Endocrine disruptors in breeding ponds and reproductive health of toads in agricultural, urban and natural landscapes

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Abstract

Many chemical pollutants have endocrine disrupting effects which can cause lifelong reproductive abnormalities in animals. Amphibians are the most threatened group of vertebrates, but there is little information on the nature and quantity of pollutants occurring in typical amphibian breeding habitats and on the reproductive capacities of amphibian populations inhabiting polluted areas. In this study we investigated the occurrence and concentrations of endocrine disrupting chemicals in the water and sediment of under-studied amphibian breeding habitats in natural, agricultural and urbanized landscapes. Also, we captured reproductively active common toads (*Bufo bufo*) from these habitats and let them spawn in a 'common garden' to assess among-population differences in reproductive capacity. Across 12 ponds, we detected 41 out of the 133 contaminants we screened for, with unusually high concentrations of glyphosate and carbamazepine. Levels of polycyclic aromatic hydrocarbons, nonylphenol and bisphenol-A increased with urban land use, whereas levels of organochlorine and triazine pesticides and sex hormones increased with agricultural land use. Toads from all habitats had high fecundity, fertilization rate and offspring viability, but the F1 generation originating from agricultural and urban ponds had reduced development rates and lower body mass both as larvae and as juveniles. Females with small clutch mass produced thicker jelly coat around their eggs if they originated from agricultural and urban ponds compared with natural ponds. These results suggest that the observed pollution levels did not compromise reproductive potential in toads, but individual

fitness and population viability may be reduced in anthropogenically influenced habitats, perhaps due to transgenerational effects and/or costs of tolerance to chemical contaminants.

Keywords: anurans, trace pollutants, emerging contaminants, human-induced environmental change, resistance to pollutants

1. Introduction

Chemical contaminants enter the hydrosphere from multiple sources. By drift, runoff and leaching, surface and ground waters receive pesticides and fertilizers from agricultural areas and various other pollutants from domestic and industrial wastewater discharges (Holt, 2000). An enormous body of literature shows that these contaminants are ubiquitously present in freshwater lakes and streams, usually in minute concentrations (Hoffman et al., 2003; Murray et al., 2010). Many hundreds of these chemicals are known to be harmful to animals and humans, even at very low concentrations, by interfering with the endocrine system and causing abnormalities in somatic and sexual development and reproductive physiology (Guillette and Edwards, 2008; Hoffman et al., 2003; Orton and Tyler, 2015). The WHO-IPCS defines exogenous substances that alter function of the endocrine system and consequently cause adverse health effects in an intact organism or its progeny or (sub)population as endocrine disruptors (Damstra et al., 2002). Such endocrine-disrupting chemicals (EDCs) can have life-long negative effects that permanently compromise reproductive potential. For example, perinatal exposure to the pesticides vinclozolin and methoxychlor increased the incidence of male infertility in adult rodents (Anway et al., 2005), whereas decreased fecundity and fertilization success was observed in adult fish after early-life exposure to ethynyl-estradiol, a synthetic estrogen found in contraceptive pills that contaminates sewage effluents (Maack and Segner, 2004).

Species that develop in water early in their life are especially vulnerable to the life-long effects of EDCs. One group at particular risk is amphibians, as most of their species have aquatic larvae, and their early ontogeny and sexual differentiation is sensitive to perturbation by water-borne substances with hormone-like activity (Orton and Tyler, 2015). Amphibians are also a group of serious conservation concern because their populations are disappearing around the globe at an alarming rate (Campbell Grant et al., 2016; Stuart et al., 2004). While the suspected reasons for these declines are linked to anthropogenic impacts such as habitat degradation, climate change and diseases, the role of chemical contaminants in amphibian diversity loss is not well understood (Campbell Grant et al., 2016; Orton and Tyler, 2015). Clarifying the latter is hindered by at least two major gaps in our knowledge.

On one hand, little information is available on the types and quantities of EDCs in the water bodies used by amphibians for spawning and larval development. One reason for this is that many amphibian species avoid flowing waters and large lakes with scarce shoreline vegetation, preferring relatively small, shallow pools instead (Wells, 2007). The input, dilution, dispersion and transformation of pollutants may be different in these water bodies than in large lakes and rivers, yet the former have been much less frequently surveyed for EDCs than the latter (Lorenz et al., 2017). Furthermore, amphibians persisting in anthropogenically influenced landscapes often breed in privately owned ponds, like angling ponds or residential garden ponds (Hassall, 2014). These kinds of water bodies are also rarely included in EDC surveys, despite the fact that they may be particularly exposed to certain contaminants, e.g. leaking from untreated wastewater (Smits et al., 2014). Even less is known about EDCs in the sediment of these ponds, even though many chemicals persist much longer in sediment than in water, and thereby sediment analyses can reveal past pollution events. Although the chemicals absorbed to sediment are considered to have little bioavailability, they can get re-suspended by disturbances to the pond bottom (Knott et

al., 2009) and re-enter the food chain via bottom-grazing and filter-feeding animals such as the larvae of many amphibian species (Wells, 2007).

On the other hand, it is poorly known if the amphibians living in habitats polluted by EDCs actually have reduced reproductive capacities. Typically, the effects of EDCs on reproductive health have been inferred in two ways (Hoffman et al., 2003; Orton and Tyler, 2015). First, laboratory ecotoxicology experiments usually expose developing larvae to EDCs and assess their reproductive anatomy, histology or physiology before or shortly after metamorphosis. Second, field studies usually look at correlations between land use or pollution levels and sex ratios or indirect indices of reproductive abilities such as sex hormone levels and secondary sexual characteristics in adults. The most frequently used endpoint in both kinds of studies is the incidence of intersex, a condition where an individual's gonads contain both female and male tissue. However, intersex can be a natural phase of ontogeny in some species (Orton and Tyler, 2015), and next to nothing is known about the reproductive success of intersex individuals (Jobling et al., 2002). Also, measurement of the above indices often requires invasive, even deadly techniques (e.g. sacrificing the animals for histological examination), which hinders such studies in dwindling populations where information on reproductive potential would be most needed.

In this study, we combined two objectives to contribute to filling these knowledge gaps. Firstly, we investigated the presence and concentration of EDCs in under-studied types of amphibian breeding habitats, i.e. ponds that are relatively small and shallow and/or privately owned. We sampled the water and sediment of these ponds in spring when amphibian larvae are in their early, sensitive stages of development, and we tested whether land use (i.e. agricultural, urban, or natural landscape) predicts the quality and quantity of EDC pollution. Secondly, we directly studied the reproductive capacity of common toads (*Bufo bufo*) that breed in these ponds.

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This species is widespread in Europe and occupies a broad range of habitat types, including landscapes influenced by agriculture and urbanization (Agasyan et al., 2009). It is protected in many countries, and although its populations are presumed large and stable, localized declines have been observed recently (Agasyan et al., 2009). Common toads are highly philopatric, with most individuals breeding in the pond in which they developed as larvae (Reading et al., 1991). Thus we hypothesized that, if breeding ponds in anthropogenically influenced landscapes are contaminated by higher levels and/or more potent kinds of EDCs than more natural ponds, then as a consequence of larval exposure to these contaminants we can detect reduced reproductive capacities in the adults breeding in anthropogenically influenced habitats. To evaluate reproductive capacity while ruling out the effects of actual exposure during breeding, we allowed adult toads to spawn in a non-polluted environment in which they could realize their full reproductive potential. We tested whether fecundity, fertilization rate, and the offspring's viability, development and growth were decreased in toads originating from habitats that are characterized by land use associated with higher pollution by EDCs.

2. Methods

2.1. Study sites and land use

We studied 12 ponds in Hungary (Table 1, Supplementary KMZ file), chosen based on three criteria: 1) common toads and other amphibians were known to breed there (from either our field experiences or personal communications by herpetologist colleagues), 2) we were able to subjectively categorize the surroundings of the pond as natural, urban or agricultural habitat, such that we had 4 ponds per habitat type; 3) logistics. The distance between ponds varied between 3.8 and 68.7 km, except for two urban ponds (Pilisvörösvár and Pilisszentiván) that were ca. 1 km

apart, separated by a fragmented landscape of roads, railroads and buildings (Supplementary KMZ file).

