NKFIH project 115402

"Environmental effects on sex in vertebrates with diverse sex-determination systems"

Final report[1]

The objective of this project was to combine empirical experiments, field surveys, theoretical modelling, and phylogenetic comparative studies to investigate the significance of human-induced environmental changes in the sex determination, sex ratios, and sex-related traits in ectothermic tetrapod vertebrates. The project started in 2016 and ended with a one-year extension in 2020.

1.) Effects of rising temperatures on sex ratios in amphibians and reptiles

1.1.) The first goal of this part of the project was to study climate-driven changes of sex ratios in reptiles with temperature-dependent sex determination (TSD). To accomplish this, we compiled an extensive database of literature data on reptile demography, including sex ratios of hatchlings/neonates and adults, sex-specific adult mortality rates and maturation ages. This vast effort revealed that the quantity and quality of information available in the literature is not sufficient for the large-scale comparative analyses of within-species temporal changes in sex ratios that we had originally planned. However, the database allowed us to tackle several questions related to the project, by putting together our own data on reptiles and amphibians with the data of Prof. Tamás Székely's research team on birds and mammals (the collaborating partner was funded by NKFIH-2558-1/2015 and ÉLVONAL-KKP 126949). Within the framework of this collaboration, we complemented our tetrapod database with further data on morphology and life history, and conducted phylogenetic comparative analyses to investigate the evolutionary changes of sex ratios and sex-dimorphic traits in taxa with TSD as well as in taxa with genetic sex determination (GSD). This part of the project resulted in a published paper and two submitted manuscripts; two further manuscripts are in preparation:

- Bókony V., Milne G., Pipoly I., Székely T., Liker A. 2019. *Sex ratios and bimaturism differ between temperature-dependent and genetic sex-determination systems in reptiles*. **BMC Evolutionary Biology** 19:57.
  We compared key demographic traits between TSD and GSD reptiles (181 species), testing a recent theoretical model that the evolution of TSD is facilitated by sex-specific fitness benefits of developmental temperatures due to bimaturism.
  Abstract: https://doi.org/10.1186/s12862-019-1386-3

- Liker A., Bókony V., Pipoly I., Lemaitre J.-F., Gaillard J.-M., Székely T., Freckleton R.P. *Evolution of large males is associated with female-skewed adult sex ratios*. Manuscript under review (in revision phase).
  Adult sex ratio (ASR) is expected to influence sexual size dimorphism (SSD), because ASR relates to both the number of competitors and available mates, which shape the intensity of mating competition and thereby facilitate SSD evolution. Using phylogenetic analyses of 462 amniote vertebrates (i.e. reptiles, birds, and mammals), we showed that male bias in SSD increases with increasingly female-biased ASR. Phylogenetic path analysis supported that higher mortality in one sex leads to skewed ASR, which in turn generates selection for SSD biased towards the rare sex. These results suggest that skewed ASR can facilitate the rarer sex to develop large size to capitalize on enhanced mating opportunities.

- Katona G., Vági B., Végvári Z., Liker A., Freckleton R. P., Bókony V., Székely T. *Are evolutionary transitions in sexual size dimorphism related to sex determination in reptiles?* Manuscript under review (in revision phase).
  Theory suggests that selection for larger size in one sex or the other may drive the evolution of different sex-determination systems. We investigated whether the type of sex determination is

[1] Note: the names of the project participants are underlined in the author lists of our papers & manuscripts.

phylogenetically associated with SSD across 250 species of reptiles. We found no relationship between SSD and the type of GSD, but species with TSDIa sex determination (in which the proportion of female offspring increases with incubation temperature) had more female-biased SSD than species with TSDII (in which males are produced at intermediate temperatures). The latter result suggests that sex-dependent developmental variations caused by environmental temperatures are linked to SSD in adulthood.

