

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. In the agile frog (*Rana dalmatina*), an emerging model species of environmental sex reversal, sex-reversed (XX) males have lower mating success in the wild compared to sex-concordant (XY) males, but the mechanisms are unclear. To address this gap, we compared sex-reversed and sex-concordant males upon 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). Furthermore, we measured waterborne concentrations of testosterone and corticosterone. Sex-reversed males were indistinguishable from sex-concordant males across all measured traits, except that the former had significantly lower testosterone concentrations. Typical male morphological appearance combined with endocrine differences might contribute to the 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, especially under the ongoing environmental change.

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

1. Introduction

Global climate change and habitat loss pose significant challenges for various taxa including reptiles [1], amphibians [2], and fish [3]. 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 [4–6]. 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 [6–9]. Amphibians were long assumed to only possess genotypic sex determination [10]. Yet, a growing body of evidence confirms that amphibian sexual development can be influenced by environmental conditions experienced during early development, including high or low temperatures and the presence of certain chemical pollutants [6,11–13]. Sex reversal may be far more common than currently known in the herpetofauna [14], particularly in areas exposed to anthropogenic land use [12,15,16]. While sex reversal may be an adaptive response to certain changes of environmental conditions [17,18], it may also have diverse population-level consequences, including shifting adult sex ratios and turnovers between different sex-determination systems [19–22]. 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.

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 behavior in sex-reversed individuals [8,9,23–25]. Nevertheless, sex-reversed animals can be fertile and reproductively active [20,23]. The progeny of sex-reversed individuals can show increased vulnerability to stress [23], 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 [21]. 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. Such comparisons, however, are rarely available from wild populations [23,26,27].

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 [28–31]. Relevant morphological traits – such as strong forearms, nuptial pads, and vocal sacs – as well as reproductive behavior, are affected by the levels of steroid hormones [28,29,32,33]. 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 color and morphology are influenced by androgens and associated with mating success in different anuran taxa [29,30]. In addition to androgens, glucocorticoids may also affect reproductive performance. In amphibians, corticosterone is the primary glucocorticoid that contributes to regulating resource allocation among competing physiological processes, including reproduction and self-maintenance, which may ultimately influence breeding success [34–36]. The relationship between glucocorticoid levels and reproduction is complex and context-dependent, with transient increases potentially supporting reproductive demands, whereas prolonged stress-induced elevation of glucocorticoid levels may lead to allostatic overload and impaired reproductive function [37]. Therefore, variation in both reproductive and glucocorticoid hormones may influence the reproductive success of males. In turn, sex reversal may affect these traits depending on how strongly they are determined by sexual genotype versus phenotype [8,38,39], but this has not yet been investigated in the wild.

The agile frog (*Rana dalmatina*) is a species with XX/XY male heterogametic sex-determination system and documented occurrence of female-to-male sex reversal in free-ranging populations [15]. In this species, elevated environmental temperatures during larval development facilitate sex reversal in genotypic (XX) females [13,17], and sex-reversal frequency increases with anthropogenic land use in free-ranging populations [15]. Sex-reversed (XX) males have lower siring success despite being similarly fertile to the sex-concordant (XY) males, and females seem to be able to differentiate between them in mate choice [23]. However, the mechanisms underlying these differences are unknown, as phenotypic traits relevant for breeding success have not been compared between the two male types except for body mass [15,23]. 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: snout-vent length (SVL), body mass, scaled mass index (SMI) as a measure of body condition, forearm length, forearm width, and nuptial pad size, and also (2) nuptial pad coloration. Furthermore, (3) we compared sex-reversed and sex-concordant males for non-invasively measured waterborne testosterone and corticosterone levels.

2. Materials and Methods

2.1. Animal collection and sexing

We captured 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. We photographed each captured animal for individual identification based on their natural pigment marks, as published elsewhere [40]. 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 [15], following the published protocol. First, we determined the Rds3 genotype for all phenotypic males by high-resolution melting (HRM) analysis. 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 genotypes were excluded from the study (N = 17 out of 538 captures). 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). The authors complied with the ARRIVE guidelines.

