

# Asymmetrical sex reversal: does the type of heterogamety predict propensity for sex reversal?

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## Abbreviations

EE2: 17 $\alpha$ -ethynylestradiol; DHT: dihydrotestosterone; E2: 17 $\beta$ -estradiol

## Abstract

Sex reversal, a mismatch between phenotypic and genetic sex, can be induced by chemical and thermal insults in ectotherms. Therefore, climate change and environmental pollution may increase sex-reversal frequency in wild populations, with wide-ranging implications for sex ratios, population dynamics, and the evolution of sex determination. We propose that re-considering the half-century old theory "Witschi's rule" should facilitate understanding the differences between species in sex-reversal propensity and thereby predicting their vulnerability to anthropogenic environmental change. The idea is that sex reversal should be asymmetrical: more likely to occur in the homogametic sex, because sex-reversed heterogametic individuals would produce new genotypes with reduced fitness. A review of the existing evidence shows that while sex reversal can be induced in both homogametic and heterogametic individuals, the latter seem to require stronger stimuli in several cases. We provide guidelines for future studies on sex reversal to facilitate data comparability and reliability.

## Introduction

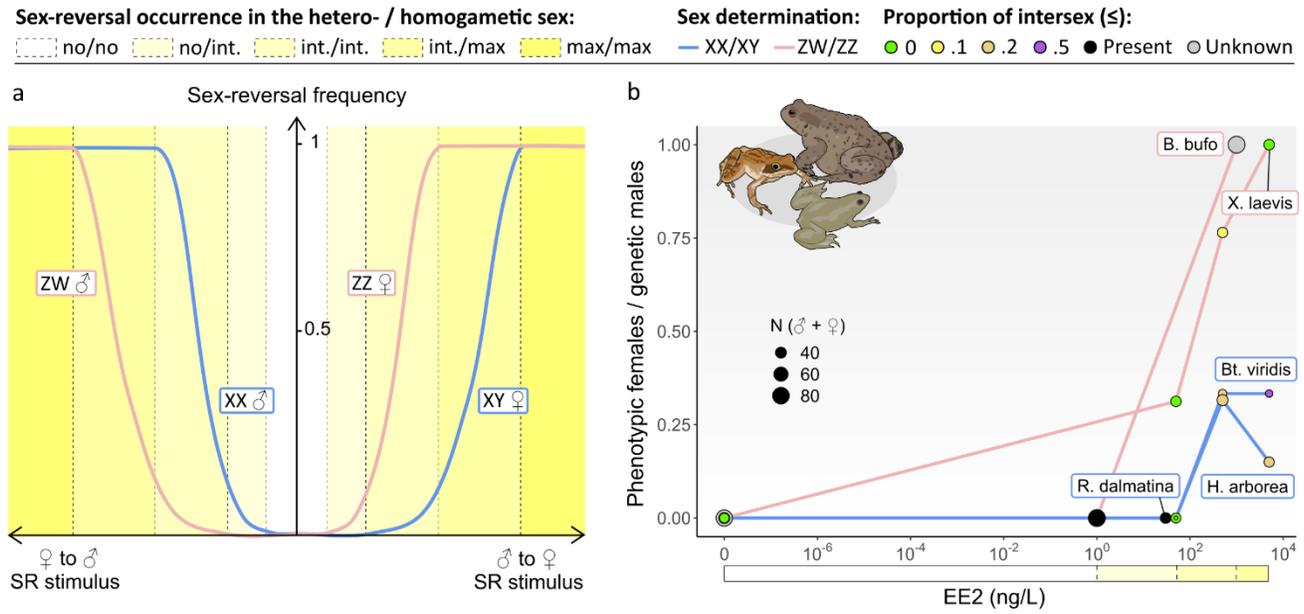
Ectothermic vertebrates feature a variety of sex-determination systems including both genetic and environmental types <sup>[1-3]</sup>. The most widespread variants of genetic sex determination are male-heterogametic (XX/XY) and female-heterogametic (ZW/ZZ) systems, displaying a variety of, often

