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Skin coloration as a possible non-invasive marker for skewed sex ratios and gonadal abnormalities in immature common toads (*Bufo bufo*)

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ABSTRACT

Environmental pollution and climate change can bias the sex ratios of animal populations in which sexual development is sensitive to environmental contaminants and temperature. Investigating these effects in field studies and ecotoxicological experiments is important but difficult when males and females cannot be distinguished without sacrificing them or applying expensive, specialized sexing methods. In this study, we examined the utility of skin coloration as a non-invasive sex marker in juvenile common toads (*Bufo bufo*) that appear sexually monomorphic. We raised toadlets from eggs in the lab, and exposed them during larval development to one of six treatments: two concentrations of two endocrine disruptor chemicals each (a glyphosate-based herbicide and 17α-ethinylestradiol, a contraceptive) and two controls. Before the first hibernation, we took a photograph of each toadlet's back, then sexed them by inspecting their gonads, and measured the hue, saturation and brightness of their dorsal skin coloration from the photographs. We found significant sexual dichromatism with males being yellower-greener (less red) and brighter than females; 34% of males and 85% of females could be categorized correctly based on objective colour measurements from photographs. The ratio of greenish and reddish individuals as categorized subjectively by human vision correlated strongly with the sex ratio of treatment groups. Treatment with 1 μg/L 17α-ethinylestradiol resulted in 100% females, with similar coloration as normal females. Intersex individuals occurred in treatment groups with 3 μg/L glyphosate and 1 ng/L 17α-ethinylestradiol; these animals were less saturated and darker reddish-brown compared to normal individuals. These results suggest that skin coloration can indicate phenotypic sex and gonadal abnormalities in common toads. Although skin colour measurement in itself is insufficient for identifying an individual's sex or the sex ratio of a single group, it can be useful for qualitative comparisons of sex ratios between groups when no other means of phenotypic sexing is possible. We propose that counting the number of greenish and reddish individuals as seen by the human eye is a cheap and fast non-invasive method for identifying natural populations or experimental groups that may have skewed sex ratios compared to other groups, and this approach is worth testing in other species to help conservation practices and non-destructive ecotoxicological experiments.

1. Introduction

With the human population growing, anthropogenic environmental changes threaten wildlife populations worldwide at an ever increasing rate. Sex ratio and reproductive health are crucial determinants of population viability, and both can be influenced by environmental conditions in several ways. First, whether an individual develops into a male or a female can depend on the environment. For example in species like many turtles and crocodiles, sex determination is temperature-dependent (Mitchell and Janzen, 2010), while other ectothermic vertebrates are prone to undergo sex reversal whereby individuals develop into the phenotypic sex opposite to their genetic sex (Flament, 2016; Holleley et al., 2016; Lambert et al., 2019; Ospina-Álvarez and Piferrer, 2008). Although sex reversal can occur under normal natural circumstances, climatic or chemical perturbations may increase its frequency (Flament, 2016; Lambert, 2015; Lambert et al., 2017b, 2018; Tamschick et al., 2016b). Second, even after sex determination is finalized gonad, development and reproductive health can be disrupted by environmental contaminants, which can decrease fertility or fecundity rates (Guillette and Edwards, 2008; Orton and Tyler, 2015). Third, in many species males and females can differ in their sensitivity to environmental stress, which can lead to sex-dependent mortality and thereby skewed sex ratios (Jones et al., 2009). The EU lists over 553 candidate substances as endocrine disruptor chemicals

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(EDC), which may alter the oestrogen, androgen, or thyroid hormone systems that are essential for normal embryonic development and reproductive activity (Orton and Tyler, 2015), and from this list 194 substances with endocrine-disrupting activity have been documented in at least one study of a living organism (Petersen et al., 2007). Through these effects, both global climate change and chemical pollution may endanger ecosystem health, so monitoring sex ratios in natural populations and investigating EDC effects in controlled experiments are important for conservation planning.

Amphibians are disappearing at a rate faster than any other vertebrate groups (Ceballos et al., 2015), and they are especially sensitive to chemical pollution because their skin is very permeable and they are exposed to agricultural, industrial, and household chemicals in both aquatic and terrestrial habitats (Orton and Tyler, 2015; Vitt et al., 1990). While their sexual development is vulnerable, i.e. easily disrupted by various environmental effects (Abdel-moneim et al., 2015; Flament, 2016; Orton and Tyler, 2015), investigation of these outcomes is hindered by the fact that sex identification in many amphibian species is often difficult. Sexual dimorphism, like larger body size in females (Shine, 1979), or secondary sexual characters like vocal sacs or nuptial pads or fangs (Sever and Staub, 2011) are, in many species, either non-existent or arise only at sexual maturity, which usually takes several years (Wells, 2010). EDC effects may be detectable much earlier than maturation (Orton and Tyler, 2015); however, due to the difficulty of sexing immature individuals, the majority of ecotoxicological studies identify phenotypic sex by sacrificing the animals and inspecting their gonads anatomically and/or histologically (e.g. Abdel-moneim et al., 2015; Griffing et al., 2017; Sharma and Patiño, 2010; Spolyarich et al., 2011). As the ultimate goal of such research is protection and conservation of the animals, there is also need to find non-destructive methods for phenotypic sexing. There exist a few less invasive methods, for example measuring the levels of sex hormones from blood, faeces or urine (Hogan et al., 2013; Narayan, 2013) or laparoscopy, i.e. inspecting the gonads through a small wound in anaesthesia (Rostal et al., 1994). A relatively novel approach for sex identification is the analysis of genetic markers from a sample taken non-invasively (e.g. a buccal swab), but this method is available for only a handful of species (Alho et al., 2010; Lambert et al., 2019; Tamschick et al., 2016b). All these non-destructive sexing methods can potentially be used on immature animals and sexually monomorphic species, but they are expensive and require special instruments and expertise. Furthermore, genetic sex does not necessarily equal phenotypic sex in amphibians due to the occurrence of sex reversals (Flament, 2016). For example in a common frog (*Rana temporaria*) population, 9% of genetically female adults expressed the male phenotype (Alho et al., 2010), whereas 2–16% of individuals was sex-reversed in 12 of 16 green frog (*Rana clamitans*) populations (Lambert et al., 2019).

A candidate method for non-invasive sex identification may be based on sexual dichromatism, i.e. differences in colour between the two sexes (Lifshitz and St Clair, 2016). In many amphibian species, adult males are more brightly coloured than adult females (Bell and Zamudio, 2012). Immatures usually appear monomorphic to the human eye, but subtle sexual dichromatism can develop before their first winter, e.g. in juvenile wood frogs (*Rana sylvatica*) males are yellower than females (Lambert et al., 2017a). A physiological link between sex and colour is suggested by further aspects of amphibian biology. Environmental factors that are known to affect sexual development such as temperature (Harkey and Semlitsch, 1988) and EDCs (Noriega and Hayes, 2000; Tamschick et al., 2016a) have been found to also influence skin colour in some cases. A few studies indicate that amphibian coloration can be controlled by sex steroids, e.g. testosterone and 17β-estradiol induced the development of adult male and female colour, respectively, in two frog species (Greenberg, 1942; Hayes and Menendez, 1999). Sex steroid receptors are present in the integument of amphibians (Sever and Staub, 2011), even in tadpoles' skin before metamorphosis (Chieffi et al., 1975). These results suggest that skin colour might indicate sex in immature individuals, even in species that seem sexually monochromatic to the human eye.