2.2. Morphometric and color measurements

On the day of capture, we measured body mass (± 0.01 g) with a digital scale, and used a caliper to measure SVL, the length and width of the left forearm, as well as the length of the left nuptial pad (± 0.1 mm). We had these morphometric data for a total of 511 capture events representing 460 individuals with unambiguous sexual genotype (i.e. some males were captured more than once as they exited and re-entered the pond during the breeding season).

We measured nuptial pad color in the hue – saturation – brightness color 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 those images on which the nuptial pad was fully visible and at sufficient resolution to delineate its borders (N = 108 individuals, comprising 42 XX males and 66 XY males; no repeated measurements). At the start of each photo session, we photographed an X-rite ColorChecker Classic Mini card (X-Rite Inc.), to enable consistent color correction across images. First, we converted the raw images to DNG file format in Adobe DNG Converter version 15.4.0.1508 [41]. Then, we used the ColorChecker Camera Calibration software version 2.2.0 [42] to create color profiles. We applied the corresponding color profile to each frog image in RawTherapee version 5.11 [43], 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 version 2.10.38 [44] and reduced the selection by five pixels to minimize potential disturbing effects of nearby areas on color measurement. Finally, we applied the “Fill with average color” plugin [45] to calculate the mean nuptial pad color and extracted the HSV values.

2.3. Hormone level evaluation

We measured waterborne levels of testosterone and corticosterone, using a minimally invasive method that has been validated in various amphibians [46–48]. These samples were collected from a subset of 40 males that were all captured on the third day of the breeding season in 2024 and used in a subsequent experiment [23]. For that experiment, we temporarily housed these males in outdoor containers (87 × 64 cm base area) 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 for 11-19 (median: 13) days until at least two gravid females arrived per day to be paired with one XX male and one XY male of 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 previously 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 placed the individual in the glass jar next to the breeding pond, where it remained undisturbed for 60 minutes. Finally, we weighed each animal again and calculated weight loss. We wore clean nitrile gloves during the procedure of hormone sampling, and we patted the frogs dry before weighing them using clean paper towels.

Water samples were stored at -20 °C and were transported to the laboratory at the Department of Behavioral and Cognitive Biology, University of Vienna while being kept frozen. We first filtered the water samples through paper filters to remove any pieces of skin or debris. We then extracted steroids 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 (CH₃OH). Steroids were then eluted with 4 mL methanol, subsequently dried down with nitrogen (N₂) and resuspended in 250 µL Assay Buffer (Arbor Assays) for the analysis of both testosterone and corticosterone. We assessed hormone concentrations of testosterone and corticosterone using a commercial Enzyme Immunoassay Kit (DetectX Testosterone ELISA Kit K032-H and DetectX Corticosterone ELISA Kit K014-H; Arbor Assays Inc), following the manufacturer's protocol. We analyzed all samples in duplicate and included control samples to evaluate intra- and inter-assay coefficients of variation (CVs). Control samples consisted of assay buffer spiked with corticosterone (Arbor Assays, catalog no. C151; 400 pg/mL) and testosterone standard (Arbor Assays, catalog no. C113; 125 pg/mL). The mean intra-assay CV was calculated based on the final concentration and was 1.3% for corticosterone and 0.7% for testosterone. Inter-assay variation was < 10% for corticosterone and < 8% for testosterone. Detection limits were calculated as the mean optical density (OD) of the maximum-binding duplicates minus three times the standard deviation of those duplicates. Across the three corticosterone assay plates, the mean detection limit corresponded to $98.0 \pm 1.2\%$ binding, equivalent to a concentration of 9.8 ± 10 pg/mL. Across the three testosterone assay plates, the mean detection limit corresponded to $89.4 \pm 3.32\%$ binding, equivalent to 15.8 ± 13.4 pg/mL.