homomorphic, sex chromosomes. Across ectotherm vertebrates, certain environmental conditions, including temperature and the presence of several chemical compounds, can favor the development of one phenotypic sex, even in species possessing genetic sex determination. This leads to sex reversal, producing genetic males with female phenotype or *vice versa*. Sex reversal has been documented in wild populations of all major ectothermic vertebrate taxa <sup>[2,4-8]</sup>. Theoretical models predict that global climate change and environmental pollution is expected to increase sex-reversal frequency in these taxa, potentially affecting sex-chromosome evolution, causing skewed sex ratios and even leading to extinction <sup>[1,9-12]</sup>. Therefore, understanding mechanisms behind the evolution of sex reversal is an important and urgent challenge. Theoretical works have shown that male- and female-heterogametic systems might respond differently to environmental changes *via* sex reversals <sup>[10,11,13]</sup>, but there is a significant knowledge gap yet to be filled with empirical data.

If sex-reversed individuals participate in breeding, new combinations of the sex chromosomes can emerge in their progeny (YY or WW). These new genotypes may possess reduced fitness due to degeneration of the genetic content of the hemizygous chromosome <sup>[13,14]</sup>, driven by accumulation of deleterious mutations <sup>[15-17]</sup> and sex-antagonistic genes <sup>[18,19]</sup> (but see <sup>[20]</sup> and <sup>[21]</sup>). These new genotypes can only be produced if the sex-reversed parent is heterogametic (XY female mating with XY male, or ZW male mating with ZW female). Thus, reduced fitness of the new genotype may lead to selection against sex reversal in the heterogametic sex. About sixty years ago, based on experiments applying exogenous sex hormones to a few amphibian species, Witschi and colleagues <sup>[22]</sup> recognized that it was predominantly the homogametic sex (XX or ZZ) that was susceptible to sex reversal (a concept sometimes referred to as Witschi's rule). Roughly twenty years later, Adkins-Regan <sup>[23]</sup> came to a similar conclusion based on reviewing data from fish, amphibians, reptiles and further taxa. However, laboratory experiments successfully produced both sex-reversed XY females and ZW males across ectotherms, and these were even fertile in some species <sup>[24-28]</sup>. Therefore, some authors see Witschi's rule as disproved <sup>[29,30]</sup>, while others maintain that sex reversal is restricted to the homogametic sex, acknowledging that there are counterexamples with no explanation <sup>[2,13,31]</sup>. Clarifying this issue empirically would be important for understanding which species are susceptible to sex reversal induced by specific environmental stimuli: Witschi's rule predicts higher vulnerability to male-to-female sex-reversing effects such as xenoestrogens in ZW/ZZ compared to XX/XY systems. By contrast, XX/XY systems should be more inclined to female-to-male sex reversal.

The apparent contradiction between Witschi's rule and empirical findings may be resolved by acknowledging that the propensity for sex reversal may vary on a gradual scale (Fig. 1a) and may be shaped by various factors. In ectotherm vertebrates, different sex-determination systems dynamically replace each other as species evolve <sup>[32]</sup>, and sex reversal of homogametic individuals may be an important driver of these transitions <sup>[13]</sup>. Degeneration of the Y or W chromosome, and thus the strength of selection for restricting sex reversal to the homogametic sex, should gradually increase with the age of the sex chromosomes. Therefore, heterogamety-based differences might be less prominent in younger sex-determination systems. Furthermore, resilience to certain external factors may have physiological limits, and consequently, increased exposure to these factors might lead to sex reversal despite the system's relative resistance to it (Fig. 1a). Comparison of sex-determination systems is further complicated by the possibility of phylogenetic inertia in sex-reversal sensitivity to different environmental conditions: for example, high temperature may cause sex reversal in either genetic females or males depending on the phylogenetic lineage <sup>[1,9,24,33]</sup>. Thus, the aim of the present article is to draw attention to the variation in sex-reversal propensity across species, and to the importance of unraveling the role of sex-chromosome systems in driving that variation. We propose that, for understanding the ecology of sex reversal (i.e. when and where does it occur and why), a relaxed

interpretation of Witschi's rule should be considered. By this relaxed interpretation, sex-reversal inducibility in homogametic and heterogametic individuals is not a matter of "yes or no", because a continuum of sex-reversal resistance is expected to occur in nature, such that in XX/XY systems, milder stimuli are enough for female-to-male sex reversal and stronger stimuli are required for male-to-female sex reversal, whereas the opposite should hold for ZW/ZZ systems. We refer to this idea as 'asymmetrical sex reversal' (borrowing this phrase from an earlier paper [13] which used it for describing Witschi's rule).



