Sexual dichromatism in agile frogs: sex-reversed males closely resemble typical male coloration

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Abstract

In species with sexual dichromatism, colouration can play an important role in intraspecific communication and affect breeding success. Communication by visual signals during the breeding season has been increasingly recognized to occur in anuran amphibians. However, most studies of sexual dichromatism have focused on consistent differences between males and females, and species with varying combinations of sexual genotypes and phenotypes have so far been overlooked. We studied dorsal and ventral colouration of the agile frog (*Rana dalmatina*), a species where genetically female (XX genotype) tadpoles experiencing warm environmental temperatures tend to undergo sex reversal. In accordance with this laboratory-based observation, phenotypic males with both XX and XY genotype occur in free-ranging populations, but 'XX males' have reduced breeding success despite being fertile. We compared different aspects of colouration between females, 'XX males' and 'XY males' to assess whether there is sexual dichromatism between the phenotypic sexes, and whether 'XX males' differ from 'XY males' in any of the measured parameters. We found that females differed from phenotypic males in both dorsal and ventral colouration. These differences were also supported by the frog vision model. Females showed a trend towards darker, more red-shifted dorsal and throat coloration, and featured substantially more ventral pigmentation near the rostral tip of the mouth relative to phenotypic males. Most females featured a red ventral pattern on the anterior part of the trunk that almost never occurred in males. Interestingly, 'XX males' showed a shift towards yellower average throat colouration relative to 'XY males'; however, the difference was small and not detectable under the frog vision model. Our results suggest that agile frogs may rely on colouration for intraspecific communication during the breeding season, similarly to other species in this genus, but visual cues are unlikely to mediate the recognition of sex-reversed males.

1. Introduction

Sexual dichromatism often facilitates intraspecific communication, affects mate choice and may also cause differential predation risk between the sexes (Hirschmann & Hödl, 2006; Bell & Zamudio, 2012; Rojas, 2017; Höbel *et al.*, 2022; Rojas *et al.*, 2023). However, sexual development in some systems is more complex than what simple differentiation between males and females could describe. Certain environmental conditions can cause sex reversal during early ontogeny in a variety of ectotherm vertebrate species (Baroiller & D'Cotta, 2001; Li *et al.*, 2016; Nemesházi & Bókony, 2023), resulting in the development of opposite gonadal sex to what is expected based on sex chromosomes. Thus, the phenotypic traits of sex-reversed individuals may theoretically differ from those of their sex-concordant counterparts, potentially resulting in differential fitness (Li *et al.*, 2016; Bókony *et al.*, 2021, 2025b; Nemesházi *et al.*, 2021; Wild *et al.*, 2023). Because their sex chromosomes correspond to one sex, whereas their ontogenetic sexual development corresponds to the other, sex-reversed individuals may resemble one sex in some traits and the other sex in other traits (Li *et al.*, 2016). In fish, laboratory experiments have reported colouration differences between sex-reversed and concordant individuals (Edmunds *et al.*, 2000). Although sex-reversal has so far been confirmed in only a handful of amphibian and reptile species, indirect evidence suggests that it may be far more widespread than currently recognized, and climate change may increase its frequency worldwide (Geffroy & Wedekind, 2020; Whiteley *et al.*, 2021; Nemesházi & Bókony, 2025). Yet, empirical data on the effects of sex reversal on visible phenotypes, physiology and behaviour in the herpetofauna remain scarce (Li *et al.*, 2016; Bókony *et al.*, 2021, 2025b; Wild *et al.*, 2023), and to our knowledge no study has assessed its effects on colouration in amphibians or reptiles (but see Ujhegyi & Bókony, 2020, where a proportion of the studied female toads were sex reversed).

The agile frog (*Rana dalmatina)* is an emerging model system for sex-reversal research (Nemesházi *et al.*, 2020; Bókony *et al.*, 2021, 2025a; Mikó *et al.*, 2021; Ujszegi *et al.*, 2022). In this amphibian, elevated environmental temperatures experienced during larval development can induce female-to-male sex reversal (Ujszegi *et al.*, 2022). While females have XX sex-chromosome genotype and sex-concordant males have XY genotype, sex-reversed males are genotypic females (XX genotype) with male primary and secondary sexual characteristics (Nemesházi *et al.*, 2020). In free-ranging agile frog populations, the proportion of sex-reversed individuals among phenotypic males increases with anthropogenic land use (Nemesházi *et al.*, 2020). Effects of sex reversal on phenotypic traits other than growth and condition in early life (Bókony *et al.*, 2021, 2025a; Mikó *et al.*, 2021; Ujszegi *et al.*, 2022) have received little

attention. Recent findings suggest that female agile frogs differentiate between sex-reversed 'XX males' and concordant 'XY males' during mating, but the basis of this differentiation is currently unknown (Bókony *et al.*, 2025b). Due to its fundamental role in both communication and camouflage in amphibians (Rojas, 2017; Rojas *et al.*, 2023), studying colouration in agile frogs may provide crucial information on the fitness-related consequences of sex reversal.

