Supporting Information for

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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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 Figure 1.

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

Locus	Accession number	Primer name	Primer sequence	Annealing (°C)	Amplicon (bp)	Sex-linked SNP (M, F)	PCR ID [a]
Rds1	MT358850-MT358851	Rd56-1F *	TGCACAAAGGGACTCCTAAACA	66	273	yes (5, 5)	seq PCR 1
		Rd56-1R	TGCCTCAGAGTGGCTGGATA				
Rds2 [b]	MT358852-MT358853	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]	MT358846-MT358849	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	MT358854-MT358861	Rd497-1F *	TGCCTTTTCCTTGCCAGCTA	62	637	no (5, 3)	seq PCR 1
		Rd497-1R	GGGTGCCCAACCTTTTGAAC				
Rdn2	MT358862-MT358863	Rd672-1F *	GTTCTCCTTGCAAGCATGTGG	64	294	no (3, 0)	seq PCR 1
		Rd672-1R	CTTTGCGTTTGAGGGACACC				
Rdn3	MT358864-MT358865	Rd972-1F *	ACCGGACATCCAGTATGGCTC	66	413	no (2, 0)	seq PCR 1
		Rd972-3R	TGAAGAGGGAGAACACTAACACT				
Rdn4	MT358866	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	35x
	[a]	30 sec	
	72°C	60 sec	
	72°C	10 min	
	10°C	hold	
seqPCR 2	94°C	2 min	
	94°C	30 sec	7x touch-down
	60-53°C	30 sec	
	72°C	60 sec	
	94°C	30 sec	25x
	53°C	30 sec	
	72°C	60 sec	
	72°C	10 min	
	10°C	hold	
sexPCR 1[b]	94°C	2 min	
	94°C	30 sec	20x touch-down
	65-63°C	30 sec	
	72°C	40 sec	
	94°C	30 sec	15x
	63°C	30 sec	
	72°C	40 sec	
	72°C	10 min	
	20°C	hold	
sexPCR 2	94°C	2 min	
	94°C	30 sec	35x
	70°C	30 sec	
	72°C	40 sec	
	72°C	10 min	
	20°C	hold	
sexHRM	95°C	15 min	
	95°C	15 sec	50x (ramp: 4.4 °C/s)
	62°C	20 sec	
	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.

Table S5. Genotype-phenotype combinations found in each clutch (note that we sampled only a small fraction of each clutch: ≤ 20 offspring out of the ca. 1000 eggs per egg mass).

Population	Clutch	Total		Offspring used in the present study				Further siblings [a]			
				Normal		Sex-reversed		Normal		Sex-reversed	
		N	N XX	XY Male	XX Female	XX Male	XY Female	XY Male	XX Female	XX Male	XY Female
Kerek-tó	K1	18	11	-	3	-	-	7	8	-	-
	K2	17	10	2	1	-	-	5	8	1	-
	K3	20	7	3	1	-	-	10	6	-	-
	K4	16	14	1	1	-	-	1	13	-	-
	K5	16	9	1	1	-	-	6	8	-	-
	K6	18	8	3	1	-	-	7	7	-	-
	K7	18	9	2	2	-	-	7	7	-	-
	K8	19	11	2	1	1[b]	-	6	9	-	-
	S8	18	12	2	2	-	-	4	10	-	-
Pilisvörösvár	P1	10	4	-	-	1[b]	-	6	2	1	-
	P2	19	12	1	3	-	-	6	9	-	-
	P3	18	12	1	2	-	-	5	10	-	-
	P4	13	6	3	1	-	-	4	5	-	-
	P5	19	9	2	2	-	-	8	7	-	-
	P6	14	6	-	1	-	-	8	5	-	-
	P7	19	8	2	2	-	-	9	6	-	-
	P8	20	15	-	4	-	-	5	11	-	-
Szárazfarkas	S1	16	7	2	2	-	-	7	5	-	-
	S2	20	11	2	2	-	-	7	9	-	-
	S3	20	14	1	3	-	-	5	11	-	-
	S4	20	12	-	4	-	-	8	8	-	-
	S5	19	9	-	3	-	-	10	6	-	-
	S6	20	12	3	1	-	-	5	11	-	-
	S7	19	12	2	2	-	-	5	10	-	-
	Zs_1	8	4	1	1	-	-	3	3	-	-
	Zs_2	18	5	5	1	-	-	8	4	-	-
	Zs_3	**9**	**9**	**-**	**3**	**-**	**-**	**-**	**4**	**2**	**-**
	Zs_4	**12**	**12**	**-**	**2**	**2**	**-**	**-**	**1**	**7**	**-**
	Zs_5	17	7	3	3	-	-	7	4	-	-
	Zs_6	12	6	2	2	-	-	4	4	-	-
	Zs_7	11	7	2	-	2	-	2	4	1	-
	Zs_8	**17**	**17**	**-**	**6**	**-**	**-**	**-**	**11**	**-**	**-**
	Zs_9	13	5	2	2	-	-	6	3	-	-
	Zs_10	12	4	3	1	-	-	5	2	1	-
Total	*34*	*555*	*316*	*53*	*66*	*6*	*0*	*186*	*231*	*13*	*0*

