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Novel genetic sex markers reveal high frequency of sex reversal in wild populations of the agile frog *(Rana dalmatina)* associated with anthropogenic land use

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Abstract

Populations of ectothermic vertebrates are vulnerable to environmental pollution and climate change because certain chemicals and high temperature can cause sex reversal during their larval development (i.e. genetically female individuals develop male phenotype or *vice versa*), which may distort population sex ratios. However, we have troublingly little information on sex reversals in natural populations, due to unavailability of genetic sex markers. Here we developed a genetic sexing method based on sex-linked single nucleotide polymorphism loci to study the prevalence and fitness consequences of sex reversal in agile frogs *(Rana dalmatina).* Out of 125 juveniles raised in laboratory without exposure to sex-reversing stimuli, 6 showed male phenotype but female genotype according to our markers. These individuals exhibited several signs of poor physiological condition, suggesting stress-induced sex reversal and inferior fitness prospects. Among 162 adults from 11 wild populations in North-Central Hungary, 20% of phenotypic males had female genotype according to our markers. These individuals occurred more frequently in areas of anthropogenic land use; this association was attributable to agriculture and less strongly to urban land use. Female-to-male sex-reversed adults had similar body mass as normal males. We recorded no events of male-to-female sex reversal either in the lab or in the wild. These results support recent suspicions that sex reversal is widespread in nature, and suggest that human-induced environmental changes may contribute to its pervasiveness. Furthermore, our findings indicate that sex-reversal is associated with stress and poor health in early life, but sex-reversed individuals surviving to adulthood may participate in breeding.

Keywords: sex reversal, masculinization, urbanization, agricultural habitats, amphibians, genetic sex marker

Introduction

Ectothermic vertebrates are highly vulnerable to climate change and chemical pollution, because several aspects of individual development, including sex differentiation depend on environmental conditions in numerous species of reptiles, amphibians and fish (Bókony, Kövér, Nemesházi, Liker, & Székely, 2017; Bókony et al., 2018; Eggert, 2004; Holleley, Sarre, O'Meally, & Georges, 2016; Orton & Routledge, 2011; Ospina-Álvarez & Piferrer, 2008; Tamschick et al., 2016). In species with genetic sex determination, thermal and chemical disturbances during embryonic or larval development can cause sex reversal, meaning that genetically female individuals become phenotypic males, or *vice versa* (Eggert, 2004; Holleley et al., 2016; Ospina-Álvarez & Piferrer, 2008). Laboratory experiments show that sex-reversed individuals of some species may have reduced reproductive success (Harris et al., 2011; Senior, Nat Lim, & Nakagawa, 2012), and theoretical studies suggest that sex reversals may lead to serious consequences for natural populations, including changes in genetic variability, distorted sex ratios, and even extinction (Bókony et al., 2017; Quinn, Sarre, Ezaz, Marshall Graves, & Georges, 2011; Wedekind, 2017). Therefore, it is imperative to gain information on the prevalence and fitness of sex-reversed individuals in natural populations, to be able to assess and forecast the effects of anthropogenic environmental changes.

For studying sex reversal, one needs to identify not only the phenotypic sex but also the genetic sex of each individual. The latter can be especially difficult in non-model organisms, due to lack of information on sex-linked DNA sequences. Because of their highly conserved sex chromosome system, universal sex-linked DNA markers have long been available for

birds and mammals (Fridolfsson & Ellegren, 1999; Griffiths & Tiwari, 1993; Shaw, Wilson, & White, 2003), making molecular sexing a routine in these taxa. However, in the majority of ectothermic vertebrates, sex chromosome turnover (i.e. the swapping of the chromosome used for genetic sex determination) is common and the sex chromosomes of many species are homomorphic (Devlin & Nagahama, 2002; Holleley et al., 2016; Jeffries et al., 2018; Miura, 2017). Consequently, there is often little homologous sex-linked variation between and sometimes even within species, making molecular sexing challenging (Ezaz, Stiglec, Veyrunes, & Marshall Graves, 2006; Perrin, 2009; Stöck et al., 2013). Furthermore, type of sex-chromosome system (i.e. male or female heterogamety) can differ between closely related species or even between different populations of the same species, especially in amphibians (Holleley et al., 2015; Rodrigues et al., 2017; Sarre, Ezaz, & Georges, 2011).

For the above reasons, genetic sexing methods need to be developed and validated species by species in amphibians. Recombination between the sex chromosomes (Ezaz et al., 2006; Perrin, 2009; Stöck et al., 2013) is expected to be reduced in the vicinity of the 'master sex-determination gene' (Bachtrog, 2006; Bachtrog et al., 2014; van Doorn & Kirkpatrick, 2007), providing a preferential target for sex marker development. Unfortunately, the master sex-determination gene remains elusive in all but a few amphibian species (Eggert, 2004; Miura, 2017; Nakamura, 2013; Yoshimoto et al., 2010), and the size of the non-recombining region around it can be small. Thus, in order to find markers which make reliable identification of the sex chromosomes possible in the species of interest, researchers must test high numbers of loci across the genome (Lambert, Skelly, & Ezaz, 2016; Olmstead, Lindberg-Livingston, & Degitz, 2010; Stöck et al., 2011). Owing to these challenges, reliable sex-linked markers only exist for a handful of amphibian species so far (Alho, Matsuba, & Merilä, 2010; Berset-Brändli, Jaquiéry, Dubey, & Perrin, 2006; Brelsford, Lavanchy, Sermier, Rausch, & Perrin,

2017; Eggert, 2004; Lambert et al., 2016; Ma, Rodrigues, Sermier, Brelsford, & Perrin, 2016; Olmstead et al., 2010; Rodrigues et al., 2017; Stöck et al., 2011).

Due to this general lack of sex markers, we know troublingly little about sex reversals in nature: how widespread they are, which environmental factors they are associated with, and how they affect individual fitness and population viability. To our knowledge, the frequency of sex reversal in the wild has been published for only two amphibian species so far: 9% of genetic females were phenotypically male in a Finnish common frog (*Rana temporaria*) population, while 8.5% female-to-male and 3% male-to-female sex reversal was found in green frogs (*Rana clamitans*) in the USA (Alho et al., 2010; Lambert, Tran, Kilian, Ezaz, & Skelly, 2019).

In this study, we investigated sex reversals in the agile frog *(Rana dalmatina)*. This species is widespread in Europe, but its population sizes show a decreasing tendency (Kaya et al., 2009). It inhabits light deciduous woodlands, but also occurs near or in urbanized areas. Similarly to most *Rana* species, its diploid karyotype consists of 26 chromosomes (Spasić-Bošković, Tanić, Blagojević, & Vujošević, 1997); its sex chromosomes were identified only recently, showing a male-heterogametic (XX/XY) sex-determination system (Jeffries et al., 2018). Because no molecular sexing method has been published for agile frogs yet, first we searched for sex-linked markers using an existing Restriction Site Associated sequencing (RADseq) dataset (Jeffries et al., 2018) and validated them to provide a reliable genetic sexing method for this species. Subsequently, we studied the occurrence of sex reversals in wild agile frog populations in North-Central Hungary, and tested if sex reversals are more common in populations associated with anthropogenic land use. Finally, we examined if sex reversal was associated with fitness costs by comparing fitness-related traits between sex-reversed and normal individuals.

Methods

Sampling and DNA extraction

We captured 162 adult agile frogs (121 males and 41 females) from 11 ponds in North-Central Hungary at the start of the breeding season in February-March in 2016 and 2017 (Table 1, Table S1). The capture sites were chosen to represent the range of habitats the species occupies, on a natural to anthropogenic scale (Table S1). Distances between capture sites varied from 4 to 60 km. Sample size varied between sites due to variation in capture success. The adults were sexed by secondary sexual characteristics (nuptial pads in males) and presence of eggs (gravid females). Buccal swab samples were taken from all wild-caught frogs for DNA extraction. Additionally, toe clip samples were also collected from 10 individuals (5 males and 5 females, from 3 ponds) for the purpose of marker finding and primer design (Table 1). We measured the adult frogs' body mass (± 0.1 g) and released them at their capture sites.

We subsequently tested the sex-linkage of our markers (see below) on 125 froglets (59 males and 66 females; from 34 clutches) collected as freshly spawned eggs in 2018 from three different ponds of the same geographical region (Table 1). These individuals were raised in laboratory under conditions that are unlikely to cause sex reversal, because the animals were not exposed to endocrine-disrupting chemicals or to extreme temperatures or to any other stressor which trigger sex reversal to our knowledge (Castañeda Cortés, Arias Padilla, Langlois, Somoza, & Fernandino, 2019; Eggert, 2004; Lambert, Smylie, Roman, Freidenburg, & Skelly, 2018). Thus, we expected that among these animals sex reversal would be absent or occur very rarely due to sex-chromosome recombination (Ezaz et al., 2006; Perrin, 2009; Stöck et al., 2013) or random processes affecting sex determination (Perrin, 2016). We are

confident that this setup provided the best conditions for ascertaining the baseline level of sex reversal in this species. Water temperature during tadpole development was 18.45 ± 0.81 (mean ± SD); all other details of animal housing and care are described in Bókony et al. (2020). Froglets were phenotypically sexed by gonad anatomy (Figure S3) during dissection 2 months after metamorphosis (ca. 16 weeks after reaching the free-swimming tadpole stage) as described in Bókony et al. (2020). At this age the gonads are well differentiated in this species (Bernabò, Gallo, Sperone, Tripepi, & Brunelli, 2011; Ogielska & Kotusz, 2004). To our knowledge, "sex races" (Rodrigues, Vuille, Loman, & Perrin, 2015) were not reported in agile frogs. From each froglet we took a tissue sample (hind feet) that we stored in 96% ethanol until DNA extraction. During dissection, we recorded several fitness-related traits (see below), and we carefully removed the gonads and fixed them in neutral-buffered 10% formalin (Sigma 1.00496) for histology. All the above procedures were approved by the Ethical Commission of the Plant Protection Institute and carried out according to the permits issued by the Government Agency of Pest County (permit numbers: PE/KTF/3596-6/2016, PE/KTF/3596-7/2016, PE/KTF/3596-8/2016, FPH061/2472-4/2017).

DNA was extracted from toe-clip samples using Geneaid Genomic DNA Extraction Kit (Thermo Fisher Scientific) for animal tissue, following the manufacturer's protocol, except that digestion time was 2 hours and 4 µl RNase was added to each sample before the binding step. From buccal swab samples, DNA was extracted either by QIAamp DNA Investigator Kit (Qiagen) or Geneaid Genomic DNA Extraction Kit (Thermo Fisher Scientific) for animal tissue following the manufacturers' instructions with a few modifications for the latter (1 hour digestion, 30 minutes lysis).