To verify our subjective categorization of the three habitat types, we quantified land use around each pond using QGIS 2.18 (QGIS Development Team, 2017) as follows. We chose a buffer-zone width of 500 m following Cothran et al. (2013), as the migration distance of common toads rarely exceeds 500 m (Reading et al., 1991; Sinsch, 1988). We created the 500 m wide buffer zone around the shoreline of each pond based on the maps of Google Satellite, Bing Aerial, and OpenStreetMap Thunderforest Outdoors, using the Projected Coordinate System for Hungary (HD72/EOV). We obtained land-use polygons in the buffer zones from the Budapest shape file of the Urban Atlas 2012 (Copernicus Land Monitoring Service, European Environment Agency), using the Geoprocessing Tool of QGIS. Then we manually updated the land-use categorization and/or the shape of some polygons based on maps of the Hungarian Land Parcel Identification System (MEPAR), the satellite imagery of Google Satellite and Bing Aerial, the Time Laps feature of Google Earth, and our field experience. We assigned each polygon into one of seven land-use categories: "natural" vegetation (e.g. woodlands, non-agricultural meadows), arable fields, pastures, residential areas, public built areas (e.g. commercial and industrial areas), roads with vehicular traffic, and railroads. We measured the area of each polygon and each buffer zone using the Geometry Tools of QGIS.

For each pond, we calculated seven landscape variables as the total area of each land-use category divided by the area of the entire buffer zone (Table 1). Using these landscape variables we performed a principal component analysis (PCA), which yielded two axes with >1 eigenvalue, explaining 80.8% of variation in total; urban landscape areas loaded positively on the first axis whereas agricultural landscape areas loaded positively on the second axis (Table 2). These two axes separated the 12 ponds clearly into three groups that matched our initial,

subjective categorization of natural, urban and agricultural habitats, except for one pond (Merzse swamp, a nature-conservation area surrounded by agricultural fields) that received higher scores on the second axis than the other three natural ponds (Fig. 1).

2.2. Toad reproductive capacity

We captured adult toads at the start of their spawning season, between 16–28 March 2017 (91 % captured over 3 days between 20–23 March). We aimed to capture at least 10 pairs from each pond; however, because the spawning season of common toads is extremely short (a few days), we could not capture gravid females at two ponds (Merzse and Gyermely). Captured animals were transported to our laboratory in Budapest, where temperature was 20 ± 1.55 °C and artificial light-dark cycles mimicked the natural photoperiod. We measured the body mass (± 0.1 g) of each toad, and housed each pair in $52 \times 37 \times 33$ cm plastic boxes filled with 15 L reconstituted soft water (RSW; 48 mg $NaHCO_3$, 30 mg $CaSO_4 \times 2$ H_2O, 61 mg $MgSO_4 \times 7$ H_2O, 2 mg KCl added to 1 L reverse-osmosis filtered, UV-sterilized tap water) and containing 4 vertical wooden sticks as spawning substrates. Each box housed one male and one female haphazardly chosen from the individuals captured at the same pond. Ninety out of 101 pairs spawned within one week (mostly within 3 days), after which all animals were released at the pond where they had been captured. Because capture success varied across ponds, and not all captured pairs spawned in the laboratory, we finally obtained 36 natural, 17 agricultural and 37 urban clutches (Table 1).

To quantify reproductive capacity, we took several measurements from each pair. On the day after spawning, we measured the parents' body mass again, and we calculated an estimate of fecundity (i.e. clutch mass before water uptake) by taking the difference between the female's pre-spawning and post-spawning body mass (note that post-hibernation toads do not feed before spawning). It is likely that the females laid all their eggs, because they appeared lean after

spawning and the males completely lost interest in them. Only a single female had an appearance after spawning that implied that she might have retained some of her eggs, but her clutch mass was not an outlier (she had the 20th lowest residual clutch mass) and our conclusions on fecundity are not altered by excluding this clutch (results not shown). We also measured the mass of the entire clutch including not only the mass of the eggs but also the water absorbed by their jelly coats (i.e. clutch mass after water uptake). The difference between clutch mass before and after water uptake provides a proxy for jelly thickness (which is very difficult to measure directly in toad egg strings), assuming that the more water the jelly absorbs the thicker it gets; this trait is important for fitness because thicker jelly coats may provide greater protection from exogenous chemical stress (Edginton et al., 2007; Licht, 1985; Shu et al., 2015).

To estimate fertilization rate and offspring survival, from each clutch we placed ca. 30 eggs, taken from 3 haphazardly chosen parts of the egg string, into a $21 \times 16 \times 12$ cm plastic box filled with 0.5 L RSW. We did not have identical numbers of eggs in all containers because ensuring that would have required longer manipulation of the egg string, risking the eggs falling out of the jelly and jeopardizing their further development. Five days after spawning, we measured fertilization rate as the proportion of eggs that started to develop, and removed the non-fertilized eggs (i.e. completely spherical eggs that had started to mold). Two weeks after spawning, when the embryos became free-swimming tadpoles (developmental stage 25, according to Gosner, 1960), we counted the proportion of embryos that survived to this stage, and started to feed the tadpoles with chopped and slightly boiled spinach. On day 17, we estimated the young tadpoles' average body mass by measuring the total mass ($\pm$ 0.01 g) of four randomly chosen tadpoles from each family and dividing it by four; then we selected one healthy-looking individual from each family and moved it into a 2-L plastic box filled with 1 L RSW (in total, 88 tadpoles from 36

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natural, 16 agricultural, and 36 urban families). The remaining eggs and tadpoles were released at the pond where their parents had been captured.

To measure the tadpoles' rate of development and growth, we raised them to metamorphosis; twice a week we changed their rearing water and fed them *ad libitum* with chopped spinach. When a tadpole started metamorphosis (i.e. appearance of forelimbs, developmental stage 42), we decreased the water level to 0.1 L and slightly tilted the container to allow the animal to leave the water. When it completed metamorphosis (i.e. disappearance of the tail, developmental stage 46), we measured its body mass ($\pm$ 0.1 mg) and moved it into a clean rearing box.

To measure juvenile survival and growth, we raised the toadlets for ca. 5 months after metamorphosis. Each rearing box contained wet paper towels as substrate and a piece of egg carton as shelter, which were changed every two weeks. Toadlets were fed *ad libitum* with springtails and small crickets, amended with a 3:1 mixture of $CaCO_3$ and Promotor 43 powder (Laboratorios Calier S.A., Barcelona, Spain) containing vitamins and amino acids. Between 6th October and 10th November 2017, we measured the toadlets' body mass ($\pm$ 0.01 g) and, as part of another experiment, we euthanized them using a water bath containing 5.4 g/L MS-222 buffered with the same amount of Na_2HPO_4. The timing of this procedure was balanced among the animals from the three habitat types such that natural, agricultural and urban individuals were systematically rotated during the one-month period. This time of the year corresponds to the time right before the beginning of first hibernation and is relevant to toadlets' fitness because their pre-hibernation body mass predicts survival during hibernation and post-hibernation body mass (Üveges et al., 2016).

All experimental procedures were carried out according to the permits issued by the Government Agency of Pest County (Department of Environmental Protection and Nature Conservation) and the Budapest Metropolitan Municipality (Department of City Administration,

FPH061/2472-4/2017). The experiments were further approved by the MTA ATK NÖVI Ethical Commission.

2.3. Water and sediment samples

During 25–27 April 2017, ca. one month after the peak spawning period of toads, we visited each pond once and collected samples for chemical analyses. The date of sampling corresponded to the early larval development of free-living toads, when the gonads are not yet differentiated (Falconi et al., 2004) and thus vulnerable to perturbations by EDCs: the tadpoles we found during sample collection were in developmental stages 26-33 (Gosner, 1960). From each pond, we collected 10 L water and 1 L sediment into 1-L amber glass bottles, and 3 L water into 1-L amber PET flasks (for measuring glyphosate, to avoid its adsorption to glass) in the areas where toad tadpoles were present and/or where the adults had been captured. Within these areas, samples were collected at several locations from the water close to the surface and from the upper layers of the bottom sediment (using a tube sampler). All samples were transported to the laboratory within 24 h, where they were stored at -18°C until analyses.

We selected relevant groups of EDCs, and specific compounds within each group, by consulting databases and reviews (Hoffman et al., 2003; Molander et al., 2009; Orton and Tyler, 2015; USEPA, 2017). We also included several pharmaceuticals that frequently occur in surface waters but their endocrine disrupting effects are poorly known. In total, we analyzed our samples for 133 potential EDCs (including some of their metabolites): 60 pesticides, 22 phenolics, 19 polycyclic aromatic hydrocarbons (PAHs), 7 polychlorinated biphenyls (PCBs), 4 phthalates, 16 pharmaceuticals, and 5 natural sex hormones (see Supplementary Material for a complete list).

