Sex-reversed males are morphologically similar but have lower testosterone levels compared to sex-concordant males in the agile frog

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Abstract

Environment-induced sex reversal – the mismatch between genetic and phenotypic sex caused by external conditions during early development – is increasingly documented in ectothermic vertebrates, yet its fitness-related consequences in natural populations remain poorly tested. To address this gap, we compared sex-reversed XX males and sex-concordant XY phenotypic males in agile frogs (*Rana dalmatina*) sampled at arrival to a breeding pond. We assessed morphometric traits that are relevant to male breeding success in anurans (snout–vent length, body mass, scaled mass index as a measure of body condition, forearm length and width, and nuptial pad size and coloration) as well as water-borne urinary concentrations of testosterone and corticosterone. Sex-reversed males were morphologically indistinguishable from sex-concordant males across all measured traits. Darker nuptial pad coloration was associated with larger body size and condition. However, sex-reversed males had significantly lower urinary testosterone concentrations than sex-concordant males. This difference persisted after controlling for body mass, sampling date, and corticosterone levels, which did not differ between groups. Our findings suggest that sex reversal in agile frogs produces a mixed phenotype, combining typical male morphological appearance with endocrine differences that may contribute to reduced paternity success previously reported in XX males. Such mismatches between various aspects of phenotype as well as with genotype deserve more attention in species susceptible to sex reversal, as ongoing climate change is expected to increase the incidence of sex reversal in natural populations.

Keywords: sex-reversed male morphology, water-borne steroid hormones, testosterone levels in XX males, male fitness, amphibian sex reversal, *Rana dalmatina*

1. Introduction

Global climate change and habitat loss pose significant challenges for various taxa including reptiles (Cox et al., 2022), amphibians (Luedtke et al., 2023), mammals and birds (Tilman et al., 2017). Ectotherms are particularly sensitive to changes in the thermal environment, which can affect

processes as fundamental as sexual development. In gonochoristic fish and reptiles, diverse sex-determination mechanisms range from environmental (most often temperature-dependent) to purely genotypic systems (Ashman et al., 2014; Baroiller & D'Cotta, 2016; Nemesházi & Bókony, 2023). More recent studies revealed that these systems are not mutually exclusive, and certain environmental conditions experienced during early ontogeny may override the effects of sex chromosomes, leading to a mismatch between sexual genotype and phenotype called sex reversal (Baroiller & D'Cotta, 2016; Li et al., 2016; Nemesházi & Bókony, 2023; Wild et al., 2023). In contrast, amphibians were long assumed to only possess genotypic sex determination (Ma & Veltsos, 2021). Yet, a growing body of evidence confirms that amphibian sexual development can be influenced by environmental conditions experienced during early ontogenetic development, including high or low temperatures and the presence of certain chemical pollutants (Edmands, 2021; Nemesházi & Bókony, 2023; Orton & Tyler, 2015; Ujszegi et al., 2022). Theoretical works suggest that temperature-induced sex reversal may explain frequent transitions between different sex-chromosome systems as well as between systems with and without sex chromosomes in ectothermic vertebrates (Holleley et al., 2015; Jeffries et al., 2018; Nemesházi et al., 2021; Sarre et al., 2011). Sex reversal may be far more common than currently known in the herpetofauna (Nemesházi & Bókony, 2025), particularly in areas exposed to anthropogenic land use (Bókony et al., 2020; Nemesházi et al., 2020, 2022; Orton & Tyler, 2015). Consequently, studying the fitness-related effects of sex reversal may be key to understanding the evolutionary forces shaping these taxa and predicting their persistence and adaptive potential under ongoing environmental change (Nemesházi et al., 2021; Nemesházi & Bókony, 2025).