[a] These siblings were exposed to chemical treatments as part of two other experiments (the one including clutches K, P and S is already published: Bókony et al. 2020). While sex reversals were double checked with a second DNA sample in lab-raised offspring that we used in the present study, we did not perform such double checks in their siblings that were treated with various chemicals. Note that sex reversal may be caused by the chemical treatment in these individuals.

[b] Sex-reversal was confirmed by the presence of testicular oogonia (see Figure S3.)

Data of clutches where all genotyped offspring were XX are highlighted in bold.

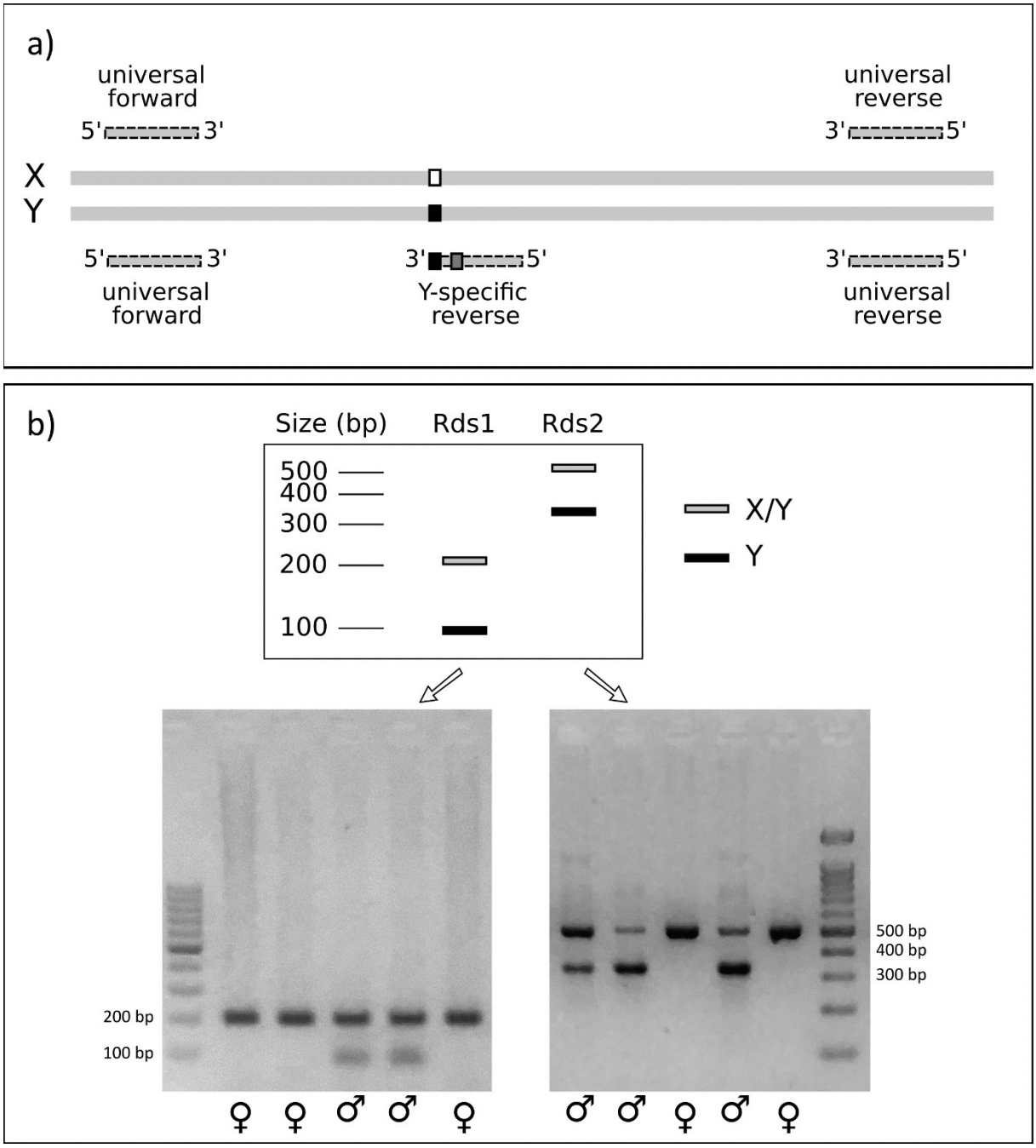


Figure S1. Molecular sexing with SNP-specific PCR primers designed for Rds1 and Rds2. a) X/Y-universal primers bind to both sex chromosomes, while the Y-specific primer (can be either forward or reverse) binds only to chromosome Y, because its binding site contains a sex-specific SNP (denoted by white 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.

