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Effects of two little-studied environmental pollutants on early development in anurans☆

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ABSTRACT

Despite intensive ecotoxicological research, we still know relatively little about the ecological impacts of many environmental contaminants. Filling these knowledge gaps is particularly important regarding amphibians, because they play significant roles in freshwater and terrestrial ecosystems, and their populations are declining worldwide. In this study, we investigated two pollutants that have been poorly studied in ecotoxicology despite their widespread occurrence in surface waters: the herbicide terbuthylazine and the pharmaceutical drug carbamazepine. We exposed two anuran species throughout their larval development to each of two environmentally relevant concentrations of each pollutant, and recorded mortality and 17 sub-lethal endpoints up to several months after exposure. Mortality was low and unrelated to treatment. In agile frogs (*Rana dalmatina*), we found that treatment with 0.3 μg/L terbuthylazine decreased tadpole activity and reduced fat bodies in juveniles, whereas treatment with 50 μg/L carbamazepine decreased spleen size and increased spleen pigmentation. In common toads (*Bufo bufo*), treatment with 0.003 μg/L terbuthylazine increased body mass at metamorphosis, treatment with 0.3 μg/L terbuthylazine increased the size of optic tecta, and treatment with 0.5 μg/L carbamazepine decreased hypothalamus size. Treatment with 50 μg/L carbamazepine reduced the feeding activity of toad tadpoles, decreased their production of anti-predatory bufadienolide toxins, and increased their body mass at metamorphosis; juvenile toads in this treatment group had reduced spleen pigmentation. Neither treatments affected the time to metamorphosis, post-metamorphic body mass, or sex ratios significantly. These results show that environmental levels of both terbuthylazine and carbamazepine can have several sub-lethal effects on anurans, which may be detrimental to individual fitness and population persistence in natural conditions. Our findings further highlight that toxic effects cannot be generalized between chemicals of similar structure, because the terbuthylazine effects we found do not conform with previously reported effects of atrazine, a related and extensively studied herbicide.

1. Introduction

Due to the ubiquitous discharge of chemical contaminants into the environment, a wide variety of potentially toxic compounds are present in aquatic systems worldwide (Loos et al., 2009; Pfluger and Dietrich, 2001). Assessing the risk posed by these pollutants to wildlife and human health is an incessant challenge as ever newer compounds and mixtures get introduced into the ecosystem. A major group of environmental contaminants is pesticides, which have received intense research and public attention since the middle of the last century (Köhler and Triebskorn, 2013). Ecotoxicological research of another major group, pharmaceuticals, has

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lagged behind, gaining momentum only about two decades ago (Pfluger and Dietrich, 2001). As the chemicals of both groups are designed and applied to influence the biology of target organisms, it is no wonder that they can have toxic effects in non-target organisms in the form of mortality and various sub-lethal effects on physiology, development, behavior, and reproduction (Hoffman et al., 2003; Orton and Tyler, 2015). Sub-lethal effects can occur at much lower contaminant concentrations than lethal effects, but may still have serious negative consequences in natural systems because they can accumulate over time and across trophic levels, interact with other stressors, and alter biotic relationships in natural communities (Köhler and Triebskorn, 2013; Relyea and Hoverman, 2006).

The effects of contaminants may vary among taxa (Hoffman et al., 2003). One vertebrate group at particular risk is amphibians, due to their permeable skin, shell-less eggs and exposure to pollutants in both aquatic and terrestrial environments. In most amphibian species, embryonic and larval stages occur in freshwater habitats, during which their somatic and sexual development is vulnerable to disruption by water-borne substances with hormone-like activity (Orton and Tyler, 2015). Amphibians are also the most threatened vertebrate group, and chemical pollution is suspected to play a role in the ongoing global loss of amphibian biodiversity (Hayes et al., 2010). Despite these concerns, amphibians have historically been underrepresented in ecotoxicology (Hoffman et al., 2003). Our aim in the present study was to contribute to understanding the effects of contaminants on amphibians by investigating two environmentally relevant pollutants that so far have been studied very sparsely in this respect: terbuthylazine and carbamazepine.

Terbuthylazine is a triazine herbicide widely used for the control of broadleaf weeds in corn and other crops (National Center for Biotechnology Information, 2019a). It is relatively persistent in the environment, with half-lives in water and soil up to 149–196 days (National Center for Biotechnology Information, 2019a). It is frequently detected in surface waters, its concentration typically being a few ng/L in European rivers (Loos et al., 2009) but can reach 0.1–0.5 μg/L in the application period (Antić et al., 2015; Wittmer et al., 2010) especially in small streams and ponds (Bókony et al., 2018b; Lorenz et al., 2017). Terbuthylazine is structurally similar to atrazine, one of the most heavily used herbicides in the US and worldwide (Rohr and McCoy, 2010). Terbuthylazine was introduced as a replacement for atrazine in the European Union where atrazine had been banned because of its persistent water-contamination levels and debated safety (Kjeldsen et al., 2013). Atrazine has been intensely researched in ecotoxicology, and while the conclusions on its overall environmental impact are heterogeneous (Bero et al., 2016; Rohr and McCoy, 2010; Van Der Kraak et al., 2014), a large number of studies reported harmful effects in aquatic vertebrates, including reduced growth and altered developmental rates, hyperactivity and compromised anti-predatory behavior, reduced olfactory and immune functions, and endocrine-disruptor effects related to sexual development and reproduction (Rohr and McCoy, 2010). In contrast, the ecotoxicology literature on terbuthylazine is strikingly scant. A handful of publications show that terbuthylazine can harmfully affect growth, development, behavior, and oxidative stress biomarkers in fish (Mikulikova et al., 2013; Pérez et al., 2012; Štěpánová et al., 2012; Živković Semren et al., 2018), and *in vitro* experiments with mammalian cell lines indicate that it may act as an endocrine disruptor by altering estradiol signaling (Kjeldsen et al., 2013). The only amphibian study we found on terbuthylazine investigated its effect on ion transport in frog skin (Cassano et al., 2006).