To date, only a handful of studies have attempted to explore the effects of ecologically relevant sex reversal on individual fitness. Early-life effects were reported on metabolism, growth rate and behaviour in sex-reversed individuals (Bókony et al., 2021; Bókony, Balogh, Mikó, et al., 2025; Li et al., 2016; Wild et al., 2023) (Bókony, Balogh, Mikó, et al., 2025; Bókony et al., 2021; Wild et al., 2023). Nevertheless, sex-reversed animals can be fertile and reproductively active (Bókony, Balogh, Ujhegyi, et al., 2025; Holleley et al., 2015). While sex reversal may be an adaptive response to certain changes of environmental conditions (Bókony, Balogh, Mikó, et al., 2025; Warner & Shine, 2008), it may also have diverse population-level consequences, including shifting adult sex ratios and turnovers between different sex-determination systems (Holleley et al., 2015; Nemesházi et al., 2021). The progeny of sex-reversed individuals can show increased vulnerability to stress (Bókony, Balogh, Ujhegyi, et al., 2025), further highlighting that sex reversal can have complex transgenerational consequences. The dynamics of population-level changes may also depend on the breeding success of the sex-reversed individuals relative to their sex-concordant counterparts (Nemesházi et al., 2021). Therefore, to better understand the evolutionary-ecological consequences of environment-induced sex reversal, it is essential to empirically compare sex-reversed individuals to their sex-concordant breeding competitors on various phenotypic traits relevant for reproductive success.

In anuran amphibians, body size and further morphometric parameters can play important roles in male breeding success, as males in many species physically compete for the females (Navas & James, 2007; Orton et al., 2020, 2023; Vági & Hettyey, 2016). Relevant morphological traits, as well as reproductive behaviour, are affected by the levels of steroid hormones (Leary, 2009; Nakamura, 2012). Testosterone, a key androgen, plays important role in the development of male secondary sexual characteristics such as vocal sacs, strong forearms and nuptial pads (Navas & James, 2007; Orton et al., 2020) and reproductive behaviour in amphibians (Leary, 2009). Males in various frog species possess strong forearms and develop nuptial pads to facilitate successful amplexus, by improving the grip on females during mating. Nuptial pad colour and morphology is influenced by the androgenic status of males, and is associated with mating success in different anuran taxa (Orton et al., 2020, 2023). Besides testosterone, corticosterone levels may also influence breeding success, as glucocorticoids modulate the allocation of resources between reproduction and survival under

stressful conditions (Crespi et al., 2013; Mausbach et al., 2022; Moore & Jessop, 2003). Therefore, levels of a variety of hormones may influence the fitness of sex-reversed compared to sex-concordant individuals of the same phenotypic sex. Theoretically, hormone levels of sex-reversed individuals may follow different patterns depending on whether phenotype or genotype exerts greater influence (Laven et al., 2025), but not all hormone concentrations in one individual necessarily follow the same trend (Gennotte et al., 2017). To our knowledge, no study assessed the effects of sex reversal on hormone levels, and only few focused on morphological parameters related to male breeding success in amphibians. One possible explanation for this knowledge gap is that genotypic sexing, which is a fundamental requirement for identifying sex-reversed individuals, has not yet been established for most amphibian species (Nemesházi & Bókony, 2023).

Genotypic sex markers are available for the agile frog (*Rana dalmatina*), a species with XX/XY male heterogametic system and documented occurrence of female-to-male sex reversal in free-ranging populations (Nemesházi et al., 2020). In this species, elevated environmental temperatures during larval development facilitate sex reversal in genotypic (XX) females (Bókony, Balogh, Mikó, et al., 2025; Ujszegi et al., 2022), and its frequency increases with anthropogenic land use in free-ranging populations (Nemesházi et al., 2020). Sex-reversed (XX) males have lower siring success despite being similarly fertile to the sex-concordant (XY) males, and females are able to differentiate between them via unknown mechanisms (Bókony, Balogh, Ujhegyi, et al., 2025). Occurrence of phenotypic differences relevant for breeding success have rarely been assessed between the two male types. The goal of this study is to evaluate if sex-reversed and sex-concordant agile frog males differ in phenotypic characteristics potentially associated with male fitness during the breeding season. Specifically, we assessed if sex-reversed males differed from sex-concordant males in (1) morphometrics relevant for male breeding success (Navas & James, 2007; Orton et al., 2020): snout-vent length (SVL),body mass, scaled mass-index (SMI) as a measure of body condition, forearm length, width, and nuptial pad size and (2) nuptial pad coloration that is also known to affect breeding success in at least some species in this genus (Orton et al., 2023). Furthermore, (3) we compared sex-reversed and sex-concordant males for testosterone and corticosterone levels, measured non-invasively from water-borne urine samples.