Both dorsal and ventral colouration differ between the sexes in several amphibian species (Todd & Davis, 2007; Bell & Zamudio, 2012; Ancillotto *et al.*, 2022; Mühlenhaupt *et al.*, 2025). In anurans, these differences can either appear dynamically for the duration of the breeding season, or can develop ontogenetically and persist across adult life, sometimes being detectable already in juveniles (Hirschmann & Hödl, 2006; Sztatecsny *et al.*, 2010; Bell & Zamudio, 2012; Ujhegyi & Bókony, 2020; Höbel *et al.*, 2022). In contrast to anurans with conspicuous aposematic colouration (Rojas, 2017), the dorsal appearance of 'true frogs' (genus *Rana*) is usually brownish, which may facilitate camouflage via background matching on leaf litter (Barnett *et al.*, 2021; Rojas *et al.*, 2023). Nevertheless, sexual dichromatism occurs in this genus too. Adult males of the moor frog (*R. arvalis*) undergo dynamic colour change from brown to conspicuous blue for the breeding season. The function of blueness is subject to debate, but it might either facilitate mating success or serve male recognition (Hettyey *et al.*, 2009b; Sztatecsny *et al.*, 2012). In the wood frog (*R. sylvatica*), males feature somewhat greener dorsal colouration compared to females already as juveniles (Lambert et al., 2017). In various anurans, including nocturnal species, conspicuous throat coloration (underside of the head) and coloration of the anterior trunk may provide important cues for mating (Gomez *et al.*, 2009; Höbel *et al.*, 2022; Robertson *et al.*, 2022). In the common frog (*R. temporaria*), sexual dichromatism only became apparent to researchers when they observed animals in their natural breeding habitat during night time (Sztatecsny *et al.*, 2010). Under these circumstances, the throat of common frog males appeared bright whitish in contrast to the darker reddish throats of females. Dorsal colouration of agile frogs can vary along a broad range of brown, yellow and grey shades, and the underside features limited pigmentation that taxonomic guides describe as white (or pink) without any characteristic patterns (AmphibiaWeb, 2026). Nevertheless, some ventral pigmentation can be detected (Fig S1). Despite being a widespread species in Europe, no systematic assessment of sexual dichromatism in agile frogs has been attempted to our knowledge.

If colouration in agile frogs, similarly to other *Rana* species plays a role in male recognition, then detectable colouration differences between sex-reversed 'XX males' and concordant 'XY

males' could be key for females to differentiate between males with different genotypes. With other words, colouration might contribute to the decreased siring success of 'XX males' in the wild (Bókony *et al.*, 2025b). In this study we had two goals. First, we tested whether adult female, sex-reversed 'XX male' and concordant 'XY male' agile frogs differed in either dorsal or ventral colouration during the breeding season. Second, we assessed whether the frog vision model predicted differentiation among the three sex categories based on overall dorsal and throat colouration. We measured the average red, green and blue reflectance for the dorsum and for the throat, and calculated hue, saturation and brightness from these values. We used the average reflectance values against the vision model of a related *Rana* species for which information was retrievable. Additionally, handling several hundred frogs, we noticed greater variation in two types of ventral patterns distinguishable by the human eye (i.e. symmetrically distributed red patterns, and dark pigmentation along the mouth). We recorded these features for each individual and assessed whether the sexes differed in them.

2. Methods

2.1. Animal collection and sexing

The agile frog breeding season in our study region (Hungary, Central Europe) starts in late February or early March, and lasts until late March or early April, depending on the weather conditions. We collected free-ranging adults at arrival for breeding from pitfall traps around a fenced breeding pond near Budapest (47.551195°N, 18.926682°E) in February-March 2024 and transported them to our nearby (ca. 700 m) experimental station in Julianna-major at the Plant Protection Institute. Each frog was photographed and released into the pond within 24 hours after falling into a pitfall trap (see section 2.2. for details on photography). The animals were weighed on a digital scale with ± 0.01 g accuracy.

We used established methods to determine the phenotypic and the genotypic sex of each individual. Male phenotype was determined by the presence of nuptial pads, and female phenotype was recognized based on large belly size (i.e. filled with eggs). For genotypic sexing, we collected buccal swabs and used E.Z.N.A. Forensic DNA Kit (Omega Bio-Tek, Inc.) to extract DNA following the manufacturer's protocol. We used sex-linked genotypic sex markers following the established agile frog sexing protocol (Nemesházi *et al.*, 2020). In short, we screened all individuals for the Rds3 marker using high-resolution melt analysis (HRM) and, in cases of mismatch between genotype and phenotypic sex, we amplified the Rds1 locus with

PCR to confirm sex reversal. Only the unambiguously genotyped males were included in this study. Based on nearly 10 years of research, agile frog females always feature the XX genotype (i.e. no XY females were ever detected; Nemesházi *et al.*, 2020; Bókony *et al.*, 2021; Mikó *et al.*, 2021), and we therefore deemed it unnecessary to determine the genotype of each female. Based on combined information on genotypic and phenotypic sex, we classified each individual as female, 'XY male', or 'XX male' (i.e. sex-reversed). Hereafter, we will refer to this combined information of genotypic and phenotypic sex as consensus sex.