Carbamazepine is a pharmaceutical drug with anticonvulsant, analgesic and mood-stabilizing properties (National Center for Biotechnology Information, 2019b). It is poorly eliminated during wastewater treatment and fairly persistent in the environment, exhibiting half-lives of 63–82 days in freshwaters and 165–439 days in wetland sediments (Conkle et al., 2012; Lam et al., 2004; National Center for Biotechnology Information, 2019b). It is ubiquitously present in surface waters (Fent et al., 2006) with a mean concentration of 0.25 μg/L in European rivers (Loos et al., 2009) and can reach much higher levels in small ponds (Bókony et al., 2018b). In humans, its side effects may include immunosuppression, cognitive impairment, excessive food intake and weight gain (Beghi and Shorvon, 2011; Lampl et al., 1991; Vermeulen and Aldenkamp, 1995). In aquatic organisms, carbamazepine typically shows low toxicity (Fent et al., 2006; Heye et al., 2019; Jos et al., 2003), but ecologically relevant sub-lethal endpoints have rarely been studied. In fishes, carbamazepine has been reported to reduce feeding (Nassef et al., 2010) and alter swimming behavior suggesting reduced anxiety (Brandão et al., 2013), and endocrine-disruptor effects were indicated by reduced fecundity, atretic oocytes and altered ovarian histology in females and decreased sex-hormone levels, courtship and sperm function in males (Galus et al., 2014, 2013). We found only four relevant papers on amphibians, reporting that carbamazepine altered the ion transport in toad skin (Suwalsky et al., 2006) but had very moderate embryotoxic effect in *Xenopus laevis* (Pfluger and Dietrich, 2001; Richards and Cole, 2006) and no chronic effect on growth and development in *Limnodynastes peronii* tadpoles (Melvin et al., 2014).

In this study, we examined the effects of terbuthylazine and carbamazepine in two anuran species, the agile frog (*Rana dalmatina*) and the common toad (*Bufo bufo*). Both species are abundant in Europe and occur in natural as well as anthropogenic habitats; the International Union for Conservation of Nature lists them in the "least concern" category but recognizes pollution as a threat for both (International Union for Conservation of Nature, 2019). We have detected several contaminants, including terbuthylazine and carbamazepine, in the ponds where both species breed in Hungary (Bókony et al., 2018b). To focus on ecological significance, we chose two environmentally relevant concentrations for each chemical, and selected a set of sub-lethal endpoints that have clear connections to individual fitness and thereby to population viability. Our treatments lasted throughout the entire larval life of the animals to mimic chronic low-level exposure during the sensitive period of early ontogeny, and we studied the treatments' effects during exposure as well as several months later. During exposure, we observed the tadpoles' behavior because higher activity increases predation risk (Skelly, 1994). In toad tadpoles we also measured the amount of bufadienolide toxins that are important for protection from predators and pathogens (Bókony et al., 2018a; Hettyey et al., 2019). At the end of exposure, we measured the time needed to finish metamorphosis and the body mass acquired by then, because both these traits influence survival to maturity in amphibians (Altwegg and Reyer, 2003; Smith, 1987). When the animals reached the age at which their reproductive organs are fully differentiated, we examined sex ratios and gonadal abnormalities because these are determinants of the populations' reproductive success (Orton and Tyler, 2015). At this time we measured their body mass again and examined their fat reserves to assess their body condition which is relevant for juvenile survival (Scott et al., 2007; Üveges et al., 2016). We also quantified the spleen's size and pigmentation in juveniles because these traits provide information on immune function and pathogen resistance in ectothermic vertebrates (Hadidi et al., 2008; Steinel and Bolnick, 2017). In a subset of juvenile toads, we also measured the size of whole brain and functionally significant brain regions, because brain size may affect

survival and reproductive success (Kotrschal et al., 2019, 2015, 2013) and toxic substances may induce adaptive or maladaptive changes in brain architecture (McClelland et al., 2018; Schulz-Mirbach et al., 2016).

2. Methods

2.1. Chemicals

Terbuthylazine and carbamazepine were obtained from Sigma (analytical standards 45678 and C4024). Stock solutions were prepared by dissolving 3 mg terbuthylazine in 10 mL 96% ethanol, and 50 mg carbamazepine in a solution of 1 mL 96% ethanol and 1 L distilled water. We chose two nominal concentrations for each chemical based on literature data as follows. For terbuthylazine, the lower concentration was 0.003 μg/L, which falls between the median (0.002 μg/L) and mean (0.009 μg/L) concentrations measured in European rivers (Loos et al., 2009); the higher concentration was 0.3 μg/L, which is the value we had detected in a Hungarian pond where several anuran species breed (Bókony et al., 2018b) and is similar to the mean value measured in small streams (0.1 μg/L) (Lorenz et al., 2017). For carbamazepine, the lower concentration was 0.5 μg/L, which was reported to have endocrine-disruptor effects in fish (Galus et al., 2014, 2013) and is close to the mean concentrations measured in European rivers (0.25 μg/L) (Loos et al., 2009); the higher concentration was 50 μg/L, which is the average value we had detected in Hungarian ponds where several anuran species breed (Bókony et al., 2018b).

To measure the actual concentrations in the experimental containers, we took samples from the rearing water of tadpoles into amber PET flasks once per week over three weeks, taking 1 L per treatment on each occasion. The samples were immediately transported to the accredited analytical laboratory of SynTech Research Hungary Kft for analysis. Extraction was performed following a protocol that had been verified for terbuthylazine (Solymosiné Majzik, 2006) and also yielded good average recoveries for carbamazepine, as assessed at 3 concentrations (1, 10 and 100 μg/L) in five replicates each (87, 103 and 99%, respectively). In short, the water samples were filtered (3–5 μm pore size) and extracted using Isolute ENV + solid phase extraction columns primed with 5 mL methanol and 10 mL MilliQ water. After sample loading, the columns were rinsed with 5 mL distilled water, dried, and eluted with 2 × 2 mL acetone:ethyl-acetate (1:1, v/v) solution. The eluent was evaporated under a gentle stream of nitrogen gas, and the residue was re-suspended in 1 mL acetonitrile:water (1:1, v/v) solution. Quantitative measurement of terbutylazine and carbamazepine was carried out by liquid chromatography employing tandem mass-spectrometric detection (LC-MS/MS), using a Sciex QTRAP 5500 LC-MS/MS system (Sciex, Concorde, Canada) equipped with two ExionLC binary gradient solvent pumps, a vacuum degasser, a thermostated autosampler, a column oven and a mass analyser with electrospray ionization (ESI/MS/MS). Samples (20 μL injection volume) were analyzed at 30 °C on an ACE UltraCore SuperC18 column (100 mm × 3 mm i.d., 2.5 μm particle size). Eluent A was MilliQ water with 0.1% formic acid and eluent B was acetonitrile with 0.1% formic acid. The flow rate was 0.3 mL/min and the gradient was as follows: 0–1 min, 5% B; 1–2 min, 5–45% B; 2–16.5 min, 45–95% B; 16.5–19 min, 95% B; 19–20 min, 5% B. MS/MS conditions were as follows: Turbo Spray ionization mode, positive polarity, MRM scan type, curtain gas 45 psi, collision gas medium, ionspray voltage 4500 V, temperature 420 °C, ion source gas 1 and 2 60 psi. Two ion transitions were monitored for each analyte, which supported the specificity of the method. The instrument parameters were checked for analyte sensitivity and resolution prior to each chromatographic run and the exact parameters were documented with each data set. The data were acquired and processed using the Analyst 1.6 software. These analyses detected neither chemical in the control treatment (lowest quantification level: 2 ng/L for both compounds), whereas the concentrations measured in samples treated with terbuthylazine or carbamazepine were close to the nominal concentrations (Table 1). The concentrations given throughout the text henceforth are nominal.