**Figure 1. Sex reversal in XX/XY and ZW/ZZ systems.** Panel **a**: Under ‘asymmetrical sex reversal’, response curves to the same sex-reversing (SR) stimuli may be shifted towards the opposite direction in the two systems, i.e. the homogametic sex is expected to be more susceptible to sex reversal than the heterogametic sex. Three response categories are indicated: no sex reversal (no), intermediate sex-reversal frequency (int.), or all individuals of the affected genetic sex undergo sex reversal (max). Panel **b**: Genetically confirmed sex reversal caused by EE2 treatment of tadpoles is in agreement with the theoretical expectations. On panel **b**, correspondence to the theoretical comparison of homogametic and heterogametic sexes from panel **a** is shown by the colored stripe under the X axis. Dot sizes are proportional to the number of animals with unambiguous sexual phenotype. Displayed anuran genera are: *Bufo* (B.), *Bufotes* (Bt.), *Hyla* (H.), *Rana* (R.) and *Xenopus* (X.). For data and references for Panel **b** see Supplementary Table 1.

### Empirical evidence for ‘asymmetrical sex reversal’ in nature

If sex reversal is asymmetrical between sex-chromosome systems, we should expect that in free-living populations mostly female-to-male sex reversal should occur in XX/XY systems, while male-to-female sex reversal should be predominant in ZW/ZZ systems. This is supported by the currently available, limited data on wild populations: genetically proven female-to-male sex reversal was frequent in four anuran species with XX/XY systems (and only rare male-to-female sex reversal occurred in one of them; [4–7]), while sex-reversal frequency was negligible in *Bufo bufo*, the only ZW/ZZ anuran with such data to our knowledge [34]. This latter finding is especially interesting because female-to-male sex reversal was frequent in *Rana dalmatina* (XX/XY) in the same habitats, so the lack of sex reversal in these *Bufo bufo* populations are not attributable to lack of female-to-male sex-reversing stimuli [34].

The pattern is similar in the two reptile species for which genetically confirmed sex reversal has been studied in the wild: XX males were found in *Acritoscincus (Bassiana) duperreyi* (XX/XY) and ZZ females in *Pogona vitticeps* (ZZ/ZW) across several free-living populations<sup>[33,35,36]</sup>. The picture is less clear in fishes<sup>[8]</sup>; however, sex-reversal research in fish has so far focused on its aquaculture aspects, and the genetic sex markers developed for captive populations (i.e. altered by artificial selection or genetic drift) are not always reliable in wild populations<sup>[8,37]</sup>. For example, because rare mutations or recombination events may cause mismatches between sexual phenotype and the genotype identified by genetic sex markers, individuals mismatching based on a single sex marker do not always represent environmental sex reversal<sup>[38]</sup>. Therefore, although *in situ* sex-reversal frequencies in both amphibians and reptiles with genetic sex determination conform to the theory of ‘asymmetrical sex reversal’, the most conclusive comparisons of sex-reversal propensity between XX/XY and ZW/ZZ systems may be made by controlled experiments that manipulate environmental conditions during sex determination.