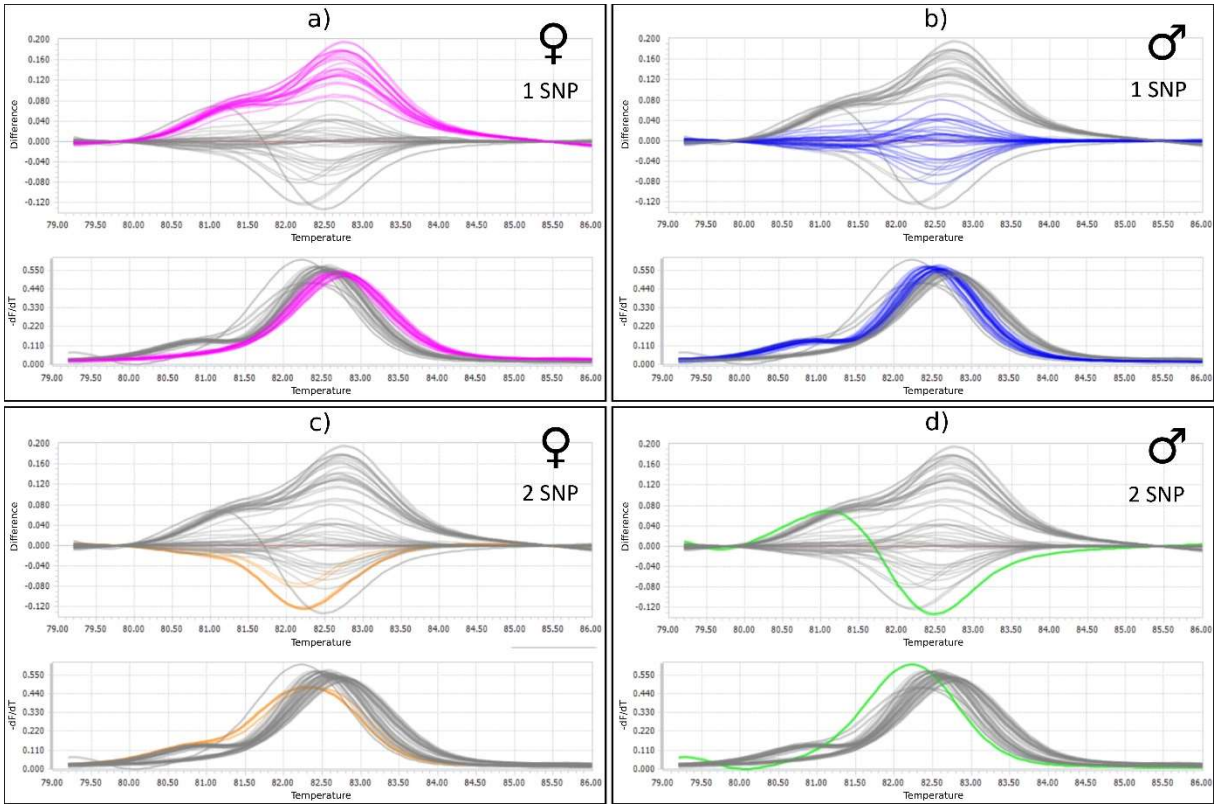


Figure S2. HRM-based genotyping on Rds3. Curves that are highlighted in colour refer to genotypes XX (a, c) and XY (b,d). The upper graph within each panel is the Difference Plot, while the bottom graph is the Normalized Melting Peaks plot drawn by Roche LightCycler®96 1.1.0.1320. Besides the SNP used 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).