Marker development

We studied putatively sex-linked sequences that were identified by RADseq from a sample of 40 agile frogs from a single clutch in Switzerland (Jeffries et al., 2018). The 92 bp long RAD tags were mapped to a genome assembly of the common frog (unpublished data, D.L. Jeffries), a species closely related to the agile frog (Pyron & Wiens, 2011), using Magic-BLAST 1.3 (Boratyn, Thierry-Mieg, Thierry-Mieg, Busby, & Madden, 2019). We concentrated on those tags that hit to the agile frog's sex chromosomes (Jeffries et al., 2018) uniquely or had an e-value of at least 5 orders of magnitude lower than the next best hit. First, we aimed to check if the putative sex-linked loci carried sex-linked single nucleotide polymorphisms (SNPs) in our study populations as well. Using the NCBI Primer-BLAST tool (Ye et al., 2012), we designed primers for a total of 14 loci based on common frog genome with aim for sequencing agile frog DNA around the sex-linked RAD tags so we could sequence DNA fragments of about 220-1100 bp length. PCRs were performed with these primers on DNA samples of morphologically sexed adult agile frogs from Hungary in the laboratory of the Conservation Genetics Group, Department of Ecology, University of Veterinary Medicine Budapest (for PCR primers used for sequencing and detailed conditions see Table S2; PCR programs are described in Table S3). Clear PCR products in the expected length range were cut and purified from 2% agarose gel, using NucleoSpin Gel and PCR Clean-up kit (Macherey-Nagel), and ran on a 3130xl Genetic Analyzer (Thermo Fisher Scientific) at BIOMI, Gödöllő, Hungary. Sequencer output files were analysed by the STADEN package (Bonfield, Smith, & Staden, 1995; downloaded from https://sourceforge.net/projects/staden/files) and sequences were checked manually. In total, primers designed for 11 loci produced strong PCR products close to the expected fragment sizes which were also suitable for cutting from agarose gel, and 7 of these yielded unambiguous DNA sequences of the target loci (Table S2). Three out of these 7 loci

contained sex-linked SNPs based on sequences from 10 agile frogs (5 males and 5 females) from Hungary (Table S2), and we denominated these Rds1, Rds2 and Rds3, according to their order on the common frog's chromosome 4 (that is corresponding to the agile frog's sex chromosome). Segregation of SNPs at all three loci matched expectations for an XX/XY sex-determination system as found in Jeffries et al. (2018). Because there is no sex chromosome sequence assembly available for the agile frog, we estimated the distances between the sex-linked SNPs based on the corresponding chromosome 4 of the common frog (unpublished data of D. L. Jeffries; reported as chromosome 5 in Jeffries et al. (2018).

We designed sexing primers for these three putatively sex-linked loci, so that for each locus two fragments could be amplified in a single PCR: one fragment amplified from both chromosomes X and Y, and a shorter fragment amplified only from Y (i.e. if a Y-specific SNP was present). The shorter amplicon is part of the X/Y-universal fragment (see Figure S1). Using this method, successful amplification of the X/Y-universal product means that the target locus is amplifiable in the investigated DNA sample (i.e. positive control). If the Y-specific fragment is amplified as well, that proves the presence of the Y-specific SNP (male genotype). We designed primers specific for the Y-SNPs so that the SNP was present at their 3' end. To increase allelic specificity, a mismatching base was artificially introduced at the 3rd position closest to the 3' end of these primers (replacing the original base in the sequence; following Liu et al. (2012). On Rds2 two sex-linked SNPs were situated 11 nucleotides apart, therefore the Y-specific primer binding to both of these SNPs did not require the introduction of any artificial mismatch. PCR conditions for each pool of sexing primers were optimized based on individuals with known DNA sequence at the concerned locus. Specificity of the primers was tested by agarose gel electrophoresis of PCR products from individuals with known DNA sequence. Sexing PCRs were carried out in a final volume of 16 µl containing

1.6 µl DreamTaq green buffer (10x, Thermo Fisher Scientific), 0.65 µl dNTP (2 mM, Thermo Fisher Scientific), primers of varying amount (Table 2), 0.065 µl DreamTaq DNA polymerase (5U/µl, Thermo Fisher Scientific) and 20-100 ng genomic DNA. PCRs were carried out on a Bioer Life ECO gene amplification instrument (TC-96/G/H(b)C). Optimized sexing PCR profiles are described in Table S3.

Because PCR optimization by the above method was insufficient for Rds3, we developed an HRM-based (high-resolution melting) method for sex-linked SNP-identification at this locus in the laboratory of the Ruminant Genome Biology Research Group, NARIC Agricultural Biotechnology Institute, Gödöllő, Hungary. Total HRM reaction volume was 15 µl, containing 3 µl 5x HOT FirePol EvaGreen qPCR Mix Plus (ROX, Solis BioDyne), 1 µl forward and 1 µl reverse primer (10 µM each; Table 2) and 80-100 ng genomic DNA. Reactions were performed in a Roche Light Cycler 96 Instrument (as described in Table S3) and the results were analysed with the Light Cycler 96 v. 1.1.0.1320 software (Roche Diagnostics International LTD). Detailed guidance for HRM-based sexing is available in Figure S2. HRM allows us to differentiate not only between individuals carrying and not carrying Y-SNP but it provides information on further differences between individual genotypes as well (i.e. presence of additional SNPs can be detected: Figure S2). Genotyping with this method was validated by comparing the assumed genotype based on HRM to DNA sequence data of 42 individuals. While PCR-based sexing allowed us to detect the presence or absence of a Y-SNP (Figure S1), the HRM method gave information on the presence of both the Y-SNP and the X-SNP (Figure S2).

Identification of sex reversal

Sex linkage of the 3 markers was tested on 125 laboratory-raised froglets. Individual molecular sexing was performed independently with each marker, and we subsequently checked if the identified genotype matched with the phenotypic sex. For each marker, we calculated the rates of female-to-male and male-to-female sex reversal, i.e. the proportion of phenotypic males among genetic females (XX) and the proportion of phenotypic females among genetic males (XY), respectively, as well as the proportion of sex-reversed individuals within each phenotypic sex. If a marker indicated sex reversal for a laboratory-raised individual, we accepted the result only if a second DNA sample extracted from the other stored foot of that individual gave the same result as the first one (i.e. to avoid false identifications of sex reversals due to human error during the molecular laboratory work). Because the second DNA sample always confirmed the assumptions from the first one, we found these genotypes to be unambiguous. Note that the 125 froglets came from an experiment in which their siblings were exposed to various treatments (Bókony et al., 2020); here we used some of the genetic sex data of those treated siblings to evaluate whether our findings of sex-reversed froglets may have been due to null alleles, sex-chromosome recombination or mutation, or being sired by a sex-reversed parent.

Because the above analyses showed that one of our three markers (Rds2) would not be suitable for sexing in our populations (see Results), we used the other two markers to evaluate two sexing methods. In Method 1, we screened all laboratory-raised individuals for the marker with the highest sex linkage (Rds3) and we accepted an individual to be normal male or female if its Rds3 genotype was in accordance with its phenotypic sex. Those individuals that seemed to be sex-reversed by Rds3 were screened for the marker with the second highest sex linkage (Rds1) as well and were accepted to be sex-reversed only if both markers confirmed sex reversal. In Method 2, all laboratory-raised individuals were screened for both

Rds1 and Rds3 and genotyping was considered to be successful only if both markers gave the same result. In both methods, individuals with discrepant genotyping results were considered to be of unknown genetic sex.

We estimated sex-reversal frequency in the wild-captured adults using Method 1, because this sexing method performed best in the laboratory-raised individuals (see Results). As females are more difficult to find and capture than males, the majority of the investigated adults were males, so we had too few females to provide a reliable estimate of female-to-male sex-reversal rate in adults. Therefore, we report the proportion of sex-reversed individuals (XX males) among the phenotypic males (hereafter referred to as XX/male ratio) as a measure of female-to-male sex-reversal frequency.

Phenotypic correlates of sex reversal

In the laboratory-raised froglets, we compared the following indices of health and fitness between sex-reversed individuals (XX males) and normal individuals (XY males and XX females): duration of larval development, body mass at metamorphosis and at dissection, size of the fat bodies, size and pigmentation of the spleen, and the mean size of the two testes. We also recorded any abnormality observed during dissection. A detailed description of the biological relevance of these traits, the methods of their measurement and statistical analysis is available in the Supporting Information (pages 11-17).

Histological analysis of the sex-reversed froglets was performed to examine if sex reversal was accompanied by intersex, a condition where both male and female tissue elements are present in the gonads (Lambert et al., 2019). Our preliminary study showed that sex categorized by gonadal anatomy matched sex categorized by histology in 100% of 32 agile frogs (17 males, 15 females) that had been raised without any chemical treatment in 2016,

using the same lab protocol as in 2018. Therefore, to minimize the costs of histological analysis, we chose to analyze gonad histology only in those lab-raised froglets from 2018 for which the identified genetic sex did not match the phenotypic sex categorized by gonad anatomy (i.e. to check if the mismatch was due to erroneous categorization of phenotypic sex). For histology, the gonads were placed in embedding cassettes and dehydrated through graded ethanol, cleared in xylene and infiltrated with paraffin wax in an Excelsior ES Tissue Processor (Thermo Fisher Scientific). Processed gonads were embedded in paraffin, sectioned into 3-4 μm longitudinal slices using a Reichert type microtome, stained with haematoxylin and eosin, and mounted on glass slides. The slides were examined and photographed using a Nikon Eclipse E600 microscope equipped with a QImaging MicroPublisher 3.3 RTV camera. For each individual, 5-6 sections were examined; ovaries were recognized by the presence of ovarian cavities, early meiotic oocytes and/or diplotenes, and testes by spermatogonia, spermatocytes and/or seminiferous cords or tubules (Figure S3).

In the adult frogs, we compared body mass between sex-reversed individuals (XX males) and normal (XY) males using a linear mixed-effects (LME) model with capture site as a random factor. Because most of the captured females were gravid, we did not include them in the analysis of adult body mass. All statistical analyses were run in R 3.5.2. environment (R Core Team, 2019), using the *nlme* package for mixed models (Pinheiro & Bates, 2019).

Human land use and sex reversal

We quantified land use in a 500-m wide belt zone around each pond using geoinformatics software as described in detail in (Bókony et al., 2018). We divided each belt zone into the following 8 land-use categories: natural vegetation (e.g. woodlands, non-agricultural meadows), arable fields, pastures, residential areas, public built areas (e.g. commercial and

industrial areas), roads with vehicular traffic, railroads, and water; and we calculated the proportion of area falling into each of these categories (Table S1). Because railroads and water were present only around 2 and 3 ponds, respectively, and covered very small areas (Table S1), we omitted these from further analyses. We used two alternative approaches to quantify the intensity of anthropogenic land use for each capture site. First, we summed the proportions of arable land, pastures, residential and public built-up areas, and roads for each pond; we will refer to this variable as "total anthropogenic land cover". In the second approach, we performed a principal components analysis (PCA) using the 6 landscape variables, which yielded two axes with >1 eigenvalue, explaining 82.1% of variation in total. Urban landscape areas loaded positively on the first axis whereas agricultural landscape areas loaded positively on the second axis (Table S4, Figure S4). We will refer to the habitat scores along these two axes as "urban PC scores" and "agricultural PC scores". We analysed the relationship between these habitat variables and XX/male ratio of the adult frogs in generalized linear models with binomial error distribution, using the *brglm* function in R package *brglm* (Kosmidis, 2019). This analysis weights each site by sample size (the number of phenotypic males in our case) and appropriately handles separation (i.e. in our dataset, there were no sex-reversed adults at certain sites) by the maximum penalized likelihood method. One model contained "total anthropogenic land cover" as the only predictor, whereas the other model contained the two PCA score variables simultaneously.