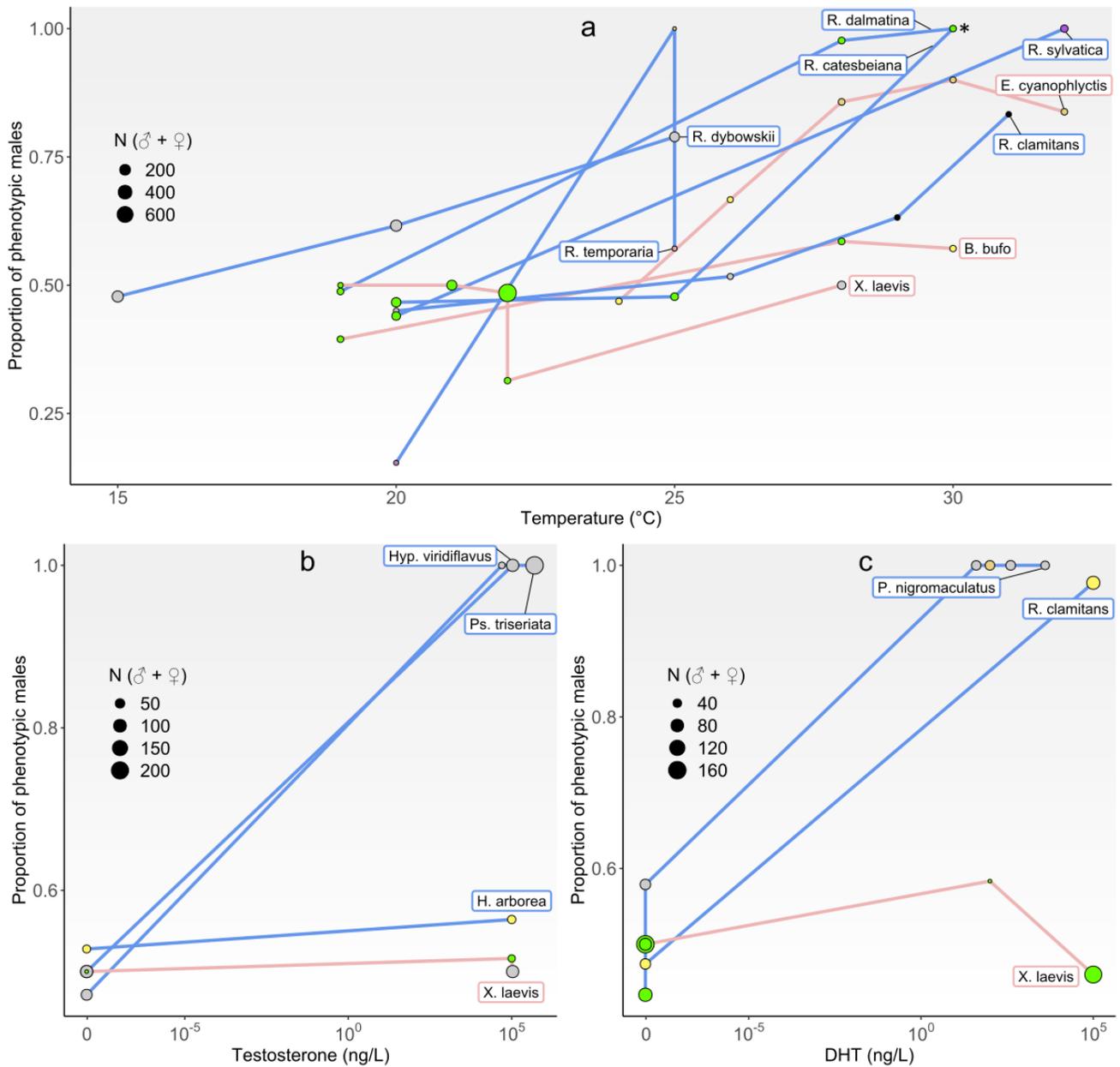
### Experimental evidence for ‘asymmetrical sex reversal’

Anuran amphibians, the taxon in which Witschi has originally discovered his rule, offers an ideal group for testing ‘asymmetrical sex reversal’, because genetic sex determination underlies sexual development in all anuran species studied so far, unlike in fishes and reptiles where many species seem to have temperature-dependent sex determination with little genetic influence<sup>[39–41]</sup>. We searched the literature for sex-reversal and phenotypic sex-ratio data from laboratory experiments that were carried out on anuran species with either XX/XY or ZW/ZZ sex-determination system, focusing on the sex-reversing effects that were most often studied in this regard: developmental temperature, sex hormones, and anthropogenic chemicals with endocrine-disrupting effects. From the latter group, we chose the two compounds that have been studied most frequently: the contraceptive 17 $\alpha$ -ethynylestradiol (EE2) and the herbicide atrazine<sup>[12]</sup>. Detailed searching methods are described in Supplement 1, and the data extracted in Supplementary Table 1. We found only four experiments in which anuran species with both male and female heterogamety were studied for sex-reversal propensity<sup>[30,42–44]</sup>, although heterogamety was not in their focus. Other studies were usually restricted to a single species. Experimental methods differed greatly across studies, including the applied concentrations of the same compounds as well as water temperature. Because genetic sex markers have been established for only a handful of amphibian species so far (e.g. <sup>[4–7,34]</sup>), in the vast majority of studies sex reversal was inferred based on biased phenotypic sex ratios produced by specific treatments. Several relevant experimental conditions, such as treatment duration or mortality rates, were unclear in numerous instances, especially among broadly-cited publications from the previous century<sup>[45–47]</sup>. For these reasons, we judged that formal meta-analyses would be unfeasible with the currently available, highly heterogeneous data.