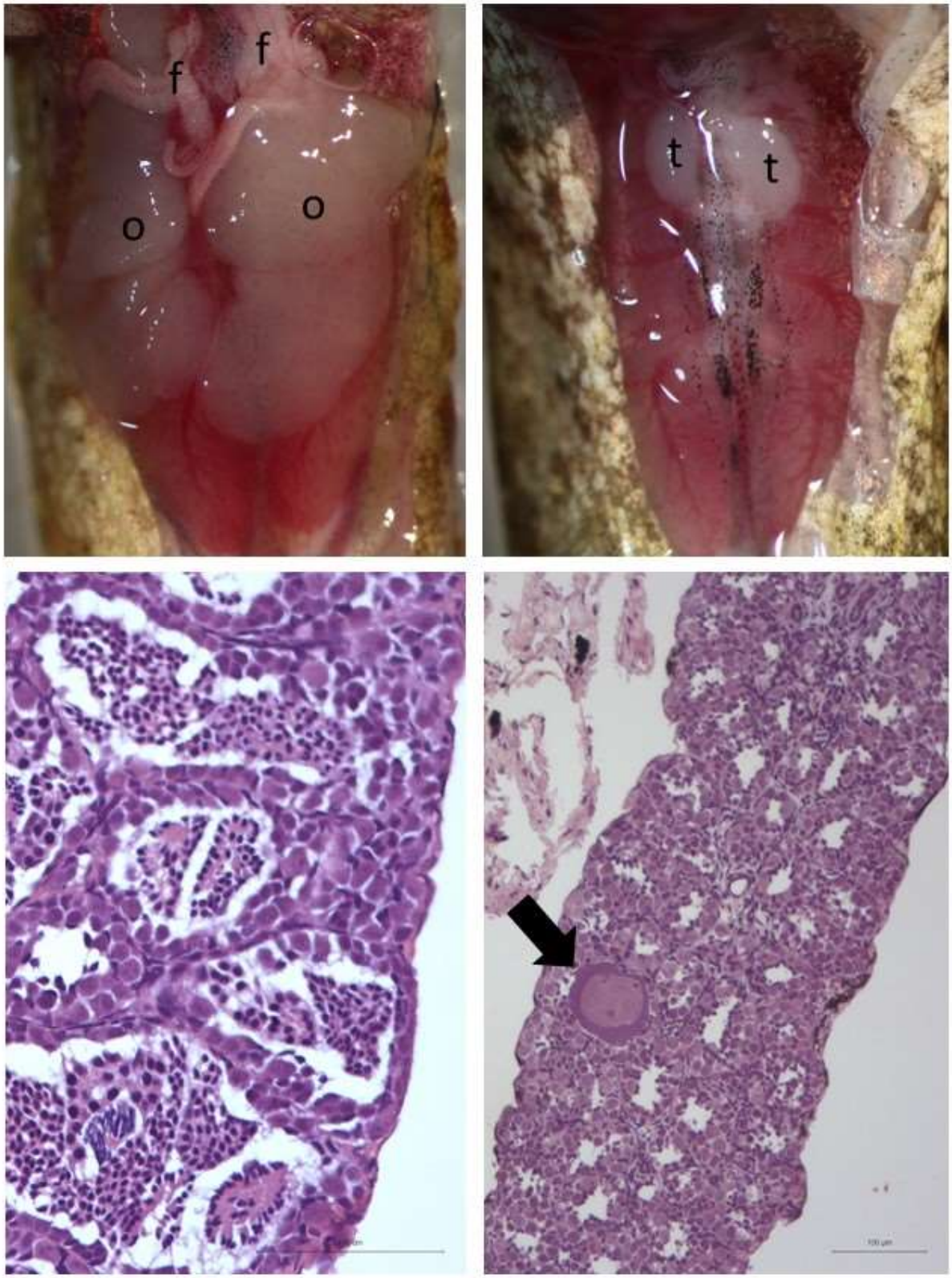


Figure S3. Gonads in juvenile agile frogs. Ovaries (o) with fat bodies (f; top left) and testes (t; top right) at 16× magnification; histological section of a well-developed testis with spermatocytes (bottom left) and a testis with an oogonium shown by an arrow (bottom right).