- Pipoly I., Bókony V., Gaillard J.-M., Lemaitre J.-F., Székely T., Liker A. *Demographic links between adult sex ratio and genetic sex-determination system in tetrapods*. Manuscript in preparation.
  We showed earlier that variation in ASR across tetrapods is related to the type of GSD, with typically more male-biased ASRs in ZZ/ZW (female-heterogametic) than in XX/XY (male-heterogametic) systems. We investigated whether this difference may be due to mechanisms that distort the sex ratios in embryos/neonates, or to differential post-natal mortality or maturation of the sexes. Using a dataset of ASR, birth sex ratio, and sex-specific juvenile and adult mortality rates and maturation ages of amphibians, reptiles, birds, and mammals, we found that birth sex ratio was not associated with GSD type, but in XX/XY species males had higher mortality rates in juvenility as well as in adulthood, and longer maturation times relative to females than in ZZ/ZW species. These results suggest that mechanisms acting after birth have an important contribution to the ASR difference between GSD systems.

- Mészáros G., Pipoly I., Vági B., Bókony V., Székely T., Liker A. *Multiple paternity and adult sex ratios in reptiles*. Manuscript in preparation.
  Multiple paternity, i.e. females mating with multiple males, is widespread in animals, yet its evolutionary-ecological causes are poorly understood. Mating opportunities for females, as well as harassment by males, may be determined by the relative frequency of males in the adult population. We tested this hypothesis with a phylogenetic comparative study of 84 reptile species. Contrary to expectation, we found no relationship between adult sex ratio and the frequency of multiple paternity. However, species with ZZ/ZW sex-chromosome systems had significantly higher rates of multiple paternity than species with either XX/XY sex-chromosome systems or TSD. This finding supports the theoretical model that sexual selection may be stronger in GSD systems with female heterogamety.

1.2.) Our second main goal was to study climate-driven sex reversal in species with thermosensitive GSD. First, we collected published data on phenotypic sex ratios in natural populations of amphibians, and compared the change of sex ratios over time between species with different types of GSD. Second, we developed a mathematical modelling framework to investigate the consequences of sex reversal under climate warming. This part of the project yielded a published paper and an accepted manuscript:

- 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.
  By theoretical modelling and meta-analysis of empirical data, we showed that XX/XY and ZZ/ZW systems differ in vulnerability to climate change due to ASR skew by temperature-induced sex reversal.
  Abstract: https://doi.org/10.1098/rstb.2016.0325

- Nemesházi E., Kövér S., Bókony V. 2021. *Evolutionary and demographic consequences of temperature-induced masculinization under climate warming: the effects of mate choice*. Preprint manuscript; accepted for publication in **BMC Ecology and Evolution** on 14 January 2021 (DOI: 10.1186/s12862-021-01747-3).
  In this theoretical study we show that climate change may affect the evolution of mate choice, which in turn may influence the evolution of sex-determination systems, sex ratios, and thereby adaptive potential and population persistence.
  Abstract: https://doi.org/10.1101/2020.06.08.139626

2.) Sex effects of environmental pollutants in amphibians

2.1.) The goal of this part of the project was to develop a molecular method for identifying genetic sex in two widespread amphibian species, the common toad (*Bufo bufo*) and the agile frog (*Rana dalmatina*), and then, using these new diagnostic tools, to study sex reversal in natural populations along pollution gradients and to experimentally investigate the effects of pollutants and climatic factors on sex reversal. We have fully accomplished these goals for agile frogs, resulting in a published paper and 4 manuscripts:

- 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.
  In this paper we published our genetic sexing method validated for agile frogs, and presented data on the fitness correlates of sex reversal and its prevalence across a gradient of anthropogenic land use.
  Abstract: https://doi.org/10.1111/mec.15596

- 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. *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?* Preprint manuscript; under review.
  We experimentally assessed the simultaneous effects of a few-days heat wave (masculinizing agent) and a widespread estrogenic pollutant (17α-ethinylestradiol; feminizing agent) on sex determination and fitness-related traits in agile frogs.
  Abstract: https://doi.org/10.1101/2020.12.29.424761