In this study, we investigated the possibility of sexing juveniles by skin colour in common toads (*Bufo bufo*). This species is abundant throughout Europe and in northern Asia, occupying various habitats including human-modified environments, so it is a useful potential indicator as it can be studied across a diversity of environmental gradients. Although it is listed in the "Least Concern" category of the IUCN Red List, its populations are potentially threatened by local habitat loss to urbanization, climate change, infections and water pollution (Aram et al., 2009). At first glance, the two sexes in common toads seem similarly brown, although a Russian field guide (Bannikov et al., 1977) and personal observations (Fig. S1) suggest that adult males have light greenish-brownish dorsal coloration whereas females are browner or reddish. Here our aim was to explore such sexual differences in toadlets before their first winter. We collected toad eggs from natural populations and reared the larvae in the laboratory until they reached the size and age for phenotypic sexing by dissection, and we quantified their dorsal skin coloration objectively from photographs. To investigate the role of environmental effects in the development of sexual dichromatism, we treated the tadpoles with one of two EDCs which had been reported to cause male-to-female sex reversal, intersex gonads, and female-skewed sex ratios: a glyphosate-based herbicide (Howe et al., 2004; Lanctôt et al., 2014) and 17α-ethinylestradiol (EE2), a contraceptive (Hayes, 1998; Tamschick et al., 2016b) that gets into natural water bodies mostly with treated wastewater, because the wastewater cleaning methods cannot eliminate it totally (Avar et al., 2016; Krantzberg and Hartley, 2018). Using these data, we examined the utility of colour based on human vision as a practical index for assessing sex ratios.

2. Material and methods

2.1. Experimental procedures

The animals used in this study were raised from eggs laid by 88 pairs of common toads. We captured the adults at 10 ponds in Hungary between 16 and 28 March 2017 and housed them in our laboratory for a few days until they spawned, as detailed in an earlier paper (Bókony et al., 2018). Throughout the study, lab temperature was 20 ± 1.55 °C and the photoperiod was set to follow the local light–dark cycles in nature. From each clutch we kept ca. 30 haphazardly chosen eggs in 0.5 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). Two weeks after spawning, when the larvae reached the free-swimming stage (developmental stage 25 according to Gosner, 1960), we selected 6 healthy-looking individuals from each family and moved each of them into a 2-L plastic box filled with 1 L RSW (one tadpole per box). The treatments (described below) began at this stage and lasted until each tadpole started to metamorphose. Throughout the treatment period, we changed the rearing water twice a week and fed the tadpoles with chopped and slightly boiled spinach *ad libitum*.

We used 4 EDC treatments and 2 controls. Within each family, we randomly assigned one tadpole to each of the 6 treatment groups; our starting sample size was 528. The control group was kept in clean RSW, whereas the solvent-control group's rearing water contained 1 μL/L ethanol. The former group served as control for the glyphosate treatments, in which a glyphosate-based herbicide formulation (Glyphogan® Classic; Monsanto Europe S.A., Brussels, Belgium; containing 41.5 w/w % glyphosate and 15.5 w/w% polyethoxylated tallowamine surfactant) was added to the rearing water to maintain a nominal concentration of either 3 μg/L or 3 mg/L glyphosate. The solvent-control group served as control for the EE2 treatments, in which the nominal concentration was either 1 ng/L or 1 μg/L EE2, obtained by dissolving EE2 powder (Sigma E4876) in 96% ethanol and adding 1 μL of this solution to each litre of

rearing water. Each treatment was renewed at every water change. Concentrations measured in 5 samples of rearing water per treatment validated that the actual concentrations were close to the nominal concentrations (Supplementary Material, Table S1). The EDC concentrations we applied are environmentally relevant: the lower concentrations represent typical levels while the higher concentrations are close to the maximum levels detected in natural water bodies (Avar et al., 2016; Bhandari et al., 2015; Bókony et al., 2018; Edwards et al., 1980).

When a tadpole started metamorphosis (i.e. appearance of forelimbs, developmental stage 42), we changed its rearing water to 0.1 L clean RSW (i.e. no chemical treatment) and slightly tilted the container to allow the animal to leave the water. When the individual completed metamorphosis (i.e. disappearance of the tail, developmental stage 46), we moved it into a clean rearing box, lined with wet paper towels and a piece of egg carton for shelter which were changed every two weeks. We fed the toadlets *ad libitum* with springtails and small crickets sprinkled with a 3:1 mixture of $CaCO_3$ and Promotor 43 multivitamin powder (Laboratorios Calier S.A., Barcelona, Spain). We raised the toadlets for ca. half a year after the metamorphosis, because at this age (i.e. before the first hibernation) their gonads are completely differentiated (Ogielska and Kotusz, 2004) and a study on wood frogs showed that skin colour becomes sexually dichromatic before the first winter (Lambert et al., 2017a).

Between 6th October and 10th November 2017, we sexed and photographed 412 toadlets as follows. By this time, our initial sample size of 528 decreased by 116 because 114 individuals died (most of them, n = 77, in the 3 mg/L glyphosate treatment) and 2 toadlets escaped from their boxes. We euthanized the toadlets by a one-hour immersion into a room-temperature water bath of 5.4 g/L MS-222 (Sigma E10521) buffered to neutral pH with the same amount of Na_2HPO_4 (Hadfield and Whitaker, 2005; Torreilles et al., 2009), in a white box similar to which they lived their entire life. We used a high concentration of MS-222 that is an effective and rapid method for euthanizing amphibians (Torreilles et al., 2009), because we wanted to minimize the time exposed to any stress associated with euthanasia. During euthanasia and photographing, the animals were kept at the same temperature as during their life. With this protocol, we aimed to minimize any effect that would change the toadlets' coloration. We photographed each animal as described in the next section (we did not take photos of younger animals like Lambert et al. (2017a) did, because our study was a first attempt to find out if there was any sexual dichromatism at all in juvenile toads). Then we determined their sex by dissection and identification of testes or ovaries under a stereomicroscope with 1-3× optical zoom. We categorized individuals with mixed-sex gonads (i.e. containing both male and female tissues based on the gross morphological observation of the gonads; Fig. 1) as intersex (Abdel-moneim et al., 2015; Sharma and Patiño, 2010; Spolyarich et al., 2011). 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 Ethical Commission of the Plant Protection Institute (ATK NÖVI).

