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# Evolutionary ecology of anthropogenic environmental change and sex reversal in amphibians

MTA DSc thesis

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Budapest
2022

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## *I. Introduction*

Our world is increasingly affected by human-induced environmental change. An estimated 77% of Earth's terrestrial habitats have already been modified by the direct effects of human activities such as agriculture and urbanization, and the remaining pristine habitats are also disappearing at a high rate (Watson *et al.*, 2018). The anthropogenic changes in land use are accompanied by a multitude of abiotic and biotic changes, including habitat fragmentation, altered biogeochemical cycles, and shifting of flora and fauna towards lower diversity and more introduced species (Alberti *et al.*, 2017; Johnson & Munshi-South, 2017; Turcotte *et al.*, 2017). Both urban and agricultural land use loads the environment with various chemical pollutants such as pesticides, heavy metals, and road deicers, which are accompanied in urban areas by noise, artificial light at night, and disturbance posed by the presence of humans and their pets (Mann *et al.*, 2009; Seress & Liker, 2015). Many of these anthropogenic environmental changes are stressful to wild animals, exposing them to unpredictable or uncontrollable stimuli that threaten their homeostasis or survival, while some species can exploit the novel resources offered by human-modified habitats and thrive there (Bonier, 2012). Besides land-use conversion, another pervasive form of human-induced environmental change is the contemporary, rapid change of climatic conditions, which includes both rises in average temperatures and an increasing frequency of extreme weather events such as heat waves and droughts (IPCC, 2014; Spinoni *et al.*, 2015; Tomczyk & Bednorz, 2019). These climatic changes are exacerbated by land-use conversion, specifically by the urban heat island effect. Mainly due to heat storage in buildings and sealed roads, built-up areas are significantly warmer than the surrounding rural areas, and heat waves can be more deadly in cities (Li & Bou-Zeid, 2013; Rizvi *et al.*, 2019). Understanding the phenotypic and genetic adaptations by which organisms cope with all these anthropogenic challenges is of paramount importance for current research in ecology, evolutionary biology, and conservation.

Starting around the turn of the millennium, a surge of studies documented wide-ranging effects of human-induced environmental changes on the morphology, physiology, behavior and life history of wild organisms (Seress & Liker, 2015; Alberti *et al.*, 2017; Johnson & Munshi-South, 2017; Turcotte *et al.*, 2017). Some of these phenotypic changes are maladaptive and contribute to population declines, while some are adaptive and help living in anthropogenic environments (Alberti *et al.*, 2017). Adaptive changes may come about by two, not mutually exclusive processes: phenotypic plasticity within individuals, and changes in population composition ("persistent effects", as opposed to phenotypic plasticity) due to genetic differentiation by natural selection (microevolution) or other trans-generational effects such as epigenetic variation (Liker, 2020). The role of plastic and persistent mechanisms in phenotypic divergence between populations can be tested with common garden (or reciprocal transplant) experiments, whereby individuals from different populations are reared in the same environment (De Villemereuil *et al.*, 2016). Such experiments, increasingly aided by genomics and transcriptomics, have revealed several cases that suggest adaptive changes by one or more of the above mechanisms in wild populations in human-modified habitats (Cothran *et al.*, 2013; Johnson & Munshi-South, 2017; Liker, 2020). In most cases, however, there is yet insufficient knowledge to identify the mechanisms responsible for human-induced phenotypic changes, although such knowledge would be crucial for predicting and dealing with the eco-evolutionary consequences of anthropogenic environmental change.

One animal group at particular risk from human activities is amphibians. This class of vertebrates is of serious conservation concern because they are disappearing at an alarming rate: 41% of amphibian species are threatened by extinction and almost 50% show population declines (IUCN, 2021). The suspected reasons for these declines are linked to anthropogenic impacts such as habitat degradation, climate change, and infectious diseases (Orton & Tyler,

2015; Campbell Grant *et al.*, 2016). Because of their biphasic life, amphibians not only play important roles in both freshwater and terrestrial ecosystems but are also exposed to environmental changes in both habitats. Their limited dispersal abilities make them vulnerable to habitat loss and fragmentation. Due to their shell-less eggs, aquatic larvae, and permeable integument, they are especially sensitive to chemical contaminants and drought. As ectothermic animals, they lack the metabolic, physiological, and anatomical mechanisms that would allow them to maintain constant body temperatures. Despite all these well-known features that render amphibians particularly endangered by climate change, land-use conversion, and chemical pollution, the thousands of extant amphibian species are under-represented in relevant research fields such as ecotoxicology and the evolutionary ecology of anthropogenic environmental change (Hoffman *et al.*, 2003; Hamer & McDonnell, 2008; Falcón *et al.*, 2020; Sordello *et al.*, 2020). The work presented here was undertaken in order to contribute to filling this knowledge gap, by investigating the phenotypic and genetic differences of amphibians in association with human-induced environmental change and the potential mechanisms behind those differences.

This dissertation is based on twelve research papers[1] whose main findings are presented in the following ten chapters. The first seven chapters form two series of empirical case studies performed with anuran amphibians in the field and in the laboratory, whereas the last three chapters consist of two theoretical models and a review of an updated hypothesis. While all these studies were conducted with a focus on amphibians, their findings are relevant on a broader taxonomic scale, as the phenomena and mechanisms being tested pertain not only to amphibians but also to other animals with similar vulnerabilities to the various forms of anthropogenic environmental change, especially reptiles and freshwater fishes, but to some extent other vertebrates or other ectothermic species as well. While I was the supervising leader in all the studies described here, each of them was realized in collaboration with other researchers, so the first-person plural is used throughout the text.

For the case studies with anurans, we sampled small water bodies in Hungary (**Table I.1**) with standing or slowly moving water, which are the typical habitats during spawning and larval development for the amphibian species in this region. The young after metamorphosis and the adults outside the mating season inhabit the surrounding woody areas, usually within a few hundred meters of their natal pond (Reading *et al.*, 1991; Ponsero & Joly, 1998). Based on the land-use characteristics of the area around each pond, we categorized each of our sampling locations into one of three categories of habitat type (**Fig. I.1**): natural (sites dominated by woodland vegetation, away from human settlements), agricultural (ponds in the vicinity of arable fields and pastures), and urban (water bodies within or right next to urbanized areas). In the small, shallow, low-velocity aquatic habitats that are preferred by many amphibians, human-induced environmental changes are relatively poorly researched. For example, surveys of water pollution are typically focused on large rivers and lakes (Lorenz *et al.*, 2017), and the urban heat island effect that is well known in the terrestrial realm has only just started to get documented in ponds (Brans *et al.*, 2018a). To address this hiatus, we monitored water temperatures in a subset of study ponds using data loggers over an entire season of tadpole development and found, in agreement with the urban heat island effect, that the ponds were significantly warmer in urbanized sites (mean ± SD: 20.57 ± 6.0, range: 6.06 – 33.85 °C) than in natural habitats (mean ± SD: 14.66 ± 4.27, range: 3.68 – 28.66 °C) by an average of 5.8 °C ± 0.03 SE (**Fig. I.2**). Also, we measured pH, conductivity, total dissolved solids, and salinity in the pond water at the locations where we collected tadpoles for the study in **Chapter II.1** (for methods, see Bókony *et al.*, 2021a). All these values tended to be higher in anthropogenic sites, especially in urban ponds, compared to natural sites (**Fig. I.3**). These changes are similar to

[1] Each paper is cited in footnotes to the title of the respective chapter. For details not included in this dissertation, the full text of the original papers and their supplementary materials are available electronically on this website: http://real.mtak.hu/ (specific links are given in each chapter).

those documented in streams draining urban catchments, collectively known as the "urban stream syndrome", suggesting elevated concentrations of nutrients and contaminants (Walsh *et al.*, 2005). To assess chemical pollution in the ponds at the time of early tadpole development, we also collected water and sediment samples during the study in **Chapter II.3** and analyzed them for 133 contaminants that frequently occur in surface waters (for methods and detailed results, see Bókony *et al.*, 2018b). We detected 41 compounds (**Fig. I.4**), almost all of which had been demonstrated to have toxic effects on reproduction-related traits in animals (see Table S1 for a non-comprehensive review in the supplementary material of Bókony *et al.*, 2018b). Several compounds were present in many or all ponds, and even the small forest ponds that represent natural breeding habitats of many amphibians were contaminated (**Fig. I.4**). The number of contaminants was higher in agricultural ponds (29.0 ± 1.4 SE) and in urban ponds (31.0 ± 1.1 SE) than in natural ponds (24.5 ± 1.9 SE), and several chemical groups showed a concentration gradient increasing from natural towards anthropogenic ponds. The intensity of urbanization (i.e. pond scores along the first principal component axis, as explained in **Fig. I.1**) correlated positively with the sediment concentrations of polycyclic aromatic hydrocarbons which are industrial and domestic combustion byproducts (Spearman rank-correlation: $r_s = 0.59$) and two phenolics widely used as industrial additives (nonylphenol and bisphenol-A; $r_s = 0.60$), whereas the intensity of agriculture (i.e. pond scores along the second PC axis, as in **Fig. I.1**) correlated positively with the sediment concentrations of estrone ($r_s = 0.62$) and pesticides, mostly banned organochlorines that were used in large amounts half a century ago ($r_s = 0.80$). Furthermore, testosterone and triazine pesticides were only detected in the water of agricultural ponds (**Fig. I.4**). The association between sex hormones and agricultural land use may be due to animal excreta used as fertilizers or originating from livestock grazing (Lange *et al.*, 2002; Kolodziej & Sedlak, 2007) or aquaculture in fish ponds (Barel-Cohen *et al.*, 2006). The concentrations we found generally fall within the range of values reported from other surface waters and sediments (see Table S1 for a non-comprehensive review in the supplementary material of Bókony *et al.*, 2018b), but we detected exceptionally high water concentrations of glyphosate, an herbicide used in very large amounts worldwide, and carbamazepine, an anti-epileptic and anti-depressant drug which is very persistent in the environment (**Fig. I.4**). This indicates that chemical contaminants may accumulate heavily in small standing waters exposed to frequent input, e.g. due to wastewater leaching and runoff, or direct deposition associated with human activities such as tourism and angling. Many chemicals can persist especially long in sediments; thus, the sediment pollution gradients we found reveal that aquatic wildlife in anthropogenic habitats have been exposed to higher pollution loads in the recent past than their counterparts in more natural habitats. Since many amphibian species can live as long as 10-15 years in nature (Smirina, 1994), lasting developmental effects of pollution may be detectable in amphibian populations even a decade after exposure. Furthermore, although the chemicals absorbed to sediment are considered to have little bioavailability, they can get re-suspended by disturbances to the pond bottom (Knott *et al.*, 2009) and re-enter the food chain via bottom-grazing and filter-feeding animals such as the larvae of many amphibian species (Wells, 2007). Altogether, these data demonstrate that anthropogenic land-use conversion is accompanied by similar environmental changes in the "amphibian ponds" as in other, better-studied habitat types, at least in terms of thermal and chemical stressors. Thus, by studying how amphibians deal with these challenges, we can also address more general questions about the biological effects of anthropogenic environmental change.

**Table I.1.** Collection sites used in the studies presented in **Chapters II.1-4** and **III.1-3**, the proportion of area belonging to each land-use type within a 500-m wide belt zone around each pond (measured with QGIS) and total anthropogenic land cover. See **Fig. I.1** for habitat type categories.

| Site* | Natural vegetation | Arable fields | Pastures | Residential areas | Public built areas | Roads | Total anthropogenic land cover | Chapters |
|---|---|---|---|---|---|---|---|---|
| Anyácsapuszta | 0.145 | 0.802 | 0.051 | 0 | 0 | 0.007 | 0.860 | II.2-4 |
| Apátkút | 0.888 | 0 | 0.080 | 0.007 | 0.008 | 0.018 | 0.113 | II.1, III.2 |
| Bajdázó-tó | 0.970 | 0 | 0.022 | 0 | 0 | 0.024 | 0.046 | II.1-4, III.1 |
| Erzsébet-ér | 0.370 | 0.015 | 0.102 | 0.324 | 0.124 | 0.063 | 0.628 | II.4, III.1 |
| Garancsi-tó | 0.859 | 0.002 | 0.056 | 0.066 | 0.001 | 0.015 | 0.140 | II.4, III.1 |
| Göd | 0.248 | 0 | 0 | 0.431 | 0.033 | 0.053 | 0.517 | II.2-4, III.1 |
| Gyermely | 0.213 | 0.735 | 0.014 | 0.001 | 0.027 | 0.011 | 0.788 | II.4 |
| Határrét | 0.284 | 0.484 | 0.137 | 0.070 | 0 | 0.026 | 0.717 | II.1-4 |
| János-tó | 0.987 | 0 | 0 | 0 | 0 | 0.012 | 0.012 | II.2-4, III.1 |
| Juliannamajor | 0.635 | 0.216 | 0.096 | 0.020 | 0.018 | 0.015 | 0.365 | II.1 |
| Kerek-tó | 0.845 | 0.150 | 0 | 0 | 0 | 0.005 | 0.155 | III.1,3 |
| Merzse-mocsár | 0.584 | 0.341 | 0.068 | 0 | 0 | 0.011 | 0.420 | II.4, III.1 |
| Nagykovácsi | 0.476 | 0.025 | 0.156 | 0.287 | 0.018 | 0.039 | 0.525 | II.1 |
| Perőcsény | 0.498 | 0.346 | 0.141 | 0 | 0 | 0.014 | 0.501 | II.1-4 |
| Pesthidegkút | 0.156 | 0.013 | 0 | 0.724 | 0.031 | 0.077 | 0.845 | II.1-4 |
| Pilisvörösvár | 0.270 | 0.004 | 0.024 | 0.531 | 0.083 | 0.077 | 0.719 | II.2-4, III.1,3 |
| Pilisszentiván | 0.282 | 0 | 0 | 0.455 | 0.173 | 0.076 | 0.704 | II.1,3-4 |
| Pisztrángos | 0.940 | 0 | 0.042 | 0.004 | 0 | 0.015 | 0.061 | III.1 |
| Szárazfarkas | 0.988 | 0 | 0 | 0 | 0 | 0.012 | 0.012 | II.2-4, III.1,3 |
| Szarvas-tó | 0.973 | 0 | 0 | 0 | 0.012 | 0.015 | 0.027 | II.1 |

*All sites are located in North-central Hungary: mostly in Pest county or Budapest, a few in Komárom-Esztergom county.

**Figure I.1.** Collection sites used in the studies presented in **Chapters II.1-4** and **III.1-3**, placed along the two dimensions of land use as quantified by principal component (PC) analysis using the six landscape variables shown in **Table I.1**. The first PC axis explains 52% variation and correlates positively with the proportion of urban areas (residential: r = 0.95, public: r = 0.84, roads: r = 0.96) and negatively with natural vegetation cover (r = -0.64); the second PC axis explains 27% variation and correlates positively with the proportion of agricultural areas (arable fields: r = 0.86, pastures: r = 0.57) and negatively with natural vegetation cover (r = -0.74). Each study site was categorized as natural (*circles*), agricultural (*squares*), or urban (*triangles*); two sites marked with *cross* were not categorized (as they were not included in analyses that required habitat categories).

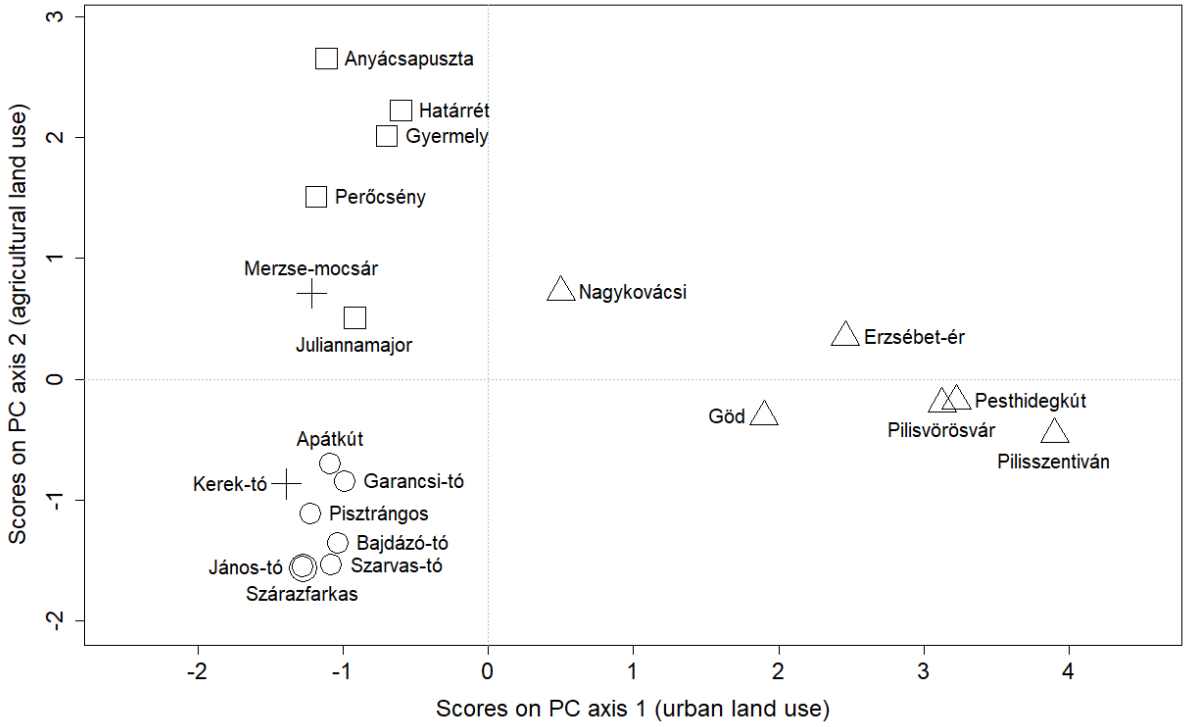



**Figure I.2.** Water temperature over the tadpole development season of 2021 in six ponds: three in natural habitats (green) and three in areas influenced by urbanization (blue).

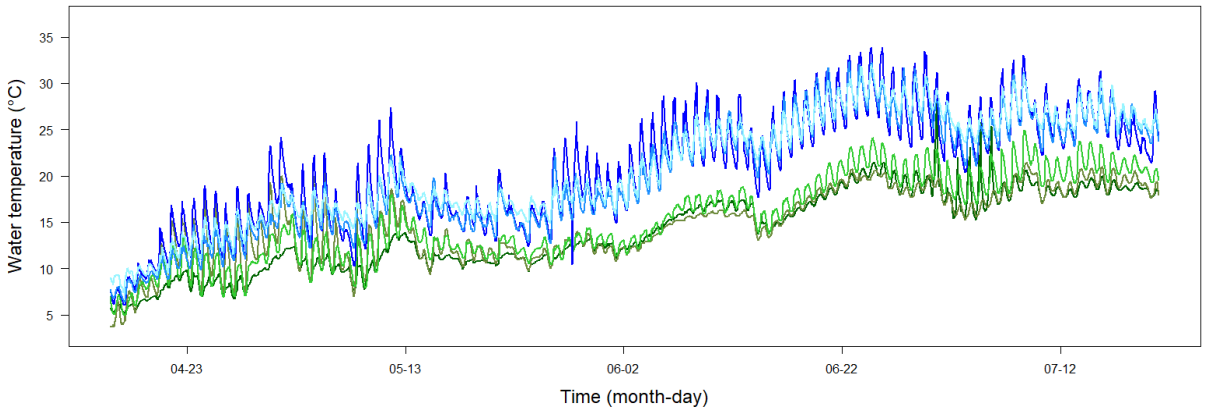
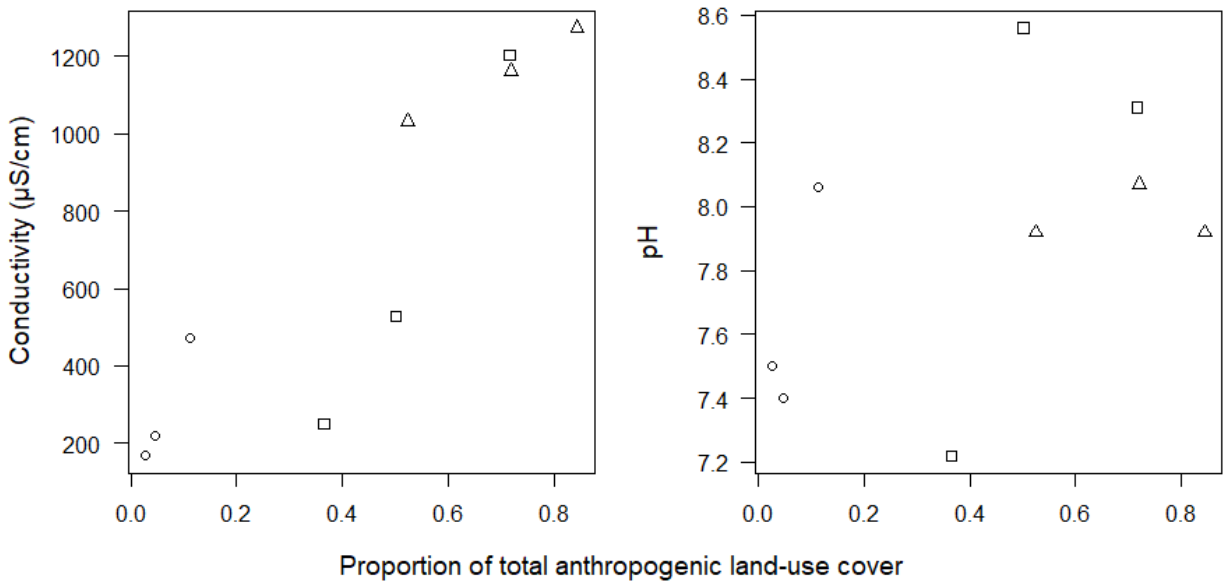


**Figure I.3.** Water conductivity and pH in natural (*circles*), agricultural (*squares*), and urban (*triangles*) ponds in relation to the proportion of area belonging to anthropogenic land use (arable fields, pastures, residential areas, public built areas, roads) within a 500-m wide belt zone around the pond. Total dissolved solids and salinity are not shown, as these values are derived from conductivity using a conversion factor and thus show the same trends.




→

**Figure I.4** (see the figure on the next page). Schematic representation of the concentrations of polluting chemical compounds in the water (µg/L) and sediment (mg/kg) of ponds in different habitats. Within each cell, the left and right symbol stand for water and sediment, respectively. Different symbols indicate different concentrations as follows:

| | |
|---|---|
| ○ | Not detected |
| ◔ | ≤0.01 |
| ◑ | ≤0.1 |
| ◕ | ≤1 |
| ● | ≤10 |
| ⓾ | >10 |
| ▪ | Detected, but below quantification limit |

| Group | Compound | Natural ponds | | | Agricultural ponds | | | | Urban ponds | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | János-tó | Szárazfarkas | Bajdázó.tó | Perősérry | Határrét | Gyermely | Anyácsapuszta | Göd | Pilisszentiván | Pilisvörösvár | Pesthidegkút |
| Polycyclic aromatic hydrocarbons | Naphthalene | ◑◑ | ◑◔ | ◑◔ | ◑◔ | ◑◔ | ◑◔ | ◑◔ | ◑◑ | ◑◑ | ◑◔ | ◑◔ |
| | 2-methyl-naphthalene | ◑◑ | ◑◔ | ◑◔ | ◑◔ | ◑◔ | ◑◔ | ◑◔ | ◑◑ | ◑◑ | ◑◔ | ◑◔ |
| | 1-methyl-naphthalene | ◑◔ | ◑◔ | ◔◔ | ◔◔ | ◑◔ | ◔◔ | ◔◔ | ◔◔ | ◔◔ | ◑◔ | ◔◔ |
| | Acenaphthylene | ◔◔ | ◔○ | ○○ | ○○ | ◔○ | ◔○ | ◔○ | ◔◔ | ◔◔ | ◔◔ | ◔◔ |
| | Acenaphthene | ◑◔ | ◔○ | ◔○ | ◔○ | ◔◔ | ◔○ | ◔○ | ◔◔ | ◔◔ | ◔◔ | ◔○ |
| | Fluorene | ◑◔ | ◕◔ | ◑◔ | ◔◔ | ◑◔ | ◔◔ | ◔◔ | ◔◑ | ◔◔ | ◑◑ | ◑◔ |
| | Phenanthrene | ◑◔ | ◕◔ | ◑◔ | ◔◔ | ◑◔ | ◔◔ | ◔◔ | ◑◑ | ◑◑ | ◑◕ | ◑◔ |
| | Anthracene | ◔◔ | ◑○ | ◔○ | ○○ | ◔◔ | ○○ | ○○ | ◔◔ | ◔◔ | ◔◑ | ◔◔ |
| | Fluoranthene | ◑◑ | ◑◔ | ◔◔ | ◔◔ | ◑◑ | ◔◔ | ◔◔ | ◔◕ | ◔◑ | ◑◕ | ◔◑ |
| | Pyrene | ◑◑ | ◔◔ | ◔○ | ◔◔ | ◔◔ | ◔◔ | ◔◔ | ◔◑ | ◔◑ | ◑◕ | ◔◑ |
| | Benz(a)anthracene | ◔◔ | ◔◔ | ◔○ | ◔◔ | ◔◔ | ◔◔ | ◔◔ | ○◑ | ◔◑ | ◔◕ | ◔◔ |
| | Chrysene | ◔◑ | ◔◔ | ◔○ | ◔◔ | ◔◔ | ◔◔ | ○◔ | ○◑ | ◔◑ | ◔◑ | ◔◔ |
| | Benzo(b)fluoranthene+ Benzo(k)fluoranthene | ◑◕ | ◔◑ | ◔◔ | ◔◔ | ◔◑ | ◔◔ | ◔◔ | ◔◕ | ◔◑ | ◑◕ | ◔◑ |
| | Benzo(e)pyrene | ◔◑ | ◔◔ | ◔○ | ◔◔ | ◔◔ | ○◔ | ◔◔ | ◔◑ | ◔◑ | ◔◑ | ◔◔ |
| | Benzo(a)pyrene | ◔◔ | ◔◔ | ○○ | ○◔ | ◔◔ | ○◔ | ○◔ | ○◑ | ○◑ | ◔◕ | ◔◔ |
| | Indeno(1,2,3-cd)pyrene | ○◑ | ○◔ | ○○ | ○○ | ○◔ | ○◔ | ○◔ | ○◑ | ○◑ | ○◑ | ○◔ |
| | Dibenzo(a,h)anthracene | ○◔ | ○○ | ○○ | ○○ | ○◔ | ○○ | ○○ | ○◔ | ○◔ | ○◑ | ○◔ |
| | Benzo(g,h,i)perylene | ○◑ | ○◔ | ○○ | ○◔ | ○◔ | ○◔ | ○◔ | ○◑ | ○◑ | ○◑ | ○◔ |
| Phtalates and phenolics | Diethyl-phthalate | ◔○ | ○○ | ○○ | ○○ | ◔○ | ◔○ | ◔○ | ◑○ | ◑○ | ◑○ | ◑○ |
| | Di-butylphthalate | ◕○ | ○○ | ◑◔ | ○◑ | ◑◔ | ◕○ | ◕◑ | ◑◔ | ◕◔ | ◑◔ | ◕○ |
| | Di(2-ethylhexyl)phthalate | ○◑ | ◕◑ | ◕◑ | ◕◑ | ○◑ | ○◔ | ◕◑ | ◕◕ | ◕◑ | ◕◑ | ◕◑ |
| | Nonylphenol | ○◕ | ○◕ | ○◕ | ○◕ | ○◕ | ○◑ | ○◑ | ○◕ | ○◕ | ○◕ | ○◕ |
| | Bisphenol-A | ○○ | ○◔ | ○◔ | ○◔ | ○◑ | ○◔ | ○◔ | ○◔ | ○◑ | ○◔ | ○◔ |
| Pesticides | o,p-DDE + p,p-DDE | ○◔ | ○◔ | ○○ | ○◑ | ○◑ | ◔◔ | ◔◕ | ○◔ | ○◔ | ○◑ | ○◔ |
| | o,p-DDD + p,p-DDD | ○○ | ○○ | ○○ | ○◔ | ○◔ | ○○ | ○◑ | ○◑ | ○◔ | ○◑ | ○◔ |
| | Aldrin | ○○ | ○○ | ○○ | ○○ | ○○ | ○○ | ○◔ | ○○ | ○○ | ○○ | ○○ |
| | Dieldrin | ○○ | ○○ | ○○ | ○○ | ○○ | ○○ | ○◔ | ○○ | ○○ | ○○ | ○○ |
| | Terbuthylazine | ○○ | ○○ | ○○ | ○○ | ○○ | ◕○ | ○○ | ○○ | ○○ | ○○ | ○○ |
| | Terbutryn | ○○ | ○○ | ○○ | ○○ | ○○ | ◑○ | ○○ | ○○ | ○○ | ○○ | ○○ |
| | Dicofol | ○○ | ○○ | ○○ | ○○ | ○○ | ○○ | ○◔ | ○◔ | ○○ | ○◔ | ○○ |
| | Glyphosate | ●○ | 10○ | ●○ | ●○ | ●○ | 10○ | 10○ | ●○ | ●○ | 10○ | ●○ |
| | Aminomethylphosphonic acid | ●○ | 10○ | ●○ | ●○ | 10○ | ●○ | ●○ | ●○ | 10○ | 10○ | 10○ |
| Pharmaceuticals and hormones | Estrone | ○○ | ○○ | ○○ | ○○ | ○◔ | ○ · | ○◔ | ○◔ | ○◔ | ○◔ | ○○ |
| | Testosterone | ○○ | ○○ | ○○ | ◔○ | ○○ | ◔○ | ○○ | ○○ | ○○ | ○○ | ○○ |
| | 17α-ethynylestradiol | ○○ | ○◔ | ○○ | ○○ | ○○ | ○○ | ○○ | ○○ | ○○ | ○○ | ○○ |
| | Caffeine | ◑○ | ○○ | ○○ | ○◔ | ◑○ | ○○ | ◑○ | ◑○ | ◑○ | ◑○ | ○○ |
| | Sulfamethoxazole | ◔○ | ○○ | ○○ | ○○ | ○○ | ◔○ | ◔○ | ○○ | ○○ | ◔○ | ○○ |
| | Carbamazepine-epoxide | ○○ | ○○ | ○○ | ○○ | · ○ | ○○ | ○○ | ○○ | · ○ | · ○ | ○○ |
| | Carbamazepine | 10○ | ◑○ | ○○ | 10○ | 10◕ | ○○ | ○○ | 10◕ | 10○ | 10◕ | ●○ |
| | Ketoprofen | ○○ | ○○ | ○○ | ○○ | ○○ | ○○ | ○○ | · ○ | ○○ | ○○ | ○○ |

**Overview of thesis structure**

The studies described in the remaining chapters are divided into three main sections, as follows:

*Section II* presents a series of field observations and captive experiments in which we investigated the phenotypic differences between common toads (*Bufo bufo*) living in natural and anthropogenic habitats, and the underlying mechanisms, for several traits that are important for individual fitness, demography and population viability. The studies described in Chapters **II.2-4** were all done on different but more-or-less overlapping subsets of the same animals collected from the natural, agricultural and urban sites shown in **Table I.1** and **Fig. I.1**, whereas the study in Chapter **II.1** used a different sample, collected in another year from a partially overlapping subset of the same sites.

The common toad is abundant throughout Europe and in northern Asia, occupying a broad range of habitat types including landscapes influenced by agriculture and urbanization. Although it is listed in the "Least Concern" category of the IUCN Red List, its populations are potentially threatened by habitat loss to urbanization, climate change, infectious diseases, and water pollution. It is protected in many countries including Hungary, and localized population declines have been observed recently (Agasyan *et al.*, 2009).

*Section III* presents a field study and a series of captive experiments in which we investigated the causes and consequences of environmentally induced sex reversal in agile frogs (*Rana dalmatina*). The field study described in **Chapter III.1** was done with animals collected from the natural, agricultural and urban sites shown in **Table I.1** and **Fig. I.1**. The laboratory data presented in **Chapters III.1** and **III.3** came from a single experiment whereas **Chapter III.2** describes another experiment with different individuals.

The agile frog is widespread in much of Europe, where it inhabits light deciduous woodlands, but also occurs near or in urbanized areas. It is protected in many countries including Hungary. Although it is listed in the "Least Concern" category of the IUCN Red List, its populations are decreasing and threatened by anthropogenic habitat alteration (Kaya *et al.*, 2009).

*Section IV* deals with general aspects of environmentally induced sex reversal using theoretical approaches and synthesis of empirical data. In **Chapter IV.1** we present a revised hypothesis with testable predictions, and a review of literature data, to explain why and how species with different sex-chromosome systems may have evolved different susceptibilities to environmentally induced sex reversal. In **Chapters IV.2-3** we use formal theoretical modelling to predict what happens when the frequency of sex reversal increases over generations, and how various factors modify the outcomes. Although both models were developed with a focus on climatic warming, their conclusions are relevant for any other persistent environmental change (e.g. increasing levels of chemical pollution) that may trigger sex reversal. For one of the models (**Chapter IV.2**) we compare the theoretical predictions with an analysis of literature data on amphibian sex ratios.

Finally, a summary of our key findings and a brief outlook is given in *Section V*.

## *II. Correlates of anthropogenic land use in common toads*

### II.1. Glucocorticoid stress response[2]

The glucocorticoid (GC) response to stress in vertebrate animals may play a crucial role in organismal adaptation to anthropogenic environments (Partecke *et al.*, 2006; Bonier, 2012). Glucocorticoids are secreted in response to stressful stimuli by the activation of the hypothalamus-pituitary-adrenal/interrenal (HPA/I) axis (Sapolsky *et al.*, 2000; Romero, 2004; Romero *et al.*, 2009) and mediate a wide variety of physiological and behavioral changes that help animals cope with stressors (Sapolsky *et al.*, 2000). However, when a stressor persists and cannot be avoided, GCs can lead to pathological effects that directly impact fitness and can ultimately result in death (Wingfield & Sapolsky, 2003; Romero & Wikelski, 2010). In such situations, dampening of the stress response or "resistance to stress" may be adaptive (Wingfield & Sapolsky, 2003). Researchers have hypothesized that wildlife populations in anthropogenic environments can escape the negative effects of chronic stress by reducing their responsiveness to stressors (Partecke *et al.*, 2006; Atwell *et al.*, 2012). This hypothesis has generated great interest, yielding a large amount of mixed results across species, mostly in birds (Bonier, 2012; Sepp *et al.*, 2018) and in reptiles (French *et al.*, 2018). That is, urban populations in some species show higher stress response than non-urban conspecifics while other species show the opposite or no difference between habitats; the reason for this heterogeneity could not yet be identified by interspecific comparative studies and meta-analyses (Murray *et al.*, 2019; Iglesias-Carrasco *et al.*, 2020; Injaian *et al.*, 2020).

Glucocorticoid negative feedback is a key component of the stress response that needs investigation to understand if and how the modulation of the stress response helps organisms cope with anthropogenic habitats (Narayan *et al.*, 2019). The stress response involves suppressive actions on the HPA/I axis which decrease the production of GCs; thus, an efficient negative feedback returns GC levels to their baseline quickly after cessation of the stressor (Sapolsky *et al.*, 2000). According to the reactive scope model of stress, the shorter the duration of the acute stress response, the lower the chances that repeated or prolonged stressors will provoke phenotypic damage; so an efficient negative feedback may allow for a strong stress response without leading to pathology (Romero *et al.*, 2009). Negative feedback efficiency varies between individuals and is modulated by environmental conditions (Vitousek *et al.*, 2019; Lattin & Kelly, 2020). Individuals with more efficient negative feedback are less likely to suffer from chronically elevated GC levels (Taff *et al.*, 2018) and are better able to cope with stressors such as starvation (Romero & Wikelski, 2010), predation risk (Zimmer *et al.*, 2019), or disturbance by noise (Soldatini *et al.*, 2015). Therefore, we hypothesize that a non-attenuated stress response coupled with efficient negative feedback may help wildlife adapt to anthropogenic habitats (Wingfield, 2013; Narayan *et al.*, 2019; Vitousek *et al.*, 2019), predicting faster recovery from acute stress in individuals in these habitats. Such a strategy might be an alternative to a suppressed stress response; thus, the heterogeneity in empirical findings might be due to different species relying on different coping strategies. So far, only a single study reported empirical data for evaluating this idea, finding that urban individuals were more responsive to stress (adrenocorticotropin hormone, ACTH injection) than conspecifics in a desert population of a bird species, but negative feedback as measured by a

[2] This chapter is based on the following publication: Bókony V., Ujhegyi N., Hamow K.Á., Bosch J., Thumsová B., Vörös J., Aspbury A., Gabor C.R. 2021. Stressed tadpoles mount more efficient glucocorticoid negative feedback in anthropogenic habitats due to phenotypic plasticity. Science of the Total Environment 753: 141896. http://real.mtak.hu/115480/

dexamethasone suppression test did not differ between the two habitats (Fokidis & Deviche, 2011). Furthermore, no study to our knowledge has assessed the role of plastic *versus* persistent mechanisms in differences of GC negative feedback efficiency between populations living in habitats with different levels of anthropogenic influence, despite the fact that common-garden and reciprocal-transplant experiments have shown that both individual plasticity and persistent population divergence can contribute to differences in baseline GC levels and stress response between urban and non-urban habitats (Partecke *et al.*, 2006; Atwell *et al.*, 2012; Ouyang *et al.*, 2019).

In this study, we had two main objectives. First, we examined whether the GC stress response and its negative feedback differed between anthropogenic and natural habitats, by testing whether toad tadpoles living in natural, agricultural, and urban habitats differ in their release rates of corticosterone (the main GC of amphibians) in response to a standardized stressor, and after a standard time allowed for recovery (i.e., negative feedback). Second, we tested the role of persistent population divergence in the different GC profiles observed between habitats, i.e. whether the differences observed in the field persist when individuals from different habitats are raised in a common garden experiment, which would indicate persistent population divergence rather than phenotypic plasticity as the main mechanism of GC responses to anthropogenic environments.

## Methods

### *Sampling in the field*

We selected 3 ponds each from natural, agricultural, and urban habitats (**Table I.1, Fig. I.1**), where common toad eggs were available in large numbers. We sampled tadpoles at each of the 9 ponds between 9 and 20 May 2019, in the early phase of larval development, before toe differentiation. We estimated that the majority (90%) of sampled tadpoles were between developmental stages 28-31 (Gosner, 1960), and had spent at least 5 weeks in their habitat since spawning. Each pond was sampled on a different day between 09:30-14:30 h; date and time of sampling were unbiased by habitat type. We used a non-invasive method of water-borne hormone sampling to take a baseline, stressed, and recovery sample from each tadpole. This method provides an integrated measure of corticosterone which is repeatable within individuals, correlates with plasma levels, and responds to ACTH challenge (Gabor *et al.*, 2013, 2016; Forsburg *et al.*, 2019; Narayan *et al.*, 2019); the post-stress rate of recovery of GC levels provides a measure of natural negative feedback (Lattin & Kelly, 2020). Upon sampling, we quickly collected 18 tadpoles from the pond with a dip net and placed each tadpole in a clean plastic insert (a perforated cup, to facilitate removal of tadpoles from beakers) in a 250 ml glass beaker containing 100 ml spring water. The animals were left undisturbed for one hour to measure "baseline" corticosterone release rates, after which we moved them (with the perforated insert) into another beaker of 100 ml spring water. Over the next hour, the tadpoles were agitated by gently shaking their beakers for one minute every 3 minutes (Forsburg *et al.*, 2019) to measure "stressed" corticosterone release rates, then moved into a third beaker with 100 ml spring water and left undisturbed again for an hour to measure "recovery" corticosterone release rates. The 3 water-borne hormone samples of each animal, and a 100-ml sample of pond water for measuring the background levels of corticosterone (Gabor *et al.*, 2018), were filtered in the field using coffee filters (equivalent to grade 4 filter paper) immediately after collection, stored on ice in the dark for 3-5 hours while being transported into the lab, and placed at -20°C until we measured corticosterone. After sampling, we transported the tadpoles to the lab in individual capped containers, measured their body mass (± 0.1 mg), and euthanized them by cooling-then-freezing (Shine *et al.*, 2019) for further studies which are not described in this dissertation. Before leaving the pond, we took a 1-L sample of pond water in an amber PET flask

for measuring the concentrations of polluting chemicals; this sample was kept in the dark and on ice during transportation, and then stored at -80°C until analysis.

***Common garden experiment***

On 12 March 2019, we set up 54 mesocosms by placing 45-L plastic tubs (56 × 39 × 28 cm) in an open outdoor area and filling them with 40 L tap water. Two days later we added 0.5 L pond water and 40 g dried beech (*Fagus sylvatica*) leaves to each tub to set up a self-sustaining ecosystem that provided nutrients and refuge for tadpoles. We repeated the pond-water inoculation two weeks later to ensure a sufficiently large population of phytoplankton and zooplankton. To prevent colonization by predators, we covered the tubs with mosquito net lids, and we removed all mosquito larvae from the pond water before inoculation.

Between 3 and 5 April, we collected toad eggs from 4 freshly spawned egg strings (ca. 30 eggs per egg string) from each of the 9 ponds, and transported them to our laboratory, where they were raised until developmental stage 25 (Gosner, 1960). Each family (sibling group) was kept in a separate plastic box (19 × 30 × 15 cm) in 2 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). Lab temperature was 19°C and we maintained a light-dark cycle that mimicked the natural photoperiod. On 13 April, we haphazardly selected 9 tadpoles from each family, pooled the 36 tadpoles that originated from the same pond, and randomly distributed them among 6 mesocosms for each pond, resulting in 6 tadpoles per mesocosm and a total number of 324 tadpoles in 54 mesocosms. Pond of origin was allocated to the mesocosms in a randomized block design.

Between 6 and 8 May, we took water-borne hormone samples from the tadpoles in the mesocosms using the same protocol as we applied in the field. At that time, the captive-reared tadpoles were in similar developmental stages as the free-living tadpoles were at hormone sampling. We sampled 3 tadpoles from each tub (18 tadpoles in total per pond of origin), totaling 162 tadpoles; 54 per day. Sampling took place between 09:00-13:30 h each day. The order of sampling of the tubs followed a randomized block design such that two tubs per pond were sampled each day, and both date and time of day were balanced among the 3 habitat types. After sampling, we measured the body mass (± 0.1 mg) of each tadpole and released them back to their tubs; after completion of the experiment, tadpoles were transported back to their ponds of origin. The water-borne hormone samples were filtered after removing the tadpoles and stored at -20°C until measuring corticosterone.

***Measuring corticosterone***

We extracted corticosterone from the water samples following an established protocol (Gabor *et al.*, 2016). We measured corticosterone concentration in duplicates for all samples using enzyme immunoassay (EIA) kits (№ 501320, Cayman Chemical Company, Inc.; assay has a range of 8.2-5000 pg/ml and a sensitivity (80% B/B0) of approximately 30 pg/ml). Sample absorbance was read on a spectrophotometer plate reader at 405 nm (BioTek 800XS). Inter-plate variation was 14.3% for the field data (13 plates; range: 0.07-17.7%) and 9.28% for mesocosms (9 plates; range: 0.31-8.08%). Because of the high cost of extracting columns and EIA kits, we aimed to analyze 16 tadpoles per pond in the field and 12 tadpoles per pond in the mesocosms (we expected lower variance in the common garden experiment than in the field). However, our final sample sizes were somewhat lower due to sample loss during sample processing and measurement (the total number of individuals for each corticosterone variable in the statistical analyses ranged 139-144 in the field and 112-120 in the mesocosms; see Table 2 in Bókony *et al.*, 2021a). We quantified corticosterone

release rates as the amount of water-borne corticosterone measured over one hour, divided by tadpole body mass (pg/g/h), and we used its 10-base logarithm in our statistical analyses. We did not correct for developmental stage because body mass is correlated with developmental stage in young tadpoles, and corticosterone levels do not differ across Gosner stages 25-40 (Glennemeier & Denver, 2002).

***Measuring water pollutants***

Due to pollution by wastewater, pharmaceutical discharge, and manure in agricultural areas, natural water bodies often contain GCs and other hormonally active compounds (Lange *et al.*, 2002; Macikova *et al.*, 2014; Gabor *et al.*, 2018). Such water-borne pollutants are taken up by wild animals, especially by those living in aquatic habitats, and may disrupt their endocrine system (Lange *et al.*, 2002; Macikova *et al.*, 2014; Pottinger, 2017). Therefore, while chemical contaminants in general may act as physiological stressors, corticoid-disrupting compounds may have specific effects on the stress response and its negative feedback, which might confound the responses of the HPA/I axis to other anthropogenic stressors. For example, progesterone, an endogenous hormone that is also used as medication, occurs in wastewaters as well as natural water bodies and can affect various components of the GC system, including expression of GC receptors and enzymatic regulation of GC biosynthesis (Macikova *et al.*, 2014). To control for these potential confounding effects of water pollutants, using the 1-L pond water samples we measured the concentration of 23 endocrine disrupting compounds that can affect glucocorticoid signaling pathways, including steroid hormones and pharmaceutical drugs that get into natural water bodies *via* wastewater and agricultural runoff (Lange *et al.*, 2002; Macikova *et al.*, 2014). Additionally, we measured the concentration of carbamazepine which is considered a general marker for wastewater pollution (Nakada *et al.*, 2008; Tran *et al.*, 2014). The full list of compounds and details of the UPLC-UniSpray™ MS/MS analysis are described in the open-access paper on which the present chapter is based (Bókony *et al.*, 2021a).

***Statistical analyses***

All statistical analyses in this dissertation were run in the R computing environment. First, we used a linear mixed-effects (LME) model ('lme' function in package 'nlme') to test if tadpoles in the field and in the mesocosms showed stress response (i.e. significant difference between baseline and stressed corticosterone release rates) and negative feedback (i.e. significant difference between stressed and recovery corticosterone release rates). We used the 3 consecutive samples of each individual as repeated measures, and we tested the fixed effects of venue (field or mesocosms), sample type (baseline, stressed, and recovery corticosterone release rate), and their interaction. Individual identity was included as a random factor. We calculated a type-2 analysis-of-deviance table to test the main effects of venue and sample, and their interaction, using the 'Anova' function in package 'car'. We extracted the model's estimated marginal means and compared pairwise the 3 categories of sample type separately for the two venues, and corrected the significance level with the false discovery rate (FDR) method (Pike, 2011), using the 'emmeans' package.

Second, we tested if baseline, stressed, and recovery corticosterone release rates, and the magnitude of stress response and negative feedback differed among the 3 habitat types (natural, agricultural, and urban) separately in the two venues. We used two variables to quantify the magnitude of stress response: the absolute stressed levels of corticosterone release rates, and the relative change of corticosterone release rate in response to stress (stress-induced change, calculated as: 100 × (stressed – baseline) / baseline). Similarly, we quantified negative feedback as the relative change from stressed to recovery levels as: 100 × (stressed – recovery) / stressed (Lattin

& Kelly, 2020). Because the values of stress-induced change were strongly right-skewed whereas the values of negative feedback were strongly left-skewed, we applied power transformations (power ¼ for the former and power 4 for the latter) to ensure that the residual distributions conform to normality and homoscedasticity. Because both variables had negative values, for each variable the |minimum value – 1| was added to all values before power transformation.

For each of the 5 dependent variables (baseline, stressed, and recovery corticosterone release rates, stress-induced change, and negative feedback), we used the 'geeglm' function in the 'geepack' package to build two generalized estimation equations (GEE) models: one for the field data and one for the mesocosm data. GEE is a population-averaging method that can handle the correlation structure of our data (i.e. tadpoles from the same pond are not independent, but the pond effect is nested within the habitat effect) appropriately and without penalizing power (Zuur *et al.*, 2009). We analyzed the venues separately because the correlation structure as well as the relevant fixed effects differed between venues. In the field, pond identity was a significant random effect (likelihood ratio, LR: 40.9, $P < 0.001$), and the relevant fixed effects included the actual levels of pollution and date as a numeric covariate. In the mesocosms, tub identity was a significant random effect (LR: 32.1, $P < 0.001$) whereas pond of origin was not (LR < 0.001, $P > 0.999$), and date had only 3 different values so it could not be used as a numeric covariate; we did not consider the pollution levels (measured in the ponds one month after taking the eggs into the common garden) as predictor for the tadpoles in the mesocosms. Therefore, in the GEE models of the field data, we tested the following fixed effects: habitat type, date (number of days since 1st May), time of day (number of hours since 08:00 h), carbamazepine concentration, and total concentration of corticoid-disrupting chemicals; the latter two variables were transformed as $\log_{10}(x+0.1)$ to ensure normal distribution and homoscedasticity of model residuals. There was no multi-collinearity between the predictors included in the models (variance inflation factor: VIF < 1.7). The random factor in these models was pond identity. In the GEE models of the mesocosm data, we tested the fixed effects of habitat type, date (as a 3-category factor), and time of day; the random factor was tub identity. All numeric predictors in the analyses were mean-centered.

**Results**

Corticosterone release rates were significantly affected by venue (LME: $\chi^2_1 = 1168.9$, $P < 0.001$), sample type ($\chi^2_2 = 197.6$, $P < 0.001$), and their interaction ($\chi^2_2 = 156.4$, $P < 0.001$). Tadpoles mounted a significant stress response (stressed levels were significantly higher than baseline levels) and then showed significant negative feedback (recovery levels were significantly lower than stressed levels) both in the field and in mesocosms (**Table II.1.1, Fig. II.1.1**). The recovery levels were significantly higher than the baseline levels in the mesocosms but did not differ significantly from baseline in the field (**Table II.1.1, Fig. II.1.1**).