2. Materials and Methods

2.1. Animal collection and sexing

We captured 513 adult males at arrival to a breeding pond near Budapest, Hungary (47°33'04.3"N 18°55'36.1"E) during the breeding season in 2023 and 2024, using a drift fence with pitfall traps. Male phenotype was determined by the presence of nuptial pads. We took buccal swab samples from all individuals and extracted DNA from the swabs using a commercial kit (Bio-Tek Omega E.Z.N.A. Forensic DNA Kit), following the manufacturer's protocol. Using the extracted DNA, we identified sex-reversed males based on two markers (Rds1 and Rds3) that were previously developed for this species (Nemesházi et al., 2020), following the published protocol. First, we determined the Rds3 genotype for all phenotypic males by high-resolution melting (HRM). Then, in those individuals where HRM revealed mismatch between the sexual phenotype and genotype for Rds3, we amplified the Rds1 locus by polymerase chain reaction (PCR) to confirm sex reversal. We only deemed those males "sex reversed" that possessed an XX genotype according to both markers. Individuals with ambiguous sexual genotype were excluded from the study. This study conforms to Directive 2010/63/EU and was approved by the Ethics Committee of the Plant Protection Institute. All procedures were permitted by the Environment Protection and Nature Conservation Department of the Pest County Bureau of the

Hungarian Government (PE/EA/295-7/2018, PE/EA/00270-6/2023, PE-06/KTF/07949-6/2023, PE-06/KTF/00754-8/2022, PE-06/KTF/00754-9/2022).

2.2. Morphometric and colour measurements

We measured body mass (± 0.01 g) with a digital scale and SVL, the length and width of the left forearm, as well as the length of the left nuptial pad (± 0.1 mm) of 108 XX males and 405 XY males with a calliper. We measured nuptial pad colour in the hue – saturation – brightness colour space (HSV; where 'V' stands for 'value', that is equivalent with brightness). To this end, we used digital photographs of a subset of the agile frogs taken with a Canon EOS R50 digital camera (Canon Inc.; equipped with a 49mm Hoya UX II slim frame circular polarization filter) under identical lighting conditions (5000K LED light; Matcheasy Banda LED 5V-U-3-5K). We photographed those males that were captured in 2024 on days when performing this extra task was logistically feasible. We manually assessed each photograph and retained only images on which the nuptial pad was fully visible and of sufficient quality to delineate its borders. In total, we deemed the nuptial pad photos of 41 XX males and 69 XY males suitable for further analysis. At the start of each photo session, we photographed an X-rite ColorChecker Classic Mini card (X-Rite Inc.), to enable consistent colour correction across images. First, we converted the raw images to DNG file format in Adobe DNG Converter (v. 15.4.0.1508). Then, we used the ColorChecker Camera Calibration software (v.2.2.0; X-Rite Inc.) to create colour profiles. We applied these profiles to the agile frog images in RawTherapee (v.5.11), along with a pre-saved exposure curve to improve nuptial pad visibility in a standardized way. On each image, we manually selected the nuptial pad using the 'Free Select' tool in GIMP (v.2.10.38) and reduced the selection by five pixels to minimise potential disturbing effects of nearby areas on colour measurement. Finally, we applied the "Fill with average colour" plugin (Ofnuts' Gimp Tools, 2017) to calculate the mean nuptial pad colour and extracted the HSV values.

2.3. Hormone level evaluation

We measured water-borne levels of testosterone and corticosterone, using a minimally invasive method that has been validated in various amphibians (Rodriguez et al., 2022; Baugh et al., 2018; Gabor et al., 2013). These samples were collected from a subset of males that were all captured on the third day of the breeding season in 2024 and used in a subsequent experiment (Bókony, Balogh, Ujhegyi, et al., 2025). For that experiment, we temporarily housed these males in outdoor containers (87 × 64 cm) placed next to the breeding pond, that were filled with pond water to ca. 15 cm depth. The animals were housed in groups of up-to 10 individuals and were kept there until at least two gravid females arrived per day to be paired with one XX male and one XY male with similar SVLs. Hormone sampling was conducted right before pairing the males with the females for the experiment. We weighed each male with a digital scale (± 0.01 g) and placed it in a glass jar (8 cm diameter and 8 cm height) that had been thoroughly cleaned with 96% ethanol and rinsed with reverse-osmosis filtered, UV-sterilized tap water, and filled with 40 ml of spring water. We left the animals undisturbed next to the breeding pond for 60 minutes, while they released hormones into the surrounding water predominantly by urinating (i.e. none of the frogs excreted faeces into the water, but they might have also released hormones through their skin (Baugh et al., 2025; Baugh & Gray-Gaillard, 2021; Gabor et al., 2013). Finally, we weighed each animal again and calculated weight loss. We wore clean nitrile gloves while handling the animals and equipment for hormone sampling, and we patted the frogs dry before weighing them using clear paper towels.