2.2. Experimental procedures

For each of the two species (referred to as “frogs” and “toads” henceforth, for simplicity), we aimed to collect eggs from 8 clutches from 3 ponds each. For frogs, we collected the eggs from Pilisvörösvár (47°36′40.02″N, 18°55′9.45″E) on 13 March, from Kerek-tó (47°38′41.22″N, 18°46′31.59″E) on 15 March, and from Szárazfarkas (47°44′4.12″N, 18°49′7.04″E) on 16 March. Because one of the clutches from the latter pond turned out to be infertile, we replaced it with a 9th clutch from Kerek-tó. For toads, we collected all eggs on 9 April, from Pesthidegkút (47°34′9.38″N, 18°57′17.09″E), Nagykovácsi (47°34′34.72″N, 18°52′8.06″E), and Garancsi-tó (47°37′25.38″N, 18°48′26.18″E). All eggs were collected freshly (the day after the night of spawning) and transported to the Evolutionary Ecology Laboratory at the Experimental Station of MTA ATK NÖVI in Julianna-major, Budapest (47°32′52″N, 18°56′05″E). Throughout the experiment, we maintained a photoperiod that mimicked the natural light-dark cycle. We kept the eggs at 19 °C, each family in a 3-L container filled with 1 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). When the hatchlings reached the free-swimming state, i.e. developmental stage 25 (Gosner, 1960), we started the experiment by randomly selecting 20 healthy-looking larvae from each clutch and placing them into the experimental containers. We reared the tadpoles at 20 °C individually in 2-L containers filled with 1 L RSW, arranged in a randomized block design to ensure that treatments were homogeneously distributed among the shelves in the lab (lighting came from lamps on the ceiling so animals on the upper shelves received slightly more light than animals on the lower shelves). Twice per week, we changed the rearing water and fed the tadpoles *ad libitum* with slightly boiled chopped spinach (commercially bought frozen spinach for human consumption, which is unlikely to be contaminated with considerable amounts of pollutants).

We distributed the tadpoles evenly across 5 treatment groups, such that we had 4 replicates in each treatment × family combination (i.e. 4 individually-housed tadpoles × 5 treatments × 8 families × 3 ponds × 2 species). For toads, we included one additional tadpole in each treatment × family combination for toxin sampling, thus we started the experiment with 600 toad tadpoles and 480 frog tadpoles. The remaining eggs and tadpoles were released at their pond of origin. In the control treatment we kept

Table 1
Concentrations (μg/L) measured in the tadpoles' rearing water (ND: neither compound detected).

treatment	week 1	week 2	week 3
control	ND	ND	ND
terbuthylazine 0.003 μg/L	0.0036	0.0039	0.0031
terbuthylazine 0.3 μg/L	0.290	0.288	0.405
carbamazepine 0.5 μg/L	0.497	0.438	0.415
carbamazepine 50 μg/L	45.7	44.7	45.0

the tadpoles in chemical-free RSW throughout the experiment, to which we added 96% ethanol as solvent control (1 μL ethanol to 1 L RSW); this ethanol concentration is much lower than those observed to harm anuran embryos or tadpoles (Fainsod and Kot-Leibovich, 2017; Peng et al., 2005; Taylor and Brundage, 2013). The other four treatment groups corresponded to the two nominal concentrations of terbuthylazine or carbamazepine as described above. The tadpoles were exposed to the treatments during the entire duration of their larval development, and we renewed the nominal concentrations at each water change.

During larval development, we observed the behavior of each tadpole using the "instantaneous sampling" method (Altmann, 1974) four times a week, yielding 20 observations for each frog tadpole and 16 observations for each toad tadpole. The observations were conducted on two days each week, twice each day, with one session in the morning and one session in the afternoon (each lasting ca. one hour). During each session, all tadpoles were scanned once in a fixed order and their instantaneous behavior was recorded as one of the following three categories: no activity, feeding, and swimming.

To quantify toxin levels in toad larvae, 3 weeks after starting the experiment we sacrificed 118 tadpoles (one from each treatment × family combination; 2 out of 120 tadpoles died before sampling) by placing each into a microcentrifuge tube filled with 1 mL HPLC-grade absolute methanol. We chose this time for toxin sampling because our earlier study showed that bufadienolide levels are at their peak at ca. 3 weeks of larval development in common toads (Üveges et al., 2017). The samples were stored at −20 °C until chemical analysis.

To measure the rate of larval development and growth, we raised the experimental tadpoles to metamorphosis, measured their body mass (±0.1 mg) at the start of metamorphosis, and recorded the length of larval development as the number of days from the start of the experiment until the start of metamorphosis. Because water temperature may influence growth and development, we quantified the spatial variation in temperature by measuring water temperature (±0.1 °C) 4 and 3 times during the larval development of frogs and toads, respectively. To avoid disturbing the animals, we measured water temperatures in 0.5-L cups placed between the tadpoles' rearing boxes: one cup for each block of six animals.

To investigate the long-term effects of larval chemical exposure, we raised the animals for several months after ending the treatments. When a tadpole reached developmental stage 42 (the start of metamorphosis, identified by the appearance of forelimbs), we decreased the water level to 0.1 L and slightly tilted the container to allow the animal to leave the water. When metamorphosis was completed (developmental stage 46, identified by the disappearance of the tail), we moved the animal into a clean rearing box that contained wet paper towels as substrate and a piece of egg carton as shelter, which we changed every other week. The metamorphosed animals were fed *ad libitum* with springtails and small (2–3 mm) crickets, sprinkled with a 3:1 mixture of Reptiland 76280 (Trixie Heimtierbedarf GmbH & Co. KG, Tarp, Germany) and Promotor 43 (Laboratorios Calier S.A., Barcelona, Spain) containing vitamins, minerals and amino-acids. Because the timing of gonad development differs between the two species (Ogielska and Kotusz, 2004), we raised froglets for 49–92 (median: 63) days and toadlets for 111–142 (median: 127) days after metamorphosis.