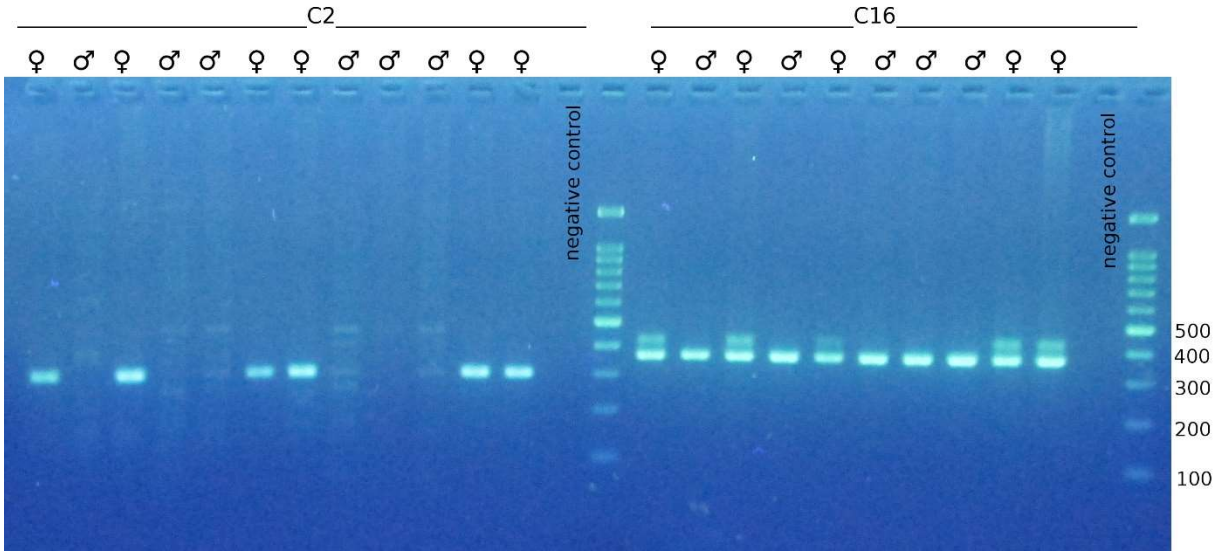
- Bókony V., Nemesházi E., Ujhegyi N., Verebélyi V., Mikó Z., Erös R., Vili N., Gál Z., Hoffmann O.I., Hettyey A. *Sex reversal and performance in fitness-related traits in agile frogs.* Manuscript in preparation.
  We compared individual performance in fitness-related traits between sex-reversed animals, concordant females, and concordant males during the early-life development of agile frogs in a laboratory experiment. The three groups did not differ in the duration of larval development and body mass at metamorphosis, but while concordant individuals showed a trade-off between these two traits, sex-reversed individuals did not. Compared to concordant individuals, sex-reversed tadpoles had reduced locomotor activity, exhibited the steepest increase in feeding rate with age, and they were the only group that responded to predation risk by escaping shorter than when startled without predator cues. Sex-reversed individuals resembled concordant males in that they both showed shorter freezing response to predation risk compared to concordant females. These results indicate that sex reversal may affect life history and behavior, which may have knock-on effects on fitness in nature.

- Ujszegi J., Bertalan R., Kásler A., Mikó Z., Nemesházi E., Szederkényi M., Ujhegyi N., Verebélyi V., Gál Z., Hoffmann O.I., Bókony V., Hettyey A. *Species-specific effects of acute thermal stress during early development on life-history traits and sex ratio in two anurans*. Manuscript in preparation.
  Climate change is expected to increase the frequency of extreme weather events like heat waves, but the effects of such events on wildlife are rarely studied. We investigated how an experimentally simulated 6-days heat wave in the early, middle, or late phase of larval development affects fitness-related traits and sex ratios in two anuran species. In agile frogs, 30°C (and to a lesser extent, 28°C) treatments resulted in higher mortality, delayed metamorphosis, and male-biased sex ratio *via* sex reversal. In common toads, heat treatments

decreased body mass at metamorphosis. These results show that climate change is likely to have species-specific negative effects on demography and population viability.