Chemical analyses were conducted at the Bálint Analitika accredited laboratory. All compounds were detected and measured by gas chromatography with mass spectrometry (GC-

MS), except that pharmaceuticals, hormones, glyphosate and its metabolite aminomethylphosphonic acid (AMPA) were analyzed by reversed-phase high-performance liquid chromatography coupled to tandem mass spectrometry (RP-HPLC-MS/MS). All GC-MS analyses were carried out according to standards (PAHs and PCBs: MSZ 1484-6:2003 and MSZ 1484-11:2003, organochlorine pesticides: EPA 8081B:2007, other pesticides, phthalates, and bisphenol-A: EPA 8270D-2007, alkyl-phenols: MSZ EN ISO 18857-1:2007, chlorophenols: MSZ-EN 12673:2000 and MSZ 21470-97:2009). All compounds were detected in selected ion monitoring (SIM) mode using Agilent 5890 and 6890 GC-MS instruments. Analysis of pharmaceutical residues was performed in accordance with the EPA 1694:2007 standard. For natural hormones, glyphosate and AMPA, in-house methods were used. In case of hormones, water samples were extracted with dichloromethane (DCM); then the organic phase was evaporated, and the residues were dissolved in methanol. Sediment samples were extracted with acetone prior to extraction with DCM to dehydrate the samples. In case of glyphosate and AMPA, a derivatization with 9-fluorenylmethyl chloroformate was applied prior to analysis. The conditions of the derivatization reaction were optimized as: pH=9 (borate), 10% acetonitrile, agitation for 60 min at room temperature; the reaction was then stopped by adding 500 µL of 10% formic acid to each sample. After filtration, the samples were analyzed using RP-HPLC-MS/MS; all compounds were detected by multiple reaction monitoring (MRM) using a Sciex 6500 QTrap mass spectrometer. Detection limits are given in the Supplementary Material.

2.4. Statistical analyses

All analyses were run in the R 3.3.1 environment (R Core Team, 2016), using the following packages: cluster (Maechler et al., 2017), vegan (Oksanen et al., 2018), psych (Revelle, 2017),

smatr (Warton et al., 2012), geepack (Halekoh et al., 2006), and lsmeans (Lenth, 2016). We set the level of statistical significance at 0.05, and we report means with standard errors.

2.4.1. Does land use predict EDC pollution?

We compared the three habitat types in a linear model to test if the number of detected EDC compounds (either in the water or in the sediment or both) differs between natural ponds and anthropogenically influenced ponds. To analyze the concentration of chemicals, we used the concentration of each EDC detected in water and in sediment as separate variables, because both the unit of measurement and the biological implications differ between water and sediment concentrations. Thereby we had 60 variables describing the concentrations of 41 EDCs either in the water or in the sediment (Table 3). Because many of these variables had zero-inflated distribution, we used non-parametric multivariate methods to analyze whether the concentrations of various EDCs correlated with urban or agricultural land use, as follows.

First we calculated the pairwise dissimilarity of all ponds based on either the 7 landscape variables or the 60 EDC variables. We used the Gower distance as metric of dissimilarity (function 'daisy' in package 'cluster'), because it does not ignore negative matches (i.e. when a certain landscape type or EDC is absent at both sites) and allows the assignment of differential variable weights. The number of compounds detected in our samples varied greatly across different chemical groups (Table 3), and we did not want our analysis to be biased by EDC groups with many compounds; therefore during the calculation of Gower distances we assigned to each compound a weight inversely proportional to the number of detected compounds in the respective group of EDCs (i.e. the total number of compounds, 60, divided by the number of compounds in the respective group). We did not apply differential weights for the landscape

variables because even a small landscape area (e.g. a sewage plant) can be a considerable source of pollution.

To assess whether the two distance matrices (i.e. dissimilarities of ponds by landscape and by EDCs) were significantly correlated, we used the Mantel-test with 999 permutations (function 'mantel' in package 'vegan'). Then, to interpret this correlation (i.e. which EDCs are associated with which landscapes), we reduced the dimensionality of the data as follows. For the landscape variables we used the two PCA axes as described above, quantifying urban and agricultural land use respectively. For the 60 EDC variables, we summed the concentrations of compounds belonging to the same group, separating the following 6 groups: PAHs, phenolics, phthalates, pesticides, pharmaceuticals, natural hormones. For each group, concentrations measured in water and in sediment were summed separately, yielding 11 variables. Then we tested the pairwise relationships between the scores along each PCA axis and the concentrations in each EDC group using Spearman rank-correlations, and calculated the 95% confidence interval of each correlation coefficient by bootstrapping with 999 permutations (function 'cor.ci' in package 'psych').

2.4.2. Does land use predict toads' reproductive capacity?

As measures of each pair's reproductive capacity, we analyzed the following 8 variables. Because fecundity is known to increase with female size (Banks and Beebee, 1986; Reading, 1986), we statistically controlled for this effect by calculating residuals from a standardized major axis regression (function 'sma' in package 'smatr') with female post-spawning body mass as the explanatory variable and clutch mass before water uptake (i.e. the female's body mass loss from before to after spawning) as the dependent variable. This approach is favorable over simple ratios or ordinary least-squares regression residuals when the goal is to obtain accurate predicted values

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rather than merely testing if the slope differs from zero (Peig and Green, 2010, 2009). We used

the residuals from this regression as measure of fecundity corrected for female size.

As a proxy for jelly thickness, we subtracted clutch mass before water uptake (i.e. female

weight loss during egg laying) from clutch mass measured after water uptake. This variable,

referred to henceforth as jelly mass, increases with the total mass of eggs, but our data indicated a

non-linear association and we had no *a priori* knowledge on the function by which this

relationship can be adequately described. Therefore, to take variation in clutch mass into account,

we categorized clutch mass into three groups (i.e. small, medium and large clutches, before water

uptake) with equal sample size, and analyzed the effects of habitat within these three groups (see

below).

We measured fertilization rate as the proportion of eggs that started embryonic development,

and embryo viability as the proportion of embryos that survived to the free-swimming tadpole

stage. We did not analyze the survival of tadpoles and juveniles because only one out of 88

tadpoles died before metamorphosis and 5 out of 87 toadlets died before the termination of the

study. Out of these 6 cases of mortality, 4 individuals were of urban origin while only one

originated from each of the other two habitat types. We analyzed offspring body mass measured

at three times: on day 17 (early tadpole stage), at completion of metamorphosis, and at

termination (ca. 5 months after metamorphosis). We also analyzed the time to metamorphosis

(number of days from spawning until the completion of metamorphosis).

Each of the above variables was used as a dependent variable in Generalised Estimation

Equations models (function 'geeglm' in package 'geepack'). In these models, we allowed for the

non-independence among pairs captured from the same pond using the 'exchangeable

correlation' (or 'compound symmetry') association structure (Zuur et al., 2009). We used habitat

type as a fixed factor, and we parameterized the design matrix such that we estimated the

differences between the natural habitat type (used as intercept) and each of the two anthropogenically influenced habitat types (i.e. agricultural and urban). We did not use the PCA scores of the landscape variables as explanatory variables because their distribution was strongly bimodal and hence unsuitable for testing linear relationships (Table 1, Fig. 1). Note, however, that our habitat categories are in good agreement with the PCA scores (Fig. 1), and we had no clutches from site Merzse which turned out to be halfway between natural and agricultural ponds along the second PCA axis. In the model of juvenile body mass, we included the individual's age (i.e. number of days from completion of metamorphosis to termination; mean-centered) as a covariate. In the models of fertilization rate, embryo viability, offspring mass, and time to metamorphosis, we also included the body mass of both parents (measured after spawning; mean-centered) as covariates. In the model of jelly thickness, we included clutch mass (categorized as small, medium and large; see above) as fixed factor and its interaction with habitat type, then we performed pairwise post-hoc comparisons by calculating linear contrasts between natural and either agricultural or urban habitat within each clutch-mass category and correcting the significance level for multiple testing by the Tukey method (function 'lsmeans' in package 'lsmeans').