2.2. Photography and colouration

We took dorsal photos of all individuals, and ventral photos of a subset of the individuals (see details in the next paragraph; examples are shown in Fig S2). Photos were taken under standard lighting conditions provided by a 5000K LED light (Matcheasy Banda LED 5V-U-3-5K). High-resolution raw images (CR3; 24.2 MP) were captured with a Canon EOS R50 digital camera (Canon Inc.) that was mounted on a tripod. Aiming to minimise glare on the frog skin, we equipped the camera with a circular polarization filter (Hoya UX II slim frame, 49mm). We operated the camera using the Canon Camera Connect smart phone application (version 3.0.10.23; Canon Inc.). We photographed a ColorChecker Classic mini card (X-Rite Inc.; hereafter colour standard) each day and each time when the camera was re-mounted on the tripod (e.g. due to battery change), approximately at the same spot where the animals were photographed. Our dataset contained no more than one ventral and one dorsal photo per individual, as ensured by photo-based individual identification (Nemesházi *et al.*, 2026). We had ventral photos for only a subset of the captured individuals, and we excluded from our dataset all photos that did not provide a view of the vast majority of the throat area. In practice, ca. up-to the size of a frog finger was accepted to obscure the throat, which was left out from the area selected for colour measurement. Furthermore, we excluded all photos showing lesions (e.g. blisters) on the frog's skin that could have potentially biased colouration measurements.

We measured average dorsal and throat colouration on the digital photographs in the Multispectral Image Calibration and Analysis toolbox (MICA) version 2.2.3 (Troscianko & Stevens, 2015) plugin in ImageJ version 1.54g (Schneider *et al.*, 2012). A single image was analysed per individual (see Fig S2 for examples). We had 45 dorsal and 27 ventral images of 'XX males'. To obtain equal representation of each combination of sexual phenotypes and genotypes, we randomly chose the same numbers of images (one per individual) featuring females and 'XY males' as well, resulting in a total of 135 dorsal and 81 ventral images used for these measurements. We opened the raw image files with the 'generate multispectral image'

function in MICA, calibrated them with the black, grey and white tiles of the colour standard (reference greyscale values were taken from Figure 2 in Gaiani *et al.*, 2017), and created a 'linear normalized reflectance stack' from each image. We manually selected the region of interest (ROI): the back area following the ridges that frame the dorsum of agile frogs (on dorsal photos), or the approximate throat area denoted by the lower jaw (on ventral photos). For each ROI, we extracted reflectance values in the RGB colour space, corresponding to the visible spectrum: red (R; ~600 to 700 nm), green (G; ~500 to 600 nm) and blue (B; ~400 to 500 nm). Based on these values, we calculated hue (the dominant wavelength; i.e. green, yellow, red etc.), saturation (the intensity of the colour), and brightness (the amount of light reflected; often denoted by V for 'value') according to the HSV colour space using the 'rgb2hsv' function of the 'grDevices' package in R version 4.5.2 (R Core Team, 2025).

As our vision model approach, we used scotopic visual-space proxy model. Frog visual space was estimated using a scotopic *Rana temporaria* proxy model to approximate low-light perception in the focal system. Spectral sensitivity functions were reconstructed with the Govardovskii visual-pigment template (Govardovskii *et al.*, 2000), parameterized to the two rod classes reported for anuran vision, with peak sensitivities at approximately 430 nm and 502 nm (Sztatecsny *et al.*, 2010). This template-based reconstruction provides a standard, biologically interpretable perspective to represent receptor sensitivity from known peak wavelengths, while avoiding the stronger claim of direct physiological measurement. Calibrated Mica RGB values were then transformed into estimated rod catches, yielding patch values in a frog-relevant perceptual space rather than in camera-native colour coordinates. Noteworthily, as the analysis is based on image-derived measurements (and not on full reflectance spectra), it should be interpreted as an ecologically reasonable proxy: it is suited for comparing relative differences among patches and sex classes under a shared calibration framework, but it does not reconstruct absolute spectral properties of the skin or the full complexity of frog colour vision. In that sense, the model provides a low-light approximation for comparative sensory ecology analyses. To quantify perceptual separation under the low-light visual proxy, we calculated just-noticeable differences (JNDs) from predicted rod catches derived from calibrated Mica RGB values using the dichromat model.

To enable further measurements and manual assessment of specific human-visible features, we processed one ventral photo per individual (of all individuals for which images were available) in additional software (75 females, 27 'XX males' and 105 'XY males'). After converting the raw image files to DNG file format in Adobe DNG Converter version 15.4.0.1508 (Knoll *et al.*,

2023), we assigned a matching colour profile and the same pre-saved exposure curve to each photo in RawTherapee version 5.9 (The RawTherapee Team, 2022). The colour profiles were created from images of the colour standard in ColorChecker Camera Calibration version 2.2.0 (X-Rite Inc., 2020). We saved the colour-corrected ventral frog photos in jpeg format with the 'best quality' option in RawTherapee. We opened these images in Fiji (running with ImageJ version 1.54g; Schneider *et al.*, 2012), and measured the relative coverage of dark pigment patterns on the ventral surface of the head, close to the tip of the mouth (hereafter referred to as 'chin mottling') by our custom-developed plugins 'frog chin selection' and 'mottling threshold' (Supplement 1). First, we manually selected the area denoted by the mouth opening (Fig S3 A-B). Then, in the 'frog chin selection' plugin we placed a point selection at the tip of the mouth, after which the plugin automatically selected the ROI (Fig S3 C-E). Finally, we used the 'mottling threshold' plugin to select those pixels which were darker than a pre-defined threshold, calculate the area of dark patches ('area%') and create a masked image for visualization (Fig S3 F). Further details on how the plugins were created, as well as a detailed user guide are available in Supplement 1. In the 'mottling threshold' plugin, we used a pixel-darkness threshold that could suitably capture the contrast between the pigment-based mottling and the surrounding skin across our ventral photos, as determined based on 10 haphazardly chosen images.