In the free-living tadpoles, we found several differences between natural and anthropogenic habitats (**Fig. II.1.1**). Tadpoles in urban ponds had higher baseline and stressed corticosterone release rates than tadpoles in natural ponds; their stress-induced change did not differ significantly (**Fig. II.1.1**). In contrast, tadpoles in agricultural ponds had similar corticosterone release rates but greater stress-induced change compared to tadpoles in natural ponds (**Fig. II.1.1**). Tadpoles in both urban and agricultural ponds exhibited stronger negative feedback, their corticosterone release rates returning to similar recovery levels, compared to tadpoles in natural ponds (**Fig. II.1.1**). Furthermore, tadpoles in ponds with higher concentrations of corticoid disruptors had higher stressed (slope ± SE: 0.209 ± 0.077, $P = 0.007$) and recovery corticosterone release rates (slope ± SE: 0.199 ± 0.050, $P < 0.001$) and greater stress-induced change (slope ± SE: 0.143 ± 0.015, $P < 0.001$; **Fig. II.1.2**), and the rate of stress-induced change also increased with carbamazepine

concentration (slope ± SE: 0.030 ± 0.012, P = 0.009; **Fig. II.1.2**). In the common garden experiment, the habitat type of the tadpoles' origin had no significant effect on any of the studied hormonal variables (**Fig. II.1.1**).

**Table II.1.1.** Pairwise comparisons (c: linear contrasts, estimated from LME model) of corticosterone release rates ($log_{10}$ pg/g/h) between sample types in each venue. P-values were corrected with the FDR method; df = 509.

| Venue | Contrast (sample types) | c ± SE | t | P |
|---|---|---|---|---|
| Field | Stressed - Baseline | 0.091 ± 0.029 | 3.13 | 0.003 |
| | Stressed - Recovery | 0.104 ± 0.029 | 3.60 | 0.001 |
| | Recovery - Baseline | -0.014 ± 0.029 | -0.47 | 0.636 |
| Mesocosms | Stressed - Baseline | 0.54 ± 0.032 | 16.96 | <0.001 |
| | Stressed - Recovery | 0.073 ± 0.032 | 2.26 | 0.024 |
| | Recovery - Baseline | 0.467 ± 0.032 | 14.59 | <0.001 |

**Figure II.1.1.** Corticosterone release rates of tadpoles in the field ("free"; filled symbols) and in the common garden experiment ("captive"; empty symbols) by habitat type of their pond of origin. The slopes of the lines connecting the error bars illustrate the rates of stress-induced change (baseline to stressed) and negative feedback (stressed to recovery). Asterisks and crosses stand for significant differences in the field between natural ponds and either agricultural or urban ponds for corticosterone release rates (asterisks above the error bars: $^{*}P < 0.05$) or for the rates of stress-induced change and negative feedback (crosses above the lines connecting error bars: $^{\dagger}P < 0.05$, $^{\dagger\dagger}P < 0.01$). For more detailed statistical results, the reader is referred to Tables 4-5 in Bókony *et al.* (2021a).

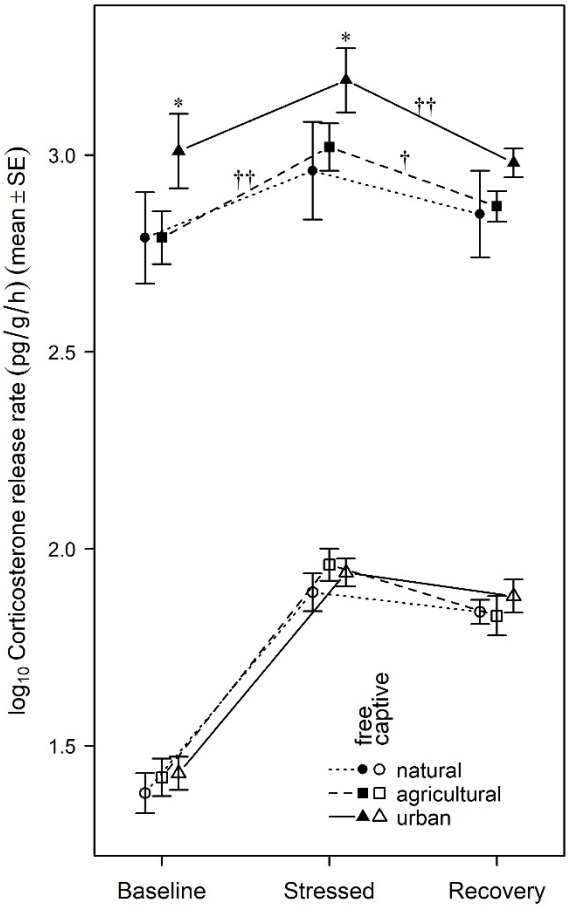
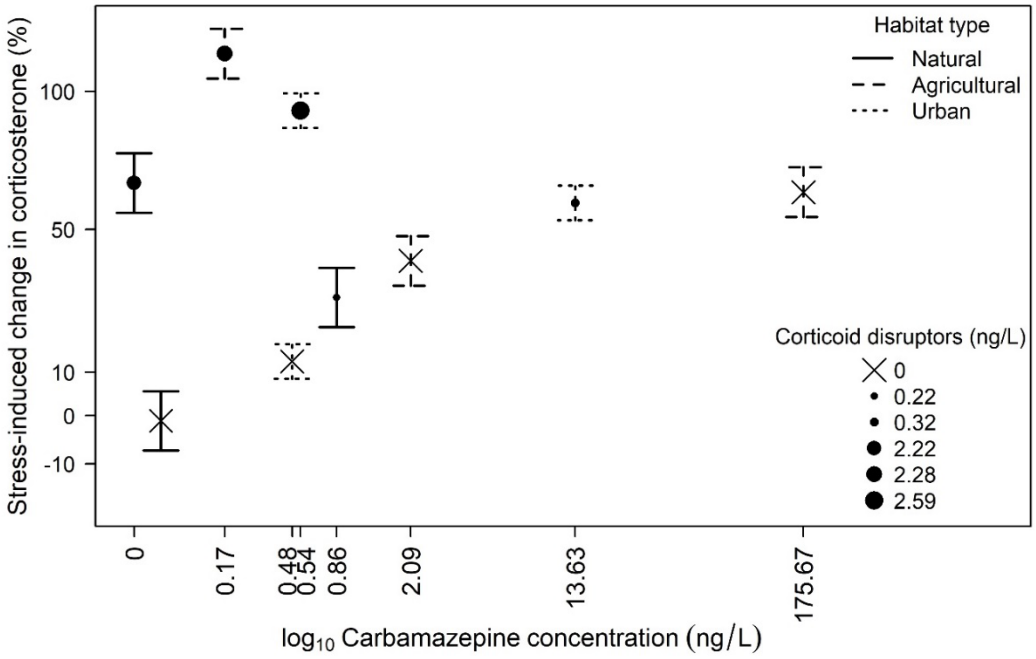


**Figure II.1.2.** Stress-induced change of corticosterone release rates (means and standard errors) in free-living tadpoles in relation to the concentrations of corticoid disruptors and carbamazepine in pond water. Stress-induced change in corticosterone release rates was calculated as 100 × (stressed – baseline) / baseline. Both axes are shown on transformed scale, as used in the analyses ($Y^{¼}$, $\log_{10}X$). For more detailed statistical results, the reader is referred to Table 4 in Bókony *et al.* (2021a).




**Discussion**

In accordance with our prediction, our study suggests stronger negative feedback in the HPI axis of common toad tadpoles in anthropogenic habitats than in tadpoles living in natural habitats. Over the one hour recovery period after the agitation test, tadpoles in both urban and agricultural ponds showed greater downregulation of corticosterone release rates relative to their stressed rates, indicating that their negative feedback had greater scope (magnitude) and/or faster speed (Taff & Vitousek, 2016) compared to tadpoles in natural habitats. This finding aligns with recent suggestions that the GC negative feedback is an important determinant of the capacity to cope with stress and thus should be favored in populations facing frequent stressors (Wingfield, 2013; Narayan *et al.*, 2019; Vitousek *et al.*, 2019). Although we have no data to assess if the stronger negative feedback is adaptive in anthropogenic habitats, empirical evidence suggests that more efficient negative feedback has fitness benefits under stressful conditions (Romero & Wikelski, 2010; Zimmer *et al.*, 2019). As high GC levels can be detrimental to early-life growth and development in amphibians (Crespi *et al.*, 2013), the ability to quickly shut down the stress response after the stressor ceased should protect the tadpoles from chronic GC elevation and its pathological consequences (Romero *et al.*, 2009; Wingfield, 2013).

This protective effect of strong negative feedback may explain why we, and several previous studies on other species (Bonier, 2012; Sepp *et al.*, 2018; Iglesias-Carrasco *et al.*, 2020), did not find attenuated stress responses in anthropogenic habitats. Although reduced stress responsiveness may also protect the organism from phenotypic damage and the "wear and tear" effect of repeated

stress responses (Romero *et al.*, 2009), a weak stress response may be insufficient for adequately dealing with stressful stimuli (Vitousek *et al.*, 2019). A strong stress response may be especially adaptive when the likelihood of unpredictable stressors is high (Romero, 2002), because stress-induced GCs have preparative actions that prepare the organism for a "better" response to a subsequent stressor (Sapolsky *et al.*, 2000), and may increase the threshold of severity necessary for the subsequent stimuli to become stressors (Vera *et al.*, 2017). Thus, instead of dampening the GC stress response, the ability to quickly "turn it on and off" may be the best strategy in stressful environments (Vitousek *et al.*, 2019). Supporting this idea, birds mounting a strong stress response coupled with strong negative feedback are less likely to abandon incubation upon stress (Zimmer *et al.*, 2019) and can breed in disturbed locations (Soldatini *et al.*, 2015). Our tadpole study aligns with these findings, because we found higher stressed corticosterone release rates in urban ponds and proportionally greater stress-induced increase in agricultural than in natural ponds. Currently, it is not clear whether the stressed levels of GCs or their stress-induced increase better predict the transcriptomic, phenotypic, and fitness effects of stress (Vitousek *et al.*, 2018). All else being equal, a higher absolute GC concentration has stronger effects (Romero, 2004), suggesting that urban but not agricultural tadpoles responded more to agitation than tadpoles in natural ponds did. However, all else is rarely equal: the effects of GCs depend on other components of the HPA/I axis, including the abundance of GC receptors, corticosteroid binding globulins, and enzymes that metabolize GCs (Breuner *et al.*, 2003; Lattin & Kelly, 2020). For example, long-term elevation of baseline GCs can be accompanied by decreased receptor production and thus diminished biological effects at a given GC concentration (Romero, 2004). Therefore, the increase from baseline to acute stressed GC levels might better express the strength of the stress response when organisms differ in their baseline levels (Vitousek *et al.*, 2018). This scenario, in contrast, suggests that agricultural but not urban tadpoles responded more to agitation than tadpoles in natural ponds did. Because urban but not agricultural tadpoles had higher baseline levels than tadpoles in natural ponds, our results on stressed levels and stress-induced change may altogether suggest stronger stress response in both types of anthropogenic habitats. We propose that stronger negative feedback allows for stronger stress responses in such habitats, offering an alternative (and potentially more advantageous) strategy for minimizing the time spent at high GC levels; and we suggest that this may be a widespread reason for not finding a dampened stress response in urban animals.

We also observed higher baseline corticosterone release rates of tadpoles in urban ponds than in the tadpoles in the other habitat types. Elevated baseline GC levels are often interpreted as a sign of chronic stress; however, chronic stress can either increase or decrease or have no effect on baseline GC levels (Dickens & Romero, 2013). Alternatively, higher baseline levels may be adaptive in several, mutually non-exclusive ways. Baseline GCs have permissive effects that allow the organism to perform better under stress (Sapolsky *et al.*, 2000), thus temporal variation in baseline GC levels has been proposed to serve as preparation for periods of high potential exposure to adverse conditions (Romero, 2002). Similarly, spatial variation in the likelihood of exposure to stressful stimuli may explain the difference we observed between tadpole populations. Furthermore, because GC levels are upregulated during times of increased energetic demands (Romero, 2002), it is possible that urban tadpoles need a higher baseline for maintaining a higher metabolic rate. For example, urban ponds are often more polluted (Bókony *et al.*, 2018b), which may favor higher detoxification rates, thereby increasing energy demands. In our present study, concentrations of a general wastewater marker, carbamazepine were relatively high in urban ponds, although they were also high in two agricultural ponds where baseline corticosterone release rates were not elevated, and we found no correlation between baseline corticosterone release rates of tadpoles and carbamazepine concentrations across our ponds. However, urban ponds had higher

conductivity, total dissolved solids, and salinity (**Fig. I.3**), suggesting contamination by other toxicants such as de-icing salts, which can increase baseline corticosterone levels in amphibians (Chambers, 2011; Hall *et al.*, 2017; Goff *et al.*, 2020). Furthermore, higher metabolic rates may also result from the urban heat island effect which makes urban ponds warmer than rural ponds (Brans *et al.*, 2018a; **Fig. I.2**).

We found no close relationship between anthropogenic land use and carbamazepine concentrations, and the total amount of corticoid-disrupting compounds varied independently of both land use and carbamazepine (reflecting overall pollution) levels. In turn, each of these three aspects of anthropogenic environmental change had some effects on the tadpoles' corticosterone profiles. The concentrations of both carbamazepine and corticoid disruptors were positively correlated with the stress-induced change, and more corticoid disruptors in pond water were accompanied by higher stressed corticosterone release rates in tadpoles. These results suggest that various chemical contaminants may contribute to altered GC physiology even in habitats where land use does not indicate strong anthropogenic influence, aligning with previous findings that even non-anthropogenic ponds can be contaminated by various endocrine disruptors (**Fig. 1.4**). Thus, the stressors faced by wild animals may vary in complex ways across gradients of anthropogenic environmental change.

Our common garden experiment showed that the differences observed in free-living tadpoles' corticosterone profiles did not persist when the animals were raised in captivity in uncontaminated water. This suggests that individual phenotypic plasticity was responsible for the differences in the field, rather than persistent divergence between populations. An alternative explanation for our results may be genotype-by-environment interaction (G×E). Specifically, it is possible that the GC differences between free-living populations were due to genetic differences that were expressed in the wild but did not get expressed in the captive environment. Although we cannot rule out this possibility, G×E is a less parsimonious explanation for our findings than phenotypic plasticity alone, because G×E requires not one, but two, processes: genetic differentiation between populations (explaining our field results) and phenotypic plasticity (explaining the lack of phenotypic differences in the common garden). While there is empirical evidence that genetic differences may be "hidden" by differences in phenotypic plasticity, increased phenotypic variability due to latent genetic variability often becomes expressed when the organisms are taken out from the environment to which they have adapted (DeWitt & Scheiner, 2004). This pattern is the opposite of what we found, since the GC differences between populations diminished, rather than increased, in the common garden. Nevertheless, further study is needed to explicitly test the role of G×E, by performing the common-garden experiment in several different environments.

Taken together, our study demonstrates marked differences between natural and anthropogenic habitats in the GC stress physiology of toad tadpoles, which were related partly to land use and partly to chemical pollution. Both urban and agricultural populations showed stronger negative feedback, and by some measures also stronger stress response, compared to populations in natural habitats, supporting the idea that dynamic regulation of the GC stress response is an important component of stress coping capacity that is favored in anthropogenic environments (Narayan *et al.*, 2019; Vitousek *et al.*, 2019). Our common garden experiment demonstrates that the differences observed between populations are unlikely to have resulted from microevolution or transgenerational effects, suggesting that toad tadpoles modulate major components of their endocrine flexibility by phenotypic plasticity in response to stressors experienced in anthropogenic environments.

## II.2. Chemical defense[3]

In the context of anthropogenic environmental change, chemical defenses in the animal kingdom are especially under-studied. Like plants, many animal species rely on defensive chemicals or toxins for protection from their natural enemies such as predators, parasites and competitors (Hettyey *et al.*, 2014). Chemical defense may have important consequences for life-history evolution and ecology, as chemically protected animals can live longer (Hossie *et al.*, 2013) and occupy a larger niche space (Arbuckle *et al.*, 2013). Changes in toxicity can affect survival not only for the defended animal (Llewelyn *et al.*, 2012) but also for other species; for example, predators can suffer serious mortality when consuming unusually toxic prey which can lead to predator population declines (Shine, 2010). Some predators can learn to avoid toxic prey and switch to other prey species which then may alter trophic interactions and community structure (Webb *et al.*, 2008; Nelson *et al.*, 2010); whereas other predators adapt to consuming toxic prey by evolving toxin resistance, leading to co-evolutionary arms races between the defended organisms and their enemies (Brodie & Brodie, 1990). Despite all this potential of defensive toxins to impact multiple populations across wildlife communities and thereby biodiversity conservation, we have very little understanding of how environmental changes influence chemical defenses in animals (Hettyey *et al.*, 2014; Bucciarelli *et al.*, 2017), and we know virtually nothing about whether and how their toxicity is altered by anthropogenic habitats.

In this study, we investigated the chemical defenses of common toads in natural and anthropogenic environments. Bufonid toads synthesize toxic steroids called bufadienolides, which they store in their skin glands including their main toxin depot, the pair of parotoid glands (Shine, 2010). These toxic compounds are potent inhibitors of $Na^+/K^+$-ATPase enzymatic activity, causing upon ingestion a bitter taste, nausea or heart failure (Deulofeu, 1948; Kamalakkannan, 2014; Fedorova *et al.*, 2015), and thereby they often repel or can even kill predators (Henrikson, 1990; Shine, 2010). Bufadienolides may also contribute to the toads' immune defense against pathogens (Barnhart *et al.*, 2017). Bufagenins, the smaller, hydrolyzed bufadienolide molecules usually have stronger cardiotoxic effects than bufotoxins, the larger bufadienolide molecules with an amino-acid side chain (Chen & Chen, 1933; Kamalakkannan, 2014). Common toads start to produce both types of bufadienolides as young tadpoles (Üveges *et al.*, 2017), and they flexibly adjust their toxin levels to larval environmental conditions like food availability (Üveges *et al.*, 2017) and competitor density (Bókony *et al.*, 2018a). Furthermore, our earlier experiments showed that chronic exposure to an agricultural pollutant increased the bufadienolide content of common toad tadpoles (**Box 1**). Because anthropogenic habitats are characterized by higher levels of chemical pollution and differ from natural habitats in many further environmental factors (see **Chapter I**) that may also affect the animals' ability and/or need to defend themselves with toxins, we hypothesized that adult toads may have altered toxin levels in agricultural and urban habitats. To test this idea, we captured free-living adult toads in different habitats and assessed two aspects of their chemical defense: parotoid size as proxy for the total amount of toxins (Llewelyn *et al.*, 2012) and the chemical composition of their parotoid secretions. Then, to infer whether the differences we observed between toads from

[3] This chapter is based on the following publications:

Bókony V., Üveges B., Verebélyi V., Ujhegyi N., Móricz Á.M. 2019. Toads phenotypically adjust their chemical defences to anthropogenic habitat change. Scientific Reports 9:3163. http://real.mtak.hu/101881/

Bókony V., Mikó Z., Móricz Á.M., Krüzselyi D., Hettyey A. 2017. Chronic exposure to a glyphosate-based herbicide makes toad larvae more toxic. Proceedings of the Royal Society B 284: 20170493. http://real.mtak.hu/119680/

natural and anthropogenic habitats were due to microevolution or phenotypic plasticity, we used a common garden experiment to compare the chemical defenses of juveniles raised from the eggs of the adults captured from different habitats.

**Box 1. Chronic exposure to a glyphosate-based herbicide makes toad larvae more toxic**

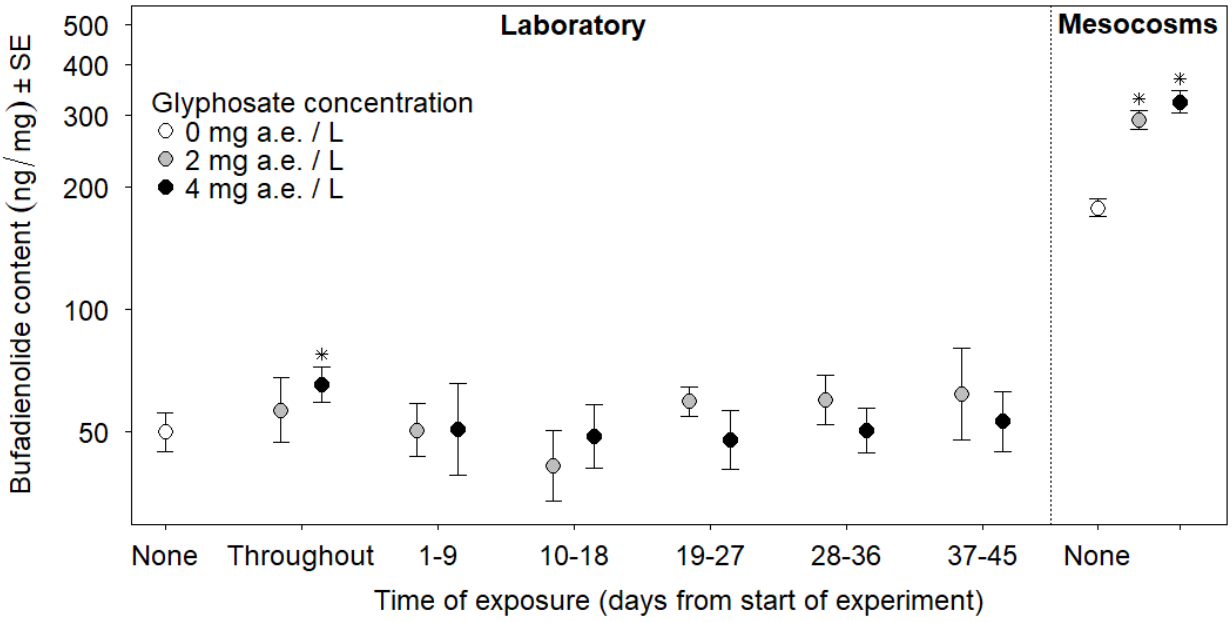
We conducted two experiments to investigate how exposure to glyphosate-based herbicides (GBH), the most widespread agrochemicals worldwide, affects the production of bufadienolides in common toad tadpoles. In both experiments, we used the popular GBH formulation Glyphogan® Classic (Monsanto Europe S.A., Brussels, Belgium) at two nominal concentrations, corresponding to 2 and 4 mg a.e./L glyphosate. The lower value is similar to the expected environmental concentration after application of certain GBHs at the maximum allowed label rate (Govindarajulu, 2008), whereas the higher value is close to the highest glyphosate concentrations observed in runoff after GBH use (Edwards *et al.*, 1980). We chose these concentrations based on earlier experiments showing that the $LC_{50}$ value over 5 days of exposure was 4.4 mg a.e./L glyphosate for toad tadpoles (Mikó *et al.*, 2017a).

In the laboratory experiment, 65 tadpoles collected as eggs from a pond in Nagykovácsi (**Table I.1**) were raised individually from developmental stage 25 until the start of metamorphosis in 13 treatment groups. In the control treatment we kept the tadpoles in 0.7 L clean RSW, whereas the other 12 treatment groups formed a 2×6 design, in which we combined the two GBH concentrations with 6 different exposure times. The tadpoles were exposed to the GBH either during the entire duration of the experiment (36-61 days) or only for a 9-days period during the 1st, 2nd, 3rd, 4th, or 5th period of their larval development (i.e. days 1-9, 10-18, 19-27, 28-36, and 37-45, respectively). During GBH exposure we renewed the initial pesticide concentration twice a week. We measured bufadienolides at developmental stage 42, using the methods described later in this chapter.

In the mesocosm experiment, we collected eggs from 16 ponds (see Table S1 in the electronic supplementary material of Bókony *et al.*, 2017b) and raised 192 tadpoles in mesocosms similarly to **Chapter II.1**. The GBH concentrations were not renewed during this experiment. We measured bufadienolides after 18 days of larval development, when the tadpoles were in stages 32-35 (mostly 34). We chose this stage to maximize the detectability of treatment effects, because in our earlier experiment we found that developing toads had the highest amount of bufadienolides around stage 34, and rearing conditions had the largest effect on toxin levels in this stage (Üveges *et al.*, 2017).

Using linear mixed models, we found significant variance among the GBH treatment groups both in the laboratory experiment ($F_{13,48} = 82.22$, $P < 0.001$) and in the mesocosm experiment ($F_{2,174} = 39.71$, $P < 0.001$). Dunnett's post-hoc tests showed that tadpoles in the laboratory exposed to the higher concentration of GBH for the entire duration of their larval development had significantly higher bufadienolide content than the control tadpoles (**Fig. II.2.1**). In the mesocosms, tadpoles exposed to the lower or the higher concentration of GBH both had significantly higher bufadienolide content than control tadpoles (**Fig. II.2.1**). This effect of GBH treatment was similar across tadpoles originating from different ponds, as the pond × treatment interaction was not significant ($F_{30,144} = 0.85$, $P = 0.685$). Thus, the two experiments consistently show that chronic exposure to GBH significantly increases the bufadienolide content of toad tadpoles. The effects are statistically large (Hedges' $d > 1$) and ecologically relevant, being comparable to bufadienolide increases in other toad species which were induced by predatory threat (Benard & Fordyce, 2003; Hagman *et al.*, 2009) and caused considerable mortality to predators (Hayes *et al.*, 2009).

**Figure II.2.1.** Mean bufadienolide content of toad tadpoles at the start of metamorphosis in the laboratory experiment and at developmental stage 34 in the mesocosm experiment. The groups marked with asterisks differ significantly ($P < 0.05$) from the control group. Note the logarithmic scale on the Y axis.




## Methods

### *Data collection*

We sampled adult toads from 9 ponds in Hungary, which were located in natural, urban or agricultural habitats, with 3 ponds per habitat type (**Table I.1, Fig. I.1**). We hand-collected toads at each pond at the start of the spawning season, between 16 and 28 March 2017. We transported the captured animals to our laboratory in Budapest, where they were kept until spawning at 20°C and artificial light-dark cycles that mimicked the natural photoperiod. We housed each pair in a plastic box (52 × 37 × 33 cm) filled with 15 L RSW and containing 4 vertical wooden sticks as spawning substrates. Each box housed one male and one female haphazardly chosen from the individuals captured at the same pond. After spawning, we measured each toad's snout-vent length (SVL) and the length and width of the left parotoid to the nearest 0.1 mm with a caliper (Phillips & Shine, 2006; Regueira *et al.*, 2017). We obtained a toxin sample from each toad by pressing the right parotoid gland and wiping off the secretion with a cotton swab (in 4 animals, both parotoids had to be sampled to get enough secretion). The swab was immediately placed into a microcentrifuge tube filled with 1 mL HPLC-grade absolute methanol. Our sample size was 166 adult toads (72, 52, and 42 from natural, urban, and agricultural habitats, respectively; the differences are due to variation in capture success among habitats); the parotoid size of one agricultural female could not be measured because it was scarred and deformed. After toxin sampling, all animals were released at the pond where they had been captured.

From each pair, we kept ca. 30 eggs in the lab until hatching. When the embryos became free-swimming tadpoles, we selected 6 healthy-looking individuals (i.e. no visible deformities or abnormal behavior) from each family and moved each tadpole into a 2-L plastic box filled with 1 L RSW; the remaining tadpoles were released to the pond where their parents had been captured.

Out of every 6 siblings, 5 tadpoles were exposed to chemical treatments during their larval development as part of another experiment (described in **Chapter II.4**) while one tadpole was kept in clean RSW as a control; only the latter were used in the present study. We raised the tadpoles to metamorphosis by feeding them with chopped and slightly boiled spinach *ad libitum* and changing their rearing water twice a week. When a tadpole started metamorphosis (i.e. 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 it completed metamorphosis (i.e. disappearance of the tail), we moved it into a clean rearing box containing wet paper towels as substrate and a piece of egg carton as shelter, which were changed every two weeks. Toadlets were fed ad libitum with springtails and small crickets, amended with a 3:1 mixture of $CaCO_3$ and Promotor 43 powder (Laboratorios Calier S.A., Barcelona, Spain) containing vitamins and amino acids. Juveniles were raised until ca. 5 months after metamorphosis (October 6 to November 10, 2017), which corresponds to the time of year when toads prepare for their first hibernation. At this age they are large enough (ca. 4 g) for measuring parotoid size (ca. 7.5 mm long and 2.5 mm wide) and their gonads are developed enough for identification of phenotypic sex. We sampled the juveniles for measuring chemical defense over a one-month period during which natural, agricultural and urban individuals were systematically rotated such that the timing of measurement was balanced among the animals from the 3 habitat types. After euthanizing the toadlets using a water bath of 5.4 g/L MS-222 buffered with the same amount of $Na_2HPO_4$ to neutral pH (Hadfield & Whitaker, 2005; Torreilles *et al.*, 2009), we photographed each juvenile in a standardized setting including a size reference. Then we determined their sex by inspecting the gonads, and we preserved their bodies in 96% ethanol. Using the preserved specimens, we measured the length and width of the left parotoid to the nearest 0.1 mm with a caliper; then we obtained a toxin sample from each juvenile by cutting out a sample of the parotoid (a small piece of tissue that constituted a major part of the gland) and we stored it in 1 mL HPLC-grade absolute methanol. We used somewhat different methods for the juveniles than for the adults because the toadlets were raised and euthanized as part of another experiment (see **Chapter II.4**), and we decided to study their chemical defenses only after finishing the analyses of adult data, when the toadlets had already been sacrificed. For the purposes of the present study, we used one offspring of each pair; 73 juveniles in total (35, 23, and 15 from natural, urban, and agricultural origin, respectively; sample sizes are lower for juveniles than for adult pairs because some pairs refused to spawn in the lab and a few offspring died before sampling).

We measured the amount of bufadienolide compounds in the adult and juvenile secretion samples by high-performance liquid chromatography and mass spectrometry following established protocols (Bókony *et al.*, 2016, 2017b, 2018a; Üveges *et al.*, 2017). From the photographs, we measured juvenile SVL using the ImageJ software with high within-observer repeatability (intra-class correlation coefficient, ICC: 0.997, $P < 0.001$).

***Statistical analyses***

First we tested if site of origin (i.e. pond within habitat type) was a significant random effect, because toads from one site may be non-independent from each other (Zuur *et al.*, 2009). For all variables we found that site of origin as a random effect did not improve model fit significantly ($\Delta AIC \leq 2$, LR tests: $P > 0.09$), so we did not include it in subsequent analyses, i.e. we treated all animals from one habitat type as one sample of independent data, regardless of the specific site they originated from (Zuur *et al.*, 2009).

As proxy for parotoid size, we estimated the base area of the left parotoid gland from its length and width by assuming the shape of an ellipse (Regueira *et al.*, 2017). Our caliper measurements of parotoid size were fairly repeatable (ICC: 0.66, $P < 0.001$) and validated by an alternative

measurement using the photographs of the juvenile toads (more details can be found in the supplementary material of the paper that forms the basis of this chapter: Bókony *et al.*, 2019). To test whether toads originating from urban and agricultural habitats differed from toads originating from natural habitats in their parotoid size, we used linear models with parotoid size as the dependent variable, habitat type as a fixed factor, and SVL as a covariate. We also added sex as a fixed effect and the interaction between sex and SVL to allow for the relationship between SVL and parotoid size to have different slope in males and females. This was needed because adult females were significantly larger (SVL: 72.3-118.0 mm, mean ± SE: 100.2 ± 0.74) than adult males (SVL: 67.7-88.5 mm, mean ± SE: 77.9 ± 0.72; Welch's t-test: $t_{114.9} = 21.3$, $P < 0.001$), although there was no sexual dimorphism in juveniles' SVL (males: 27.5-38.7 mm, mean ± SE: 34.85 ± 0.34, females: 28.5-38.5 mm, mean ± SE: 34.45 ± 0.39, Welch's t-test: $t_{65.7} = 0.76$, $P = 0.448$).

To analyze toxin composition, we estimated the concentration of each compound in the parotoid secretion as the marinobufotoxin-equivalent quantity of each bufadienolide compound per 1 mg dry mass of toxin sample (see details in the supplementary material of Bókony *et al.*, 2019). First, we included all bufadienolide compounds in a single linear mixed-effects model (one for adults, one for juveniles) using the 'nlme' package. In both models, the fixed effects were habitat type, compound, sex, all their two-way interactions and their three-way interaction. Because there were large differences among compounds in their quantities' range, and some compounds were not detected (i.e. had zero quantity) in some individuals, we transformed the bufadienolide quantities as $\log_{10}$(value + the lowest non-zero value across all samples in that age group) to ensure that the distribution of residuals would not violate the LME models' requirements, and we allowed for heteroscedasticity using the 'varIdent' variance structure (Zuur *et al.*, 2009). These analyses showed that the differences between habitat types varied significantly among compounds (type-2 analysis-of-deviance; habitat × compound interaction, adults: $\chi^2_{60} = 305.3$, $P < 0.001$; juveniles: $\chi^2_{60} = 130.4$, $P < 0.001$). This means that the effect of habitat depends on the compound (and also on sex in adults; sex × compound interaction: $\chi^2_{30} = 8884.5$, $P < 0.001$; sex × habitat × compound interaction: $\chi^2_{60} = 125.4$, $P < 0.001$). Thus, the habitat effect cannot be efficiently tested with commonly used statistics like t-tests for each compound or post-hoc tests from a linear model, because the large number of tests would seriously inflate type-1 error. Therefore we used within-study meta-analysis (Nakagawa & Santos, 2012) to estimate the overall effect of habitat type on bufadienolide concentrations and to identify moderator variables that influence the habitat effects.

As the first step of our meta-analysis, we calculated effect sizes as Hedges' d, i.e. unbiased Hedges' g (Nakagawa & Cuthill, 2007) for the differences of each compound's concentration between the animals originating from natural habitats and the animals originating from either urban or agricultural habitats (i.e. one urban and one agricultural effect size for each compound). Hedges' d is a standardized difference in means, i.e. difference between the means of two groups divided by their pooled and weighted standard deviation, multiplied by a correction factor for sample size (Nakagawa & Cuthill, 2007). For the adults, we calculated the effect sizes for each compound for the two sexes separately, because 6 out of 31 compounds were not detected in the majority (86-100%) of males (we did not calculate effect sizes for the latter 6 compounds in males). Furthermore, we omitted one compound because it was not detected in 84% of adults (99% of males and 69.1% of females). Thus, we had 108 effect sizes for the adults. In the juveniles, all detected compounds were found in the majority of individuals and there were no sex differences in bufadienolide concentrations (sex main effect: $\chi^2_1 = 0.6$, $P = 0.421$; sex ×compound interaction: $\chi^2_{19} = 15.0$, $P = 0.721$; sex × habitat × compound interaction: $\chi^2_{38} = 41.2$, $P = 0.334$), so we calculated the urban and agricultural effect sizes for all toadlets regardless of sex, resulting in 40 effect sizes for 20 compounds. Note that this approach has the further benefit of keeping the sample sizes for effect-

size estimation relatively constant, as we had toxin data for ca. twice as many adults (N = 166) as juveniles (N = 73).

In the second step, we analyzed the effect size estimates (i.e. standardized difference between natural and anthropogenic habitats for each compound) in meta-analysis models, which are similar to weighted regression analyses, i.e. they account for the uncertainty of estimates (Nakagawa & Santos, 2012). We conducted separate meta-analyses for adults and juveniles using the 'metafor' package. In each meta-analysis, compound was included as a random intercept to take into account the non-independence of effect sizes comparing the data of urban and agricultural animals to the same animals of natural origin for any given compound. For both age groups, we ran 3 meta-analyses. The first was a simple meta-analytical model (an intercept-only model without any moderators) on all available data to examine the overall effect of anthropogenic habitats on the concentrations of bufadienolides. In the second model we added habitat type as a moderator to test if urban and agricultural habitats had different effects. In the third model we added the interaction of habitat type and toxin type to test if the effects of each habitat type differed between bufotoxins and bufagenins. Point estimates from statistical models were considered significantly different from zero when their 95% confidence intervals (CI) did not overlap zero.

**Results**

Parotoid area of adults was significantly larger in animals captured from urban as well as agricultural habitats compared to toads from natural habitats (**Table II.2.1, Fig. II.2.2**). In contrast, juveniles originating from agricultural habitats had significantly smaller parotoids than juveniles from natural habitats (**Table II.2.1, Fig. II.2.2**), while toadlets from urban habitats had similar parotoid size as toadlets from natural habitats (**Table II.2.1, Fig. II.2.2**).

Regarding the toxin composition of adults, the meta-analytic mean effect of anthropogenic habitats on the concentrations of bufadienolide compounds did not differ from zero (**Table II.2.2**), but the effect sizes varied significantly by habitat type (moderator effect of habitat type, agricultural compared to urban: $0.25 \pm 0.05$, $P < 0.001$). Compared to the toxin samples of adults from natural habitats, the overall concentration of bufadienolides was significantly higher in agricultural samples (**Table II.2.2**) and lower in urban samples, although the latter effect was not quite significant (**Table II.2.2**). The interaction of habitat type and toxin type was also significant ($P < 0.001$): compared to toads from natural habitats, toads from both anthropogenic habitats had more bufagenins (**Table II.2.2, Fig. II.2.3**), but while urban toads had less bufotoxins (**Table II.2.2, Fig. II.2.3**), toads from agricultural habitats had slightly (although not significantly) more bufotoxins per unit dry mass of parotoid secretion (**Table II.2.2, Fig. II.2.3**).

Similarly to the adults, the juveniles' meta-analytic mean effect of anthropogenic habitats on the concentrations of bufadienolide compounds did not differ from zero (**Table II.2.2**), but the effect sizes varied significantly by habitat type (moderator effect of habitat type, urban compared to agricultural: $0.42 \pm 0.09$, $P < 0.001$). However, the direction of these differences was opposite to what we found in adults. Compared to the toxin samples of juveniles originating from natural habitats, the overall concentration of bufadienolides tended to be lower in agricultural samples and higher in urban samples, although neither of these effects was significant (**Table II.2.2**). In contrast with the adults, in the juveniles there was no significant interaction between habitat type and toxin type ($P = 0.706$; **Table II.2.2, Fig. II.2.3**), and juveniles from agricultural habitats had significantly lower concentrations of bufotoxins compared to their conspecifics originating from natural ponds (**Table II.2.2, Fig. II.2.3**).

**Table II.2.1.** Parameter estimates of linear models for parotoid size (measured with caliper in $mm^2$) in 165 adults and 73 juveniles. Significant habitat effects are highlighted in bold.

| Age group | Model parameters | Estimate | SE | t | P |
|---|---|---|---|---|---|
| Adults | Intercept (natural, male) | 95.229 | 5.989 | 15.90 | <0.001 |
| | SVL (mm, mean-centered) | 1.332 | 0.482 | 2.76 | 0.006 |
| | Sex (female) | 14.030 | 6.642 | 2.11 | 0.036 |
| | **Habitat (agricultural)** | **8.161** | **3.664** | **2.23** | **0.027** |
| | **Habitat (urban)** | **7.491** | **3.349** | **2.24** | **0.027** |
| | Sex × SVL | 1.447 | 0.536 | 2.70 | 0.008 |
| Juveniles | Intercept (natural, male) | 16.154 | 0.561 | 28.78 | <0.001 |
| | SVL (mm, mean-centered) | 0.749 | 0.212 | 3.54 | 0.001 |
| | Sex (female) | 0.648 | 0.630 | 1.03 | 0.307 |
| | **Habitat (agricultural)** | **-1.854** | **0.850** | **-2.18** | **0.033** |
| | Habitat (urban) | 0.012 | 0.802 | 0.02 | 0.988 |
| | Sex × SVL | -0.193 | 0.270 | -0.71 | 0.478 |

**Table II.2.2.** Results of meta-analyses of toxin composition in wild-caught adult toads and their captive-reared offspring. Meta-analytic means (Hedges' d) with 95% confidence intervals (CI; in brackets) represent the standardized differences between natural and anthropogenic habitats in bufadienolide concentrations (amount of each compound per unit dry mass of toxin sample). CIs not including zero are highlighted in bold.

| Meta-analysis | Effect | Adults | Juveniles |
|---|---|---|---|
| Model 1 | Anthropogenic habitats | 0.013 (-0.102, 0.127) | 0.005 (-0.215, 0.225) |
| Model 2 | Urban habitats | -0.106 (-0.231, 0.019) | 0.184 (-0.050, 0.418) |
| | Agricultural habitats | **0.144 (0.018, 0.271)** | -0.236 (-0.481, 0.008) |
| Model 3 | Urban habitats | | |
| | Bufotoxins | **-0.343 (-0.469, -0.217)** | 0.117 (-0.140, 0.374) |
| | Bufagenins | **0.377 (0.198, 0.555)** | 0.451 (-0.064, 0.967) |
| | Agricultural habitats | | |
| | Bufotoxins | 0.117 (-0.012, 0.246) | **-0.321 (-0.590, -0.052)** |
| | Bufagenins | **0.204 (0.022, 0.387)** | 0.101 (-0.437, 0.639) |

**Figure II.2.2.** Parotoid size in adult toads captured from different habitats, and in their offspring reared in the common garden experiment, as estimated by the models in **Table II.2.1**.

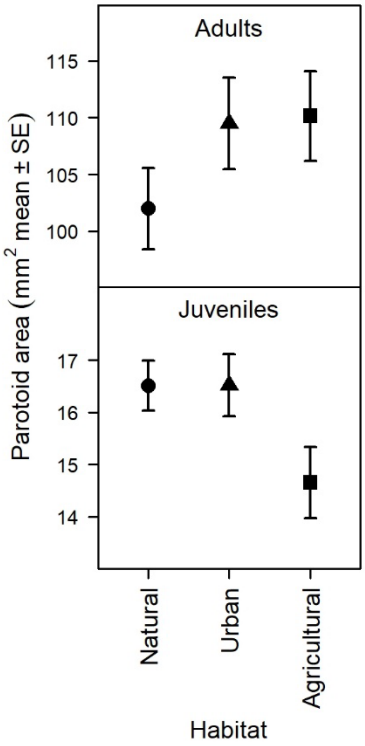
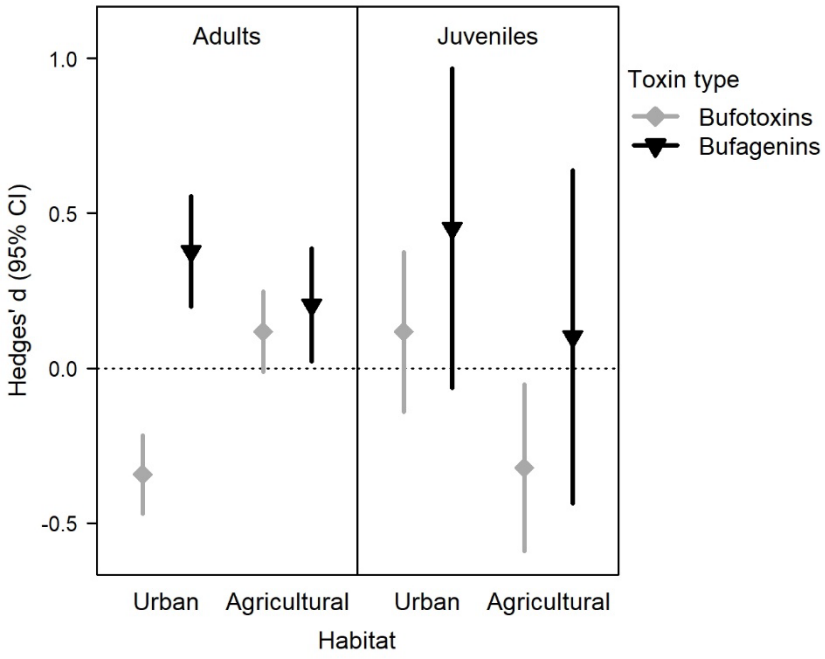



**Figure II.2.3.** Effects of anthropogenic habitats on toxin composition in wild-caught adult toads and their captive-reared offspring. Meta-analytic means with 95% confidence intervals (CI) express the standardized differences between habitats in bufadienolide concentrations (amount of each compound per unit dry mass of toxin sample). Thus, a positive value means larger concentration of the given type of bufadienolides in animals from the anthropogenic habitat than in animals from the natural habitat.




**Discussion**

The parotoid is by far the largest toxin gland in toads, and larger parotoids contain more toxins (Deulofeu, 1948; Llewelyn *et al.*, 2012). Thus, our finding that adult toads had larger parotoids in urban and agricultural habitats than in natural habitats suggests that the total amount of stored toxins was higher in the animals captured from anthropogenic habitats. Furthermore, the overall amount of bufadienolides per unit dry mass of parotoid secretion was higher in adult toads from agricultural habitats, suggesting that their toxin secretion was more potent. These results parallel our experimental findings on toad tadpoles that herbicide exposure increases their bufadienolide content (**Box 1**). Altogether, one potential explanation for these results is that chemical pollutants in anthropogenic habitats may enhance the production of bufadienolides, which may be either a non-adaptive constraint or an adaptive response. On the one hand, bufadienolides are synthesized from the same precursor as glucocorticoid stress hormones and steroid sex hormones, starting with the same initial steps (Fedorova *et al.*, 2015). Thus, elevated bufadienolide levels may be by-products of physiological stress and/or of the endocrine-disrupting effects known to be elicited by many anthropogenic pollutants (Bókony *et al.*, 2017b). Notably, elevated levels of stress hormones

have been found in aquatic amphibians in urban areas (Gabor *et al.*, 2018), and our results presented in the previous chapter (i.e. that free-living toad tadpoles seem to mount stronger glucocorticoid stress responses; **Chapter II.1**) are also in line with this. On the other hand, increasing the levels of bufadienolides might also be an adaptive response to chemical stressors, because bufadienolides may act as regulators of the amphibian skin's ion transport (Lichtstein *et al.*, 1992) which can be altered by several pollutants (Cassano *et al.*, 2006; Suwalsky *et al.*, 2006; Bellantuono *et al.*, 2014). Both of these potential mechanisms can apply to the adult toads in our present study, because in a joint study we found that our ponds contained larger numbers and higher concentrations of endocrine-disrupting chemicals in anthropogenic habitats than in natural habitats (see **Chapter I**, **Fig. I.4**). Experimental studies are needed to test whether these chemicals or other pollutants affect bufadienolide levels by any of the two mechanisms proposed above.

However, pollution and physiological stress are not the only features of anthropogenic habitats that may contribute to the higher levels of toad toxins. Deterrence of predators is considered the main function of amphibian toxins, and experiments with newts and common toads showed that individuals can upregulate their chemical defenses in response to higher predation risk (Bucciarelli *et al.*, 2017; Hettyey *et al.*, 2019). Although we have no data on predator densities at our study sites, ample literature shows that generally both urban and agricultural environments differ from natural habitats in the composition of predator fauna (Chalfoun *et al.*, 2002; Sorace & Gustin, 2009). While larger, specialist predators typically avoid anthropogenic habitats, mammalian and avian generalist mesopredators like corvids, badgers, martens, raccoons, skunks and foxes (all known as predators of toads) are often more abundant in urban habitats, as are domestic cats and dogs (Sorace & Gustin, 2009; Bateman & Fleming, 2012). Similarly, some corvids (Andrén, 1992) and herons (Fasola, 1986) occur at higher densities in agricultural habitats, and some toad-eating snakes can spend up to 80% of their time in crop fields (Wisler *et al.*, 2008). Therefore, toads living in anthropogenic habitats may perceive higher overall predation risk and may adjust their toxin levels accordingly. This scenario is supported by our results that bufagenins, the typically more toxic compounds (Chen & Chen, 1933; Kamalakkannan, 2014), were present in higher concentrations in the parotoid secretion of adult toads from urban as well as agricultural habitats, compared to their counterparts from natural habitats. This difference was particularly prominent for urban habitats (i.e. almost twice as large effect size as for agricultural habitats), where the increase in bufagenins seems to have come at the expense of bufotoxins. It is thus possible that urban toads invest more heavily into producing and storing higher amounts of the more potent compounds even at the price of having to deal with higher autotoxicity (Deulofeu, 1948). Alternatively, toads might not store the bufagenins but rather produce them upon gland discharge by enzymatically cutting the side chain off of the bufotoxins (Deulofeu, 1948; Kamalakkannan, 2014). In this case, our urban toads may have had a faster machinery for this process, allowing them to respond more rapidly to a predator attack (i.e. a human squeezing their parotoids). Clarifying the avenues by which anthropogenic environments affect toad chemical defenses will take more detailed studies on their toxin physiology.

Our common garden experiment showed that the differences found in adult toads were not retained in their offspring when the latter were raised in the lab under identical environmental conditions. When we compared the animals originating from anthropogenic and natural habitats, the significant differences seen in adults were either not present in the juveniles or even showed the opposite direction. In contrast with the larger parotoids and higher bufadienolide concentrations in adults from agricultural habitats, their offspring had smaller parotoids and lower bufotoxin concentrations. Similarly, the juveniles originating from urban habitats showed neither the larger parotoids nor the higher concentration of bufagenins and lower concentration of bufotoxins that

we found in their parents. These results indicate that genetic or epigenetic changes are not likely to be responsible for the differences we observed in the adult toads' chemical defenses between anthropogenic and natural habitats. Cautionarily, we cannot exclude the possibility that the differences seen in the adults are genetically determined but are only expressed after maturity and/or during the breeding season. However, the juveniles had well differentiated gonads by the time of sampling and, in other toad species, parotoids acquire the morphological and histochemical characteristics of adults before sexual maturity (Regueira *et al.*, 2017) and little if any seasonal variation has been found in parotoid size and toxin composition (Phillips & Shine, 2006; Petroselli *et al.*, 2018). Also, we cannot rule out that some similarity in habitat effects between the two age groups might have been masked by the minor differences in the methods applied to adults and juveniles to measure parotoids and sample toxins. However, detailed validation of both measurements (see the supplementary material of Bókony *et al.*, 2019) suggests that the disparity between adult and juvenile habitat differences in chemical defenses is unlikely to be a methodological artefact. Thus, had the same differences by habitat of origin been present in juveniles as in adults, it is likely that we would have been able to detect them (notably, we did find differences between the same offspring of toads from natural and anthropogenic habitats in traits other than toxicity, as explained later in **Chapter II.3**). Therefore, our findings suggest that phenotypic plasticity at the level of individuals may play an important role in the enhanced chemical defenses of toads living in anthropogenic habitats. This mirrors our findings presented in the previous chapter that glucocorticoid stress physiology also seems to vary between habitats due to phenotypic plasticity (**Chapter II.1**).