3. Results

3.1. Does land use predict EDC pollution?

Out of the 133 EDCs screened for, we detected 41 compounds in at least one of the 12 ponds: 19 PAHs, 3 phthalates, 2 phenolics, 9 pesticides, 6 pharmaceuticals, and 2 natural hormones (Table 3). Out of these, 21 compounds were present both in water and sediment samples; 9 compounds were found only in water while another 11 compounds were found only in sediment (Table 3). No polychlorinated phenols or PCBs were detectable in any of the ponds. The number of EDCs

was slightly higher in agricultural ponds (29.0 ± 1.4; p = 0.066) and significantly higher in urban

ponds (31.0 ± 1.1; p = 0.014) than in natural ponds (24.5 ± 1.9; Table 3).

Pond dissimilarity based on EDC concentrations in water and sediment correlated

significantly with pond dissimilarity based on landscape variables (Mantel-test: r = 0.41, p =

0.008). Pairwise correlations showed that the intensity of urbanization (i.e. pond scores along the

first PCA axis) correlated positively with the sediment concentrations of PAHs and phenolics

(nonylphenol and bisphenol-A), whereas the intensity of agriculture (i.e. pond scores along the

second PCA axis) correlated positively with the sediment concentrations of organochlorine

pesticides and estrone (Table 4). Furthermore, testosterone and triazine pesticides were only

detected in the water of agricultural ponds (Table 3).

3.2. Does land use predict toads' reproductive capacity?

Fecundity corrected for female size did not differ between the three habitat types (Table 5, Fig.

2a). Jelly mass was significantly larger in agricultural and urban clutches when clutch mass was

small, whereas medium and large clutches had relatively large jelly mass irrespective of habitat

type (Table 6, Fig. 2b). Pairs from agricultural and urban ponds had similarly high fertilization

rate (Table 5, Fig. 2c) and embryo viability as pairs from natural ponds (Table 5, Fig. 2d).

Tadpoles from natural ponds were larger at the start of larval life than tadpoles with urban

and agricultural origin (Table 5, Fig. 2e). Despite taking less time to develop into metamorphosed

toadlets (Table 5, Fig. 2f), tadpoles from natural ponds did not have smaller mass at

metamorphosis compared to tadpoles from anthropogenically influenced ponds (Table 5, Fig.

2g). Furthermore, toadlets from natural ponds reached larger body mass by October compared to

the animals with agricultural or urban origin (Table 5, Fig. 2h).

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4. Discussion

Our chemical analyses of pond water and sediment revealed a complex picture on the EDC levels in amphibian breeding habitats. On one hand, 92 out of 133 pollutants were not present in detectable quantities in any of the studied water bodies (Supplementary Material), including many compounds known as dangerous EDCs, such as PCBs and various pesticides (Hoffman et al., 2003). On the other hand, several EDCs were present in many or all ponds, and even the small forest ponds that represent natural breeding habitats of many amphibians were contaminated. The concentrations we found generally fall within the range of values reported from other surface waters and sediments (see Table S1 for a non-comprehensive review), with a few notable exceptions. The levels of glyphosate, an herbicide used in very large amounts worldwide and also in Hungary, were higher in our ponds than those typically measured in large rivers and lakes; they were similar to the concentrations observed in urban runoff (Table S1). This finding, coupled with the ubiquity of glyphosate in our ponds irrespective of land use, suggests that a significant part of the glyphosate load in surface waters originates not only from agricultural applications but from other anthropogenic sources like weed control of railways, roadsides, and backyards (Hanke et al., 2010). Carbamazepine, an anti-epileptic and anti-depressant drug which is very stable in the environment, was found at extraordinarily high concentrations in some of our ponds, in 5 samples exceeding the highest level reported for European rivers (Table S1). This indicates that persistent contaminants can accumulate heavily in small standing waters exposed to frequent input, e.g. due to wastewater leaching and runoff, or direct deposition associated with human activities such as tourism and angling.

Whether the detected EDC concentrations are dangerous to amphibians is difficult to assess due to a general paucity of data (reviewed in Table S1). Although almost all compounds detected in our ponds have been demonstrated to have reproduction-related EDC effects (Hoffman et al.,

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2003), there is large variation among chemical groups in the amount of experimental data, especially on aquatic vertebrates. For some intensively studied compounds, like bisphenol-A and ethynyl-estradiol, there is evidence that even very small, environmentally relevant concentrations can interfere with the sexual development of amphibians (Tamschick et al., 2016a, 2016b, 2016c). For some other compounds, like phthalates and glyphosate, only a few experiments were published so far on their effects on amphibian sexual development, which applied much higher concentrations than those found in our ponds and in other water bodies (Table S1). Disquietingly, we found no relevant publications at all on amphibians for most of the compounds we detected, including PAHs, the non-hormonal pharmaceuticals, and the pesticides excluding glyphosate and DDT-derivatives. Given the widespread occurrence of these chemicals in aquatic habitats (Lorenz et al., 2017; Murray et al., 2010), we urgently need more data on their endocrine-disrupting effects at environmentally realistic concentrations.

Most of the EDCs we found in sediments are environmentally persistent, and several groups of these chemicals showed a concentration gradient increasing from natural towards anthropogenically influenced ponds. Specifically, urban land use was associated with higher levels of PAHs (industrial and domestic combustion byproducts) and two phenolics widely used as industrial additives (nonylphenol and bisphenol-A), whereas agricultural land use was associated with higher levels of pesticides (mostly banned organochlorines that were used in large amounts 50 years ago) and natural sex hormones. The latter may be due to animal excreta used as fertilizers or originating from livestock grazing (Kolodziej and Sedlak, 2007; Lange et al., 2002) or aquaculture in fish ponds (Barel-Cohen et al., 2006). These pollution gradients demonstrate that aquatic wildlife in anthropogenically influenced habitats have been exposed to higher pollution loads in the recent past than their counterparts in more natural habitats. Since many amphibian species can live as long as 10-15 years in nature (Smirina, 1994) and common

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toads live up to 6-12 years (Hemelaar, 1988), adverse effects of pollution may be detectable in the reproductive health of amphibian populations even a decade after exposure.

Our results on the toads' reproductive capacities are also complex. On the bright side, pairs from all ponds had high fecundity, fertilization rate and offspring viability under ideal environmental conditions, suggesting that the populations living in urban and agricultural habitats are not suffering from long-term reproductive impairments despite the more frequent occurrence and higher concentrations of EDCs in these habitats. This means that individual reproductive success and population viability may also be high if acute exposure to pollutants during breeding does not compromise the adults' reproductive output or the offspring's survival and development. Embryos and young larvae can be particularly sensitive to chemical insults (Hoffman et al., 2003; Mikó et al., 2017), so it may be adaptive for females to provide their spawn with extra protection in environments where contamination load is higher. This might explain our finding that female toads from agricultural and urban ponds produced large jelly mass even when clutch size was relatively small, as if the minimum thickness of jelly needed was larger than for females from natural ponds. The jelly coat around the eggs can restrict the uptake of waterborne pollutants and thereby reduce embryo mortality (Edginton et al., 2007; Licht, 1985), so we hypothesize that females living in polluted habitats may produce thick jelly coats as a pre-emptive measure to buffer the effects of expectable contamination events. Interestingly, another environmental stressor, acidification has been found to exert strong local selection on embryonic acid tolerance in frog populations, which is mediated by the jelly's enhanced ability to retain water due to its increased content of negatively charged glycans (Shu et al., 2016, 2015). It would be worth investigating whether chemical pollution favors similar alterations in the macromolecular composition of egg jelly.

On the down side, however, our results indicated reduced performance in the offspring originating from agricultural and urban ponds compared to those originating from natural ponds. Despite being raised in a contaminant-free environment that allowed for maximal investment into development and growth, individuals originating from anthropogenically influenced habitats took longer to complete metamorphosis and had smaller body mass both as larvae and as juveniles. These traits are critical determinants of fitness and population size in amphibians (Wells, 2007): for example, early metamorphosis increases survivorship to maturity by allowing to reach reproductive size earlier (Smith, 1987), and larger juveniles are more likely to survive the first hibernation (Üveges et al., 2016) and maintain their size advantage as adults (Berven, 1990) which in turn affects female fecundity and male mating success (Banks and Beebee, 1986; Davies and Halliday, 1979; Höglund, 1989; Reading, 1986). Thus, our results suggest that the offspring of toads in anthropogenically influenced habitats have reduced chances of becoming successfully reproducing adults. Similarly, recent studies on British populations of the common toad reported that embryos collected from agricultural habitats grew slower in captivity than embryos from reference sites (Orton and Routledge, 2011), and adult males were smaller at higher intensity of anthropogenic land use (Orton et al., 2014).