Using established genetic sex markers, sex reversal caused in anurans by different concentrations of EE2 administered in the rearing water has been tested in two species with ZW/ZZ, and three species with XX/XY system (Fig. 1b). The reported sex-reversal frequencies are in agreement with the theory of ‘asymmetrical sex reversal’: genetic males became phenotypic females in ZW/ZZ species at lower EE2 concentrations compared to XX/XY species. We found no treatments other than EE2 for which genetically proven sex-reversal rates were published for both ZW/ZZ and XX/XY species. In the species *Glandirana rugosa*, different populations feature different sex-determination systems<sup>[48]</sup>. Using a variety of sex hormones, sex reversal could be induced only in ZZ individuals in the ZW/ZZ populations, while it was absent in the population with heteromorphic X and Y chromosomes, and it occurred in both XX and XY individuals in populations with homomorphic sex chromosomes<sup>[48]</sup>. All these data on proven cases of sex reversals support that the homogametic sex has higher propensity to undergo sex reversal, especially when the two sex chromosomes are more diverged. Although a study

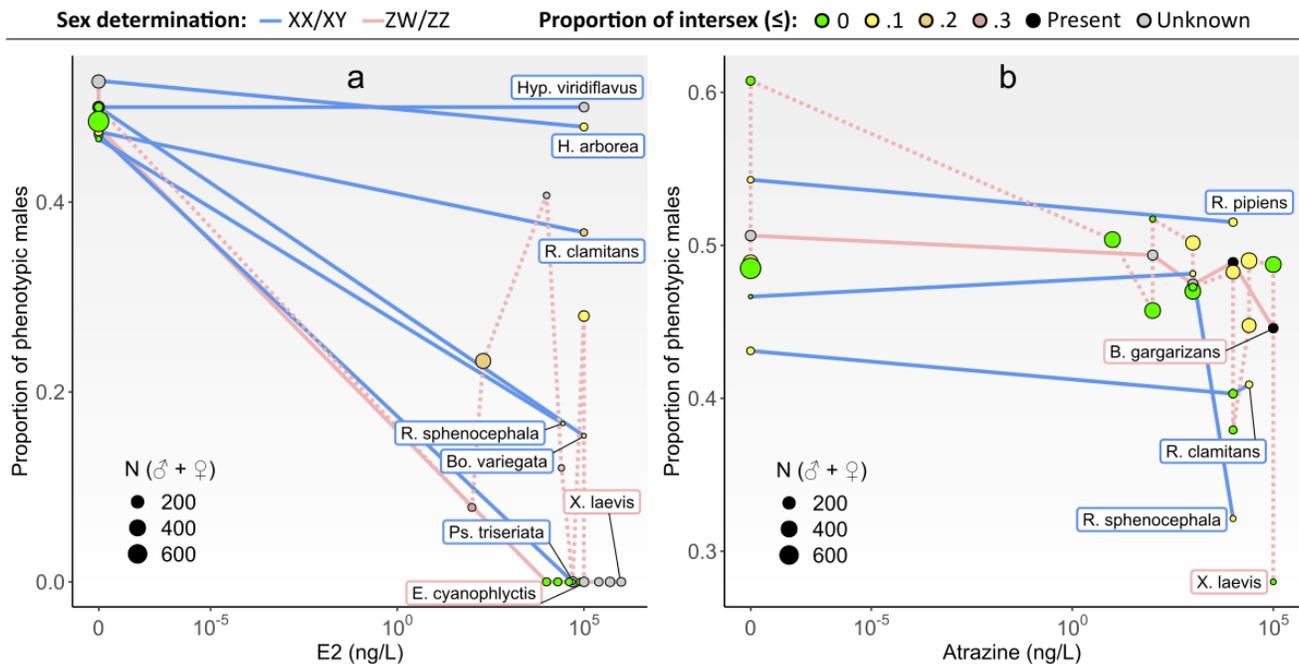
on reptiles <sup>[49]</sup> concluded that sex-chromosome heteromorphy does not constrain the sensitivity to sex reversal, this conclusion was based on the finding that high doses of E2 injected into the eggs caused 100% female phenotype in two turtle species with XX/XY sex determination, regardless of their sex chromosomes being heteromorphic or homomorphic. Low sample sizes and the lack of genetic sexing both limit the interpretation of these results.

