due to its endocrine disrupting potency, it van result in indirect adverse effects on reproductive physiology and behavior of various vertebrates [9,27–32]. In female salamanders, DDE increased Müllerian duct epithelial areas by exhibiting estrogenic MOA [20]. Accordingly, DDE was shown to be able to increase the female:male sex ratio in amphibians [33] and in alligators [34], while it failed to do so in Nigerian male guppies [31]. However, a DDE exposure resulted in altered and suppressed adult male courtship and reproductive behavior in several species, including birds [32], fishes [29,30] and reptiles [27], suggesting a reduced reproductive fitness of DDE contaminated animals. In previous studies, we could show that estrogenic as well as antiandrogenic EDC can adversely affect the mate calling behavior of adult male South African clawed frogs (*Xenopus laevis*) in a MOA-specific manner [35,36]. However, it has not yet been tested whether DDE can alter this endpoint, too. In this study we exposed adult male *X. laevis* to different concentrations of DDE, as well as to the estrogenic EDC ethinylestradiol (EE2) and the antiandrogenic fungicide vinclozolin (VIN) and analyzed the calling behavior of the frogs for four consecutive nights. By doing so, we aimed to assess whether DDE exposure results in estrogen-specific or antiandrogen-specific alterations of the mate calling behavior of *X. laevis*.

2. Material and methods

Male *X. laevis* (5 years of age; weight: 63.3 g ± 15.6 g; length: 8.9 cm ± 0.9 cm) were exposed to DDE, EE2 and VIN in a flow-through system and their calling behavior was recorded and analyzed as described previously [35–38]. In short, animals were transferred individually into the test tanks of the flow through system. One hydrophone (Technology SQ 26, Nauta, Milano, Italy) was placed into each test tank and recordings of vocalizations were performed trigger-controlled using a fire-wire audio interface (Saffire Pro 40, Focusrite, High Wycombe, United Kingdom), a desktop computer and Avisoft Recorder software (Avisoft Bioacoustics, Berlin, Germany). Test tanks were insulated with acoustic foam plates to ensure visual and acoustic isolation of test animals. Vocalizations of male *X. laevis* comprise five spectrally and temporally distinct call types [35,39] that are produced under water by laryngeal muscle contractions [40]. One contraction results in a click sound and several clicks are then assembled to compose the different call types. Advertisement calls are usually produced to attract females. They consist of a slow trill part, which is followed by a fast trill. Chirping is frequently uttered by males while clasping a female [39]. Growling, on the other hand, is elicited when males are clasped by another male. Growling and ticking serve as release calls as well [39]. Rasping is a recently discovered call type [35] which is thought to broadcast a sexually unaroused state of the male, since it is rarely uttered by sexually aroused male frogs [35]. Analyses of stored recordings were performed using Avisoft SasLab software (Avisoft Bioacoustics, Berlin, Germany) as described by Hoffmann and Kloas [35], resulting in the following measured parameters per frog and night: absolute calling activity, absolute and relative amounts of each of the five call types, as well as various temporal and spectral parameters of the advertisement calls, such as call and slow/fast trill duration [41], click duration, inter click interval (ICI), click rate, number of (accentuated) clicks, peak frequency and bandwidth.

To induce a basic mate calling behavior [42], experimental frogs were injected with 100 units human chorionic gonadotropin (hCG) in the dorsal lymph sack prior to the respective exposure. Subsequently, frogs were exposed to the particular EDC in three different exposure experiments. First, 16 male frogs (n = 8) were exposed to EE2 at the environmentally relevant concentration of 10^{-10} M (29.6 ng/L) [43] and a respective solvent control. In a later experiment, another 16 male *X. laevis* (n = 8) were exposed to VIN at an environmentally relevant concentration of 10^{-10} M (28.6 ng/L) [44,45] and a respective solvent control. In a third experiment, 30 male frogs (n = 10) were exposed to two different DDE concentrations (10^{-9} M; 318.0 ng/L and 10^{-11} M; 3.18 ng/L; both environmentally relevant concentrations [12,46]) and a solvent control. Exposure duration was always 96 h. Exposure chemicals were obtained from Sigma Aldrich (Steinheim, Germany). Dimethyl sulfoxide (DMSO) and ethanol (EtOH), respectively, served as solvent and solvent concentrations in the test tanks were 0.00001%. During all test runs frogs were fed a commercial fish diet (Metabolica, Aller Aqua, Golßen, Germany; 2 mm pellets) every other day and water temperature was measured daily (21.6 ± 0.2 °C). Further parameters, such as nitrate and ammonium concentrations, pH and conductivity were measured every other day to ensure an optimal water quality during the whole exposure experiment. The light:dark cycle was 12:12 h. At the end of the exposure period, all frogs were returned to the animal husbandry of the institute.

To identify statistical differences between single treatments of the individual experiments, behavioral data was analyzed using general linear mixed models (GLMM) and Sidak post-hoc tests as described previously [36,47]. Normal distribution of data or residuals was ensured with Kolmogorov-Smirnov tests.

For verifying the desired test concentrations, water samples of each tank were taken and analyzed at the beginning and end of the experiment. Water samples were concentrated 1000-fold using octadecyl C18 cartridges as recently described by Efosa, Kleiner [47] and Garmshausen, Kloas [48]. EE2 samples were then dissolved in 1 mL 10% methanol, while VIN and DDE samples were dissolved in 500 μL hexane. EDC concentrations were analyzed using enzyme-linked immunosorbent assays (ELISAs) for EE2 determination as described by Garmshausen, Kloas [48], and gas chromatography (GC) for determination of VIN and DDE concentrations. GC measurements were conducted using an Agilent 7890 B with electron capture detector and DB 5MS (60 m × 0,25 mm × 0,25 μm) columns (initial temperature: 70 °C; 40 grd/min ramp to 160 °C, held for 10 min; 2 grd/min to 235 °C, held for 25 min). Helium was used as carrier gas at a constant flow of 1 mL/min. The detector temperature was 225 °C.

3. Results

3.1. Analytical water analyses

None of the supply tanks of the three control groups contained any EE2, VIN and DDE, respectively. Water samples from the tanks of the 10^{-10} M EE2 treatment group (desired concentration: 29.6 ng/L EE2) contained 18.1 ng/L (16.4 ng/L–20.5 ng/L) EE2 (median (interquartile range)).

Due to the relatively low sensitivity of the GC testing method, only water samples from the supply tank of the 10^{-10} M VIN treatment could be analyzed. Water samples from the supply tanks of this treatment group (desired concentration: 10^{-8} M = 2610 ng/L VIN) contained 2603.5 ng/L (2058.6–2863.9 ng/L VIN). The solution of those supply tanks were directly used to produce the exposure solution in the test tanks (10^{-10} M VIN). Because the amount of VIN-solution, as well as the volume of dilution water was measured daily, it is reasonable