The lower mass and slower development of toads from anthropogenically influenced habitats may be due to several, mutually non-exclusive phenomena. One possibility is that reduced offspring performance is a lasting consequence of chemical pollution. It is now well established that EDCs can have transgenerational effects, such that early-life exposure of parents leads to adverse health outcomes in offspring and later generations (Anway et al., 2005; Bhandari et al., 2015). Such effects can occur even when the exposed parents show no phenotypic abnormalities (Bhandari et al., 2015). Alternatively, reduced offspring size may be a cost of an adaptive resistance to contaminants. For example, several urban fish populations have evolved tolerance to

toxic pollutants (Meyer and Di Giulio, 2003; Whitehead et al., 2012), but their offspring had reduced growth rates in clean water and were more susceptible to other stressors compared with the offspring of conspecifics from a non-contaminated site (Meyer and Di Giulio, 2003). Similarly, in frogs, evolved pesticide tolerance along an agricultural land-use gradient was found to be associated with susceptibility to parasites (Hua et al., 2017). Similar trade-offs may have been responsible for poor performance in the offspring of our toads from anthropogenically influenced habitats. For example, if the latter produce more or different jelly material to provide tolerance against EDCs or other pollutants (see above), they might have less resources to allocate into the eggs (Podolsky, 2004), and their offspring might not be able to catch up from this initial handicap in egg size or quality (Loman, 2002). Furthermore, because oocytes mature while females are on their post-spawning feeding grounds (Wells, 2007), egg nutrient content may be affected by terrestrial EDC exposure of females; research on such effects on vitellogenesis is virtually absent and highly needed.

Besides pollution, several other environmental factors may differ between anthropogenically influenced and natural habitats, and many of these environmental differences may select for local adaptation in life-history traits. For example, common-garden experiments have shown that tadpole development is faster in populations breeding in temporary pools where desiccation favors earlier metamorphosis than in permanent pools (Lind et al., 2008), and growth efficiency is higher in populations living in harsh environments such as northern latitudes and high altitudes (Lindgren and Laurila, 2005). If anthropogenically influenced ponds are less likely to dry out or if larval competition is low (e.g. due to small population size in fragmented landscapes), this may relax the selection on growth and developmental rates. Also, compared to natural forest habitats, ponds in urbanized and agricultural landscapes may have less closed-canopy vegetation and shade, which would result in higher temperatures, more food for tadpoles, and altered predator

fauna, all of which might result in relaxed selection on growth and development speed (Van

Buskirk and Arioli, 2005). Disentangling these possible effects of human-induced habitat changes

on life-history evolution, and thereby population viability, is an important challenge for ecology

and conservation biology.

Taken together, we have documented that small water bodies and private ponds representing

typical amphibian breeding habitats contain various EDCs, sometimes in remarkably high

concentrations, and show distinct pollution gradients with increasing influence by agriculture and

urbanization. This contamination is relevant not only for amphibians but also for other aquatic

animals, terrestrial wildlife, and even human health. Although we found no sign of pollution-

related reproductive failure in adult toads, our results suggest reduced vigor in offspring

originating from anthropogenically influenced habitats. Our study thus highlights that

investigating transgenerational EDC effects and resistance to contaminants may be crucially

important for furthering our understanding of the consequences of chemical pollution from

evolutionary, ecological and conservationist points of view.

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Table 1. Characteristics of the 12 ponds in the study. See Supplementary KMZ file for a map of locations.

| Pond | Habitat type | Pond size (ha) | Proportion of landscape cover | | | | | | | Number of EDCs | Number of toad clutches in lab |
			Arable fields	Pastures	Vegetation	Residential	Public, built	Roads	Rail-roads		
Bajdázó (B)	natural	0.39	0	0.022	0.970	0	0	0.024	0	19	7
Szárazfarkas (S)	natural	0.05	0	0	0.988	0	0	0.012	0	25	15
János-tó (J)	natural	0.46	0	0	0.987	0	0	0.012	0	28	14
Merzse (M)	natural	5.11	0.341	0.068	0.584	0	0	0.011	0	26	0
Határrét (H)	agricultural	5.31	0.484	0.137	0.284	0.070	0	0.026	0	31	11
Perőcsény (P)	agricultural	5.43	0.346	0.141	0.498	0	0	0.014	0	25	4
Anyácsapuszta (A)	agricultural	3.38	0.802	0.051	0.145	0	0	0.007	0	31	2
Gyermely (GY)	agricultural	17.10	0.735	0.014	0.213	0.001	0.027	0.011	0	29	0
Pesthidegkút (PH)	urban	0.45	0.013	0	0.156	0.724	0.031	0.077	0	28	8
Pilisszentiván (PS)	urban	5.15	0	0	0.282	0.455	0.173	0.076	0.015	31	10
Pilisvörösvár (PV)	urban	9.47	0.004	0.024	0.270	0.531	0.083	0.077	0.014	33	7
Göd (G)	urban	0.70	0	0	0.248	0.431	0.033	0.053	0.011	32	12

Table 2. The first two axes (PC1 and PC2) of the principal component analysis, and their Pearson correlations with the seven landscape variables.

	PC1	PC2
Eigenvalue	2.00	1.28
% variance explained	57.34	23.47
Correlation of PC scores and landscape variables:		
Natural vegetation	-0.41	-0.88
Arable fields	-0.58	0.74
Pastures	-0.52	0.54
Public built areas	0.86	0.13
Residential areas	0.91	0.11
Roads	0.96	0.07
Railroads	0.87	0.07

Table 3. EDC concentrations in a) water and b) sediment ('nd': not detected; abbreviations of pond names as in Table 1).

a) Chemicals in water (ng/L; *µg/L)		Natural ponds				Agricultural ponds				Urban ponds		
Group	Compound	B	S	J	M	H	P	A	GY	PH	PS	PV	G
Polycyclic aromatic hydrocarbons (PAH)	naphthalene	15	80	90	23	67	19	17	22	23	20	78	25
	2-methyl-naphthalene	15	62	94	13	47	15	15	18	13	16	62	15
	1-methyl-naphthalene	6	25	39	7	21	6	6	8	6	8	26	8
	acenaphthylene	nd	2	5	1	3	nd	1	1	1	2	9	1
	acenaphthene	1	4	43	1	4	1	1	1	1	2	6	2
	fluorene	40	149	19	3	13	3	3	3	44	7	18	5
	phenanthrene	25	176	34	9	24	6	6	6	42	11	37	11
	anthracene	2	20	3	nd	2	nd	nd	nd	8	1	2	1
	fluoranthene	2	15	18	3	15	2	3	3	5	4	22	4
	pyrene	1	7	11	2	8	1	1	2	3	2	11	2
	benz(a)anthracene	1	4	7	1	5	1	1	1	2	1	6	nd
	chrysene	1	3	5	1	3	1	nd	1	1	1	3	nd
	benzo(b)fluoranthene+ benzo(k)fluoranthene	1	8	17	2	8	1	1	2	4	2	14	1
	benzo(e)pyrene	1	6	7	nd	3	1	1	nd	2	1	6	1
	benzo(a)pyrene	nd	2	3	nd	2	nd	nd	nd	1	nd	4	nd
Phthalates	diethyl-phthalate	nd	nd	10	10	10	nd	10	10	30	20	30	20
	dibutyl-phthalate	30	nd	250	nd	40	nd	120	140	180	110	90	80
	di-ethylhexyl phthalate	730	180	nd	300	nd	520	270	nd	310	230	240	570
Pesticides	o,p-DDE + p,p-DDE	nd	nd	nd	nd	nd	nd	3	1	nd	nd	nd	nd
	terbuthylazine	nd	nd	nd	nd	nd	nd	nd	330	nd	nd	nd	nd
	terbutryn	nd	nd	nd	nd	nd	nd	nd	30	nd	nd	nd	nd
	glyphosate*	5.18	15	2.36	10.9	8.85	7.06	10.4	14.8	6.52	8.12	10.9	9.5
	aminomethylphosphonic acid*	2.62	25.9	3.39	6.21	17.6	6.67	7.33	9.94	14	19.1	15.2	4.98
Hormones	testosterone	nd	nd	nd	nd	nd	10	nd	9	nd	nd	nd	nd
Pharmaceuticals	caffeine	nd	nd	90	40	60	nd	40	nd	nd	50	70	90
	sulfamethoxazole	nd	nd	1	nd	nd	nd	1	1	nd	nd	1	nd
	carbamazepine*	nd	0.06	90.5	nd	276	11.2	nd	nd	1.3	50.2	65.3	16.1
	carbamazepine epoxide	nd	nd	nd	nd	<5	nd	nd	nd	nd	<5	<5	nd
	ketoprofen	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	<5