Waterborne hormone sampling provides an integrated measurement of hormones released over time by various means, including urine, feces, and skin secretions [47,49,50]. We expected urine to represent the main source of waterborne hormones in our study for the following reasons. None of the frogs excreted feces, as agile frogs typically do not feed before mating. Bladder urine volume can represent a considerable proportion of body mass in large terrestrial anurans [51], and water contact can stimulate urination [52]. Previous studies raised the possibility that individual differences in urine excretion rates might make waterborne hormone measurements unreliable [49,51]. Therefore, we accounted for the individual variation in surmised urine release rates as follows. We estimated urine volume during waterborne hormone sampling, assuming that body mass loss during sampling equaled the mass of urine released, and we used this estimate to express waterborne testosterone concentrations per gram of body-mass loss (pg hormone / g body-mass loss). For simplicity, we are referring to the resulting variables as testosterone and corticosterone concentrations hereafter.

Out of the 40 males, we excluded eight individuals, for the following reasons. Some of the water sample of one individual was accidentally spilled. We could not infer urine mass for 7 individuals, out of which four individuals were weighed with a scale that later proved to be inaccurate, and three individuals did not lose any weight during the sampling. In total, we could obtain reliable estimates for 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 and Green [53,54]. 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. We calculated this equation using the body mass and SVL data of all 511 capture events in our dataset. The regression slope was 3.16, and average SVL was 54.3 mm; we therefore calculated SMI as $\text{body mass} \times (54.3/\text{SVL})^{3.16}$.

We performed all statistical analyses in R version 4.5.2 [55]. First, we tested whether sex-reversed males differed from sex-concordant males in various aspects of body size. For SVL, body mass, and SMI, we fitted a linear mixed-effects 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). Individual identity was added as a random intercept to accommodate the repeated data. 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 color. 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.

For analyzing 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 in all these models, based on model diagnostics. To test for differences in hormone concentrations between sex-reversed and sex-concordant males, the models included genotypic sex, body mass, and the number of days from capture to sampling as fixed effects. Additionally, to test whether variations in testosterone levels could be explained by differences in corticosterone levels, we re-ran the analysis of testosterone by adding corticosterone as an explanatory variable into the above model. To assess whether nuptial pad brightness was associated with testosterone levels, we ran a separate model instead of adding nuptial pad brightness into the above models, because testosterone levels and morphological traits were strongly correlated. To avoid multi-collinearity, this latter model included nuptial pad brightness as the only fixed effect.

For model diagnostics for all analyses, 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.

3. Results

XX and XY males did not differ significantly in SVL, body mass, SMI, forearm width and length corrected for SVL, or nuptial-pad length corrected for SVL (**Error! Reference source not found.**). Nuptial pad colors of sex-reversed and sex-concordant males did not differ significantly in any of the three measured HSV variables (**Error! Reference source not found.**). Across all males, irrespective of genotype, males with redder (lower hue), less saturated, and darker colored nuptial pads tended to have longer SVL (Table 1). In addition, males with higher SMI had darker nuptial pads (Table 1).

Waterborne testosterone concentrations were significantly lower in XX males (mean $\pm$ SE: 173 ± 45.8 pg/g) than in XY males (451 ± 40.1 pg/g; **Error! Reference source not found.**, Fig. 1). Corticosterone concentrations did not differ significantly between sex-reversed and sex-concordant males (**Error! Reference source not found.**, Figure 1), and corticosterone concentration was not a significant predictor of testosterone concentration (Table S1). Nuptial pad brightness was negatively associated with testosterone levels (Table S2), indicating that males with brighter nuptial pads had lower testosterone concentrations.