Capturing ventral images, we noticed the presence of roughly symmetrical red patterns (patches and dots) on the throat and anterior part of the trunk of a considerable proportion of the individuals (hereafter 'red ventral pattern'; Fig S2 D). To assess whether this feature could be sex-linked, one author (E.N.) categorized the extent of 'red ventral pattern' on each individual's photo on an ordinal scale using a photo guide (Fig S4) as follows: no red patterns at all ('level 0'), animals with suspected but dubious presence of faint red patches or dots ('level 1'), animals on which some scattered red patches or dots were unambiguously present but did not form a clear pattern ('level 2'), and animals on which several red patches or dots formed clearly visible pattern on the anterior part of the trunk and the throat ('level 3'). Unavoidably, the sex of the individuals could be detected by the observer on the photos. Although the observer deliberately ignored sex when assigning the 'red ventral pattern' categories, we cannot exclude the possibility that this information unconsciously affected the decisions. We could not crop the images in a standard way that would provide a complete view of the area of interest while excluding all potentially visible secondary sexual characteristics. First, the presence or absence of nuptial pads would be visible on all photos where the animals held their front limbs partially

in front of the trunk or throat. Second, the dark pigmentation along the mouth can also provide a clue about the sex of the animal (see the results on 'chin mottling'). To validate that the subjectively assigned 'red ventral pattern' categories also corresponded with objectively measured differences (i.e. average throat colouration), we performed additional statistical analyses as described in Supplement 2.

To make sure that the 'red ventral pattern' is not a peculiarity of our focal population, we manually browsed several hundred agile frog observations available from across the species range on the iNaturalist platform (https://www.inaturalist.org/). We focused on photos on which the throat of the animal was visible, and the phenotypic sex could be determined based on the presence of nuptial pads in males and either a large belly or visible eggs (if the animal was found dead) in females. Additionally, we received a few photos of agile frogs of known phenotypic sex taken by colleagues during their fieldwork. Altogether, we retrieved images of 22 phenotypic females and 14 phenotypic males with unambiguously determined sex. The visibility of the anterior part of the trunk and throat greatly varied between these images, preventing accurate quantification. Therefore, we report the presence or absence of the 'red ventral pattern' as detected on the photos, without performing statistical analysis of these data.

2.3. Statistical analyses

Statistical analyses were performed in R 4.5.2 (R Core Team, 2025). As the main interest of our study was to evaluate whether females, 'XX males' and 'XY males' differed from each other in the measured features of colouration, first we performed models where the consensus sex reflecting the genotype-phenotype combinations was the only predictor. We used linear models with the 'lm' function for the hue, saturation and brightness variables, with two exceptions. For ventral hue and ventral saturation, homoscedasticity was not met, so we used generalized least squares model by the 'gls' function of the 'nlme' package (Pinheiro & Bates, 2025) to allow for group-specific variances ('varIdent' argument). We analysed the differences between the consensus sex categories in the proportion of 'chin mottling' using beta distribution with logit link using the 'glmmTMB' function of the 'glmmTMB' package (Brooks *et al.*, 2026). Because of heteroscedasticity, we allowed for different variances between sex categories in the latter model too ('dispformula' argument). Additionally, we also performed a sensitivity test for each model to assess whether the differences between sexes were robust enough to persist when further potential predictors were included. As additional predictors, we used body mass and day of arrival to the breeding pond (i.e. the day of the year at photo capturing). Because females are larger than males, and males often arrive much earlier to the breeding pond

than females, to avoid multicollinearity, we centred both predictors by subtracting the mean of the relevant phenotypic sex from the original value of each individual. Because sample collection was not designed to enable assessing the effects of these additional predictors, interactions were not included in the sensitivity tests. For every model, we used the 'emmeans' function of the 'emmeans' package (Lenth & Piaskowski, 2026) to calculate pairwise contrasts between the consensus sex categories, and we applied Benjamini-Hochberg false discovery rate (FDR) correction for the 3 p-values from each model (Pike, 2011). Model diagnostics were performed for all models by the 'simulateResiduals' function of the 'DHARMa' package (Hartig, 2024), whenever this was possible. Because 'DHARMa' is not suitable for the 'gls' models, for those we calculated model residuals and fitted values with the default R functions called 'resid' and 'fitted', respectively, and visualized them by the 'plot' and 'qqnorm' functions for evaluation of model fitting.

We used linear models with the 'lm' function to test whether frog vision enabled differentiation between the consensus sex categories based on either average dorsal or average ventral colouration, respectively. JNDs were estimated from predicted rod catches using a computational low-light visual proxy, and group differences were evaluated using bootstrapped dichromat distance estimates.