b) Chemicals in sediment (µg/kg)		Natural ponds				Agricultural ponds					Urban ponds		
Group	Compound	B	S	J	M	H	P	A	GY	PH	PS	PV	G
Polycyclic aromatic hydrocarbons (PAH)	naphthalene	6	8	20	7	8	5	7	8	7	20	10	20
	2-methyl-naphthalene	5	6	16	6	7	4	5	7	6	12	9	18
	1-methyl-naphthalene	2	3	9	3	4	2	3	3	3	7	5	10
	acenaphthylene	nd	nd	1	1	nd	nd	nd	nd	1	2	5	2
	acenaphthene	nd	nd	1	nd	1	nd	nd	nd	nd	1	7	5
	fluorene	1	1	2	1	3	1	1	1	1	3	13	12
	phenanthrene	1	3	10	7	4	1	3	3	6	27	115	35
	anthracene	nd	nd	1	1	1	nd	nd	nd	1	4	23	10
	fluoranthene	1	6	30	19	11	2	6	5	18	69	226	146
	pyrene	nd	4	16	13	7	1	4	3	14	42	164	99
	benz(a)anthracene	nd	2	9	8	4	1	2	2	9	26	112	57
	chrysene	nd	3	13	9	3	1	3	3	4	23	67	56
	benzo(b)fluoranthene+ benzo(k)fluoranthene	1	14	139	25	17	3	9	7	24	98	261	153
	benzo(e)pyrene	nd	6	42	10	7	1	4	3	9	37	96	56
	benzo(a)pyrene	nd	3	10	8	5	1	2	2	10	27	107	50
	indeno(1,2,3-cd)pyrene	nd	4	34	5	6	nd	2	2	8	27	82	46
	dibenzo(a,h)anthracene	nd	nd	4	1	1	nd	nd	nd	1	5	15	5
	benzo(g,h,i)perylene	nd	5	24	9	7	1	3	2	8	28	70	48
Phthalates	dibutyl-phthalate	10	nd	nd	nd	10	20	20	nd	nd	10	10	10
	di-ethylhexyl phthalate	30	30	60	50	60	20	30	10	30	100	40	140
Phenolics	nonylphenol	127	135	124	113	194	143	94	70	172	323	179	306
	bisphenol A	3	7	nd	4	43	4	4	5	4	16	5	8
Pesticides	o,p-DDE + p,p-DDE	nd	1	3	1	25	79	139	8	6	5	11	8
	o,p-DDD + p,p-DDD	nd	nd	nd	nd	5	4	11	nd	1	2	40	13
	dicofol	nd	nd	nd	nd	nd	nd	1	nd	nd	nd	4	3
	aldrin	nd	nd	nd	nd	nd	nd	7	nd	nd	nd	nd	nd
	dieldrin	nd	nd	nd	nd	nd	nd	7	nd	nd	nd	nd	nd
Hormones	estrone (E1)	nd	nd	nd	nd	1.03	nd	0.94	<0.5	nd	1.53	0.78	0.55
Pharmaceuticals	17α-ethynylestradiol (EE2)	nd	4.78	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
	caffeine	nd	nd	nd	nd	nd	2.76	nd	nd	nd	nd	nd	nd
	carbamazepine	nd	nd	nd	nd	332	nd	nd	nd	nd	nd	514	106

Table 4. Spearman rank-correlations (with 95% confidence intervals; CI) between EDC

concentrations and PCA scores of landscape composition. Correlation coefficients for which the

CI excludes zero are highlighted in bold.

EDC group	PC1 (urbanization)	PC2 (agriculture)
PAHs in water	0.52 (-0.18, 0.89)	-0.55 (-0.94, 0.13)
PAHs in sediment	**0.59 (0.03, 0.90)**	-0.19 (-0.7, 0.43)
Phenolics in sediment	**0.60 (0.09, 0.97)**	-0.06 (-0.61, 0.66)
Phthalates in water	0.22 (-0.43, 0.69)	-0.17 (-0.84, 0.66)
Phthalates in sediment	-0.21 (-0.72, 0.64)	0.53 (-0.04, 0.90)
Pesticides in water	0.32 (-0.25, 0.80)	0.24 (-0.46, 0.93)
Pesticides in sediment	-0.29 (-0.78, 0.45)	**0.80 (0.44, 0.96)**
Pharmaceuticals in water	0.24 (-0.33, 0.72)	0.08 (-0.57, 0.70)
Pharmaceuticals in sediment	0.11 (-0.50, 0.69)	0.09 (-0.47, 0.68)
Hormones in water	-0.47 (-0.83, 0.00)	**0.38 (0.04, 0.78)**
Hormones in sediment	0.22 (-0.49, 0.78)	**0.62 (0.10, 0.85)**

Table 5. Reproductive capacity of toad pairs in relation to habitat type (Generalised Estimation Equations models).

Dependent variable	Model parameters	Estimate ± SE	p
Fertilization rate (logit)	natural habitat, mean	2.012 ± 0.245	<0.001
	agricultural - natural	0.265 ± 0.520	0.610
	urban - natural	-0.06 ± 0.291	0.840
	mother's body mass	0.001 ± 0.009	0.950
	father's body mass	0.006 ± 0.069	0.930
Embryo viability (logit)	natural habitat, mean	4.097 ± 0.235	<0.001
	agricultural - natural	-0.053 ± 0.300	0.860
	urban - natural	-0.132 ± 0.254	0.600
	mother's body mass	-0.007 ± 0.006	0.250
	father's body mass	0.010 ± 0.020	0.610
Tadpole mass (mg)	natural habitat, mean	40.855 ± 1.183	<0.001
	agricultural - natural	-5.320 ± 2.705	0.049
	urban - natural	-4.355 ± 2.137	0.041
	mother's body mass	0.007 ± 0.037	0.853
	father's body mass	0.028 ± 0.136	0.838
Time to metamorphosis (days)	natural habitat, mean	57.17 ± 0.209	<0.001
	agricultural - natural	2.585 ± 0.289	<0.001
	urban - natural	0.876 ± 0.284	0.002
	mother's body mass	0.008 ± 0.010	0.461
	father's body mass	-0.011 ± 0.035	0.747
Mass at metamorphosis (mg)	natural habitat, mean	164.294 ± 4.351	<0.001
	agricultural - natural	-7.637 ± 6.448	0.240
	urban - natural	-4.030 ± 6.364	0.530
	mother's body mass	0.063 ± 0.160	0.700
	father's body mass	-0.734 ± 0.496	0.140
Juvenile mass (g)	natural habitat, mean	4.561 ± 0.049	<0.001
	agricultural - natural	-0.269 ± 0.065	<0.001
	urban - natural	-0.436 ± 0.070	<0.001
	age at mass measurement	0.036 ± 0.007	<0.001
	mother's body mass	0.001 ± 0.002	0.778
	father's body mass	0.031 ± 0.011	0.006

Sample sizes: 90 for fecundity and fertilization rate, 88 for embryo viability, 87 for tadpole mass, 86 for time to and mass at metamorphosis, and 82 for juvenile mass. The juveniles' age at mass measurement (days), and the mother's and father's body mass (g) were mean-centered before analysis.

Table 6. Jelly mass (g) in relation to habitat type (Generalised Estimation Equations model, n = 90 clutches).