2.2. Measuring skin coloration

Before the euthanasia, one researcher (N.U.) categorized each toadlet subjectively into one of seven groups based on their skin colour as black, green, brown, and four categories of red: reddish brown, brownish red, red, extremely red (Fig. S4). We used 4 categories of red, because the colour of the toadlets is usually brownish-reddish, so we expected the majority of variation along the brown–red continuum. The observer was unaware of the animals' sex. Although treatment group was included in each animal's identification code, the observer did not consider this information during colour scoring and had no presumption of what effect each treatment should have on skin coloration, so her categorizations were unbiased.

After the euthanasia and before the dissection we photographed each toadlet in a standardized setting to measure their dorsal skin coloration (Fig. S2). All photographs were taken with a Canon PowerShot SX50 HS digital camera, with a pixel count of 12 megapixels. We used manual settings for integration time and lens aperture (F-stop: f/5 with a shutter speed 1/13 s, ISO 80), macro mode without flash, 2-sec self-timer, and the white balance was set to 'fluorescent'. Photos were taken in a windowless room, lit only by an OSRAM L36W/965 BIOLUX fluorescent lamp (rated lamp efficacy 64 lm/W; colour temperature 6500 K; colour rendering index Ra ≥ 95). The camera was fixed on a tripod 50 cm above the toadlets, right below the lamp. Images were saved as uncompressed Canon Raw Version 2 (CR2) RAW image files, because the RAW format is 12-bit and allows less information loss than the JPG format (Stevens et al., 2007). Along with each toadlet we photographed a ColorChecker Classic mini card (X-Rite, Munsell Color Laboratories), which was used to standardize the reflectance values obtained from digital photographs (Myers, 2010; Plavcan, 2004).

We analysed the photos in Adobe Photoshop (version CS6, Adobe Systems Inc., San Jose, California, USA). To obtain the reference colour levels corresponding to each square of the ColorChecker chart, we converted one of our RAW images using the software of ColorChecker Passport into an Adobe Digital Negative (DNG) file (Bowman, 2016). Then we used the obtained colour profile in Camera Raw 10.3 plug-in for Adobe Photoshop. We used the Quick Selection Tool to cut the body from the photo (i.e. head and back without the legs), then we cut off a 5-pixel wide buffer band along the contours of the body to ensure that we did not select pixels belonging to the background. Then we used the Average Blur function to obtain the average colour of the pixels in this selected body region, and we used the Color Picker Tool to obtain the hue, saturation, and brightness values of this average colour (Lambert et al., 2017a). Hue represents the shade of the colour (values closer to 0° meaning more red while those closer to 60° meaning more yellow),

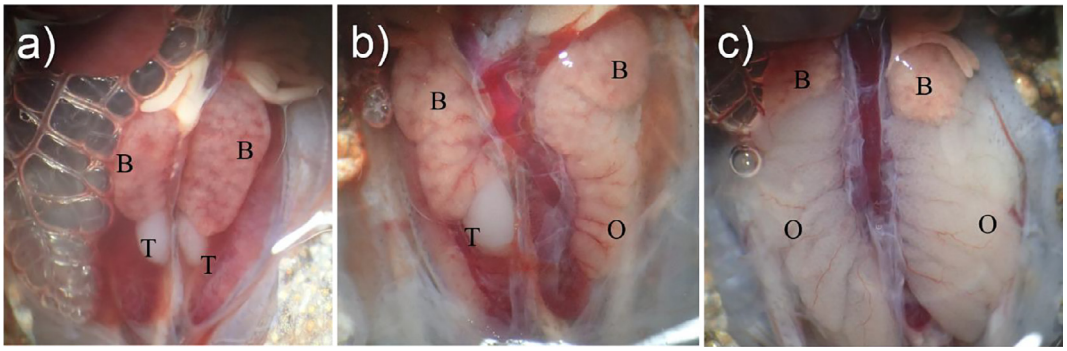

Fig. 1. Gonads of juvenile toads: a) normal morphology of testes (T); b) an intersex individual with one testis and one ovary; c) normal morphology of ovaries (O). Both sexes have a pair of Bidder's organ (B), an ovary-like formation with unknown function.

saturation represents the intensity of the colour (percentage of grey relative to hue; higher values meaning "more colourful"), and brightness represents the amount of light reflected by the colour (percentage of white). We chose these parameters because they quantify colours based on human perception, and ultimately our goal was to develop an easy method for toad sexing by human vision. However, these parameters can be relevant also for animal vision by correlating with the amount of various pigments that produce the coloration (McGraw et al., 2005; Saks et al., 2003).

2.3. Statistical analyses

All analyses were run in the R 3.5.0 computing environment (R Core Team, 2016), using the following packages: nlme (Jose et al., 2018), car (Fox et al., 2018), lsmeans (Lenth, 2016), MASS (Venables and Ripley, 2003), multcompView (Graves et al., 2015).

We used linear mixed-effects (LME) models to test whether each colour variable (hue, saturation, and brightness) differed across treatments and sexes. In each model, treatment (6 groups) and sex (3 groups) were used as fixed factors without interaction and family was used as random factor. We also considered capture site as a random factor but found it non-significant (see Supplementary Material, Table S2) and therefore excluded it from the models presented here (Zuur et al., 2009). Because graphical examination of the data showed heteroscedasticity, we allowed the variances to differ between sexes and treatment groups in all models using the 'varIdent' function (Zuur et al., 2009). Then we calculated linear contrasts (i.e. differences between group means) from the model estimates to compare the three sex categories with each other pairwise and to compare each treatment group to its respective control (pre-planned post-hoc tests; see Ruxton and Beauchamp, 2008). The significance level of these comparisons was corrected for the number of tests using the false-discovery rate (FDR) method (Pike, 2011).

We used a subset of the data to examine whether the interaction between sex and treatment had a significant effect on hue, saturation, and brightness. We excluded intersex animals and the 3 mg/L glyphosate treatment group, because the sample size in these groups was too low for testing the interaction effect. We also excluded the 1 μg/L EE2 treatment group because all animals in this group were females (see Results). With this reduced dataset, we used the same LME model structure as described above, but we also included the treatment × sex interaction and tested its significance with an analysis-of-deviance test (type-2 ANOVA). We used another subset to compare the hue, saturation and brightness between the 1 μg/L EE2 treatment group and the females of the solvent-control group, to test if skin colour differed between normal females and a group containing both normal females and male-to-female sex-reversed individuals (all animals in the 1 μg/L EE2 treatment group had ovaries, so we assume that there must be male-to-female sex-reversed individuals among them; see Results). We calculated this contrast from an LME model including only females, with treatment as the fixed effect, family as the random effect, and allowing the variance to differ among the 6 groups (Zuur et al., 2009).

We used a discriminant function analysis to test which colour variables contributed significantly to distinguishing between males and females. Discriminant analysis is a classification technique that generates a linear combination of variables which maximizes the probability of correctly assigning observations to pre-determined groups. We used sex as the response variable (excluding intersex individuals) and hue, saturation, and brightness as predictor variables (each mean-centred and divided by its standard deviation, to provide standardized coefficients).