A further important question is whether the elevated levels of chemical defense are costly in terms of fitness. Although experiments with common toad tadpoles did not reveal any cost of bufadienolide production (Kurali *et al.*, 2016; Üveges *et al.*, 2017), studies with another toad species suggest energetic trade-offs between toxin production and both reproduction and energy metabolism (Blennerhassett *et al.*, 2019). Thus, the adults in our present study might have traded-off some resources for increased toxin synthesis. For example, if toads from anthropogenic habitats converted the common steroid precursor into bufadienolides at the expense of sex hormones (Fedorova *et al.*, 2015), they might have suffered reduced reproductive success. In line with this idea, we found significantly reduced offspring performance in our toads from anthropogenic habitats, as detailed in the next chapter (**Chapter II.3**), which might also explain the lower bufotoxin levels of juveniles from agricultural habitats.

Finally, our results may also have implications for conservation biology. Invasive toad species, such as the cane toad (*Rhinella marina*) in Australia (Shine, 2010) and the Asian common toad (*Duttaphrynus melanostictus*) in Madagascar (Licata *et al.*, 2019) pose serious threats to the native fauna, mainly because their toxins kill predators upon ingesting or mouthing toads or their eggs. As these invasive toads occupy a wide range of habitats and prefer anthropogenically altered sites (Shine, 2010), they may often come into contact with various pollutants and pesticides, which might contribute to the spatial heterogeneity in their toxicity (Phillips & Shine, 2006). Furthermore, increased toxicity of native toad species may also have far-reaching consequences for animal communities, for example by driving their predators to switch to more palatable prey (Gunzburger & Travis, 2005). Therefore, understanding how widespread the anthropogenic effects on toad toxicity are, and what mechanisms govern them, may prove important for the conservation and population management of toads and their predators alike.

## II.3. Reproductive capacity[4]

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

As shown in **Chapter 1** (**Fig. I.4**), small water bodies representing typical amphibian breeding habitats contain various EDCs, sometimes in remarkably high concentrations, and exhibit distinct pollution gradients with increasing influence by agriculture and urbanization. However, it is poorly known if the amphibians living in habitats polluted by EDCs have reduced reproductive capacities. Typically, the effects of EDCs on reproductive health have been inferred in two ways (Hoffman *et al.*, 2003; Orton & Tyler, 2015). First, laboratory ecotoxicology experiments usually expose developing larvae to EDCs and assess their reproductive anatomy, histology, or physiology before or shortly after metamorphosis. Second, field studies usually look at correlations between land use or pollution levels and sex ratios or indirect indices of reproductive abilities such as sex hormone levels and secondary sexual characteristics in adults. The most frequently used endpoint in both kinds of studies is the incidence of intersex, a condition where an individual's gonads contain both female and male tissues. However, intersex can be a natural phase of ontogeny in some species (Orton & Tyler, 2015), and next to nothing is known about the reproductive success of intersex individuals (Jobling *et al.*, 2002; Harris *et al.*, 2011; Fuzzen *et al.*, 2015). Also, measurement of the above indices often requires invasive, even deadly techniques (e.g. sacrificing the animals for histological examination), which hinders such studies in dwindling populations where information on reproductive potential would be most needed.

In this study, we investigated the reproductive capacity of common toads that breed in agricultural, urban, or natural habitats. This species is highly philopatric, with most individuals breeding in the pond in which they developed as larvae (Reading *et al.*, 1991). Thus we hypothesized that, if breeding ponds in anthropogenically influenced landscapes are contaminated by higher levels and/or more potent kinds of EDCs than more natural ponds, then as a consequence of larval exposure to these contaminants we can detect reduced reproductive capacities in the adults

[4] This chapter is based on the following publication: Bókony V., Üveges B., Ujhegyi N., Verebélyi V., Nemesházi E., Csíkvári O., Hettyey A. 2018. Endocrine disruptors in breeding ponds and reproductive health of toads in agricultural, urban and natural landscapes. Science of the Total Environment 634: 1335-1345. http://real.mtak.hu/79319/

breeding in anthropogenic habitats. To evaluate reproductive capacity while ruling out the effects of actual exposure during breeding, we allowed adult toads to spawn in a non-polluted environment in which they could realize their full reproductive potential. We tested whether fecundity, fertilization rate, and the offspring's viability, development and growth were decreased in toads originating from habitats that are characterized by anthropogenic land use associated with higher pollution by EDCs.

## Methods

### *Data collection*

The toads used for this study were the same as those in the previous chapter, with the addition of a fourth urban population (Pilisszentiván). Initially, we aimed to capture at least 10 pairs each from 4 ponds per habitat type; however, because the spawning season of common toads is extremely short (a few days), we could capture gravid females from 10 ponds (3 natural, 3 agricultural, 4 urban; **Table I.1**, **Fig. I.1**). We captured the adults at the start of their spawning season (91% over 3 days between 20–23 March 2017), and transported them to our laboratory in Budapest, where they were weighed (± 0.1 g) and allowed to spawn as described in **Chapter II.2**. Ninety out of 101 pairs spawned within one week (mostly within 3 days), after which all animals were released at the pond where they had been captured. Because capture success varied across ponds, and not all captured pairs spawned in the laboratory, we finally obtained 36 natural, 17 agricultural and 37 urban clutches (egg strings).

To quantify reproductive capacity, we took several measurements from each pair. On the day after spawning, we measured the parents' body mass again, and we calculated an estimate of fecundity (i.e. clutch mass before water uptake) by taking the difference between the female's pre-spawning and post-spawning body mass (note that post-hibernation toads do not feed before spawning). It is likely that the females laid all their eggs, because they appeared lean after spawning and the males completely lost interest in them. Only a single female had an appearance after spawning that implied that she might have retained some of her eggs, but her clutch mass was not an outlier (she had the 20$^{th}$ lowest residual clutch mass) and our conclusions on fecundity are not altered by excluding this clutch (results not shown). We also measured the mass of the entire clutch including not only the mass of the eggs but also the water absorbed by their jelly coats (i.e. clutch mass after water uptake). The difference between clutch mass before and after water uptake provides a proxy for jelly thickness (which is very difficult to measure directly in toad egg strings), assuming that the more water the jelly absorbs the thicker it gets; this trait is important for fitness because thicker jelly coats may provide greater protection from exogenous chemical stress (Licht, 1985; Edginton *et al.*, 2007; Shu *et al.*, 2015).

To estimate fertilization rate and offspring survival, from each clutch we placed ca. 30 eggs, taken from 3 haphazardly chosen parts of the egg string, into a 21 × 16 × 12 cm plastic box filled with 0.5 L RSW. We did not have identical numbers of eggs in all containers because ensuring that would have required longer manipulation of the egg string, risking the eggs falling out of the jelly and jeopardizing their further development. Five days after spawning, we measured fertilization rate as the proportion of eggs that started to develop, and removed the non-fertilized eggs (i.e. completely spherical eggs that had started to mold). Two weeks after spawning, when the embryos became free-swimming tadpoles (developmental stage 25 according to Gosner, 1960), we counted the proportion of embryos that survived to this stage, and started to feed the tadpoles with chopped and slightly boiled spinach. On day 17, we estimated the young tadpoles' average body mass by measuring the total mass (± 0.01 g) of 4 randomly chosen tadpoles from each family and dividing it by 4; then we selected 6 healthy-looking individuals from each family and moved each tadpole

into a 2-L plastic box filled with 1 L RSW. Only one of 6 siblings were used for the present study (in total, 88 tadpoles from 36 natural, 16 agricultural, and 36 urban families); the rest were used for the study presented in **Chapter II.4**. The remaining eggs and tadpoles were released at the pond where their parents had been captured.

To measure the rate of development and growth in tadpoles and juveniles, we raised the animals to metamorphosis and for ca. 5 months thereafter as described in the previous chapter (**Chapter II.2**). For each individual we recorded the start of metamorphosis (i.e. appearance of forelimbs, developmental stage 42), the end of metamorphosis (i.e. disappearance of the tail, developmental stage 46), and body mass at the end of metamorphosis (± 0.1 mg) and at termination of the experiment (± 0.01 g), i.e. in juveniles between 6$^{th}$ October and 10$^{th}$ November 2017. This time of the year corresponds to the time before the beginning of first hibernation and is relevant to toadlets' fitness because their pre-hibernation body mass predicts survival during hibernation and post-hibernation body mass (Üveges *et al.*, 2016).

***Statistical analyses***

As measures of each pair's reproductive capacity, we analyzed the following 8 variables. Because fecundity is known to increase with female size (Banks & Beebee, 1986; Reading, 1986), we statistically controlled for this effect by calculating residuals from a standardized major axis regression (function 'sma' in package 'smatr') with female post-spawning body mass as the explanatory variable and clutch mass before water uptake (i.e. the female's body mass loss from before to after spawning) as the dependent variable. This approach is favorable over simple ratios or ordinary least-squares regression residuals when the goal is to obtain accurate predicted values rather than merely testing if the slope differs from zero (Peig & Green, 2009, 2010). We used the residuals from this regression as measure of fecundity corrected for female size.

As a proxy for jelly thickness, we subtracted clutch mass before water uptake (i.e. female weight loss during egg laying) from clutch mass measured after water uptake. This variable, referred to henceforth as jelly mass, increases with the total mass of eggs, but our data indicated a non-linear association and we had no *a priori* knowledge on the function by which this relationship can be adequately described. Therefore, to take variation in clutch mass into account, we categorized clutch mass into 3 groups (i.e. small, medium and large clutches, before water uptake) with equal sample size, and we analyzed the effects of habitat on jelly mass within these 3 groups (see below).

We measured fertilization rate as the proportion of eggs that started embryonic development, and embryo viability as the proportion of embryos that survived to the free-swimming tadpole stage. We did not analyze the survival of tadpoles and juveniles because only one out of 88 tadpoles died before metamorphosis and 5 out of 87 toadlets died before the termination of the study. Out of these 6 cases of mortality, 4 individuals were of urban origin while only one originated from each of the other two habitat types. We analyzed offspring body mass measured at three times: on day 17 (early tadpole stage), at completion of metamorphosis, and at termination (ca. 5 months after metamorphosis). We also analyzed the time to metamorphosis, calculated as the number of days from spawning until the completion of metamorphosis.

Each of the above variables was used as a dependent variable in GEE models (function 'geeglm'). In each model, we allowed for the non-independence among pairs captured from the same pond using the "exchangeable correlation" (or "compound symmetry") association structure (Zuur *et al.*, 2009). We used habitat type as a fixed factor, and we parameterized the design matrix such that we estimated the differences between the natural habitat type (used as intercept) and each of the two anthropogenic habitat types (i.e. agricultural and urban). In the model of juvenile body

mass, we included the individual's age (i.e. number of days from completion of metamorphosis to termination; mean-centered) as a covariate. In the models of fertilization rate, embryo viability, offspring mass, and time to metamorphosis, we also included the body mass of both parents (measured after spawning; mean-centered) as covariates. In the model of jelly mass, we included clutch mass (categorized as small, medium and large; see above) as fixed factor and its interaction with habitat type, then we performed pairwise comparisons by calculating linear contrasts between natural and either agricultural or urban habitat within each clutch-mass category and correcting the significance level for multiple testing by the FDR method (function 'emmeans').

**Results**

Fecundity corrected for female size did not differ significantly between the 3 habitat types (**Table II.3.1**, **Fig. II.3.1a**). Jelly mass was significantly larger in agricultural and urban clutches when clutch mass was small, whereas medium and large clutches had relatively large jelly mass irrespective of habitat type (**Table II.3.2**, **Fig. II.3.1b**). Pairs from agricultural and urban ponds had similarly high fertilization rate (**Table II.3.1**, **Fig. II.3.1c**) and embryo viability as pairs from natural ponds (**Table II.3.1**, **Fig. II.3.1d**).

Tadpoles originating from natural ponds were larger at the start of larval life than tadpoles with urban or agricultural origin (**Table II.3.1**, **Fig. II.3.1e**). Despite taking less time to develop into metamorphosed toadlets (**Table II.3.1**, **Fig. II.3.1f**), tadpoles from natural ponds did not have smaller mass at metamorphosis compared to tadpoles from anthropogenic ponds (**Table II.3.1**, **Fig. II.3.1g**). Furthermore, toadlets from natural ponds reached larger body mass by October compared to the animals with agricultural or urban origin (**Table II.3.1**, **Fig. II.3.1h**).

**Discussion**

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

**Figure II.3.1.** Reproductive capacity of common toad pairs in relation to habitat type (N: natural, A: agricultural, U: urban). Boxplots show the distribution of observed data (thick middle line: median, box: interquartile range; whiskers extend to the most extreme data points within 1.5 × interquartile range from the box; empty circles are data points falling outside of the latter range); grey error bars show the mean ± SE fitted by GEE models.

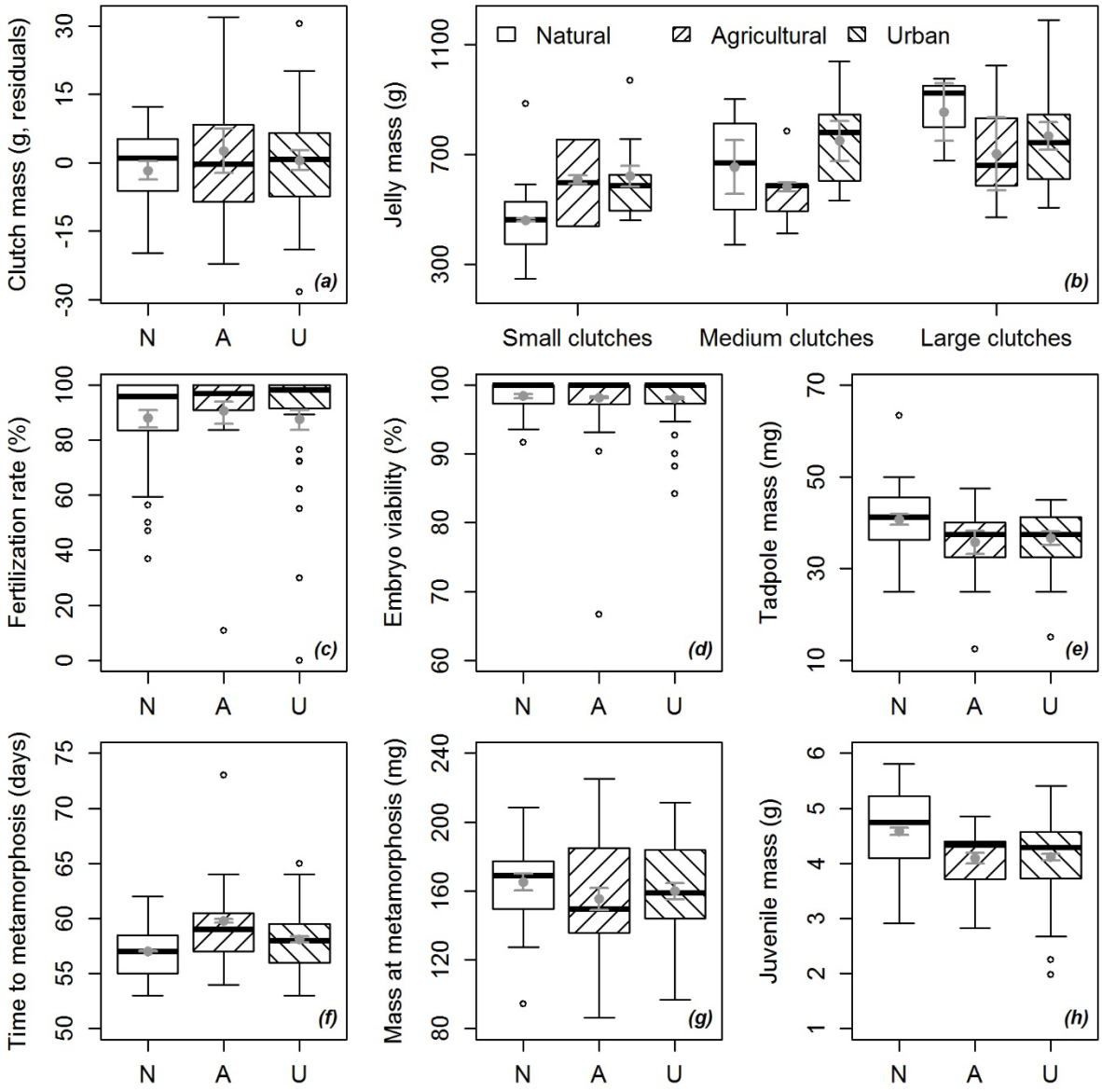


**Table II.3.1.** Reproductive capacity of toad pairs in relation to habitat type (GEE models).

| Dependent variable | Model parameters | Estimate ± SE | P |
|---|---|---|---|
| Clutch mass (g, residuals) | natural habitat, mean | -1.690 ± 2.020 | 0.400 |
| | agricultural - natural | 4.310 ± 5.270 | 0.410 |
| | urban - natural | 2.270 ± 2.940 | 0.440 |
| Fertilization rate (logit) | natural habitat, mean | 2.012 ± 0.245 | <0.001 |
| | agricultural - natural | 0.265 ± 0.520 | 0.610 |
| | urban - natural | -0.06 ± 0.291 | 0.840 |
| | mother's body mass | 0.001 ± 0.009 | 0.950 |
| | father's body mass | 0.006 ± 0.069 | 0.930 |
| Embryo viability (logit) | natural habitat, mean | 4.097 ± 0.235 | <0.001 |
| | agricultural - natural | -0.053 ± 0.300 | 0.860 |
| | urban - natural | -0.132 ± 0.254 | 0.600 |
| | mother's body mass | -0.007 ± 0.006 | 0.250 |
| | father's body mass | 0.010 ± 0.020 | 0.610 |
| Tadpole mass (mg) | natural habitat, mean | 40.855 ± 1.183 | <0.001 |
| | agricultural - natural | -5.320 ± 2.705 | 0.049 |
| | urban - natural | -4.355 ± 2.137 | 0.041 |
| | mother's body mass | 0.007 ± 0.037 | 0.853 |
| | father's body mass | 0.028 ± 0.136 | 0.838 |
| Time to metamorphosis (days) | natural habitat, mean | 57.17 ± 0.209 | <0.001 |
| | agricultural - natural | 2.585 ± 0.289 | <0.001 |
| | urban - natural | 0.876 ± 0.284 | 0.002 |
| | mother's body mass | 0.008 ± 0.010 | 0.461 |
| | father's body mass | -0.011 ± 0.035 | 0.747 |
| Mass at metamorphosis (mg) | natural habitat, mean | 164.294 ± 4.351 | <0.001 |
| | agricultural - natural | -7.637 ± 6.448 | 0.240 |
| | urban - natural | -4.030 ± 6.364 | 0.530 |
| | mother's body mass | 0.063 ± 0.160 | 0.700 |
| | father's body mass | -0.734 ± 0.496 | 0.140 |
| Juvenile mass (g) | natural habitat, mean | 4.561 ± 0.049 | <0.001 |
| | agricultural - natural | -0.269 ± 0.065 | <0.001 |
| | urban - natural | -0.436 ± 0.070 | <0.001 |
| | age at mass measurement | 0.036 ± 0.007 | <0.001 |
| | mother's body mass | 0.001 ± 0.002 | 0.778 |
| | father's body mass | 0.031 ± 0.011 | 0.006 |

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

**Table II.3.2.** Jelly mass (g) in relation to habitat type (FDR-corrected linear contrasts from GEE model, N = 90 clutches).

| Clutch mass | Contrast | Estimate ± SE | P |
|---|---|---|---|
| Small | natural - agricultural | -147.0 ± 7.8 | <0.001 |
| | natural - urban | -158.5 ± 19.1 | <0.001 |
| Medium | natural - agricultural | 72.7 ± 49.0 | 0.138 |
| | natural - urban | -94.6 ± 60.6 | 0.138 |
| Large | natural - agricultural | 152.2 ± 84.7 | 0.138 |
| | natural - urban | 87.5 ± 58.1 | 0.138 |

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

The lower mass and slower development of toad offspring from anthropogenic habitats may be due to several, mutually non-exclusive phenomena. One possibility is that reduced offspring performance is a lasting consequence of chemical pollution. It is now well established that EDCs can have transgenerational effects, such that early-life exposure of parents leads to adverse health outcomes in offspring and later generations (Anway *et al.*, 2005; Bhandari *et al.*, 2015b). Such effects can occur even when the exposed parents show no phenotypic abnormalities (Bhandari *et al.*, 2015b). Alternatively, reduced offspring size may be a cost of an adaptive resistance to contaminants. For example, several urban fish populations have evolved tolerance to toxic pollutants (Meyer & Di Giulio, 2003; Whitehead *et al.*, 2012), but their offspring had reduced growth rates in clean water and were more susceptible to other stressors compared with the offspring of conspecifics from a non-contaminated site (Meyer & Di Giulio, 2003). Similarly, in frogs, evolved pesticide tolerance along an agricultural land-use gradient was found to be associated with susceptibility to parasites (Hua *et al.*, 2017). Similar trade-offs may have been responsible for poor performance in the offspring of our toads from anthropogenic habitats. For example, if the latter produce more or different jelly material to provide tolerance against EDCs or other pollutants (see above), they might have less resources to allocate into the eggs (Podolsky, 2004), and their offspring might not be able to catch up from this initial handicap in egg size or

quality (Loman, 2002). Furthermore, because oocytes mature while females are on their post-spawning feeding grounds (Wells, 2007), egg nutrient content may be affected by terrestrial EDC exposure of females; research on such effects on vitellogenesis is virtually absent and highly needed.

Besides pollution, several other environmental factors may differ between anthropogenic and natural habitats, and many of these environmental differences may select for local adaptation in life-history traits. For example, common-garden experiments have shown that tadpole development is faster in populations breeding in temporary pools where desiccation risk favors earlier metamorphosis compared to permanent pools (Lind *et al.*, 2008), and growth efficiency is higher in populations living in harsh environments such as northern latitudes and high altitudes (Lindgren & Laurila, 2005). If anthropogenic ponds are less likely to dry out or if larval competition is low (e.g. due to small population size in fragmented landscapes), this may relax the selection on growth and developmental rates. Also, compared to natural forest habitats, ponds in urbanized and agricultural landscapes may have less closed-canopy vegetation and shade, which would result in higher temperatures, more food for tadpoles, and altered predator fauna, all of which might result in relaxed selection on growth and development speed (Van Buskirk & Arioli, 2005). Disentangling these possible effects of human-induced habitat changes on life-history evolution, and thereby population viability, is an important challenge for future research.

Taken together, while we found no sign of pollution-related reproductive failure in adult toads, our results suggest reduced vigor in offspring originating from anthropogenic habitats. Our study thus highlights that investigating transgenerational EDC effects and resistance to contaminants may be crucially important for furthering our understanding of the consequences of chemical pollution from evolutionary, ecological and conservationist points of view.

## II.4. Sex reversal[5]

In ectothermic animals, various environmental stimuli can cause a developmental effect rarely seen in endotherms: sex reversal, whereby individuals exposed to such stimuli during their embryonic or larval life develop the sexual phenotype opposite to their genetic sex (Baroiller & D'Cotta, 2016; Flament, 2016; Whiteley *et al.*, 2021a). This phenomenon is similar to (and can be considered a form of) environmental sex determination, such as the temperature-dependent sex determination of many turtles and crocodilians; however, sex reversal pertains to species whose sex-determination system involves not only environmental sensitivity but also a strong genetic component such as sex chromosomes. Sex reversal occurs in fish, amphibians, and reptiles in nature (Alho *et al.*, 2010; Baroiller & D'Cotta, 2016; Lambert *et al.*, 2019; Whiteley *et al.*, 2021a; Xu *et al.*, 2021), and theoretical studies caution that it may have far-reaching consequences including skewed sex ratios, sex-chromosome evolution, and even population extinction (Perrin, 2009; Grossen *et al.*, 2011; Wedekind, 2017; Schwanz *et al.*, 2020). Laboratory experiments show that sex reversal can be induced by anthropogenic stressors like chemical pollution and elevated temperature (Flament, 2016; Tamschick *et al.*, 2016a). Thus, we may expect that the contemporary and future increase in the levels of anthropogenic stressors will influence the rates of sex reversal in free-living populations of ectothermic vertebrates. Whether this influence would be an increased or decreased sex-reversal frequency in anthropogenic environments is not a trivial question, for the following reasons.

On the one hand, sex reversal may happen more often in areas where the chemical and thermal stimuli triggering it are more pervasive, such as in agricultural areas polluted by pesticides and in urban heat islands. This may be simply a consequence of sex-reversing stimuli being more frequent in such habitats. Alternatively or additionally, sex reversal might also be an adaptive response to anthropogenic environments, given that adjusting phenotypic sex to environmental conditions can be adaptive (Geffroy & Douhard, 2019), and sex reversal might be an evolved mechanism for achieving the sexual phenotype that best matches the environment, similarly to environmental sex determination (Schwanz & Georges, 2021). Thus, selection may facilitate the spread of sex reversal in populations persisting in anthropogenic environments, especially because the propensity to develop into one sex or the other can exhibit genetic or epigenetic inheritance (McGaugh & Janzen, 2011; Piferrer & Anastasiadi, 2021).

On the other hand, sex reversal may be costly in terms of fitness. Due to their sex-chromosome genotype, sex-reversed individuals may be unable to produce daughters or sons (Wedekind, 2017), and therefore may be selected against by sex-ratio selection (Schwanz & Georges, 2021). Also, sex-reversed individuals may perform poorly in traits that influence survival or reproductive success (Pandian & Sheela, 1995; Senior *et al.*, 2012). In such situations, we can expect resistance to sex reversal to be adaptive in environments where sex-reversing stressors are pervasive. As a result of such adaptation, populations exposed to sex-reversing environments might maintain the same or similar (low) frequency of sex reversal as unexposed populations.

Assessing sex-reversal frequencies in wild populations has been hindered by the difficulty of diagnosing sex reversal in non-model organisms. Due to the high evolutionary lability and homomorphy of sex chromosomes in ectothermic vertebrates, genetic sexing methods are available

[5] This chapter is based on the following publication: Nemesházi E., Sramkó G., Laczkó L., Balogh E., Szatmári L., Vili N,, Ujhegyi N., Üveges B., Bókony V. 2022. Novel genetic sex markers reveal unexpected lack of, and similar susceptibility to, sex reversal in free-living common toads in both natural and anthropogenic habitats. Molecular Ecology 31: 2032-2043. http://real.mtak.hu/145523/

only for a small fraction of species (e.g. Alho *et al.*, 2010; Baroiller & D'Cotta, 2016; Tamschick *et al.*, 2016; Lambert *et al.*, 2019; Whiteley *et al.*, 2021; Xu *et al.*, 2021). In two of such species, recently developed genetic sex markers have been used to investigate whether sex reversal is more prevalent in anthropogenic habitats, and they reported contradictory answers: yes in one frog species (see later in **Chapter III.1**) but no in another (Lambert *et al.*, 2019). Furthermore, no study to our knowledge has yet tested whether animal populations living in anthropogenic habitats have increased or reduced inherent propensity for sex reversal.

In this study, we first aimed to produce a reliable molecular marker set for diagnosing genetic sex in the common toad. This species has a female-heterogametic (ZZ/ZW) sex-chromosome system (Dufresnes *et al.*, 2020), and is liable to chemically induced sex reversal (Hayes, 1998). Then, using our novel marker set, we investigated whether the frequency of sex reversal in toads differed between natural, agricultural, and urban habitats. Finally, we performed a common garden experiment to test whether toads originating from these different types of habitat differ in their susceptibility to sex reversal induced by chemical pollutants. We focused on the sex-reversing effects of two EDCs with high prevalence in surface water in agricultural and urban areas, respectively: glyphosate, the most used herbicide worldwide (Brovini *et al.*, 2021), and 17α-ethinylestradiol (EE2), a common ingredient of contraceptives that pollutes natural water bodies *via* wastewater (Bhandari *et al.*, 2015a). Both EDCs may cause male-to-female sex reversal based on their effects on estrogenic enzymatic activities, female-skewed sex ratios, and intersex gonads observed in amphibians (Howe *et al.*, 2004; Lanctôt *et al.*, 2014; Bhandari *et al.*, 2015a; Tamschick *et al.*, 2016b). As both chemicals have been in use for about half a century, we can expect resistance to have potentially evolved in populations chronically exposed to these pollutants. Similar, rapid evolutionary changes due to anthropogenic habitat alterations have been documented in various taxa, including evolved tolerance to lethal effects of pollutants (Cothran *et al.*, 2013; Reid *et al.*, 2016; Johnson & Munshi-South, 2017; Marques da Cunha *et al.*, 2019; Brans *et al.*, 2021). Here we tested for altered susceptibility to a sub-lethal EDC effect (sex reversal) in common toad populations living in anthropogenic habitats.

## Methods

### *Data collection*

We captured 352 adult toads during the spawning seasons of 2016 and 2017 at 14 breeding sites that represent natural, agricultural, and urban habitats, with 4-5 sites per habitat type (**Table I.1, Fig. I.1**). We identified phenotypic males by their nuptial pads (N=216) and phenotypic females by the presence of eggs (N=136). We took a buccal swab or tissue sample from each individual and stored it in 96% ethanol for DNA extraction.

In 2017, we transferred 89 pairs of the captured adults to captivity and allowed them to spawn there, as described in **Chapters II.2-3**. Depending on the availability of females and their willingness to spawn in captivity, we had 1-15 egg strings (families) from each of 11 sites out of the 14 sites sampled for adult DNA (36, 16, and 37 families from natural, agricultural, and urban sites, respectively). When the captive-laid eggs became free-swimming tadpoles, we haphazardly selected 6 individuals from each family, distributed them among 6 treatments (control, solvent control, and 4 EDC treatments; N=534), and raised them for ca. 5 months as described in **Chapters II.2-3**. The control group was kept in clean RSW, and 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 tallow amines) 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, in which the rearing water

contained 1 µL/L ethanol, 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 liter of rearing water. Actual EDC concentrations were close to the nominal concentrations (Ujhegyi & Bókony, 2020). Both EDCs are documented to occur in our actual study ponds (see **Fig. I.4**). The lower and higher concentrations we used for each EDC represent the typical and maximum concentrations, respectively, reported from surface waters (Bhandari *et al.*, 2015a; Avar *et al.*, 2016; Bókony *et al.*, 2018b; Brovini *et al.*, 2021). The treatments lasted throughout the entire larval period for each individual and were renewed twice a week at each water change.

When the toadlets (N=417) reached the age by which their gonads are fully differentiated (Ogielska & Kotusz, 2004), we euthanized them using MS-222, and identified whether each individual had testes or ovaries by dissection. We stored the gonads in 10% buffered formalin and later examined them histologically by routine sectioning and staining procedures. In a few cases when we could not unambiguously categorize the gonads as testes or ovaries based on gross anatomy and histology, we treated the phenotypic sex as uncertain. We stored the body of dissected toadlets in 96% ethanol until extracting DNA from a foot sample.

***Marker development***

As this dissertation focuses on evolutionary-ecological results, the reader is referred to Nemesházi *et al.* (2022) for the full details of how we developed and validated the genetic sex marker set for common toads. In short, we applied Restriction-site Associated DNA Sequencing (RADseq) to identify sex-specific markers following Feron et al. (2021), using 24 toadlets with known phenotypic sex (11 males, 13 females) from the control group of the common garden experiment. Since these animals had not been exposed to any stimuli that are expected to cause sex reversal, they are likely to have phenotypic sex concordant with their genetic sex. We designed sequencing primers for polymerase chain reaction (PCR) amplification of potentially sex-linked loci to obtain Sanger sequences from 3 females and one male. Focusing on those loci where we obtained unambiguous sequences and found sex-linked differences, we sequenced 5 males and 5 females for the purpose of designing diagnostic sexing primers. For 4 sex-linked loci (c2, c5, c12 and c16), we optimized PCR primers for detection on agarose gel. Then we tested the performance of the latter 4 sex markers in 46 males and 36 females with unambiguous sexual phenotype from the control group, all being non-siblings and representing all 11 study sites used in the experiment.

***Identification of sex reversals***

We used our novel sexing primers to distinguish sex-reversed individuals from the animals with concordant genetic and phenotypic sex both among free-living adults and their juvenile offspring raised in our common-garden experiment. Detailed methods of DNA extraction, PCR settings, and agarose gel electrophoresis are described in Nemesházi *et al.* (2022). First, we used sex marker c16 for screening all those individuals from the common garden experiment that had not been sexed during the marker testing phase as well as the wild-caught adults (we chose c16 because both the Z-linked and W-linked PCR products of c16 were similarly bright on the agarose gel, and no third primer or enzymatic restriction was necessary for this marker). If the c16 genotype of an individual did not match its phenotypic sex, we genotyped the individual for sex marker c12 as well, to ensure correct assignment of the genetic sex. Individuals with uncertain sexual phenotype were sexed for at least two additional markers besides c16.

## Results

### *Marker development and validation*

RADSeq analysis identified 17 significantly sex-linked markers, out of which 13 showed female-biased pattern (as expected under ZW/ZZ sex determination), but two of the latter were suspected to be paralogue sequences. During marker development, we concentrated on the remaining 11 RAD loci and designed sequencing primer pairs for each. Out of these, 9 primer pairs produced bright PCR products of the expected fragment size, and we obtained unambiguous sequences from 7 loci (for details, see the supplementary material of Nemesházi *et al.*, 2022). We found sex-linked differences in the sequences of 4 loci (NCBI GenBank accession numbers: OK507208-15). Sexual genotypes based on each of our newly devised markers matched the sexual phenotype in all 82 non-sibling individuals chosen for marker validation, yielding 100% reliability for genetic sexing for each of the 4 markers.

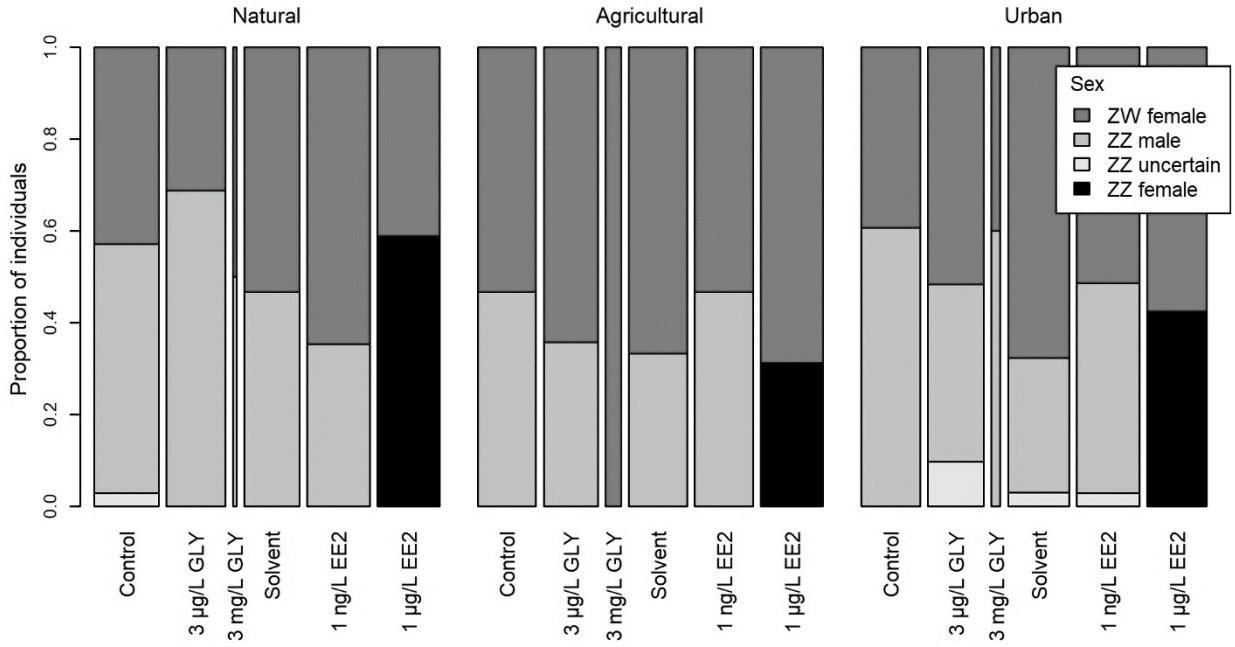
### *Sex reversal in the wild*

We successfully genotyped 349 wild-caught adults from 14 breeding ponds, while PCRs failed in 3 individuals. We found 135 concordant ZW females, 213 concordant ZZ males, and a single sex-reversed individual: a phenotypic male from an agricultural site (Határrét; see **Fig. I.1**). This toad was diagnosed as genetic female (ZW) by 3 out of 4 markers (c16, c2, and c12), while repeated PCRs with marker c5 gave ambiguous results.

### *Sex reversal in the common garden*

We successfully genotyped all 417 toadlets that survived until phenotypic sexing in the common garden experiment. We detected no sex reversal in any of the treatment groups, except for the higher concentration of EE2 (**Fig. II.4.1**). However, detection of sex reversal in the group treated with high concentration of the glyphosate-based herbicide might have been hindered by the high mortality rate in this group (**Fig. II.4.1**). In the high concentration EE2 treatment, all genetic males developed into phenotypic females, regardless of their original habitat type (**Fig. II.4.1**). The ovaries of the male-to-female sex-reversed individuals (ZZ females) were anatomically and histologically indistinguishable from the ovaries of concordant (ZW) females (for color pictures, see Fig. S3 in the supplementary material of Nemesházi *et al.*, 2022). Additionally, 6 individuals with uncertain sexual phenotype were found to be genetically males (ZZ). Four of these toadlets, all originating from urban ponds, had intersex gonads (one testis and one ovary, or testes containing oogonia); three of them had been treated with the lower concentration of the glyphosate-based herbicide and one with the lower concentration of EE2 (**Fig. II.4.1**). In the remaining two toadlets we could not unambiguously ascertain if the gonads were intersex or normal testes (for color pictures of the intersex and uncertain gonads, see Fig. 3 in Nemesházi *et al.*, 2022).

**Figure. II.4.1.** Proportion of concordant (ZW female and ZZ male) and sex-reversed (ZZ female) individuals in each treatment group (GLY: glyphosate, EE2: 17α-ethinylestradiol) by habitat type of the parents' capture site. Phenotypic sex is either uncertain or intersex in individuals designated as ZZ uncertain. Bar widths are proportional to sample size, which varies between 2 and 35 due to differences in survival (detailed sample sizes are given in Table S2 in the supplementary material of Nemesházi *et al.*, 2022).




**Discussion**

The novel sex markers developed in our study confirmed that Hungarian populations of the common toad are female heterogametic, echoing recent findings from Switzerland (Dufresnes *et al.*, 2020). Our new marker system enables accurate identification of the genetic sex in this species. While the majority of amphibian species to which genetic sexing methods have been established feature XX/XY sex determination (Yoshimoto *et al.*, 2008; Alho *et al.*, 2010; Baroiller & D'Cotta, 2016; Lambert *et al.*, 2019; Nemesházi *et al.*, 2020; Xu *et al.*, 2021), our new marker set provides a cheap and easy-to-use method for future studies aiming to understand sex-reversal mechanisms in an anuran species with ZW/ZZ sex determination.

Our field study on several hundreds of adult toads found only a single case of sex reversal: 3 out of our 4 sex markers confirmed that the W chromosome was present in the DNA sample of one adult male captured at an agricultural site. The fourth marker (c5) gave inconclusive result, cautioning against this marker to be preferred for genetic sexing in future studies. In the lack of further swab samples from this animal, we cannot completely exclude the possibility of DNA contamination. Nevertheless, we had the highest number of phenotypic male samples from the pond where this individual was captured; thus, finding a single sex reversal at this site is compatible with the idea of an existing but very low frequency of sex reversal in the studied common toad populations. This almost complete lack of sex reversal is surprising, because we found many EDCs in the studied ponds, with higher concentrations in anthropogenic areas (see **Chapters I** and **II.1**).

Furthermore, we found a considerable number of female-to-male sex-reversed agile frogs in some of these ponds in the same years (see later in **Chapter III.1**). Thus, the lack of sex reversal in toads cannot be explained by the general lack of sex-reversing effects in the studied sites. Instead, this result may suggest that toad populations living in more polluted areas might have evolved resistance to sex reversal, thereby showing the same undisrupted sex development as their conspecifics in natural habitats. However, this interpretation is not supported by the results of our common garden experiment, because the effects of sex-reversing EDC treatments on the offspring of the studied toads did not depend on their original habitat type. Instead, they either all showed no sex reversal at low-concentration of both EDCs, or showed 100% male-to-female sex reversal in the presence of high EE2 concentration. We only found a slight indication of habitat dependence of EDC susceptibility, suggesting that toads originating from anthropogenic habitats may be more, not less, susceptible to disrupted sex development: only urban toadlets displayed intersex gonads in a few cases when treated with ecologically realistic, low EDC concentrations. It remains to be tested if other EDC compounds or other concentrations within the range of the realistically low and close-to-maximum values that we applied here would reveal habitat-dependent sex-reversal probabilities in toads or any other species liable to sex reversal. Nevertheless, since most EDCs found in amphibian breeding habitats have estrogenic potential (Bókony *et al.*, 2018b), our treatments provide a good overall representation of the estrogenic EDC effects likely present in the field.

A possible explanation for our results could be that survival rate of sex-reversed juveniles might be low in the wild, resulting in low prevalence of sex-revered individuals among adults. The environmental stimuli that cause sex reversal may have other developmental effects that might reduce survival; for example, heat stress in agile frogs increases both sex-reversal rate and mortality (see later in **Chapter III.2**). However, a meta-analysis found no significant relationship between chemically induced sex reversal and mortality in aquaculture fish (Senior et al., 2012). Similarly, in our present study, toadlet survival was not reduced in the treatment group that showed 100% male-to-female sex reversal (see Table S2 in the supplementary material of Nemesházi *et al.*, 2022), although this might not be representative of their survival chances in the wild. Mortality can be especially high during the first winter hibernation (Üveges *et al.*, 2016), which was not assessed in the present study. Moreover, sex-reversed individuals may behave differently compared to sex-concordant conspecifics (Li et al., 2016; Senior et al., 2015). If their altered behavior also affects their microhabitat use or activity during the breading season, these individuals might be harder to find by conventional capturing methods. Next to nothing is known about the survival and behavior of sex-reversed individuals in nature (Wild *et al.*, 2022), so testing the above ideas will require further research.

As an alternative explanation that is not mutually exclusive with the above hypotheses, we speculate that the common toad may be relatively resistant to sex reversal, regardless of habitat type. In all other anuran species studied so far for sex reversal in free-living populations, female-to-male sex reversal was found in noticeable numbers (Alho *et al.*, 2010; Lambert *et al.*, 2019; Xu *et al.*, 2021), and a few cases of male-to-female sex reversal were also indicated (Lambert *et al.*, 2019). There are several differences between the common toad and the previously studied anuran species, which might contribute to the apparent difference in sex-reversal frequencies found in their wild populations. First, all species studied so far belong to the family Ranidae, whereas the common toad is a member of Bufonidae; and different phylogenetic lineages may show different sensitivity for certain sex-reversing conditions (Hayes, 1998; Chardard *et al.*, 2004; Orton & Tyler, 2015; Tamschick *et al.*, 2016b). Second, toads produce their defensive toxins from cholesterol, the precursor of steroid hormones (Daly, 1995), and they have been selected for resisting autotoxicity

(Moore *et al.*, 2009). This might have conferred them tolerance to other chemical perturbations which mimic the effects of steroid hormones (including estradiols and "stress hormones"), similarly to the cross-resistance provided by tolerance to certain pesticides in other anurans (Hua *et al.*, 2014). Third, sex reversal may be triggered by not only chemical but also thermal stimuli, and different species may have adapted to different temperatures. If sex reversal in free-living amphibians occurs mostly due to extreme temperatures (Lambert *et al.*, 2018), the lack of sex reversal in common toads might be explained by potentially high tolerance to heat. For example, tadpole survival at 27°C water temperature was found much higher in common toads than in Rana species (Morand *et al.*, 1997); similar inter-specific differences may exist in terms of susceptibility to heat-induced sex reversal (as discussed later in **Chapters III.2** and **IV.1**).

What makes different populations and species more or less susceptible to sex reversal is an important question for evolutionary ecology as well as for conservation biology. Possible reasons include constraints such as the degree of sex-chromosome heteromorphy (Miura *et al.*, 2016) and direct or indirect selection pressures. For example, artificial selection for increased fecundity in females can indirectly affect male sensitivity to estrogenic disruption of testis development and spermatogenesis (Spearow *et al.*, 1999). Such selection pressures may force populations to evolve or plastically modulate any element involved in the biochemical pathway that translates environmental stimuli into sex (Castelli *et al.*, 2020), e.g. by mutation of genes encoding hormone receptors (Castañeda Cortés *et al.*, 2019; Hamilton *et al.*, 2020). Thus, the vulnerability of phenotypic sex development may be shaped by multiple forces, which might explain why researchers so far had mixed success in finding clear-cut relationships of sex-reversal rate with environmental factors such as climate (e.g. Castelli et al., 2021 vs. Dissanayake et al., 2021) and urbanization (Lambert et al., 2019 vs. **Chapter III.1**) or with taxonomy (Senior et al., 2013). Even when a clear correlation is present, the underlying mechanisms are difficult to ascertain: for example, estrogenic pollution in river stretches is associated with high frequency of intersex in fish but not with polymorphisms in genes involved in responses to EDCs (Hamilton *et al.*, 2020). Our present results with common toads add to this complex picture, emphasizing the need for further research on sex reversal in a wide diversity of species. The remaining chapters revolve around this issue.

# *III. Sex reversal in agile frogs*

## III.1. Anthropogenic land use[6]

As explained in **Chapter II.4**, ectothermic vertebrates are highly vulnerable to climate change and chemical pollution because thermal and chemical disturbances during embryonic or larval development can cause sex reversal (Eggert, 2004; Ospina-Álvarez & Piferrer, 2008; Holleley *et al.*, 2016), which theoretically can lead to serious consequences for natural populations, including changes in genetic variability, distorted sex ratios, and even extinction (Quinn *et al.*, 2011; Wedekind, 2017; see also later in **Chapters IV.2-3**). Therefore, it is imperative to gain information on the prevalence and fitness of sex-reversed individuals in natural populations, for being able to assess and forecast the effects of anthropogenic environmental changes.

For studying sex reversal, identifying genetic sex can be especially difficult in non-model organisms, due to lack of information on sex-linked DNA sequences. In the majority of ectothermic vertebrates, sex chromosome turnover (i.e. the swapping of the chromosome used for genetic sex determination) is common, and the sex chromosomes of many species are homomorphic (Devlin & Nagahama, 2002; Holleley *et al.*, 2016; Miura, 2017; Jeffries *et al.*, 2018). Consequently, there is often little homologous sex-linked variation between and sometimes even within species, making molecular sexing challenging (Ezaz *et al.*, 2006; Perrin, 2009; Stöck *et al.*, 2013). Furthermore, the type of sex-chromosome system (i.e. male or female heterogamety: ZZ/ZW or XX/XY) can differ between closely related species or even between different populations of the same species, especially in amphibians (Sarre *et al.*, 2011; Holleley *et al.*, 2015; Rodrigues *et al.*, 2017).

For the above reasons, genetic sexing methods need to be developed and validated species by species in amphibians. Recombination between the sex chromosomes (Ezaz *et al.*, 2006; Perrin, 2009; Stöck *et al.*, 2013) is expected to be reduced in the vicinity of the "master sex-determination gene" (Bachtrog, 2006; van Doorn & Kirkpatrick, 2007; Bachtrog *et al.*, 2014), providing a preferential target for sex marker development. Unfortunately, the master sex-determination gene remains elusive in all but a few amphibian species (Eggert, 2004; Yoshimoto *et al.*, 2010; Nakamura, 2013; Miura, 2017), and the size of the non-recombining region around it can be small. Thus, in order to find markers which make reliable identification of the sex chromosomes possible in the species of interest, researchers must test high numbers of loci across the genome (Olmstead *et al.*, 2010; Stöck *et al.*, 2011; Lambert *et al.*, 2016). Owing to these challenges, reliable sex-linked markers only exist for a handful of amphibian species so far (Eggert, 2004; Berset-Brändli *et al.*, 2006; Alho *et al.*, 2010; Olmstead *et al.*, 2010; Stöck *et al.*, 2011; Lambert *et al.*, 2016; Ma *et al.*, 2016; Brelsford *et al.*, 2017; Rodrigues *et al.*, 2017). Due to this general lack of sex markers, we know troublingly little about sex reversals in nature: how widespread they are, which environmental factors they are associated with, and how they affect individual fitness and population viability. While we found almost zero sex reversal across a wide range of habitats in common toads (see **Chapter II.4**), 9% of genetic females were phenotypically male in a Finnish common frog (*Rana temporaria*) population, and 8.5% female-to-male and 3% male-to-female sex reversal was found in green frogs (*Rana clamitans*) in the USA (Alho *et al.*, 2010; Lambert *et al.*, 2019).

[6] This chapter is based on the following publication: Nemesházi E., Gál Z., Ujhegyi N., Verebélyi V., Mikó Z., Üveges B., Lefler K.K., Jeffries D.L., Hoffmann O.I., Bókony V. 2020. Novel genetic sex markers reveal high frequency of sex reversal in wild populations of the agile frog (*Rana dalmatina*) associated with anthropogenic land use. Molecular Ecology 29: 3607-3621. http://real.mtak.hu/115403/

In this study, we investigated sex reversal in the agile frog. In this species, sex chromosomes were identified only recently, showing a male-heterogametic (XX/XY) sex-determination system (Jeffries *et al.*, 2018). First, we developed a genetic sexing method for this species; then we studied the occurrence of sex reversal in wild agile frog populations in North-Central Hungary. We also tested if sex-reversed individuals were more common in populations associated with anthropogenic land use. Finally, we examined if sex reversal was associated with fitness costs by comparing fitness-related traits between sex-reversed and sex-concordant individuals.

**Methods**

***Data collection***

We captured 162 adult agile frogs (121 males and 41 females) from 11 ponds at the start of the breeding season in February-March in 2016 and 2017. The capture sites were chosen to represent the range of habitats the species occupies, on a natural to anthropogenic scale (**Table I.1, Fig. I.1**). Distances between capture sites varied from 4 to 60 km. Sample size varied between sites due to variation in capture success. The adults were sexed by secondary sexual characteristics (nuptial pads in males) and presence of eggs (gravid females). Buccal swab samples were taken from all wild-caught frogs and stored in 96% ethanol until DNA extraction. We measured the adult frogs' body mass (± 0.1 g) and released them at their capture sites.