The occurrence of the 'level 3' category of the 'red ventral pattern' variable was limited to one consensus sex category, preventing us from finding a well-fitting model. Therefore, we applied Kruskal-Wallis rank sum test using the 'kruskal.test' base function to evaluate whether females, 'XX males' and 'XY males' significantly differed from one another in 'red ventral pattern'. We performed pairwise comparisons of the consensus sex categories by Mann-Whitney U tests with the 'wilcox.test' function, and finally applied FDR correction for the p-values using the 'p.adjust' function ('stats' package; R Core Team, 2025).

In all analyses, we used 95% confidence levels. For illustrative purposes, in the figures we plotted mean estimates with 84% confidence intervals (CI) for each group, because non-overlapping 84% CIs correspond approximately to statistical significance at $p < 0.05$ in pairwise comparisons (Payton *et al.*, 2003).

3. Results

Regarding the average dorsal colouration, the main linear models revealed that females featured significantly more reddish colour (hue), lower saturation, and appeared overall darker (i.e. lower brightness) than both 'XX males' and 'XY males' (Fig 1 A; see detailed results in Supplement 3). 'XX males' did not differ from 'XY males' in any of these variables. The sensitivity tests confirmed that all these differences between females and 'XX males' as well as between females and 'XY males' were robust (remained significant; Supplement 3).

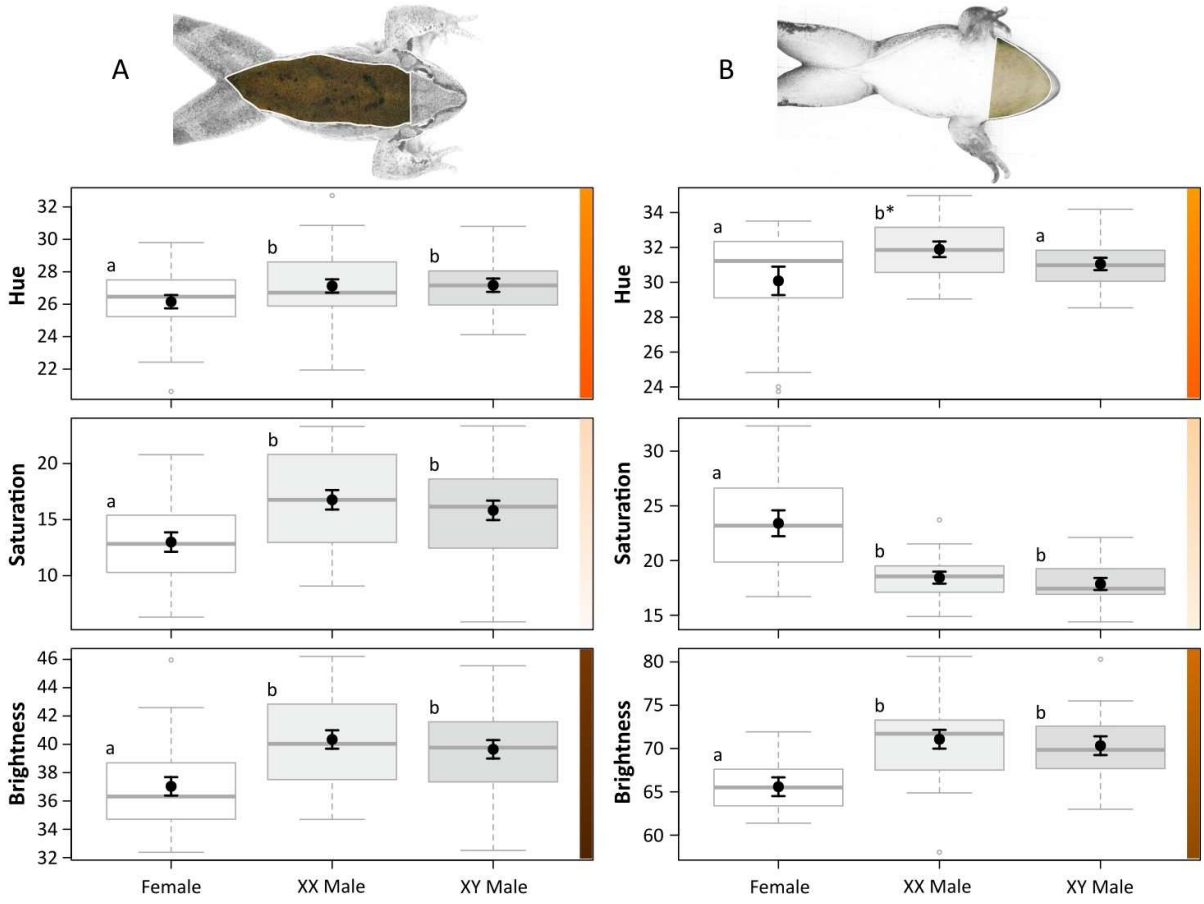

Fig 1. Average dorsal (A) and throat (B) hue, saturation and brightness. Colour bars on the right illustrate human-visible differences along the corresponding Y axis. Letters at the top of the boxes indicate statistically significant differences within each model (groups with different letters differ at $P < 0.05$ after FDR correction). The only exception is the letter marked with asterisk on panel B, where the FDR-corrected P-value for the 'XX M' vs. 'XY M' comparison was marginally nonsignificant ($p = 0.0566$; note that the corresponding p-value was 0.026 in the sensitivity test). Black dots represent model-estimated marginal means, and black error bars indicate 84% confidence intervals. In each boxplot, the median is denoted by the thick grey line, the interquartile range (IQR) is shown by the box, IQR ± 1.5 × IQR is denoted by grey

whiskers, and data points more extreme than these latter values are represented by empty circles.