When the animals reached the minimum age that allows phenotypic sexing by gross gonad anatomy, we measured their body mass (±0.01 g) and euthanized them using a water bath containing 6.6 g/L MS-222 buffered to neutral pH with the same amount of Na_2HPO_4. After dissection (N = 440 froglets and 458 toadlets), we examined the internal organs under an Olympus SZX12 stereomicroscope and recorded any abnormalities seen at 16 × magnification. We cut out the entire digestive tract and measured its mass (±0.01 g), because many animals' guts contained food despite that we had not fed them for 2–4 days before dissection. Thus, the animal's total body mass minus gut mass provides a proxy measure for lean body mass (i.e. without food). We photographed the spleen at 45 × magnification with a Canon EOS 60D camera attached to the stereomicroscope, and saved the pictures in JPG format (Fig. S1). We recorded whether the animal had fat bodies (Fig. S2) at the cranial end of the kidneys, and whether it had testes (male) or ovaries (female) or abnormally looking gonads (uncertain sex). Finally, we stored the dissected bodies in 4% formaldehyde solution (Sigma 1.00496); a subsample of 60 toadlets (one per each treatment group from each of 12 families) were fixed in 4% formalin − 0.1 M phosphate-buffered saline solution for brain measurements.

All experimental procedures were approved by the Ethical Commission of the Plant Protection Institute and 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).

2.3. Analysis of toad toxins

For quantifying bufadienolide levels, we applied our previously described protocol for sample preparation (Üveges et al., 2017) and HPLC (Bókony et al., 2019). In short, each tadpole was homogenized and dried in vacuum to measure dry mass. The samples were redissolved in 1 mL HPLC-grade absolute methanol, and filtered using nylon syringe filters (0.22 μm). We applied high-performance liquid chromatography with diode-array detection and mass spectrometry (HPLC-DAD-MS; LC-MS-2020, Shimadzu, Kyoto, Japan) to identify and quantify bufadienolide compounds in each sample. We recognized bufadienolides by their characteristic UV spectrum, and identified them by comparing their peak retention time and m/z (mass-to-charge ratio) values to those of standards (8 commercially available compounds and a marinobufotoxin standard obtained as courtesy from Professor Robert Capon, University of Queensland, Australia) and to the peaks present in a toxin sample obtained from the parotoid glands of juvenile common toads. We used the calibration curve of the marinobufotoxin standard to express the marinobufotoxin-equivalent concentration of each bufadienolide compound per sample; then we summed these values and divided the sum by tadpole dry mass to obtain the total amount of bufadienolides per tadpole mass (μg/mg). Henceforth we refer to this variable as bufadienolide content.

2.4. Spleen measurements

We evaluated the spleen photos of all dissected animals and recorded the presence of polysplenia (multiple spleens; Fig. S1) or other spleen abnormalities. If the animal had a single, not deformed spleen, and image quality was good, we measured the area of the spleen with the 'freehand selections' tool and the percentage area of pigmented spots (Fig. S1) on the spleen with the 'threshold' tool of the ImageJ software (Schneider et al., 2012). The measurements were converted from pixels to mm^2 by photographing a size standard at the same magnification as the spleens. The image analyses were done by two persons (VV and EV), with one person analysing the photos of terbuthylazine-treated animals and the other person analysing the photos of carbamazepine-treated animals; both persons measured all photos of control animals. Using the latter measurements, we calculated between-person repeatability as the intra-class correlation coefficient (ICC) and its 95% confidence

interval (CI) for N = 80 photos (Nakagawa and Schielzeth, 2010). This showed high repeatability for spleen size (ICC = 0.993, 95% CI: 0.988–0.995, $F_{79,80}$ = 266, P < 0.001) as well as pigmentation (ICC = 0.882, 95% CI: 0.822–0.923, $F_{79,80}$ = 16, P < 0.001). We also measured within-person repeatability between two different file formats (JPG and lossless CR2; N = 37 spleens measured by VV), and we found it high for spleen size (ICC = 0.989, 95% CI: 0.978–0.994, $F_{36,37}$ = 173, P < 0.001) as well as pigmentation (ICC = 0.856, 95% CI: 0.739–0.923, $F_{36,37}$ = 12.9, P < 0.001).

2.5. Brain measurements

The whole brains were dissected from the cranium of the animals stored in fixative solution under a Zeiss SteREO Discovery.V8 microscope, placed on glass beads, and photographed from dorsal, lateral (left) and ventral views (Fig. S3) with a Canon 600D digital camera. All photographs were analyzed using TpsDig 2.31 software (https://life.bio.sunysb.edu/ee/rohlf/software.html) by a single researcher (SO). For the whole brain as well as for each brain region (bulbus olfactorius, telencephalon, tectum optica, medulla oblongata, diencephalon, and hypothalamus), height, length, and width were measured as the greatest distance enclosed by the given structure in the given direction, converted to mm using a size reference included in each picture. From these three size dimensions, we estimated the volume of the brain and each brain region according to the ellipsoid model following the formulas (including the correction factor) of Pollen et al. (2007). The same researcher measured 15 randomly chosen brains three times; these measurements showed high repeatability (for all brain variables: ICC ≥ 0.877, P < 0.001; Table S1). The medulla got damaged during dissection in two animals and the hypothalamus in another two, so we had 56 data points for each of these regions and also for total brain size.

2.6. Statistical analyses

All statistical analyses were run with R 3.4.1 (R Core Team, 2018), using the packages 'nlme' and 'MASS'. An overview of the analyses and sample sizes is given in Table S2. We tested the effects of treatments using general and generalized linear mixed-effects models, analyzing frogs and toads separately. In each analysis, we included the fixed effect of treatment and other, potentially confounding variables as detailed below. Covariates (i.e. numeric predictor variables) were mean-centered before the analyses. In each model, we used family nested within population (pond of origin) as random factors. For behavioral data, we had repeated measures for each individual, so in the analyses of tadpole behavior we also included individual identity (nested within family) as a third random factor. For numeric dependent variables (time to metamorphosis, body mass, toxin content, spleen size and pigmentation, and brain size) we used Gaussian error distribution, whereas for binary dependent variables (swimming, feeding, presence/absence of fat bodies, sex) we used quasi-binomial error distribution with logit link function. In the Gaussian models, we allowed the variances to differ among treatment groups, except for brain analyses where sample size was smaller than in the other analyses and likelihood-ratio tests showed that model fit was not improved significantly by treatment-specific variances. Additionally, in the analyses of time to metamorphosis and body mass, we also allowed the variances to differ among families (note that this was not possible in the other analyses where some or all family × treatment combinations had N ≤ 1).