For sex marker development, we used 125 froglets (59 males and 66 females; from 34 clutches) collected as freshly spawned eggs in 2018 from three different ponds of the same geographical region (Kerek-tó, Pilisvörösvár, and Szárazfarkas; **Table I.1**). These individuals were raised in laboratory under conditions that are unlikely to cause sex reversal, because the animals were not exposed to endocrine-disrupting chemicals or to extreme temperatures or to any other stressor that would trigger sex reversal to our knowledge (Eggert, 2004; Lambert *et al.*, 2018; Castañeda Cortés *et al.*, 2019). Thus, we expected that among these animals sex reversal would be absent or occur very rarely due to sex-chromosome recombination (Ezaz *et al.*, 2006; Perrin, 2009; Stöck *et al.*, 2013) or random processes affecting sex determination (Perrin, 2016). Water temperature during tadpole development was 18.45 ± 0.81 (mean ± SD) °C; all other details of animal housing and care were similar to those used for laboratory-raised toad tadpoles in **Chapters II.2-4** and are described in detail in an open-access paper (Bókony *et al.*, 2020). Froglets were phenotypically sexed by gonad anatomy (see Fig. S3 in the supplementary material of Nemesházi *et al.*, 2020) during dissection 2 months after metamorphosis (ca. 16 weeks after reaching the free-swimming tadpole stage). At this age the gonads are well differentiated in this species (Ogielska & Kotusz, 2004; Bernabò *et al.*, 2011). We weighed the froglets (± 0.01 g) and euthanized them as we did with toadlets (**Chapters II.2-4**). During dissection, we cut out the entire digestive tract and weighed it to be able to calculate lean mass (i.e. body mass minus gut mass, to eliminate differences due to food remains). We recorded any visceral abnormality observed in the animals, and we collected the following data as proxies for fitness. Frogs have fat reserves attached to the cranial end of the gonads in the form of finger-like fat bodies, which are important for overwinter survival (Scott *et al.*, 2007). We categorized the size of the fat bodies in each froglet into one of four subjective categories: none, small, medium, or large. Because the size and pigmentation of the spleen provide information on immune function and pathogen resistance in anamniotes (Hadidi *et al.*, 2008; Steinel & Bolnick, 2017), we photographed the spleen of each froglet at 45× magnification with a camera attached to the stereomicroscope. We also photographed the males' testes at 16× magnification, and we carefully removed all gonads and fixed them in neutral-buffered 10% formalin for histological examination. We took a tissue sample (hind feet) from each froglet that we stored in 96% ethanol until DNA extraction.

***Marker development***

As this dissertation focuses on evolutionary-ecological results, the reader is referred to Nemesházi *et al.* (2020) for the full details of how we developed and validated the genetic sex marker set for agile frogs. In short, we used 14 putatively sex-linked sequences that were identified by RADseq from a sample of 40 agile frogs from a single clutch in Switzerland (Jeffries *et al.*, 2018), and a genome assembly of the common frog to design sequencing primers for our agile frogs. Sanger sequencing of 5 male and 5 female adults yielded 3 loci that contained sex-linked (Y-specific) single-nucleotide polymorphisms (SNPs). For these loci we designed sexing primers, and we tested each marker on the 125 laboratory-raised froglets by PCR (markers Rds1 and Rds3) or high-resolution melting (HRM, for marker Rds3). Based on the results of this analysis, we used the following method to identify sex-reversed individuals (juveniles and adults). First, we screened all individuals for the marker with the highest sex linkage and we accepted an individual to be concordant male or female if its genotype was in accordance with its phenotypic sex. Those individuals that seemed to be sex-reversed by this approach were additionally screened for the marker with the second highest sex linkage, and were accepted to be sex-reversed only if both markers confirmed sex reversal. Individuals with discrepant genotyping results were considered to be of unknown genetic sex.

***Human land use and sex reversal***

We quantified the intensity of anthropogenic land use for each capture site in two ways, based on the geoinformatics measurements of landscape variables in a 500-m wide belt zone around each pond (**Table I.1, Fig. I.1**). First, we calculated "total anthropogenic land cover" by summing the proportions of arable land, pastures, residential and public built-up areas, and roads for each pond. In the second approach, we performed a principal components analysis using the six landscape variables, which yielded two PC axes with >1 eigenvalue, explaining 82.1% of variation in total. Based on the PC loadings (**Fig. I.1**), we designated the habitat scores along these two axes as "urban PC scores" and "agricultural PC scores".

As females are more difficult to find and capture than males, the majority of the investigated adults were males, so we had too few females to provide a reliable estimate of female-to-male sex-reversal rate (i.e. the proportion of phenotypic males among genetic females) in adults. Therefore, we report the proportion of sex-reversed individuals (XX males) among the phenotypic males (hereafter referred to as XX/male ratio) as a measure of female-to-male sex-reversal frequency. We analyzed the relationship between the numeric habitat variables and XX/male ratio of the adult frogs in generalized linear models with binomial error distribution, using the 'brglm' function in R package 'brglm'. This analysis weights each site by sample size (the number of phenotypic males in our case) and appropriately handles separation (i.e. there were no sex-reversed adults at certain sites) by the maximum penalized likelihood method. One model contained total anthropogenic land cover as the only predictor; the other model contained the two PC score variables simultaneously.

***Phenotypic correlates of sex reversal***

In the adult frogs, we compared body mass between sex-reversed individuals (XX males) and concordant (XY) males using a linear mixed-effects model with capture site as a random factor (using the 'lme' function). Because most of the captured females were gravid, we did not include them in the analysis of adult body mass.

In the laboratory-raised froglets, we compared the following indices of health and fitness between sex-reversed individuals (XX males) and concordant individuals (XY males and XX

females): size of the fat bodies, size and pigmentation of the spleen, mean size of the two testes, and the frequency of visceral abnormalities observed during dissection (see **Chapter III.3** for further analyses on fitness-related traits, including growth and development, as a function of sex reversal in agile frogs). From the photographs, we measured the area ($mm^2$) of the spleen and each testis with the "freehand selections" tool and the percentage area of pigmented spots on the spleen with the "threshold" tool of the ImageJ software (Schneider *et al.*, 2012). These measurements had high repeatability (ICC: 0.856 – 0.993) both between and within persons (Bókony *et al.*, 2020). For the statistical analysis of fat-body size, we used cumulative link mixed (CLM) model (function 'clmm' from package 'ordinal') with family (clutch) as random factor, whereas for spleen and testis measurements we used generalized least-squares (GLS) models ('gls' function in 'nlme' package; in these models we did not include family as random factor, as most families were represented by one or a few individuals because some photos could not be measured due to insufficient image quality). In all these models, we included lean body mass as a covariate, and sex as a three-category fixed factor (XX males, XY males, and XX females). Since visceral abnormalities were very rare, we used Fisher's exact tests to compare their frequency between sex-reversed and concordant individuals (males and females pooled).

Histological analysis of the sex-reversed froglets' gonads was performed by routine sectioning and staining procedures to examine if sex reversal was accompanied by intersex. Our preliminary study showed that sex categorized by gonadal anatomy matched sex categorized by histology in 100% of 32 froglets (17 males, 15 females) that had been raised without any chemical treatment in 2016, using the same lab protocol as in 2018. Therefore, to minimize the costs of histological analysis, we chose to analyze gonad histology only in those lab-raised froglets from 2018 for which the identified genetic sex did not match the phenotypic sex categorized by gonad anatomy (i.e. to check if the mismatch was due to erroneous categorization of phenotypic sex).

**Results**

***Novel sex markers***

All of the 125 laboratory-raised froglets were successfully genotyped with all three markers. The strongest sex-linkage was shown by Rds3 (95% match between phenotypic sex and genotype at the locus), followed by Rds1 (89% match) and finally Rds2 (70% match). Because we had not exposed the laboratory-raised froglets to sex-reversing effects, we concluded that Rds2 is not suitable for genetic sexing in our populations. Based on Rds3 and Rds1, 6 out of the 125 froglets qualified as sex-reversed (all XX males), yielding a female-to-male sex-reversal rate of 8%, and an XX/male ratio of 10%. Four out of these 6 sex-reversed animals had both XX and XY siblings (making it unlikely that they were identified as XX due to the presence of null alleles or as an outcome of recombination or mutation, i.e. X-SNPs on Y), whereas two of them came from a family in which we found only XX siblings (N=12), suggesting that the latter family might have been fathered by an XX male.

***Sex reversal in nature***

Out of 162 wild-caught adults, 152 were genotyped unambiguously: we identified 89 concordant males (XY), 41 concordant females (XX), and 22 sex-reversed XX males, but no male-to-female sex reversal (for 10 phenotypic males, genetic sex could not be identified: all were XX based on Rds3, but two were XY based on Rds1, while Rds1 genotyping failed in the other 8). The overall XX/male ratio was 20% across wild populations, being two times higher than in the laboratory-raised animals.

Among the wild-caught adults, XX/male ratio increased significantly with total anthropogenic land cover (**Table III.1.1, Fig. III.1.1**). Similarly, XX/male ratio increased significantly with higher "agricultural PC scores", and it showed a marginally non-significant positive relationship with "urban PC scores" (**Table III.1.1, Fig. III.1.1**). Notably, sex reversal occurred even at the least anthropogenic sites (**Fig. III.1.1**), and XX/male ratio increased on average from 12.8% to 29.3% as total anthropogenic land cover increased from zero to 50% (**Table III.1.1, Fig. III.1.1**).

***Fitness correlates of sex reversal***

Among the wild-caught adults, sex-reversed XX males had similar body mass as concordant XY males (LME, difference: 1.47 ± 1.29 g, $t_{98}$ = 1.15, P = 0.254; **Fig. III.1.2**). However, among the lab-raised animals we found increased spleen size in the sex-reversed XX males, while their spleen pigmentation, fat-body size, and mean testis size did not differ from those of concordant individuals (**Table III.1.2**, **Fig. III.1.3-4**). Liver abnormalities occurred more frequently in sex-reversed than in concordant froglets (stunted or missing liver lobes: 33% *vs.* 0.8%, P = 0.009; greyish liver coloration: 50% *vs.* 6.4%, P = 0.006), and there was a similar, marginally non-significant difference in the incidence of strong visceral pigmentation (50% *vs.* 15.2%, P = 0.067), which is a general sign of physiological stress in anamniotes. In two out of the 6 froglets that were genetically female but had testes with normal anatomy, histological analysis revealed oogonia in otherwise normal testicular tissue (see Fig. S3 in the supplementary material of Nemesházi *et al.*, 2020), in contrast to the 17 males dissected in 2016 that all had testes without oogonia. These two intersex individuals had small testes relative to their body size and age (**Fig. III.1.3**). The remaining 4 sex-reversed individuals showed completely normal testicular histology in the examined sections. In those two individuals that had XX siblings only (possibly sired by an XX male; see above), testis size was large relative to their body size and age (**Fig. III.1.3**), and testis histology showed a more mature developmental stage than in the rest of the histologically examined individuals.

**Figure III.1.1.** Relationship between the XX/male ratio in adult agile frogs and human land use across 11 breeding ponds. The curves show the probabilities that a phenotypic male sampled in a breeding pond is genetically female, in relation to the proportion of anthropogenic area (**a**), the urban PC score (**b**), and the agricultural PC score (**c**), as estimated from the models in **Table III.1.1**.

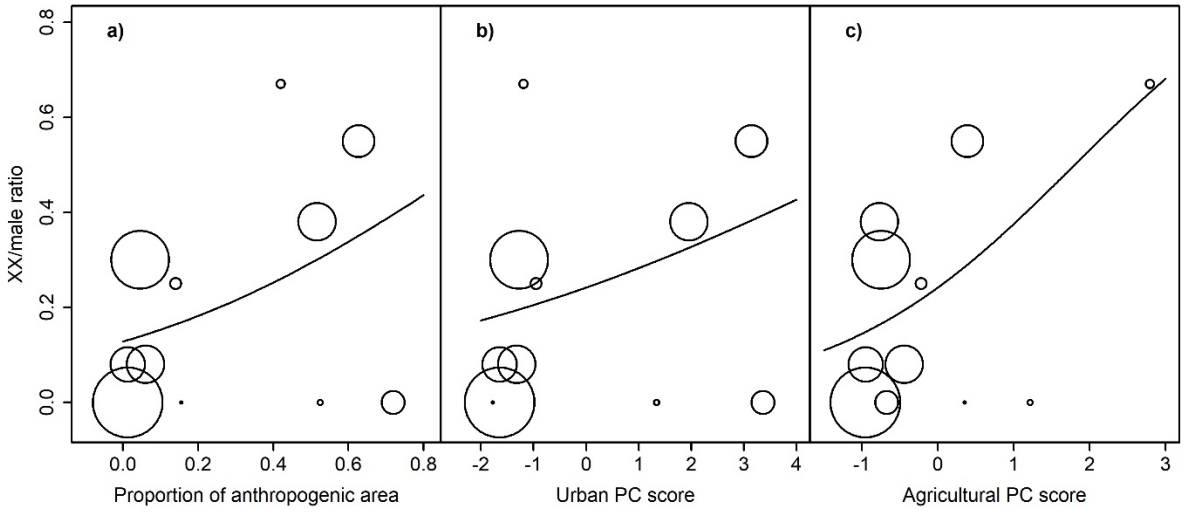


**Table III.1.1.** Parameter estimates (*b*) of the binomial models relating the proportion of XX males in all phenotypic males to the land use of the capture site.

| Model | Parameters | *b* | SE | z | P |
|---|---|---|---|---|---|
| Model 1 | Intercept | -1.917 | 0.357 | -5.365 | <0.001 |
| | Total anthropogenic land cover | 2.076 | 0.856 | 2.424 | 0.015 |
| Model 2 | Intercept | -1.144 | 0.273 | -4.195 | <0.001 |
| | Urban PC | 0.212 | 0.121 | 1.756 | 0.079 |
| | Agricultural PC | 0.634 | 0.292 | 2.175 | 0.030 |

The parameter estimates are on logit scale. Inverse logarithmic transformation of the intercept ($e^b$) gives the odds of a phenotypic male being a genetic female when the value of the predictor variables is zero; for the remaining parameter estimates, exp-transformation gives the proportional change in this odds value (i.e., the odds ratio) for one unit change of the predictor variable.

**Table III.1.2**. Parameter estimates (*b*) of the statistical models comparing female-to-male sex-reversed and concordant froglets.

| Dependent variable | Model parameter | *b* | SE | t | P |
|---|---|---|---|---|---|
| Size of fat bodies* | Body mass | 0.840 | 0.590 | 1.425 | 0.154 |
| (N = 6 + 66 + 53) | Sex-reversed – XX females | 0.085 | 0.830 | 0.103 | 0.918 |
| | Sex-reversed – XY males | 0.215 | 0.842 | 0.255 | 0.799 |
| Spleen size ($mm^2$) | Sex-reversed XX males | 0.763 | 0.078 | 9.822 | < 0.001 |
| (N = 4 + 19 + 15) | – XX females | -0.154 | 0.087 | -1.776 | 0.085 |
| | – XY males | -0.212 | 0.087 | -2.428 | 0.021 |
| | Body mass | 0.404 | 0.108 | 3.754 | 0.001 |
| Spleen pigmentation (%) | Sex-reversed XX males | 2.785 | 0.614 | 4.540 | 0.000 |
| (N = 5 + 18 + 14) | – XX females | -0.590 | 0.710 | -0.830 | 0.413 |
| | – XY males | -0.046 | 0.720 | -0.064 | 0.950 |
| | Body mass | -0.552 | 0.908 | -0.608 | 0.547 |
| Testes size ($mm^2$) | Sex-reversed XX males | 1.611 | 0.084 | 19.182 | < 0.001 |
| (N = 6 + 0 + 24) | – XY males | 0.085 | 0.188 | 0.453 | 0.654 |
| | Body mass | 1.309 | 0.291 | 4.504 | < 0.001 |

For each model, sample size (N) is given as the number of sex-reversed individuals + number of concordant females + number of concordant males. All covariates were mean-centered before the analyses. Therefore, the parameter "Sex-reversed XX males" refers to the mean value of sex-reversed individuals, and the parameters "– XX females" and "– XY males" give the difference between the respective group and sex-reversed individuals.

*Cumulative link mixed model; the test statistic is z (for all other dependent variables, t values from GLS models are shown). Number of individuals in each category of fat-body size (none, small, medium, large), respectively: 0, 4, 0, 2 in sex-reversed individuals, 7, 15, 39, 5 in concordant females, and 7, 14, 22, 10 in concordant males.

**Figure III.1.2.** Body mass of concordant XY males (N=89) and sex-reversed XX males (N=21) among wild-caught adult agile frogs. In each boxplot, the thick middle line and the box represent the median and interquartile range, respectively; the whiskers extend to the minimum and maximum.

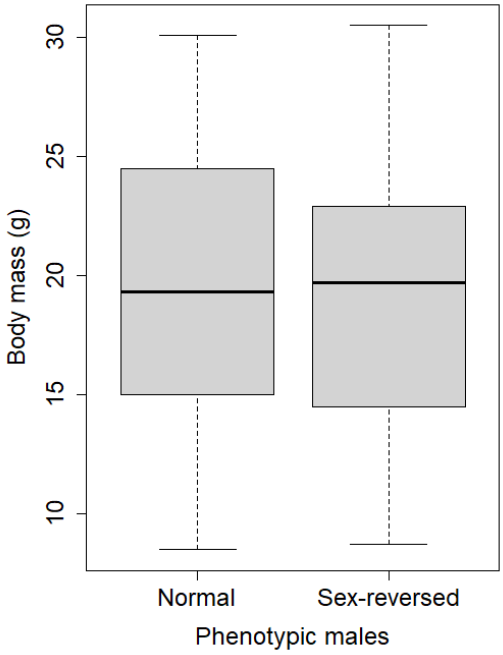



**Figure III.1.3**. Testis size in concordant (circles) and sex-reversed (squares) male froglets. The solid line is a regression line fitted for all phenotypic males. Two sex-reversed males with testicular oocytes are marked with black squares. Two other sex-reversed males that had no XY siblings (possibly sired by an XX male) are marked with grey squares.

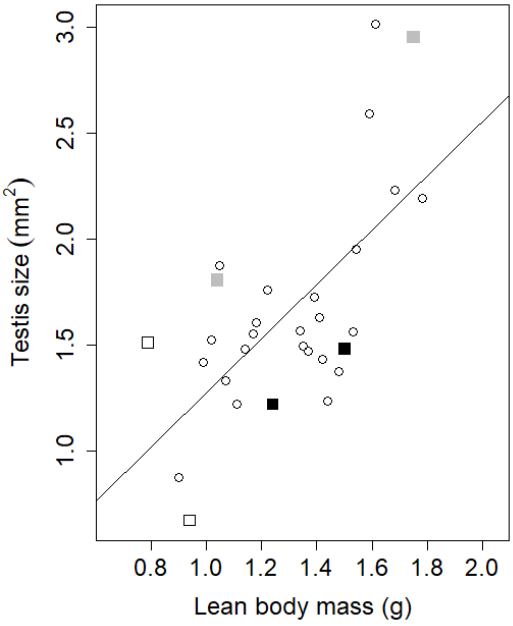
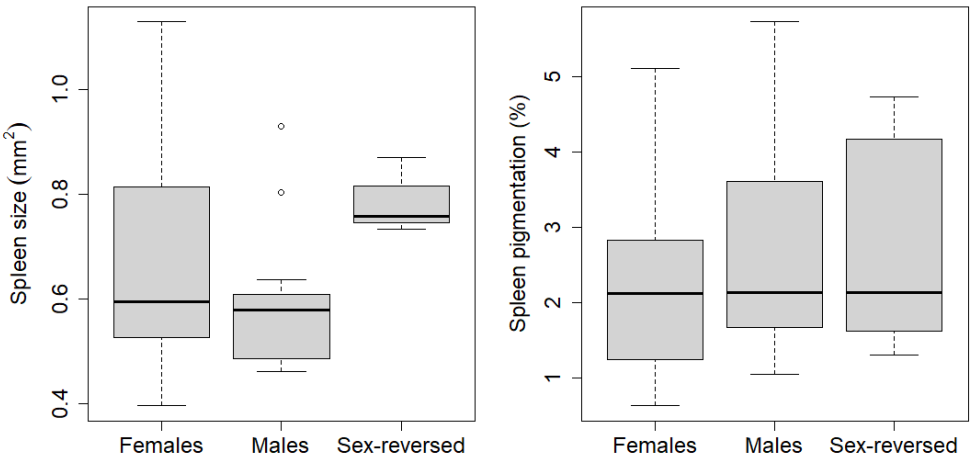



**Figure III.1.4.** Spleen size and pigmentation in lab-raised froglets (concordant XX females, concordant XY males, and sex-reversed XX males). Boxplot interpretation is as in **Fig. II.3.1**.



**Discussion**

Our marker set of Rds1 and Rds3 enables genetic sexing in agile frogs with high confidence (at least 95 %; see next paragraph). Based on a genome sequence assembly of a closely related species, the common frog, it seems that these two markers cover a suitably large region of the sex chromosomes, being at 112 million nucleotides distance from each other. Using two sex-linked markers that are located relatively far from each other on the sex chromosomes makes it highly unlikely that we would misdiagnose rare mutation or recombination events as sex reversal, which can happen when only one marker is used (Toli *et al.*, 2016). Because amphibian sex determination can vary even within species (Rodrigues *et al.*, 2014; Miura, 2017), the reliability of our sex markers should be tested before applying them in other, especially distant populations (Rodrigues *et al.*, 2014; Lambert *et al.*, 2016). However, genetic diversity of the agile frog is in general very low across Europe (Vences *et al.*, 2013), suggesting that our markers may be sex-linked in other agile frog populations as well, facilitating sex-reversal studies on this declining species potentially throughout its distribution range.

According to our markers, 6 out of 125 laboratory-raised froglets were genetically females (XX) with male phenotype (testes), despite being raised under controlled conditions with presumably no sex-reversing effects. There are several potential explanations for these mismatches. First, phenotypic sex might have been erroneously categorized; however, we can exclude this possibility because the phenotype based on gonad morphology was corroborated by histology in the mismatching individuals. Second, the presence of "sex races" could result in false assumption of sex reversal; for example, in the common frog, some individuals develop ovaries first that turn into testes later (Rodrigues *et al.*, 2015). This would cause overestimation of the proportion of XY females, which we did not find in our study at all. Third, the mismatches may have been due to recombination or mutation (Ezaz *et al.*, 2006; Perrin, 2009; Stöck *et al.*, 2013); however, 4 out of the 6 concerned froglets had XY siblings in our sample, suggesting that both Rds3 and Rds1 genotypes of chromosome Y were normal in their families. Although multiple paternity occurs in agile frogs, it is rare (Lodé & Lesbarrères, 2004; Lodé *et al.*, 2005). Thus, this explanation requires the combination of two rare events: multiple paternity, and recombination or mutation on the Y chromosome of one of the fathers. The fourth interpretation is that the mismatching individuals were indeed sex-reversed, which we consider most likely. Recent studies suggest that sex reversal may be a natural phenomenon in ectothermic vertebrates (Lambert, 2015; Holleley *et al.*, 2016; Lambert *et al.*, 2019), and stochastic variation in gene expression levels may lead to sex reversal even in the absence of environmental effects on sex determination (Perrin, 2016). Alternatively, but not mutually exclusively, sex reversal may result not only from random variation but also from stressful stimuli, as experiments with fishes showed that various forms of physiological stress can induce sex reversal, and glucocorticoid "stress hormones" (activated by the hypothalamus-pituitary-interrenal glands axis) mediate this process (Fernandino *et al.*, 2013; Castañeda Cortés *et al.*, 2019). Therefore, we suspect that a few of our lab-raised animals might have experienced relatively high levels of physiological stress despite the generally favorable lab conditions, and this led to sex reversal. Their developmental abnormalities may have been either the cause or the consequence of the stress that ultimately caused their sex reversal; in either case, our findings suggest that sex reversal can be associated with poor health. For example, enlarged spleen may indicate infections (Hadidi *et al.*, 2008), while changes in liver size and coloration may result from malnutrition or hypoxia (Harper & Wolf, 2009).

Despite the latter findings suggesting that sex-reversed individuals might have poor viability in nature, we found a relatively high number of sex-reversed adults in free-living agile frog populations. Genetically XX phenotypic males made up ca. 20% of phenotypic males and ca. 35%

of genetic females, although the latter rate of female-to-male sex reversal is probably overestimated because we had relatively low capture success (small sample size) for females. These numbers are relatively high compared to those reported for natural populations of two other frog species in the Ranidae family (Alho *et al.*, 2010; Lambert *et al.*, 2019). Interestingly, we found no difference in body mass between sex-reversed and concordant adult males. This suggests that those sex-reversed individuals that survive to adulthood in nature may be able to mate, because male body size influences success in competition for mates (Vági & Hettyey, 2016). Their reproduction might still fail, however, if sex reversal reduces fertility, as reported in fish (Senior *et al.*, 2012) and indicated by some of our findings with the lab-raised froglets, i.e. three sex-reversed juveniles had small testes and two of them had testicular oogonia (intersex). However, other findings of our study suggest that at least some of the sex-reversed individuals might be fertile. First, four out of six sex-reversed froglets showed normal testicular histology, and three of them had relatively large testes. Second, we found one family that was likely to be sired by an XX male: 12 laboratory-raised animals that were randomly chosen as eggs from a single clutch were all XX individuals, which would have a very low chance of happening merely by accidental sampling if the clutch had the theoretically expected 1:1 sex ratio. Sex-reversed individuals were found to be fertile in some ectothermic vertebrates (Edmunds *et al.*, 2000; Devlin & Nagahama, 2002; Holleley *et al.*, 2015), and in common frogs XX males appear to be fertile and as successful in mating as XY males (Alho *et al.*, 2010; Veltsos *et al.*, 2019). If sex-reversed individuals do reproduce in nature, the biased sex ratios of their progeny may lead to changes in the population sex ratio, sex-chromosome frequencies, and ultimately the sex-determination system (Quinn *et al.*, 2011; Wedekind, 2017; see also later in **Chapters IV.2-3**). Furthermore, the offspring of sex-reversed individuals may themselves be more susceptible to sex reversal, as suggested by empirical results from laboratory experiments (Piferrer & Anastasiadi, 2021).

We found higher female-to-male sex-reversal frequency in breeding populations exposed to anthropogenic land use, and our results suggest that both urbanization and agriculture may contribute to this relationship. Both kinds of anthropogenic habitats are polluted by various chemicals (**Fig. I.4**), many of which have demonstrated sex-reversing effects (Reeder *et al.*, 1998; Kloas *et al.*, 1999; Hayes *et al.*, 2002; Eggert, 2004; Nakamura, 2013; Tamschick *et al.*, 2016b). Our result that sex reversal occurred even in the least anthropogenic habitats concurs with our earlier finding that those habitats are not devoid of chemical pollutants either (**Fig. I.4**). Furthermore, the increased female-to-male sex-reversal rate that we found in some urban agile frog populations may as well be due to the urban heat island effect (**Fig. I.2**; Brans *et al.*, 2018a), given that high temperature during larval development is a known inducer of sex reversal in amphibians (Chardard *et al.*, 2004; Lambert *et al.*, 2018). Variation in chemical, thermal, and potentially other stressors might complicate the relationship between sex-reversal rate and anthropogenic land use. In line with this, no correlation was found between sex-reversal frequency and urbanization along a forest-suburban gradient in green frogs (*Rana clamitans*; Lambert et al., 2019), although the frequency of testicular oocytes increased with urban land cover (Skelly *et al.*, 2010). Similarly, several but not all studies found a positive association between agricultural land use and amphibian intersex (Orton & Tyler, 2015), laryngeal demasculinization (Zlotnik *et al.*, 2019) and reduced spermatogenesis (McCoy *et al.*, 2017). These reports together with our results emphasize the need for further studies on sex-reversal frequency and its causes and consequences in wildlife populations with environmentally sensitive sex determination. Developing novel sex markers for non-model species will be a key step in this endeavor.

## III.2. Heat and estrogenic pollution[7]

As discussed in **Chapters II.4** and **III.1**, both climate change and environmental contaminants pose a special threat to species that are liable to sex reversal. Exposure to high temperatures during the sensitive period of sexual development usually leads to female-to-male sex reversal in amphibians and fish, and also in some reptiles (Edmands, 2021), and this phenomenon is expected to become more frequent as a result of global climate change. Water temperature in ponds, where the young of many aquatic vertebrates develop, can already reach as high as 30-50 °C during spring and summer, even under temperate and highland-Mediterranean climates (Lambert et al., 2018; Lindauer et al., 2020; **Fig. I.2**), and heat waves may have particularly strong effects on populations living in urbanized areas because of the urban heat island effect (Brans *et al.*, 2018a; Lambert *et al.*, 2019). Accordingly, trends observed in phenotypic sex ratios suggest that the expected increase in heat-induced masculinization has already started in some species with environmentally sensitive sex determination (Grayson *et al.*, 2014; see later in **Chapter IV.2**).

In addition to climatic challenges, several pollutants that are released into the environment act as endocrine disruptor chemicals, which can affect somatic and sexual development by interfering with the hormonal system of animals (Orton & Tyler, 2015). The interactions (i.e. non-additive effects) of these two major environmental factors, i.e. temperature and EDCs, are being investigated on various phenomena with increasing intensity (Noyes & Lema, 2015; DeCourten *et al.*, 2019), as climate change may modify the sensitivity of organisms to EDCs, while the chemicals may damage the capacity of organisms to respond to rapidly changing climatic conditions (Hooper *et al.*, 2013; Noyes & Lema, 2015). Although simultaneous effects of temperature and pollutants on sex have been studied in taxa with temperature-dependent sex determination (Mizoguchi & Valenzuela, 2016; Decourten & Brander, 2017), very little is known about the combined effects of heat and EDCs on sex reversal, specifically. Results from experiments on fish showed that high temperature amplified the effect of clotrimazole, a female-to-male sex-reversing EDC present in many fungicides (Brown *et al.*, 2015), while treatment with the female sex hormone 17β-estradiol completely neutralized the female-to-male sex-reversing effect of high temperatures (Kitano *et al.*, 2007, 2012). However, these studies used high concentrations of EDCs, which exceeded environmentally relevant concentrations by an order of magnitude.

In natural waters, hormonally active chemical agents typically occur in low concentrations (Loos *et al.*, 2009), although many of the more persistent EDCs may be enriched in shallow, small water bodies and in their sediments (**Fig. I.4**, Bókony *et al.*, 2018b). As discussed in **Chapter II.4**, 17α-ethinylestradiol is often found in surface waters and ground waters worldwide, typically in concentrations of a few ng/L (Bhandari *et al.*, 2015a), and has a strong male-to-female sex-reversing effect (Bhandari *et al.*, 2015a; Tamschick *et al.*, 2016b). Sensitivity to this effect differs

[7] This chapter is based on the following publications:

Mikó Z., Nemesházi E., Ujhegyi N., Verebélyi V., Ujszegi J., Kásler A., Bertalan R., Vili N., Gál Z., Hoffmann O.I., Hettyey A., Bókony V. 2021. Sex reversal and ontogeny under climate change and chemical pollution: are there interactions between the effects of high temperature and 17α-ethinylestradiol on early development in agile frogs? Environmental Pollution 285: 117464. http://real.mtak.hu/129485/

Ujszegi J., Bertalan R., Ujhegyi N., Verebélyi V., Nemesházi E., Mikó Z., Kásler A., Herczeg D., Szederkényi M., Vili N., Gál Z., Hoffmann O.I., Bókony V.*, Hettyey A.* 2022. "Heat waves" experienced during larval life have species-specific consequences on life-history traits and sexual development in anuran amphibians. Science of the Total Environment 835: 155297. http://real.mtak.hu/145522/

*Joint senior authors

among species, with effective concentrations varying from as low as 1.8 ng/L (Berg *et al.*, 2009; Gyllenhammar *et al.*, 2009) to as high as 500 ng/L (Tamschick *et al.*, 2016b) or even 1 µg/L (Mackenzie *et al.*, 2003). It is unclear if the low concentrations of EE2 that occur in nature can compensate for the female-to-male sex-reversing effect of high temperatures that occur during heat waves, and if there are any other interactions between the effects of heat and EE2 that may influence the ecological consequences of climate change in polluted waters.

In this study, our aim was to investigate the simultaneous effects of high temperature and EE2 on the sexual development of the agile frog. We performed an experiment to mimic environmentally realistic scenarios in which we exposed tadpoles for a few days to high temperature (simulating a heat wave) or EE2 (simulating a short-duration point source pollution) or both. We chose a temperature and EE2 concentration that are relatively high within the range of contemporary environmental conditions, but can be expected to occur with increasing frequency in nature because of climate change (Spinoni *et al.*, 2015; Tomczyk & Bednorz, 2019) and ongoing urbanization. Furthermore, because both high temperature and EE2 are known to influence mortality, growth, and somatic development during early ontogeny in aquatic vertebrates (Hogan *et al.*, 2008; Gyllenhammar *et al.*, 2009; Orizaola & Laurila, 2009; Tompsett *et al.*, 2012; Lambert *et al.*, 2018; Hu *et al.*, 2019; Marques da Cunha *et al.*, 2019), we also investigated whether there is interaction between the effects of high temperature and EE2 on these fitness-related traits besides sexual development. Since sex ratios can be biased not only by sex reversal but also by sex-specific tolerance to lethal environmental stressors (Medina *et al.*, 2002; Afonso *et al.*, 2003; Liwanag *et al.*, 2018), it is important to distinguish between environmental effects on sex-specific mortality *versus* on sex determination (Geffroy & Wedekind, 2020).

## Methods

### *Data collection*

On 8 March 2019 we collected 60 agile frog eggs from each of 4 freshly laid clutches (families) from 3 ponds located in a hilly woodland (Apátkút in **Table I.1**, and two further natural sites 3.4-4 km away: Ilona-tó and Katlan, both with zero anthropogenic land cover). Eggs were transported to our laboratory where we kept them at 16.3 ± 0.3 °C (mean ± SD), each of the 12 families separately in 5-L containers (24 × 16 ×13 cm) filled with 1.3 L RSW. To ensure sufficient oxygenation, we aerated the water in the containers with aquarium air pumps. At hatching (17 March), we raised the lab temperature to 19 °C. When the hatchlings reached the free-swimming stage 4 days after hatching, we started the experiment by randomly selecting 48 healthy-looking larvae from each of the 12 families (N=576 in total) and placing them into individual rearing containers (18 × 13 × 12.5 cm) where they were kept until the end of the study. The remaining eggs and tadpoles were released at their ponds of origin.

We combined 2 temperature treatments (19 °C or 30 °C) with 2 hormone treatments (0 or 30 ng/L EE2) and applied their combinations over one of 3 treatment periods. We replicated each treatment combination (temperature × EE2 × period) 4 times per family. We chose the nominal concentration of 30 ng/L EE2 for the following reasons. First, this value is environmentally realistic; for example, the average EE2 concentration in ponds, lakes and rivers is a few ng/L (Avar *et al.*, 2016; Mina *et al.*, 2018; Rechsteiner *et al.*, 2020), but higher concentrations up to 98.33 ng/L occur at point sources of pollution (Jakab *et al.*, 2020). Across small streams in our study region, the highest measured EE2 concentration was 27.97 ng/L (Kondor *et al.*, 2022). Also, in a previous study (Bókony *et al.*, 2018b) we found EE2 in the sediment of a pond where agile frogs breed (**Fig. I.4**). Second, a similar concentration (0.09 nM, equivalent to 27 ng/L) was shown to cause female-biased sex ratios in the common frog, a close relative of agile frogs (Pettersson & Berg, 2007). We

chose the 30 °C temperature treatment because it represents an environmentally relevant extreme that occurs in the ponds where we collected eggs for this experiment (e.g. the highest water temperature we recorded in Ilona-tó in 2020 was 38.16 °C) and in other water bodies where amphibians breed and develop in climates similar to ours (Lambert *et al.*, 2018; Lindauer *et al.*, 2020).

Outside the treatment period, we reared the tadpoles at 20.1 ± 1.1 °C (mean ± SD), their containers arranged in a randomized block design where each block contained all members of one family. The general housing and maintenance procedures were the same as in our previous tadpole experiments (**Chapters II.2-4** and **III.1**). Each tadpole was exposed to a treatment period of 6 days, starting either 2, 8, or 14 days after start of the experiment (period 1: developmental stages 25-28, period 2: developmental stages 29-32, period 3: developmental stages 33-37). During the treatment period, treated tadpoles experienced the following changes in rearing conditions. The volume of rearing water was increased from 1 to 1.7 L, and the container was placed in an 80 × 60 × 12 cm tray filled with ca. 18 L tap water (see Fig. S3 in the supplementary material of Ujszegi *et al.*, 2022). The tap water in the tray was circulated using an aquarium water pump and heated using an aquarium heater. This arrangement ensured homogeneous water temperatures in the tray and among the tadpole rearing containers. Each tray hosted 12 containers, one from each family, resulting in 4 trays in each treatment group. During the treatment period we changed the water of treated animals every other day with temperature-adjusted and chemical-administered RSW (according to experimental treatments) and fed the tadpoles with a reduced amount of spinach to prevent water fouling in the heat treatments. During treatment, the water of all treated tadpoles contained a minute amount of ethanol (1 µL 96 % ethanol in 100 L RSW) because this solvent was necessary for EE2 treatment. This ethanol concentration is much lower than those that were shown to damage tadpoles (Peng *et al.*, 2005; Taylor & Brundage, 2013; Fainsod & Kot-Leibovich, 2017) and in a previous experiment we showed that the same ethanol concentration did not result in skewed sex ratio or any gonadal abnormalities in agile frogs (Bókony *et al.*, 2020).

At the beginning of each treatment period, we filled the trays with 19 °C water and placed the tadpoles' containers (with freshly changed RSW) in the trays. In case of treatments involving elevated temperature, we subsequently turned on the heating. Thereby, water temperature gradually increased to 30 °C over the course of two hours and thereafter was maintained at 29.9 ± 0.2 °C (mean ± SD) in the tadpoles' containers. After 6 days of treatment, we turned off the heating in the trays, replaced the water in the rearing containers with 1 L fresh 30 °C RSW, and transferred them back to their original place in the laboratory. These procedures ensured that the tadpoles were not exposed to a sudden change in temperature. In the 19 °C treatments, we applied the same protocol, except that the water in the trays was not heated, only circulated; water temperature in these treatments was 18.8 ± 0.3 °C (mean ± SD) in the tadpole containers.

We exposed the tadpoles to waterborne EE2 similarly as in **Chapter II.4**. To determine how EE2 concentration changed during treatment, we took samples from the rearing water of treated tadpoles 3 times during the experiment. On each occasion, we collected one sample from each EE2 treatment right before water change. UPLC-UniSpray™ MS/MS analysis (for detailed methods see Bókony et al., 2021) showed that, over two days of treatment, the initial (nominal) 30 ng/L concentration has approximately halved at 19 °C (measured concentrations in the 3 replicate samples: 14.27, 15.93, 16.6 ng/L), and dropped to about a quarter at 30 °C (6.42, 7.53, 7.99 ng/L). This means that the EE2 concentrations experienced by each tadpole oscillated over a relatively large range of environmentally relevant values.

By starting the treatments 6, 12, or 18 days after hatching, we aimed to cover the majority of the larval period without risking that tadpoles would start to metamorphose during treatment,

because a preliminary experiment showed that exposure to 30 °C close to the metamorphic climax may result in increased mortality in agile frogs. We had no prior information on whether and when agile frogs have a sensitive time window for sex determination, but studies on other closely related species indicated such a time window either during early (Hogan *et al.*, 2008) or mid-late larval development (Lambert *et al.*, 2018). For each individual, we recorded the time to and body mass (± 0.1 mg) at metamorphosis (emergence of forelimbs) and raised them after metamorphosis as in our previous experiments (**Chapters II.2-4** and **III.1**).

We dissected the animals 6-8 weeks after metamorphosis (14-16 weeks after they reached the free-swimming tadpole stage) and measured lean body mass (without the digestive tract) using the same protocol as in **Chapter III.1**. We recorded whether the animal had fat bodies and categorized phenotypic sex as male (testes), female (ovaries), or uncertain (abnormally looking gonads). We stored the gonads in neutral-buffered 10 % formalin for histological analysis, and a tissue sample (hind feet) from each froglet in 96 % ethanol for DNA extraction. In light of the results in **Chapter III.1**, we analyzed gonad histology only in those froglets for which phenotypic sex was ambiguous based on gonad anatomy. In these cases, when phenotypic sex could be clearly identified by histology, the animal was considered as female or male; if mixed-sex tissues were found, the individual was considered as intersex. We used the method described in **Chapter III.1** to diagnose genetic sex.

***Statistical analyses***

We analyzed the effects of treatments using pre-planned comparisons (Ruxton & Beauchamp, 2008). For each dependent variable, we ran a model containing the three-way interaction of temperature treatment, EE2 treatment, and treatment period; block (family) was entered as random factor in each model. Then, using the model estimates we tested the overall effects of temperature, EE2, and their interaction within each treatment period by calculating *a priori* linear contrasts ('emmeans' function), correcting the significance of linear contrasts for multiple testing with the FDR method.

For the analysis of survival, we used Cox's proportional hazards model ('coxme' function of the 'coxme' package). Survival was categorized as 1: died during treatment, 2: died after treatment but before the start of metamorphosis, 3: died during metamorphosis, 4: died after metamorphosis before dissection, 5: the froglet survived until dissection (the latter group was treated as censored observations). Animals that died before their respective treatment period (16 individuals) were not included in this analysis.

To analyze time to metamorphosis, body mass at metamorphosis, and lean mass at dissection, we used linear mixed-effects models ('lme' function). In the analysis of time to metamorphosis, we allowed the variances to differ among treatment groups using the 'varIdent' function, because graphical model diagnostics indicated heterogeneous variances. When analyzing lean mass at dissection, we included the individual's age (number of days from finishing metamorphosis until dissection) as a covariate and the combination of genetic and phenotypic sex (i.e. a 3-category factor: concordant male, concordant female, or sex-reversed individual) as a fixed factor. From the latter analysis, we excluded 7 individuals for which genetic sex was unknown and 3 individuals that had intersex gonads.

For the analyses of the presence/absence of fat bodies and phenotypic sex ratio, we used binomial mixed-effects models ('glmmPQL' function of the 'MASS' package). The 7 individuals with unknown genetic sex and the 3 with intersex gonads were excluded from both models. In the model of fat bodies, the combination of genetic and phenotypic sex was added as fixed factor, and age of the froglets was included as a covariate.

For the analysis of sex reversal, we could not apply the same modeling framework as for sex ratio, because there was separation (i.e. the number of sex-reversed individuals was zero in certain treatment groups). Therefore, we used Firth's bias-reduced logistic regression ('logistf' function of the 'logistf' package), which yields less biased estimates when separation is present in the data as compared to logistic regression. Because this method does not accommodate random effects and linear contrasts, we could not include block as a random factor, and we obtained our pre-planned comparisons as the model estimates from 3 separate analyses, one for each treatment period. We restricted this analysis to genetically female (XX) individuals, and the dependent variable was phenotypic sex, i.e. whether or not the individual was sex-reversed.

To test if sex-reversed individuals differed from their concordant siblings in fitness-related traits, we repeated the above analyses of time to metamorphosis and body mass at metamorphosis by using only the combination of genetic and phenotypic sex as fixed factor (we did not add this factor into the full models above, because it would have decreased the sample size for testing the treatment effects: 476 animals reached metamorphosis but only 425 could be sexed). For the presence/absence of fat bodies and lean mass at dissection, we also repeated the above analyses by using only the combination of genetic and phenotypic sex as fixed factor, because the results indicated strong multi-collinearity between sex reversal and heat treatment.

**Results**

Regardless of treatment timing, phenotypic sex ratio and sex-reversal rate were both significantly affected by heat treatment ($P \leq 0.002$ in all periods), whereas EE2 and its interaction with heat had no significant effects ($P \geq 0.148$ in all periods). Heat treatment significantly increased the proportion of phenotypic males by inducing sex reversal in genetically female individuals (**Fig. III.2.1**): overall, 58 out of 199 genetically female individuals became phenotypic males, 55 of which received heat treatment. This female-to-male sex-reversing effect was strongest when heat was applied in the third period and weakest when it was applied in the second period (heat treatment × period: $P = 0.008$; **Fig. III.2.1**).

Additionally, the genetic sex ratio of froglets surviving to dissection was also male-biased for animals that received heat treatment in the first period (11 XX to 27 XY individuals, binomial test: $P = 0.014$); all other treatment groups had roughly 1:1 genetic sex ratios (ranging from 46.3 to 63.2 % males). Furthermore, we found 3 intersex individuals which came from different treatment groups: one received heat but no EE2 in the second period, one received EE2 but no heat in the third period, and one received both EE2 and heat in the third period. These animals were genetic females with abnormally looking gonads that were histologically identified as ovotestes (testes containing oogonia). We could not identify the genetic sex of 7 phenotypically male individuals that received heat treatment, because in all these cases the Rds3 genotype (corresponding to the locus with the highest sex linkage in our marker set) indicated sex reversal from female to male, whereas the Rds1 genotype suggested the genetic sex to be male.

Heat treatment significantly decreased survival (**Fig. III.2.1**), delayed metamorphosis and decreased body mass at metamorphosis (**Fig. III.2.2**), and increased the proportion of animals that had no fat bodies at all (without heat treatment: 4.5-13.2% per group, 7.2% in total; with heat treatment: 21.6-47.4% per group, 30.9% in total; $P < 0.010$). Most of these effects were similar across the 3 treatment periods (heat × period: all $P \geq 0.149$), except for the time to metamorphosis, which was delayed significantly more when heat was applied to younger tadpoles ($P = 0.019$; **Fig. III.2.2**). For lean mass of froglets at dissection, besides the main effect of heat, the three-way interaction (heat × EE2 × period) was also significant ($P < 0.001$). Heat applied in the first period increased froglet mass regardless of EE2 treatment, but for the other two periods the heat × EE2

interaction was significant (**Fig. III.2.2**). Without heat, EE2 applied in the second period tended to increase lean mass, whereas EE2 applied in the third period decreased mass, but these effects were reversed in the heat-treated groups (**Fig. III.2.2**). Thus, only the chemically untreated (no EE2) animals in the second period and the EE2-treated animals in the third period showed significant increase in lean mass in response to heat (**Fig. III.2.2**).

Compared to concordant males and females, sex-reversed individuals metamorphosed significantly later (by 4.9 ± 0.7 days; $t_{409} = 6.7$, $P < 0.001$) and with smaller body mass (by 32.7 ± 8.8 mg; $t_{409} = 3.7$, $P < 0.001$), and more often lacked fat bodies (odds ratio: 0.32 ± 0.11, $t_{406} = 3.4$, $P < 0.001$). Overall, there was no such difference in lean mass at dissection ($P = 0.814$); however, when the significant three-way interaction of treatment effects was taken into account, sex reversal reduced froglet mass by 5 % on average, to 1.15 ± 0.03 g compared to 1.22 ± 0.02 g in concordant females ($P = 0.020$) and 1.19 ± 0.02 g in concordant males ($P = 0.090$) across all treatment groups (see Fig. S5 in the supplementary material of Mikó *et al.*, 2021b). More detailed statistical results of all the above analyses are available in the supplementary material of Mikó *et al.* (2021b).

**Figure III.2.1.** Effects of heat (30 °C *versus* 19 °C) and EE2 (30 ng/L marked as EE2, *versus* none marked with a Ø symbol) treatments on phenotypic sex ratio and female-to-male sex-reversal in juvenile agile frogs treated in one of 3 different periods of larval development. Letters above the bars indicate the significant differences between treatment groups (in each panel, groups marked with different letters differ significantly with $P > 0.05$, whereas groups marked with the same letter do not differ according to FDR-corrected P-values from pairwise comparisons). Differences in sample size between treatment groups are due to mortality (without heat treatment: 6.3-16.7% mortality per group, 9.6% in total; with heat treatment: 19.1-58.7% mortality per group, 34.9% in total).

