

Supplementary Information

Title:

**Sex reversal assessments reveal different vulnerability to endocrine disruption
between deeply diverged anuran lineages**

Authors:

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Supplementary Table 1: Geographic coordinates for origin of European tree frogs (*Hyla arborea*) and European green toads (*Bufo viridis*).

Family ID	Species	Locality	Long.	Lat.	Number of individuals
Ha_Mix	<i>H. arborea</i>	Greece, Crete, Thrapsano	35.185	25.303	40 siblings and 40 mixed offspring
		Greece, Crete, Mochos	35.247	25.442	
Ha_Mo×La	<i>H. arborea</i>	Greece, Crete Mochos (mother)	35.247	25.442	Mother, father, 80 siblings
		Greece, Crete Lasithi Plateau (father)	35.193	25.432	
Bv_Mix	<i>B. viridis</i>	Greece, Crete, Thrapsano	35.185	25.303	40 and 32 siblings
		Greece, Naxos	36.975	25.493	
Bv_GR13_11x12	<i>B. viridis</i>	Greece, Crete, Lasithi Plateau	35.193	25.432	Mother, father, 80 siblings

Supplementary Table 2: Measured ethinylestradiol (EE2) concentrations. In weeks 4, 5 and 8 only stock solutions were quantified; chart in Supplementary Fig. 1.

Concentration EE2 ng/L	Week 1	Week 2	Week 3	Week 6	Week 7	Week 9	Week 10	Average
5,000	2950	3030	4030	5000	4910	4680	3850	4064
500	670	1270	1275	260	480	357		719
50	80	110	40	100	30	30	20	59

Supplementary Table 3: Primer information and PCR protocols for molecular sexing of *Xenopus laevis*, *Hyla arborea* and *Bufo viridis*. (a) Marker with corresponding accession numbers, sequences, labels and sizes for all three species. (b), (c) PCR ingredients and cyclers protocol for genes *DMRT1* and *DM-W* for *X. laevis*. (d), (e) PCR ingredients and cyclers protocol for microsatellite markers WHa5-201 and Ha-H108 for *H. arborea*. (f), (g) PCR ingredients and cyclers protocol for microsatellite marker C201 for *B. viridis*. (h), (i) PCR ingredients and cyclers protocol for microsatellite markers HNRNPD and CHD1 for *B. viridis*.

(a)

Species	Locus	GenBank Acc. number	Primer sequences (5'=>3')	Fluorescence label	Allele size (bp)
<i>X. laevis</i>	<i>DMRT1</i>	AB259777	F: AACAGGAGCCCAATTCTGAG R: AACTGCTTGACCTCTAATGC	none	206
<i>X. laevis</i>	<i>DM-W</i>	AB365520	F: CCACACCCAGCTCATGTAAAG R: GGCAGAGTCACATATACTG	none	260
<i>B. viridis</i>	C201	HQ386137	F: AGGACCCAGGATTTCCAT R: GCTTCTACCAAAGACTGTTCC	HEX (green)	94 - 122
<i>B. viridis</i>	HNRNPD	SAMN03993917 (Bioproject)	F: AGCGTGATGGAGGCCGCCAGCG R: CTTGCTGGCGTTGATCTTC	FAM (blue)	128 - 140
<i>B. viridis</i>	CHD1	SAMN03993917 (Bioproject)	F: TCCCTGCATTCTGTCAAGAAC R: TGGACGGTATGAGGAACCAT	HEX (green)	299 - 301
<i>H. arborea</i>	Ha-H108	EU029102	F: GGGGGTGAGTAAGGGTTAAATC R: GCCACTGTATAGTCCCTCCCTA	FAM (blue)	234 - 261
<i>H. arborea</i>	WHa5-201	AJ403999	F: TCATGGACTGTCGTCATGGT R: AGGTAAATGGAATCTGGGTGTG	HEX (green)	228 - 253

(b)

	Volume per well in μ l
ddH ₂ O	19.25
10x Buffer (15 mM MgCl ₂)	2.5
dNTP Mix (2,5 mM)	0.25
Taq polymerase	0.2
Primer <i>DM-W</i> for	0.2
Primer <i>DM-W</i> rev	0.2
Primer <i>DMRT1</i> for	0.2
Primer <i>DMRT1</i> rev	0.2
Total Volume	23.00
DNA	2.00

(c)

Cycle	Temperature	Time period	Process
1	95°C	4 min	Denaturation
2	95°C	1 min	Denaturation
3	60°C	1 min	Annealing
4	72°C	1 min	Extension
5	go to 2	30 x	
6	72°C	10 min	Extension
7	10°C	forever	

(d)

	Volume per well in μl
ddH ₂ O	3.18
Multiplex Mastermix	3.00
Primer Ha5-201 for	0.33
Primer Ha5-201 rev	0.33
Primer H108 for	0.08
Primer H108 rev	0.08
Total Volume	7.00
DNA	3.00

(e)

Cycle	Temperature	Time period	Process
1	95°C	15 min	Denaturation
2	94°C	30 s	Denaturation
3	58°C	1.5 min	Annealing
4	72°C	1.0 min	Extension
5	go to 2	32 x	
6	60°C	30 min	Extension
7	10°C	For ever	

(f)

	Volume per well in μl
ddH ₂ O	1.86
Multiplex Mastermix	3.00
Primer D214 for	0.07
Primer D214 rev	0.07
Primer C201 for	1.50
Primer C201 rev	1.50
Total Volume	8.00
DNA	2.00

(g)

Cycle	Temperature	Time period	Process
1	95°C	15 min	Denaturation
2	94°C	30 s	Denaturation
3	48°C	1.5 min	Annealing
4	72°C	1.0 min	Extension
	go to 2	35 x	
5	60°C	30 min	Extension
6	10°C	For ever	

(h)

	Volume per well in μl
ddH ₂ O	2.90
10x Buffer (15 mM MgCl ₂)	1.00
Sol Q	1.00
dNTP Mix (2,5 mM)	1.00
Top Taq DNA polymerase	0.10
Primer HNRNPD for	0.50
Primer HNRNPD rev	0.50
Primer CHD1 for	0.50
Primer CHD1 rev	0.50
Total Volume	9.00
DNA	1.00

(i)

Cycle	Temperature	Time period	Process
1	95°C	3 min	Denaturation
2	94°C	30 sec	Denaturation
3	59,5°C	60 sec	Annealing
4	72°C	40 sec	Extension
	go to 2	35 x	
5	72°C	10 min	Extension
6	10°C	For ever	

Supplementary Fig. 1: Measured 17 α -ethinylestradiol (EE2) concentrations. Nominal EE2 concentrations comprised 50 (green line), 500 (red line) and 5,000 (blue line) ng/L; for numeric measured values: Supplementary Table 2.