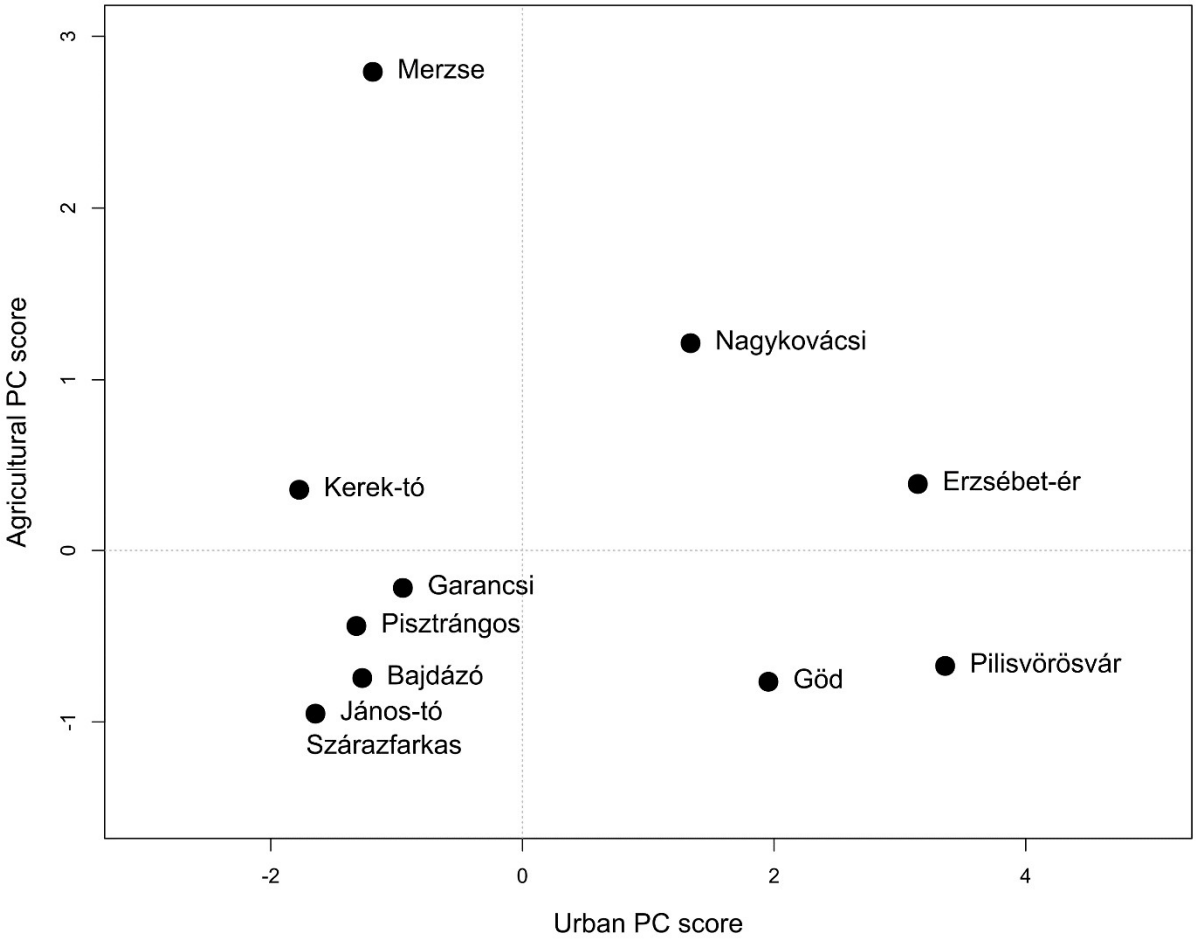


Figure S4. Distribution of the breeding ponds along the „urban PC" and the „agricultural PC". Note that two ponds (János-tó and Szárazfarkas) overlap.

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 S6, Figure S5).

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 S6, Figure S6). 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 S6). 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 S6).

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 S6), 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 S6). 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 S6, Figure S5). Spleen pigmentation did not differ significantly between the three groups (Table S6, Figure S5).