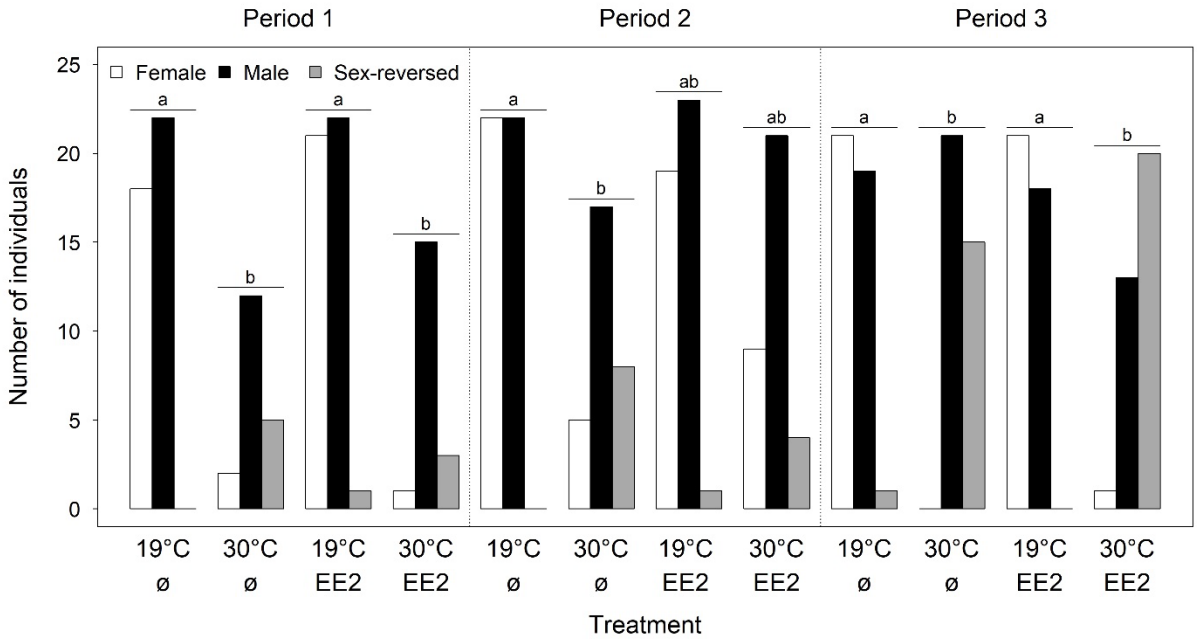
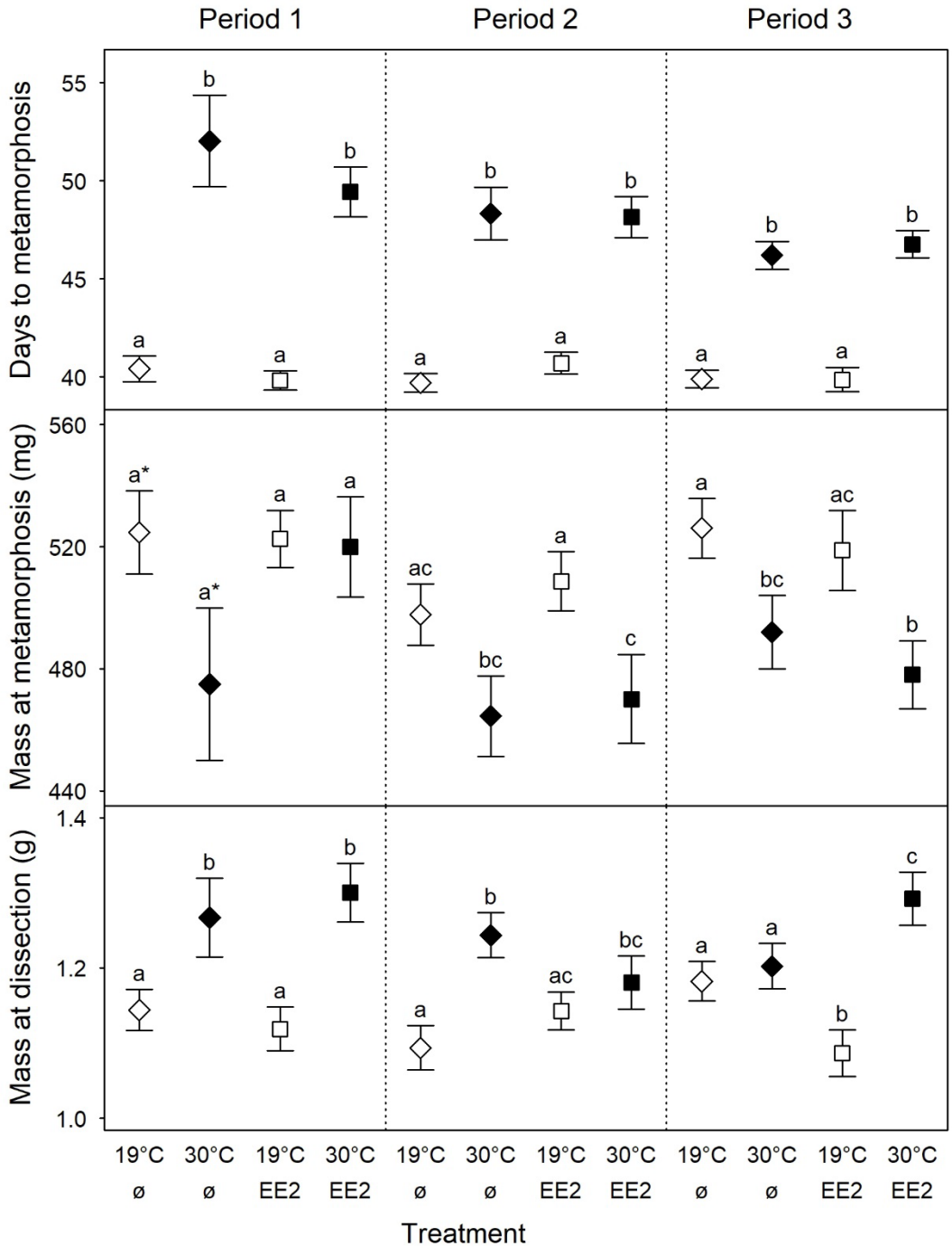


**Figure III.2.2.** Effects of heat (30 °C *versus* 19 °C) and EE2 (30 ng/L marked as EE2, *versus* none marked with a Ø symbol) treatments on larval developmental time, body mass at metamorphosis, and lean mass of juvenile frogs at dissection. Error bars show the means and standard errors estimated from the statistical models. Letters above error bars indicate the significant differences between treatment groups (in each panel, groups marked with different letters differ significantly whereas groups marked with the same letter do not differ according to FDR-corrected *P*-values from pairwise comparisons). The two groups marked with asterisk differ marginally (P = 0.064).



**Discussion**

In this study, we investigated the simultaneous effects of ecologically relevant larval exposure to high temperature and EE2 on the sexual development, survival, growth, and somatic development of the agile frog. We found that all measured variables were significantly affected by the heat treatment regardless of its timing, whereas EE2 and its interaction with heat had no significant effects except for froglet body mass. The simulated heat wave caused female-to-male sex reversal and had long-term negative effects on several fitness-related traits, suggesting that populations of the agile frog (and other species with similar sensitivity to heat) face multiple threats from climate change due to skewed sex ratios and poor survival, which are not countered by environmental concentrations of male-to-female sex-reversing pollutants like EE2.

In our experiment, the lack of EE2 effects on sex was unexpected, given that a similar concentration (27 ng/L) caused female-biased sex ratios in the closely related common frog (Pettersson & Berg, 2007). This discrepancy confirms that EE2 sensitivity can vary not only among phylogenetically distant lineages (Tamschick *et al.*, 2016b) but also within a genus (Mackenzie *et al.*, 2003). A notable difference is that previous studies showing female-biased sex ratios in response to EE2 in amphibians either exposed individuals throughout their entire larval development and/or used doses much higher than the environmentally realistic concentrations (Mackenzie *et al.*, 2003; Pettersson & Berg, 2007; Hogan *et al.*, 2008; Berg *et al.*, 2009; Gyllenhammar *et al.*, 2009; Tompsett *et al.*, 2013). Our treatments were ecologically relevant in terms of both magnitude and duration, as EE2 typically occurs in surface waters in the ng/L concentration range and its presence is usually not constant, possibly due to photolysis and adsorption to suspended solids and sediment (Bhandari *et al.*, 2015a; Avar *et al.*, 2016; Jakab *et al.*, 2020). Therefore, our findings suggest that population persistence in the agile frog is threatened more by climate change than by xenoestrogens. However, this conclusion does not necessarily apply to other species, as our results from a parallel experiment on common toads highlight that species can differ greatly in vulnerability to heat, too (**Box 2**).

Our experimental simulation of a 6-days heat wave confirmed that high temperatures during early ontogeny can result in male-biased sex ratios, which was found for several other amphibian and fish species in previous studies. However, these earlier experiments usually applied heat treatment throughout the entire larval period (Dournon *et al.*, 1990; Chardard *et al.*, 2004; Eggert, 2004; Ospina-Álvarez & Piferrer, 2008; Flament, 2016; Lambert *et al.*, 2018), which may not represent ecologically realistic temperature regimes in natural water bodies under current climatic conditions (Lambert *et al.*, 2018; Lindauer *et al.*, 2020; see also **Fig. I.2**). The bias towards phenotypic males in our agile frog experiment was mostly due to sex reversal, as 30-100 % of genetically female individuals (depending on treatment) developed testes after experiencing the heat weave. Thus, our study shows that even a relatively short hot spell, lasting only 6 days, can lead to a preponderance of males via sex reversal. Theoretical models suggest that such sex-ratio skews, due to increasing frequency of climate-driven female-to-male sex reversal, may have detrimental consequences for population viability (Wedekind, 2017; Schwanz *et al.*, 2020; see later in **Chapters IV.2-3**). Additionally, it seems that the high temperature in our experiment caused sex-dependent mortality in the youngest tadpoles, because genetic sex ratio at dissection was also male-biased when heat was applied in the first period. This highlights the importance of molecular sexing methods for diagnosing sex reversals and disentangling the mechanisms by which anthropogenic disturbances cause skewed sex ratios, i.e. sex reversal *versus* sex-biased mortality (Lambert *et al.*, 2016; Geffroy & Wedekind, 2020).

**Box 2. Heat waves are less harmful in toads than in frogs**

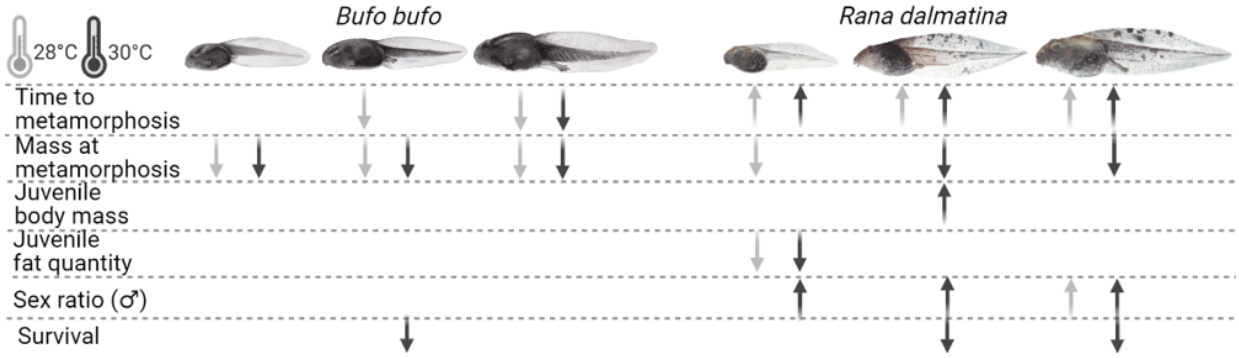
Using the same methods as in **Chapter III.2**, we studied the effects of a 6-days long exposure to either 28 °C or 30 °C in 3 consecutive larval developmental periods in both of our study species. These temperature extremes occurred in ponds that we monitored in 4.2% and 1.7% of recordings taken every 30 minutes from April to July when tadpoles develop there (**Fig. I.2**). While the experimental heat wave had negative effects on survival, somatic and sexual development, metamorphic mass, and juvenile fat stores in agile frogs[8] (as detailed in **Chapter III.2**), most of these effects were absent or even reversed in common toads (**Fig. III.2.3**). The only significant effect of heat in toads was a reduction of the time needed to reach metamorphosis, which led to smaller body mass at metamorphosis (**Fig. III.2.3**), whereas we observed no effects on toadlet body mass, fat stores, phenotypic sex ratio, or survival (apart from a slight increase in mortality in the toads that had been treated with 30°C in the middle period). In both species, treatment with 28 °C had similar effects as did treatment with 30 °C, but the latter reached statistical significance more often for than the former (**Fig. III.2.3**).

These results highlight that even sympatric species that are relatively similar in their ecology may be affected very differently by heat waves. The lower thermosensitivity of common toads aligns with the fact that their distribution range is wider, extending further into both hotter and cooler regions where agile frogs cannot be found (Agasyan *et al.*, 2009; Kaya *et al.*, 2009). Sympatrically, the breeding season starts ca. one month later in spring for common toads than for agile frogs in our study region, so tadpoles are likely to experience higher temperatures in the former than in the latter species. These spatio-temporal differences might have selected in common toads for higher physiological heat tolerance, and for adaptive phenotypic plasticity such as speeding up larval development to escape desiccating hot ponds even at the cost of smaller metamorphic mass (Richter-Boix *et al.*, 2011). Furthermore, the lower thermosensitivity of common toads compared to agile frogs may also explain why we found sex-reversed individuals in free-living populations of the latter (**Chapter III.1**) but not the former (**Chapter II.4**).

Our finding that phenotypic sex ratios in common toads were unaffected by heat treatment as high as 30 °C contradicts the frequently cited study by Piquet (1930), 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 common toads and spiny toads (*Bufo spinosus*), two species that were thought to be one at the time (Recuero *et al.*, 2012; Arntzen *et al.*, 2013; Dufresnes *et al.*, 2020). Notably, the spiny toad features an XX/XY sex-chromosome system (Skorinov *et al.*, 2018). Thus, it is likely that the study of Piquet (1930) is incorrectly cited in several reviews (e.g. Wallace *et al.*, 1999; Eggert, 2004; Ruiz-Garciá *et al.*, 2021) as evidence for temperature-induced sex reversal in common toads, and her findings potentially reflect female-to-male sex reversal induced in homogametic XX instead of heterogametic ZW individuals. Thus, our results caution that the information published earlier about the sensitivity of different species to various sex-reversing effects may need revisiting. **Chapter IV.1** elaborates this issue further.

[8] Note that EE2 was not applied to common toads in this experiment, and the comparisons depicted in **Fig. III.2.3** reflect the results of analyses restricted to hormonally untreated agile frogs but include those treated with 28 °C. The difference between the agile frog results presented in **Fig. III.2.3** and **Fig. III.2.2.** in froglet mass is due to the fact that the model for the latter included the effects of EE2 and sex reversal. Also, fat-body size was analyzed as four categories for **Fig. III.2.3** but as presence/absence for **Fig. III.2.2**.

**Figure III.2.3.** Changes in life-history traits and sex ratios in response to a 6-days experimental heat wave applied during the early, middle, or late period of larval development (represented by small, medium, and large tadpole images, respectively) in common toads and agile frogs. Arrows pointing up or down indicate significant positive (increase) or negative (decrease) effects, respectively. Detailed statistical results are available in Ujszegi *et al.* (2022).




In many ectothermic vertebrates, there is a thermosensitive period (TSP), a time window of limited length during early development, during which environmental temperatures can influence whether the bipotent gonad commits to male or female development (Eggert, 2004; Ospina-Álvarez & Piferrer, 2008; Mitchell & Janzen, 2010; Flament, 2016). In our experiment, tadpoles were between 6 and 24 days after hatching (developmental stages 25-37) during the treatment periods, and heat-induced sex reversal occurred in all three 6-days periods when the treatment temperature was 30°C. However, when it was 28°C, only the treatment applied in the third period triggered sex reversal (**Figure III.2.3**), suggesting that the most sensitive period in agile frogs occurs between developmental stages 33 and 37. We propose that TSP length and timing deserve more attention in ectothermic vertebrates that are susceptible to sex reversal, because a longer and later-occurring TSP may increase the risk that it coincides with extreme temperatures (e.g. summer heat waves) and thereby causes sex reversal or gonadal abnormalities.

Notably, 3 individuals in our experiment, out of 253 that did not receive heat treatment, showed a mismatch between genetic and phenotypic sex. This low "baseline" rate of sex reversal (1.2 % in the current study, which is similar to the 4.8 % found in **Chapter III.1**) aligns with the hypothesis that, besides genes and the environment, random processes can also affect sex determination (Perrin, 2016). In the wild, however, the frequency of female-to-male sex-reversed agile frogs was considerably higher, especially in anthropogenic habitats including urban ponds (**Chapter III.1**). The latter findings combined with our current experimental results suggest that anthropogenic stressors, such as heat waves exacerbated by the urban heat island effect, may increase sex-reversal rates above their baseline in natural populations.

Our current study also provides indirect but much-needed information about the relationship between sex reversal and individual fitness prospects. The results of our agile frog experiment show that, when sex reversal is triggered by heat stress, it is accompanied by reduced survival, slower development, lower body mass at metamorphosis, and less fat before the first winter hibernation. These changes suggest that sex reversal is associated with inferior fitness in agile frogs, corroborating **Chapter III.1** in which another, much smaller sample of spontaneously female-to-male sex-reversed froglets showed some signs of poor condition. These findings are in

line with the emerging view that sex reversal is mechanistically linked with physiological stress, as studies on fish and reptiles suggest that extreme temperature may be one out of many stressors that influence sex development *via* the activation of the hypothalamus-pituitary-interrenal axis (Fernandino *et al.*, 2013; Castañeda Cortés *et al.*, 2019) or cellular pathways of the calcium and redox regulation system (Castelli *et al.*, 2020). However, empirical data on the relative fitness of sex-reversed individuals in nature are very few and controversial (Senior *et al.*, 2012; Holleley *et al.*, 2016; Wild *et al.*, 2022). For example, despite the apparently poor health of sex-reversed agile frogs in the laboratory, body mass of sex-reversed adults did not differ from concordant males' in free-living populations (**Chapter III.1**). See the next chapter for more on this issue.

In our current experiment on agile frogs, the negative effect of heat treatment on metamorphic size disappeared after ca. two months: body mass at dissection was larger in heat-treated animals than in their control siblings. This result might be explained with compensatory growth (Squires *et al.*, 2010; Hector *et al.*, 2012) or by selective mortality of the smallest heat-treated animals (i.e. individuals that died between the onset of metamorphosis and dissection had smaller body mass at metamorphosis than conspecifics that survived until the end of the experiment; Welch's test: $t_{32}$ = 3.54, P = 0.001). Alternatively, the environmental matching hypothesis claims that individuals that developed under poor conditions can have higher fitness later in life than those that developed under good conditions, if adult environmental conditions are also poor (Monaghan, 2008). Whatever mechanism allowed for faster growth after metamorphosis, it pertained only to those heat-treated individuals that developed concordant sex; those that underwent sex reversal had reduced body mass as juveniles. This suggests that heat-induced sex reversal constrains juvenile growth, or alternatively, individuals with the least inherent potential for juvenile growth performance are the most likely to respond to heat with sex reversal. This latter possibility might even be an adaptive strategy if reduced growth is more detrimental for fitness in females than in males (Baroiller & D'Cotta, 2016; Schwanz *et al.*, 2016), which may be true for agile frogs and many other ectothermic vertebrates where mature females are larger than mature males.

Juvenile body mass was the only trait in our study that was affected by EE2, but only in some treatment combinations. Although differences in EE2 effects between heat treatments might have been due to the difference in EE2 degradation rate (which resulted in lower concentrations after 2 days at 30°C than at 19°C), we found no consistent pattern to support this: the EE2 effects were not consistently higher or lower in the heated group than in the control group. When treatment was applied in the second period, EE2 counteracted the heat-induced increase in body mass, whereas in the third period, EE2 decreased mass without heat but contributed to mass increase when combined with heat. The mechanisms behind these interactions are unclear, but both high temperature and estrogens may influence the hormonal regulation of growth, including thyroid hormones, prolactin, and growth hormone (Hogan *et al.*, 2008; Hu *et al.*, 2019). For example, a study on salamander larvae found that high temperature decreased the gene expression of growth hormone and its brain receptors during treatment but increased it after treatment (Hu *et al.*, 2019). In amphibians, growth is governed by different hormones in different stages of development, which might explain the stage-dependent effects of EE2 (Hogan *et al.*, 2008). Altogether, our results indicate that heat waves and EE2 pollution in water bodies may have non-additive effects on fitness *via* influencing body mass even when EE2 does not cause sex reversal in environmentally relevant concentrations. These findings highlight that climate change and chemical pollution may have intricate consequences for individual fitness and population persistence in species with environmentally sensitive sex determination.

## III.3. Performance in fitness-related traits during early life[9]

Sex is a fundamental aspect of individual state in all sexually reproducing organisms. Having testes *versus* ovaries often comes with a diverse set of differences in physiology, morphology, life history, and behavior, including mating and parental strategies often labelled as "sex roles" (Schärer *et al.*, 2012; Immonen *et al.*, 2018). In species with genetic sex determination, where the process of gonad development is triggered by genomic elements, males and females also often differ in their genetic make-up. For example, the chromosome restricted to one sex (e.g. Y in male-heterogametic systems) is inclined to undergo degeneration, which may lead to sex differences in mortality rates and senescence (Marais *et al.*, 2018). In species with environmental sex determination, where the fate of the gonads is decided by external factors such as temperature during early ontogeny, sex ratios and hence population viability may be particularly vulnerable to environmental changes (Mitchell & Janzen, 2010). Thus, the way males and females come to be has crucial implications for population dynamics and thereby biodiversity conservation.

As discussed in **Chapters II.4** and **III.1-2**, environmental influences can override the effect of genes during sex determination in early life, resulting in sex reversal whereby individuals develop phenotypic sex discordant with their genetic sex. One of the most urgent questions regarding sex reversal is how sex-reversed individuals compare to concordant males and females in terms of performance in fitness-related traits. Understanding this issue would be highly valuable for several reasons. *Firstly*, it would facilitate forecasting the effects of sex reversal on demography and evolution, because many of these theoretically predicted effects critically depend on the viability and reproductive success of sex-reversed individuals (Grossen *et al.*, 2011; this will be elaborated in **Chapters IV.2-3**). *Secondly*, it would provide insight into the ultimate and/or proximate drivers of sex reversal. On the one hand, sex reversal may be an adaptive sex-allocation strategy that allows individuals to develop the sex that is most beneficial under the prevailing environmental conditions (Geffroy & Douhard, 2019), similarly to temperature-dependent sex determination (Schwanz *et al.*, 2016). In this case, sex-reversed individuals may perform at least as well or even better than concordant individuals, as found for fecundity in a reptile (Holleley *et al.*, 2015). On the other hand, sex reversal may be a mechanistic consequence of endocrine disruption due to early-life stress, as demonstrated in fish (Senior *et al.*, 2012; Baroiller & D'Cotta, 2016). In this case, sex-reversed individuals may display reduced performance in important life-history traits due to early-life stress, as suggested by the results presented in **Chapters III.1-2**. Yet another alternative is that sex reversal may arise by random sex determination (Perrin, 2016): in absence of strong genetic and environmental triggers, sex may be determined by stochastic variability in gene expression levels. This process may coexist with genetic and environmental sex determination, and can explain considerable proportion of phenotypic variance (Perrin, 2016). Thus, random variation (i.e. no systematic difference) between sex-reversed and concordant individuals in fitness-related performance might indicate random sex determination. *Thirdly*, because sex reversal de-couples genetic and phenotypic sex, it allows for evaluating the relative importance of sex-linked genes *versus* gonadal effects (sex hormones and other sex-specific modifiers that orchestrate sex-biased gene expression) in the development of sex-specific life histories and behaviors. Despite all these reasons for studying the consequences of sex reversal, we have very little empirical information on the fitness of sex-reversed individuals, apart from fish in

[9] This chapter is based on the following publication: Bókony V., Ujhegyi N., Mikó Z., Erös R., Hettyey A., Vili N., Gál Z., Hoffmann O.I., Nemesházi E. 2021. Sex reversal and performance in fitness-related traits during early life in agile frogs. Frontiers in Ecology and Evolution 9: 745752. http://real.mtak.hu/133662/

aquaculture (Senior *et al.*, 2012), where sex reversal is artificially induced for industrial purposes and thus may not necessarily be ecologically relevant. Researchers have only just begun to investigate the relationship between ecologically relevant sex reversal and individual performance, and most of the little knowledge that exists comes from a single reptilian species, where high incubation temperatures produce male-to-female sex-reversed individuals that display a complex combination of male-like, female-like, "supermale" and "superfemale" traits (Holleley *et al.*, 2015; Li *et al.*, 2016; Jones *et al.*, 2020).

Here we address the fitness-related performance of sex-reversed individuals by using data from previous experiments (Bókony *et al.*, 2020; Mikó *et al.*, 2021a) on agile frogs. Both experiments were designed to test sub-lethal effects of larval exposure to environmentally relevant concentrations of chemical pollutants on early-life traits related to fitness. Neither of the chemical treatments affected phenotypic sex ratios significantly (Bókony *et al.*, 2020; Mikó *et al.*, 2021a), but as we report here, sex-reversed individuals occurred in both experiments, indicating that some agile frog tadpoles spontaneously undergo sex reversal even in the absence of any sex-reversing chemical or thermal treatment, in accordance with **Chapters III.1-2**. Therefore, these data offered us the opportunity to examine whether genetic and phenotypic sex and the combination thereof (i.e. reversed *vs.* concordant sex) are associated with differences in life history and behavior during early ontogeny. The larval phase is of critical importance in amphibian life history because mortality during this stage can be extremely high (Riis, 1991), mainly due to predation, pond desiccation, and limited food availability. Thus, larval survival depends to a large extent on the behavioral strategies (predator avoidance, foraging activity) adopted by tadpoles (Skelly, 1994) and the speed of their development (Griffiths, 1997). Also, the rates of development and growth until metamorphosis can have life-long effects on fitness in amphibians (Smith, 1987; Berven, 1990; Altwegg & Reyer, 2003). However, sex differences in larvae are very rarely investigated due to the difficulties of phenotypic sex identification in immature animals (Ujhegyi & Bókony, 2020) and genetic sexing in amphibians overall (**Chapters II.4** and **III.1**). In the agile frog, males typically start reproducing one year earlier than females (Riis, 1991; Sarasola-Puente *et al.*, 2011), and larger males are more successful in male-male competition (Vági & Hettyey, 2016). Therefore, we predicted to observe higher growth rate and faster larval development in males. Fast growth and development require high food intake, so we predicted that male tadpoles would spend more time feeding and, in trade-off, take higher predation risk (Urszán *et al.*, 2015). At least some of these sex differences might be sex-chromosome-linked; this would make the female-to-male sex-reversed individuals (XX males) resemble concordant XX females. However, the agile frog sex chromosomes are homomorphic (Jeffries *et al.*, 2018), suggesting limited genetic differentiation between the sexes. By this latter logic, female-to-male sex-reversed individuals may be more likely to resemble concordant XY males, due to the presence of testes which produce androgen hormones that stimulate the expression of male phenotypic traits (Guarino & Bellini, 1993). We evaluated these predictions by comparing early-life development, growth, and behavior among three groups: males and females with concordant genetic and phenotypic sex, and female-to-male sex-reversed individuals.

## Methods

### *Data collection*

The detailed methods of the two experiments have been published in two open-access papers (Bókony *et al.*, 2020; Mikó *et al.*, 2021a). Here, only a brief description of each experiment is given, focusing on those aspects that are directly relevant for the above objectives. For both experiments, we collected freshly spawned agile frog eggs in March 2018 from ponds in natural

woodland habitats: from 8 egg masses from each of three ponds for experiment 1 (Kerek-tó, Pilisvörösvár, Szárazfarkas; **Table I.1**), and further 10 egg masses from one of these ponds for experiment 2 (Szárazfarkas). The eggs were taken into our laboratory, where the two experiments were conducted simultaneously in the same room, rearing the animals with the same general protocol as in the previous chapters. The control groups of these two experiments are the same animals as those used for developing genetic sex markers and investigating the physiological health of 6 female-to-male sex-reversed agile frogs in **Chapter III.1**.

In both experiments, we exposed the tadpoles to environmentally relevant concentrations of widespread water-polluting chemicals for which sex-related endocrine-disrupting effects had been reported. In experiment 1, we applied two concentrations each of carbamazepine (0.5 and 50 µg/L), a pharmaceutical drug (Galus *et al.*, 2013, 2014), and terbuthylazine (0.003 and 0.3 µg/L), a herbicide frequently used in Europe (Kjeldsen *et al.*, 2013). In experiment 2, we applied two concentrations of chlorpyrifos (0.5 and 5 µg/L), a broad-spectrum insecticide (Bernabò *et al.*, 2011). The applied two concentrations for each chemical correspond to the mean (or median) and close-to-maximum values reported from surface waters, respectively (Bókony *et al.*, 2020; Mikó *et al.*, 2021a). In both experiments, the control group of tadpoles was kept in clean RSW to which we added ethanol as solvent control (1 µL 96% ethanol to 1 L RSW). All other treatment groups also contained this amount of ethanol as vehicle. In experiment 1, we distributed 480 tadpoles (20 from each family) evenly across 5 treatment groups with 4 replicates in each treatment × family combination (4 tadpoles × 24 families × 5 treatments). In experiment 2, we distributed 144 tadpoles evenly across 3 treatment groups (48 tadpoles per treatment, with 4-6 tadpoles in each treatment × family combination).

We exposed tadpoles to the treatments over the entire duration of their larval development (from Gosner stage 25 to 42). Twice a week, we renewed each treatment by changing the rearing water; the actual concentrations were close to the nominal concentrations (Bókony *et al.*, 2020; Mikó *et al.*, 2021a). We collected different kinds of data on tadpole behavior in the two studies (see below), with our focus being on foraging activity in experiment 1 and anti-predatory response in experiment 2. We recorded the time to and body mass (± 0.1 mg) at metamorphosis (stage 42). On average 15 weeks (96-136 days) after starting the experiment, we weighed the animals to the nearest 0.01 g and dissected them to measure lean mass (body mass without gut mass) and record phenotypic sex, using the same methods as in **Chapters III.1-2**. Throughout both experiments we monitored survival daily, and we stored a tissue sample from each animal that died before dissection, as well as from all dissected animals, in 96% ethanol. To diagnose genetic sex (465 individuals in experiment 1 and 140 individuals in experiment 2), we used the method described in **Chapter III.1**.

Phenotypic sex was uncertain by gross gonad morphology in two individuals, and one of them also had uncertain genetic sex; we excluded the latter animal from all statistics presented here, yielding 439 individuals in experiment 1 and 135 individuals in experiment 2 for phenotypic sex. For those individuals that we identified as sex-reversed, we also examined the gonads histologically to make sure that the mismatch was not due to incorrect categorization of phenotypic sex. For one individual, the sections failed to include gonadal tissue. For all other individuals examined, the gonads were clearly identifiable by histology either as testes (N=14) or ovotestes containing a few oogonia (N=6, including the individual whose phenotypic sex had been uncertain based on gross gonad anatomy). Therefore, in the analyses we treated these individuals as phenotypic males (female-to-male sex-reversed individuals).

***Behavioral observations and video analysis***

In experiment 1, we observed the behavior of each tadpole in their rearing boxes containing *ad libitum* food, using the "instantaneous sampling" method (Altmann, 1974) four times a week during the larval period, totaling 20 observations per tadpole. Four researchers conducted the observations on two days each week, in one morning session and one afternoon session (each lasting ca. one hour) each day. During each session, we scanned all tadpoles once in a fixed order, recorded their instantaneous behavior as inactive, feeding, or swimming, and we also categorized the location of the tadpole within the box as on the bottom, next to the wall (i.e. within one tadpole distance from the wall, but not on the bottom), or in the open (i.e. not near the bottom or wall). In each session, a single observer scanned all tadpoles, and the identity of observers was rotated between sessions.

In experiment 2, we video-recorded tadpole behavior one month after starting the experiment and 3 days thereafter. As we had a limited number of cameras, we video-recorded only a subsample (83%) of individuals (40 from each chemical treatment group). On each occasion, we transferred the tadpoles into new containers identical to their rearing boxes, but with no food (to facilitate automatic tracking). On the first occasion, the box contained RSW with the same chemical treatment the individual was reared in, whereas on the second occasion all tadpoles were moved into clean RSW for video recording. Each time, we recorded the tadpoles' behavior for 20 minutes, and then exposed them to a startling stimulus by abruptly pouring 40 ml RSW into their water. Typically, the tadpoles reacted by a short burst of swimming ("escape"), followed by a period of motionlessness ("freezing"). To allow for measuring these responses we continued recording for another 20 minutes after the stimulus. As a main objective of experiment 2 was to assess anti-predator responses, half of the tadpoles within each chemical treatment group received clean RSW as startling stimulus on both occasions, whereas the other half received chemical cues indicating predation risk. The chemical cues were prepared as described in Hettyey et al. (2016), using water from the tank of European perch (*Perca fluviatilis*) that had been feeding on agile frog tadpoles. As the European perch is a native predator in our region, agile frog tadpoles respond to its chemical cues by decreasing their activity, even if they are predator-naïve (Hettyey *et al.*, 2016).

From the video recordings, we collected data on tadpole activity using the automatic tracking software ToxTrac (Rodriguez *et al.*, 2018). All tracking results were manually checked for quality; all tracking data used here were error-free. We calculated the following variables from the first 20 minutes of each recording (i.e. before the addition of predator cues): total distance moved as a measure of locomotor activity, proportion of area used as a measure of exploration rate, and proportion of time spent near the wall (within a 50-pixel wide stretch from the side of the box; the tadpoles had an average snout-to-vent length of ca. 40 pixels in the videos) as a measure of risk aversion. For quantifying the startle response, the second 20 minutes of each video recording (i.e. after the addition of predator cues) were analyzed manually by a single observer, who recorded the following variables: the intensity of immediate reaction to the startle ("startle response"), categorized on a 0-3 scale (0: no movement, 1: slight movement, 2: swimming away apparently calmly, 3: swimming around fervently); the duration of escape, measured as the time spent moving continuously from the startle stimulus until the first stop; and the duration of freezing, measured as the time spent motionless after the escape until the first movement thereafter. Note that the duration of escape and freezing were quantifiable only in those individuals whose startle response had a non-zero intensity score.

***Statistical analyses***

First, we tested whether sex-reversal rate was independent of chemical treatment, by analyzing the phenotypic sex of genetic females (XX individuals) in a generalized linear mixed-effects model with binomial error and logit link, including treatment type as fixed factor, and family nested in experiment as random factors. Two treatment groups lacking sex-reversed individuals had to be excluded from this analysis because such separation in logistic models results in unreliable estimates; thus, we used Fisher's exact tests to compare sex-reversal rate in these two groups with the respective control group.

We tested whether survival rate over the entire duration of the experiments differed between genetic males and genetic females using a Cox's proportional hazards model ('coxme' function), including family nested in experiment as random effects, and treating the dissected individuals as censored observations (i.e. these animals survived until the end of the study). In all remaining models (see below), we included sex as a three-category factor (concordant XY male, concordant XX female, or sex-reversed XX male), thus individuals with missing data on either genetic or phenotypic sex were excluded.

We analyzed the duration of larval development (number of days from starting the experiment at stage 25 until the start of metamorphosis at stage 42), body mass at metamorphosis, and juvenile body mass (measured at dissection ca. 2 months after metamorphosis; excluding gut mass) by pooling the data of the two experiments, taking into account chemical treatment as a fixed factor and family nested in experiment as random factors. Duration of larval development was analyzed with a Cox's proportional hazards model, whereas both mass variables were analyzed with linear mixed-effects models ('lme' function), allowing for heteroscedasticity among the 3 sex categories. In the model of juvenile body mass, we also included age (number of days from starting the experiment until dissection) as a covariate. To investigate the trade-off between development and growth in tadpoles in the 3 sex categories, we added the interaction between the duration of larval development and sex into the model of mass at metamorphosis; in this model we allowed for heteroscedasticity also among families because residual diagnostics indicated that a few families exhibited outliers in the relationship between development and growth.

We analyzed tadpole behavior separately for the two experiments. For experiment 1, we analyzed two variables. We compared the proportion of observations in the open among the 3 sex categories with a Fisher's exact test, because separation in the data precluded the use of logistic models (thus, in this statistical test we could not control for potential effects of other predictors such as chemical treatment). To analyze the proportion of individuals feeding, we used a generalized linear mixed-effects model with binomial error and logit link ('glmmPQL' function). For experiment 2, we analyzed the pre-startle distance moved, exploration rate, and time spent near the wall with LME models, allowing for heteroscedasticity among the 3 sex categories. We analyzed the intensity of startle response using a cumulative-link mixed model with logit link ('clmm' function), and the durations of escape and of freezing with Cox's proportional hazards models excluding those tadpoles that did not react with movement. All models of behavioral variables included individual nested in family as random factors, chemical treatment as a fixed factor, and the fixed effects of date (a covariate in experiment 1, expressing the number of days from starting the experiment; and a two-category factor in experiment 2) and time of day (a two-category factor in experiment 1, and a covariate in experiment 2, expressing the order of video recordings which were done in 7 consecutive bouts per day). Additionally, the model of experiment 1 included shelf height and observer identity as fixed factors and water temperature as a covariate (detailed explanation on these covariates can be found in Bókony et al., 2020). To investigate the change of feeding rate over time in the 3 sex categories, we added the interaction between date and

sex into the model of feeding rate (we expected that young tadpoles with undifferentiated gonads would behave similarly, whereas feeding rate may diverge between sexes in later stages as the gonads become differentiated). For experiment 2, the model of exploration rate included the total distance moved as a covariate, because we aimed to investigate the percentage of area used independently of locomotor activity. The 3 models of post-startle variables also included the total distance moved as a covariate, because post-startle activity may depend on pre-startle activity. Furthermore, the latter 3 models included stimulus type (i.e. whether the stimulus water contained predator cues or not) as a fixed factor, and its interaction with sex to test whether the effect of predator cues on behavior differed between sex categories.

In each model of development, growth, and behavior, we tested the effect of sex by pre-planned comparisons (Ruxton & Beauchamp, 2008) using the 'emmeans' function. Specifically, we extracted 3 linear contrasts from each model: genetic males *versus* genetic females (the latter including sex-reversed individuals), phenotypic females *versus* phenotypic males (the latter including sex-reversed individuals), and sex-reversed *versus* concordant individuals (the latter including males and females). We provide these contrast estimates with 95% confidence intervals as non-standardized measures of effect size (Nakagawa, 2004; Nakagawa & Cuthill, 2007); we interpret CIs excluding zero (or one, in case of odds ratios and hazard ratios) as statistically significant. Further, to test whether the sexes differed in the trade-off between development and growth, we estimated the slope of relationship between the duration of larval development and body mass at metamorphosis for each sex category, and then applied the above 3 linear contrasts to the slopes. We used the same approach to compare the slope of change over time in feeding rate among the sexes. To test whether the sexes differed in the effect of predator cues on post-startle behaviors, for each sex category we estimated the difference in each behavioral variable between animals startled with *versus* without predator cues, and again we applied the above 3 linear contrasts to these predator-effect estimates.

## Results

### *Sex ratios and sex reversal*

In the total sample, there was significant female bias in genetic sex ratio (344/605 = 56.9% females, 95% CI = 0.53 – 0.61) but not in phenotypic sex ratio (305/574 = 53.1% females, 95% CI = 0.49 – 0.57). Out of the 571 individuals for which both genetic and phenotypic sex was identifiable, 21 were female-to-male sex-reversed (**Table III.3.1**), 16 of which originated from the pond sampled for both experiments (9.8% sex-reversal rate), and 5 from the two ponds sampled only for experiment 1 (2.4% and 3.1% sex-reversal rate, respectively). The sex-reversed individuals came from 8 (out of 34) different families (5-75% of individuals sex-reversed per family), which does not conform to a homogeneous distribution of sex reversal among families ($\chi^2_{33} = 233$, $P < 0.001$). In 3 out of the 34 families we found no genetic males at all; two of these families exhibited 30% and 75% sex-reversal rate, respectively (see Table S1 in the supplementary material of Bókony *et al.*, 2021b). The frequency of sex reversal among genetic females was independent of chemical treatments (6.4% overall; **Table III.3.1**). Survival rate until dissection did not depend on genetic sex (18 females and 16 males died; hazard ratio: 0.84, 95% CI: 0.60 – 2.38).

**Table III.3.1.** Sample sizes by sex in each treatment group, and odds ratios (OR) with 95% confidence intervals (CI) for sex-reversal rate in genetic females (XX genotype) between the control group and each treatment group.

| Experiment | Treatment | Sex-reversed (XX male) | Concordant | | Died* | | | |
|---|---|---|---|---|---|---|---|---|
| | | | XX female | XY male | XX | XY | Other† | OR (95% CI) |
| 1 | Control | 2 | 45 | 35 | 5 | 6 | 0 | – |
| | Carbamazepine 0.5 µg/l | 0 | 55 | 34 | 2 | 1 | 1 | 0 (0, 4.53)‡ |
| | Carbamazepine 50 µg/l | 0 | 53 | 37 | 2 | 1 | 0 | 0 (0, 4.70)‡ |
| | Terbuthylazine 0.003 µg/l | 1 | 38 | 48 | 4 | 3 | 1 | 0.46 (0.03, 6.22) |
| | Terbuthylazine 0.3 µg/l | 2 | 50 | 36 | 2 | 3 | 1 | 0.41 (0.04, 4.20) |
| 2 | Control | 5 | 23 | 19 | 1 | 0 | 0 | – |
| | Chlorpyrifos 0.5 µg/l | 6 | 21 | 20 | 0 | 0 | 0 | 1.61 (0.30, 10.69) |
| | Chlorpyrifos 5 µg/l | 5 | 20 | 16 | 2 | 2 | 0 | 1.80 (0.21, 8.95) |

The table excludes 15 individuals for which we had no data on sex (7 escaped before dissection and DNA sampling; 8 died before dissection and could not be sexed genetically).

*Phenotypic sex could not be diagnosed for the individuals that died before dissection.

†One phenotypic male in each treatment group had unknown genetic sex due to marker disagreement (Rds3 genotype was female whereas Rds1 genotype was male).

‡These two odds ratios were taken from Fisher's exact tests; the rest from a single binomial mixed model (overall effect of treatment: Wald test, $\chi^2_4 = 1.24$, $P = 0.871$).

***Development and growth***

We found no significant difference between any combination of genetic and phenotypic sex in the length of larval development and body mass at metamorphosis or at dissection (**Table III.3.2**). However, the relationship between the length of larval development and mass at metamorphosis varied significantly with sex (**Table III.3.2**, **Fig. III.3.1A**): animals that metamorphosed later had higher body mass in both concordant males and concordant females (**Table III.3.3**), whereas in sex-reversed individuals the slope of this relationship did not differ significantly from zero (**Table III.3.3**). Some sex-reversed individuals metamorphosed relatively early and with large mass; most of them came from those families where we detected no genetic males at all (**Fig. III.3.1A**). Other sex-reversed individuals metamorphosed relatively late and with small mass; most of them had testicular oogonia (**Fig. III.3.1A**).

**Table III.3.2.** Differences in life-history and behavioral traits by genetic and/or phenotypic sex, shown as non-standardized effect sizes with 95% confidence intervals (CI). Significant differences (i.e. CI excluding 0 or, in case of odds ratios and hazard ratios, 1) are marked with bold text. The comparisons marked with an asterisk are calculated from the effect sizes given in **Table III.3.3**.

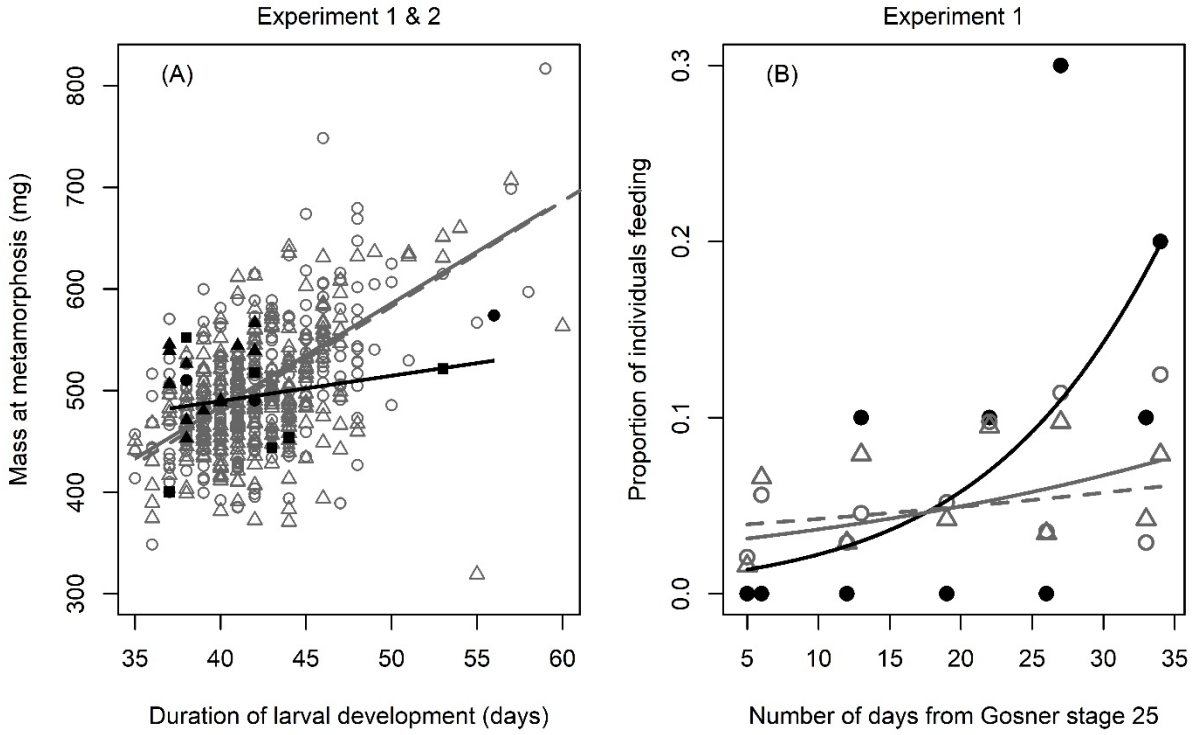
| Trait | Sex-reversed *vs.* concordant individuals[§] | Genetic females *vs.* genetic males[†] | Phenotypic females *vs.* phenotypic males[‡] |
|---|---|---|---|
| Duration of larval development (hazard ratio) | 1.30 (0.75, 2.26) | 1.07 (0.79, 1.47) | 0.82 (0.61, 1.12) |
| Body mass at metamorphosis (mg) | 7.24 (-16.40, 30.90) | 9.16 (-5.09, 23.4) | 1.92 (-12.00, 15.80) |
| Trade-off between larval development and growth (mg) * | **-7.79 (-14.4, -1.19)** | **-4.02 (-7.74, -0.30)** | **3.76 (0.15, 7.37)** |
| Juvenile body mass (g) | -0.05 (-0.11, 0.02) | -0.02 (-0.06, 0.02) | 0.03 (-0.01, 0.07) |
| Frequency of feeding (odds ratio) | 1.63 (0.71, 3.74) | 1.31 (0.84, 2.04) | 0.81 (0.52, 1.25) |
| Change of feeding rate with age (odds ratio) * | 1.08 (0.98, 1.18) | 1.05 (1.00, 1.10) | 0.97 (0.93, 1.02) |
| Total distance moved (kilopixels) | **-5.73 (-11.10, -0.40)** | -2.90 (-7.86, 2.06) | 2.83 (-1.66, 7.32) |
| Exploration rate (% of area visited) | -2.30 (-12.10, 7.47) | -4.43 (-12.3, 3.40) | -2.13 (-9.27, 5.01) |
| Time spent near wall (%) | -4.93 (-17.3, 7.47) | 0.75 (-8.42, 9.92) | 5.68 (-2.02, 13.4) |
| Intensity of startle response (cumulative odds ratio) | 2.42 (0.61, 9.51) | 1.02 (0.36, 2.90) | 0.42 (0.17, 1.05) |
| Effect of predator cues on intensity of startle response (cumulative odds ratio) * | 0.23 (0.01, 3.93) | 0.97 (0.11, 8.41) | 4.22 (0.66, 27.08) |
| Duration of escape (hazard ratio) | 0.74 (0.36, 1.52) | 1.15 (0.63, 2.11) | 1.56 (0.90, 2.70) |
| Effect of predator cues on escape duration (hazard ratio) * | **0.25 (0.07, 0.89)** | 0.66 (0.21, 2.12) | 2.68 (0.97, 7.46) |
| Duration of freezing (hazard ratio) | 1.69 (0.86, 3.29) | 0.99 (0.54, 1.84) | 0.59 (0.34, 1.03) |
| Effect of predator cues on freezing time (hazard ratio) * | 0.91 (0.27, 3.05) | 2.06 (0.66, 6.45) | 2.26 (0.82, 6.22) |

[§]Concordant individuals include XX females and XY males.

[†]Genetic females include sex-reversed individuals and concordant females.

[‡]Phenotypic males include sex-reversed individuals and concordant males.

**Figure III.3.1.** Relationships between mass at metamorphosis and duration of larval development (A) and between feeding rate and tadpole age (B) in sex-reversed individuals (XX males; black filled symbols and black lines), concordant females (XX females; gray open circles and gray solid lines), and concordant males (XY males; gray triangles and gray dashed lines). The lines represent the slopes given in **Table III.3.3**. To facilitate graph readability, panel A was cropped to exclude an outlier point (a concordant male with 373.4 mg mass at metamorphosis and 78 days of larval development; note that this individual was included in all analyses and thus also in the estimation of the regression lines fitted here). Among the filled black symbols in panel A, squares represent individuals with ovotestes (testicular tissue containing oogonia), whereas triangles stand for the two families that contained only XX genotypes; circles mark all other sex-reversed individuals. Note that the symbols represent individuals in panel A, whereas each symbol in panel B is a group of same-sex individuals within an observation session.




***Tadpole behavior***

In experiment 1, none of the 5 sex-reversed individuals were ever observed in the open; 0.37% and 0.39% of observations of concordant females and concordant males, respectively, were in the open. This difference was not significant (Fisher's exact test: P = 0.900). Also, feeding frequency did not vary significantly with sex (**Table III.3.2**). The proportion of tadpoles feeding increased slightly as the tadpoles aged and this increase appeared greatest in sex-reversed individuals and smallest in concordant males (**Fig. III.3.1B**); however, the slopes had wide confidence intervals (**Table III.3.3**) and did not differ significantly between any combination of genetic and phenotypic sex (**Table III.3.2**).

In experiment 2, the total distance moved was significantly shorter in sex-reversed tadpoles (14.2 kpx ± 2.3 SE) than in concordant individuals (males: 20.0 ± 2.0, females: 19.9 ± 1.6; **Table**

**III.3.2**), but exploration rate and time spent near the wall did not vary significantly with sex (**Table III.3.2**). Similarly, we found no significant differences in the intensity of startle response and the durations of escape and freezing between any combination of genetic and phenotypic sex (**Table III.3.2**). However, the presence of predator cues modified these behaviors in sex-dependent ways (**Table III.3.3**). Among concordant females, those that received predator cues responded less intensely to the disturbance than those that received clean water (**Table III.3.3**, **Fig. III.3.2**); there was no such difference in concordant males or in sex-reversed individuals (**Table III.3.3**, **Fig. III.3.2**). The duration of escape was shorter in sex-reversed individuals if they received predator cues than when they did not (**Table III.3.3**, **Fig. III.3.3**); there was no such difference in concordant individuals (**Table III.3.3**, **Fig. III.3.3**). The duration of freezing after the escape reaction was longer in concordant females in the presence of predator cues than in clean water (**Table III.3.3**, **Fig. III.3.3**); the similar trends in concordant males and sex-reversed individuals were not statistically significant (**Table III.3.3**, **Fig. III.3.3**). Out of all these sex differences in the effects of predator cues, only the one for escape duration was statistically significant (**Table III.3.2**).