Because somatic development, growth, and behavior may have been affected by the slight variation in temperature and light across our lab, we used the average water temperature per each block of six animals as a covariate and shelf height (ranging from 1 to 5, with 5 being the topmost, best-lit shelf) as a fixed factor for all dependent variables excepting sex. When analyzing the toxin content of toad tadpoles, only treatment and temperature were used as fixed effects, because all tadpoles raised for toxin sampling were kept on shelf 6. For all measurements taken at dissection (body mass, presence of fat bodies, spleen and brain measurements) we also included age (number of days from finishing metamorphosis) as a covariate. We also tested the effect of sex on these variables, but omitted it from the models presented here if its effect was non-significant (as it was in all but one case), because including sex decreased sample size due to the small number of animals with uncertain sex.

We analyzed tadpole behavior using two binary dependent variables: swimming versus no locomotion (which included inactivity as well as feeding; note that tadpoles feed stationarily while moving their tails only), and feeding versus no feeding (which included inactivity as well as swimming). The fixed effects in these analyses were treatment, temperature, shelf height, tadpole age (number of days from the start of the experiment), time of day (morning or afternoon), observer identity, and the number of days since the last water change (when the tadpoles got fresh food).

Because spleen measurements were obtained by two researchers, and there was a small but significant difference between their pigmentation measurements (paired *t*-test: t_{79} = 2.75, P = 0.007), we analyzed the effects of carbamazepine and terbuthylazine in two separate models for each species, using the control animals' measurements obtained by the respective researcher in each model. Besides treatment, temperature, shelf height, and age (number of days from finishing metamorphosis), these analyses included body mass as a covariate.

Similarly, in the analysis of total brain size, the fixed effects were treatment, temperature, shelf height, age, and body mass. These same predictors were included in the model of each brain region, and total brain size was also used as a covariate. We graphically examined the relationship between total brain size and body mass, and also between each brain region and total brain size, both with and without logarithmically transforming the variables, and we found no indication that power-function relationships would fit the data better than linear relationships, so we used the latter (Pollen et al., 2007).

For analyzing sex ratios, we used two approaches. First, we tested whether treatment predicted sex (i.e. whether sex ratio differed between the control group and any other treatment group) using a linear mixed-effects model with quasi-binomial error distribution, including treatment as the only fixed effect. Second, we tested whether the sex ratio of each treatment group differed significantly from 1:1 using binomial tests. In these analyses, we used only those animals whose sex could be categorized unambiguously as male or female.

The full parameter-estimates table of each model is presented in the supplementary material (Table S3). In the main text, we only present the treatment effects, i.e. the differences between the control group and each treatment group. For estimates of mean, we provide the standard error (±SE). We use 95% confidence levels, and we interpret effects with P < 0.05 as statistically significant.

3. Results

Mortality was low over the entire experiment (5.28%): out of the 1080 animals in total, 35 frogs and 22 toads died for unknown reasons. All but 8 deaths occurred post-treatment (after the start of metamorphosis). Neither terbuthylazine nor carbamazepine treatments increased the mortality rate relative to the control group (Table 2).

Table 2
Number of animals that died or survived in each treatment group. In each cell of the table, the first number refers to frogs and the second to toads that were raised until dissection unless they died. A third number in parentheses refers to toads that were raised for toxin sampling until 3 weeks of tadpole age. "Other" refers to froglets that escaped from their home boxes before dissection and toad tadpoles that were euthanized after 68 days because they did not develop at all.

treatment	died				other	survived
	during treatment (as larva)	during metamorphosis	after metamorphosis	total		
control	2 + 0	9 + 0	3 + 3	14 + 3	0 + 0	82 + 93 (+24)
terbuthylazine 0.003 μg/L	1 + 1 (+1)	1 + 0	6 + 4	8 + 5 (+1)	0 + 1	88 + 90 (+23)
terbuthylazine 0.3 μg/L	0 + 1	3 + 0	2 + 2	5 + 3	2 + 1	89 + 92 (+24)
carbamazepine 0.5 μg/L	0 + 1	1 + 1	3 + 3	4 + 5	2 + 0	90 + 91 (+24)
carbamazepine 50 μg/L	0 + 0 (+1)	2 + 0	2 + 4	4 + 4 (+1)	1 + 0	91 + 92 (+23)
total	3 + 3 (+2)	16 + 0	16 + 16	35 + 20 (+2)	5 + 2	440 + 458 (+118)

3.1. Sub-lethal effects during exposure

During larval development, treatment with 0.3 μg/L terbuthylazine significantly reduced the swimming activity of frog tadpoles, from 5.5 ± 0.6% to 4.1 ± 0.5% (Table 3), whereas treatment with 50 μg/L carbamazepine significantly reduced the feeding activity of toad tadpoles, from 34.8 ± 1.7% to 29.2 ± 1.6% (Table 4). The lower concentrations had no significant effect on tadpole behavior in either species (Tables 3–4).

In toad larvae, the number of bufadienolide compounds per tadpole ranged from 28 to 32, with the majority (80 tadpoles out of 118) containing 31 compounds. Out of a total of 33 compounds, 26 was detected in all tadpoles. Bufadienolide content was significantly reduced in the treatment group exposed to 50 μg/L carbamazepine (Table 4) from 2.56 ± 0.201 to 2.20 ± 0.168 μg/mg; the other treatments had no significant effect on toxin content (Table 4).

Larval development time was not significantly influenced by any treatment in either species (Tables 3–4). Similarly, in frog tadpoles, body mass at metamorphosis was not significantly affected by any treatment (Table 3). However, in toad tadpoles, body mass at metamorphosis was significantly increased in two treatment

Table 3
Mean ± SE of each outcome variable in each treatment group in *Rana dalmatina* frogs, as estimated by the models shown in Table S3. Values that differ significantly from the control are highlighted with bold type and an asterisk ($P < 0.05$).