We extracted steroids from the water using silica gel-filled solid-phase extraction (SPE) columns (Chromabond C18 ec, 45 µm, 3 mL/500 mg; Macherey-Nagel GmbH & Co KG), which were first primed with methanol (CH_3OH). Then, the samples were extracted through the columns and subsequently 4 ml methanol was used to elute the steroids. After evaporating the methanol, we resuspended the samples in assay buffer for subsequent analysis. We assessed hormone

concentrations of testosterone and corticosterone using a commercial Enzyme Immunoassay Kit (DetectX Corticosterone Multi-Format ELISA Kit and DetectX Testosterone ELISA Kit; Arbor Assays Inc), following the manufacturer's protocol. Samples were quantified in duplicates. The detection limits were determined as the mean optical density of the maximum binding duplicates minus three times the standard deviation between the duplicates. Urinary hormone concentrations (pg hormone / g urine) were subsequently estimated under the assumption that body mass loss during the sampling equalled the amount of urine released. Out of the 40 males, we excluded eight individuals because the urinary concentrations of the measured hormones could not be determined. Out of these, four individuals were weighed with a scale that later proved to be inaccurate, and some of the water sample of one individual was accidentally spilled. Additionally, three individuals did not lose any weight during the trial, therefore the amount of urine as well as urine hormone concentrations could not be calculated for them. In total, we could obtain reliable estimates for urine hormone concentrations for 16 XX males and 16 XY males.

2.4. Statistical analysis

To quantify the frogs' body condition as body mass relative to body size, we calculated the scaled mass index (SMI) following (Peig & Green, 2009, 2010). This index adjusts individual body mass to a value expected at a common body length, using the equation of the linear regression of log-mass on log-length estimated by standardized major axis regression. For the calculation of this equation, we used the body mass and SVL data of all 511 males in our dataset. The regression slope was 3.15, whereas average SVL was 54.3 mm, thus we calculated SMI as body mass × $(54.3/SVL)^{3.15}$.

We performed all statistical analyses in R (v.4.5.2). First, we tested whether sex-reversed males differed from sex-concordant males in various aspects of body size. For these analyses, we used the data of 511 males that were captured in 2023 and 2024. For SVL, body mass, and SMI, we fitted a linear model for each, with genotypic sex as the fixed factor and sampling date as a numeric covariate (expressed as the number of days since the first capture in that year). For forearm length, forearm width, and nuptial pad length, we ran similar models, but we also added SVL as a numeric covariate to account for varying body size between the individuals.

Second, we tested whether sex-reversed males differed from sex-concordant males in nuptial pad colour. For hue, saturation, and brightness of the nuptial pad, we ran a linear model each with genotypic sex as the fixed factor, and SVL, SMI, and sampling date as covariates. In these analyses, we included all males for which we had an acceptable-quality photograph of the nuptial pad (n=110).

To test for differences in hormone concentrations between sex-reversed and sex-concordant males, we used the hormone data from 32 males. For analysing urine concentrations of testosterone and corticosterone as response variables, we ran generalized least squares models to account for heteroscedasticity. As variance in hormone concentrations increased with body mass, we applied an exponential variance function structure, based on model diagnostics. As fixed effects, the models included genotypic sex, body mass, and the number of days from capture to sampling. Additionally, to test whether variations in testosterone levels could be explained by differences in corticosterone levels (Eikenaar et al., 2012; Leary & Harris, 2013), we re-ran the analysis of testosterone by adding corticosterone as an explanatory variable into the above model.

For model diagnostics, we inspected the relevant residual graphs to check for homoscedasticity, normality, and outliers, and we calculated the variance inflation factor to check for multicollinearity (<2 in all models). We used 95% confidence levels throughout. Our dataset and R script are available from the corresponding author upon request.