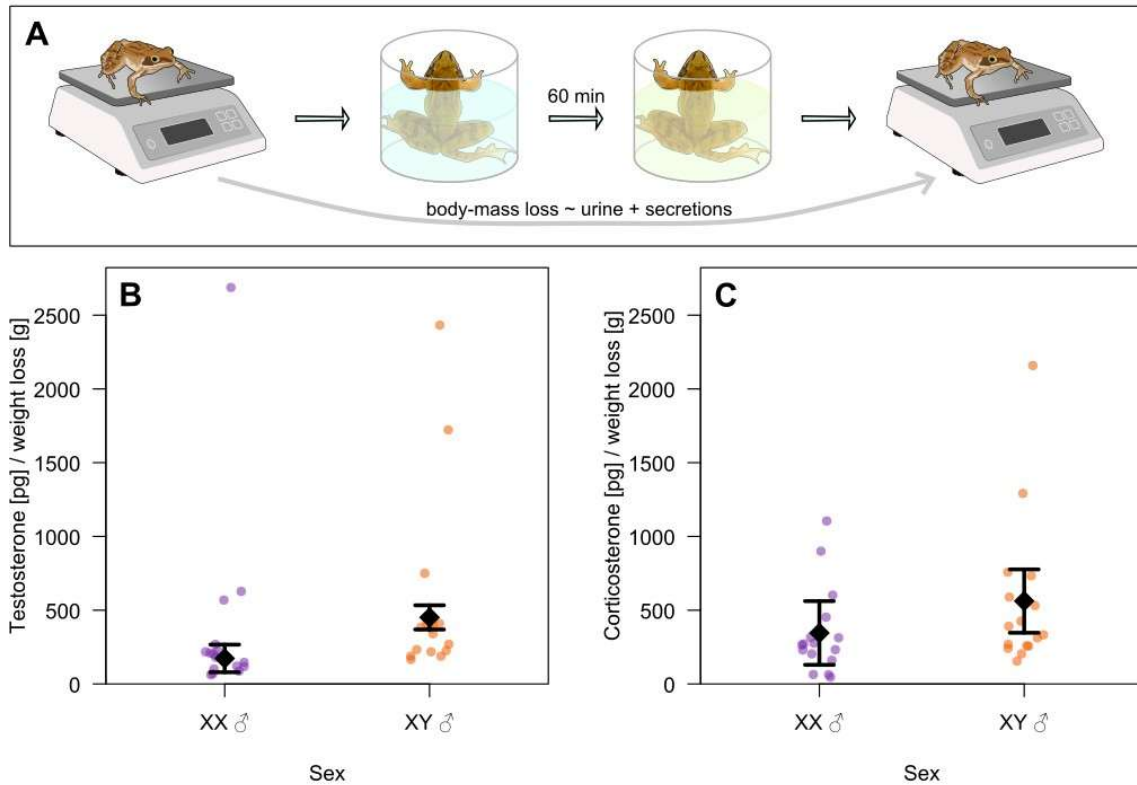


Figure 1: Waterborne hormone sampling procedure and resulting testosterone and corticosterone concentrations in sex-reversed (XX) and sex-concordant (XY) males. (A) Schematic of the waterborne hormone collection procedure, (B) testosterone and (C) corticosterone concentrations (pg/g) in XX and XY males (n = 16 each) with individual data points jittered horizontally. Black diamonds and error bars show means ± 95% confidence intervals estimated from generalized least squares models, adjusted for body mass and for the number of days between capture and hormone sampling.

Table 1: Differences of sex-reversed males from sex-concordant males as estimated from multi-predictor statistical models, with standard error (SE).