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 S6); 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 S6).

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 S6 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, at the average age or average body mass of froglets measured at dissection.

*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 S5. Larval growth and development speed, and juvenile spleen size and pigmentation in lab-raised froglets.

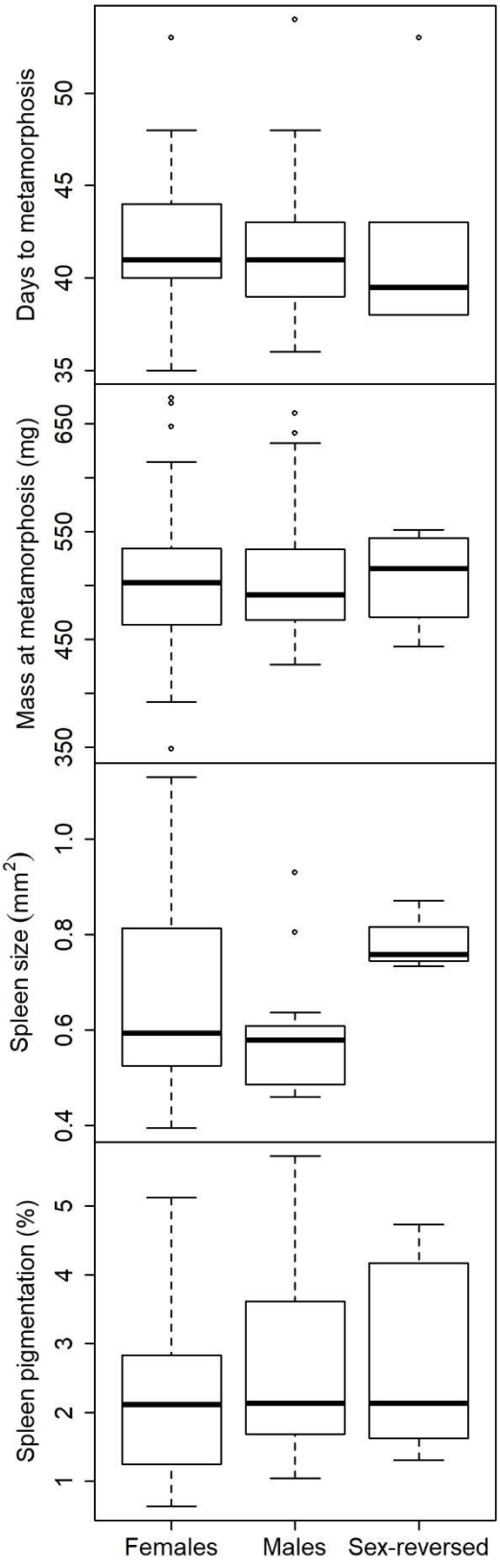


Figure S6. Froglets' body mass (without gut mass) at dissection in normal females (empty gray triangles), normal males (empty black circles), and sex-reversed individuals (filled squares; colours identify individuals to facilitate comparisons with Figure S7). The solid line is a regression line fitted for all animals.