3. Results

3.1. Morphometric measurements of sex-reversed and sex-concordant males

Sex-reversed and sex-concordant males did not differ significantly in SVL, body mass, SMI, left forearm width and length corrected for SVL, or left nuptial-pad length corrected for SVL (**Hiba! A hivatkozási forrás nem található.**). Nuptial pad colours of sex-reversed and sex-concordant males did not differ significantly in any of the three measured HSV variables (**Hiba! A hivatkozási forrás nem található.**). Across both genotypic groups, males with higher hue, saturation, and brightness (i.e., lighter-coloured nuptial pads) tended to have shorter SVL and arrived later at the breeding site (**Hiba! A hivatkozási forrás nem található.**). In addition, males with lower SMI had brighter nuptial pads (**Hiba! A hivatkozási forrás nem található.**).

3.2. Comparison of steroid hormone levels between sex-concordant and sex-reversed male agile frogs

Testosterone concentrations were significantly lower in sex-reversed males than in sex-concordant males (**Hiba! A hivatkozási forrás nem található.**), with estimated mean concentrations (mean ± SE) of 173 ± 45.8 pg/g in XX males and 451 ± 40.1 pg/g in XY males. Corticosterone concentrations did not differ significantly between sex-reversed and sex-concordant males (**Hiba! A hivatkozási forrás nem található.**), and corticosterone was not a significant predictor of testosterone in the model (β = 0.099 ± 0.070 SE, t = 1.417, p = 0.168).

Table 1: *Differences of sex-concordant males from sex-reversed males as estimated from multi-predictor statistical models, with standard error (SE). Note that each model included several other predictors, see Table 2.*

	Dependent variable	***Difference***	***SE***	***t***	***p***
	SVL (mm)	0.585	0.428	1.366	0.173
	Body mass (g)	0.458	0.460	0.995	0.320
	SMI (Scaled Mass Index; g)	-0.249	0.238	-1.043	0.297
	Left forearm length (mm)	-0.042	0.092	-0.455	0.650
	Left forearm width (mm)	0.050	0.095	0.521	0.603
	Left nuptial pad length (mm)	0.058	0.057	1.008	0.314
	Nuptial pad hue	-0.135	0.524	-0.258	0.797
	Nuptial pad saturation	-0.482	0.979	-0.492	0.624
	Nuptial pad brightness	-0.660	0.875	-0.754	0.452
	Testosterone in urine (pg/g)	278.201	21.310	13.055	<0.001
	Corticosterone in urine (pg/g)	216.191	146.755	1.473	0.152

Table 2: *Slopes of the effects of SVL (snout–vent length), date (days since start), and SMI (scaled mass index) in the multi-predictor statistical analyses. Note that each model also included genotypic sex, whose effects are presented separately in Table 1. Additional model results for size and hormone data are available from the corresponding author upon request.*

	Dependent variable	***Difference***	***SE***	***t***	***p***
Hue	SVL (mm)	-0.199	0.066	-3.007	0.003
	Days since start	0.076	0.044	1.735	0.086
	SMI (Scaled Mass Index; g)	-0.18	0.129	-1.391	0.167
Saturation	SVL (mm)	-0.613	0.124	-4.952	<0.001
	Days since start	0.156	0.081	1.913	0.058

	SMI (Scaled Mass Index; g)	-0.202	0.241	-0.837	0.404
Brightness (Value)	SVL (mm)	-0.512	0.111	-4.63	<0.001
	Days since start	-0.693	0.073	-1.873	0.064
	SMI (Scaled Mass Index; g)	-0.66	0.215	-3.222	0.002