We investigated whether the human eye can see the sexual dichromatism in toadlets, and whether the colours scored by human vision can be used to infer the sex ratio of groups. To this end, first we used LME models to test whether each colour variable was related to the colour score as seen by the human eye. In these models, the fixed effect was the subjective categories of skin coloration, the response variable was the objective colour variable (hue, saturation, or brightness), and we used family as a random factor. We compared pairwise the subjective colour categories with each other, correcting P-values with the FDR method.

To analyse the relationship between sex and human-scored colour, first we used binomial tests to investigate if sex ratio deviated from 1:1 in each subjective colour category. Then, we used a generalized linear mixed model with quasi-binomial error to estimate the probability of an individual being female in each subjective colour category, and to compare the sex ratios between the subjective colour groups. In this model, sex was the dependent variable, the toadlet's colour category was the predictor variable, and family was random factor. Because the results of these analyses suggested that the "green" and "extremely red" colours provided the most reliable information for assessing sex ratios (see details in Results), and these two colour groups were the most distinguishable based on hue (see Results), we chose these two colour groups for our next analyses. We used two approaches to test if group sex ratio can be predicted by the ratio of red and green individuals in each treatment group. First, we used binomial tests to investigate if the ratio of "extremely red" and "green" individuals deviated from 1:1 in each treatment group, and qualitatively compared these results with the results of binomial tests of the real sex ratios observed in each treatment group. Second, we used a generalized linear model with quasi-binomial error to investigate if differences between group sex ratios can be predicted by differences in the ratio of red and green animals. In this model, we used the proportion of females out of all sexable (i.e. not intersex) animals as the dependent variable, and the predictor variable was the percentage of "extremely red" individuals out of the sum of "green" and "extremely red" animals in each group. Each data point (i.e. group) was weighted by sample size (i.e. the sum of males and females).

2.4. Validation of the colour measurement method

In 2019 we performed a follow-up study to validate two aspects of our colour measurement methods. For this study we used toadlets that were raised for the purpose of another experiment, applying similar protocols and lab conditions as in 2017 (without EDC treatments). We reared the animals for two and a half months after metamorphosis (four months after the start of tadpole development), because we found in a similar study in 2018 that gonad development at this age is already sufficient for phenotypic sexing (Bókony et al., 2020). We used these toadlets to address the reproducibility of subjective colour scoring and the effect of euthanasia on skin colour, as follows.

First, to test the reproducibility of categorizing toadlet colour by the human eye, we asked 14 volunteers (11 women, 3 men) to score the skin coloration of 310 toadlets; 13 observers were naïve to this task. We used a modified set of categories, for the following reasons. First, our findings in the 2017 study showed that hue ranging from green to red was the most important aspect of coloration in discriminating between the two sexes (see Results). Second, our experience in 2017 revealed that it was difficult to separate five categories between brown and red (e.g. only 60 out of 412 animals were categorized as reddish brown or brownish red by our original scoring system). Third, we also found in 2017 that some animals would be best categorized between brown and green. Therefore, we switched to the following six subjective colour categories: green, greenish, brown, black, reddish, and red. Each observer scored each animal once, and we tested the reproducibility between observers using the method of Culp et al. (2018), as implemented in the "ordinalRR" package of R. This method is an extension of the model of de Mast and Van Wieringen (2010), which assumes that observers classify the test subjects into ordinal categories along a continuum of a latent property (in our case, "true colour"), and observers may differ from each other in their category boundaries (i.e. the cut-off value of "true colour" between neighbouring categories). For this

analysis, we converted our colour categories into ordinal scores representing a continuum from green to red (1 = green, 2 = greenish, 3 = brown or black, 4 = reddish, 5 = red). We treated our 14 observers as a random sample from a larger population of observers, and estimated their cut-off values using the Bayesian approach of Culp et al. (2018) and the default settings of "ordinalRR". We calculated reproducibility as the proportion of matches between observer pairs averaged over all toadlets (equation 25 in Culp et al., 2018). Additionally, to assess the utility of the 14 observers' scores for predicting toadlets' sex by their colour, we ran a linear model with quasi-binomial error for each observer, using toadlet sex as dependent variable and the colour scores from 1 to 5 as numeric predictor variable. Sex was identified by dissection after colour assessments, using the same euthanasia protocol as in 2017. We note here that because the colour categories were different, this follow-up study cannot directly validate the usefulness of colour categories used in the 2017 experiment. However, our aim was to evaluate, in more general, whether different observers categorize the same objects similarly, and whether such subjective scores predict toadlet sex.

Second, we tested the effect of euthanasia on skin coloration. Although temperature, light and substrate background were standardized during the experiments and euthanasia, it is possible that handling stress or death changed the colour of the animals (Kindermann et al., 2013). We made two photographs of each of 110 toadlets dissected in 2019. We took the first photo right before putting them in the MS-222 solution, when they were still alive, and the second photo one hour later when they were dead. We measured their objective skin colour variables the same way as in 2017. To test if death changed coloration, we compared the hue, saturation and brightness between live and dead animals using paired t-tests. We used LME models to test if the sex differences that we found in 2017 can be detected in the live animals as well. Our dependent variables were hue, saturation and brightness, our independent variables were the status of the individuals (alive or dead) and sex (excluding one intersex individual) and their two-way interaction, and we used animal ID as a random factor. We tested the significance of the interaction with an analysis-of-deviance test (type-2 ANOVA) to assess if sexual dichromatism differed between live and dead animals. Then we calculated linear contrasts from the model estimates to compare the two sexes before and after death.

3. Results

3.1. Sex ratios in EDC treatment groups

The ratio of male and female toadlets did not deviate significantly from 1:1 in the control group or in any of the two glyphosate treatments (Table 1). Although there were almost twice as many females as males in the 3 mg/L glyphosate treatment (Table 1), the power of detecting skewed sex ratio in this group was low because of small sample size due to high mortality (87.64%). While the sex ratio was slightly female-biased in the solvent-control group, it did not deviate significantly from 1:1 in the 1 ng/L EE2 treatment, but 100% of animals in the 1 μg/L EE2 treatment became females (Table 1). We found 1 and 3 intersex individuals, respectively, in the 1 ng/L EE2 and 3 μg/L glyphosate treatment groups (Table 1).

Table 1
The number of male, female, and intersex individuals in each treatment group. P-values show whether the group's sex ratio (i.e. proportion of females among non-intersex individuals) differed significantly from 1:1 according to binomial tests. Each treatment group started with 88 tadpoles. Total sample size at dissection was 412 toadlets.

Treatment	Male	Female	Intersex	P
Control	46	36	0	0.320
3 μg/L glyphosate	39	35	3	0.728
3 mg/L glyphosate	4	7	0	0.549
Solvent control	30	48	0	0.054
1 ng/L ethynilestradiol	35	46	1	0.266
1 μg/L ethynilestradiol	0	82	0	< 0.001

Table 2
Sex differences in objective colour variables measured from photos (n = 412 toadlets).