outcome variable	treatment				
	control	terbuthylazine 0.003 μg/L	terbuthylazine 0.3 μg/L	carbamazepine 0.5 μg/L	carbamazepine 50 μg/L
% of time spent swimming	5.50 ± 0.63	5.74 ± 0.64	**4.11 ± 0.51***	5.34 ± 0.60	5.36 ± 0.60
% of time spent feeding	6.01 ± 0.59	6.20 ± 0.60	4.95 ± 0.52	5.91 ± 0.57	5.06 ± 0.52
time to metamorphosis (days)	43.1 ± 1.14	43.7 ± 1.10	43.1 ± 1.11	42.8 ± 1.10	42.7 ± 1.10
body mass at metamorphosis (mg)	496 ± 6.65	488 ± 5.91	505 ± 5.55	489 ± 6.13	494 ± 5.04
body mass at dissection (mg)	1221 ± 17.1	1231 ± 16.6	1222 ± 16.4	1227 ± 16.7	1213 ± 17.4
% of animals having fat bodies	88.4 ± 3.52	88.5 ± 3.39	**77.3 ± 4.93***	91.8 ± 2.86	87.6 ± 3.55
spleen size[a] (mm^2)	0.67 ± 0.03	0.62 ± 0.02	0.63 ± 0.02		
	0.67 ± 0.04			0.68 ± 0.04	**0.60 ± 0.03***
% of spleen surface pigmented[a]	2.63 ± 0.40	2.57 ± 0.34	2.79 ± 0.36		
	1.81 ± 0.25			1.96 ± 0.22	**2.38 ± 0.22***

[a] Spleen measurements were conducted by two different persons, so two estimates are given for the control group.

Table 4
Mean ± SE of each outcome variable in each treatment group in *Bufo bufo* toads, as estimated by the models shown in Table S3. Values that differ significantly from the control are highlighted with bold type and asterisks (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$).

outcome variable	treatment				
	control	terbuthylazine 0.003 μg/L	terbuthylazine 0.3 μg/L	carbamazepine 0.5 μg/L	carbamazepine 50 μg/L
% of time spent swimming	19.1 ± 1.54	16.9 ± 1.45	21.5 ± 1.62	18.1 ± 1.50	20.6 ± 1.59
% of time spent feeding	34.8 ± 1.69	33.5 ± 1.67	32.2 ± 1.66	32.7 ± 1.66	**29.2 ± 1.63****
bufadienolide content (μg/mg)	2.56 ± 0.20	2.39 ± 0.18	2.66 ± 0.32	2.74 ± 0.32	**2.20 ± 0.17***
time to metamorphosis (days)	35.3 ± 0.33	35.5 ± 0.35	35.4 ± 0.34	35.2 ± 0.35	35.2 ± 0.36
body mass at metamorphosis (mg)	258 ± 6.56	**286 ± 6.78*****	265 ± 6.43	267 ± 6.87	**281 ± 6.51*****
body mass at dissection (mg)	3420 ± 110	3458 ± 110	3436 ± 111	3536 ± 108	3479 ± 107
% animals having fat bodies	98.8 ± 1.01	98.4 ± 1.07	98.2 ± 1.19	99.1 ± 0.75	99.3 ± 0.64
spleen size[a] (mm^2)	1.18 ± 0.05	1.16 ± 0.05	1.18 ± 0.05		
	1.19 ± 0.04			1.17 ± 0.04	1.19 ± 0.04
% of spleen surface pigmented[a]	1.59 ± 0.22	1.47 ± 0.19	1.71 ± 0.23		
	1.54 ± 0.23			1.24 ± 0.19	**1.12 ± 0.19***
total brain volume (mm^3)	33.4 ± 0.67	33.7 ± 0.73	34.1 ± 0.71	33.6 ± 0.64	34.0 ± 0.64
bulbus olfactorius volume (mm^3)	1.95 ± 0.10	1.94 ± 0.11	1.89 ± 0.11	1.96 ± 0.10	1.93 ± 0.10
telecephalon volume (mm^3)	3.29 ± 0.08	3.35 ± 0.09	3.42 ± 0.08	3.40 ± 0.08	3.43 ± 0.08
tectum optica volume (mm^3)	1.91 ± 0.04	1.93 ± 0.04	**2.04 ± 0.04***	1.92 ± 0.04	1.94 ± 0.04
medulla oblongata volume (mm^3)	5.77 ± 0.17	5.85 ± 0.19	5.86 ± 0.18	5.56 ± 0.16	6.08 ± 0.16
diencephalon volume (mm^3)	4.17 ± 0.11	4.43 ± 0.12	4.47 ± 0.12	4.19 ± 0.10	4.38 ± 0.10
hypothalamus volume (mm^3)	1.19 ± 0.06	1.21 ± 0.07	1.10 ± 0.06	**1.03 ± 0.06***	1.14 ± 0.06

[a] Spleen measurements were conducted by two different persons, so two estimates are given for the control group.

groups (Table 4): compared to an average 258 ± 6.56 mg in the control group, body mass at the start of metamorphosis was 286 ± 6.78 mg in the 0.003 μg/L terbuthylazine treatment and 281 ± 6.51 mg in the 50 μg/L carbamazepine treatment.

3.2. Sub-lethal effects after metamorphosis

Body mass at dissection was not significantly influenced by any treatment in either species (Tables 3–4). Similarly, in toadlets, the proportion of animals with or without fat bodies did not differ significantly between the control group and any other treatment (Table 4). However, in froglets, treatment with 0.3 μg/L terbuthylazine significantly reduced the percentage of animals that had fat bodies, from 88.4 ± 3.5% to 77.3 ± 4.9% (Table 3).

Incidence of spleen abnormalities did not differ significantly among treatment groups (χ^2 tests; froglets: $\chi^2_4 = 1.96$, $P = 0.743$, toadlets: $\chi^2_4 = 3.87$, $P = 0.423$), although the highest incidence occurred in the 50 μg/L carbamazepine treatment (Table 5). Spleen size in toadlets was not significantly affected by any treatment (Table 4), whereas in froglets, treatment with 50 μg/L carbamazepine significantly reduced spleen size, from 0.67 ± 0.04 mm^2 to 0.60 ± 0.03 mm^2 (Table 3). Spleen pigmentation was not influenced significantly by terbuthylazine treatments in either species (Tables 3–4); however, treatment with 50 μg/L carbamazepine significantly increased the pigmentation of froglets' spleens from 1.81 ± 0.25% to 2.38 ± 0.22% (Table 3) and decreased the pigmentation of toadlets' spleens from 1.54 ± 0.23% to 1.12 ± 0.19% (Table 4).

In toadlets, brain volume as well as the volume of various brain regions was not affected by treatments (Table 4), with two exceptions. First, treatment with 0.3 μg/L terbuthylazine significantly increased the volume of tectum optica from 1.91 ± 0.04 mm^3 to 2.04 ± 0.04 mm^3 (Table 4). Second, treatment with 0.5 μg/L carbamazepine significantly decreased the volume of hypothalamus from 1.19 ± 0.06 mm^3 to 1.03 ± 0.06 mm^3 (Table 4). However, this latter result might be an artefact of light conditions, because in the sample of animals we used for brain analysis the treatment groups happened to be non-homogenously distributed among the five shelves (Fig. S4), and the difference in hypothalamus size was no longer significant when shelf height was removed from the model (Table S4).