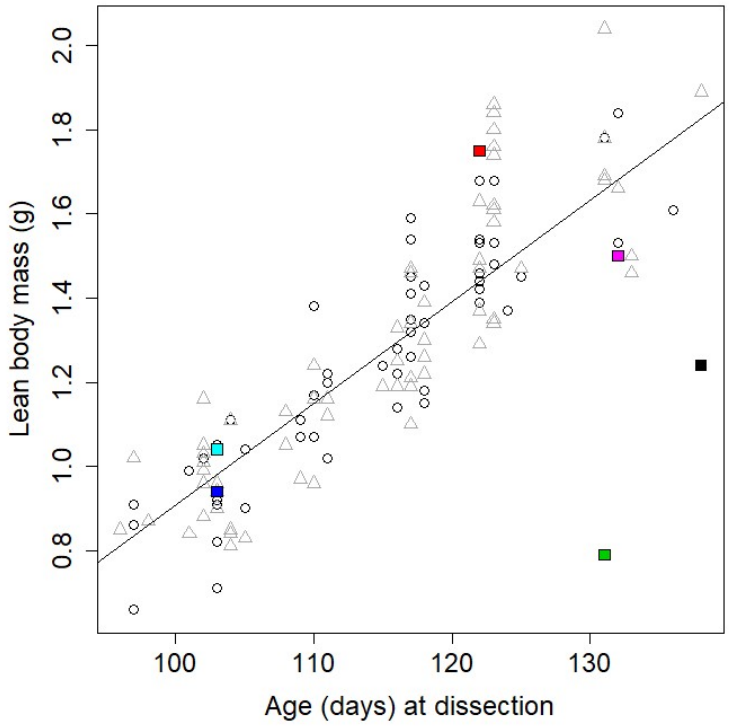
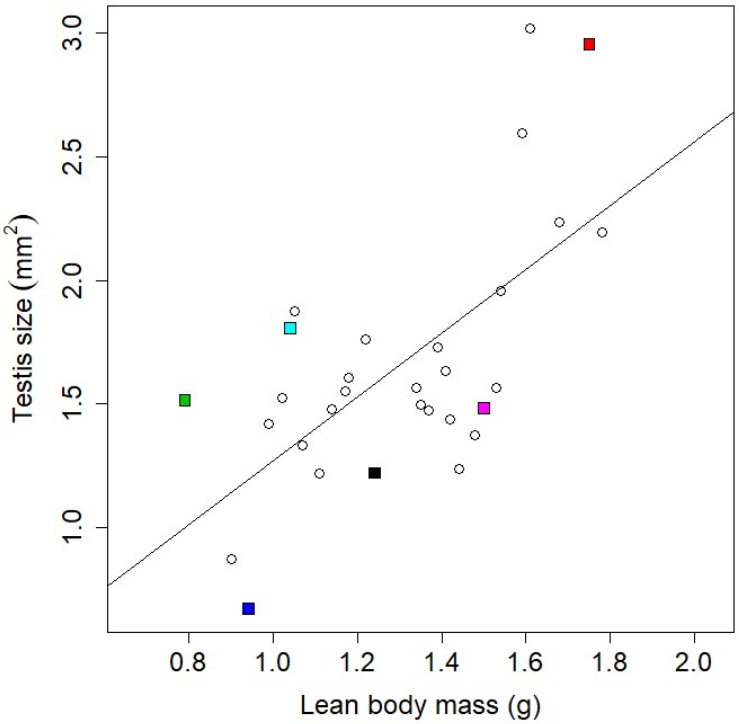


Figure S7. Froglets' testis size in normal males (empty circles) and sex-reversed individuals (filled squares; colours identify individuals to facilitate comparisons with Figure S6). The solid line is a regression line fitted for all phenotypic males. Two sex-reversed males with testicular oocytes (intersex) 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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