Dependent variable	Contrast	Estimate ± SE	t	P
Hue	male – intersex	3.93 ± 0.84	4.68	< 0.001
	male – female	1.99 ± 0.25	7.67	< 0.001
	intersex – female	−1.94 ± 0.85	−2.28	0.023
Saturation	male – intersex	5.02 ± 1.71	2.92	0.010
	male – female	1.99 ± 0.25	0.28	0.778
	intersex – female	−1.94 ± 0.85	−2.73	0.010
Brightness	male – intersex	2.09 ± 0.76	2.75	0.019
	male – female	0.69 ± 0.30	2.29	0.033
	intersex – female	−1.39 ± 0.77	−1.81	0.071

Linear contrasts (differences between means) were estimated from linear mixed-effects models with sex and treatment as fixed factors and family as random factor; df = 317. P-values were corrected with the FDR method.

3.2. Sexual dichromatism in toadlets

The LME models showed that the colour variables differed significantly between the sexes (Table 2, Fig. 2). Skin coloration had higher hue and higher brightness in males than in females, whereas saturation was similar in the two sexes (Table 2, Fig. 2). The higher hue values of males correspond to a greenish-yellowish brown whereas the lower hue values of females correspond to a deep reddish brown (Fig. S2). Intersex individuals had significantly lower hue values, lower saturation, and lower brightness than males and females, although the difference in brightness between females and intersex individuals was marginally non-significant (Table 2, Fig. 2). Neither the treatments' main effects nor the treatment × sex interaction was significant for any of the three colour variables (Table 3, Table 4, Fig. S3). The all-female group of 1 μg/L EE2 treatment did not differ from the females in the solvent-control group in hue (linear contrast: b ± SE = 0.132 ± 0.420, t_{161} = 0.314, P = 0.754), saturation (b ± SE = 0.218 ± 1.187, t_{161} = 0.184, P = 0.854) or brightness (b ± SE = 0.346 ± 0.468, t_{161} = 0.741, P = 0.460, Fig. S3).

The discriminant analysis produced one highly significant discriminant function (Wilk's λ = 0.897, P < 0.001), in which the standardized coefficient of hue (−1.02) was much larger than that of saturation (0.11) and brightness (−0.09). Using this function, the toadlets could be classified as males or females with an overall accuracy of 65.7% (53 out of 154 males, 34% and 215 of 254 females, 84% correctly classified); 88.5% of sexable (i.e. not intersex) toadlets with hue ≤25 were females (Table S3).

3.3. Scoring by human vision

Variation among animals in all three objective colour variables was detected by the human eye (Fig. 3). Hue was significantly higher in the "green" group than in all other subjective colour groups (P ≤ 0.005; Fig. 3). For saturation, the "black" group had significantly lower values, and the "red" and "extremely red" groups had significantly higher values than all the other groups (P < 0.001; Fig. 3). Similarly, for brightness the "black" group had significantly lower values and the "extremely red" group had significantly higher values than all the other groups (P < 0.001; Fig. 3). Investigating whether the subjective colour categories can provide information about sex, we found that the "green" group had significantly male-biased sex ratio, while significantly female-biased sex ratio was found in the "extremely red" and "black" groups (note, however, that the sample size was very small in the latter group), and there was a trend for female bias in the "brown"

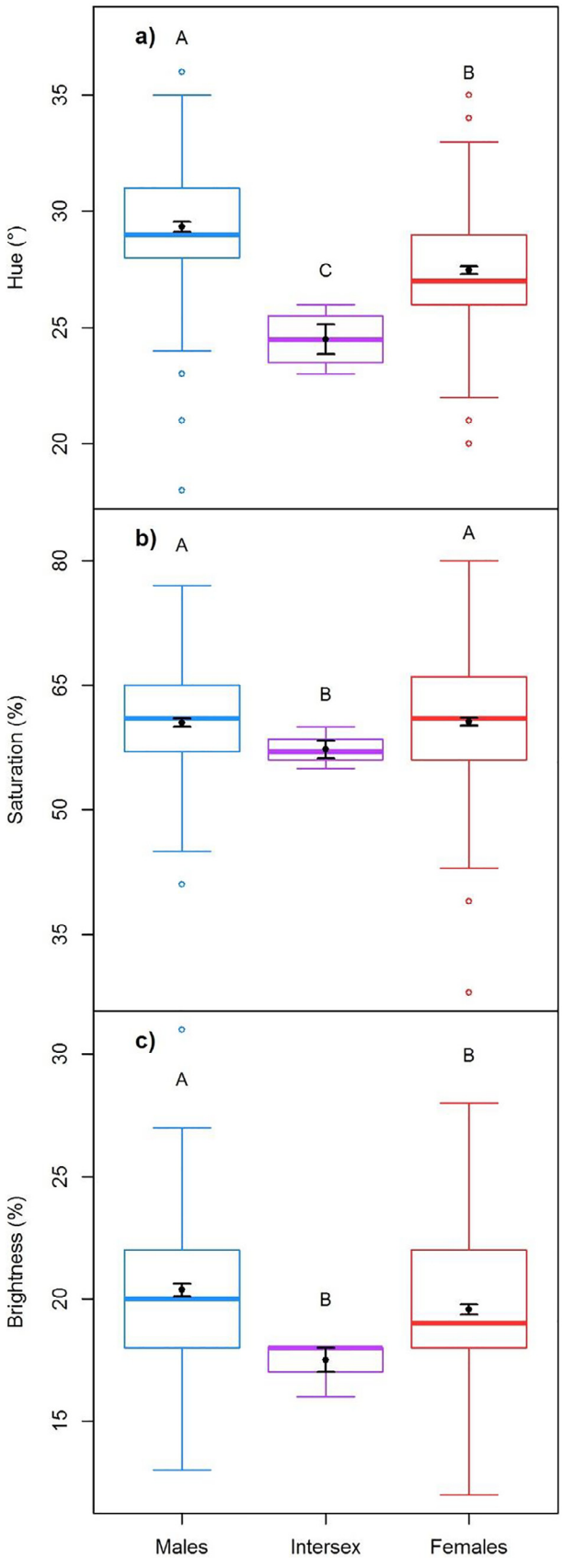

Fig. 2. Dorsal skin coloration of toadlets (a: hue, b: saturation, c: brightness) in relation to sex. Error bars show the mean ± SE values; boxplots show the distribution of data (thick middle line: median, box: interquartile range; whiskers extend to the most extreme data points within 1.5 × interquartile range from the box). Groups marked by the same letters do not differ from each other significantly (P > 0.05 after FDR correction).

Table 3
Differences between treatment groups in objective colour variables measured from photos (n = 412 toadlets).