Sex determination: — XX/XY — ZW/ZZ Proportion of intersex (≤): ● 0 ● .1 ● .2 ● .3 ● .4 ● .5 ● .6 ● Present ○ Unknown



**Figure 2. Phenotypic sex ratios (i.e. proportion of males among individuals with unambiguous sexual phenotype) reported from anurans exposed to different temperatures (a), or different concentrations of testosterone (b) or DHT (c) as tadpoles.** Within each panel, dot sizes are proportional to the number of animals with unambiguous sexual phenotype; dot colors indicate the proportion of intersex individuals among all animals examined for intersexuality. Displayed anuran genera are: *Bufo* (B.), *Euphlyctis* (E.), *Hyla* (H.), *Hyperolius* (Hyp.), *Pelophylax* (P.), *Pseudacris* (Ps.),

*Rana* (R.) and *Xenopus* (X.). Two overlapping data points are marked by an asterisk: the proportion of intersex individuals at 30°C was 0 in *Rana dalmatina* and 0.56 in *Rana catesbeiana* (a). For data and references see Supplementary Table 1.



**Figure 3. Phenotypic sex ratios reported from anurans exposed to different concentrations of E2 (a) or atrazine (b) as tadpoles.** Within each panel, dot sizes are proportional to the number of animals with unambiguous sexual phenotype; dot colors indicate the proportion of intersex individuals among all animals examined for intersexuality. Displayed anuran genera are: *Bombina* (Bo.), *Bufo* (B.), *Euphlyctis* (E.), *Hyla* (H.), *Hyperolius* (Hyp.), *Pseudacris* (Ps.), *Rana* (R.) and *Xenopus* (X.). For better visualization, data of *X. laevis* are connected with a dotted line. For data and references see Supplementary Table 1.

Lacking data on genetically confirmed sex reversals in most species, tentative speculations can be made based on phenotypic sex ratios (Figs 2, 3). Out of 18 anuran species for which we found data on the effects of water temperature, sex hormones, or atrazine on sex ratios, only four featured ZW/ZZ sex-determination system. Treatments with testosterone, dihydrotestosterone (DHT) and high temperature caused sex-ratio bias towards males, but complete or near-complete elimination of phenotypic females ( $\geq 98\%$  males) at higher treatment values was achieved only in XX/XY species (Fig. 2). Treatments with 17 $\beta$ -estradiol (E2) and atrazine tended to cause sex-ratio bias towards females, and ZW/ZZ species produced the strongest responses: only ZW/ZZ species reached 100% female sex ratios for E2 (excepting a single XX/XY species, *Pseudacris triseriata*) and high female bias ( $<30\%$  males) for atrazine (Fig. 3). Majority of the studies we overviewed accounted for the presence of intersex individuals, although the definition of intersexuality differed between articles: in general, it included individuals with one ovary and one testis, or gonads with mixed-sex tissue based on either gross morphology or histology. The proportion of intersex individuals can vary greatly between and within species (Figs. 1b, 2 and 3), sometimes even exceeding 50% of the treated individuals; many of these cases are likely signs of incomplete sex reversal and might indicate limited sex-reversal ability in the genetic sex affected by the applied treatment. However, intersex and sex reversal may also occur

independently of each other <sup>[5,50]</sup>, as intersex may be a natural phase of gonad development in some species of amphibians as well as fish <sup>[12,51]</sup>.