**Figure III.3.2.** Intensity of response to the startling stimulus with and without predator cues in sex-reversed individuals (XX males), concordant (XX) females and concordant (XY) males in experiment 2 (scored as 0: no movement, 1: slight movement, 2: swimming away apparently calmly, 3: swimming around fervently).

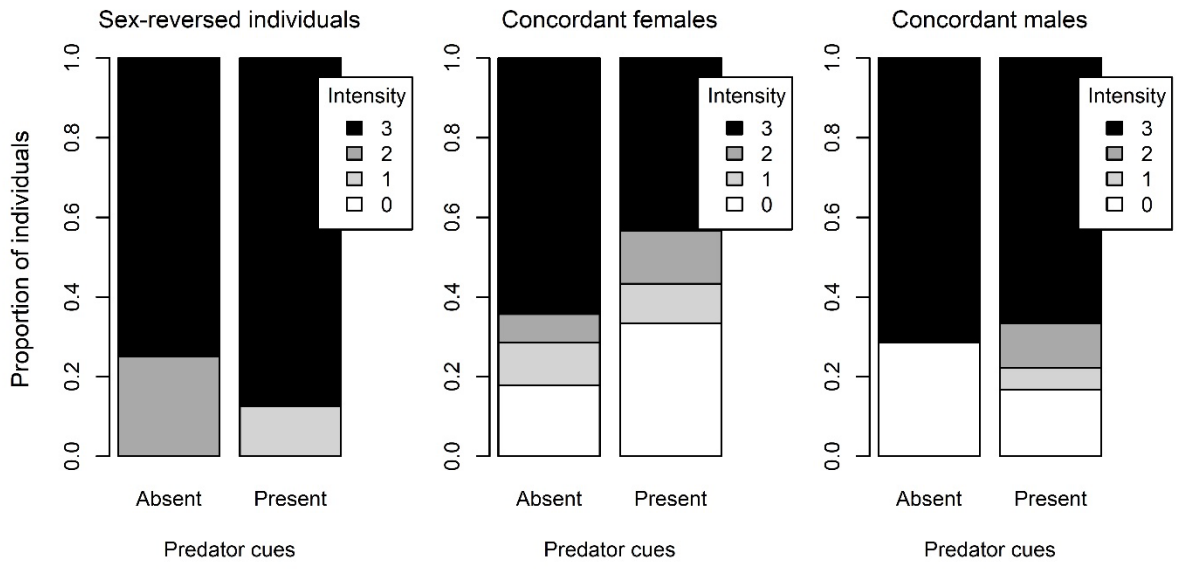


**Figure III.3.3.** Duration of escape and freezing after the startle stimulus with and without predator cues in sex-reversed individuals (XX males), concordant (XX) females and concordant (XY) males in experiment 2, excluding those individuals that did not respond to the stimulus by moving. Boxplot interpretation is as in **Fig. II.3.1**.

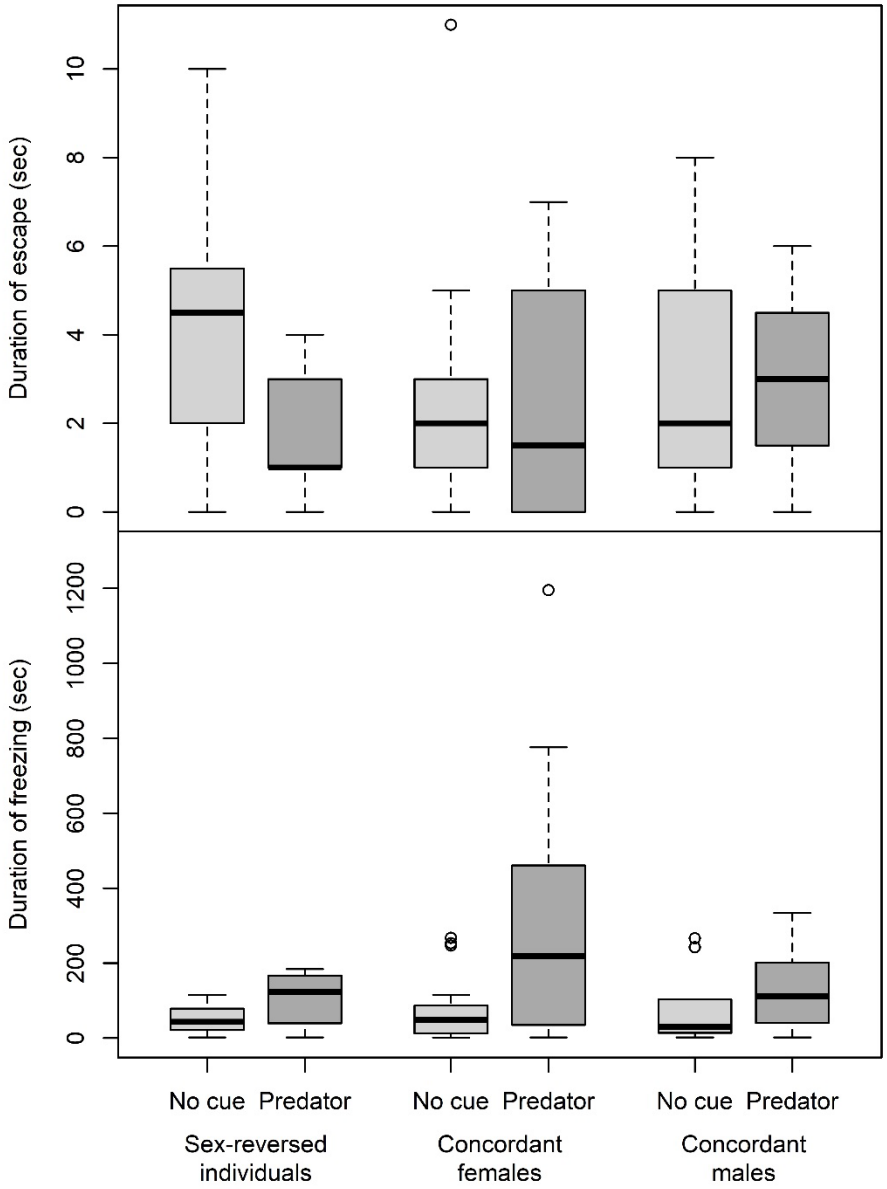


**Table III.3.3.** Trade-off between larval development and growth, change of feeding rate with age, and effects of predator cues on behavior in the 3 sex categories, shown as non-standardized effect sizes with 95% confidence intervals (CI). Significant effects (i.e. CI excluding 0 or, in case of odds ratios and hazard ratios, 1) are marked with bold text.

| Effect | Sex-reversed individuals (XX males) | Concordant (XX) females | Concordant (XY) males |
|---|---|---|---|
| Slope of relationship between duration of larval development and mass at metamorphosis (mg) | 2.49 (-4.06, 9.04) | **10.19 (8.74, 11.64)** | **10.36 (8.57, 12.16)** |
| Slope of relationship between feeding rate and age (odds ratio) | 1.63 (0.71, 3.74) | 1.31 (0.84, 2.04) | 0.81 (0.52, 1.25) |
| Effect of predator cues on intensity of startle response (cumulative odds ratio) | 0.47 (0.03, 6.53) | **3.24 (1.09, 9.68)** | 1.26 (0.25, 6.37) |
| Effect of predator cues on escape duration (hazard ratio) | **0.31 (0.10, 0.93)** | 1.51 (0.74, 3.08) | 1.03 (0.37, 2.83) |
| Effect of predator cues on freezing time (hazard ratio) | 2.22 (0.77, 6.39) | **4.07 (1.78, 9.29)** | 1.46 (0.58, 3.62) |

**Discussion**

This study confirmed that female-to-male sex reversal occurs in agile frogs at a relatively low rate (6.4%) in the absence of thermal stress, and demonstrated that this rate was independent of chemical treatments representing ecologically relevant concentrations of carbamazepine, terbuthylazine, and chlorpyrifos. Because the animals were raised in the laboratory at benign temperatures with *ad libitum* food and no predators, and their sex development was not altered by the chemical treatments, it seems likely that these instances of sex reversal occurred independently of any obvious environmental stressor. Furthermore, the sex-reversed individuals in this study were similar to their sex-concordant siblings in almost all morphological, life-history and behavioral traits that we examined. Taken together, these results may be explained by two, not mutually exclusive ideas. First, the theory of random sex determination (Perrin, 2016) postulates that, in the lack of strong genetic and environmental effects on sex, developmental noise (i.e. random fluctuations in the expression of sex-determining genes) decides the sexual fate of individuals. Second, the threshold model of sex determination (Quinn *et al.*, 2011; elaborated later in **Chapter IV.3**) assumes that phenotypic sex depends on whether the amount of "male signal" (i.e. expression of male-producing developmental signals, which can be influenced by both genotype and environment) exceeds the individual's threshold for male development, a trait encoded by genetic elements. Thus, for individuals who happen to have genes encoding high "male signal" levels and/or low threshold levels, even a small elevation of environmentally induced "male signal" expression may result in female-to-male sex reversal. This theory is supported in the present study

by the non-random distribution of sex reversal among agile frog families, and by the high rate of sex reversal in those families where we detected only genetic females and no genetic males at all. The latter fits the threshold theory because the agile frog has an XX/XY sex determination system, so families containing 100% female offspring suggest that the sire in those families had been a female-to-male sex-reversed individual (i.e. an XX male, mating with a concordant XX female) who may have passed on his alleles encoding high propensity for sex reversal to his offspring. Such a combination of genetic variation and random environmental noise might explain at least some occurrences of sex reversal in natural populations, especially where sex-reversal rate is not correlated with environmental factors such as the level of urbanization (Lambert *et al.*, 2019), climate (Castelli *et al.*, 2021), or elevation (Phillips *et al.*, 2020).

The fact that the sex-reversed individuals in this study did not differ from concordant individuals in growth and development contrasts with some findings of **Chapter III.1**, i.e. that a subset of the animals analyzed here (specifically, the control groups of experiments 1 and 2, used for sex-marker development) exhibited signs of physiological stress associated with sex reversal. However, that sample was tiny (6 sex-reversed froglets), and the number of health indices that did *versus* did not show association with sex reversal was identical (3 indices each). More strikingly, the results of the present chapter stand in stark contrast with our findings from **Chapter III.2**, where sex reversal was induced by a 6-days "heat wave" treatment during larval development. Heat resulted in high rates of female-to-male sex reversal, but also reduced survival, development, growth, and fat reserves (**Chapter III.2**). Thus, in that experiment, sex reversal was strongly associated with signs of developmental stress and poor fitness prospects, similarly to what has been reported about some aquaculture fishes (Senior *et al.*, 2012; Baroiller & D'Cotta, 2016). Combining those findings with our current results, we speculate that the fitness of sex-reversed individuals may depend on the etiology of sex reversal. When sex reversal arises by stochastic variation in the biochemical processes of sex determination or in individual sensitivity to environmental effects on sex, it might not be systematically accompanied by changes in fitness-related traits. In contrast, when sex reversal is triggered by strong environmental effects and/or high physiological stress, it might be associated with poor health or reduced performance in life-history traits. This association may arise by the same stressor affecting both sex and fitness-related traits, perhaps mediated by stress-induced glucocorticoid hormone effects (Geffroy & Douhard, 2019) or cellular calcium-redox regulation (Castelli *et al.*, 2020). For example, a meta-analysis concluded that the poor fitness of fish that underwent chemically induced sex reversal were not due to sex reversal *per se*, but were the result of the chemical treatments themselves (Senior *et al.*, 2012). Additionally, the association between sex reversal and fitness might be exacerbated by sex reversal itself directly affecting some fitness-related traits (as shown by the reduced body mass of sex-reversed froglets regardless of treatment in **Chapter III.2**) or by making the offspring of sex-reversed individuals more susceptible to environmentally induced sex reversal (Piferrer & Anastasiadi, 2021). If one sex can do better than the other under stressful conditions, environment-induced sex reversal may serve as an adaptive sex-allocation strategy (Geffroy & Douhard, 2019). However, in agile frogs that spawn in early spring and develop in cool waters, high temperatures during larval development might not have been frequent enough in their evolutionary past for such an adaptive strategy to evolve. Nevertheless, the findings in **Chapter III.1** indicate that sex-reversed agile frogs occur more frequently in anthropogenic habitats, and phenotypic sex ratios have become more male-biased in some amphibian species since the start of contemporary climate change (as shown later in **Chapter IV.2**), suggesting that sex reversals might be shifting from mostly spontaneous or stochastic to increasingly stress-induced incidences. These speculations would deserve further empirical testing.

In the few instances where we found significant differences between sex-reversed and sex-concordant individuals in the present study, the former stood out by having lower locomotor activity and responding to disturbance with shorter escape duration when predator cues were present. Furthermore, the natural trade-off between larval development speed and growth rate seemed to be lacking in sex-reversed individuals. These findings support neither higher nor lower performance in terms of overall fitness for sex-reversed animals. First, while low activity may constrain foraging success, sex-reversed tadpoles were feeding at least as often as concordant individuals. Second, while short escape duration may lower the probability of being noticed by predators, it may be disadvantageous for escaping predators if they are already in pursuit. Third, although both early metamorphosis and large metamorphic mass are considered beneficial for amphibians in general (Smith, 1987; Berven, 1990; Altwegg & Reyer, 2003), sex-reversed individuals in our study tended to perform either well or poorly in both traits. Those sex-reversed individuals that did well in these traits tend to conform to the theory of heritable, random variation in the propensity for sex reversal, because most of them originated from two families with only XX genotypes (suggesting a sex-reversed sire; see above). Almost all sex-reversed individuals that did poorly in both development and growth appeared unsuccessful also in executing sex reversal completely, as they had oogonia in their testes. This supports the above idea that sex-reversed individuals may represent a heterogeneous group whose life history and health might depend on the etiology of sex reversal.

When comparing males and females (either genetically or phenotypically), we found no difference in development and growth, and only a few differences in behavior. Concordant females were the only group that reacted to predator cues by less intense startle response and longer freezing. This may indicate lower risk taking by females, which may agree with the behavior of adult agile frogs observed in nature, where females forage less in open areas than males do (Cicort-Lucaciu *et al.*, 2011). Phenotypic males, including sex-reversed individuals and concordant males, did not show the same responses to predator cues as females did, suggesting that sex differences in these aspects of risk-taking behavior may not be genetically determined, but rather may develop after sex determination, e.g. by sex hormones. However, because none of the male-female differences in our study were statistically significant despite the relatively large sample for both sexes, we conclude that most of the divergent life histories and behaviors making up sex roles in agile frogs do not seem to arise in their larval life. In this species, males search and compete actively for females at high densities (Lodé *et al.*, 2004), whereas at lower densities males maintain territories and females appear to choose males by their call characteristics (Lesbarrères *et al.*, 2008), but both parents abandon the eggs after spawning. It would be very interesting to perform similar studies with species where either the male or the female parent takes the risky job of providing care to the offspring, as the developmental determinants of sex roles and therefore the effects of sex reversal may vary greatly between traditional and sex-role reversed systems.

Since sex reversal occurs relatively rarely under natural circumstances, most of our existing knowledge about ecologically relevant sex reversal comes from studies that include relatively small numbers of sex-reversed individuals in each population, year or treatment group (Li *et al.*, 2016; Lambert *et al.*, 2019; Jones *et al.*, 2020; Wild *et al.*, 2022). The present study is no exception to this constraint. However, the fact that sex-reversed individuals do not make up a large proportion of current populations does not mean that they are merely a curiosity: they may be powerful catalyzers of evolutionary change (Holleley *et al.*, 2016), as elaborated in the following **Chapters IV.2-3**.

# *IV. Theoretical considerations on environment-induced sex reversal*

## IV.1. Asymmetrical sex reversal: a hypothesis revised[10]

Evolutionary changes in sex-reversal liability are difficult to study empirically, yet the resulting differences between species in their sensitivity to sex-reversing environmental factors, including anthropogenic stressors, are important from the perspectives of both basic science and conservation practice. One particular factor that may affect the propensity to undergo certain types of sex reversal is the type of genetic sex-determination system. About 60 years ago, based on experiments applying exogenous sex hormones to a few amphibian species, Witschi and colleagues (Witschi *et al.*, 1958) recognized that it was predominantly the homogametic sex (i.e. genotype with XX or ZZ sex chromosomes) that was susceptible to sex reversal – a concept sometimes referred to as Witschi's rule. Roughly 20 years later, Adkins-Regan (1981) came to a similar conclusion based on reviewing data from fish, amphibians, reptiles and further taxa. However, laboratory experiments across ectothermic vertebrates successfully produced sex reversal in heterogametic individuals, yielding both XY females and ZW males, and these were even fertile in some species (Devlin & Nagahama, 2002; Chardard *et al.*, 2004; Chen *et al.*, 2014; Roco *et al.*, 2015; Veltsos *et al.*, 2019). Therefore, some authors see Witschi's rule as disproved (Wallace *et al.*, 1999; Piprek *et al.*, 2012), while others maintain that sex reversal is restricted to the homogametic sex, acknowledging that there are counterexamples with no explanation (Schwanz *et al.*, 2013; Ruiz-Garciá *et al.*, 2021; Whiteley *et al.*, 2021a). Clarifying this issue empirically would be important for understanding which species are susceptible to sex reversal induced by specific environmental stimuli. For example, 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. Furthermore, whether the heterogametic sex is resistant to sex reversal or not is important also for our theoretical understanding of the evolution of sex-determination systems: some models dealing with transitions between these systems assumed that only certain sex-chromosome genotypes can undergo sex reversal (Schwanz *et al.*, 2013; **Chapter IV.2**), while others made no such assumption (Bull, 1981, 1985; Grossen *et al.*, 2011; Quinn *et al.*, 2011; Schwanz *et al.*, 2020; **Chapter IV.3**).

After knowledge kept gathering on the evolution of sex chromosomes and sex-determination systems during the past century, it was suggested that natural selection could cause the pattern described by Witschi's rule (Schwanz *et al.*, 2013). The key seems to be that if sex-reversed individuals participate in breeding, new combinations of the sex chromosomes (YY or WW) can emerge in their progeny. These new genotypes may have reduced fitness due to degeneration of the genetic content of the hemizygous chromosome (Bull, 1985; Schwanz *et al.*, 2013), driven by accumulation of deleterious mutations (Charlesworth & Charlesworth, 1997; Charlesworth, 2021; Peona *et al.*, 2021) and sex-antagonistic genes (Rice, 1987; Van Doorn & Kirkpatrick, 2010; but see Perrin, 2021 and Jeffries *et al.*, 2021). 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. As a result, the homogametic sex may be more susceptible to sex reversal compared to the heterogametic sex. This potential evolutionary mechanism has been mentioned in

[10] This chapter is based on the following publication: Nemesházi E., Bókony V. 2022. Asymmetrical sex reversal: does the type of heterogamety predict propensity for sex reversal? BioEssays 44: 2200039. http://real.mtak.hu/145524/

several reviews on environmental sex reversal (e.g. Ruiz-Garciá *et al.*, 2021; Schwanz & Georges, 2021; Whiteley *et al.*, 2021a); however, none of these papers offered a robust explanation why some species conformed to Witschi's rule while others did not.

We propose that 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. IV.1.1a**) and may be shaped by various factors. In ectothermic vertebrates, different sex-determination systems dynamically replace each other as species evolve (Sarre *et al.*, 2011). 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 evolutionary 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. IV.1.1a**). Comparison between 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 (Chardard *et al.*, 2004; Ospina-Álvarez & Piferrer, 2008; Holleley *et al.*, 2015; Edmands, 2021). Thus, the aim of the present chapter 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 it occurs 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 (which will be referred to as "*masculinization*" henceforth for brevity) and stronger stimuli are required for male-to-female sex reversal (which will be referred to as "*feminization*"), 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 (Schwanz *et al.*, 2013) which used it for describing a pattern corresponding with Witschi's rule.

**Empirical evidence for asymmetrical sex reversal in nature**

If sex-reversal propensity is asymmetrical between sex-chromosome systems, we should expect that in free-living populations mostly masculinization should occur in XX/XY systems, while feminization should be predominant in ZW/ZZ systems. This is supported by the currently available, limited data on wild populations: genetically proven masculinization was frequent in 4 anuran species with XX/XY systems (Alho *et al.*, 2010; Lambert *et al.*, 2019; Xu *et al.*, 2021; **Chapter III.1**) and only rare feminization occurred in one of them (Lambert *et al.*, 2019), while sex-reversal frequency was negligible in *Bufo bufo*, the only ZW/ZZ anuran with such data to our knowledge (**Chapter II.4**). 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* (formerly *Bassiana*) *duperreyi* (XX/XY) and ZZ females in *Pogona vitticeps* (ZW/ZZ) across several free-living populations (Holleley *et al.*, 2015; Castelli *et al.*, 2021; Dissanayake *et al.*, 2021). The picture is less clear in fishes (Baroiller & D'Cotta, 2016); 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 (Senior & Nakagawa, 2013; Baroiller & D'Cotta, 2016). 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 (Toli *et al.*, 2016). 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.

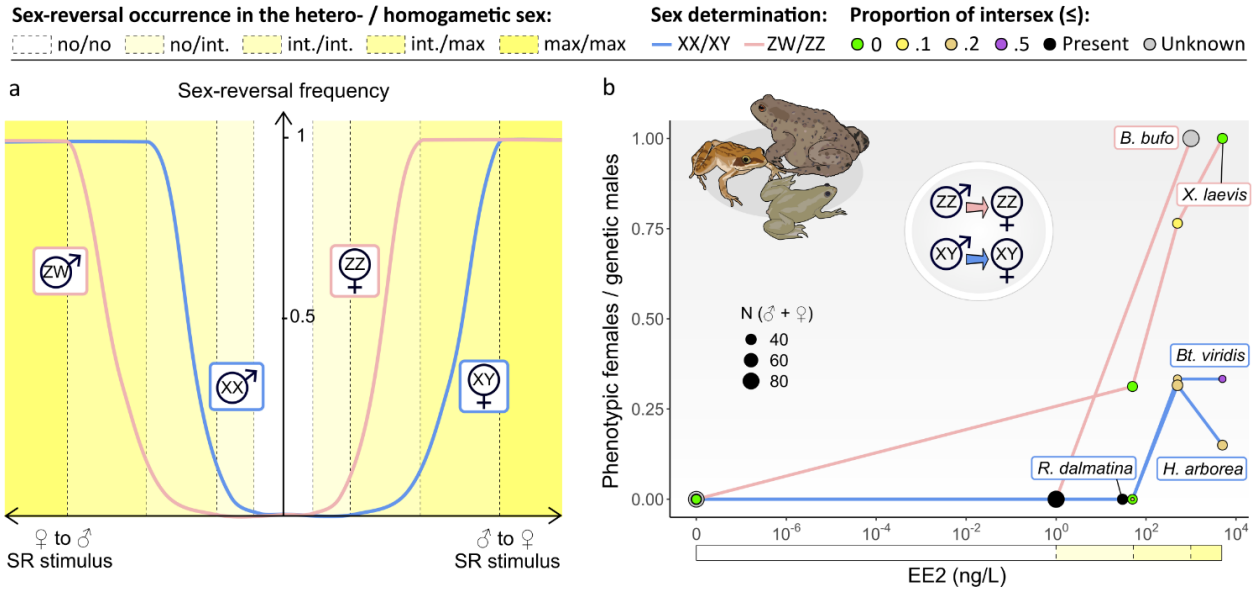
**Experimental evidence for asymmetrical sex reversal**

Amphibians, the taxon in which Witschi has originally discovered his rule, offers an ideal group for testing asymmetrical sex reversal. Genetic sex determination underlies sexual development in all amphibian species studied so far, unlike in fishes and reptiles where many species seem to have temperature-dependent sex determination with little genetic influence (Ezaz *et al.*, 2006; Ashman *et al.*, 2014; Ma & Veltsos, 2021). To evaluate the existing experimental evidence for asymmetrical sex reversal, we searched the literature for data on sex reversal and phenotypic sex ratio from laboratory experiments that were carried out on anuran amphibian species with either XX/XY or ZW/ZZ sex-determination system. We focused 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 EE2 and the herbicide atrazine (Orton & Tyler, 2015). Detailed searching methods are described in the supplementary material of Nemesházi & Bókony (2022). We found only 4 experiments in which anuran species with both male and female heterogamety were studied for sex-reversal propensity (Hayes, 1997; Piprek *et al.*, 2012; Tamschick *et al.*, 2016b; Ujszegi *et al.*, 2022), 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 (Alho *et al.*, 2010; Lambert *et al.*, 2019; Xu *et al.*, 2021; **Chapters II.4** and **III.1**), in the vast majority of studies sex reversal was inferred based only 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 (e.g. Piquet, 1930; Mintz, 1948; Petrini & Zaccanti, 1998). For these reasons, we judged that formal meta-analyses would be unfeasible with the currently available, highly heterogeneous and limited data.

The only treatment type where sex reversal was confirmed by genetic sex markers in both male- and female-heterogametic anurans was the administration of EE2 in the rearing water. Such studies have been carried out in 2 species with ZW/ZZ and 3 species with XX/XY system (**Fig. IV.1.1b**). 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 (**Fig. IV.1.1b**). A further relevant case is the anuran *Glandirana rugosa*, in which different populations feature different sex-determination systems (Miura *et al.*, 2016). 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 (Miura *et al.*, 2016). All these data on proven cases of sex reversal support that the homogametic sex has higher propensity to undergo sex reversal, especially when the pair of sex chromosomes is more diverged. Although a study on reptiles (Freedberg *et al.*, 2006) concluded that sex-chromosome heteromorphy does not constrain the sensitivity to sex reversal,

this conclusion was based on the finding that high doses of 17β-estradiol (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 latter results.

**Figure IV.1.1.** 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. Direction of sex reversal in panel **b)** is denoted in the large white circle where the symbols ♂ and ♀ stand for male and female phenotype, respectively. 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.).



Lacking data on genetically confirmed sex reversal in most species, tentative speculations can be made based on phenotypic sex ratios. Out of 18 anuran species for which we found sex-ratio data from water-temperature, sex-hormone, or atrazine treatments, only 4 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 (≥ 98% males) at higher treatment values was achieved only in XX/XY species (**Fig. IV.1.2**). Treatments with 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. IV.1.3**). The majority of overviewed studies 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 (**Fig. IV.1.1b-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 (Lambert *et al.*, 2019), as intersex may be a natural phase of gonad development in some species of amphibians as well as fish and reptiles (Bahamonde *et al.*, 2013; Orton & Tyler, 2015; Whiteley *et al.*, 2021b).

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 (*P. triseriata* and *Hyperolius viridiflavus*) was strongly affected by exogenous testosterone treatment, it was not distorted in *Hyla arborea,* the third XX/XY species, by 100 000 ng/L, twice the concentration that already caused 100% male phenotype in *P. triseriata* (**Fig. IV.1.2b**). However, this outlying lack of sex-ratio bias in *H. arborea* might be a treatment-specific outcome, since testosterone can be modified into estrogen by α-aromatase in the endocrine system; thus, testosterone treatments may cause "paradoxical" feminization in some species, while non-aromatizable androgens, such as DHT, cause masculinization in them (Kuntz *et al.*, 2003; Ogielska, 2009). Similar heterogeneity was found for fishes where methodological differences across studies accounted for much more inter-specific variation in sex-reversal inducibility than did biological differences (Senior & Nakagawa, 2013). Furthermore, even within-species differences can occur in apparent sex-reversal inducibility: for *Xenopus laevis,* the best-studied amphibian, sex ratios observed after similar treatments greatly differed between studies (e.g. Kloas *et al.*, 2009 vs. Oka *et al.*, 2008). Such differences may stem from discrepancies in the experimental set-up, sample size, or other methodological details (see below). Thus, while the patterns in **Fig. IV.1.1b-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 to sex reversal than others.

**Figure IV.1.2.** Phenotypic sex ratios (i.e. proportion of males among individuals with unambiguous sexual phenotype) reported from anurans exposed to **(a)** different temperatures, or different concentrations of **(b)** testosterone or **(c)** DHT 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. Supposed direction of sex reversal across all panels is denoted in the large white circle in panel **a**), where the symbols ♂ and ♀ stand for male and female phenotype, respectively. 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 *R. dalmatina* and 0.56 in *R. catesbeiana* **(a)**.

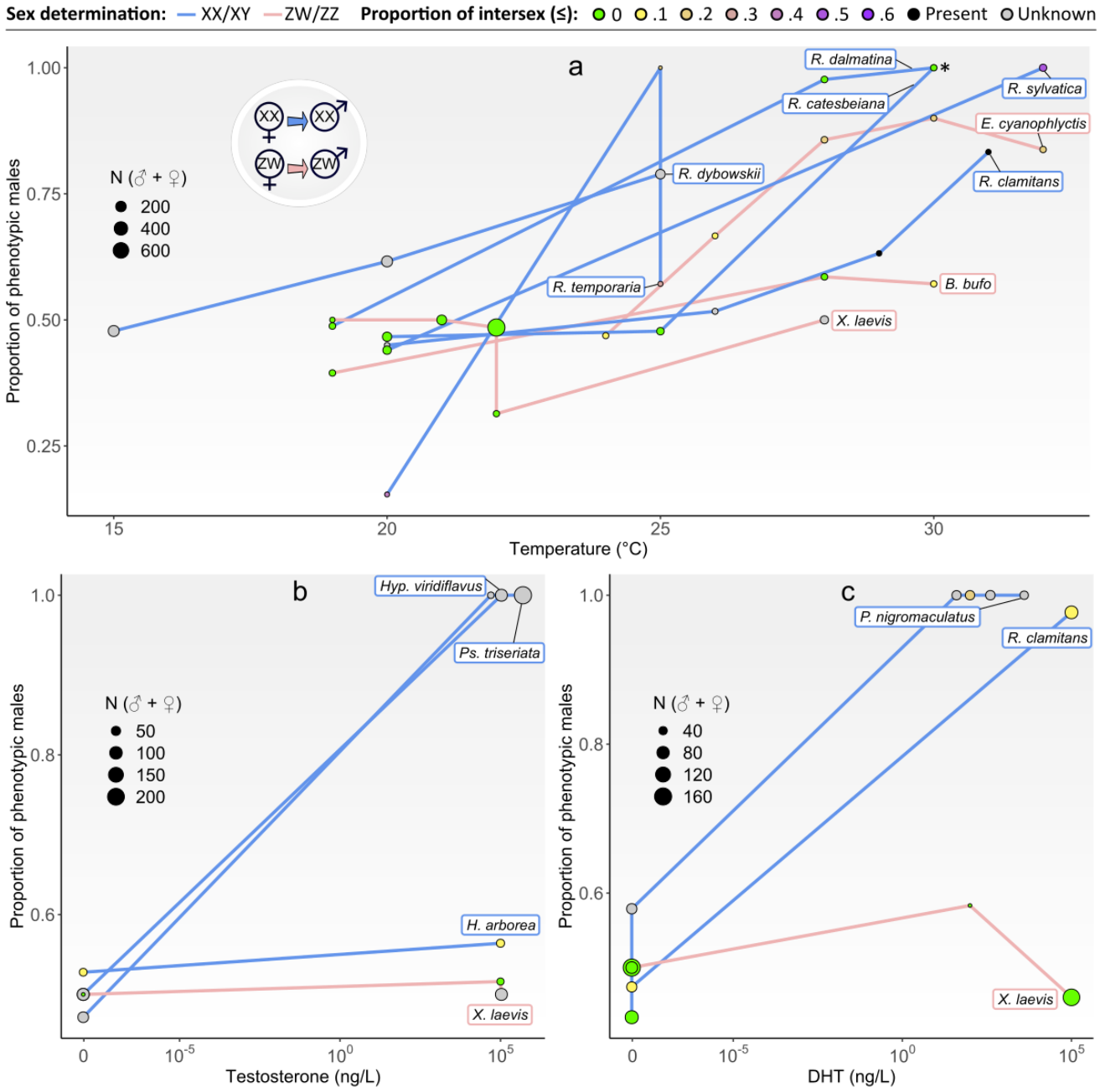
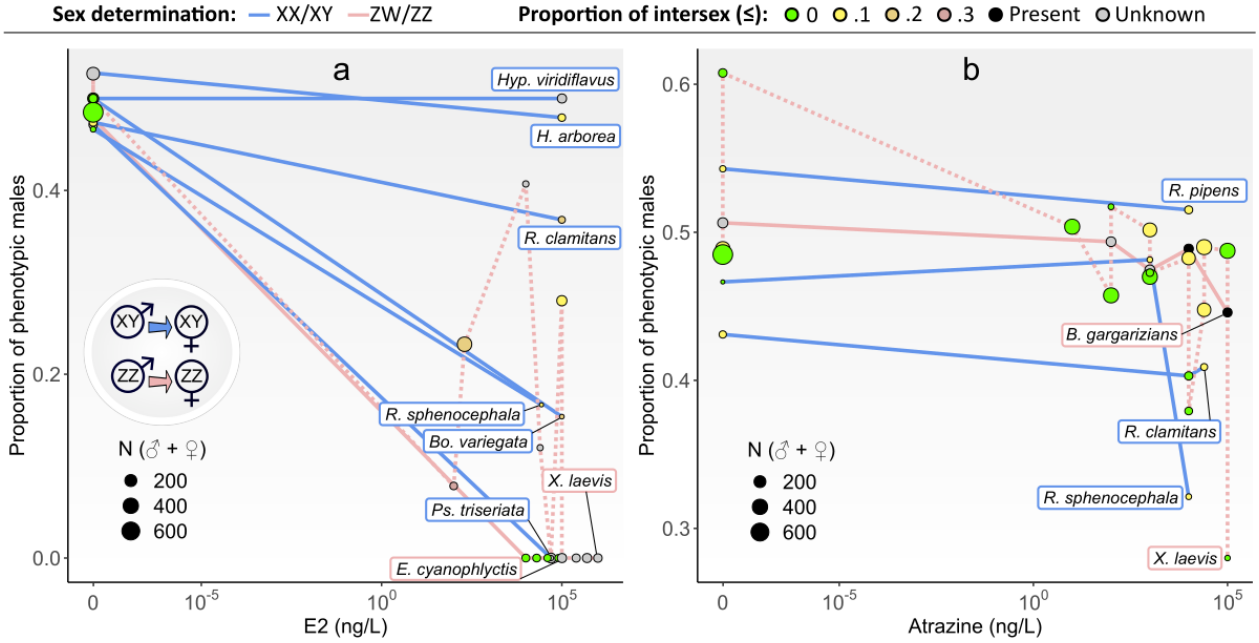


**Figure IV.1.3.** Phenotypic sex ratios reported from anurans exposed to different concentrations of **(a)** E2 (17β-estradiol) or **(b)** atrazine as tadpoles. Within each panel, dot sizes are proportional to the number of animals with unambiguous sexual phenotype; dot colors indicate the proportion of intersex individuals among all animals examined for intersexuality. Supposed direction of sex reversal across both panels is denoted in the large white circle in panel **a**, where the symbols ♂ and ♀ stand for male and female phenotype, respectively. 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.




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

In order to enable systematic comparison of responsiveness to sex-reversing effects between different sex-determination systems, 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. 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. IV.1.1a**). Pairwise comparison of closely related XX/XY and ZW/ZZ species (or populations of the same species; Miura *et al.*, 2016) would be best fitting for this purpose. 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 affected by sex-biased mortality (Geffroy & Wedekind, 2020; Lambert *et al.*, 2021) 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, 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 (Piprek *et al.*, 2012; Gramapurohit & Phuge, 2015; Miura *et al.*, 2016). Therefore, treatment periods should include the time frame when sex reversal may be induced. 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 (as in **Chapter III.2**). 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, whereas 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 ectothermic vertebrates undergo an ovary-like phase before differentiating into ovaries or testes, and the pace of this process also differs between species (Eggert, 2004; Oldfield, 2005; Ogielska, 2009; Roco *et al.*, 2021) or even within the same species (Rodrigues *et al.*, 2015). Furthermore, the relative pace of gonadal and somatic development may vary between species and treatments (Ogielska & Kotusz, 2004; Lambert *et al.*, 2018); 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 (Orton & Tyler, 2015) or impossible due to undifferentiated gonads. Apparently the same methodological issues led to earlier conclusions that sex reversal was only temporary in some amphibians (Piquet, 1930; Chang, 1955). Therefore, for phenotypic sexing to be reliable, it should be performed at a sufficiently late age, which is usually well after metamorphosis in amphibians (Ogielska & Kotusz, 2004).

Because temperature can affect sexual development, experiments on chemically induced sex reversal should also pay attention to rearing temperatures. On one hand, different species may adapt to different temperatures; thus, keeping the animals within their range of optimal temperatures is favored to prevent unexpected sex reversal 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 (Silva *et al.*, 2012; DeCourten *et al.*, 2019). 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 evolve to better survive and exploit conditions that vary across habitats, such as temperature (Bachmann, 1969) or anthropogenic chemical pollution (Cothran *et al.*, 2013); similar adaptations might also increase or decrease the likelihood of sex reversal (**Chapter II.4**). 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.

**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 adult sex ratios, sex-specific aging rates and life spans (Pipoly *et al.*, 2015; Xirocostas *et al.*, 2020; Cayuela *et al.*, 2021). 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 under 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 masculinization (Eggert, 2004; Ospina-Álvarez & Piferrer, 2008; Edmands, 2021), 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 feminization. Furthermore, in species where temperature elevation induces feminization, such as some reptiles (Whiteley *et al.*, 2021a) and caudate amphibians (Chardard *et al.*, 2004), ZW/ZZ systems may be more threatened by climate change. Therefore, more empirical research on sex reversal is needed to assess the vulnerability of ectotherms to both climate change and environmental pollution.

## IV.2. Climate-driven sex reversal: the role of sex-chromosome system[11]

As we have seen in the previous chapters, the environment can influence the development of phenotypic sex in wildlife, and this effect may depend on the type of sex determination. How do these phenomena influence the consequences of environmental changes such as the contemporary rapid change of climatic conditions? The concern that climate change may distort the sex ratio of species with temperature-dependent sex determination (TSD), where offspring sex is determined by post-fertilization environmental temperatures during a susceptible period of development, has been raised repeatedly (Ospina-Álvarez & Piferrer, 2008; Urban *et al.*, 2014; Pezaro *et al.*, 2016), as mounting evidence shows that unusually warm years yield hatchling sex ratios that are skewed towards the sex produced near the upper limit of tolerated incubation temperatures, which can result in biased sex ratio of the adult population (Mitchell & Janzen, 2010). It has even been proposed that past climate-change effects on sex ratios might have played a role in dinosaur extinctions (Miller *et al.*, 2004; but see Silber *et al.*, 2011). However, populations with genetic sex-determination systems (GSD) may also be affected by climate change *via* temperature-induced sex reversal (e.g. **Chapters III.2** and **IV.1**). Theoretical models show that sex reversals can lead to biased sex ratios (Hurley *et al.*, 2004; Cotton & Wedekind, 2007, 2009; Alho *et al.*, 2010), with far-reaching consequences for population viability (Cotton & Wedekind, 2007, 2009; Senior *et al.*, 2013) and the evolution of sex-determining mechanisms (Pen *et al.*, 2010; Grossen *et al.*, 2011; Quinn *et al.*, 2011; Schwanz *et al.*, 2013; Holleley *et al.*, 2015). For example, sex reversals can either extirpate the population or boost its size (Cotton & Wedekind, 2007, 2009), and they can propel evolutionary transitions from GSD to TSD (Pen *et al.*, 2010; Schwanz *et al.*, 2013; Holleley *et al.*, 2015) and between male-heterogametic (XX/XY) and female-heterogametic (ZZ/ZW) sex-chromosome systems (Grossen *et al.*, 2011; Quinn *et al.*, 2011).

Intuitively, the impact of temperature-induced sex reversal on the population sex ratio may differ between the two major types of GSD. For example, if high temperatures cause masculinization, as is characteristic of amphibians and fish (Wallace *et al.*, 1999; Chardard *et al.*, 2004; Ospina-Álvarez & Piferrer, 2008), in species with XX/XY systems the resulting XX males will mate with XX females, producing 100% XX offspring which may counter the sex-ratio distorting effect of masculinization. In contrast, in species with ZZ/ZW systems, masculinized ZW individuals will mate with ZW females, producing 25% male offspring (or 33% males if the WW genotype is not viable); thus this system may have lower capacity to compensate for masculinization by female-biased offspring sex ratios. Although a few previous theoretical models indicated that the two types of GSD might differ in their susceptibility to the effects of sex reversal on population demography (Cotton & Wedekind, 2007; Senior *et al.*, 2013), no study has yet formally compared the performance of the XX/XY and ZZ/ZW systems to test whether, and under what circumstances, they are differently affected by temperature-induced sex reversal.

Here we employed a two-pronged approach to study climate-driven sex-ratio shifts in ectotherms with GSD. First, we developed an individual-based theoretical framework to investigate the effects of temperature-induced sex reversal on population sex ratios under various conditions, and to contrast these effects between the two types of GSD. Second, we compiled empirical data from the literature to analyze the temporal changes of sex ratios in natural populations, and to contrast these findings with the predictions of our model. We focused on amphibians, although our

[11] This chapter is based on the following publication: Bókony V., Kövér S., Nemesházi E., Liker A., Székely T. 2017. Climate-driven shifts in adult sex ratios via sex reversals: the type of sex determination matters. Philosophical Transactions of the Royal Society B 372: 20160325. http://real.mtak.hu/119679/

model is applicable to any taxon with GSD and naturally occurring masculinization. Out of the amphibian species studied so far, all have GSD (Ashman *et al.*, 2014) and almost all can be experimentally masculinized with high temperatures during larval life (Wallace *et al.*, 1999; Chardard *et al.*, 2004). While sex reversal is difficult to study in the field due to a general lack of genetic sexing methods in amphibians (**Chapters II.4** and **III.1**), phenotypic sex ratios of adults have been studied extensively in some species over the past decades. Therefore, we tested whether adult sex ratios (ASR) changed over the last 60 years in parallel with climate warming (Seneviratne *et al.*, 2014), whether this change differed between species with different GSD systems, and whether the empirical results can be explained by increasing masculinization rates according to our model.

**Methods**

***Theoretical modelling***

We modelled the effects of increasing masculinization rate using an individual-based model in a diploid population with overlapping generations and either XX/XY or ZZ/ZW sex determination. Our specific parameter values are explained in detail in Table S1 in the supplementary material of Bókony *et al.* (2017a). Each year, a maximum number of $N_{max}$ offspring were allowed to complete metamorphosis; thereby we assumed that survival from eggs to metamorphosis is density-dependent due to limited carrying capacity of the environment. The actual number of newly metamorphosed offspring in each year was calculated as $N = \min(N_{max}; fert \times N_{female})$ where $N_{female}$ is the number of adult females and *fert* is the average number of offspring each female can recruit in the absence of density-dependence (i.e. when larval density is low due to scarcity of breeding females). Metamorphs' survival during the first winter was independent of phenotypic sex, but we allowed for an effect of genotype according to the "unguarded sex chromosome hypothesis" such that heterogametic individuals (XY or ZW) could have reduced survival, due to rare mutations on the X or Z chromosome that we assumed to exert deleterious effects primarily during early ontogeny.

After surviving the first winter, the individuals turned into juveniles with an annual survival rate independent of both genotypic and phenotypic sex. When reaching the age of maturity, which was allowed to differ between phenotypic males and females, juveniles turned into adults with an annual survival rate independent of age and genotype but dependent on phenotypic sex. For simplicity, we assumed a fixed life span; all adults died upon reaching this age. Each year, adults participated in a single breeding event, during which the mother of each of the $N$ offspring was chosen randomly out of all phenotypically female adults, while the fathers were chosen from among the phenotypically male adults according to their mating success ($\alpha$) which was allowed to vary with genotype.

Each model was run with these baseline settings and without masculinization for 50 years to allow the population structure (age and sex ratios) to stabilize. Then, each model was run for a further 350 years, during which the probability of masculinization increased linearly with some stochasticity as $p_{masc} = b_{masc} \times t \pm sd_{masc}$ where $b_{masc}$ is the year-to-year increase in the average probability of masculinization while $sd_{masc}$ is its standard deviation. Each year, the sex of newly produced, genotypically female (XX, ZW and WW) offspring was reverted to the male phenotype by the probability $p_{masc}$. Thus, we assumed that with rising average temperatures, the chances of stochastic variation resulting in temperatures high enough to trigger sex reversal will increase as well. We assumed no individual variability in the likelihood of masculinization and we did not allow resistance to sex reversal to evolve because we were primarily interested in the effects over an evolutionarily short time span to facilitate comparison with available empirical data (see below).

However, in the ZZ/ZW system, neofemales with the WW genotype (resulting from the matings between concordant ZW females and sex-reversed ZW males) were either allowed to get masculinized with the same probability as ZW females, or not at all. Masculinized individuals were then allowed to reproduce according to their phenotype and produce gametes according to their genotype, with no epigenetic inheritance of sex reversal.

We ran the model with 32 different combinations of parameter settings (hereafter scenarios, see below), each repeated in 100 runs. We examined two alternative sex-specific life histories: one characterizing urodelans (i.e., the age of first reproduction and adult survival being similar in both sexes), whereas the other corresponding to anurans (i.e., males maturing earlier but experiencing higher mortality than females). With each of these two life histories, the models were run with 16 scenarios, representing 4 different effects of genotypic sex (see panel rows in **Fig. IV.2.1**) combined with 4 different effects of phenotypic sex (see panel columns in **Fig. IV.2.1**). Specifically, the 4 different scenarios of genotypic sex effects assumed, respectively, that 1) genotypic sex had no effect on survival or masculinization, 2) the heterogametic sex suffered extra mortality early in life due to the "unguarded sex chromosome" effect, 3) the WW genotype was lethal, and 4) WW females were unable to masculinize. Each of these 4 scenarios was run with 4 different phenotypic sex effects, assuming that the mating success of masculinized individuals (i.e. phenotypic males with the XX, ZW or WW genotype) was 100%, 75%, or 1% compared to concordant males, or linked to the Y or Z chromosomes (i.e. 50% in ZW males and 1% in WW and XX males).

For each scenario, we report the results by plotting the qualitative effects of increasing masculinization rate on the phenotypic ASR and the relative frequency of each genotype until $p_{masc}$ reaches 1. From the same models, we statistically analyzed the phenotypic ASR over the first 60 years for comparison with the empirical data (see below). For each scenario we extracted the regression slope of ASR change over 60 years from each run and calculated its 95% confidence interval for the two GSD systems. We compared the slope of ASR change between the XX/XY and ZZ/ZW system within each scenario by Welch's t-tests and corrected the P-values for FDR. Since the corresponding "urodelan" and "anuran" scenarios yielded similar results, only the former are presented here; the latter can be found in the supplementary material of Bókony *et al.* (2017a).

***Empirical study***

We compiled data on the ASR (proportion of males in the adult population) from the literature as follows. We searched in Google Scholar for the terms "species name" and "sex ratio" for all amphibian species for which either male or female heterogamety has been demonstrated according to the Tree of Sex database (Ashman *et al.*, 2014). We excluded studies where the authors stated or speculated that their data may not represent the population sex ratio, or when the methods were not described in enough detail to assess the adequacy of the study (for more details on collecting, filtering and validating the data, see Pipoly *et al.*, 2015). We found data on natural populations for 39 species; the repeatability of ASR among populations within species was 0.56 (Pipoly *et al.*, 2015). However, long-term data were not available; for the vast majority of the studied populations, ASR has been reported for 1 or 2 years (maximum: 6 years). From each study we extracted ASR records for each year per population, keeping only the records with $N > 20$ individuals. We restricted our analyses to species for which there were at least 10 records and at least 10 years between the earliest and latest study year. If a study did not report annual ASR but provided average ASR over several (2-4) years, we assigned the midpoint of the study period as study year of that record.

The data that fit these criteria totaled 125 records of 6 species (2 anurans and 4 urodelans) from 51 studies conducted between 1953 and 2011. Since the ASR data for each species came from several populations, we collected data for 3 potentially confounding variables that may influence ASR: latitude, altitude, and morph. As amphibian demography and life history can vary along geographical gradients (Morrison & Hero, 2003), we extracted the latitude and altitude of the study location for each ASR record. As urodelans can be either metamorphic or paedomorphic, and the two morphs can differ in sex ratio (Denoël *et al.*, 2005), we categorized morph for each record as the presence or absence of pedomorphic adults (i.e. sexually mature individuals that retain larval somatic traits) in the population.

To analyze the relationship between ASR and time expressed as the number of years since 1950, we used a mixed-effects modelling framework. By estimating random intercepts and random slopes, we allowed the 6 species to differ in average ASR and in the slope of the change of ASR with time, respectively. As fixed effects we included time, GSD, and the time × GSD interaction to test whether species with XX/XY and ZZ/ZW systems differed in the slope of ASR change over the years. We also included the potentially confounding effects of latitude and altitude as covariates, and morph as a fixed factor. This approach assumes that each ASR record is a random sample taken from the pool of each species' all populations at various points in time and space, testing whether time (a proxy for climate warming) or geographical gradients have a systematic effect on amphibian sex ratios (see Gibbs & Steen, 2005 for a similar approach applied to turtle sex ratios).

We implemented the mixed-effects model by Bayesian approach, using the R package 'MCMCglmm' (Hadfield, 2010). We ran 4 MCMC chains with inverse Wishart priors ($V = 1$, $nu = 0.002$), each with 7,000,000 iterations, a thinning interval of 500 and a burn-in of 2,000,000 iterations, yielding 10,000 samples per chain, from which we calculated the posterior distribution of all parameter estimates (fixed and random effects). We tested the convergence of model parameters among the chains using the Gelman-Rubin statistic; the potential scale reduction factor was 1 in all cases, showing that model convergence was appropriate. Autocorrelation was < 0.02 for all estimated parameters. We report the parameter estimates (posterior means) and their 95% credibility intervals (CI) from the first chain. This approach is similar, although not identical, to meta-analysis; however, a mixed model assuming that variance decreases with increasing sample size did not fit the data better (difference in the deviance information criterion: 0.006) and yielded qualitatively identical results as those presented here.