Dependent variable	Contrast*	Estimate ± SE	t	P
Hue	SC – C	0.09 ± 0.36	0.26	0.795
	g – C	0.22 ± 0.35	0.64	0.750
	G – C	−0.32 ± 0.61	−0.53	0.750
	e – SC	−0.23 ± 0.35	−0.66	0.750
	E – SC	0.19 ± 0.37	0.52	0.750
Saturation	SC – C	1.17 ± 0.86	1.35	0.443
	g – C	0.45 ± 0.82	0.55	0.581
	G – C	−2.61 ± 1.74	−1.50	0.443
	e – SC	−0.58 ± 0.99	−0.59	0.581
	E – SC	0.62 ± 1.04	0.60	0.581
Brightness	SC – C	−0.11 ± 0.42	−0.28	0.837
	g – C	−0.08 ± 0.40	−0.21	0.837
	G – C	−1.16 ± 0.89	−1.30	0.744
	e – SC	0.29 ± 0.42	0.71	0.798
	E – SC	0.44 ± 0.42	1.04	0.744

Linear contrasts (differences between means) were estimated from linear mixed-effects models with sex and treatment as fixed factors and family as random factor; df = 317. P-values were corrected with the FDR method.

* Treatment groups are abbreviated as C: control, SC: solvent control, g: 3 μg/L glyphosate, G: 3 mg/L glyphosate, e: 1 ng/L EE2, E: 1 μg/L EE2.

Table 4
ANOVA tables of the LME models testing the effect of the treatment × sex interaction on the three objective colour variables, with family as random factor.

Dependent variable	Model terms	χ^2	df	P
Hue	Sex	55.56	1	< 0.001
	Treatment	1.42	3	0.701
	Sex × Treatment	0.72	3	0.869
Saturation	Sex	0.56	1	0.455
	Treatment	2.03	3	0.565
	Sex × Treatment	2.76	3	0.430
Brightness	Sex	6.59	1	0.010
	Treatment	0.40	3	0.940
	Sex × Treatment	1.05	3	0.790

Intersex individuals and the 3 mg/L glyphosate treatment group were excluded because the sample size in these groups was too low. The 1 μg/L EE2 treatment group was also excluded, because all these animals were females.

group (Table 5). The probability that an individual is female was significantly lower in the "green" group than in every other subjective colour category; it was highest in the "extremely red" and "black" groups (Table 5). Based on these results, we chose "green" and "extremely red" as the potentially most informative categories for inferring sex ratios.

Focusing on "green" and "extremely red" individuals, we found that their ratio deviated from 1:1 significantly in the 1 μg/L EE2 treatment group but not in any other treatment groups (Table S4). This result is qualitatively identical to what we found with the real sex ratios (Table 1). We found a significant positive relationship between the proportion of females and the ratio of "extremely red" and "green" individuals across the six treatment groups (binomial model: b ± SE = 0.077 ± 0.024, P = 0.031, Fig. 4). This relationship was not exclusively due to the female-only group, because the result did not change qualitatively when we excluded the 1 μg/L EE2 treatment group (b ± SE = 0.033 ± 0.007, P = 0.022). In groups with > 50%

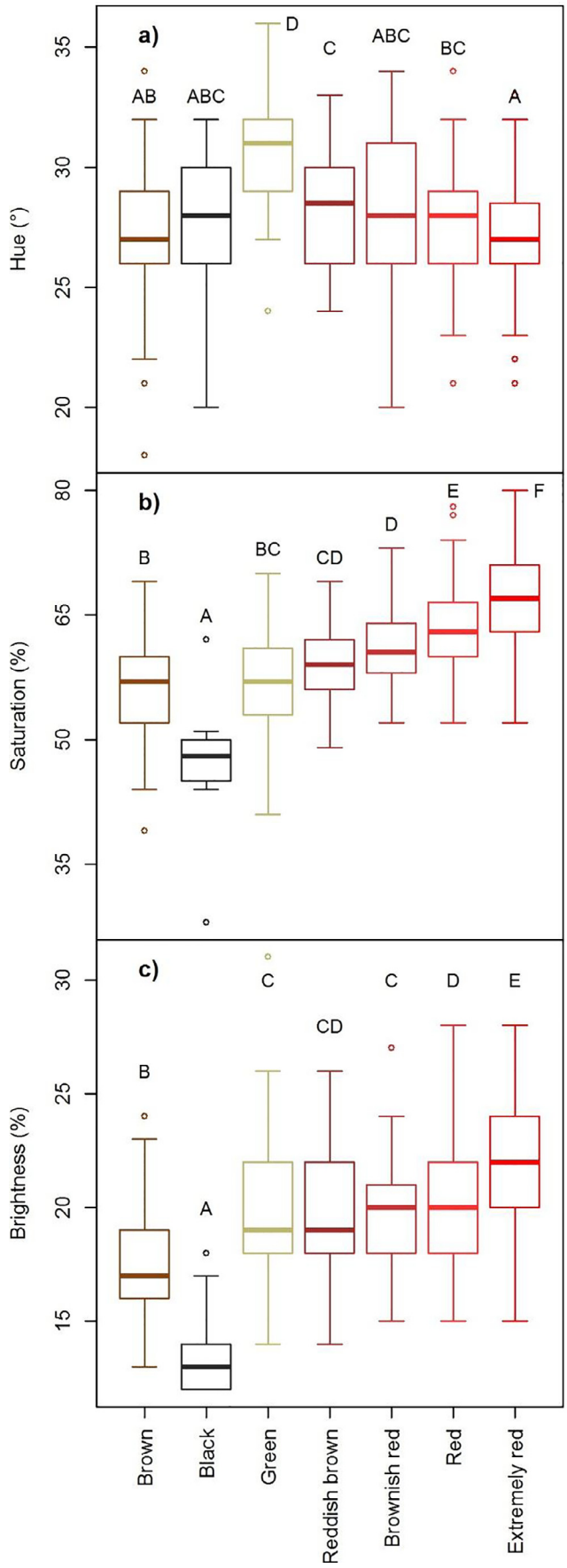

Fig. 3. Dorsal skin coloration of toadlets (a: hue, b: saturation, c: brightness), measured objectively from photos, in relation to their coloration categorized subjectively by human vision. Groups marked by the same letters do not differ from each other significantly ($P > 0.05$ after FDR correction). In each boxplot, the thick middle line and the box show the median and the interquartile range, respectively; whiskers extend to the most extreme data points within 1.5 × interquartile range from the box.

Table 5
Number of males, females, and intersex individuals assigned into each subjective colour category. "P (♂:♀)" shows the P-value from binomial tests that analyse if the group sex ratio deviates from 1:1; "♀ probability" gives the predicted probability of being female in each subjective colour group, as estimated from a quasi-binomial model. Asterisks indicate significant differences from the "green" group (*P < 0.05, **P < 0.01, ***P < 0.001).