Sex-ratio data suggest that some species might be less susceptible to sex reversal compared to others with the same sex-determination system. For example, while phenotypic sex ratio in two other XX/XY species (*Pseudacris triseriata* and *Hyperolius viridiflavus*) was strongly affected by exogenous testosterone treatment, it was not distorted in *Hyla arborea*, the third such species, by 100 000 ng/L, twice the concentration that already caused 100% male phenotype in *Pseudacris triseriata* (see Fig. 2b). Similar heterogeneity was found for fishes where methodological differences across studies accounted for much more inter-specific variation in sex reversal inducibility than biological differences <sup>[37]</sup>. Furthermore, even within-species differences can occur in apparent sex-reversal inducibility: for the best-studied species, *Xenopus laevis*, sex ratios observed after similar treatments greatly differed between studies (e.g. <sup>[52]</sup> vs. <sup>[53]</sup>). Such differences may stem from discrepancies in the experimental set-up, sample size, or other methodological details (see below). Thus, while the patterns in Figs 1b, 2 and 3 are largely in agreement with the idea that the homogametic sex is more susceptible to sex reversal, there is also noise in these patterns, and understanding the sources of this variation would be important for understanding what makes certain animals more susceptible than others to sex reversal.

### **The devil in the details: how to choose suitable methods?**

In order to enable systematic comparison of the responsiveness of different sex-determination systems to sex-reversing effects, future studies should apply the same experimental design in both XX/XY and ZW/ZZ species concurrently. This will minimize the risk that differences between species are confounded by uncontrolled differences in the circumstances (such as varying relationships between nominal and actual treatment concentrations; Supplementary Table 1). Ideally, such experiments should include multiple treatments within the range of ecologically relevant concentrations or temperatures, to facilitate the recognition of ranges where sex-reversal inducibility differs between the two sex-determination systems (see Fig. 1). Once we have enough data from such experiments, quantitative meta-analyses of the within-experiment differences will be executable to ascertain whether the type of heterogamety is a consistent determinant of sex-reversal propensity.

Even when it is not possible to include more than one species in an experiment on sex reversal, there is much researchers can do to make future findings more directly comparable among each other and clearer to interpret. We should endeavor to identify sex reversal correctly. When the conclusions are drawn solely from phenotypic sex ratios, it should be born in mind that such conclusions can be strongly affected by sex-biased mortality <sup>[50,54]</sup> and stochasticity stemming from low sample sizes. Therefore, mortalities and sample sizes should always be clearly reported. Preferably, sex-reversed individuals should be identified by genetic sexing <sup>[42,50,54]</sup>, and for this, development of genetic sex markers for those many thousands of species where such markers are not yet available is an inevitable challenge.

When designing sex-reversal experiments and reporting the data, several methodological aspects should be considered explicitly. Different species can have very different pace of ontogeny and the sensitive period to sex-reversing effects can also vary between them <sup>[30,48,50,55]</sup>. Therefore, treatment periods should include the time frame when sex reversal may be induced in each species to be compared. To ensure this, we need data on the timing of the sensitive window of each species. When such information is lacking, exposure to the sex-reversing treatment should either last for a long period during embryonic/larval development or applied in several different, shorter periods. However, we should also keep in mind that shorter treatments may be more environmentally relevant when applying

some sex-reversing stimuli such as heat waves <sup>[56,57]</sup>, while longer treatments may better simulate natural conditions with others such as persistent chemical pollutants. Another issue of timing is the diagnosis of phenotypic sex. In sex-reversal experiments, phenotypic sex is usually identified based on gonad morphology of dissected young animals, e.g. at or shortly after metamorphosis in amphibians. The timing of dissection may significantly influence the results of sexing, because gonads in several amphibian and fish species undergo an ovary-like phase before differentiating into ovaries or testes, and the pace of this process also differs between species <sup>[3,58–60]</sup> or even within the same species <sup>[61]</sup>. Furthermore, the relative pace of gonadal and somatic development may vary between species and treatments <sup>[62,63]</sup>; thus, treatment effects on somatic development (e.g. earlier metamorphosis at high temperatures) may lead to premature dissection and thereby sex assignment may be false <sup>[12]</sup> or impossible (undifferentiated gonads: Supplementary Table 1). Therefore, for phenotypic sexing to be reliable, it should be performed at a sufficiently late age, which is usually well after metamorphosis in amphibians <sup>[62]</sup>.