Results

Novel sex markers

Marker development yielded 3 agile frog loci with sex-linked SNPs in 10 individuals (Table 2). Rds1 and Rds3 contained one sex-linked SNP each, while Rds2 contained 3 sex-linked

SNPs. Accordingly, Y-SNPs were present in all 5 males at each locus, and absent from all 5 females at Rds3, but at Rds1 and Rds2, they were also present in one female each (different individuals). X-SNPs were present in all 5 females and 5 males at Rds1, and were absent from two males at Rds2 and one male at Rds3. Based on common frog genome data, SNPs of Rds1 and Rds3 are located more than 112 million nucleotides away from each other (positions 199806348 and 312650318, respectively), and even the distance between SNPs of the two closest markers Rds2 and Rds3 is more than 6 million nucleotides (Rds2 SNP position closest to Rds3 is 306051765).

All of the 125 laboratory-raised froglets were successfully genotyped with all three markers. The strongest sex-linkage was shown by Rds3 (95% match between phenotypic sex and genotype at the locus), followed by Rds1 (89% match) and finally Rds2 (70% match) (Table 3). Because we had not exposed the laboratory-raised froglets to sex-reversing effects, we concluded that Rds2 is not suitable for genetic sexing in our populations, as the 30% mismatch rate is much higher than the sex-reversal rates reported from natural populations of other species (Alho et al., 2010; Lambert et al., 2019). When we used Method 1 for identifying sex reversals based on Rds3 and Rds1, six out of the 125 froglets qualified as sex-reversed (all XX males), yielding a female-to-male sex-reversal rate of 8%, and an XX/male ratio of 10% (Table 1). Four out of these 6 sex-reversed animals had both XX and XY siblings (making it unlikely that they were identified as XX due to the presence of null alleles or as an outcome of recombination or mutation, i.e. X-SNPs on Y), whereas two of them came from a family in which we found only XX individuals (N=12), suggesting that the latter might have been fathered by an XX male. Due to discrepancies between Rds1 and Rds3 (Table 3), Method 2 failed to assign genetic sex to 8 individuals (6.4% of all froglets). These failures resulted in slightly higher estimates of both the female-to-male sex-reversal rate (9%)

and the XX/male ratio (11%) for Method 2. These discrepancies occurred in 4 families from 3 ponds (not in the same families that contained sex-reversed individuals), the discrepant genotypes being XY with Rds1 and XX with Rds3 in all but one family, with the Rds3 genotype being always concordant with the phenotype. Because Rds3 showed the highest sex-linkage, and Rds1 results matched the Rds3 results for all putative sex-reversed individuals based on Rds3, we concluded that Method 1 allows reliable genetic sexing while also keeping sex-identification failures at minimum, whereas cases where phenotypic sex matches Rds3 but not Rds1 are likely to result from recombination rather than from sex reversal.

Sex reversal in nature

Out of 162 wild-caught adults, 152 were genotyped unambiguously (Table 1): using Method 1 we identified 89 normal males (XY), 41 normal females (XX), and 22 sex-reversed XX males, but no male-to-female sex reversals (Table 1). The overall XX/male ratio was 20% across wild populations, being two times higher than in the laboratory-raised animals (Table 1). The geographical distribution of sex reversals across capture sites is shown in Figure S5.

Among the wild-caught adults, XX/male ratio increased significantly with total anthropogenic land cover (Figure 1, Table 4). Similarly, XX/male ratio increased significantly with higher "agricultural PC scores", and it showed a marginally non-significant positive relationship with "urban PC scores" (Figure 1, Table 4). Notably, sex reversals occurred even at the least anthropogenic sites (Figure 1), and XX/male ratio increased on average from 12.8% to 29.3% as total anthropogenic land cover increased from zero to 50% (Table 4).

Fitness correlates of sex reversal

Among the wild-caught adults, XX sex-reversed males had similar body mass as normal males (LME, slope ± SE = 1.47 ± 1.29, t_{98} = 1.15, p = 0.254; Figure 2). However, among the

lab-raised animals we found several signs of reduced fitness and/or increased physiological stress in the XX sex-reversed males, including reduced body mass, increased spleen size, and liver abnormalities (see details in Supporting Information, pages 11-12, Table S5, Figures S6-7). In 2 out of the 6 individuals that were genetically female but had testes with normal anatomy, histological analysis revealed oogonia in otherwise normal testicular tissue (Figure S3), in contrast to the 17 males dissected in 2016 that all had testes without oogonia. These two individuals had small testes relative to their body size and age (Figure S8). The remaining 4 sex-reversed individuals showed completely normal testicular histology in the examined sections (Figure S3). In those two individuals that had XX siblings only (possibly sired by an XX male; see above), testis size was large relative to their body size and age (Figure S8), and testis histology showed a more mature developmental stage than in the rest of the histologically examined individuals (Figure S3).

Discussion

We identified three loci carrying sex-linked SNPs in agile frog populations in Hungary. Based on a genome sequence assembly of a closely related species, the common frog, we assume that the sex markers reported here cover a suitably large region of the sex chromosomes (Rds1 and Rds3 being at 112 million nucleotides from each other). Furthermore, genetic sex based on Rds3 corresponded to the sexual phenotype in 95% of all laboratory-raised individuals, and all discrepancies were found to be likely cases of sex reversal (as discussed in more detail below). Therefore, we conclude that parallel usage of the best performing markers Rds1 and Rds3 is suitable for molecular sexing in the North-Central Hungarian populations, yielding at least 95% confidence for individual sexing (allowing for the possibility that the

5% mismatch between Rds3 genotype and phenotypic sex had been caused by recombination; Ezaz et al., 2006; Perrin, 2009; Stöck et al., 2013) and good statistical power for comparing populations or experimental groups. Because amphibian sex determination can vary even within species (Miura, 2017; Rodrigues, Merilä, Patrelle, & Perrin, 2014), the reliability of our sex markers should be tested before applying them in other, especially distant populations (Lambert et al., 2016; Rodrigues et al., 2014). However, genetic diversity of the agile frog is in general very low across Europe (Vences et al., 2013), suggesting that our markers may be sex-linked in other agile frog populations as well. Thus, our genetic sexing method enables further studies on environment-induced sex reversal in this declining species, potentially throughout its distribution range.

According to our markers, 6 out of 125 laboratory-raised froglets were genetically females (XX) with male phenotype (testes), despite being raised under controlled conditions with presumably no sex-reversing effects. There are several potential explanations to consider for these mismatches. First, phenotypic sex might have been erroneously categorized; however, we can exclude this possibility because the phenotype based on gonad morphology was corroborated by histology in the mismatching individuals. Second, the presence of sex races could result in false assumption of sex reversal; for example, in the common frog, some individuals develop ovaries first that turn to testes later Rodrigues et al. (2015). This would cause overestimation of the proportion of XY females, which we did not find in our study at all. Third, the mismatches may have been due to recombination (Ezaz et al., 2006; Perrin, 2009; Stöck et al., 2013); however, 4 out of the 6 concerned froglets had XY siblings in our sample, suggesting that both Rds3 and Rds1 genotypes of chromosome Y were normal in their families. Furthermore, all mismatching individuals showed some signs of poor condition, and we are not aware of any reason why recombination would be associated with

the deficits we detected. The fourth interpretation is that the mismatching individuals were indeed sex-reversed, which we consider most likely. Recent studies suggest that sex reversal may be a natural phenomenon in ectothermic vertebrates (Holleley et al., 2016; Lambert, 2015; Lambert et al., 2019), due to dosage-dependent sex determination where stochastic variation in gene expression levels may lead to sex reversal (Perrin, 2016). Alternatively, but not mutually exclusively, sex reversals may result not only from random variation but also from stressful stimuli, as experiments with fishes showed that various forms of physiological stress can induce sex reversal, and "stress hormones" (activated by the hypothalamus-pituitary-interrenal glands axis) mediate this process (Castañeda Cortés et al., 2019; Fernandino, Hattori, Moreno Acosta, Strüssmann, & Somoza, 2013). Therefore, we suspect that a few of our lab-raised animals experienced relatively high levels of physiological stress despite the generally favourable lab conditions, and this led to sex reversal. Their developmental abnormalities may have been either the cause or the consequence of the stress that ultimately caused their sex reversal; in either case, our findings suggest that sex reversal can be associated with reduced health and poor fitness prospects. For example, enlarged spleen may indicate infections (Hadidi, Glenney, Welch, Silverstein, & Wiens, 2008), and small body mass predicts low chances of surviving the winter hibernation (Üveges et al., 2016) and low future reproductive success (Reading & Clarke, 1995; Vági & Hettyey, 2016). Despite the above findings suggesting that sex-reversed individuals might have poor viability in nature, we found a relatively high number of sex-reversed adults in free-living agile frog populations. Genetically XX phenotypic males made up ca. 20% of phenotypic males, and ca. 35% of genetic females, although the latter rate of female-to-male sex reversal is probably overestimated because we had relatively low capture success (small sample size) for females. These numbers are relatively high compared to those reported for natural populations of two

other frog species (Alho et al., 2010; Lambert et al., 2019). Interestingly, we found no difference in body mass between sex-reversed and normal adult males, despite the fact that some of the sex-reversed juveniles in the lab had seriously reduced body mass. This suggests that those sex-reversed individuals that survive to adulthood in nature may be able to mate, because male body size influences success in competition for mates (Vági & Hettyey, 2016). Their reproduction might still fail, however, if sex reversal reduces fertility, as reported in fish (Senior et al., 2012) and indicated by some of our findings with the lab-raised froglets, i.e. three sex-reversed juveniles had small testes and two of them had testicular oogonia (intersex). However, other findings of our study suggest that at least some of the sex-reversed individuals may be fertile. First, four out of six sex-reversed froglets showed normal testicular histology, and three of them had relatively large testes. Second, we found one family that was likely to be sired by an XX male: 12 laboratory-raised animals that were randomly chosen as eggs from a single clutch were all XX individuals, which would have a very low chance of happening merely by accidental sampling if the clutch had the theoretically expected 1:1 sex ratio (ca. 0.0002 probability). Sex-reversed individuals were found to be fertile in some ectothermic vertebrates (Devlin & Nagahama, 2002; Edmunds, McCarthy, & Ramsdell, 2000; Holleley et al., 2015), and in common frogs XX males appear to be fertile and as successful in mating as XY males (Alho et al., 2010; Veltsos et al., 2019). If sex-reversed individuals do reproduce in nature, the biased sex ratios of their progeny may lead to changes in the population sex ratio, sex-chromosome frequencies, and ultimately the sex-determination system (Bókony et al., 2017; Quinn et al., 2011; Wedekind, 2017). Furthermore, the offspring of sex-reversed individuals may themselves be more susceptible to sex reversal, as suggested by empirical results from lab experiments (Holleley et al., 2015; Shao et al., 2014).