<i>Dependent variable</i>	<i>Model parameter</i>	<i>Estimate</i>	<i>SE</i>	<i>t</i>	<i>p</i>
SVL (mm) *	Intercept	54.41	0.203	268.686	<0.001
	Date	-0.162	0.026	-6.198	<0.001
	Genotypic sex	-0.575	0.436	-1.319	0.188
Body mass (g) *	Intercept	19.708	0.219	89.824	<0.001
	Date	-0.16	0.027	-5.860	<0.001
	Genotypic sex	-0.487	0.471	-1.032	0.302
SMI (Scaled Mass Index; g) *	Intercept	19.341	0.110	175.106	<0.001
	Date	-0.006	0.015	-0.392	0.697
	Genotypic sex	0.236	0.24	0.985	0.325
Left forearm length (mm) *	Intercept	13.667	0.0427	320.25	<0.001
	SVL	0.209	0.01	21.805	<0.001
	Date	-0.035	0.006	-5.903	<0.001
	Genotypic sex	0.045	0.093	0.483	0.629
Left forearm width (mm) *	Intercept	7.544	0.045	168.083	<0.001
	SVL	0.155	0.01	15.567	<0.001
	Date	-0.029	0.006	-4.822	<0.001
	Genotypic sex	-0.03	0.097	-0.312	0.755
Left nuptial pad length (mm) *	Intercept	6.96	0.027	259.333	<0.001
	SVL	0.103	0.006	17.214	<0.001
	Date	-0.018	0.004	-4.861	<0.001
	Genotypic sex	-0.062	0.058	-1.07	0.285
Nuptial pad Hue [†]	Intercept	31.919	0.355	90.023	<0.001
	SVL	-0.2	0.068	-2.955	0.004
	Date	0.075	0.044	1.712	0.09
	SMI	-0.185	0.130	-1.423	0.158
	Genotypic sex	0.199	0.528	0.378	0.706
Nuptial pad Saturation [†]	Intercept	60.954	0.657	92.72	<0.001
	SVL	-0.613	0.125	-4.895	<0.001
	Date	0.148	0.081	1.83	0.07
	SMI	-0.209	0.242	-0.866	0.389
	Genotypic sex	0.445	0.978	0.455	0.65
Nuptial pad brightness [†]	Intercept	29.868	0.6	50.629	<0.001
	SVL	-0.501	0.112	-4.459	<0.001
	Date	-0.137	0.073	-1.878	0.063
	SMI	-0.687	0.217	-3.168	0.002
	Genotypic Sex	0.57	0.878	0.65	0.517
Waterborne testosterone (pg/g) [‡]	Intercept	455.03	44	10.346	<0.001
	Mass	18.639	14.402	1.294	0.206
	Days until hormone sampling	10.175	17.283	0.589	0.561
	Genotypic sex	-278.201	21.31	-13.055	<0.001
Waterborne corticosterone (pg/g) [‡]	Intercept	570.589	105.743	5.396	<0.001
	Mass	34.684	26.051	1.331	0.194
	Days until hormone sampling	29.077	37.465	0.776	0.444
	Genotypic sex	-216.191	146.755	-1.473	0.152

Model coefficients are given as estimated means with standard errors (SE). The intercept refers to XX males with average values in all other explanatory variables. The remaining coefficients refer to the change in response to a unit change in the explanatory variables.

* Linear mixed-effects model; 511 observations of 460 individuals

† Linear model; n= 108 individuals

‡ Generalized least-squares model; n= 32 individuals

4. Discussion

The present study revealed an endocrine difference but no measurable morphological differences between XX and XY male agile frogs. Using waterborne hormone samples, we found that sex-reversed males released significantly lower 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 behavior in male anurans [32]. The lower testosterone concentrations observed in XX males therefore may indicate a functionally meaningful endocrine difference between sex-reversed and sex-concordant males. The mechanisms underlying this endocrine difference remain unknown and may involve genotypic effects, developmental processes associated with sex reversal, or other physiological differences that were not assessed in the present study. 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 [56]. In free-ranging populations of the agile frog, sex-reversed males are as fertile as sex-concordant males, yet the former feature lower success in siring offspring [23], suggesting that factors beyond fertility such as behavioral traits may underlie these differences. The functional consequences of the testosterone level difference during the breeding season may manifest primarily through behavioral traits such as calling rate and call characteristics [32,57–59], which play an important role in mate choice in agile frogs [60], highlighting the need for future studies investigating this possibility. It should be noted 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 [36,37]. Our results do not suggest that this mechanism is responsible for lower testosterone levels, however, because corticosterone levels did not explain the testosterone variation among individuals and corticosterone levels did not differ between XX and XY males. These latter findings are in line with previous studies on breeding male anurans that found either no [57,58] or sometimes even positive [59] associations between the two hormones, indicating that their relationship is context dependent [61].