Because temperature can affect sexual development <sup>[7,24,50]</sup>, experiments on chemically induced sex reversal should also pay attention to rearing temperatures. On one hand, different species may adapt to different temperatures <sup>[64]</sup>; thus, keeping the animals within their range of optimal temperatures is favored to prevent unexpected sex reversals or the above-mentioned methodological problems of sex-biased mortality and premature dissection. On the other hand, temperature may affect the solubility, uptake and degradation rate of the administered chemicals and ultimately their effects on sex <sup>[50,65,66]</sup>. Therefore, rearing temperatures should be monitored, taken into account, and reported even when temperature effects are not the focus of the experiment. Different species and even populations within species might differ in their sex-reversal propensity regardless of their sex-determination system. Local or species-specific adaptations in various traits may be developed to better survive and exploit conditions that vary across habitats, such as temperature <sup>[64]</sup> or anthropogenic chemical pollution <sup>[67]</sup>; similar adaptations might also increase or decrease the likelihood of sex reversal <sup>[34]</sup>. Therefore, the source of the experimental animals, such as the climatic and land-use conditions of the collection sites or the specificities of the used breeding stocks (e.g. in *Xenopus*), should be clearly described in sex-reversal studies. When the experiments include both ZW/ZZ and XX/XY species, ideally these should be collected from the same sources or from similar habitats in order to improve the comparability of the two systems' response to specific sex-reversing conditions.

Also, earlier information may need revisiting and updating. For example, we recently found that sex ratio in the toad *Bufo bufo* (ZW/ZZ) was not affected by exposure to 30°C, a temperature high enough to cause up to 100% female-to-male sex reversal in the frog *Rana dalmatina* (XX/XY) in the same experiment <sup>[44]</sup>. This contradicted the broadly cited study by Piquet <sup>[46]</sup>, where 25°C produced male excess in *Bufo vulgaris* (a synonym for *Bufo bufo*). The contradiction can be resolved by realizing that Piquet captured her animals near Geneva, in a hybrid zone of *Bufo bufo* and *Bufo spinosus* <sup>[68]</sup>, two species that were thought to be one at the time <sup>[69,70]</sup>. Notably, *Bufo spinosus* features XX/XY system <sup>[71]</sup>. Thus, it is likely that the study of Piquet <sup>[46]</sup> is incorrectly cited in several reviews (e.g. <sup>[3,29,31]</sup>) as evidence for temperature-induced sex reversal in *Bufo bufo*, and her findings potentially reflect female-to-male sex reversal induced in homogametic XX instead of heterogametic ZW individuals. With recent developments in molecular methods and resulting increase in the quantity and quality of data on taxonomy, sex chromosomes, and genetic sex markers, the time is now ripe for clarifying the role of heterogamety in sex-reversal susceptibility across ectothermic vertebrates.

## Conclusions

Heterogamety is a fundamental aspect of organismal biology that, according to recent research, has far-ranging consequences on life histories and population dynamics, including sex ratios, sex-specific aging rates and life spans<sup>[72–74]</sup>. Here we have highlighted that heterogamety may further influence the fate of ectothermic vertebrates by affecting their propensity to undergo environmental sex reversal. By considering ‘asymmetrical sex reversal’, a relaxed interpretation of Witschi’s rule, we can generate testable predictions regarding the differences in sex-reversal propensity between populations with different sex-chromosome systems induced by different environmental conditions. Empirical tests of these predictions are promising but so far scanty and difficult to integrate due to methodological heterogeneity behind the currently available results. Still, multiple findings suggest that in taxa like anurans and fish where high temperatures usually cause female-to-male sex reversal<sup>[1,3,9]</sup>, climate change and urban heat islands may potentially pose greater risk to XX/XY compared to ZW/ZZ systems. By contrast, ZW/ZZ species may be more vulnerable to several chemical pollutants that can induce male-to-female sex reversal. Therefore, more research on sex reversal is needed in order to assess the vulnerability of ectotherms to both climate change and environmental pollution. We hope that, by considering the methodological guidelines that we have provided in the present article, future studies will enable a systematic comparison of sex-reversal propensities in different sex-chromosome systems, and help to better understand the evolution of sex determination and its consequences in the Anthropocene.

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## Author Contributions

The main concept of this manuscript was conceived by EN. Literature data were collected and the manuscript was written by EN and VB.

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**Conflict of Interest**

The authors declare no conflict of interest.

**Supporting Information**

Detailed searching methods are described in Supplement 1. Detailed references and the collected data on sex reversal, sex bias and sex determination are shown in Supplementary Table 1.