The average throat colouration in females was significantly darker (lower brightness) and more saturated compared to both ‘XX males’ and ‘XY males’ (Fig 1 B; see detailed results in Supplement 3). The sensitivity tests confirmed that these differences were robust (Supplement 3). The throat of ‘XX males’ had was relatively more yellowish (statistically significant difference in hue) compared to females, and somewhat also compared to ‘XY males’, but the latter was just above the significance threshold (P = 0.0566) after FDR correction. However, this latter difference became significant (p = 0.026) when body mass and arrival date were also considered in the model (Supplement 3).

The vision model suggested that the frog vision system is likely able to detect the average differences in dorsal and ventral colouration between females and males regardless of their genotypes (Supplement 4). By comparison, the divergence between ‘XX males’ and ‘XY males’ was small and likely below the threshold of reliable discrimination (Supplement 4).

The proportion of ‘chin mottling’ was significantly larger in females compared to both ‘XY males’ and ‘XX males’, whereas ‘XX males’ did not differ from ‘XY males’ according to the main model (beta regression with logit link; see Fig 2 and detailed results in Supplement 2). Results of the sensitivity test were qualitatively the same (Supplement 2)

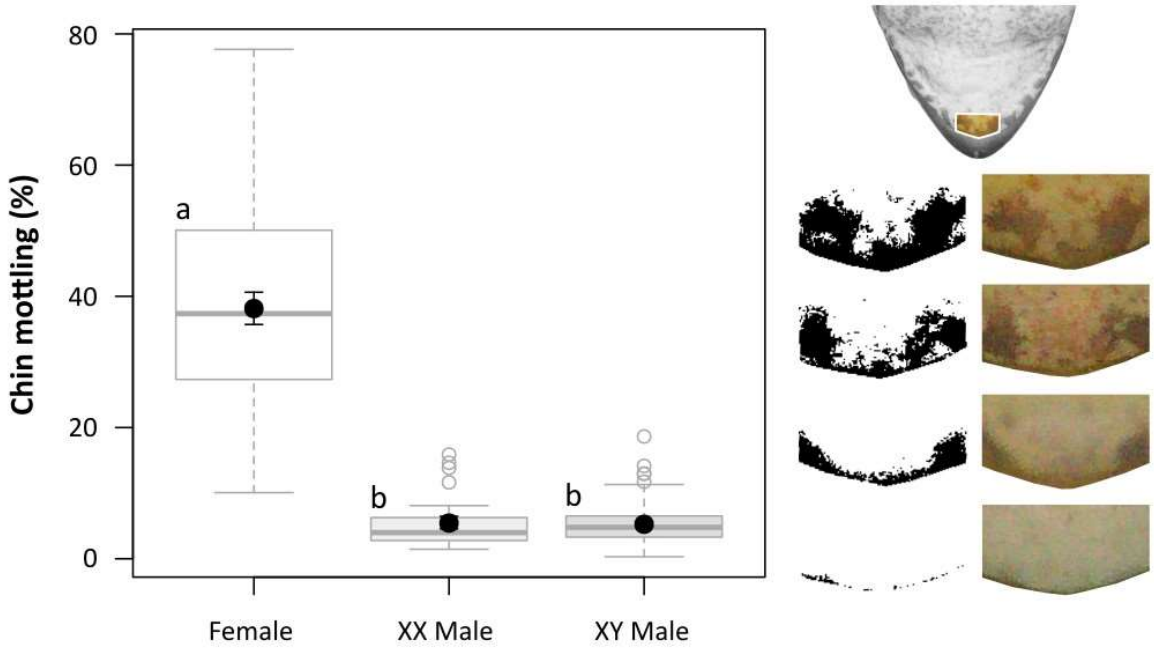

Fig 2. Percentage of dark ventral pigmentation near the frontal middle part of the mouth ('chin mottling'). Example images on the right illustrate the masked view (saved in Fiji) and the original view on frogs with varying 'chin mottling' proportions (from bottom to top: 1.5, 20.3, 40.5 and 59.4%, respectively). The ROI is denoted on the head photo on top. Letters on the top of the boxes indicate statistically significant differences within each plot (groups with different letters differ at $P < 0.05$ after FDR correction). Black dots and error bars, respectively, represent estimated marginal means and 84% confidence intervals, both back-transformed from the logit scale to the original percentage scale. See Fig 1 for the interpretation of box plots.

The Kruskal-Wallis test revealed that females significantly differed from both 'XY males' and 'XX males' in the occurrence of the 'red ventral pattern' while there was no difference between 'XX males' and 'XY males' (Fig 3 A; see detailed results in Supplement 2). The 'level 3' category, representing an unambiguous red patterning, was unique to females and it occurred in 52% of them (Fig 3 A). The subjectively assigned 'red ventral pattern' categories corresponded with differences in objectively measured average throat colouration (see Fig 3 B and the Supplement 2) and coincided with decreasing throat brightness from 'level 0' to 'level 3'. Additionally, the throat of animals with the 'level 3' category featured much higher average saturation, as well as overall more reddish colour compared to all other individuals (Fig 3 B). Animals in the 'level 2' category also tended to have relatively higher saturation (Fig 3 B).