We found higher female-to-male sex-reversal frequency in breeding populations exposed to anthropogenic land use. However, due to the availability of agile frog populations, our capture sites with different levels of anthropogenic land use were unequally distributed such that most sites West/South of the river Danube had little anthropogenic influence whereas most sites East/North of the Danube were highly anthropogenic (Figure S5). Also, the lab-raised animals that we used for validating the markers originated from three western populations (Table 1). Therefore we cannot exclude the possibility that the differences we observed in genotype-phenotype mismatches among the free-living populations were due to phylogenetic correlation, i.e. an inherited tendency for more frequent sex reversal in populations East/North of the Danube, or different patterns of linkage disequilibrium between our markers and the master sex-determination gene in these populations (e.g. higher recombination rate in the eastern populations). For example, in common frogs, sex-chromosome differentiation in Switzerland is mainly explained by a major alpine ridge separating the populations (Phillips, Rodrigues, Jansen van Rensburg, & Perrin, 2020). Alternatively, a mutation on chromosome Y at Rds3 (e.g. at the primer binding site, resulting in null allele) or a more complicated sex-determination system present on the East side could cause spatial genetic population structure (Oike et al., 2017; Rodrigues et al., 2014). These alternative explanations could be ruled out using phylogeographic information based on neutral autosomal loci in our populations, or by sexing lab-raised froglets from the eastern populations as well, but unfortunately such data are not available. However, in the region we studied, there are no high mountains or other likely geographical barriers to gene flow between these populations, because rivers like the Danube are not expected to be significant barriers for migration in species like the agile frog (Decout, Manel, Miaud, & Luque, 2012). Further, our study sites lie relatively close to each other, mostly within ca. 40 km; genetic structure at such small spatial scale in agile frogs is more

likely the outcome of habitat fragmentation than isolation by distance (Lesbarrères et al. 2006, Sarasola-Puente et al. 2012). Highways pose migration barriers for agile frogs (Lesbarrères et al. 2006, Sarasola-Puente et al. 2012); however, the distribution of main roads and highways between our study sites is more likely to reduce migration along a North-South cline than separating East from West Figure S5). Taken together, we have little reason to expect an East-West population differentiation in our study. Our only population East/North from the Danube that had low anthropogenic land cover (pond "B" in Figure S5) had high frequency of XX males; however, this pond was created from a closed quarry and was subjected to reconstruction works about a decade ago. In our study of chemical pollutants in anuran habitats in 2017, we found the highest concentration of phthalates in this latter pond (Bókony et al., 2018). Therefore, we believe that our results reflect a genuine effect of anthropogenic environmental change on sex-reversal frequencies.

Our results suggest that both urbanization and agriculture may contribute to the observed relationship between sex-reversal frequency and anthropogenic land use. Both kinds of anthropogenic habitats are polluted by various chemicals, many of which have demonstrated sex-reversing effects (Eggert, 2004; Hayes et al., 2002; Kloas, Lutz, & Einspanier, 1999; Nakamura, 2013; Reeder et al., 1998; Tamschick et al., 2016). Our result that sex reversal occurred even in the least anthropogenic habitats concurs with our earlier finding that those habitats are not devoid of chemical pollutants either (Bókony et al., 2018; see also Figure S5). Furthermore, the increased female-to-male sex-reversal rate that we found in urban agile frog populations may as well be due to the urban heat island effect which makes urban ponds warmer than rural ponds (Brans, Engelen, Souffreau, & De Meester, 2018), given that high temperature during larval development is a known inducer of sex reversal (Bókony et al., 2017; Chardard, Penrad-Mobayed, Chesnel, Pieau, & Dournon, 2004; Lambert et al., 2018).

This variety of chemical, thermal, and potentially other stressors might complicate the relationship between sex-reversal rate and anthropogenic land use. In line with this, no correlation was found between sex-reversal frequency and urbanization along a forest-suburban gradient in green frogs (*Rana clamitans*; Lambert et al., 2019), although the frequency of testicular oocytes was found to increase with urban land cover (Skelly, Bolden, & Dion, 2010). Similarly, several but not all studies found a positive association between agricultural land use and amphibian intersex (Orton & Tyler, 2015), laryngeal demasculinization (Zlotnik, Gridi-Papp, & Bernal, 2019) and reduced spermatogenesis (McCoy, Amato, Guillette, & St. Mary, 2017). These reports together with our results emphasise the need for further studies on sex-reversal frequency and its causes in wild populations of vertebrates with environmentally susceptible sex determination. Adult sex ratio has shifted towards males over the last decades in some amphibian species (Bókony et al., 2017), and this skew might be a consequence of sex reversals becoming more common due to anthropogenic environmental changes of land use and climate. The high frequency of female-to-male sex-reversal we found in this study suggests male-biased sex ratios and consequently reduced effective population sizes that might especially affect populations living in anthropogenic environments. Thus, we urgently need data on the survival and reproduction of sex-reversed individuals and their demographic effects on natural populations; developing novel sex markers for non-model species will be a key step in this endeavour.

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Data Accessibility

Genotype sequences of all 7 loci that we checked for sex-linked SNPs are available in the GenBank (NCBI, accession numbers: MT358846-MT358866). Individual genotype and measurement data are available on FigShare at https://doi.org/10.6084/m9.figshare.12776216

Author contributions

EN and VB designed the study, performed all statistical analyses, and drafted the manuscript. Adult frogs were captured and morphologically sexed, and DNA samples were taken from them by VV, BÜ, and VB. Eggs were collected by VB, BÜ, NU, VV and EN. Froglets in the laboratory were raised by VB, ZM, NU, EN and VV. Dissection and phenotypic sexing was performed by NU and VV, and histological samples were prepared and analysed by KKL. DNA extractions were performed by EN. Putatively sex-linked sequences were identified by DLJ, out of which loci were chosen by EN and VB for further analyses based on genome data provided by DLJ. PCR primers were designed, PCR conditions were optimized and PCR-based sexing was performed by EN, while HRM primers were designed and HRM conditions were optimized by OIH and ZG, and HRM-based sexing was performed by ZG. Genotype

analyses were performed by EN, and geoinformatics analyses were performed by NU. All authors proofread the manuscript, EN, VB, DLJ and BÜ revised the first draft, and all authors gave final approval for publication.

Table 1. Sample sizes (N) and results of molecular sexing by Method 1 across laboratory-raised and wild-caught agile frogs.

Dataset	Site	Anthropogenic cover	N	XX female	XY male	XX male	?? male	XX/ male ratio
Lab-raised	Kerek-tó [a]	0.155	30	13	16	1	0	0.06
	Pilisvörösvár [a]	0.719	25	15	9	1	0	0.1
	Szárazfarkas [b]	0.012	70	38	28	4	0	0.13
	Total		125	*66*	*53*	*6*	*0*	*0.10*
Wild-caught	Bajdázó	0.046	30	10	14	6	0	0.3
	Erzsébet-ér	0.627	17	1	5	6	5	0.55
	Garancsi-tó [c]	0.141	12	8	3	1	0	0.25
	Göd	0.517	22	5	8	5	4	0.38
	János-tó	0.012	13	1	11	1	0	0.08
	Kerek-tó	0.155	1	0	1	0	0	0
	Merzse-mocsár	0.420	7	3	1	2	1	0.67
	Nagykovácsi-tó [c]	0.524	5	3	2	0	0	0
	Pilisvörösvár	0.719	8	0	8	0	0	0
	Pisztrángos [c]	0.060	13	0	12	1	0	0.08
	Szárazfarkas	0.012	34	10	24	0	0	0
	Total		*162*	*41*	*89*	*22*	*10*	*0.20*

Anthropogenic cover: proportion of anthropogenic areas within a 500-m wide belt zone around the pond. For further land-use data and geographical coordinates, see Table S1.
?? male: number of phenotypic males for which genetic sex could not be identified. Out of these, 2 were XX based on Rds3 and XY based on Rds1, while Rds1 genotyping failed in the other 8 individuals (all were XX based on Rds3).
[a] N=8 families from each site.
[b] N=18 families; sex-reversed individuals were found in 2 families.
[c] In total, 5 males and 5 females captured at these 3 sites were used for marker finding.

Table 2. Sex markers and sexing primers.

Locus	Accession number	Primer	Primer sequence	µl in mix [a]	T (°C)	PCR ID	X/Y (bp)	Y (bp)
Rds1	MT358850-MT358851	Rds1-F	F: GACAGGATAGATATGTAAATAGTAGC	1.3	65-63 TD	sex PCR 1	207	97
		Rds1-R	R: GATACCCTGGCCTGAATTTCC	0.1				
		Rds1-Y-R	R: GGCCTGGTTAGTTGGTAT**G**T**A**	2.5				
Rds2	MT358852-MT358853	Rds2-F	F: CGACCCCCAGGTTAAGAATCA	1.3	70	sex PCR 2	507	341
		Rds2-R	R: CCGGTGCATGAGTCTATCCC	0.6				
		Rds2-Y-R	R: AGCGGGC**A**GCACTAACTT**G**T	0.7				
Rds3 [b]	MT358846-MT358849	Rds3-F	F: TGGTTGTAACATGACAAAATGTGGA	0.2	70-65 TD	sex PCR 1	218	166
		Rds3-Y-F	F: CAAGGCACTGTACCTG**G**T**T**	2				
		Rds3-R	R: GTCCATGTCAATGGATGCTGC	1.5				
		Rds3-HRM-F	F: AAAGTTCTAGGGGTATGAATACTTTTT	1	62	sex HRM	99	-
		Rds3-HRM-R	R: GGGACCCCAGAAGTAGAGTATTG	1				

[a] Concentration of each primer was 10 µM. PCR-based sexing (Rds1 and Rds2) was carried out in a total volume of 16 µl, while HRM (Rds3) was carried out in 15 µl reaction mixture.
[b] PCR-based sexing of Rds3 performed best under the conditions shown here. Binding of the Y-primer was SNP-specific, but band intensities on agarose gel were often insufficient (i.e. neither the X/Y universal nor the Y-specific products were detectable in many cases), therefore we used the HRM method instead.
Primer: primer names follow the logic shown in Figure S1, where F means universal forward, R means universal reverse and Y-F and Y-R means Y-specific forward and reverse primers, respectively.
PCR ID: PCR programs are described in Table S3.
Y-SNPs are denoted with bold underlined letters and artificial mismatches (Liu et al., 2012) are bold.

Table 3. The number of phenotype-genotype combinations found across 125 laboratory-raised agile frogs by each marker and relative frequencies of mismatches between phenotypic and genetic sex based on each locus.

Locus	XX female	XY male	XY female	XX male	XY/ female	XX/ male	MF rate	FM rate	Sex reversal
Rds1	60	51	6	8	0.09	0.14	0.11	0.12	0.11
Rds2	30	58	36	1	0.55	0.02	0.38	0.03	0.30
Rds3	66	53	0	6	0.00	0.10	0.00	0.08	0.05

XY/female: proportion of XY genotypes among phenotypic females (XY-female ratio).
XX/male: proportion of XX genotypes among phenotypic males (XX-male ratio).
MF rate: male-to-female sex-reversal rate calculated as the number of females among genetic males (XY).
FM rate: female-to-male sex-reversal rate calculated as the number of males among genetic females (XX).
Sex reversal: overall proportion of individuals with mismatch between their phenotypic and genetic sex.

Table 4. Parameter estimates (*b*) of the binomial models relating the proportion of XX males in all males to the land use of the capture site.