Clutch mass	Contrast	Estimate ± SE	p
Small	natural - agricultural	-147.0 ± 7.8	<0.001
	natural - urban	-158.5 ± 19.0	<0.001
Medium	natural - agricultural	72.7 ± 49.0	0.590
	natural - urban	-94.6 ± 60.6	0.531
Large	natural - agricultural	152.2 ± 84.7	0.363
	natural - urban	87.5 ± 58.1	0.572

Figure 1. Grouping of 12 ponds along the first two axes of the principal component analysis; symbol shape indicates the original, subjective habitat categorization (circles: natural, squares: agricultural, triangles: urban).
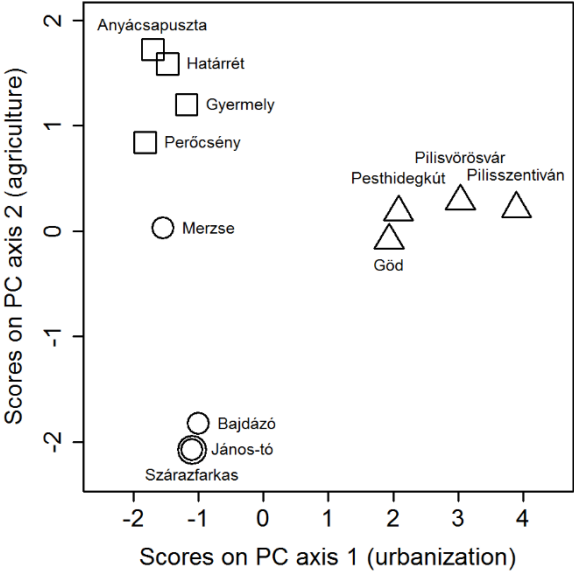
Figure 2. Reproductive capacity of toad pairs in relation to habitat type (N: natural, A: agricultural, U: urban). Boxplots show the distribution of observed data (thick middle line: median, box: interquartile range; whiskers extend to the most extreme data points within 1.5 × interquartile range from the box); error bars show the mean ± SE fitted by Generalised Estimation Equations models (see Tables 5-6).
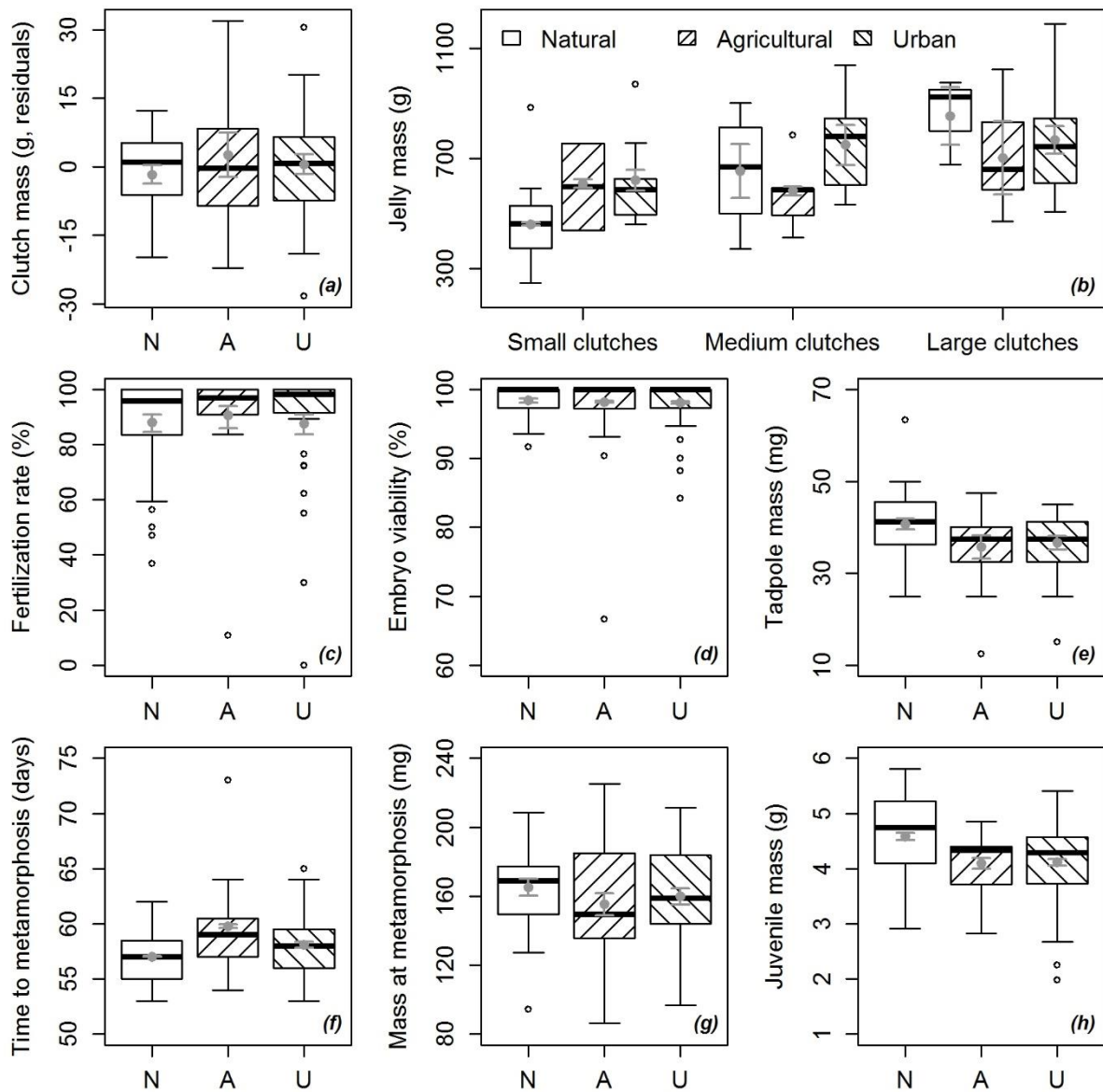
for

Endocrine disruptors in breeding ponds and reproductive health of toads in agricultural, urban and natural landscapes

by

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List of all EDC compounds analyzed, and their detection limits (DL):

Polycyclic aromatic hydrocarbons (PAH); DL in water: 0.0005 µg/l, in sediment: 0.0005 mg/kg
naphthalene
2-methyl-naphthalene
1-methyl-naphthalene
acenaphthylene
acenaphthene
fluorene
phenanthrene
anthracene
fluoranthene
pyrene
benz(a)anthracene
chrysene
benzo(b)fluoranthene+
benzo(k)fluoranthene
benzo(e)pyrene
benzo(a)pyrene
indeno(1,2,3-cd)pyrene
dibenzo(a,h)anthracene
benzo(g,h,i)perylene

Polychlorinated biphenyls (PCB); DL in water: 0.0001 µg/l, in sediment: 0.0001 mg/kg
2,4,4'-trichlorobiphenyl
2,2',5,5'-tetrachlorobiphenyl
2,2',4,5,5'-pentachlorobiphenyl
2,3',4,4',5-pentachlorobiphenyl
2,2',4,4',5,5'-hexachlorobiphenyl
2,2',3,4,4',5'-hexachlorobiphenyl
2,2',3,4,4',5,5'-heptachlorobiphenyl

Phthalates; DL in water: 0.01 µg/l, in sediment: 0.01 mg/kg
diethyl-phthalate (DEP)
dibutyl-phthalate (DBP)

butyl-benzyl-phthalate (BBP)
di-ethylhexyl phthalate (DEHP)

Phenolics; DL in water: 0.001 µg/l, in sediment: 0.001 mg/kg
nonylphenol
octylphenol
bisphenol A
3-chlorophenol
4-chlorophenol
2-chlorophenol
2,6-dichlorophenol
3,5-dichlorophenol
2,5-dichlorophenol
2,4-dichlorophenol
3,4-dichlorophenol
2,3-dichlorophenol
2,4,6-trichlorophenol
2,3,6-trichlorophenol
2,4,5-trichlorophenol
2,3,5-trichlorophenol
3,4,5-trichlorophenol
2,3,4-trichlorophenol
2,3,5,6-tetra-chlorophenol
2,3,4,6-tetra-chlorophenol
2,3,4,5-tetra-chlorophenol
pentachlorophenol

Organochlorine pesticides; DL in water: 0.0005 µg/l, in sediment: 0.0005 mg/kg
a,b,δ–HCH
γ–HCH (lindane)
hexachlorobenzene
heptachlor
heptachlor epoxide
o,p-DDE + p,p-DDE
o,p-DDD + p,p-DDD
o,p-DDT + p,p-DDT
cis-trans-chlordane
oxychlordane
endosulfan I+II
endrin
aldrin
dieldrin
methoxychlor
mirex
dicofol

Other pesticides; DL in water: 0.005 µg/l, in sediment: 0.005 mg/kg

chlorpyrifos
chlorpyrifos-methyl
diazinone
fenitrothion
fosdrin
malathion
parathion-ethyl
parathion-methyl
atrazine
atrazine-desethyl
simazine
propazine
terbuthylazine
secbumeton
metribuzin
simetryn
ametryn
prometryn
terbutryn
propachlor
trifluralin
benfluralin
acetochlor
alachlor
propisochlor
metalaxyl
metolachlor
metazachlor
difenamid
chlorobenzilate
pendimethalin
cyprodinil
vinclozolin
triadimefon
iprodione
procymidone
fenarimol
permethrin
prochloraz
cypermethrin
fenvalerate
glyphosate – DL in water: 0.3 µg/l, in sediment: 0.0002 mg/kg
aminomethylphosphonic acid – DL in water: 0.5 µg/l, in sediment: 0.0003 mg/kg

Natural hormones; DL in water: 0.001 µg/l, in sediment: 0.0002 mg/kg
17β-estradiol
estrone

progesterone
testosterone
androstenedione

Pharmaceuticals; DL in water: 0.001 µg/l, in sediment: 0.00001 mg/kg
atenolol
caffeine
cyclophosphamide
erythromycin
sotalol
sulfamethoxazole
carbamazepine epoxide
carbamazepine
ketoprofen
paracetamol
metoprolol
fenofibrate
diclofenac
ibuprofen
levonorgestrel – DL in sediment: 0.0002 mg/kg
17α-ethynylestradiol – DL in sediment: 0.0002 mg/kg

Table S1. Concentrations of EDC compounds detected in more than one pond in our study, examples of concentrations found in earlier studies, and endocrine-disrupting effects on amphibian sexual development or reproductive health.