Skin colour	Male	Female	Intersex	P (♂:♀)	♀ probability
brown	25	55	2	0.064	0.687***
black	1	8	0	0.039	0.889*
green	56	29	0	< 0.001	0.341
reddish brown	6	15	1	0.238	0.714**
brownish red	13	25	0	1	0.658**
red	32	51	1	0.902	0.614***
extremely red	21	71	0	0.006	0.772***

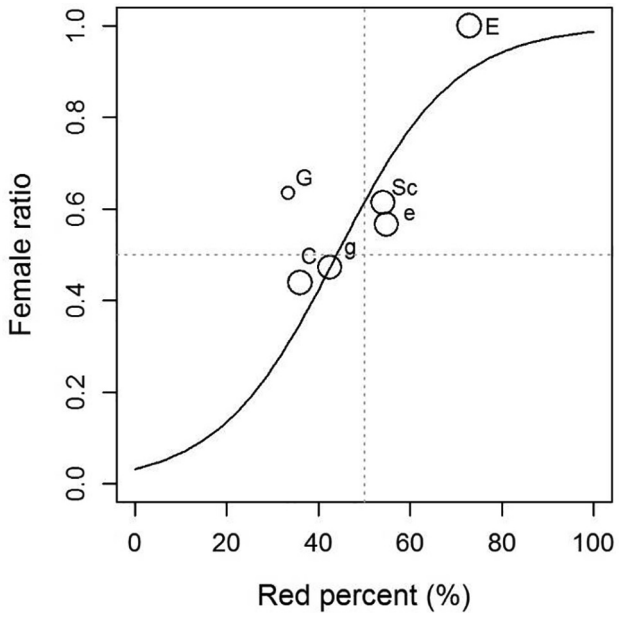

Fig. 4. Sex ratio (proportion of females among non-intersex animals) in relation to the proportion of "extremely red" individuals among "extremely red" and "green" toadlets across the six treatment groups (C: control, Sc: solvent control, g: 3 μg/L glyphosate, G: 3 mg/L glyphosate, e: 1 ng/L EE2, E: 1 μg/L EE2). The size of the circles is proportional to $\log_{10}$(sample size). The solid line was fitted from a quasi-binomial model; the dotted lines indicate the 1:1 sex ratio and 1:1 ratio of red and green toadlets.

females, there were more red than green individuals, whereas the opposite was true for the groups with > 50% males (Fig. 4), excepting the 3 mg/L glyphosate treatment group in which sample size was very small (Table 1). Neither "green" nor "extremely red" animals occurred among intersex individuals (Table 5).

3.4. Validation of the colour measurements

The estimated boundaries between subjective colour categories showed considerable variation among the 14 observers: the cut-off values between neighbouring categories were misaligned (Fig. S5).

Reproducibility was 0.47 when calculated as the proportion of perfect matches; it was 0.87 when we allowed for mismatches between neighbouring categories (e.g. one observer scoring "red" and another scoring "reddish" was accepted as a match). For all 14 observers, the relationship between colour scores and toadlet sex showed an increasing likelihood of being female as the scores increased from green to red (logit slope ranging from 0.095 to 0.437); this relationship was significant ($P < 0.05$) for 9 observers and marginally non-significant ($0.05 < P < 0.095$) for 3 observers (Fig. S5).

We found that the hue values increased significantly after euthanasia (paired *t*-test: $t_{109} = 3.63$, $P < 0.001$), but the sex difference was not changed significantly by death (non-significant sex × status interaction, Table S5): females had significantly lower hue values than males both before and after euthanasia (Table S6). Saturation decreased significantly after euthanasia ($t_{109} = 9.51$, $P < 0.0001$), but it did not differ between sexes either before or after death (Table S5, Table S6). Brightness did not change significantly after death ($t_{109} = 1.03$, $P = 0.302$), but the sex difference did (significant sex × status interaction, Table S5): males had significantly higher brightness values than females when alive; the direction of this difference was the same after death but became reduced and non-significant (Table S6).

4. Discussion

Our study has provided four main findings. First, we have detected significant colour differences between male and female toadlets with objective photographing methods. Second, these differences were also visible to the human eye, such that differences in group sex ratio were predicted by the ratio of green and red individuals. Third, two EDCs did not affect coloration *per se*, but larval treatment with 1 μg/L EE2 resulted in 100% female toadlets with normal female coloration. Fourth, intersex individuals occurred in both treatments with low EDC concentrations, and their objective colour variables differed from those of both males and females. We discuss each of these findings in detail below.

Using objective measurements from photographs, we found sexual dichromatism in half-year old common toads: males were yellower and brighter while females were redder and darker. Our follow-up study showed that these differences are more prominent in live animals than after euthanasia, and already detectable in two-months old toadlets. Although the majority of known cases of sexual dichromatism in anurans develop at sexual maturity or during the breeding season (Bell and Zamudio, 2012), our results show that dichromatism can appear even before the first hibernation, as has been found also in another species (Lambert et al., 2017a). In many dichromatic species, the male colour is yellower and/or brighter than the female colour (Bell and Zamudio, 2012; Lambert et al., 2017a), suggesting that this kind of dichromatism may be widespread in frogs and toads perhaps due to female sensory bias or developmental constraints. In the common toad, we found that sex differences in hue and brightness were significant after euthanasia in half-year old toadlets, but only in hue and not in brightness in two-months old toadlets, although this difference might have been due to the smaller sample size of the latter. In the future it would be worth to find the earliest time when dichromatism becomes reliably detectable, to minimize the rearing time needed for getting colour data.

Our results show that these objective colour differences can be detected by human vision, as toadlets seen "green" had higher hue and those seen "extremely red" had higher brightness than all other colour groups, and toadlets seen "green" had the lowest probability of being female. We found that different observers ranked the toadlets consistently from green to red, but they differed in the threshold values they used to break up the continuous scale of colour variation into subjective colour categories. Allowing for mismatches due to this variation in thresholds, we found good between-observer reproducibility (87%) in subjective colour scoring. All 14 observers tended to assign more red scores to female toadlets, and this relationship was significant or close to significant in almost all observers, without any training before colour scoring. These findings suggest that, even though individual observers do not necessarily agree if a toadlet is brown or reddish, they often agree which out of any two toadlets is redder. Also, their scores agree in showing that redder toadlets are more likely to be female.

We found that skin coloration by itself does not allow confident identification of toadlet sex at the individual level, because only 66% of juveniles could be correctly sexed based on the hue (and to a lesser extent, brightness and saturation) of their dorsal skin from photographs, and males were less readily identifiable (34%) than females (84%). Similarly, when categorized by human vision, ca. 30% of toadlets in the male-dominated "green" category and in the female-dominated "extremely red" category belonged to the opposite sex. Due to this relatively large uncertainty of individual sexing by colour, the sex ratio of any single population cannot be quantitatively estimated by colour either. Thus, this approach cannot be used to accurately identify the phenotypic sex of individual toadlets nor of cohort sex ratios. However, the information that there is a statistically significant relationship between sex and colour can be useful in certain situations, as we explain below.

First, skin colour might be a useful indicator combined with other phenotypic traits. For example, the colour differences we observed may be due to the distribution of pigment cells such as xanthophores and erythrophores that are responsible for yellow and red coloration, respectively, while melanophores modulate darkness (Bell and Zamudio, 2012). It is possible that the density of pigment cells may differ between male and female skin and might be measurable by skin biopsy (although this method may be more expensive or complicated, and more invasive than photographing, it might perhaps provide more accurate information on sex). Additionally, other aspects of sexual dimorphism may also become detectable before sexual maturation, as has been found for toxin gland size in juvenile toads (Chen et al., 2017). It would be worth investigating if secondary sexual traits like forearm size or head shape differ not only in adults but also in immatures. Combining such traits with colour scores into an indicator set might enable more reliable phenotypic sexing than each indicator alone.