Based on gonad anatomy, we could identify the sex of almost all animals; the number of individuals with uncertain sex did not exceed one per treatment group (Table 6). Sex ratio did not differ significantly between the control group and any of the treatment groups in either species ($P > 0.124$; Table S3). In toadlets, sex ratio was close to 1:1 in every treatment group (Table 6), whereas in froglets, sex ratio was slightly female-biased in all treatment groups except for the treatment group exposed to 0.003 μg/L terbuthylazine, in which the sex ratio was slightly male-biased (Table 6). Sex ratio did not differ significantly from 1:1 in any group, except for the froglets treated with 0.5 μg/L carbamazepine, where females significantly outnumbered males (Table 6).

4. Discussion

We have studied lethal effects and 17 sub-lethal endpoints in two anuran species with two environmentally realistic concentrations each of terbuthylazine and carbamazepine. Mortality was low and evenly distributed among the treatment groups in our experiment, corroborating previous results in other aquatic organisms that these two contaminants do not tend to have lethal effects at concentrations below the mg/L range (Heye et al., 2019; Jos et al., 2003; Pfluger and Dietrich, 2001; Štěpánová et al., 2012). In our study, only 11.5% of the outcomes (combinations of treatment, species, and endpoint) showed statistically significant effects, and almost all of these occurred at the higher concentrations. Altogether, these results suggest that the concentrations of terbuthylazine and carbamazepine typically found in surface waters may not be overtly dangerous to the two studied species. However, as discussed below, we observed a handful of sub-lethal effects that can be harmful in natural ecosystems.

Our experiment showed that the effects of terbuthylazine cannot be predicted from the previously observed effects of atrazine, despite the structural similarity of the two chemicals. First, we found no terbuthylazine effects on the time to metamorphosis, spleen measurements, and olfactory bulb size, whereas atrazine is known to alter larval developmental rates, immune system and olfactory functions in aquatic vertebrates (Rohr and McCoy, 2010), although many of the latter results came from studies that tested higher atrazine concentrations than our highest terbuthylazine concentration. Second, in opposite to the hyperactivity and reduced growth often caused by atrazine (Rohr and McCoy, 2010), we found reduced activity and increased body mass at metamorphosis in some of our terbuthylazine treatments (as elaborated below). These discrepancies caution that the ecotoxicological effects of terbuthylazine deserve the same scrutiny as has been focused on atrazine, given that the latter has been replaced by the former in the

Table 5
Number of animals with normal or abnormal spleen in each treatment group. In each cell of the table, the first number refers to froglets and the second to toadlets.

treatment	normal spleen	polysplenia	abnormal shape	haemosiderotic
control	75 + 86	4 + 4	1 + 3	0 + 0
terbuthylazine 0.003 μg/L	76 + 88	9 + 1	1 + 1	0 + 0
terbuthylazine 0.3 μg/L	80 + 86	8 + 4	1 + 2	0 + 0
carbamazepine 0.5 μg/L	80 + 84	9 + 3	0 + 2	1 + 0
carbamazepine 50 μg/L	80 + 83	10 + 3	1 + 5	0 + 0

Table 6
Number of males (♂), females (♀) and animals with uncertain sex (⚥; abnormal gonads) in each treatment group in each species, and P-values from binomial tests for deviation from 1:1 sex ratio. A significant P-value is highlighted with bold type and an asterisk. See Table S3 for comparisons between the control group and the other treatments.

treatment	*Rana dalmatina*			*Bufo bufo*		
	N	% male	P	N	% male	P
control	37 ♂, 45 ♀, 0 ⚥	45.12%	0.380	45 ♂, 47 ♀, 1 ⚥	48.91%	0.917
terbuthylazine 0.003 μg/L	50 ♂, 38 ♀, 0 ⚥	56.82%	0.241	41 ♂, 48 ♀, 1 ⚥	46.07%	0.525
terbuthylazine 0.3 μg/L	38 ♂, 50 ♀, 1 ⚥	42.70%	0.241	50 ♂, 42 ♀, 0 ⚥	54.35%	0.466
carbamazepine 0.5 μg/L	35 ♂, 55 ♀, 0 ⚥	38.89%	**0.045***	45 ♂, 45 ♀, 1 ⚥	50.00%	>0.999
carbamazepine 50 μg/L	37 ♂, 54 ♀, 0 ⚥	40.66%	0.113	44 ♂, 47 ♀, 1 ⚥	48.35%	0.834

European Union.

In agile frog tadpoles, we found that the higher concentration of terbuthylazine (0.3 μg/L) decreased locomotor activity by ca. 25% (accompanied by a similar but smaller, non-significant trend for decreased feeding activity). This result parallels the impairment of swimming motion and apathy observed in fishes at much higher terbuthylazine concentrations applied for much shorter exposure times (3.3–16.2 mg/L for 24–96 h) in previous studies (Mikulikova et al., 2013; Pérez et al., 2012). Generally, agile frog larvae have low activity, which helps predator avoidance in their natural habitats (Chovanec, 1992; Skelly, 1994); further reduction from this already low baseline level of activity may be costly in such habitats by decreasing foraging success and thereby slowing down growth and development. We did not find such consequences in our experiment with *ad libitum* food, but despite their apparently normal growth and development, froglets treated with 0.3 μg/L terbuthylazine were ca. 13% more likely to have no fat bodies at all. Lack of fat stores means poor body condition and low chances of surviving the winter hibernation (Scott et al., 2007; Üveges et al., 2016). Thus, these results suggest that levels of terbuthylazine occurring in natural water bodies in its application period (Antić et al., 2015; Bókony et al., 2018b; Lorenz et al., 2017; Wittmer et al., 2010) may decrease the rate of successful recruitment in agile frog populations, which might contribute to their decline.