Based on the images obtained from outside the study population (Table S1), the 'red ventral pattern' was present in at least 18 out of the 22 females ('level 3' in 16 individuals, and 'level 2' in two individuals). For the remaining four females, throat visibility did not allow a confident categorization based on the photos. Throat visibility allowed for the categorization of 12 out of the 14 males. Among these, no sign of the 'red ventral pattern' was detected in 11 males ('level 0'), and it was present at limited extent ('level 2') in a single male. The females for which categorization was possible were photographed in seven different countries (Austria, Croatia, France, Greece, Hungary, Italy and Ukraine). The male observations came from five countries (Austria, France, Hungary, Italy and Slovakia). Three photos of females and two photos of males were taken during autumn, while all others were taken during spring, around the breeding season. During autumn, all three females had 'level 3' 'red ventral pattern', and one of the two males was the only male with some 'red ventral pattern' ('level 2') detected in this dataset.

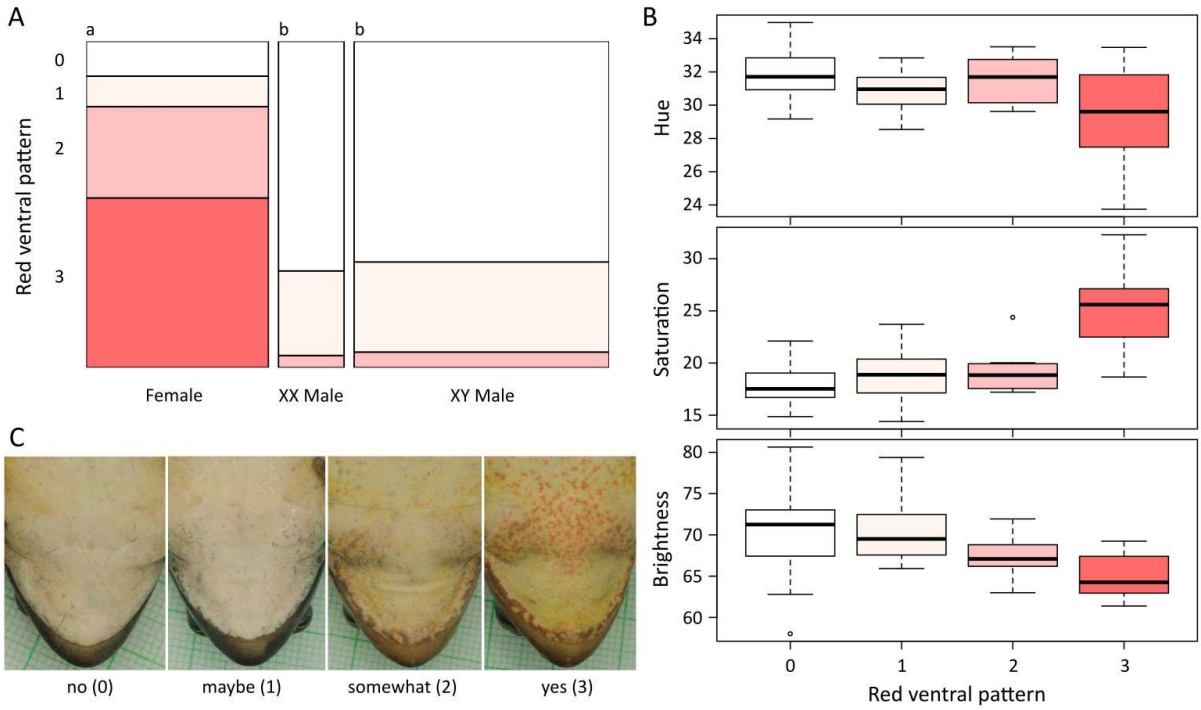

Fig 3. Distribution of the 'red ventral pattern' categories in females, 'XX males' and 'XY males' (A), and the relationships of these ordinal categories with the average colouration of the throat (B). The photos show one example per category (C). Small letters on the top of the mosaic plots (A) indicate statistically significant differences (groups with different letters differ at $P < 0.05$ after FDR correction).

4. Discussion

The agile frog is conventionally assumed to have a white ventral colouration without pigment patterns and features of interest (AmphibiaWeb, 2026). However, our study revealed sexual dichromatism in breeding adults that is visible by the human eye: phenotypic males and females significantly differed in average saturation and brightness of the throat area, the percentage of 'chin mottling' and the presence and extent of 'red ventral pattern'. Furthermore, in line with the frog vision model, males and females appear to be distinguishable from one another on the basis of their average dorsal and average throat colour.