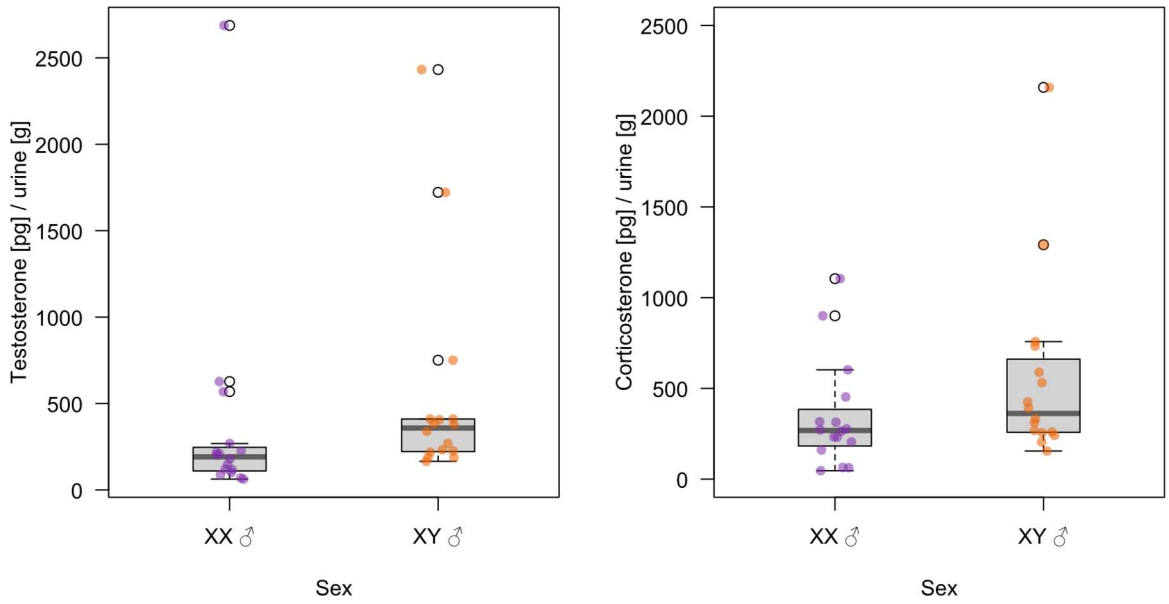

Figure 1: Urine testosterone (**left**) and corticosterone (**right**) concentrations (pg/g) in sex-reversed XX males (purple, n = 16) and sex-concordant XY males (orange, n = 16). Boxplots show the distribution of observed data (thick middle line: median, box: interquartile range; whiskers extend to the most extreme data points within 1.5 × interquartile range from the box).

4. Discussion

In free-ranging populations of the agile frog, sex-reversed XX males are as fertile as sex-concordant XY males, yet the former feature lower success in siring offspring, and differential female mating preferences at least partly contribute to this contradiction (Bókony, Balogh, Ujhegyi, et al., 2025). Consequently, phenotypic differences are expected to persist between phenotypic males with different sexual genotypes. In this study, we assessed the occurrence of such differences between sex-reversed and sex-concordant agile frog males by comparing both morphological and hormonal traits that are linked to male fitness in anurans. Using water-borne hormone samples collected during a 60-min period, we found that sex-reversed males exhibited significantly lower urinary testosterone concentrations than sex-concordant males, independent of sampling date, body size, and corticosterone levels. Testosterone is the primary androgen, regulating secondary sexual traits and reproductive behaviour in male anurans (Leary, 2009). The lower testosterone concentrations observed in XX males therefore may indicate a functionally meaningful endocrine difference between sex-reversed and sex-concordant males. Such differences might contribute to the reduced siring success of XX males reported in free-living agile frog populations (Bókony, Balogh, Ujhegyi, et al., 2025). It should be noted that the hormone dataset of the current study included 32 males, and

different sample size might play a role in the difference between the hormone and morphometric results. A further caveat is that the animals in our study were maintained in captivity for 11-19 days before sampling. If sex-reversed and sex-concordant males differed in their hormonal stress response to captive conditions, that may have contributed to the observed differences in testosterone levels. In vertebrates, elevated corticosterone can suppress testosterone secretion via inhibition of the hypothalamo-pituitary-gonadal axis (Moore & Jessop, 2003; Wingfield & Sapolsky, 2003). Our results do not support that this mechanism would be responsible for lower testosterone levels observed in sex-reversed males and sex-concordant males, and corticosterone did not explain the testosterone variation among individuals. An alternative explanation could therefore be that the lower testosterone levels of sex-reversed males arise instead from intrinsic differences in gonadal or neuroendocrine function related to genotypic sex, a hypothesis that warrants further testing. This pattern could be consistent with the theoretical framework outlined by (Laven et al., 2025), who predicted that hormone profiles of sex-reversed individuals may follow intermediate patterns, which did not fully correspond to either the genotype or to their phenotype. A comparable pattern was described in female sex-reversed Nile tilapia (*Oreochromis niloticus*), where most hormone concentrations corresponded to the phenotypic sex, yet certain steroid levels remained anomalous in sex-reversed individuals (Gennotte et al., 2017).