Model	Parameters	*b*	SE	z	p
Model 1	intercept	-1.917	0.357	-5.365	<0.001
	total anthropogenic land cover	2.076	0.856	2.424	0.015
Model 2	intercept	-1.144	0.273	-4.195	<0.001
	urban PC	0.212	0.121	1.756	0.079
	agricultural PC	0.634	0.292	2.175	0.030

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

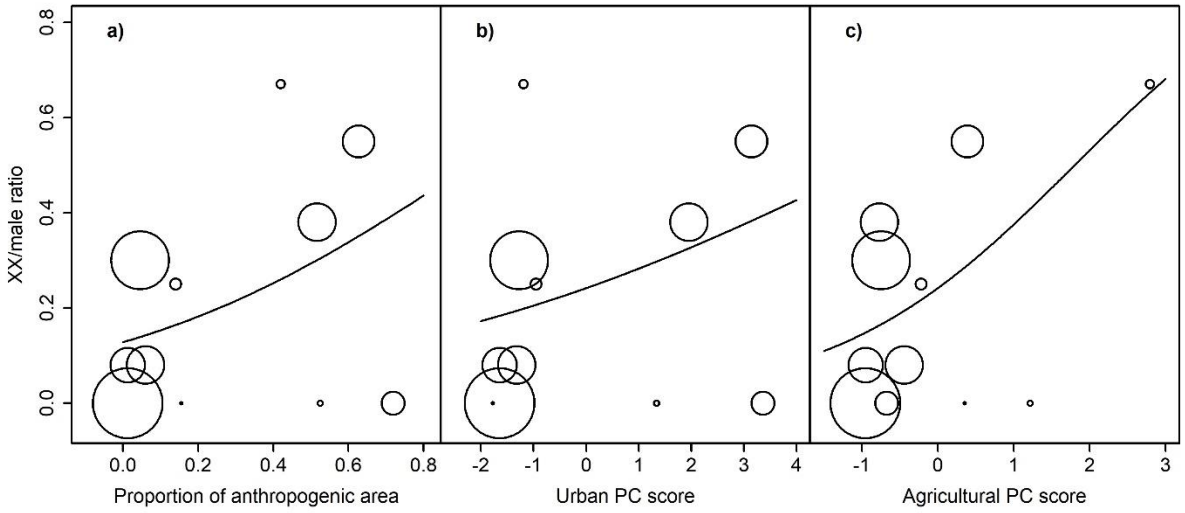


Figure 1. Relationship between the XX/male ratio and human land use across 11 breeding ponds. The curves show the probabilities that a phenotypic male sampled in a breeding pond is genetically female, in relation to the proportion of anthropogenic area (a), the urban PC score (b), and the agricultural PC score (c), as estimated from the models in Table 4.

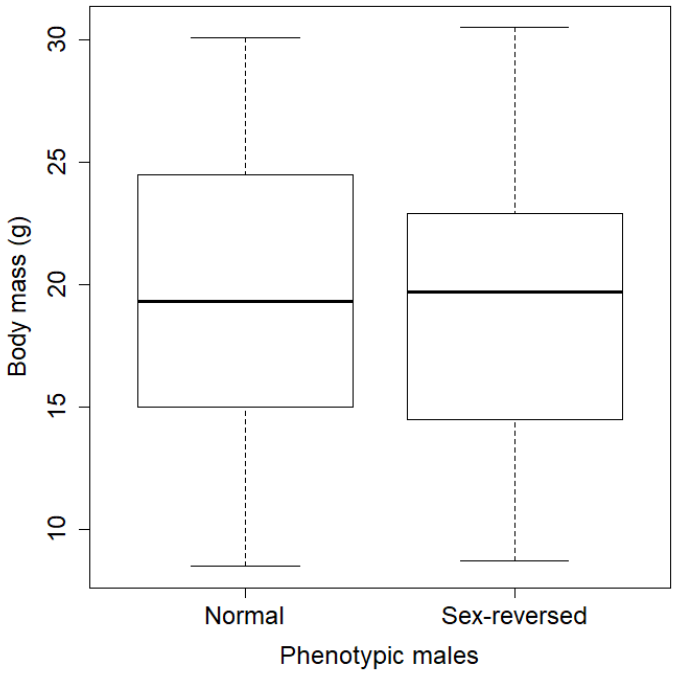


Figure 2. Body mass of normal XY males (N=89) and sex-reversed XX males (N=21) among wild-caught adults. In each box plot, the thick middle line and the box show the median and interquartile range, respectively; whiskers extend to the minimum and maximum.

Supporting Information

Table of contents:

I. Identification of sex reversal and assessing its relationships with human land use

Table S1. Sampling locations and land-use variables (proportion of land cover in a 500-m wide belt around each pond).

Pond	Abbrev.	Latitude	Longitude	Arable field	Pastures	Natural vegetation	Residential built-up	Roads	Public built-up	Railways	Water
Bajdázó	**B**	47°54'12.87"N	18°58'41.47"E	0	0.022	0.970	0	0.024	0	0	0.001
Erzsébet-ér	**E**	47°25'43.65"N	19°8'3.61"E	0.015	0.102	0.370	0.324	0.063	0.124	0	0.003
Garancsi-tó	**Ga**	47°37'25.38"N	18°48'26.18"E	0.002	0.056	0.859	0.066	0.015	0.001	0	0
Göd	**Gö**	47°41’5.16"N	19°7'48.5"E	0	0	0.248	0.431	0.053	0.033	0.011	0.225
János-tó	**J**	47°42'50.04"N	19°1'10.43"E	0	0	0.987	0	0.012	0	0	0
Kerek-tó	**K**	47°38'41.22"N	18°46'31.59"E	0.150	0	0.845	0	0.005	0	0	0
Merzse-mocsár	**M**	47°26'44.5"N	19°17'0.7"E	0.341	0.068	0.584	0	0.011	0	0	0
Nagykovácsi	**N**	47°34'34.72"N	18°52'8.06"E	0.025	0.156	0.476	0.287	0.039	0.018	0	0
Pilisvörösvár	**Pv**	47°36'40.02"N	18°55'9.45"E	0.004	0.024	0.270	0.531	0.077	0.083	0.014	0
Pisztrángos	**Pt**	47°46'0.79"N	18°58'53.25"E	0	0.042	0.940	0.004	0.015	0	0	0
Szárazfarkas	**Sz**	47°44'4.12"N	18°49'7.04"E	0	0	0.988	0	0.012	0	0	0

Abbrev.: Abbreviations for the studied ponds used in Fig. S5.

Table S2. Putative sex-linked PCR targets successfully sequenced in agile frogs with primers designed based on common frog sequences

Locus	Primer name	Primer sequence	Annealing (°C)	Amplicon (bp)	Sex-linked SNP (M, F)	PCR ID [a]
Rds1	Rd56-1F *	TGCACAAAGGGACTCCTAAACA	66	273	yes (5, 5)	seq PCR 1
	Rd56-1R	TGCCTCAGAGTGGCTGGATA				
Rds2 [b]	Rd524-3 F	TTCTAGTGCCGTGACCCCTT	59	834		seq PCR 1
	Rd524-3 R	CCTGCCTCTGCTAAGCCATTC				
	Rd524-4 F *	GATCAAGTGACCCCTGGCAA	65-53 TD	431	yes (5, 5)	seq PCR 2
	Rd524-3 R	CCTGCCTCTGCTAAGCCATTC				
Rds3 [b]	Rd524-1 F	GCCACTCTTCCATAAAGGCCA	59	985		seq PCR 1
	Rd524-1 R	AAGTCCTGCTGTCCATGTCA				
	Rd524-2 F *	GGCACTTTGTGTTGGTCTATCAC	65-53 TD	318	yes (5, 5)	seq PCR 2
	Rd524-1 R	AAGTCCTGCTGTCCATGTCA				
Rdn1	Rd497-1F *	TGCCTTTTCCTTGCCAGCTA	62	637	no (5, 3)	seq PCR 1
	Rd497-1R	GGGTGCCCAACCTTTTGAAC				
Rdn2	Rd672-1F *	GTTCTCCTTGCAAGCATGTGG	64	294	no (3, 0)	seq PCR 1
	Rd672-1R	CTTTGCGTTTGAGGGACACC				
Rdn3	Rd972-1F *	ACCGGACATCCAGTATGGCTC	66	413	no (2, 0)	seq PCR 1
	Rd972-3R	TGAAGAGGGAGAACACTAACACT				
Rdn4	Rd2546-1F	TGGGGGCTCCTATATGCTCA	64	226	no (1, 0)	seq PCR 1
	Rd2546-1R *	GCCAAACTAGTGGTGCTGGA				

Locus: arbitrarily given names to loci sequenced in Hungarian agile frogs.
M, F: the number of males and females used for initial screening for sex-linked SNPs in the Hungarian agile frogs. Note that XY males are expected to be heterozygotes for sex-linked SNPs. Therefore, only one male was sequenced with each primer pair first, and further individuals were sequenced only if the presence of at least one SNP was detected.
TD: touch-down
[a] PCR reaction mixture in 50 µl final volume: 5 µl DreamTaq buffer (10x, ThermoFisher Scientific), 2.1 µl $MgCl_2$ (25 mM), 2.1 µl dNTP (2 mM), 2 µl forward primer (10 µM), 2 µl reverse primer (10 µM), DreamTaq DNA polymerase (5 U/µl, ThermoFisher Scientific) and 40-250 ng DNA. See Table S3 for PCR programs.
[b] Before sequencing Rds2 and Rds3, nested PCRs were performed. In the second PCR, 0.9 µl product from the first PCR was used as template in the 50 µl reaction.
* Primers used for sequencing.

Table S3. PCR programs used for sequencing and molecular sexing.

PCR ID	PCR program		
seqPCR 1	94°C	2 min	
	94°C	30 sec	
	[a]	30 sec	35x
	72°C	60 sec	
	72°C	10 min	
	10°C	hold	
seqPCR 2	94°C	2 min	
	94°C	30 sec	
	60-53°C	30 sec	7x touch-down
	72°C	60 sec	
	94°C	30 sec	
	53°C	30 sec	25x
	72°C	60 sec	
	72°C	10 min	
	10°C	hold	
sexPCR 1[b]	94°C	2 min	
	94°C	30 sec	
	65-63°C	30 sec	20x touch-down
	72°C	40 sec	
	94°C	30 sec	
	63°C	30 sec	15x
	72°C	40 sec	
	72°C	10 min	
	20°C	hold	
sexPCR 2	94°C	2 min	
	94°C	30 sec	
	70°C	30 sec	35x
	72°C	40 sec	
	72°C	10 min	
	20°C	hold	
sexHRM	95°C	15 min	
	95°C	15 sec	
	62°C	20 sec	50x (ramp: 4.4 °C/s)
	72°C	15 sec	
	95°C	60 sec	
	40°C	60 sec	ramp: 2.2 °C/s
	65°C	1 sec	ramp: 2.2 °C/s
	95°C	1 sec	ramp: 0.07 °C/s
	37°C	30 sec	ramp: 1 °C/s

[a] Annealing temperature differed between primers, as described in Table S2.

[b] For PCR-based sexing with Rds3, the best performing program was sexPCR 1 modified as follows: annealing temperature decreased from 70 to 65°C during the touch-down period, and it remained 65°C for 20 more cycles (instead of 15).

Table S4. Loadings of land-use variables in the principal components.

	Urban PC		Agricultural PC	
Land-use type	**Loading**	**p**	**Loading**	**p**
arable land	-0.139	0.376	0.774	0.073
pasture	0.200	0.426	0.562	<0.001
natural vegetation	-0.472	<0.001	-0.245	0.452
residential built-up	0.498	<0.001	-0.09	0.894
roads	0.507	<0.001	-0.129	0.944
public built-up	0.462	<0.001	-0.021	0.597
Eigenvalue	*1.93*		*1.095*	
Proportion of variance explained	*0.62*		*0.2*	

P-values were calculated from Pearson correlations between the PCA scores and the land-use variables.