EDC group	Compounds detected	Number of ponds	Concentrations in our ponds	Concentrations elsewhere	Sex effects in amphibians
Polycyclic aromatic hydrocarbons (PAH)	naphthalene, 2-methyl-naphthalene, 1-methyl-naphthalene, anthracene, acenaphthylene, acenaphthene, fluorine, phenanthrene, pyrene, fluoranthene, chrysene, benz(a)anthracene, benzo(b)fluoranthene+, benzo(k)fluoranthene, benzo(e)pyrene, benzo(a)pyrene (BaP), indeno(1,2,3-cd)pyrene, dibenzo(a,h)anthracene, benzo(g,h,i)perylene	12	1-176 ng/L, 1-261 µg/kg per compound; ΣPAH 56-563 ng/L, 17-1380 µg/kg	In shallow lakes in Spain, 80-600 ng/L and 0.5-646 µg/kg per compound; ΣPAH 80-2400 ng/L and 4-4286 µg/kg (Hijosa-Valsero et al., 2016). In sediments of small, shallow lakes in Canada, ΣPAH (42-3300 µg/kg) showed a weak urban-rural gradient (Wong et al., 2009). In Hungary, ΣPAH in river Danube: 25-1745 ng/L, 8.3-1202.5 µg/kg (Szabó Nagy et al., 2013); in Lake Balaton: 170-720 ng/L, 30-360 µg/kg (Kiss et al., 1998).	A 24-h experiment with Xenopus adults found no effect of 10 µg/L BaP on sex-related endpoints (Regnault et al., 2016). No other data available on amphibians. Review of PAH effects on fish: (Nicolas, 1999).
Phenolics	nonylphenol	12	70-323 µg/kg	Sediment concentrations from around the world: from 3.6 µg/kg to 72 mg/kg (Careghini et al., 2015).	Sex ratios feminized and intersex increased by larval exposure to 10 and 100 µg/L in 2 anuran species (Mackenzie et al., 2003). In a third species, elevated testosterone levels in tadpoles after 20-days exposure to 2 µg/L (Yang et al., 2005).
	bisphenol-A (BPA)	11	3-43 µg/kg	Sediment concentrations from around the world: between 3.2 and 163 µg/kg (Careghini et al., 2015).	Abnormal gonads after larval exposure to 0.023-228 µg/L in 3 anuran species (Tamschick et al., 2016). Review of BPA effects on aquatic wildlife: (Bhandari et al., 2015).

Phthalates	dibutyl-phthalate (DBP)	9	30-250 ng/L, 10-20 µg/kg	In European freshwaters (mostly rivers), DBP 70-8800 ng/L and 60-2080 µg/kg, DEP 50-6980 ng/L, DEHP 80-97800 ng/L and 210-8440 µg/kg (Fromme et al., 2002; Gao and Wen, 2016).	Abnormal gonads after larval exposure to DBP (0.1-10 mg/L) in 2 anuran species (Lee and Veeramachaneni, 2005; Ohtani et al., 2000).
	diethyl-phthalate (DEP)	9	10-30 ng/L		
	di-ethylhexyl phthalate (DEHP)	12	180-730 ng/L, 10-140 µg/kg		
Pesticides	o,p-DDE + p,p-DDE	11	1-3 ng/L, 1-139 µg/kg	In French fish ponds, ΣDDTs 0.2-2.3 µg/kg (Thomas et al., 2012). In Danube Delta, 0.4-5.3 µg/kg DDE, 0.3-7.9 µg/kg DDD (Covaci et al., 2006). In shallow lakes in Spain, 3-65 µg/kg DDE (Hijosa-Valsero et al., 2016). In sediments of small, shallow lakes in Canada, ΣDDTs 0.2-472 µg/kg (Wong et al., 2009).	DDT (0.1 mg/L), DDE (1 mg/L) and DDD (1 mg/L) prematurely induced adult female coloration in juvenile frogs (Noriega and Hayes, 2000). In larval salamanders, DDT and DDE (0.01 mg/L) interfered with gonaduct development (Clark et al., 1998). DDT and dicofol effects in reptiles: (Guillette et al., 1994).
	o,p-DDD + p,p-DDD	7	1-40 µg/kg		
	dicofol	3	1-4 µg/kg		
	glyphosate	12	2.36-15 µg/L	In small Belgian farmland ponds: glyphosate and AMPA 0.06-2.075 µg/L (Mandiki et al., 2014). In large Hungarian surface waters: 0.035-0.68 µg/L glyphosate (Mörtl et al., 2013). In urban stormwater runoff: 0.36-90 µg/L glyphosate (Botta et al., 2009; Lamprea and Ruban, 2008).	Intersex and feminized sex ratios after larval exposure to 0.6-1.8 and 2.89 mg/L glyphosate, respectively, in 2 anuran species (Howe et al., 2004; Lanctôt et al., 2014).
	aminomethylphosphonic acid (AMPA)	12	2.62-25.9 µg/L		
Hormones	estrone	6	0.55-1.53 µg/kg	In UK river sediments, 0.40-3.3 µg/kg (Labadie and Hill, 2007). In a US watershed, from <0.12 to 2.4 µg/kg (Luo et al., 2013).	Male sex hormones including testosterone (0.02-1.7 µM) have masculinizing effects, female sex hormones including estrone

	testosterone	2	9-10 ng/L	In French surface waters, 0.3-26.3 ng/L (Vulliet and Cren-Olivé, 2011). In US surface waters on grazing rangelands, up to 4.3 ng/L (Kolodziej and Sedlak, 2007). In fish pond effluents, 5-10 ng/L (Barel-Cohen et al., 2006).	(concentrations cannot be calculated because they were reported in unconvertable units and/or the animals were injected) have feminizing effects on primary sex differentiation in amphibians (Hayes, 1998).
Pharmaceuticals	caffeine	7	40-90 ng/L, 2.76 µg/kg	In US urban ponds, 0-286 (mean: 48) ng/L (Smits et al., 2014). In Swiss lakes and rivers, 6-250 ng/L (Buerge et al., 2003). In Eropean rivers, up to 39.8 µg/L, mean: 0.96 µg/L (Loos et al., 2009). In sediments of a US river, 0.2-24.38 µg/kg (Yang et al., 2015).	No data available on amphibians. Limited data on fish: 2 mg/L caffeine induced vitellogenesis (Li et al., 2012).
	sulfamethoxazole	4	1 ng/L	In US groundwater-fed ponds, 0.63-2.2 ng/L (Standley et al., 2008). In Eropean rivers, up to 4.1 µg/L (Loos et al., 2009). In French surface waters, up to 11 ng/L (Vulliet and Cren-Olivé, 2011).	No data available on amphibians. Limited data on fish: 0.2 mg/L reduced reproductive success (Yan et al., 2016), 10 mg/L induced vitellogenesis (Li et al., 2012).
	carbamazepine	8	0.06-276 µg/L, 106-514 µg/kg	In US groundwater-fed ponds, 0.63-2.4 ng/L (Standley et al., 2008). In Eropean rivers, up to 11.6 µg/L (mean: 0.25 µg/L) (Loos et al., 2009). In French surface waters, up to 41.6 ng/L (Vulliet and Cren-Olivé, 2011). In sediments of a US river, 0.1-32.89 µg/kg (Yang et al., 2015).	No data available on amphibians. Limited data on fish: 0.5-10 µg/L negatively affected several reproductive endpoints (Galus et al., 2014, 2013).

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