## Results

### *Theoretical modelling*

As the rate of masculinization increased over several hundreds of years, the model predicted increasingly male-biased ASR in most scenarios (**Fig. IV.2.1**). ASR started to shift later if the sex-reversed individuals had relatively high mating success (**Fig. IV.2.1**). When the masculinized individuals reproduced just as well as concordant males, the initial male genotype went extinct in most scenarios by the time masculinization rate reached 100%, and the initial GSD transitioned into a TSD system in which all individuals had female genotypes (XX, or WW and ZW) and phenotypic males were produced solely by temperature-induced masculinization (**Fig. IV.2.1***a,e,i,m*). WW lethality sped up the ASR shift while slowing down the change of genotype frequencies (**Fig. IV.2.1***i,j,k,l*). When WW females could not be masculinized, ZW females and ZZ males were replaced by WW females and ZW males, respectively, thus leading to a switch from ZZ/ZW to XX/XY system (**Fig. IV.2.1***m,n,p*). Similar changes occurred, albeit more slowly, when the mating success of masculinized individuals was 75% compared to concordant males (**Fig.**

**IV.2.1***b,f,j,n*). In contrast, when the mating success of masculinized individuals was close to zero, both systems shifted rapidly to male-biased ASR with little change in genotype frequencies (**Fig. IV.2.1***c,g,k,o*). For the XX/XY system, this latter scenario is equivalent to the cases when male mating success is Y-linked, whereas for the ZZ/ZW system, Z-linked mating success predicted the same, albeit slower, trends as did the scenarios with 75% mating success of masculinized individuals (**Fig. IV.2.1***d,h,l,p*).

Over the first 60 years after masculinization rate had started to increase, both GSD systems shifted toward significantly more male-biased ASR in most scenarios (**Fig. IV.2.2**). This shift was significantly stronger in the XX/XY than in the ZZ/ZW system in 7 of the 8 scenarios when male mating success was Y/Z linked (**Fig. IV.2.2**). In contrast, the shift was significantly stronger in the ZZ/ZW than in the XX/XY system in 3 scenarios: when masculinized individuals had high mating success (100% or 75% of concordant males) and the WW genotype was lethal (**Fig. IV.2.2***i,j*), or when masculinized individuals had 100% mating success and heterogametic offspring suffered extra mortality early in life (**Fig. IV.2.2***e*). The slope of ASR change never differed between the two GSD systems when the masculinized individuals had very low mating success (**Fig. IV.2.2**).

***Empirical study***

The change of ASR over the years differed significantly between species with different GSD (**Table IV.2.1**). The two species with female heterogamety (ZZ/ZW) shifted significantly towards more male-biased ASR over time (**Table IV.2.2**, **Fig. IV.2.3**), with an average yearly increase of 0.4 in the percentage of males (CI: 0.03, 0.77). In contrast, the 4 species with male heterogamety (XX/XY) showed no significant change in ASR over the years (**Table IV.2.2**, **Fig. IV.2.3**), with an average decrease of 0.17 % per year (CI: -0.43, 0.08). This observed pattern, i.e. increasing male bias in ZZ/ZW and no change in XX/XY, is qualitatively consistent with the theoretical scenarios where $\alpha = 1$ and the WW genotype is lethal or the "unguarded" heterogametic offspring suffer extra mortality (**Fig. IV.2.2***e,i*). ASR did not vary systematically with latitude and altitude, while it was significantly lower in populations containing pedomorphic adults (**Table IV.2.1**). The difference among species or between the two GSD systems was not attributable to spatial differences in the rate of climate warming (see Fig. S4 in the supplementary material of Bókony *et al.* 2017a).

**Table IV.2.1.** Linear mixed-effects model for empirical ASR in relation to time (years since 1950) and GSD (genetic sex-determination system) in 6 amphibian species. ASR is expressed as percentage (%) of males in the adult population to avoid very small model parameter values.

| | | 95% CI | | |
|---|---|---|---|---|
| Model parameters | Posterior mean | Lower limit | Upper limit | P |
| Intercept (ZZ/ZW, metamorphic) | 55.140 | 39.030 | 70.760 | 0.001 |
| Latitude | -0.205 | -0.621 | 0.240 | 0.348 |
| Altitude | -0.003 | -0.007 | 0.001 | 0.123 |
| Morph (difference of pedomorphic) | -17.860 | -26.310 | -9.185 | <0.001 |
| GSD (difference of XX/XY from ZZ/ZW) | -0.140 | -19.310 | 21.210 | 0.964 |
| Time (slope in ZZ/ZW species) | 0.403 | 0.018 | 0.766 | 0.036 |
| Time × GSD (difference of slope) | -0.581 | -1.010 | -0.103 | 0.016 |

**Figure IV.2.1.** Model-predicted changes over 350 years in ASR (proportion of phenotypic males) and relative frequencies of genotypes, in scenarios with no sex difference in maturation age and adult survival. The width of each curve shows the 95% confidence band from 100 runs. The average rate of masculinization increases from zero by 0.003 each year; $\alpha$ denotes the mating success of masculinized individuals (i.e. phenotypic males with the XX, ZW or WW genotype) relative to concordant males. Note that the parameter settings for the XX/XY system are the same in the scenarios "$\alpha$ = 0.01" and "$\alpha$ Y-linked".

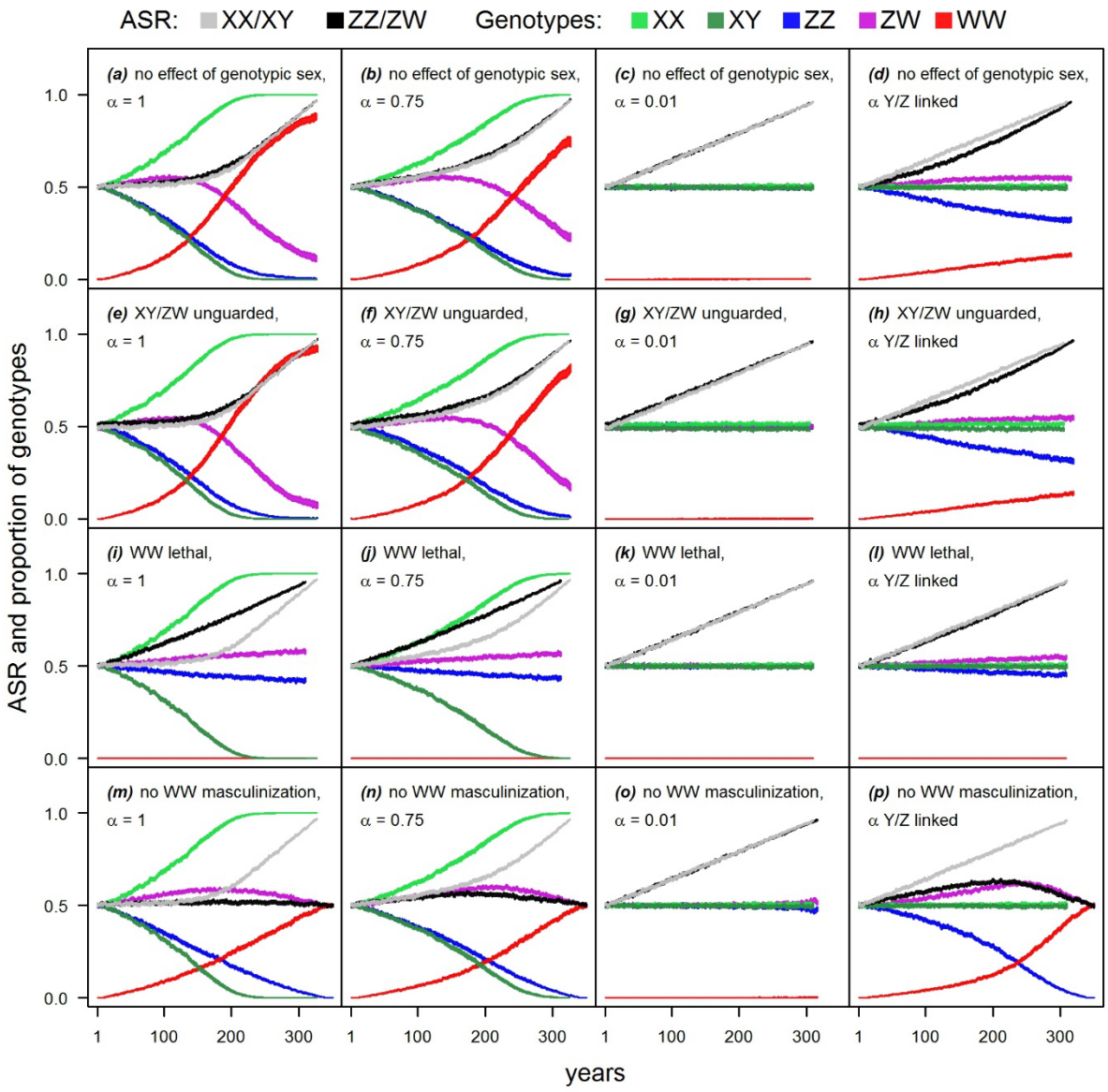


**Figure IV.2.2.** Model-predicted changes of adult sex ratio over the first 60 years in the scenarios with no sex difference in maturation age and adult survival. Black and grey stripes show the 95% confidence bands of the slopes in ZZ/ZW and XX/XY systems, respectively. Asterisks mark the scenarios in which the slopes differ significantly between the two systems ($P < 0.05$ after correction for false discovery rate). The average rate of masculinization increases from zero by 0.003 each year; $\alpha$ denotes the mating success of masculinized individuals relative to concordant males. Note that the parameter settings for the XX/XY system are the same in the scenarios "$\alpha = 0.01$" and "$\alpha$ Y-linked".

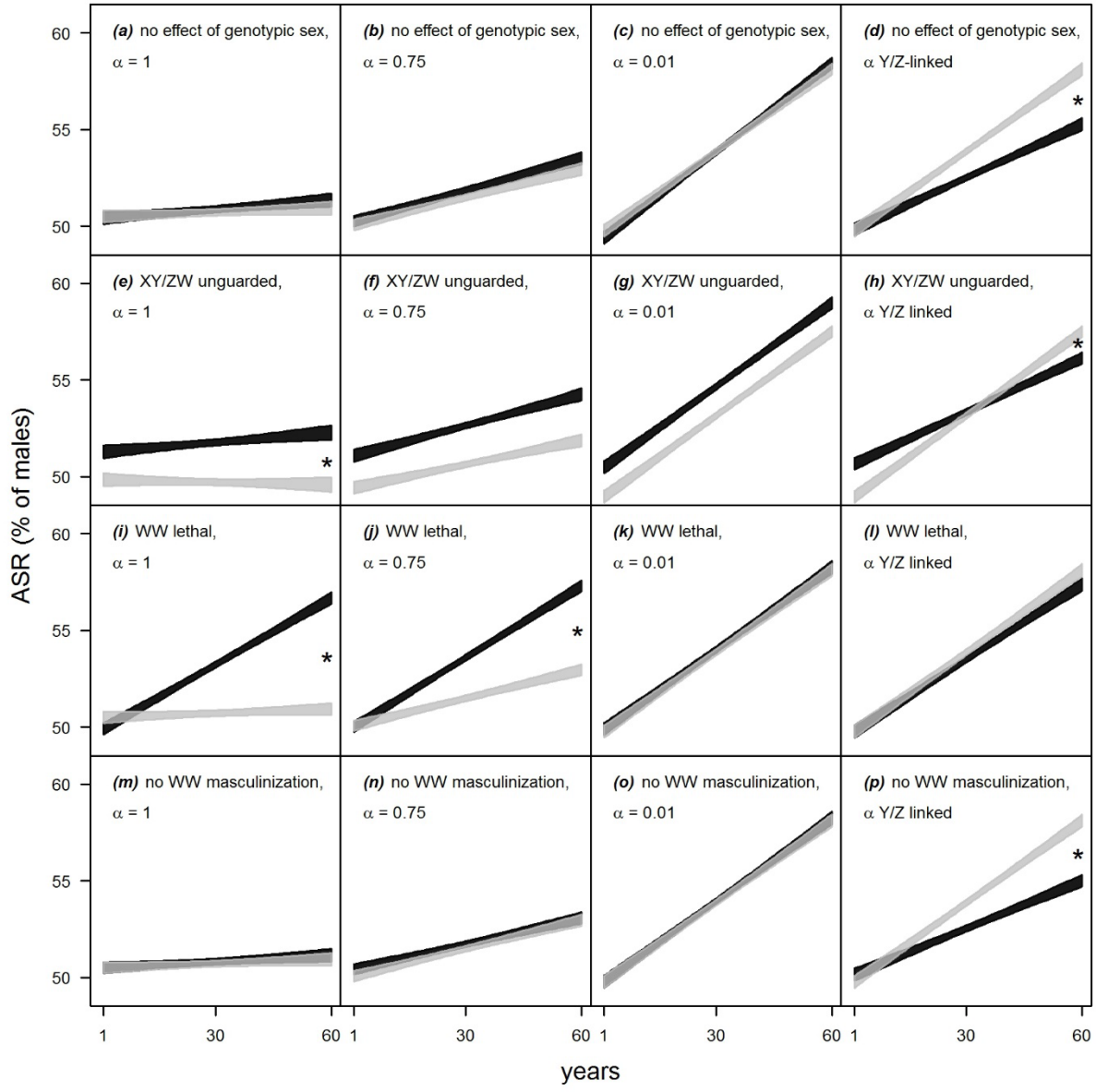
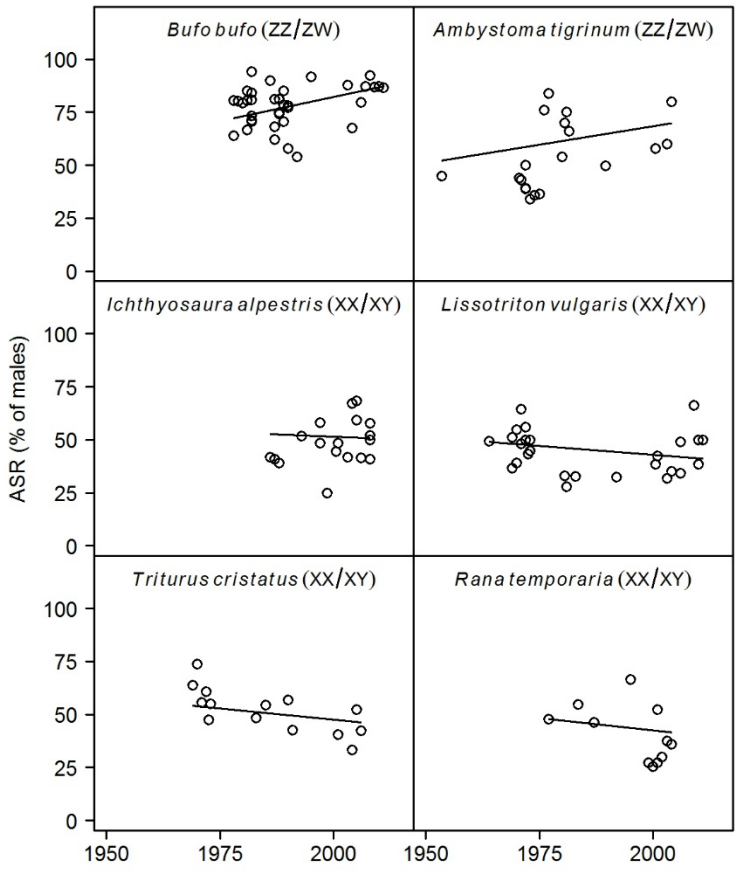


**Figure IV.2.3.** Empirical adult sex ratios over time in amphibians with either XX/XY or ZZ/ZW sex-determination systems. Slopes are fitted from the model in **Table IV.2.1**; species are ordered by slope from most positive to most negative.




**Discussion**

This study demonstrated by theoretical models, and indirectly supported by the empirical results, that the XX/XY and ZZ/ZW systems can differ remarkably in their susceptibility to climate change. Our models showed that the direction and extent of this difference varies with the mating success of sex-reversed individuals, the nature of genotype-dependent differences in offspring viability, and the ability of WW individuals to sex-reverse. The empirical data showed that the ASR shifted towards males in ZZ/ZW but not in XX/XY species of temperate-zone amphibians over the past decades while climate has been warming. Comparing these changes of ASR (**Fig. IV.2.3**) to the patterns predicted by our model (**Fig. IV.2.2**), the theoretical scenario that qualitatively best matches the empirical data is the case where masculinized individuals can reproduce as well as concordant males but offspring survival is genotype-dependent, due to either WW lethality or extra mortality of the "unguarded" heterogametic offspring. In both of these scenarios, the potential to dampen the effects of increasing masculinization rate by the production of female-biased offspring is constrained in the ZZ/ZW system compared to the XX/XY system, because early-life mortality of the genotypes is female-biased in the former but not in the latter. To explore whether these conditions are met in natural populations, and to be able to predict the impacts of climate change, detailed knowledge has to be accumulated on each species regarding the type of sex-determination system, the reproductive success of sex-reversed individuals, and the effects of genotypic sex on

offspring mortality. As most of this information is lacking for the majority of ectothermic vertebrates, this challenging task will hinge on the development of genetic sexing methods.

Our model corroborates several findings from earlier theoretical studies which showed that sex ratios can vary with climate warming in species with GSD via temperature-induced sex reversals. Firstly, if masculinized individuals are not handicapped in reproduction, higher than 50% masculinization rates lead to the extinction of the male genotype, and phenotypic males are produced solely by temperature-induced masculinization (Hurley *et al.*, 2004). Thus, the system switches from GSD to TSD even in the absence of selection for TSD, i.e. without any sex-specific fitness benefit favoring sex-specific developmental temperatures (Grossen *et al.*, 2011). Secondly, if sex-reversed individuals have reduced reproductive success, masculinization shifts ASR towards males more rapidly, while genotypic sex ratios change more slowly (Cotton & Wedekind, 2009). Thirdly, WW lethality can affect the outcome (Schwanz *et al.*, 2013) by saving the Z chromosome from extinction but rapidly shifting the ASR towards males (Hurley *et al.*, 2004). Finally, if WW females are resistant to masculinization, for example due to accumulation of male-antagonistic alleles on the W chromosome (Schwanz *et al.*, 2013), increasing masculinization rates trigger a switch between GSD systems from ZZ/ZW to XX/XY (Quinn *et al.*, 2011).

Notably, these changes occurred in our models over several hundreds of years; and similarly, theoretical studies that allowed the sex-determination system to evolve *via* mutations (which was not permitted in our models) found such switches after hundreds or thousands of years (Pen *et al.*, 2010; Grossen *et al.*, 2011; Schwanz *et al.*, 2013). Given the rapidity of contemporary environmental change (Seneviratne *et al.*, 2014), the question remains whether species can adapt fast enough (Holleley *et al.*, 2015; Pezaro *et al.*, 2016). The latest evolutionary transition between XX/XY and ZZ/ZW systems in amphibians is dated ca. 1 MYA (Miura, 2008), and the youngest neo-sex chromosome, presumably representing a switch from a mixed GSD-TSD system to a pure XX/XY system, has been documented from a lineage that diverged ca. 10 KYA (Rodrigues *et al.*, 2016). Nevertheless, several empirical findings suggest ample potential for adaptation. Even individuals with the same sex chromosomes under identical environmental conditions can vary in their propensity to develop into male or female, as pointed out by a recent review (Perrin, 2016) and by studies reporting that extreme temperatures do not always induce sex reversal in all individuals (Chardard *et al.*, 2004; see also **Chapter III.2**). Heritable variation in sex-reversal propensity (Piferrer & Anastasiadi, 2021) would allow sex-determination thresholds to evolve, and this may fundamentally change the effects of climate change as well (Pen *et al.*, 2010; Quinn *et al.*, 2011; Schwanz *et al.*, 2013). Thus, future models should allow for adaptation when comparing the responses of XX/XY and ZZ/ZW systems.

A further limitation of our theoretical study is that it did not allow for phenotypically plastic adjustments of parental or offspring behavior in choice of thermal environment. For example, many species have been responding to climate change by breeding earlier or changing nest depth (Urban *et al.*, 2014); it is unclear whether or not such behavioral adjustments can counter-balance the effects of climate change on sex ratios (Mitchell & Janzen, 2010; Urban *et al.*, 2014; Pezaro *et al.*, 2016). Because the thermal environment has critical effects on growth and developmental rates as well as on the exposure to pathogens, predators and competitors, the consequences of changing the time or place of offspring development are non-trivial and can be counter-intuitive. For example, a shift of spawning to earlier dates in a fish species has led to increasingly colder environment for the offspring at the time of sex determination, because water temperatures rose more slowly in early spring than later in the season (Wedekind & Küng, 2010). Future theoretical studies of sex reversal should account for such effects of phenotypic plasticity.

Over 60 years of climate warming, our model predicted small, although ecologically still relevant increases in ASR, averaging an additional 0.08% of males per year, which adds up to an extra ca. 5% of males in total over six decades. The data observed in nature showed relatively rapid changes in ASR, adding up to an extra 21-27% of males in ZZ/ZW species over 60 years, although these estimates inevitably had relatively high uncertainty. Changes of such magnitude are significant from evolutionary and conservationist viewpoints alike, as sex ratios have far-reaching consequences for sexual and parental behaviors, demography, and population viability (Donald, 2007; Liker *et al.*, 2013, 2014; Székely *et al.*, 2014). For example, male-biased ASR may reduce effective population size and the population's intrinsic rate of increase (Mitchell & Janzen, 2010; Pezaro *et al.*, 2016), intensify sexual competition including sexual coercion and male-male contest that can be harmful or even fatal to females (Hailey & Willemsen, 2000; Wells, 2007; Bonnet *et al.*, 2016), or induce homosexual behaviors (Bonnet *et al.*, 2016). Since we controlled for geographical variability and morph differences in our analyses, the contrasting temporal trends we found are unlikely to be artefacts of different populations being studied in different years, although among-population heterogeneity is a likely cause for the high uncertainty of our slope estimates. Due to the lack of long-term datasets, the number of species in our analysis is very small especially for the ZZ/ZW species, so further evidence will be needed to corroborate the pattern we found. Nevertheless, the 2 ZZ/ZW species have little in common in terms of life history or phylogeny that would make them stand apart from the 4 XX/XY species, suggesting that the difference we found may be related to the sex-determination system. It is also notable that in two additional ZZ/ZW species for which we found data with at least 10 years between the earliest and latest study year (2 records for each species), ASR increased from 41% in 1958-1959 to 64% in 2002-2003 in *Duttaphrynus melanostictus* and from 34% in 1981-1984 to 42% in 1997-2002 in *Crinia signifera* (Berry, 1964; Williamson & Bull, 1996; Lauck, 2005; Gramapurohit & Radder, 2012).

We caution that the observed changes of ASR do not necessarily result from global warming; direct evidence for the role of climate change and sex reversal in these empirical trends should come from long-term studies of sex ratios in various age groups within natural populations coupled with extensive genetic analyses. Until such data become available, it must be born in mind that the temporal changes of ASR may be influenced by factors unrelated to temperature. As detailed in the previous chapters, chemical pollutants have the potential to interfere with sexual development and induce sex reversal, and species can differ substantially in their susceptibility to sex reversal by the same chemicals. Furthermore, because both temperature and chemicals can disrupt the same endocrine pathway that is responsible for sexual differentiation (Chardard *et al.*, 2004; Mann *et al.*, 2009), and high temperatures can exacerbate pesticide toxicity (Mann *et al.*, 2009; Köhler & Triebskorn, 2013), chemical pollution and climate change may interact to affect sex ratios in complex ways. Such synergistic effects (see also **Chapter III.2**) may be difficult to predict unless both temperature and chemicals are considered simultaneously in theoretical and empirical studies.

Sex-determining mechanisms exhibit astonishing diversity among taxa (Bachtrog *et al.*, 2014), and evidence is accumulating that this diversity contributes profoundly to variation in demographic traits such as ASR (Pipoly *et al.*, 2015) and evolutionary processes such as adaptive radiation (Organ *et al.*, 2009) and genome evolution (Valenzuela & Adams, 2011). Our results highlight that the type of GSD can also influence the species' susceptibility to sex-ratio shifts driven by environmentally induced sex reversals. Thus, understanding the interactions between genetic and environmental effects on sex determination will be essential for predicting the among-species variation in vulnerability and adaptability to environmental changes such as climate warming. Well-informed predictions about these issues, in turn, may prove crucial in the future for the conservation of ectothermic species.

## IV.3. Climate-driven sex reversal: the role of female mate choice[12]

Earlier theoretical models of climate-driven sex reversals, including our model in the previous chapter, assumed that sex-reversed individuals are not recognized during mating, their reproductive success depending only on their fertility (Grossen *et al.*, 2011; Quinn *et al.*, 2011; Schwanz *et al.*, 2013, 2020). However, because sex-reversed individuals can differ from sex-concordant conspecifics in fecundity and offspring sex ratio (Senior *et al.*, 2012; Holleley *et al.*, 2015), individuals may benefit from taking sex reversal into account during mate choice. Choosy females then could influence the genetic sex ratio of their offspring. While mating with a concordant male would result in 50% male (XY or ZZ) and 50% female (XX or ZW) offspring, a sex-reversed (XX or ZW) male would produce 100% (XX) or 75% (ZW and WW) female offspring. Such mate-choice decisions might adjust offspring sex ratios to compensate for the distorted population sex ratio and, ultimately, might save the population from extinction. Hence, preference for sex-reversed males may potentially alter the outcomes of climate change. To our knowledge, this idea has never been addressed by theoretical studies, despite the possibility that sex-reversed individuals may be recognized by conspecifics and distinguished from concordant individuals during mate choice (Moreau *et al.*, 2001; Kirankumar & Pandian, 2002). Because sex reversal is challenging to study empirically, and only in recent years has it started to draw attention from field biologists and behavioral ecologists, no empirical study has yet tested the role of temperature-induced sex-reversal in mating success. However, increasing evidence shows that sex-reversed and concordant individuals can differ in morphology, physiology, and behavior (see **Chapters III.1-3**), all of which may affect mate choice. Furthermore, the sex-chromosome genotype of sex-reversed individuals differs from that of concordant individuals of the same phenotypic sex, so they can be distinguished on the basis of phenotypic traits linked to sex chromosomes. For example, there are several taxa with sex-chromosome-linked (hereafter referred to as sex-linked) body color genes (Lindholm & Breden, 2002; McKinnon & Pierotti, 2010; Kottler & Schartl, 2018). In such species, females may recognize sex-reversed (genetically female) males by the absence of a Y-linked male color trait (in XX/XY system) or by the presence of a W-linked female color trait (in ZW/ZZ system). Many other sexually selected traits, such as pheromones, body size, and song are also often sex-linked (Reinhold, 1998; Kirkpatrick & Hall, 2004). All this suggests that the implications of sex reversal for mate choice by sex-linked traits need to be addressed.

In this study we developed an individual-based theoretical model to investigate the role of female choice in the evolutionary and demographic consequences of temperature-induced sex reversal in a warming climate. We focused on masculinization because this seems to be the more frequent response to high developmental temperatures across ectothermic vertebrates (Chardard *et al.*, 2004; Eggert, 2004; Ospina-Álvarez & Piferrer, 2008; Holleley *et al.*, 2016). We assumed that females could distinguish between concordant and sex-reversed males by sex-linked traits, and some females tend to mate with sex-reversed males due to a genetically encoded preference. We hypothesized that mating preference for sex-reversed males could be beneficial for females and, consequently, spread in the population under climate warming, because females that chose sex-reversed males would produce more daughters, relative to those choosing concordant males, in the increasingly male-biased population. We tested this prediction, and we investigated how preference for sex-reversed males influences the adult sex ratio, the evolutionary changes in the sex-

[12] This chapter is based on the following publication: Nemesházi E., Kövér S., Bókony V. 2021. Evolutionary and demographic consequences of temperature-induced masculinization under climate warming: the effects of mate choice. BMC Ecology and Evolution 21: 16. http://real.mtak.hu/129486/

determination system, and the duration of population persistence. Although populations can no longer persist once climate has become so hot that all females get masculinized, we predicted that sexual selection may cause earlier extinction if it biases the adult sex ratio toward males. Alternatively, if sexual selection leads to less male-biased sex ratios, it may protect the population from demographic and environmental stochasticity and thereby from premature extinction.

## Methods

### *The model*

Our model parameters are explained in full detail in Fig. S1 and Table S2 in the supplementary material of open-access Nemesházi *et al.* (2021). We followed the approach of recent theoretical models on the evolution of sex determination, where sex has been assumed to be a threshold trait: a phenotypically discrete trait (i.e. male or female) determined by individual threshold sensitivity for the endogenous level of a non-discrete factor referred to as "male signal" (Grossen *et al.*, 2011; Quinn *et al.*, 2011; Schwanz *et al.*, 2013). In our model, individuals are diploid and carry a pair of sex chromosomes and two autosomal loci; all three loci are inherited independently of each other. Sex chromosomes are denoted by *A* and *a*, corresponding to Z and W in a ZW/ZZ system and to Y and X in an XX/XY system, respectively. Each sex chromosome harbors a sex-determinant locus that encodes male signal: *A* causes production of the male signal at 1.5 times higher level ($sig_A$) than *a* does ($sig_a$) under the same environmental conditions (for graphical explanation, see Fig. 2 in Nemesházi *et al.*, 2021). Male signal expression increases with environmental temperature, such that the level of male signal ($sig_{indiv}$) in an *Aa* individual is calculated with equation 1:

$$sig_{indiv} = sig_A + sig_a + sig_{env} \quad (1)$$

where $sig_{env}$ is the exogenous level of male signal due to environmental temperatures (see below). All genotypes have the same temperature sensitivity in male signal production (i.e. parallel reaction norms). We followed Grossen *et al.* (2011) in assuming that male signal increases monotonically with temperature, based on the empirical observations of masculinizing effects of high temperatures and opposite effects of low temperatures in several amphibians and fish (Devlin & Nagahama, 2002; Eggert, 2004), although we note that counter-examples exist and non-linear temperature reaction norms are also possible (Schwanz *et al.*, 2013, 2020).

The autosomal locus *thr* encodes the individual threshold for $sig_{indiv}$ that needs to be exceeded in order to develop male reproductive organs (otherwise, the individual becomes female). We calculated the individual's threshold value as the sum of the values of the two alleles that the individual carried at the *thr* locus. Although sex determination in temperature-sensitive systems is often assumed to have a polygenic basis (Bull *et al.*, 1982; Janzen, 1992), there is very little empirical evidence either *pro* or *contra* (Chandler *et al.*, 2009; Wessels *et al.*, 2014; Schroeder *et al.*, 2016); and the findings of the seminal models of temperature-sensitive sex determination were not sensitive to the assumption of one *versus* more loci (Bull *et al.*, 1982). Therefore, for simplicity we assumed that only two *thr* alleles at a single locus are present in the population; however, we explored additional simulations with 10 *thr* alleles as sensitivity tests. Allele values on locus *thr* are set according to the sex-determination system operating in the initial population. Threshold value of homozygotes for the $thr_{low}$ allele is above the average male signal level of the initial female genotype (*Aa* in system ZW/ZZ and *aa* in system XX/XY) but just below its maximum signal level realized at the temperature range before climate warming (Schwanz *et al.*, 2013). Value of the $thr_{high}$ allele is set so that threshold in $thr_{high}$ homozygotes equals the average of male signal levels that are determined by the initial female and male genotypes (*Aa* and *AA*, or *aa* and *Aa*, respectively, in ZW/ZZ and XX/XY system) under the temperature variation before climate warming. All simulations start with both *thr* alleles present in the population at 0.5 frequency.

We ran simulations where the initial sex-determination system was either XX/XY or ZW/ZZ. We assumed that sex-reversed males (XX, ZW or WW) are as viable and fecund as concordant males, following previous models and empirical data (Chardard & Dournon, 1999; Devlin & Nagahama, 2002; Senior *et al.*, 2016). Note that our previous, simpler model predicted that 25% decrease in reproductive success of masculinized individuals had little effect on adult sex ratios and sex-chromosome frequencies, whereas their sterility lead to the ZW/ZZ system behaving exactly like the XX/XY system (**Chapter IV.2**). Further, we assumed that the WW genotype (*aa* in ZW/ZZ system) is phenotypically equivalent to concordant ZW females (i.e. has the same viability, fecundity, and ability to masculinize). Empirical data support that WW individuals can be viable (Kallman, 1984; Wallace *et al.*, 1999; Devlin & Nagahama, 2002; Chardard *et al.*, 2004; Parnell & Streelman, 2013), able to reproduce (Wallace *et al.*, 1999; Chardard *et al.*, 2004; Roco *et al.*, 2015) and can also be able to develop into functional males (Kallman, 1984; Chardard & Dournon, 1999). Viability and fertility of sex-reversed males and WW individuals is likely in ectothermic vertebrates because in these taxa the sex chromosomes are usually homomorphic (Ezaz *et al.*, 2006). However, in some species the WW genotype is lethal (Wallace *et al.*, 1999), so we explored the effects of reduced WW viability in additional simulations. We did not allow new mutations to occur on the *thr* locus or the sex-determinant locus, because appearance of a new mutant allele would be unlikely in our simulations due to the realistically small population size (starting with 200 adults) and relatively short evolutionary time (according to rapid contemporary climate change). Note, however, that evolution is still possible due to standing variation in temperature sensitivity (Schwanz *et al.*, 2020), which was allowed in our simulations by changes in relative frequencies of the *thr* alleles.

In our model, climate is warming linearly over time, each year increasing the average temperature that the population is exposed to during the breeding season. In each year, each offspring may experience a different environmental temperature during the sensitive period of its ontogeny due to spatiotemporal variation in microclimatic conditions. This variation in temperature between and within years is incorporated into our model by a set of parameters defining the exogenous levels of male signal, such that in year $t$ the $sig_{env}$ level for each individual is calculated as described in equation 2:

$$sig_{env}(t) = b_{sig} + \varepsilon_B + \varepsilon_W \qquad (2)$$

where $b_{sig}$ is the slope of the yearly increase in mean $sig_{env}$ levels in the population, $\varepsilon_B$ is the normally distributed error of the yearly mean $sig_{env}$ levels (between-year climatic variance), and $\varepsilon_W$ is the normally distributed error of individual developmental temperatures (within-year climatic variance). We did not vary the values of $b_{sig}$, $\varepsilon_B$, and $\varepsilon_W$ in our simulations, but fixed each at a likely value based on empirical data. We set $b_{sig} = 0.003$ (0.3%) assuming no masculinization before 1970 (the approximate start of contemporary, human-induced climate warming) and an increase to 9% masculinization by 2000, based on the first report of sex reversal in natural amphibian populations (Alho *et al.*, 2010). We set $\varepsilon_B = 0.01$ based on the standard deviation of temperature anomalies observed in the Northern Hemisphere between 1970 and 2000 (Morice *et al.*, 2012). We set $\varepsilon_W = 0.05$, a value five times higher than $\varepsilon_B$, which is realistic based on empirical data for reptiles (Schwanz *et al.*, 2020). These settings ensure that, before the start of climate warming, a stable genetic sex-determination system (either XX/XY or ZW/ZZ) persists, with only occasional events of sex reversal in individuals experiencing unusually high temperatures (resulting in ca. 0.2% masculinization rate before the start of climate warming). We allowed this stable state to persist for 50 years (i.e. burn-in period, with $b_{sig} = 0$), after which we simulated climate warming by increasing the mean value of $sig_{env}$ each year by $b_{sig}$. We chose this relatively rapid, linear increase

in masculinization rate to model the climate warming observed in the recent past and expected in the near future (Morice *et al.*, 2012). Note that our model does not include temperature *per se*, only its effects on male signal production.

Besides the sex-determinant male signal locus, each sex chromosome harbors another locus that encodes a sexually dimorphic phenotypic trait, which for simplicity we will refer to as color and model it as a binary trait. We assume that females can recognize concordant males by a "male color" expressed in the presence of chromosome Y (*A*) when we start with an XX/XY sex determination system, and by the absence of a "female color" encoded on chromosome W (*a*) when the initial system is ZW/ZZ. The autosomal locus *C* determines preference in females for mating partners based on sex-linked color and is not expressed in males. We used an autosomal *C*, because empirical data suggest that female mating preference is more often autosomal than sex-linked (see supplementary information in Muralidhar, 2019), and sex-linkage would raise a complex problem because the outcome can depend on the sex chromosome to which the preference gene is linked (Reeve & Pfennig, 2003; Muralidhar, 2019). Values of *C* alleles determine the probability that a female would choose a concordant male if both concordant and sex-reversed males were equally available. We allowed a maximum of two *C* alleles in the population: allele $C_N$ encoding a strong but non-exclusive preference (0.9) for concordant males, and allele $C_R$ encoding the same extent of preference for sex-reversed males (i.e. 0.1 probability of choosing a concordant male). In our simulations, inheritance of *C* is fully dominant/recessive, where heterozygotes show the same choosiness as homozygotes for the dominant allele do.

Starting with either XX/XY or ZW/ZZ sex-determination system, we investigated 3 different sexual-selection scenarios. In scenario 0% $C_R$, no preference allele for sex-reversed males exists (all females are $C_NC_N$ homozygotes). In scenario 10% $C_R$, the $C_R$ allele is dominant and rare (relative frequency 0.1 in the starting population), thus initially only 19% of females (genotypes $C_RC_R$ and $C_RC_N$) express preference for sex-reversed males. This scenario could be realistic if, for example, males with female-like coloration occurred somewhat regularly in the near past e.g. due to randomly occurring sex-reversals (Perrin, 2016; Lambert *et al.*, 2019), maintaining a certain level of variance in female mating preferences. In scenario 90% $C_R$, the $C_R$ allele is recessive and widespread (relative frequency 0.9), thus initially 81% of females ($C_RC_R$) express preference for sex-reversed males. This latter scenario is possible if allele $C_R$ has spread in the initial population by neutral processes or due to a sensory bias for the "female color" that was generally not expressed in males while sex reversal was very rare (Ryan & Keddy-Hector, 1992). This can be seen as a similar case to the famous experiment of Basolo (Basolo, 1995) where 93% of females of the swordless fish (*Priapella olmeacae*) preferred males with artificial swords. For simplicity, we only investigated the effects of relatively low and high $C_R$ frequency, assuming that these two scenarios represent the range of potential effects (i.e. the effects of an initial $C_R$ frequency between 10% and 90% may fall between the effects presented here). We chose to set the initially rare *C* allele to dominant because rare recessive alleles are easily lost by drift. However, we additionally examined several other scenarios, including intermediate inheritance and a *C* allele encoding lack of preference (indiscriminate mating), to assess the sensitivity of our results to these settings.

We aimed to build a relatively realistic model where a number of parameters affect the life history and demography of iteroparous animals, including the age of maturation, annual survival rates differing across life stages, fertility, environmental carrying capacity, and limited number of mating events for each individual per breeding season. We set these parameters to be representative for many amphibians using empirical data from the literature, mostly following our previous model (**Chapter IV.2**), although similar parameter settings are representative for other temperature-

sensitive taxa such as fish or reptiles. Every year, the population produces *N* offspring calculated using equation 3:

$$N = \min(Nmax; Nmother \times fert_f), \quad (3)$$

where *Nmax* is the carrying capacity, *Nmother* is the number of adult females that found a mating partner, and $fert_f$ is the average number of offspring each female can recruit in the absence of density-dependence. For simplicity, we assume that the annual survival rate of juveniles and adults, age of maturity, and maximum life span were independent of both genotypic and phenotypic sex, except for scenarios with reduced WW viability. In our previous model on climate-driven sex reversal, the resulting changes in sex ratios and sex-chromosome genotype frequencies were not affected grossly by changing the parameters to genotypic-sex-dependent mortality and phenotypic-sex-dependent life history (**Chapter IV.2**), so we did not vary these parameters' values in the present model. Each year, adults participate in a single breeding event, during which mate choice is constrained by the availability of mating partners: within a breeding season, each female can mate with one male, while each male can mate with maximum 3 females ("libido"). Females in a randomized order, one after another, choose a single mate from the pool of still available phenotypic males according to the females' preference and relative frequency of the available male genotypes, resulting in altogether *Nmother* parent pairs. Thus, in an XX/XY system for example, a female with a dominant $C_R$ allele would mate with an XY male with a probability of $C_R \times P_{XY}$, where $C_R$ is the strength of preference for concordant males and $P_{XY}$ is the proportion of mating opportunities with XY males out of all available matings (i.e. number of available males multiplied by their remaining "libido"). Parent pair for each of the *N* offspring is chosen randomly, and each offspring randomly inherits one sex chromosome, one *thr* and one $C_R$ allele from each parent, and receives a $sig_{env}$ value, based on which its phenotypic sex is determined.

***Statistical analyses***

We performed 100 runs for each scenario. We compared the dynamics of sex-determination system evolution, sex ratios, and population persistence among scenarios by calculating the following values from each run. First, to identify transitions between different sex-determination systems, we defined the XX/XY system (i.e. *aa* females, *Aa* males) such that at least 95% of the adult phenotypic males have the *Aa* genotype, while less than 5% are sex-reversed (*aa*). Similarly, a ZW/ZZ system (i.e. *Aa* females, *AA* males) persists as long as less than 5% of the adult males carry chromosome *a*. By these definitions, a mixed sex-determination system ("GSD+TSD") is operating when more than 5% of phenotypic males are sex-reversed individuals. We defined the endpoint of each sex-determination period as the first year after which the above conditions did not prevail anymore for 50 consecutive years. For example, an XX/XY period ended and a mixed system began when the proportion of sex-reversed males increased above 5% and did not drop below this value for 50 consecutive years. In practice this meant that the proportion of a given genotype would never exceed or fall below the specified value again, as masculinization rate of each genotype kept increasing in the continuously warming environment. We chose the 50-year time window for identifying the start and end of each sex-determination period because there was a notable annual fluctuation in genotype frequencies among adult males due to the stochastic nature of our model. For each simulation, we calculated the length of each sex-determination period in years, and the year of extinction which occurred when no females were left in any age group in the population.

We also investigated changes in adult sex ratio (proportion of phenotypic males among adults) over evolutionary time in our simulations. We identified the sex-determination periods in which ASR deviated from 0.5 by visually inspecting our results, and for this period in each run we

calculated the average of yearly ASR values, and the first year when average ASR across 5 consecutive years increased above 0.6.

For each variable above (genotype frequencies, ASR, length of each sex-determination system and population persistence), we compared the 3 scenarios (0% $C_R$, 10% $C_R$ and 90% $C_R$) pairwise, starting the simulations either from ZW/ZZ or XX/XY system. To this end, we entered the values calculated from each run as a dependent variable in a linear model, using scenario as fixed factor, and we calculated 3 linear contrasts among the 3 scenarios using the function 'lsmeans' in package 'lsmeans' (the predecessor of 'emmeans'). All P-values of all these linear contrasts were adjusted simultaneously by Bonferroni correction ('bonferroni' method in the R function 'p.adjust'). We chose this strict correction method to be conservative about statistical significance in our analyses. For scenarios in the sensitivity analysis with 25% WW viability, where the distribution of data did not meet the requirements of linear models, we used pairwise median tests with Bonferroni correction.

For scenarios 10% $C_R$ and 90% $C_R$ we evaluated if the relative frequency of allele $C_R$ changed during the period when the following criteria were met: 1) co-occurrence of concordant and sex-reversed males allowed females to choose between them (i.e. both concordant and sex-reversed males were available with >5% frequency among phenotypic males), and 2) the effect of drift (due to reduced effective population size, see below) on allele frequencies did not exceed the strength of sexual selection. For each run, we calculated the average relative frequency of allele $C_R$ among adults across 5 years at the start and end of this period, and we compared the start and end values with a paired t-test across all simulations within each scenario. The 4 P-values from these t-tests were adjusted simultaneously by Bonferroni correction.

To better understand the forces behind the changes of $C_R$ frequency, we recorded for each year in each run the relative frequency of allele $C_R$ among adult males, among adult females, and among the offspring's preference alleles inherited from fathers and mothers separately. Also, we calculated the following values (as detailed in the supplementary material of Nemesházi *et al.,* 2021). For each year in each run we calculated the effective population size ($N_e$), the selection coefficient ($s$) of $C_R$ resulting from sex-ratio selection, linkage disequilibrium (LD) between allele $C_R$ and chromosome $A$, and LD between $C_R$ and the $thr_{low}$ allele. For each run, we recorded the year when the ultimate population decline started, i.e. the year in which $N_e$ permanently decreased below the average $N_e$ of the 10 years before the start of climate warming when the population was in a stable state. Because our populations had overlapping generations, we estimated generation time (T) as the mean age of reproduction; T was about 3 years in our simulations in all scenarios.

## Results

### *Consequences of climate warming without preference for sex-reversed males*

When we assumed that all females preferred concordant males (scenario 0% $C_R$), a continuous rise in environmental temperatures resulted in changes in population structure in terms of genotypes and phenotypic sexes, leading to evolutionary switches between sex-determination systems and, ultimately, to population extinction (**Fig. IV.3.1a,d**). When starting with an XX/XY system, the increasing frequency of masculinized individuals quickly skewed the ASR towards phenotypic males and resulted in a mixed sex-determination system (hereafter referred to as "final period") in which phenotypic males with both *Aa* (XY) and *aa* (XX) genotypes were present (**Fig. IV.3.1a**). Because individuals possessing one or two copies of the $thr_{high}$ allele were less susceptible to temperature-induced masculinization, and thus were able to retain female phenotype during the earliest stages of climate warming, this allele spread and usually became fixed in the population (**Fig. IV.3.1a**, see also Fig. S2a in the supplementary material of Nemesházi *et al.,* 2021). However,

even homozygotes for the $thr_{high}$ allele started to masculinize as climate got increasingly hotter, leading to population extinction after ca. 42 generations (**Fig. IV.3.1a**).

When starting with a ZW/ZZ system, the earliest stages of climate warming caused similar increases in ASR and frequency of the $thr_{high}$ allele as in the XX/XY system (**Fig. IV.3.1d**). However, the ZW/ZZ system adapted to climate change by transitioning into an XX/XY system as follows. Increasing frequency of masculinization led to a mixed sex-determination system in which phenotypic males with both *AA* (ZZ) and *Aa* (ZW, corresponding to XY) genotypes were present (**Fig. IV.3.1d**). During this transitional mixed sex-determination period, the ASR became strongly male-biased, which decreased the effective population size such that in some runs the population barely escaped extinction (see Fig. S3d in the supplementary material of Nemesházi *et al.,* 2021). Under male-biased ASR, sex-ratio selection favored the reproduction of non-preferred *Aa* males (masculinized individuals) because those produced less male-biased offspring for two reasons. First, mating between two *Aa* individuals produced fewer genotypic males (25% *AA* offspring, in contrast to 50% from "normal" matings). Second, such mating events produced 25% *aa* (WW) offspring, a novel genotype in the ZW/ZZ system which was resistant to temperature-induced masculinization as long as climate warming was mild, due to its genetically low levels of male signal. These "resistant females" rapidly accumulated because increasingly high numbers of sex-reversed males could reproduce (as the proportion of masculinized individuals increased among phenotypic males, more and more females were forced to accept them). Because all females preferred concordant *AA* males, and the female phenotype became more and more restricted to the *aa* genotype, dis-assortative mating occurred between the two homozygote genotypes. This produced an excess of *Aa* genotypes, while genotype *AA* disappeared from the population because *aa* females could not produce *AA* offspring. This way, the population transitioned to an XX/XY system, where all phenotypic females had the *aa* genotype (WW became XX) and all phenotypic males had the *Aa* genotype (ZW became XY), producing 0.5 progeny sex ratios and returning the ASR to 0.5 (**Fig. IV.3.1d**). This system persisted until *aa* individuals started to get masculinized by the ever-increasing temperatures, once again skewing the ASR towards phenotypic males. During this "final period", the frequency of mating between masculinized and concordant *aa* individuals increased, thereby chromosome *A* (Y) became rare or even extinct. When no phenotypic females were left, the population died out, ca. 98 generations after the start of climate warming (**Fig. IV.3.1d**).

***Effects of preference for sex-reversed males***

When we allowed females to vary in mating preference, the presence of allele $C_R$ significantly changed the temporal dynamics of the above processes and the magnitude of ASR skew (**Fig. IV.3.1-4**). The evolutionary switches between sex-determination systems happened faster, resulting in shorter initial period in both starting systems, shorter transition period between ZW/ZZ and XX/XY, and longer final period, whereas the duration of the XX/XY system that evolved from the ZW/ZZ system was not affected (**Fig. IV.3.2**). These changes were greater when the initial frequency of the $C_R$ allele was higher (**Fig. IV.3.2**). Ultimately, these changes did not alter the population's extinction time when starting from a ZW/ZZ system (although a few populations in the 90% $C_R$ scenario died out prematurely; see Fig. S3f in the supplementary material of Nemesházi *et al.,* 2021), but the XX/XY system starting with a widespread $C_R$ allele survived slightly longer (**Fig. IV.3.3**). However, all these differences in duration were biologically small, averaging only a few generations (see Table S3 in the supplementary material of Nemesházi *et al.,* 2021).

Average ASR was not affected by the presence of allele $C_R$ during the initial ZW/ZZ and XX/XY periods, nor during the XX/XY period evolved from the ZW/ZZ system (see Table S3 in

the supplementary material of Nemesházi *et al.,* 2021). As these periods were defined by the scarcity of sex-reversed males and thus little variation in female choice, ASR remained near 0.5 (**Fig. IV.3.1**). However, during the mixed sex-determination period following the initial sex-chromosome system, the presence and frequency of allele $C_R$ significantly influenced the timing and extent of ASR skew towards males (**Fig. IV.3.1**, **Fig. IV.3.4**). When the initial frequency of the $C_R$ allele was 10%, it had little effect on ASR in the XX/XY system (**Fig. IV.3.1b**), but populations starting with ZW/ZZ system had significantly less male-biased ASR during the transitional period between ZW/ZZ and XX/XY (on average, 63% males instead of 72%) and reached 0.6 ASR about 3 generations later than populations where all females preferred concordant males (**Fig. IV.3.1e**, **Fig. IV.3.4**). When the initial frequency of allele $C_R$ was 90%, it had even greater effects on ASR (**Fig. IV.3.1f**, **Fig. IV.3.4**). First, in both systems, ASR decreased slightly below 0.5 temporarily after the end of the initial sex-determination system, returning then to 0.5 (**Fig. IV.3.1c,f**). After that, ASR remained close to 0.5 throughout the transition period following the ZW/ZZ system, keeping the population in a balanced sex ratio for ca. 50 generations longer compared to the other two scenarios (**Fig. IV.3.1d-f**, **Fig. IV.3.4a**). Starting with the XX/XY system, ASR increased to 0.6 slightly later when allele $C_R$ was widespread in the initial population compared to scenario 0% $C_R$ (**Fig. IV.3.1a-c**, **Fig. IV.3.4a**), although the slope of ASR increase was alternatingly steeper and shallower before shifting ultimately to 1 (**Fig. IV.3.1a-c**).