For toads, we encountered several unexpected difficulties during marker development, which delayed our progress. We found no sex-linked markers in this species with microsatellites that had been published as sex-linked in other, related species, and neither with extensive attempts using the AFLP method. We then had a RAD-seq analysis conducted; this yielded only one putative marker, which turned out not to be usable for genetic sexing. Finally, a second, different RAD-seq analysis has provided 17 candidate markers; we designed primers for evaluating these markers in a larger number of animals and found consistently sex-specific PCR products for three markers (Fig. 1). Applying this genetic sexing marker set to the DNA samples we have collected from free-living toads (ca. 350 individuals from 14 populations), we can assess the rate of sex reversal in this species and investigate the relationship of sex reversal with the intensity of anthropogenic habitat modification. This work is underway and we hope to publish its result before the end of 2021.

Figure 1: An example gel electrophoresis of PCR products of two sex-linked markers (C2 and C16) in common toads. For C2, a primer specific to the W chromosome was used, which amplifies only in females. For C16, a "unisex" primer was used, which yields one product (from the Z chromosomes) in males and two different-sized products (Z and W) in females.



2.2.) To compensate for the above-mentioned delay in this part of the project (i.e. genetic sexing of toads), we conducted several further, originally not planned field studies and lab experiments to investigate the effects of various forms of chemical pollution on endocrine physiology and reproductive health in anurans. This resulted in 6 published papers and 2 manuscripts:

- 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.
  In two experiments we found that larval exposure to glyphosate-based herbicides, the most widespread agrochemicals worldwide, increases the production of bufadienolides, the main compounds of chemical defense in common toads.
  Abstract: https://doi.org/10.1098/rspb.2017.0493

- 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.
  We investigated the nature and quantity of endocrine-disrupting pollutants in under-studied, typical amphibian breeding ponds, and the reproductive capacities of toads inhabiting urban and agricultural areas as compared to natural habitats.
  Abstract: https://doi.org/10.1016/j.scitotenv.2018.03.363

- 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.
  With field data and a common-garden experiment, we found that toads' chemical defenses respond to the challenges of anthropogenic environments via phenotypic plasticity. Adults in agricultural and urban habitats had larger toxin glands and more potent toxin secretion.
  Abstract: https://doi.org/10.1038/s41598-019-39587-3

- Bókony V., Verebélyi V., Ujhegyi N., Mikó Z., Nemesházi E., Szederkényi M., Orf S., Vitányi E., Móricz Á.M. 2020. *Effects of two little-studied environmental pollutants on early development in anurans*. **Environmental Pollution** 260: 114078.
  We investigated two pollutants that have been poorly studied in ecotoxicology despite their widespread occurrence in surface waters: the herbicide terbuthylazine and the pharmaceutical drug carbamazepine. We found several sub-lethal effects in agile frogs and common toads.
  Abstract: https://doi.org/10.1016/j.envpol.2020.114078

- Ujhegyi N., Bókony V. 2020. *Skin coloration as a possible non-invasive marker for skewed sex ratios and gonadal abnormalities in immature common toads* (*Bufo bufo*). **Ecological Indicators** 113: 106175.
  We presented evidence that skin coloration can serve as a non-invasive marker of phenotypic sex and gonadal abnormalities in juvenile toads, and can be useful for qualitative comparisons of sex ratios between groups when no other means of phenotypic sexing is possible.
  Abstract: https://doi.org/10.1016/j.ecolind.2020.106175

- 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.
  With field data and a common-garden experiment, we found that toad tadpoles in urban and agricultural habitats increased their glucocorticoid flexibility via phenotypic plasticity, coupling of stronger stress response and stronger negative feedback, which may be an important mechanism for coping with anthropogenic environmental change.
  Abstract: https://doi.org/10.1016/j.scitotenv.2020.141896

- Mikó Z., Bókony V., Ujhegyi N., Nemesházi E., Verebélyi V., Erös R., Hettyey A. *Effects of chlorpyrifos on anti-predatory behavior and early development in agile frogs*. Manuscript in preparation.
  We studied the effects of two environmentally relevant concentrations of chlorpyrifos (CPF; 0.5 and 5 µl/L), a common insecticide, on larval fitness and gonad development in agile frogs. We also tested if acute or chronic exposure disrupts the tadpoles' activity and behavioral responses to olfactory cues of predation risk. We found that chronic exposure to CPF did not affect time to metamorphosis and phenotypic sex ratio, but tadpoles exposed to 5 µl/L CPF over their development had reduced body mass at metamorphosis. While control tadpoles reacted to predation risk by freezing, none of the CPF-treated tadpoles showed this adaptive response, even when CPF exposure was as short as 20 minutes. This suggests that CPF may affect predator-prey interactions and increase tadpole mortality by predation.