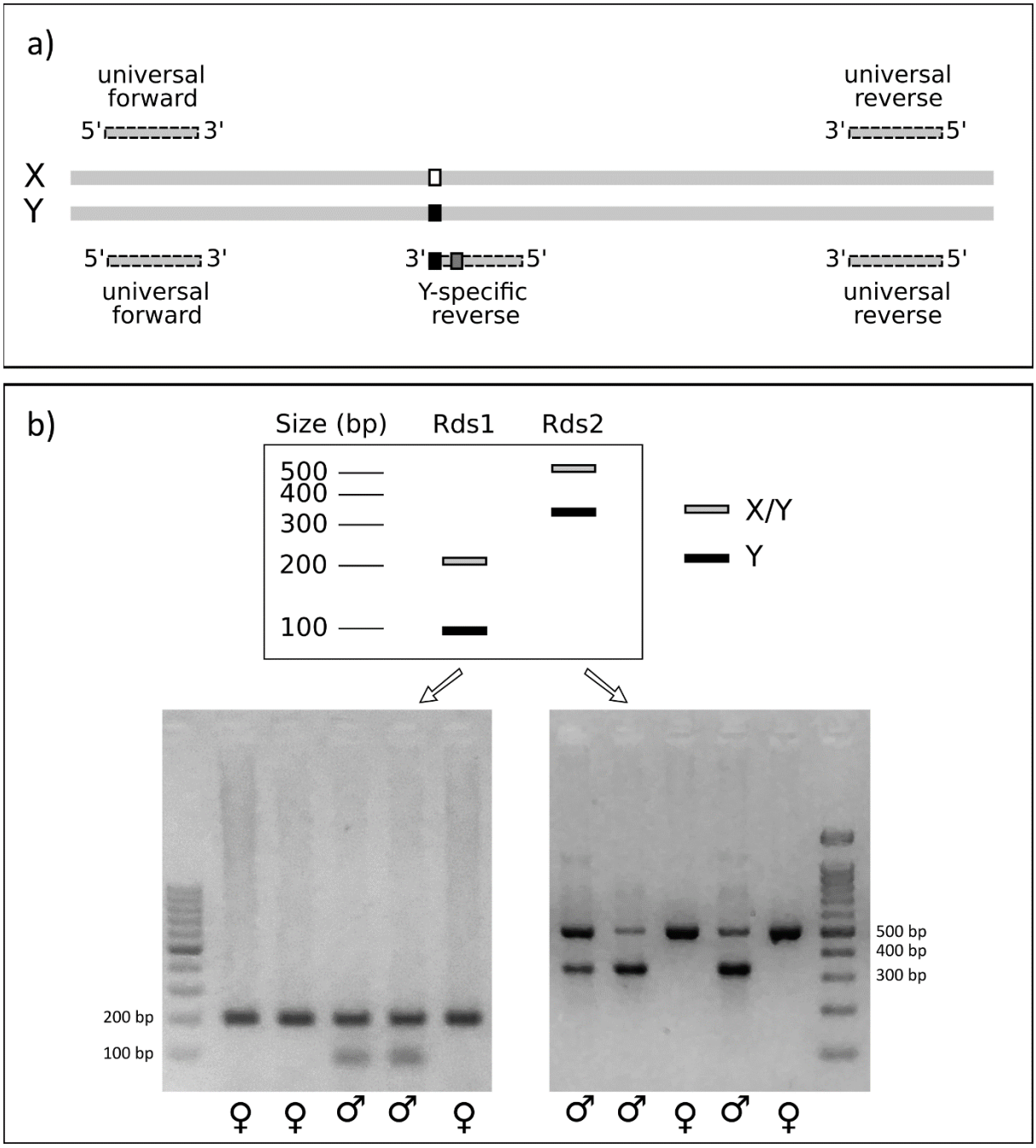


Figure S1. Molecular sexing with SNP-specific PCR primers designed for Rds1 and Rds2. a) X/Y-universal primers bind to both sex chromosomes, while the Y-specific primer (can be either forward or reverse) binds only to chromosome Y, because its binding site contains a sex-specific SNP (denoted by white and black squares). Specificity of the Y-specific primer was further improved for Rds1 by an artificially introduced mismatch (indicated by a dark grey square); this was not necessary for Rds2 because the Y-specific primer's binding site contained two sex-lined SNPs. b) As a result of this design, multiplex PCR with the three primers produces a single band in females (i.e. Y-SNP missing) and two bands in males (i.e. Y-SNP present). Note that after testing sex-specificity of the markers on laboratory-raised froglets, Rds2 was found to be not reliable for genetic sexing in the Hungarian populations.

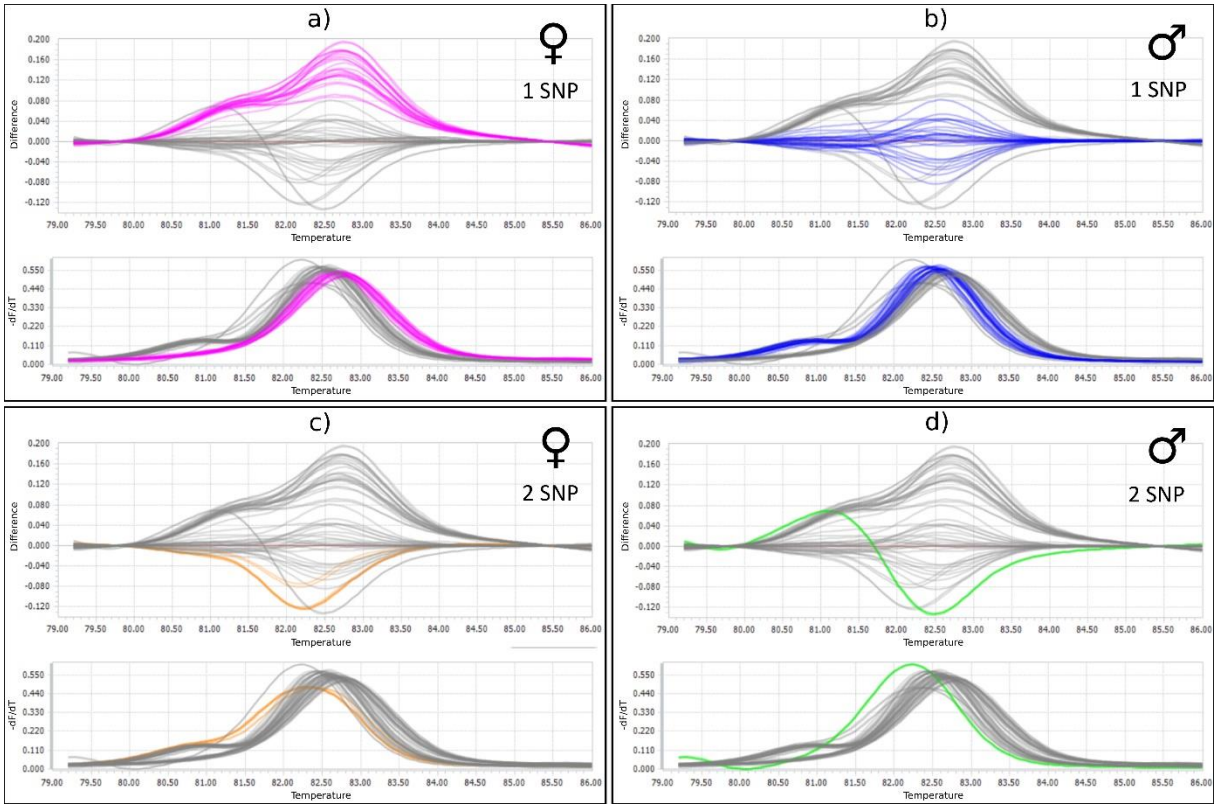


Figure S2. HRM-based genotyping on Rds3. Curves that are highlighted in colour refer to genotypes XX (a, c) and XY (b,d). The upper graph within each panel is the Difference Plot, while the bottom graph is the Normalized Melting Peaks plot drawn by Roche LightCycler®96 1.1.0.1320. Besides the SNP used for sexing, in some individuals a second SNP occurs 16 base positions apart from the first one, causing alterations in the curves' shape (c, d). Curves on the Difference Plot differ significantly between the genotypes of XX without (a) and with the second SNP (c), and also between XY without (b) and with the second SNP (d). Because Difference Plot curves are similar between XX with a second SNP (c) and XY without it (b), inspection of the Normalized Melting Peaks is also necessary for sexing. The Normalized Melting Curves of XY genotypes have two peaks (b, d), with the smaller one being shifted left in the presence of the second SNP (d). In genotype XX, Normalized Melting Curves consist of a single peak which, compared to the single-SNP XX curves (a), is shifted left if the second SNP is present as well (c). The latter curve is easy to mistake for the single-SNP XY curve (b); note that the two curves (blue in panel b, orange in panel c) overlap until the single-SNP XY curve reaches its first, smaller peak, where it remains at a plateau for a while (blue) whereas the two-SNP XX curve keeps rising (orange).

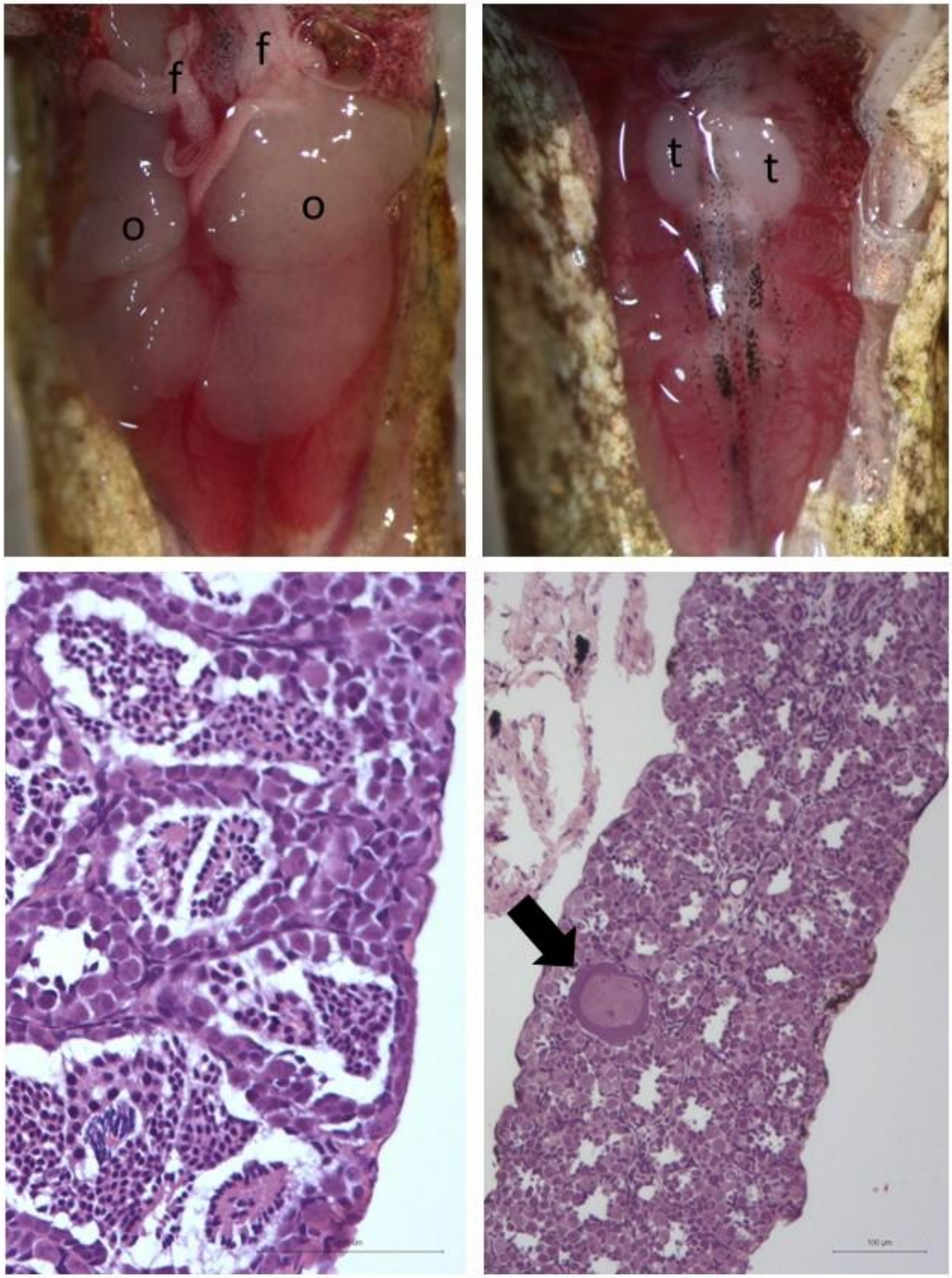


Figure S3. Gonads in juvenile agile frogs. Ovaries (o) with fat bodies (f; top left) and testes (t; top right) at 16× magnification; histological section of a well-developed testis with spermatocytes (bottom left) and a testis with an oogonium shown by an arrow (bottom right).