Second, our results indicate that judging skin coloration by the naked eye may facilitate qualitative comparisons of sex ratios between natural populations or experimental groups. In our experiment, there was good correlation between the experimental groups' sex ratios and the ratio of red and green toadlets in each group (except for a group with very small sample size): the ratio of red to green individuals increased as the proportion of females in the group increased. Furthermore, the ratio of red to green individuals deviated significantly from 1:1 only in the single group in which the sex ratio also deviated strongly and significantly from 1:1. Thus, such subjective categorization may be a cheap and fast method for identifying populations which have skewed sex ratios compared to others, e.g. a high ratio of red toadlets may be an indicator of strong female bias relative to other populations. This method may be useful in monitoring of natural populations for conservation management and in field or lab projects that aim to assess and/or manipulate sex ratios without applying invasive sex-identification procedures (e.g. longitudinal ecotoxicological studies, captive breeding programs or wildlife conservation programs). For example, a change over time in the ratio of green and red toadlets in a single population, or a difference between a polluted site and a reference site in the ratio of green and red toadlets may indicate a difference in sex ratio, which could serve as a basis for further research or conservation action. Such comparisons within each study should be done using the colour scores of a single observer, due to the differences in colour perception between individual humans. However, we caution that the uncertainty of the sex-colour relationship does not allow for assigning sex to an individual or a sex ratio value to a toadlet group; it only provides information about the probability of being female or having female-

biased sex ratio. When interpreted carefully, keeping the method's inaccuracy in mind, this information can be valuable when no other information on sex is obtainable.

Sex ratio did not deviate from 1:1 in any of the treatment groups except for the 1 μg/L EE2 treatment and a marginally significant female bias in the solvent control group. The latter might be due to natural fluctuation within the limited sample of 78 surviving individuals, or perhaps the very low concentration of ethanol (0.000001 v/v%) might have affected sex. In the future it would be worth testing the EDC impacts of very low ethanol concentrations, because ethanol is often used as solvent in ecotoxicological experiments. Although there were almost twice as many females than males in the 3 mg/L glyphosate treatment group, this sample was too small for powerful statistical analysis because survival was very low (4 males and 7 females). The high mortality rate was probably caused by the polyethoxylated tallowamine (POEA) additives that are present in many glyphosate-based herbicides (Howe et al., 2004; Lanctôt et al., 2014; Rissoli et al., 2016) like the formulation we used. Nevertheless, glyphosate itself might have endocrine disruptor effects at relatively high concentrations, as indicated by previous studies that found female-biased sex ratios and higher incidence of intersex in treatments with glyphosate or its formulations (Howe et al., 2004; Lanctôt et al., 2014). In low concentrations, neither the glyphosate-based herbicide nor EE2 skewed the sex ratios of our toadlets, which is in accordance with previous findings in amphibians (Pettersson and Berg, 2007; Tamschick et al., 2016b). However, in the 1 μg/L EE2 treatment group, all the surviving 82 toadlets developed into females (i.e. had ovaries), which means that this concentration caused sex reversal in 100% of genetically male individuals, as reported in other species (Tamschick et al., 2016b). The coloration of toadlets did not differ significantly between the 1 μg/L EE2 treatment group (including sex-reversed individuals, i.e. genetic males that developed into females) and normal females (in the solvent-control group). This result has two implications. First, because EE2 is a synthetic hormone that mimics the effects of endogenous oestrogens, our result suggests that the development of toad skin colour is regulated by sex hormones, similarly to what was found in other anuran species, (Greenberg, 1942; Hayes and Menendez, 1999). Second, it suggests that skin colour in common toads may only indicate phenotypic sex but not genetic sex or sex reversal; developing genetic sex markers will be needed to address this question. The EDC treatments themselves had no effect on coloration within each sex, implying that skin colour does not indicate EDC exposure directly, but it may indicate endocrine disruptor effects indirectly *via* altering gonad development and thereby probably the levels of sex hormones.

In both low-concentration treatment groups we found a very low percentage of intersex individuals, which did not occur in either control groups, similarly to the results of previous studies on EE2 and glyphosate-based herbicides (Howe et al., 2004; Sharma and Patiño, 2010; Tamschick et al., 2016b). The coloration of the intersex individuals was significantly different from the normal males and females: they had less saturated and darker skin. Although their hue was redder, they appeared pale brown or reddish-brown to the human eye. Because of our small sample size of intersex individuals, we cannot assess the utility of skin colour as a phenotypic marker for intersex, but we urge future studies into this question. Such a marker would be very useful because intersex individuals have been found in nature in several amphibian species, especially in anthropogenic habitats (Griffing et al., 2017; Orton and Tyler, 2015; Skelly et al., 2010), and we know nothing about their reproductive success and their effects on population viability, because so far intersex could only be detected by dissection (Orton et al., 2014).

Interestingly, in our study, the parents of all four intersex toadlets were captured from urban ponds (four families from three different ponds; see Bókony et al., 2018). Although we did not examine the histology of toadlet gonads, our finding is in accordance with the higher incidence of abnormal testes (containing testicular oocytes) in suburban and urban ponds that was observed in green frogs (Skelly et al., 2010), although this was not confirmed by another, larger study (Lambert et al., 2019). Because our toadlets were raised in the lab from eggs, and animals originating from different habitats were exposed to the same chemicals, it is possible that parental exposure to anthropogenic environments made the offspring susceptible to abnormal sexual development which might have been amplified by our low-concentration EDC treatments (Orton and Routledge, 2011). Such low concentrations often occur in natural waters (Avar et al., 2016; Bhandari et al., 2015; Edwards et al., 1980; Kumar et al., 2016), so intersex and other gonadal abnormalities may become more and more frequent with human-induced environmental change.

Taken together, we have found sexual dichromatism in juvenile common toads that is detectable objectively from photographs as well as by human vision. Our results show that skin colour cannot be used as an unambiguous indicator of an individual's sex or as a direct proxy of a population's sex ratio. However, our findings suggest that counting the number of greenish and reddish individuals can provide qualitative information on phenotypic sex ratios, thus this can be a cheap, fast and non-invasive method for identifying populations or experimental groups that may have skewed sex ratios relative to others. Furthermore, our findings suggest that skin coloration might also indicate abnormalities of sexual development (intersex). We propose that the utility of sexing by colour is worth exploring in other species as well, and combining coloration with other phenotypic markers may be a fruitful approach for developing indicator sets for identifying the sex of each individual reliably. Replacing destructive or invasive sexing methods with non-invasive indicator sets will help the conservation of wild populations.

Authors contributions

NU and VB designed the study and raised the animals. NU performed dissections and colour measurements. NU and VB analyzed the data and wrote the manuscript.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ecolind.2020.106175.

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