Sex-reversed and sex-concordant males were statistically indistinguishable across all measured morphological traits. This might contradict the hormonal result, because higher androgen levels potentially could have resulted in larger size measurements. 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 [62,63], 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 [32,64], with both XX and XY males exceeding the androgen threshold required for full trait expression. Alternatively, testosterone concentrations measured during the breeding season may not accurately reflect androgen exposure during the period when forearm musculature and nuptial pads develop. As male agile frogs breed shortly after emerging from hibernation, these traits are being developed before hibernation, potentially decoupling their morphology from testosterone concentrations measured during the breeding season [65,66]. Thus, sex-reversed males may have experienced sufficient androgen exposure

before hibernation to develop normal forearm musculature and nuptial pads, while lower testosterone concentrations after emergence could affect reproductive behaviors during the mating season. One further possible explanation is 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. We hormone-sampled the males at the start of the breeding season, when typically the largest males arrive [67], and sex-hormone levels can also differ between early and late arriving males [68]. Such complexity might have contributed to the heterogeneity of results observed both within our study and compared to other studies [25,39]. Therefore, more research is needed to elucidate hormonal variation over the season in sex-reversed animals, especially in the wild.

Contrary to our expectations and despite the difference in testosterone, we found no significant differences between XX and XY males in nuptial pad coloration. However, darker nuptial pads were associated with higher testosterone levels in our dataset, which mirrors previous findings in other anuran species [29,30]. Therefore, nuptial pad coloration may provide information about male quality, potentially explaining why it was associated with male mating success in the common frog (*Rana temporaria*), a species closely related to the agile frog [30]. Therefore it was suggested that nuptial-pad morphology may be seen as a biomarker of reproductive health in anurans [29]. Here, we found that nuptial pad coloration in agile frogs was associated with male quality indicators independently of genotypic sex: males with lower hue (redder), less saturated, and darker colored nuptial pads tended to be larger in SVL and arrived earlier at the breeding site, and males with higher SMI had darker nuptial-pad color. These results are consistent with findings in the common frog [30] and suggest that nuptial pad coloration may function as a condition-dependent signal of male quality in agile frogs, warranting further study.

Finally, we observed no differences between free-ranging adult sex-reversed and sex-concordant males in SVL, body mass and SMI. Our results are consistent with previous findings that adult male body mass does not vary with genotypic sex in wild agile frog populations [15,17]. Also, this result fits with earlier experimental findings on the species in captivity. Specifically, sex reversal in agile frogs is primarily explained by early-life heat stress [13,17,23,69], which can have long-lasting negative effects on growth and other life-history traits [13,15,24,69]. However, heat-exposed individuals that undergo sex reversal seem to compensate for this developmental cost by faster early-life growth, which allows them to reach body masses comparable to those of heat-unexposed sex-concordant males by a few months after metamorphosis [17]. Our current results corroborate and extend this picture by showing that, among adults that grew up in the wild, sex-reversed males match sex-concordant males in a set of morphometrics that are relevant in mating competition. These results suggest that the reduced breeding success of sex-reversed males is not explained by smaller body size or poorer body condition, consistent with observations that these males can outcompete sex-concordant males for females in captive trials [23].

Conclusion

Our findings contribute to the growing recognition that sex reversal in ectotherms does not simply produce individuals physiologically 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 [8,9,14,24,56]. We demonstrated that sex-reversed agile frog males release significantly lower 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 [23]. As ongoing environmental changes pose growing threat to ectotherms worldwide [2,70] and may elevate the frequency of sex reversal in free-living populations [15,71],

understanding these nuanced fitness costs becomes increasingly important for predicting the demographic and evolutionary consequences of sex reversal in nature.

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