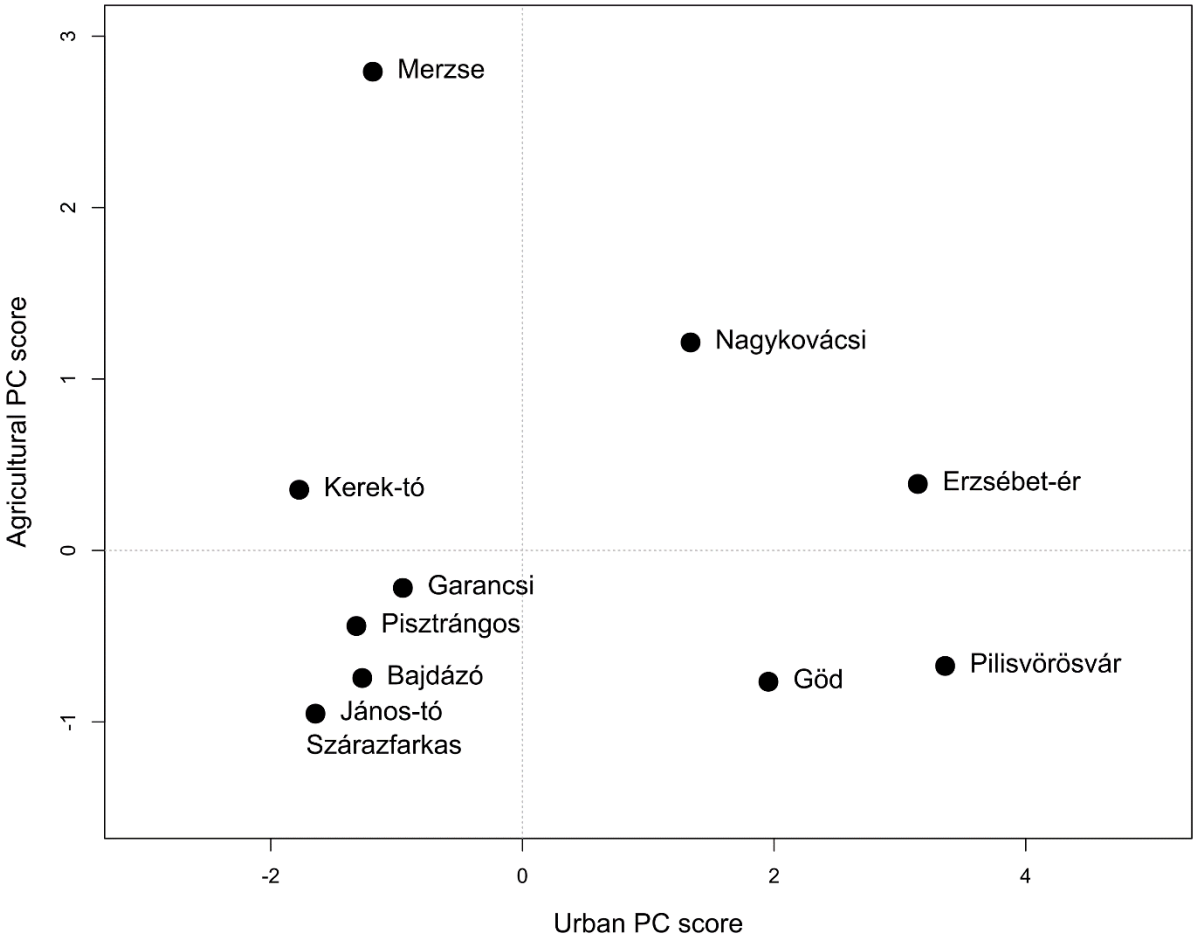


Figure S4. Distribution of the breeding ponds along the „urban PC" and the „agricultural PC". Note that two ponds (János-tó and Szárazfarkas) overlap.

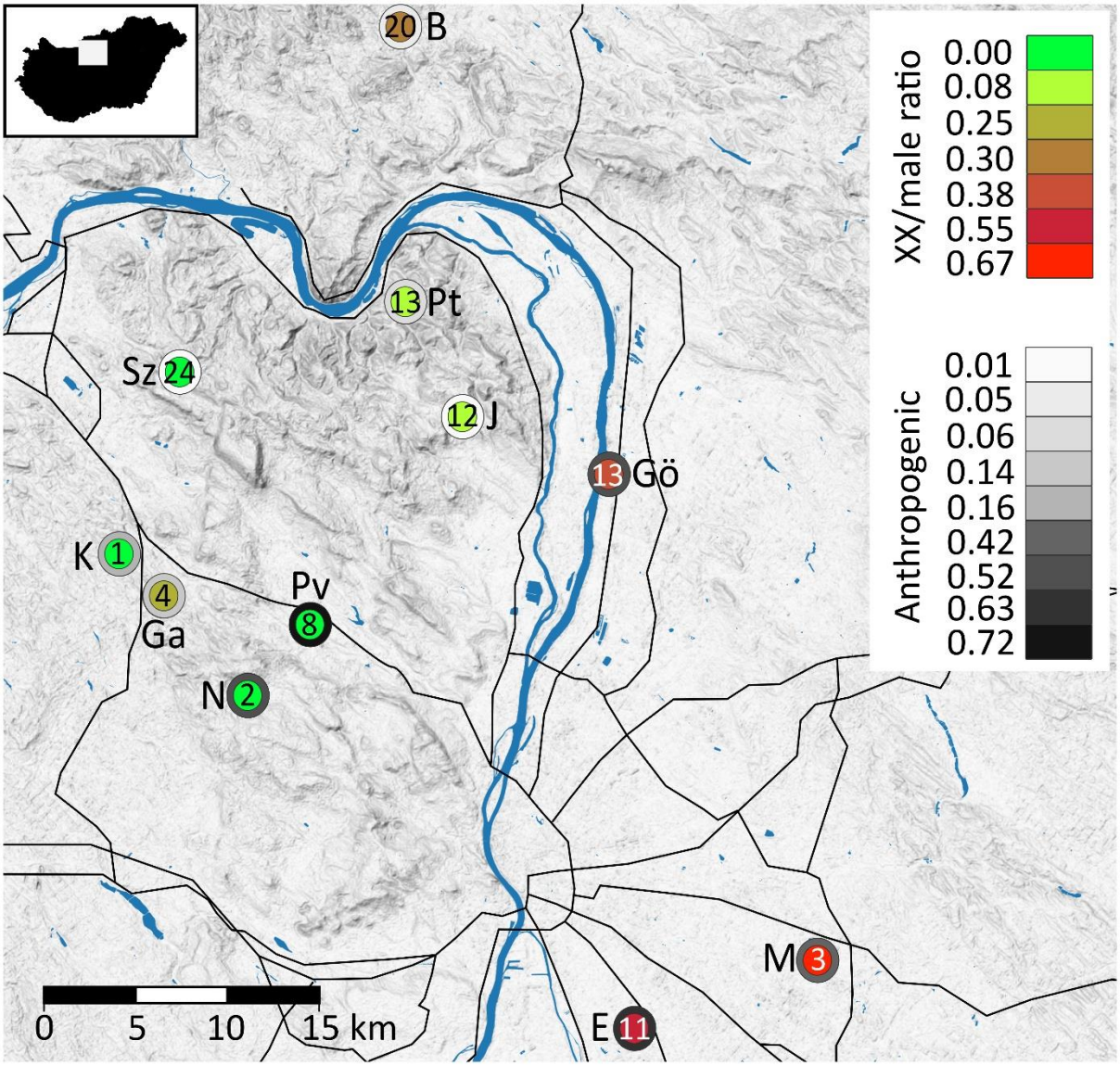


Figure S5. Geographical distribution of our capture sites on both sides of the river Danube in Hungary. Location within the country is shown by the white square in the inlet figure in the top-left corner. Proportion of anthropogenic land cover is indicated on a grey scale in the boundary of each circle representing a pond. XX/male ratio (proportion of sex-reversed XX males out of all successfully genotyped phenotypic males) found in each pond is indicated on a green to red scale. The number in each circle indicates the number of successfully genotyped phenotypic males in each pond. Abbreviations of the pond names are the same as in Table S1. Main roads and highways are indicated with black lines. The highest altitude on the map is about 700 m above sea level.

II. Developmental abnormalities in lab-raised sex-reversed froglets

The froglets we raised in the laboratory correspond to the control group of the experiment described in (Bókony et al., 2020); all details of their housing and handling are given in that open-access paper. When the tadpoles started metamorphosis, we measured their body mass (± 0.1 mg) and for each animal we recorded the duration of larval development as the number of days between developmental stages 25 (start of the free-swimming, foraging larval life phase according to (Gosner, 1960) and 42 (appearance of front limbs). We analysed these two variables using a linear mixed-effects (LME) model with capture site as a random factor, and we found no significant difference between sex-reversed individuals (XX males) and either normal (XY) males or normal (XX) females (Table S5, Figure S6).

At dissection, we measured body mass (right before euthanasia) and the mass of the entire digestive tract (± 0.01 g) because the latter contained varying amount of food remains; we calculated lean body mass as the animal's total body mass minus gut mass. We analysed this variable with an LME model with family as random factor, and we included age at dissection as a covariate, because the froglets were dissected at 96-138 days of age (from the start of larval development; 49-92 days after metamorphosis). This model indicated that sex-reversed individuals had significantly smaller body mass compared to both normal males and normal females (Table S5, Figure S7). However, variance in body mass was much higher among sex-reversed individuals than among normal males and females (likelihood ratio test: $\Delta AIC=33.03$, $P<0.001$), and allowing for this heterogeneity the differences in average body mass were no longer significant (Table S5). Graphical examination of the data showed that these results were due to the fact that 2 out of 6 sex-reversed individuals had much smaller body mass than what would be expected based on their age (Figure S7).

Frogs have fat reserves in the form of finger-like fat bodies attached to the cranial end of the gonads (Figure S3). We categorized the size of the fat bodies in each individual into one of four subjective categories: none, small, medium, or large, and we analysed it using a cumulative link mixed model with family as random factor. Due to the multi-collinearity between age and body mass (Table S5), we only included body mass as a covariate. We found that sex-reversed individuals had similar amounts of fat as normal males and females did (Table S5). Among the 6 sex-reversed individuals, the fat bodies were small in 4 and large in 2 animals; whereas among the 53 normal males and 66 normal females, the fat bodies were small in 14 and 15, medium in 22 and 39, large in 10 and 5, and no fat body was detected in 7 and 7, respectively.