Sex-reversed and sex-concordant males were statistically indistinguishable across all measured morphological traits, suggesting that sex-reversed individuals feature typical male morphology despite reduced testosterone levels. Forearm musculature and nuptial pad morphology are important secondary sexual traits that affect the success of maintaining amplexus. Although these traits are known to be androgen-dependent (Epstein & Blackburn, 1997; Lynch & Blackburn, 1995), differences in testosterone levels between XX and XY males did not translate into detectable differences in nuptial pad morphology in this study. The lack of morphological differences in forearm and nuptial pad morphology may indicate that these traits respond to androgens in a threshold-dependent or saturating manner (Leary, 2009; McDermott & Safran, 2021), with both XX and XY males likely exceeding the androgen threshold required for full trait expression. The functional consequences of the testosterone difference may therefore manifest primarily through behavioural traits such as calling rate and call characteristics, which play an important role in mate choice in agile frogs (Leary, 2009; Lesbarrères et al., 2008), but were beyond the scope of this study. Reduced calling performance could potentially contribute to the reduced paternity rates and apparent female aversion reported in sex-reversed males (Bókony, Balogh, Ujhegyi, et al., 2025), highlighting the need for future studies investigating this possibility.

Contrary to our expectations and despite the difference in testosterone, we found no significant differences between XX and XY males in nuptial pad colour. Darker nuptial pads can be associated with higher testosterone levels in male frogs (Orton et al., 2020), and thus nuptial pad colour may provide information about male quality and can be associated with male mating success as demonstrated in the common frog *(Rana temporaria),* (Orton et al., 2023). It was previously suggested that nuptial-pad morphology may be seen as a biomarker of reproductive health in anurans (Orton et al., 2020). Here, we found that nuptial pad colouration in agile frogs was associated with male quality indicators independently of genotypic sex: males with higher hue, saturation, and brightness (i.e., lighter colouration) tended to be smaller in SVL and arrived later at the breeding site, and males with lower SMI had brighter nuptial-pad colour (**Hiba! A hivatkozási forrás nem található.**). These results are consistent with findings in the common frog by Orton et al. (2023) and suggest that nuptial pad colouration may function as a condition-dependent signal of male quality in agile frogs, warranting further study.

Further, we observed no differences between free-ranging adult sex-reversed and sex-concordant males in SVL, body mass and SMI. This result contradicts previous reports on differential growth rate during larval and juvenile development in agile frogs raised in captivity (Bókony, Balogh, Mikó, et al., 2025; Bókony et al., 2021), but is consistent with the finding that adult male body mass does not vary with genotypic sex in wild populations (Bókony, Balogh, Mikó, et al., 2025; Nemesházi et al., 2020). This discrepancy may reflect differences between early developmental stages and adulthood, as growth rate divergences observed during larval and juvenile phases may diminish or disappear by the time animals reach reproductive age (e.g. catch-up growth). Additionally, captive conditions likely impose different physiological constraints than natural environments, potentially amplifying or masking genotypic effects on growth that would not manifest under free-ranging conditions. Body size and condition can affect the outcome of male-male competition, and larger males may be more successful in physical combat (Vági & Hettyey, 2016). However, our results suggest that reduced breeding success (Bókony, Balogh, Ujhegyi, et al., 2025) of sex-reversed males is not caused by smaller body size or lower body condition, which fits with observations that they are capable of outcompeting sex-concordant males for females in captive trials (Bókony, Balogh, Ujhegyi, et al., 2025).

Conclusion

Our findings contribute to the growing recognition that sex reversal in ectotherms does not simply produce individuals equivalent or opposite to their phenotypic sex, but rather individuals with a complex and subtle suite of trait differences that may not be apparent from external morphology alone (Bókony et al., 2021; Nemesházi & Bókony, 2025). We demonstrated that sex-reversed agile frog males have significantly lower urine testosterone concentrations than sex-concordant males, while being phenotypically indistinguishable in all measured morphological traits. Together, these findings suggest that sex reversal in agile frogs produces a mixed phenotype, with typical male morphology combined with endocrine differences that might contribute to the reduced reproductive success of XX males reported in wild populations (Bókony, Balogh, Ujhegyi, et al., 2025). As ongoing environmental changes poses growing threat to amphibians worldwide (Hamer & McDonnell, 2008; Luedtke et al., 2023) and may elevate the frequency of sex reversal in free-living populations (Nemesházi et al., 2020; Bókony et al., 2017), understanding these nuanced fitness costs becomes increasingly important for predicting the demographic and evolutionary consequences of sex reversal in nature.

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