***Background of preference effects: sex-ratio selection and sexual selection***

Starting with XX/XY system and a rare (10%) dominant $C_R$ allele, the accumulation of masculinized individuals was slightly sped up by the presence of females preferring them. Because individuals possessing the $thr_{low}$ allele were the first to masculinize, they were the ones chosen by females possessing $C_R$, leading to positive LD between $thr_{low}$ and $C_R$ (**Fig. IV.3.5a**) and keeping $thr_{low}$ from decreasing for several decades (**Fig. IV.3.1b**). This resulted in earlier transition into the mixed system of the final period. However, the rare $C_R$ allele had little if any effect on ASR, because its spread was selected against for the following reasons. During the first decades, masculinized individuals (*aa* males) and the females choosing them (females with $C_R$) produced female-biased offspring (all *aa* genotypes, facing relatively low masculinization rates at this early stage), which was not beneficial because the ASR was still close to 0.5 (**Fig. IV.3.6a**). As ASR became more and more male-biased, sex-ratio selection increasingly favoured $C_R$ (**Fig. IV.3.6a**), but sexual selection acted against it for the following reasons. Because females carrying $C_R$ preferred to mate with sex-reversed males (*aa*, i.e. those without chromosome *A*), negative LD arose between $C_R$ and *A* (**Fig. IV.3.5a**). As the majority of females did not possess allele $C_R$ and thus preferred concordant males, sexual selection favoured males with chromosome *A*, and thereby acted against $C_R$ due to the negative LD between $C_R$ and *A.* Thus, males carrying $C_R$ were less likely to become fathers than those without $C_R$ (i.e. proportion of $C_R$ was lower among the alleles passed on by fathers than among the alleles in adult males; see light and dark blue lines in **Fig. IV.3.6a**). Therefore, the frequency of allele $C_R$ decreased significantly (on average by 0.032, 95% CI: 0.025-0.039, $t_{99} = 8.56$, $P < 0.001$) from the start of the final mixed-system period until the beginning of ultimate population decline, and the $C_R$ allele generally vanished before population extinction (**Fig. IV.3.1b**, see also Fig. S4a in the supplementary material of Nemesházi *et al.,* 2021).

When the XX/XY system started with a common (90%) recessive $C_R$ allele, more drastic changes occurred. Since most females preferred sex-reversed males, as soon as the latter started to spread, they flooded the population with female-biased offspring that skewed the ASR towards females (**Fig. IV.3.1c**) and passed on $C_R$ and $thr_{low}$. This increased the frequency of both alleles

significantly (**Fig. IV.3.1c**, **Fig. IV.3.6b**) and created LD between $C_R$ and $A$ and between $C_R$ and $thr_{low}$ (**Fig. IV.3.5b**). Due to the widespread preference for *aa* males and the positive LD between $a$ and $C_R$, the relative frequency of $C_R$ was higher in males becoming fathers than in the adult male population (**Fig. IV.3.6b**). Therefore, sexual selection spread $C_R$ (**Fig. IV.3.6b**; average increment in $C_R$ frequency was 0.029, 95% CI: 0.020-0.039, $t_{99} = 6.132$, $P < 0.001$) despite the disadvantage of $C_R$ in sex-ratio selection while climate warming was relatively mild. The spread of $C_R$ was accompanied by a rapid shift towards male-biased ASR because the widespread preference for sex-reversed males increased the frequency of $thr_{low}$ to ca. 80% (**Fig. IV.3.1c**). Notably, sexual selection for the lack of chromosome $A$ (Y) eradicated it from the population about 20 generations after the start of climate warming, leaving only *aa* genotypes (**Fig. IV.3.1c**) and ending all selection on $C_R$ (**Fig. IV.3.6b**). At this point, the role of the sex-chromosome-linked sex-determining locus was taken over by the autosomal threshold locus, as individuals possessing (more copies of) $thr_{high}$ could resist masculinization for some time. During this time, sex-ratio selection favored $thr_{high}$ and started to spread it until fixation, which temporarily slowed the increase of masculinization rate and ASR (**Fig. IV.3.1c**, see also Fig. S5c in the supplementary material of Nemesházi *et al.,* 2021). This resulted in maintaining somewhat higher effective population size compared to scenarios with no or rare $C_R$ (see Fig. S3 and Fig. S9 in the supplementary material of Nemesházi *et al.,* 2021), and thereby slightly increased the population's survival time (**Fig. IV.3.5a**).

Starting with ZW/ZZ system and dominant $C_R$ allele at 10% frequency, the simulations resulted in similar LD of $C_R$ with chromosome $a$ (W) and allele $thr_{low}$ (**Fig. IV.3.5c**) as described above. This slowed the decrease of $thr_{low}$ and accelerated the accumulation of *aa* genotypes and thereby the transition into XX/XY system (**Fig. IV.3.1e**). Because not all females preferred concordant (*AA*) males, this scenario produced a milder excess of *Aa* genotypes during the transition period (**Fig. IV.3.1e**) compared to the scenario without $C_R$ (**Fig. IV.3.1d**). The slightly better mating success of masculinized individuals resulted in faster accumulation of "resistant females" (*aa*), slowing the ASR increase and keeping it less male-biased during the transition period (**Fig. IV.3.1e**, **Fig. IV.3.4b**). Nevertheless, ASR was skewed enough so sex-ratio selection favored parents that produced less male-biased offspring, i.e. females choosing masculinized individuals, thus the frequency of $C_R$ almost doubled by the end of the transition period (**Fig. IV.3.6c**; during ZW/ZZ to XX/XY transition, relative frequency of allele $C_R$ increased on average by 0.076, 95% CI: 0.059-0.093; paired t-test: $t_{99} = 9.0$, $P < 0.001$). Once the *AA* genotype went extinct, both sex-ratio selection for allele $C_R$ (and chromosome $a$; **Fig. IV.3.5c**) and sexual selection against it ended, and the further processes followed a similar course as in the scenario without $C_R$ (**Fig. IV.3.1d-e**).

When the ZW/ZZ system started with the recessive $C_R$ allele at 90% frequency, the effects seen in the previous scenario became stronger, *via* similar mechanisms. Since most females preferred masculinized individuals, the accumulation of *aa* genotypes and the transition to XX/XY system were even faster (**Fig. IV.3.1f**, **Fig. IV.3.2b**). During the transition period, there was only a small excess of *Aa* genotypes (**Fig. IV.3.1f**) because *AA* males were soon replaced by *Aa* males as the latter spread and were preferred by most females (who increasingly had the *aa* genotype). The frequency of $thr_{low}$ increased greatly due to the widespread preference for individuals with low masculinization thresholds (**Fig. IV.3.1f**). Since masculinized individuals enjoyed high mating success, ASR became slightly female-biased at the start of the transition period, but soon returned to 0.5 and remained close to it afterwards (**Fig. IV.3.1f**, **Fig. IV.3.4b**) because the production of "resistant females" compensated for the increasing masculinization rate. The frequency of allele $C_R$ slightly increased during the transition period (by 0.021 on average, 95% CI: 0.012-0.030, $t_{99} = 4.707$, $P < 0.001$; **Fig. IV.3.6d**) due to two reasons: 1) sexual selection (i.e. the more preferred sex-

reversed males carried allele $C_R$ and passed it on to the next generation with higher than expected probability: **Fig. IV.3.6d**), and 2) LD with chromosome *a* (W) and allele $thr_{low}$ (**Fig. IV.3.5d**). Overall preference for sex-reversed males kept the frequency of $thr_{low}$ around 80% until nearly 75 generations after the start of climate warming, causing a relatively early and sudden ASR increment after the XX/XY system ceased to persist (**Fig. IV.3.1f**). Thereafter, sex-ratio selection favored allele $thr_{high}$ over $thr_{low}$, but could not prevent a relatively early population decline compared to the other scenarios (**Fig. IV.3.1f**, see also Fig. S3f in the supplementary material of Nemesházi *et al.,* 2021). Thus, when chromosome *A* went extinct and the role of the sex-determining locus was taken over by the autosomal threshold locus, $thr_{high}$ was already close to fixation and so this new sex-determination system persisted for only a few generations before the population died out (**Fig. IV.3.1f**).

***Sensitivity analyses***

The above-described effects of $C_R$ were very similar in the following cases: 1) when we assumed a multiallelic *thr* locus (instead of biallelic) or 2) intermediate inheritance of *C* alleles (instead of fully dominant/recessive), or 3) when the value of $C_N$ was set to 0.5, encoding indiscriminate mating, or 4) when viability of WW offspring was reduced by 25% or 50%. Changes in relative frequency of $C_R$ were also very similar in these cases, except that it showed little if any change over time when initial frequency of $C_R$ was 10% and $C_N$ encoded indiscriminate mating. All these results are presented in Fig. S10-S15 in the supplementary material of Nemesházi *et al.* (2021).

However, when WW viability was reduced by 75%, presence or absence of $C_R$ made a significant difference (**Fig. IV.3.3b**). When $C_R$ was absent, the population could transition to an XX/XY system and persist for ca. 270 years in only 26% of runs; the rest died out after ca. 120 years (**Fig. IV.3.3b**). When $C_R$ was present, it saved the population from early extinction by enabling the switch to XX/XY system in 56% of runs when $C_R$ was rare and in 100% of runs when $C_R$ was widespread (**Fig. IV.3.3b**). Due to high mortality of WW offspring, relative frequency of $C_R$ started to decrease in both scenarios, but it increased rapidly just before the end of transition when ASR became highly male-biased (see Fig. S16 in the supplementary material of Nemesházi *et al.,* 2021).

In contrast, when we assumed that the WW genotype was not viable at all, the ZW/ZZ system always behaved like the XX/XY system (no transition in sex determination; population extinction after ca. 120 years of warming) and the presence of $C_R$ had very little effect on the shifting of ASR towards males. Relative frequency of both the rare and the widespread $C_R$ decreased steadily in these scenarios, as females mating with sex-reversed males lost 25% of their offspring because of being WW (see Fig. S17 in the supplementary material of Nemesházi *et al.,* 2021).

**Figure IV.3.1.** Changes in relative frequency of males (ASR), sex-chromosome genotypes, and threshold alleles among adults. On the left: simulations starting with XX/XY system with scenarios 0% $C_R$ (a), 10% $C_R$ (b) and 90% $C_R$ (c). On the right: simulations starting with ZW/ZZ system with scenarios 0% $C_R$ (d), 10% $C_R$ (e) and 90% $C_R$ (f). Each curve indicates median values calculated from 100 runs. Vertical dotted lines indicate the end of each sex-determination period, and dashed lines indicate the start of ultimate population decline. Year zero is the start of climate warming.

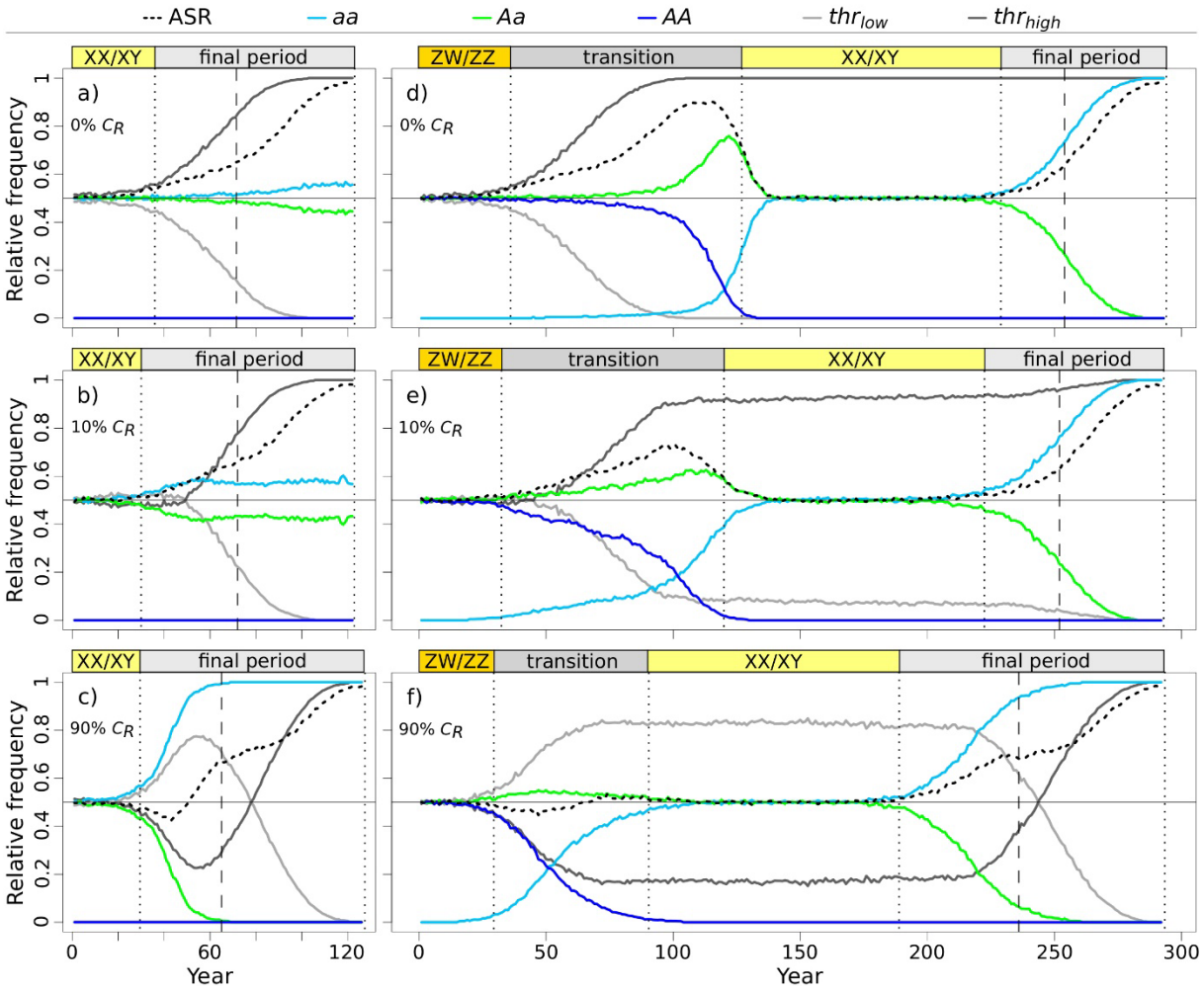


**Figure IV.3.2.** Length of consecutive sex-determination periods in each scenario starting with either a) XX/XY system or b) ZW/ZZ system. Each boxplot shows the distribution of the results from 100 runs (boxplot interpretation is as in **Fig. II.3.1**). Significant pairwise differences are indicated above the boxplots as: * 0.01 < P < 0.05, *** P < 0.001. Year zero is the start of climate warming.

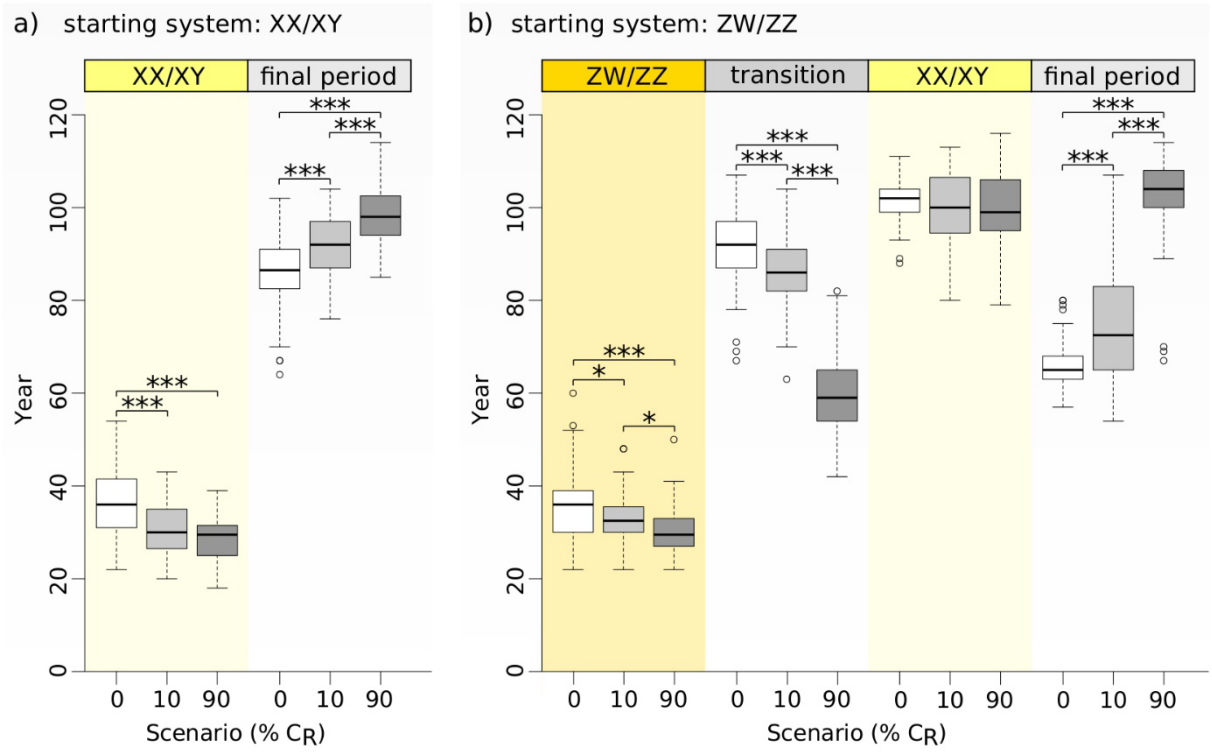
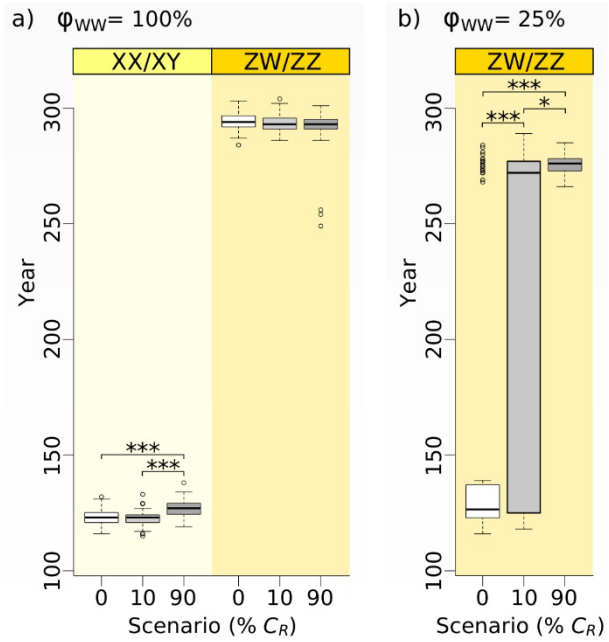



**Figure IV.3.3.** Population persistence a) in our main scenarios (starting with either XX/XY or ZW/ZZ system) and b) in the sensitivity test with ZW/ZZ initial system in which the survival of the WW genotype ($\varphi_{WW}$) was reduced to 25% compared to the survival of genotypes ZW and ZZ. Interpretation of boxplots, asterisks, and year zero is the same as in **Fig. IV.3.2**.



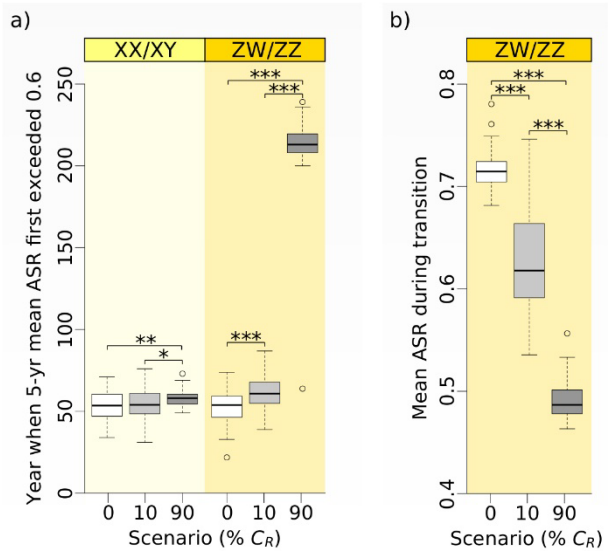
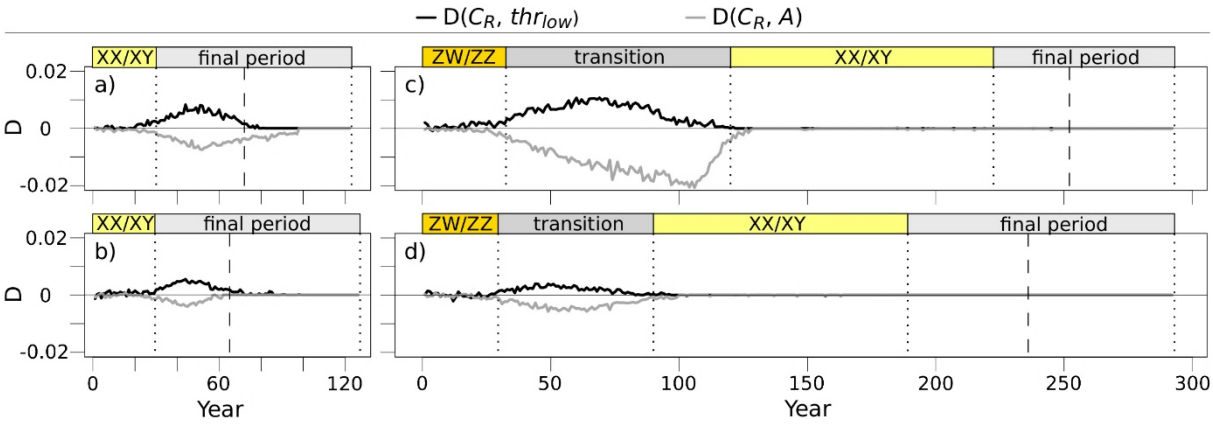
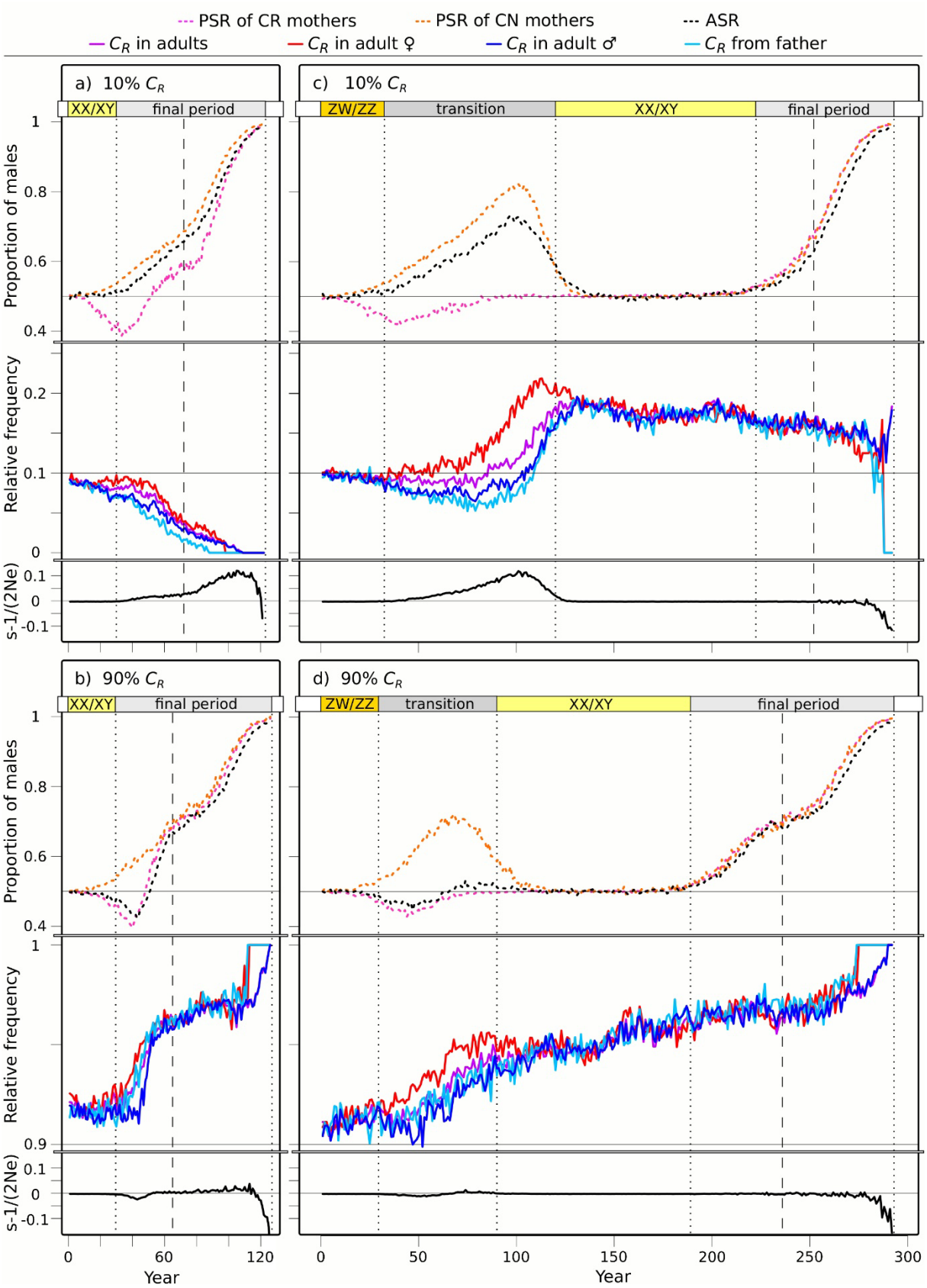



**Figure IV.3.4.** Differences in adult sex ratios (ASR, proportion of males) among scenarios. Each boxplot shows the distribution across 100 runs for a) the year when mean ASR across five consecutive years first exceeded 0.6, starting with either XX/XY or ZW/ZZ system, and b) the mean ASR during the transition period between the initial ZW/ZZ and the subsequent XX/XY system. Significant pairwise differences are indicated above the boxplots as: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. Interpretation of boxplots is the same as in **Fig. IV.3.2**.

**Figure IV.3.5.** Linkage disequilibrium coefficient (D) between preference allele $C_R$ and either the threshold allele $thr_{low}$ or sex chromosome $A$. On the left: simulations starting with XX/XY system with scenarios 10% $C_R$ (a) and 90% $C_R$ (b). On the right: simulations starting with ZW/ZZ system with scenarios 10% $C_R$ (c) and 90% $C_R$ (d). Each curve indicates median values calculated from 100 runs. Vertical dotted lines indicate the end of each sex-determination period, and the dashed lines indicate the start of ultimate population decline. Year zero is the start of climate warming. Note that negative linkage disequilibrium (LD) with $A$ is equivalent to positive LD with $a$.




PSR of CR mothers
PSR of CN mothers
ASR
$C_R$ in adults
$C_R$ in adult ♀
$C_R$ in adult ♂
$C_R$ from father
a) 10% $C_R$
c) 10% $C_R$
b) 90% $C_R$
d) 90% $C_R$
XX/XY
final period
ZW/ZZ
transition
Proportion of males
Relative frequency
s-1/(2Ne)
Year

←

**Figure IV.3.6** (see the figure on the previous page). Consequences of mate choice: progeny sex ratio, inheritance of allele $C_R$, and sex-ratio selection acting on allele $C_R$. On the left: simulations starting with XX/XY system with scenarios 10% $C_R$ (a) and 90% $C_R$ (b). On the right: simulations starting with ZW/ZZ system with scenarios 10% $C_R$ (c) and 90% $C_R$ (d). In each panel, the top 3 curves show the changes in adult sex ratio (ASR) and progeny sex ratio (PSR) of mothers preferring sex-reversed and concordant males (CR and CN mothers, respectively); the 4 curves in the middle show the relative frequency of allele $C_R$ across and among sexes ($C_R$ in all adults, $C_R$ in adult females, $C_R$ in adult males) and the relative frequency of $C_R$ among preference alleles inherited by offspring from their fathers ($C_R$ from father; note how it differs from the $C_R$ in adult males). Mothers passed on allele $C_R$ with a relative frequency corresponding to its presence among adult females (not shown). The bottom curve of each panel shows the strength of sex-ratio selection ($s$) relative to the effective population size ($N_e$), expressed as $s$–$1/(2N_e)$ (this value is shown instead of $s$ itself, because the effect of genetic drift can override $s$ in small populations). Each curve indicates median values calculated from 100 runs. Note that the top, middle, and bottom part of each panel has 3 different Y axes with different scales to facilitate visibility. Vertical dotted lines indicate the end of each sex-determination period, and dashed lines indicate the start of ultimate population decline. Year zero is the start of climate warming.

**Discussion**

This chapter investigated how female preference for sex-reversed males would affect evolution in an increasingly masculinizing environment. Our simulations showed that the presence and frequency of such a preference allele ($C_R$) influenced both the temporal dynamics of evolution of sex-determination systems and the changes in adult sex ratio across a wide range of circumstances, and under certain conditions it also affected the timing of population extinction. Furthermore, we found that a rare, dominant $C_R$ allele may spread in populations with ZW/ZZ sex-determination system more likely than in populations with XX/XY system, as discussed in more detail below.

In our simulations, increasing masculinization rate under continuous climate warming resulted in a process where different sex-determination systems replaced one another. This agrees with the findings of previous models (Grossen *et al.*, 2011; Quinn *et al.*, 2011; Schwanz *et al.*, 2013; **Chapter IV.2**) and experimental data (Holleley *et al.*, 2015) suggesting that climate change may cause turnovers between different sex-determination systems and thus may have contributed to the variability of sex-determination systems across ectothermic vertebrates (Hillis & Green, 1990; Devlin & Nagahama, 2002; Miura, 2017). Our present results demonstrate for the first time that the speed of these turnovers may be enhanced by sexual selection if females can recognize and prefer to mate with sex-reversed males. Specifically, when the $C_R$ allele was present in our simulations, the initial sex-determination system (either XX/XY or ZW/ZZ) evolved sooner into a mixed sex-determination system, and when the initial system was ZW/ZZ, the transitional mixed system also evolved sooner into an XX/XY system. These effects were stronger when the relative frequency of $C_R$ was higher. Furthermore, a widespread $C_R$ allele facilitated a turnover in the XX/XY system that was not seen when $C_R$ was rare or absent: the original male-determining sex chromosome went extinct, and its role was taken over by an autosome (harboring the original threshold locus). These results suggest the idea that, over the evolutionary past with alternatingly warmer and colder climates, variation in female preferences for sex-linked traits might have catalyzed the diversification of sex-determination systems in taxa that are liable to sex reversal.

Further models could test this idea by simulating climate warming followed by a period of stable climate (Schwanz *et al.*, 2020).

Because WW individuals were able to resist masculinization longer, production of viable WW offspring was key to the transition from ZW/ZZ to XX/XY system in our model. Therefore, reduced WW viability had major impacts on the outcomes in our sensitivity tests. Complete WW lethality erased any difference in ASR and persistence between ZW/ZZ and XX/XY systems and any effect of $C_R$. By contrast, 25% WW viability resulted in strikingly divergent fates with and without $C_R$, whereby most populations possessing $C_R$ persisted for more than twice as long as populations without $C_R$. This shows that sexual selection for sex-reversed males may prevent premature extinction in certain circumstances. However, when the WW genotype was at least fairly viable (50% or higher viability), $C_R$ had very little effect on population persistence in our simulations. The latest possible extinction time was determined by the values of allele $thr_{high}$ and $b_{sig}$ (which were fixed in our simulations): when climate warming caused masculinization in all $thr_{high}$ homozygotes, no more female offspring could be produced, after which the population could persist only as long as the remaining females survived. Populations with ZW/ZZ initial system persisted longer when they could transition into an XX/XY system, but within the same simulation type (i.e. initial system) females disappeared from the populations at roughly the same time in all scenarios, regardless of the frequency of the $C_R$ preference allele. The only exception was that population extinction happened a few years later when $C_R$ had high frequency in populations with XX/XY initial system. In this scenario, after chromosome Y disappeared, only individuals with low endogenous male signal levels (i.e. XX) remained, and therefore there were slightly more females (i.e. non-masculinized XX individuals) in the final years before population extinction. Although population persistence may be prolonged by occurrence of new, mutant alleles causing lower male signal levels or higher threshold levels, we did not allow for such mutations in our model because, based on preliminary simulations, we assumed that the rapid climate change would not provide enough time for new mutants to appear and spread before population extinction, for the following reasons. First, the realistically small population size in our model would restrict the number of new mutants to less than one (assuming a mutation rate of $10^{-5}$ over 100 generations in a population of 200 adults). Second, rare new mutant alleles would have a high chance of random loss, and even if they spread, they would be positively selected only in periods with unbalanced ASR, whereas they would face counter-selection in periods with balanced ASR. In accordance with this, the time needed for evolutionary changes of sex-determination systems was typically long (hundreds or thousands of generations) in previous models that allowed for new mutations (Grossen *et al.*, 2011; Quinn *et al.*, 2011; Schwanz *et al.*, 2013). Bearing this caveat in mind, our results suggest that female preference for sex-reversed males alone cannot grant much longer persistence for a population under continuous, rapid climate warming if the WW genotype does not suffer from markedly increased mortality rate. However, in our simulations, population persistence was not threatened by anything other than climate-driven masculinization. In reality, further effects of climate change or other perturbations may also harm the populations (Hayes *et al.*, 2010), and resilience against these perturbations might be affected by $C_R$, as discussed next.

We found that female preferences can have a significant effect on changes of adult sex ratio during climate warming. While the male to female ratio was around 1:1 during ZW/ZZ and XX/XY sex-determination periods, it markedly shifted towards males in the mixed periods in scenarios where all females favored concordant males. By contrast, presence of allele $C_R$ prolonged the time before the ASR became strongly male-biased (except when starting from an XX/XY system with a rare $C_R$). Furthermore, during the transition from ZW/ZZ to XX/XY system, $C_R$ kept the ASR less male-biased (when $C_R$ was rare or when WW had reduced viability) or close to 0.5 (when $C_R$

was widespread). This way, the presence of allele $C_R$ helped maintain a higher effective population size when populations without $C_R$ suffered population bottlenecks. Populations with less biased ASR and higher effective population size are more likely to survive environmental perturbations such as anthropogenic habitat loss and disease outbreaks that currently parallel and interact with the effects of global climate change (Hayes *et al.*, 2010). In this respect, our results suggest that ZW/ZZ populations with widespread preference for sex-reversed males might have the highest adaptive potential and the best chances to cope with contemporary climate change.

Our present results support the previous findings that XX/XY and ZW/ZZ systems may differ in their responses to climate change (Schwanz *et al.*, 2013; **Chapters IV.1-2**). Which of the two systems is more resilient depends on several conditions, such as the fertility of sex-reversed males and the viability of WW individuals (**Chapter IV.2**). Here we found that the ZW/ZZ system may maintain healthier sex ratios for longer, and persist more than two times longer, than the XX/XY system if the sex-reversed males can reproduce like concordant males and produce viable, fertile WW offspring that are resistant to masculinization. These conditions stand in various taxa (Kallman, 1984; Wallace *et al.*, 1999; Devlin & Nagahama, 2002; Chardard *et al.*, 2004; Parnell & Streelman, 2013; Roco *et al.*, 2015; Veltsos *et al.*, 2019), although our empirical knowledge on WW or masculinized individuals in nature is very scarce. Furthermore, our results show that the two systems respond to climate warming identically when the WW genotype is lethal, and also when WW viability is poor and $C_R$ is absent, but the ZW/ZZ system is more likely to outlive the XX/XY system even with poorly viable WW if $C_R$ is present. Thus, the frequency of female preference for sex-reversed males is a further condition that may lead to different effects of climate change in the two sex-chromosome systems.

A further difference between the two systems in our model was seen in the changes in $C_R$ frequency when $C_R$ was competing with an allele encoding preference for concordant males. We found that a rare $C_R$ allele spread in the population when the initial sex-determination system was ZW/ZZ, but tended to disappear instead when sex determination was initially XX/XY. The major difference between the two systems was that sex-ratio selection that favored allele $C_R$ was stronger in the ZW/ZZ system, because females carrying $C_R$ produced WW offspring that were resistant to masculinization, thus their progeny sex ratios were more advantageous when ASR was male-biased. This stronger sex-ratio selection in the ZW/ZZ system counteracted the prevailing sexual selection that acted against $C_R$ in both systems due to the widespread preference for concordant males and to the LD between $C_R$ and the sex chromosomes. Thus, our results suggest that a rare autosomal preference for sex-reversed males is more likely to spread in ZW/ZZ than XX/XY systems. This finding parallels previous theoretical studies that showed that the ZW/ZZ system is particularly prone to evolve sex-linked preferences for sexually antagonistic traits (Muralidhar, 2019) and a new male ornament is more protected against random loss in ZW/ZZ compared to XX/XY systems (Reeve & Pfennig, 2003).

By contrast, when $C_R$ was widespread in the population, sexual selection in both systems favored sex-reversed males and consequently allele $C_R$ that was more frequent in such males. In these scenarios, sex-ratio selection played virtually no role in the spread of $C_R$, because adult sex ratio hardly deviated from 0.5 as long as both concordant and sex-reversed males were present. Both sexual selection and sex-ratio selection have long been known to be major driving forces of evolution (Charnov, 1975; Maynard Smith, 1991; Ryan & Keddy-Hector, 1992), and these forces together can even lead to speciation (Seehausen *et al.*, 1999). Occurrence of novel combinations of genetic sex and sex-linked coloration under sexual selection can lead to rapid sympatric speciation (Lande *et al.*, 2001); thus, our findings raise the possibility that climate-driven sex reversals might contribute to speciation. Because sex-ratio selection, which spread $C_R$ in our

simulated populations, was due to the masculinizing effect of rising environmental temperature, our results demonstrate that climate change may influence the evolution of female mate choice. This finding parallels the conclusions of a modelling study showing that environmental pollution may disrupt sexual selection and thereby decrease population fitness (McNair Senior *et al.*, 2014). Taken together, these theoretical results highlight that various forms of ongoing anthropogenic environmental change worldwide may be driving changes in mating preferences, which then can have knock-on consequences for adaptive potential and population viability.

Our main conclusion is that sexual selection for sex-linked traits may influence the effects of climate change on the demography and evolution of populations with temperature-sensitive sex development. This provokes several further questions for future studies. On the one hand, the genetic architecture of sexually selected traits and the genetic and environmental determinants of sex are poorly known for many taxa. To assess how much wildlife is at risk by climate change, we need more empirical information filling these knowledge gaps. Our study highlights that mate choice for sex-linked traits may have crucial consequences in species with sex reversal, so we urgently need empirical research to test if conspecifics recognize sex-reversed individuals and behave differently towards them. On the other hand, we also need theoretical studies on how further factors affect our projections. For example, in species where the temperature reaction norm is non-linear, ZZ individuals develop into females at high temperatures, and surprisingly, these sex-reversed females can enjoy a fecundity advantage over concordant females (Holleley *et al.*, 2015). In such cases, males might prefer sex-reversed females, which may complicate the effects of climate warming similarly to what we found here for female choice. Spatially heterogeneous temperatures may further complicate these outcomes, either by high dispersal dampening the sex-ratio bias at the metapopulation level, or by low dispersal leading to restricted habitat use and reduced population growth rate (Harts *et al.*, 2014). As growing evidence suggests that sex reversal is more likely widespread in ectotherms rather than a rare oddity (Holleley *et al.*, 2016), exploring the complexity of its consequences is an important emerging research avenue.

## *V. Concluding remarks*

**Summary of novel findings**

The research presented in this thesis contributes to the growing knowledge about the effects of human-induced environmental changes on wildlife, by the following main results:

**Chapters II.1** and **II.2** showed that in agricultural and urban habitats, common toad tadpoles had stronger glucocorticoid stress response and more efficient negative feedback along the hypothalamus-pituitary-interrenal axis, whereas adults had larger parotoid glands with larger quantities and/or more poisonous composition of bufadienolide toxins compared to their counterparts living in natural habitats. As none of these differences were detected in common garden experiments, the most parsimonious explanation is phenotypic plasticity whereby individuals respond to the challenges presented by anthropogenic environments. One potential driver of this plasticity might be chemical pollution, as two experiments demonstrated that chronic exposure to a glyphosate-based herbicide increased bufadienolide production in toad tadpoles.

**Chapters II.3** and **II.4** compared common toads from natural, agricultural and urban habitats under identical captive conditions, and found that they had similar fecundity, fertility, embryo survival, and propensity to undergo sex reversal upon larval exposure to feminizing pollutants (a xenoestrogen and a glyphosate-based herbicide). In agreement with these signs of uncompromised reproductive capacity and sex development, we found only one sex-reversed individual out of 349 free-living adult toads across the 3 habitat types. However, females from anthropogenic habitats produced thick jelly coats around their eggs regardless of clutch size, and their offspring exhibited reduced tadpole development rates and lower body mass both as larvae and as juveniles. This suggests that females may protect their embryos from pollution but this protection (and potentially further mechanisms of tolerance to chemical contaminants or other anthropogenic environmental stressors) might come at a cost of reduced offspring performance in fitness-related traits.

**Chapter III.1** showed that, in contrast to common toads (above), agile frogs in the same habitats had a relatively high sex-reversal rate especially in agricultural and urban ponds, with 20% of adult males being genetically female. This may be due to a difference between the two species in sensitivity of sex development to thermal stressors such as heat waves and the urban heat island effect, because the experiment presented in **Chapter III.2** demonstrated that a 6-days exposure to 28 or 30 °C during tadpole development caused female-to-male sex reversal in agile frogs but not in common toads. The same experiment found no sex-reversing effect in agile frogs by an ecologically relevant concentration of a widespread xenoestrogen, suggesting that environmentally occurring feminizing pollutants are unlikely to counteract heat-induced sex reversal in this species.

We also investigated whether sex-concordant and female-to-male sex-reversed agile frogs differed in traits related to fitness. In **Chapter III.1** we detected no such difference in the body mass of free-living adult males. In contrast, among the lab-raised animals of **Chapter III.2** heat-induced masculinization was associated with slower larval development, reduced body mass, and lack of fat reserves; and some of these effects were modified by xenoestrogen exposure depending on developmental timing. A small sample of spontaneously masculinized froglets in **Chapter III.1** also exhibited some signs of poor health, such as increased spleen size and more frequent liver abnormalities. However, a somewhat larger sample of spontaneously masculinized individuals in **Chapter III.3** did not support an overall fitness difference from concordant conspecifics based on an extensive comparison of early life history and tadpole behavior. Based on these contrasting findings, we formulated the hypothesis that the fitness consequences of sex reversal may depend on the mechanism that caused it.

The final section of the thesis contemplated on general differences between species in the rate of environmentally induced sex reversal: how these differences might have arisen through the evolutionary past and how they may affect what will happen in the future loomed over by climate warming and other forms of human-induced environmental changes. **Chapter IV.1** revised a hypothesis with a review of empirical evidence that the type of heterogamety may confer asymmetrical susceptibility to sex reversal, such that species with XX/XY sex chromosomes would be more be more liable to masculinization while ZZ/ZW systems would be more liable to feminization. **Chapter IV.2** continued this line of thought by a theoretical modelling study, showing that the type of sex determination also influences the way adult sex ratios respond to increasing rates of sex reversal. Our "meta-analysis" of amphibian sex ratios supported this prediction, indicating that the adult sex ratios shifted towards males in two ZZ/ZW species over the past decades but did not change systematically in four XX/XY species. **Chapter IV.3** developed our theoretical model further to demonstrate that the population-level outcomes of masculinization *via* climate warming are affected by female mating preferences for sex-chromosome-linked phenotypic traits, and these effects also vary by the type of heterogamety.

**Perspectives**

The fact that we found barely any sign of microevolutionary changes in common toads inhabiting anthropogenic land is intriguing, given the increasing number of studies reporting such changes across wild organisms (Johnson & Munshi-South, 2017; Liker, 2020). Out of those that have investigated the mechanisms by which anthropogenic environments influence the animals' physiological and behavioral responses to risk (Partecke *et al.*, 2006; Atwell *et al.*, 2012; Miranda *et al.*, 2013) or chemical stress (Whitehead *et al.*, 2012; Brans *et al.*, 2018b), the majority suggested microevolution or other transgenerational effects. For example, two previous studies on birds suggested that evolutionary divergence or other transgenerational (e.g. epigenetic or maternal) effects were responsible for generating differences in glucocorticoid stress response between urban and non-urban populations (Partecke *et al.*, 2006; Atwell *et al.*, 2012), although a third avian study found that phenotypic plasticity played a more important role in generating such differences in baseline glucocorticoid levels (Ouyang *et al.*, 2019). Both glucocorticoid physiology and toad chemical defenses are heritable (Hudson *et al.*, 2017, 2021; Guindre-Parker, 2018), so in this sense their evolutionary adaptation should not be constrained. A potential explanation for relying on phenotypic plasticity rather than on microevolution is that anthropogenic environments may exert complex and variable selection forces because of spatio-temporal heterogeneity in the frequency and type of stressors such as pollution (Bókony *et al.*, 2018b) and predation risk (Sorace & Gustin, 2009); such heterogeneity should favor the evolution and maintenance of phenotypic plasticity (Bradshaw & Hardwick, 1989; Moran, 1992; Sultan & Spencer, 2002). Similarly, phenotypic plasticity had a ubiquitous role across species in promoting phenotypic changes in response to climatic variation (Urban *et al.*, 2014), although some of these responses are maladaptive (Urban *et al.*, 2014) or may be insufficient for adaptation (Radchuk *et al.*, 2019). The changes we observed in agricultural and urban toads in stress physiology and chemical defenses seem adaptive, and might even contribute to their uncompromised reproductive health, but further research will be needed to test the adaptive value of these phenotypic changes. More generally, the fate of populations of wild organisms will depend on the net outcome of adaptive and maladaptive plastic and microevolutionary responses, or lack thereof, to the multitude of challenges posed by human-induced environmental alterations.

Among the potential responses to environmental changes, sex reversal is an emerging topic. What we have learnt so far indicates that environmentally induced sex reversal may have wide-

ranging significance in wild populations of ectothermic vertebrates (Baroiller & D'Cotta, 2016; Holleley *et al.*, 2016; Whiteley *et al.*, 2021a) as well as invertebrates (where it is often referred to as "imposex"; Oetken *et al.*, 2004; Ford, 2012). However, most of the early research focused on proximate issues of endocrine disruption and aquaculture techniques, while the evolutionary-ecological aspects of sex reversal have just begun to gain wider attention, with only a few research groups around the world concentrating on it, mostly using reptilian model species and theoretical modelling (Grossen *et al.*, 2011; Holleley *et al.*, 2016; Lambert *et al.*, 2019; Schwanz *et al.*, 2020). Recognizing this hiatus, I aimed to establish amphibian model systems for studying sex reversal from the viewpoint of evolutionary ecology and conservation biology. With our newly developed molecular sex markers for agile frogs and common toads, we have contributed to the recent realization that sex reversal can have high ecological relevance in amphibians, too. Our experiments on agile frog sex reversal are also among the first few to test how ecologically relevant scenarios of heat events and chemical pollution may translate into changes in sex and thereby, potentially, in individual fitness. Similarly, our theoretical work is among the first to highlight that understanding why and how different species or populations vary in their susceptibility to sex reversal by various environmental stressors is important for predicting the effects of anthropogenic environmental change on wildlife, including their demography, population persistence, sex-determination systems, and mating preferences. As in all research every new finding raises new questions and ideas, we are now at an exciting start to unravel these lines of inquiry further. Building on our accumulated understanding from laboratory experiments on how environmental perturbations affect sex and from theoretical models on how sex reversal may impact population dynamics and evolution, the time is now ripe for empirical studies on the causes and consequences of sex reversal across wild populations in the Anthropocene.

### *VI. Acknowledgements*

The studies presented in this thesis are the results of collaborations with many researchers and students, all of whom I am thankful to for their indispensable contributions. Much obliged to the past and present members and students of the former Lendület Evolutionary Ecology Research Group (now Department of Evolutionary Ecology, ATK NÖVI) for providing a cheerful and inspiring work environment; special thanks to Edina Nemesházi for being my "first mate" in the sex-reversal project. Several colleagues from my previous institutions (the Department of Ecology at the University of Veterinary Medicine Budapest and the Department of Limnology at the University of Pannonia) also have been helpful in my professional progress, one way or another contributing to this thesis as well. I am most grateful to my mentor András Liker: our many years of brainstorming and working together has been a truly life-forming experience for my scholar development and personal growth. I am also endlessly grateful to Attila Hettyey who made it possible for me to re-boot my scientific career and taught me how to work with amphibians.

The research presented in the dissertation was financed by the National Research, Development and Innovation Office of Hungary ("OTKA" grants 115402 and 135016; 2019-2.1.11-TÉT-2019-00026), with additional funding from the Hungarian Academy of Sciences (János Bolyai Scholarship, Lendület program, Young Researcher program) and the New National Excellence Program of the Ministry for Innovation and Technology from the source of the National Research, Development and Innovation Fund (ÚNKP-20-5 & ÚNKP-21-5). All the work involving animals presented in this thesis was ethically approved and permitted by the Environment Protection and Nature Conservation Department of the Pest County Bureau of the Hungarian Government.

Finally, but most importantly, I am indebted to my family for their support in all possible ways, especially to my parents and grandparents who raised me to love nature and learning, and to my husband who has been a cornerstone of my life since my very first steps of becoming a researcher.

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