For each animal, we photographed the spleen at 45× magnification with a camera attached to the stereomicroscope, and we analysed the photos as described in (Bókony et al., 2020). In short, we measured spleen size (mm^2) and the total area of pigmented spots on the spleen (%), which are two commonly used indices of immune function in amphibians and fish (Bókony et al., 2020). Sample size was reduced in this analysis because some spleens could not be measured due to insufficient image quality; therefore, we did not include family as random factor because most families were represented by one or a few individuals. Thus, we used generalized least-squares models with body mass as a covariate. These analyses showed that spleen size was significantly larger in sex-reversed individuals than in normal males, and there was a similar, marginally non-significant difference from normal females (Table S5, Figure S6). Spleen pigmentation did not differ significantly between the three groups (Table S5, Figure S6).

Similarly, we photographed the males' testes at 16× magnification and measured the size (mm^2) of the left and right testis, and we analysed the mean of the two measurements in a generalized least-squares model with body mass as a covariate. We found no significant difference in average testes size between sex-reversed and normal males (Table S5); however, graphical examination of the data revealed a non-random pattern: the sex-reversed individuals had either relatively large or relatively small testes compared to normal males (Figure S8).

During dissection, we recorded the following abnormalities in at least one of the 6 sex-reversed individuals: small or poorly developed liver (N=2), greyish liver coloration (N=3), strong visceral pigmentation (N=3). We compared the frequency of each of these phenomena between sex-reversed and normal individuals (males and females pooled; N=125) using Fisher's exact tests. We found that both kinds of liver abnormalities occurred more frequently in sex-reversed than in normal individuals (small size: in 1 normal individual, P = 0.009; greyish coloration: in 8 normal individuals, P = 0.006), and there was a similar, marginally non-significant difference in visceral pigmentation (in 19 normal individuals, P = 0.067).

Table S5. Parameter estimates (b) of the statistical models comparing sex-reversed and normal froglets.

Dependent variable	Model parameter	b	SE	t	p
Time to metamorphosis (days)					
(N = 6 + 66 + 53)	Sex-reversed	43.050	1.375	31.300	< 0.001
	- Normal females	-1.056	1.400	-0.754	0.453
	- Normal males	-1.313	1.401	-0.937	0.351
Body mass at metamorphosis (mg)					
(N = 6 + 66 + 53)	Sex-reversed	508.452	24.286	20.936	< 0.001
	- Normal females	0.183	25.345	0.007	0.994
	- Normal males	-2.836	25.580	-0.111	0.912
Body mass at dissection (g)					
(N = 6 + 66 + 52)	Sex-reversed	1.050	0.069	15.286	< 0.001
	- Normal females	0.227	0.071	3.201	0.002
	- Normal males	0.227	0.071	3.184	0.002
	Age	0.024	0.001	16.412	< 0.001
Body mass at dissection (g)*					
(N = 6 + 66 + 53)	Sex-reversed	1.036	0.185	5.592	< 0.001
	- Normal females	0.246	0.186	1.324	0.189
	- Normal males	0.241	0.186	1.297	0.198
	Age	0.025	0.001	20.525	< 0.001
Size of fat bodies**					
(N = 6 + 66 + 53)	Body mass	0.840	0.590	1.425	0.154
	Sex-reversed - normal females	0.085	0.830	0.103	0.918
	Sex-reversed - normal males	0.215	0.842	0.255	0.799
Spleen size (mm^2)					
(N = 4 + 19 + 15)	Sex-reversed	0.763	0.078	9.822	< 0.001
	- Normal females	-0.154	0.087	-1.776	0.085
	- Normal males	-0.212	0.087	-2.428	0.021
	Body mass	0.404	0.108	3.754	0.001
Spleen pigmentation (%)					
(N = 5 + 18 + 14)	Sex-reversed	2.785	0.614	4.540	0.000
	- Normal females	-0.590	0.710	-0.830	0.413
	- Normal males	-0.046	0.720	-0.064	0.950
	Body mass	-0.552	0.908	-0.608	0.547
Testes size (mm^2)					
(N = 6 + 0 + 24)	Sex-reversed	1.611	0.084	19.182	< 0.001
	- Normal males	0.085	0.188	0.453	0.654
	Body mass	1.309	0.291	4.504	< 0.001

For each model, sample size is given as the number of sex-reversed individuals + number of normal females + number of normal males. All covariates were mean-centered before the analyses. Therefore, the parameter "Sex-reversed" refers to the mean value of sex-reversed individuals, and

the parameters "- Normal females" and "- Normal males" give the difference between the respective group and sex-reversed individuals.
*In this model, sex-reversed individuals, normal females and normal males were allowed to differ in variance (using the 'varIdent' function).
**Cumulative link mixed model; the test statistic is z instead of t.

Figure S6. Larval growth and development speed, and juvenile spleen size and pigmentation in lab-raised froglets.

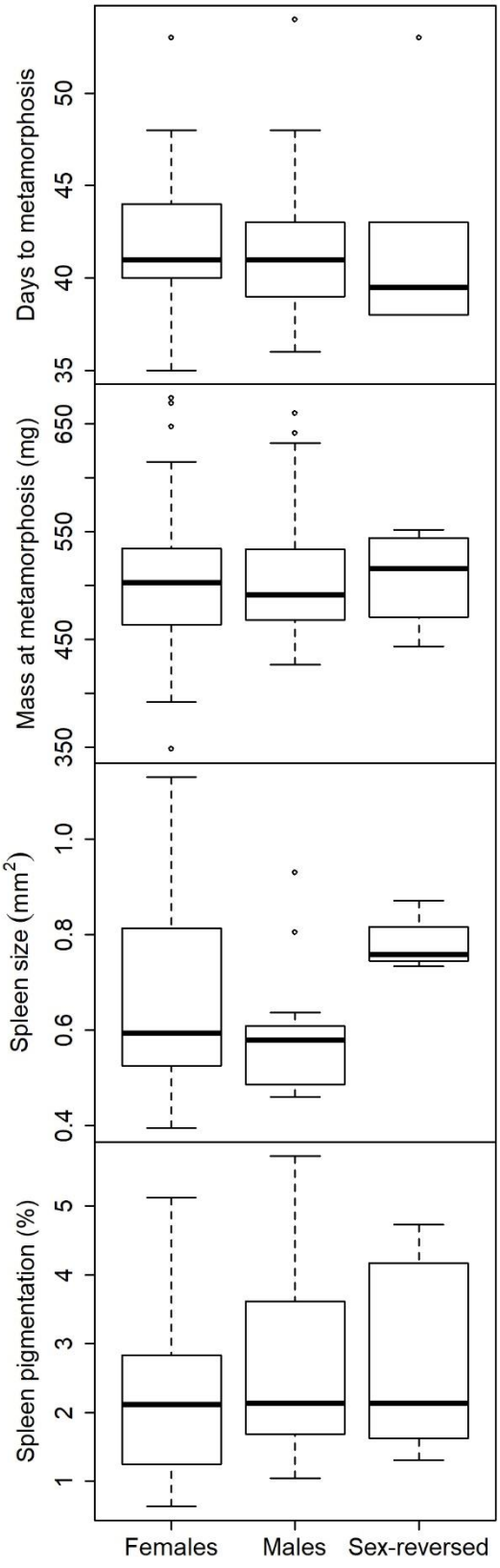


Figure S7. Froglets' body mass (without gut mass) at dissection in normal females (empty gray triangles), normal males (empty black circles), and sex-reversed individuals (filled squares; colours identify individuals to facilitate comparisons with Figure S8). The solid line is a regression line fitted for all animals.

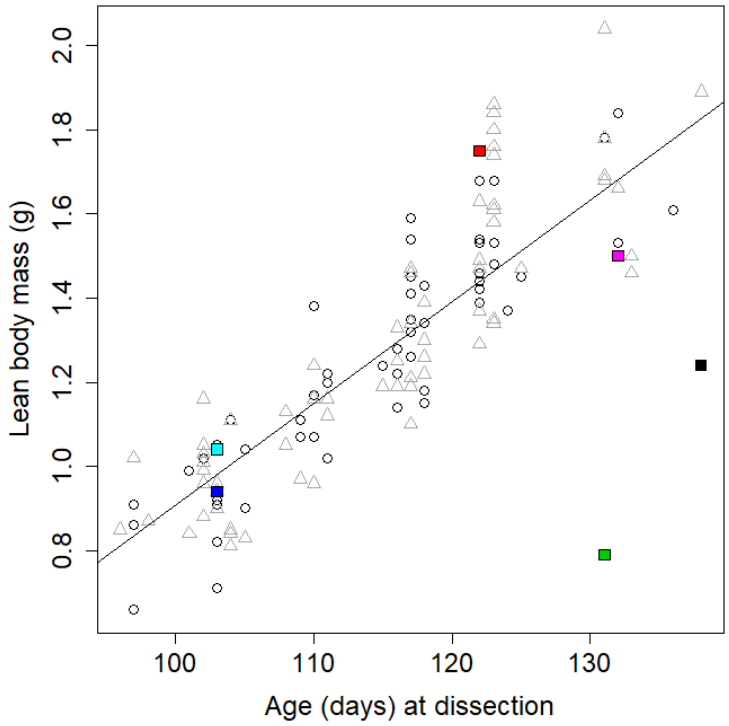
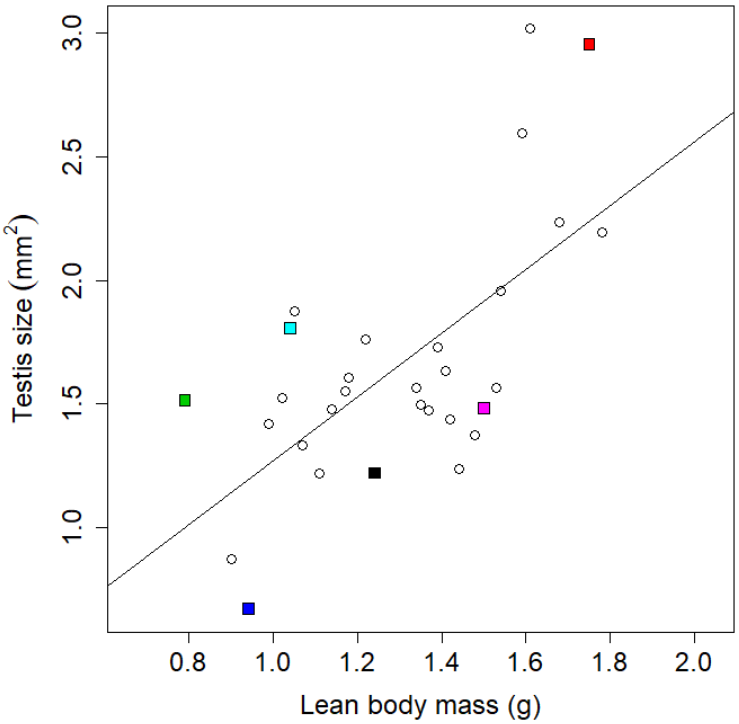


Figure S8. Froglets' testis size in normal males (empty circles) and sex-reversed individuals (filled squares; colours identify individuals to facilitate comparisons with Figure S7). The solid line is a regression line fitted for all phenotypic males. Two sex-reversed males with testicular oocytes (intersex) are marked with black and pink square, respectively. Two other sex-reversed males that had no XY siblings (possibly sired by an XX male) are marked with red and light blue square, respectively.



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