In common toads, the effects of terbuthylazine in our experiment are more challenging to interpret. We observed a small (ca. 9%) but highly significant increase in their body mass at metamorphosis at the lower concentration only, which might indicate a non-linear dose-response relationship. Although higher metamorphic mass is beneficial to survival in amphibians (Altwegg and Reyer, 2003; Smith, 1987), we are reluctant to interpret this as a positive outcome of terbuthylazine treatment, because no difference in body mass was present any longer in the juvenile toads at dissection. Further, we found enlarged optic tecta in the toadlets treated with the higher concentration of terbuthylazine, although the difference was small (6.8%). Neurodevelopment is known to respond adaptively to environmental conditions in aquatic vertebrates (Schulz-Mirbach et al., 2016; Trokovic et al., 2011); however, the effect we observed may be more likely due to apoptosis and neural swelling, similarly to those caused by another pesticide, chlorpyrifos in rodents and amphibians (McClelland et al., 2018). This would mean that larval exposure to application-period levels of terbuthylazine might have long-term negative effects on the vision of common toads.

The higher concentration of carbamazepine (50 μg/L) in our experiment had effects on the spleen of both species, suggesting that this chemical may modulate immune system activity in amphibians as it does in mammals (Beghi and Shorvon, 2011). In agile frogs in this treatment group, we found a ca. 10% decrease in spleen size and ca. 31% increase in spleen pigmentation. In amphibians and fish, the spleen is a lymphopoietic organ that is a widely accepted index of immunocompetence, as larger spleen size predicts resistance to infection (Hadidi et al., 2008). The spleen contains aggregations of melano-macrophages, pigmented cells that play important roles in humoral as well as cellular immunity and phagocytosis of non-infectious toxic materials; their size and/or numbers increase in response to infections, chemical pollution, and other stressors (Steinel and Bolnick, 2017). Because smaller spleen size implies lower pathogen resistance (Hadidi et al., 2008) and increased melano-macrophage centers indicate infection and physiological stress (Steinel and Bolnick, 2017), our results suggest that agile frogs exposed to 50 μg/L carbamazepine had poor immune defenses and may have been suffering from infections, perhaps by the facultatively pathogenic microbiota that can be present under laboratory conditions. Because amphibians are globally threatened by serious infectious diseases (Scheele et al., 2019), the potentially immunosuppressive effects of carbamazepine are troubling and need further research.

In common toads, the effects of carbamazepine in our experiment were more complex. The 50 μg/L treatment decreased spleen pigmentation by ca. 27% without altering spleen size. Similar decrease in melano-macrophage centers was observed in the liver in amphibians and fish exposed to other pollutants, the mechanisms of which have remained unclear but apoptosis as well as disrupted melanin synthesis were suspected (De Oliveira et al., 2017). Also, our toad tadpoles showed ca. 16% decrease in feeding activity and ca. 9% increase in body mass at metamorphosis in response to 50 μg/L carbamazepine, although the latter effect disappeared in juvenile toads. These results align with the previous finding that carbamazepine influences the expression of many genes in the fish brain, targeting similar processes as in humans, especially those associated with pituitary hormones such as pro-opiomelanocortin which is a regulator of feeding, homeostasis, and melanin synthesis (Hampel et al., 2014). Furthermore, we found ca. 14% decrease in bufadienolide content in the toad tadpoles exposed to 50 μg/L carbamazepine, indicating lower rates of anti-predatory toxin production. Because toad tadpoles respond to risky and stressful environments with increased bufadienolide synthesis (Bókony et al., 2018a, 2017; Hettyey et al., 2019; Üveges et al., 2017), our result suggests that carbamazepine might decrease anxiety in this species similarly to humans and fish (Brandão et al., 2013). Finally, since the hypothalamus controls many of the neuroendocrine pathways underlying defensive behaviors, fear processing, feeding, and weight gain (Pollen et al., 2007), the changes we observed in these endpoints might be related to altered hypothalamic function. However, the ca. 13% decrease in hypothalamus size we found in response to the lower carbamazepine concentration was not detected at the higher concentration. Larger hypothalamus in the lower carbamazepine group or the lack of such effect in the higher carbamazepine group might have been due to an inadvertent imbalance in light conditions in the subsample of animals examined for brain architecture, given that dark environment has been shown to increase hypothalamic volume in fish (Schulz-Mirbach et al., 2016). Nevertheless, our findings altogether highlight that carbamazepine at its highest environmentally relevant concentrations has the potential for a range of neuro-endocrine disruptor effects, the prevalence and mechanisms of which need further research on non-target organisms.

A further endocrine-disruptor endpoint is sex ratio, because amphibian sex determination and sexual development take place during larval life and can be perturbed by pollutants (Orton and Tyler, 2015). Although we found no significant differences from the control group' sex ratio in any of our treatments, it must be noted that statistical tests of ratios require large sample size, and even though we started our experiment with $N = 96$ per treatment group, the ca. 6% mortality decreased our power to detect small but biologically relevant changes in sex ratios. Bearing this caveat in mind, we find it noteworthy that, despite the generally slightly female-biased sex ratio of agile frogs, the treatment with 0.003 μg/L terbuthylazine produced a slightly male-biased sex ratio (57% males as opposed to ca. 58% females in all other treatment groups combined). This contrasts with several findings on atrazine that suggested a feminizing effect in amphibians (Rohr and McCoy, 2010). On the other hand, agile frog sex ratio was significantly female-biased in the treatment with 0.5 μg/L carbamazepine while there was no significant deviation from 1:1 in the control group. These results raise the need for further studies on the potential sex-

disruptor effects of environmentally realistic concentrations of terbuthylazine and carbamazepine, as reproductive health includes a range of histological, physiological and behavioral aspects, many of which may be affected negatively by such pollutants (Galus et al., 2014, 2013).

Taken together, our study revealed that terbuthylazine and carbamazepine, both occurring ubiquitously in surface waters, may have several potentially harmful effects in two anuran amphibians, despite their low toxicity in terms of direct mortality in environmentally relevant concentrations. The two species reacted entirely differently to each of the two chemicals, demonstrating that ecotoxicological effects cannot be generalized even across relatively closely related species with similar ecology. Furthermore, almost all effects of terbuthylazine were in conflict with the previously reported effects of atrazine, a related chemical that was much more intensely studied. Thus, our results highlight the importance of meticulous ecotoxicological research across a wide range of pollutants and non-target organisms.

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Veronika Bókony: Conceptualization, Investigation, Formal analysis, Writing - original draft, Writing - review & editing, Supervision, Funding acquisition. **Viktória Verebélyi:** Investigation. **Nikolett Ujhegyi:** Investigation, Writing - review & editing. **Zsanett Mikó:** Investigation, Writing - review & editing. **Edina Nemesházi:** Investigation, Writing - review & editing. **Márk Szederkényi:** Investigation. **Stephanie Orf:** Investigation, Writing - review & editing. **Evelin Vitányi:** Investigation. **Ágnes M. Móricz:** Investigation, Writing - review & editing.

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Appendix A. Supplementary data

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