- Üveges B., Kalina C., Szabó K., Móricz Á.M., Gabor C.R., Hettyey A., Bókony V. *Chronic stress influences defensive toxin production in toad tadpoles*. Manuscript in preparation.
  Physiological regulation of chemical defense in vertebrates is poorly understood. We tested the hypothesis that production of bufadienolide toxins may be stimulated either by corticosterone (CORT), the main GC hormone of amphibians, or by the same upstream regulators that stimulate CORT. We found that CORT release rates of tadpoles were increased strongly by CORT treatment (125 nM) and less strongly by metyrapone treatment (110 μM), but both treatments decreased bufadienolide content. These findings suggest that bufadienolide synthesis is not stimulated by CORT; rather, it may respond to stressors via some other regulatory compound that is involved in the stress response. Thus, environmental stress in general, and endocrine-disrupting chemicals in specific, may interfere with toxin production, which may affect the fitness of toads and their predators and competitors.

Finally, we also published popular science papers to disseminate our research findings to the Hungarian public:

- Verebélyi V., Bókony V., Üveges B. 2018. Békanász a városban és a szántóföldeken: *Kétéltűink és a környezetszennyezés.* Élet és Tudomány 2018(36): 1126-1128.
- Hettyey A., Bertalan R., Ujszegi J., Ujhegyi N., Bókony V. 2020. *A klímaváltozás hatása kétéltűek ivari fejlődésére: Eltűnnek a békalányok?* Élet és Tudomány 75(29): 905-907.
- Ujhegyi N. 2021. *Alkalmazkodás szennyezett világunkhoz: Stresszes varangyok.* Élet és Tudomány 76(3): 73-75.

Changes in personnel:

The project started with six participants. Two young researchers (Ivett Pipoly, Zoltán Tóth) left the project before its completion. However, three young researchers joined the project after its start (Edina Nemesházi, Nikolett Ujhegyi, and Bálint Üveges), resulting in a higher than planned FTE for the project overall. Thus, these changes altogether allowed us to diversify our research into additional directions. All these personnel changes were approved by the NKFIH.

Changes in budget:

In total, the realized budget was the same as planned. Over the course of the project, we requested and were granted the following changes to increase the personnel costs:

- In the first year the government increased the minimum wages of public servants, so we re-allocated 2,470,150 HUF from the operational and capital expenditures to cover the salary of the young researcher employed full-time by the project.
- In the 2nd and 3rd years, we re-allocated in total 570,000 HUF from operational expenditures and per diem to cover the fees of temporary assistants hired each year for helping experimental work (we had to pay higher than planned fees due to inflation).
- By the end of the 4th year, we had not spent 4,832,994 HUF, which we moved to a 5th year that we were granted for finishing the development of toad sex markers and the analyses of DNA samples. We allocated the majority of this sum to covering the operational costs (1,531,628 HUF) and personnel costs (3,037,548 HUF) of DNA laboratory work. The personnel costs were shared among 3 contracts:
  - Edina Nemesházi, the young researcher who led the genetic part of the project, was no longer employed full-time by the project in 2020, but continued to participate substantially by designing primers, evaluating sequencing results, isolating DNA, and performing PCRs.
  - Another young researcher, Zoltán Gál (not a project participant) was hired to perform the high-resolution melting (HRM) analyses that are needed for using our agile frog sex markers. We do not have the necessary equipment and experience with HRM, so this task was outsourced.
  - A student participant of the project, Viktória Verebélyi, assisted the researchers to tackle the large amount of laboratory work.

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