Agile frog males keep territories and use advertisement calls to attract females when breeding density is low (Lesbarrères *et al.*, 2008), but when breeding density is high (which is often the case, including the focal breeding pond of our study), they engage in scramble competition (Hettyey *et al.*, 2014). Female agile frogs can enforce their mate preferences when being

clasped by either conspecific or heterospecific males (Hettyey *et al.*, 2009a; Vági & Hettyey, 2016; Bókony *et al.*, 2025b). First, they can refuse to lay eggs for a long period, while a more suitable partner might replace the male momentarily clasping them. Second, they can lay a subset of their eggs for the unwanted male, encouraging it to end the amplexus, and potentially giving opportunity to a more desirable partner to fertilize the rest of the eggs. It is currently unknown which male features females take into account for mate preference aside from certain call characteristics (Lesbarrères *et al.*, 2008), but colouration is a reasonable candidate, as visual communication can be key for successful breeding in various amphibians (Hirschmann & Hödl, 2006; Bell & Zamudio, 2012; Rojas, 2017; Höbel *et al.*, 2022; Rojas *et al.*, 2023). Despite being fertile, sex-reversed 'XX males' have lower breeding success compared to concordant 'XY males' in the wild, and females tend to lay their eggs in two portions more often when paired with 'XX males' (Bókony *et al.*, 2025b). Based on reports on other frog species, the appearance of the male throat might influence breeding success (Sztatecsny *et al.*, 2010; Höbel *et al.*, 2022), and therefore females might potentially differentiate between males based on throat colouration. The only feature in which 'XX males' appeared to differ from 'XY males' in our dataset was the hue of the throat, that was somewhat skewed towards yellow compared to the relatively redder hue the 'XY males'. However, the visual modelling analysis suggested that this colour difference would not be detectable under the applied computational low-light proxy, indicating that throat coloration is unlikely to explain the reduced mating success of 'XX males' in agile frogs. While ours is the first systematic assessment of the colouration of sex-reversed and concordant individuals in an amphibian, differences in throat colouration among male individuals has been previously speculated to reflect sex reversal in the green frog (*Lithobates clamitans*; Stephenson & Christensen, 2023). This speculation is in accordance with our findings in the agile frog.

Most of the females included in our study featured the 'red ventral pattern' to some extent, while it was largely missing from males. Qualitative comparison of the images of adult agile frogs from a broad geographical range also confirmed that the 'red ventral pattern' is a common feature in this species, and that it occurs predominantly in females. It seems surprising that this human-visible feature was overlooked by herpetologists so far. The simplest explanation is that ventral colouration, as in other amphibians as well, received much less attention compared to dorsal colouration that is far more apparent from the human eye view (Plewnia *et al.*, 2024). Alternatively, it is also possible that researchers who noticed the presence of the 'red ventral pattern' assumed that it was a symptom of a disease instead of a potentially sex-linked feature,

due to its dubious appearance. We did not perform histological analysis and therefore cannot exclude the possibility that the 'red ventral pattern' was caused by skin lesions. However, we are unaware of any infectious amphibian disease that cause symmetrical skin lesions restricted to the frontal half of the ventral surface of the body, or that cause symptoms largely restricted to one sex (Chai, 2015; Pessier, 2018). Colour and patterns on the skin of vertebrates are produced by the combination of different cell types and pigments (Rojas *et al.*, 2023). Red colouration, for instance, can be produced by certain carotenoids, and sometimes even by haemoglobin (Mills & Patterson, 2009). Therefore, taking the currently available data together, the 'red ventral pattern' is most likely a normal feature of agile frog colouration. This pattern is largely missing in adult males, that in turn feature a much brighter throat. Similar sexual dichromatism was reported in common frogs, where breeding males also featured bright whitish throat compared to the dark reddish throat of females (Sztatecsny *et al.*, 2010). This species was observed to engage in heterospecific amplexus with agile frogs in shared breeding ponds, despite the fact that they do not hybridize (Hettyey *et al.*, 2014). Similarly to what was hypothesised for the common frog (Sztatecsny *et al.*, 2010), the bright throat of agile frog males might facilitate sex recognition and decrease the probability that (conspecific) males would mistakenly engage in amplexus with one another. Misdirected amplexus is a widespread phenomenon which can have negative consequences on the fitness of both participants (i.e. missing out on actual breeding opportunities; Mollov *et al.*, 2010; Vági & Hettyey, 2016; Serrano *et al.*, 2022; Brischoux & Lorrain-Soligon, 2024). Furthermore, in rarer cases, amplexus may even lead to the death of the clasped individual (Mollov *et al.*, 2010), although conspecific males normally respond to release calls by stopping the amplexus (Marco & Lizana, 2002). Alternatively, presence or absence of a 'red ventral pattern' might also affect male fitness if female agile frogs rely to some extent on throat colouration for mate preference. In line with the logic of any of the above hypotheses, the lack of 'chin mottling' in males might also increase fitness by enlarging the conspicuously bright surface on the throat. However, we are not aware of studies assessing sex-linked differences in pigmentation along the mouth of amphibians, and on the potential function of such patterns.

In conclusion, colouration may play a role in conspecific communication in the agile frog, and our study took the first step towards exploring this aspect of evolution in an amphibian with thermal sex reversal. We found differences between males and females in several aspects of colouration, and even found subtle difference between sex-reversed and concordant males in average throat hue. However, because this difference is not detectable within the limits of the

applied frog-vision proxy, we believe that throat coloration alone is unlikely to explain the observed limited breeding success of sex-reversed males.

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AI usage

The 'frog chin selection' Fiji plugin was built with the assistance of ChatGPT (version GPT-4) and it was manually corrected for syntax errors. The R code used for vision model extraction was implemented using GPT-5.4-codex.

Supporting information

The dataset, as well as supplementary figures, supplementary information on the methods and results and R codes are available in FigShare at https://doi.org/ 10.6084/m9.figshare.31647649.

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