Electronic supplementary material

Sex reversal and ontogeny under climate change and chemical pollution: are there interactions between the effects of elevated temperature and a xenoestrogen on early development in agile frogs?

Zsanett Mikó, Edina Nemesházi, Nikolett Ujhegyi, Viktória Verebélyi, János Ujszegi, Andrea Kásler, Réka Bertalan, Nóra Vili, Zoltán Gál, Orsolya I. Hoffmann, Attila Hettyey, Veronika Bókony

Supplementary Methods

Measurements validating temperature in heat treatments

Following the setup of trays (filled with ca. 18 L tap water, resulting in a water depth of 8 cm) to heat treatments, but before the start of animals' exposure, we tested the heating system with boxes that were the same as individuals' rearing containers (filled with 1.7 L RSW, resulting in a water depth of 10 cm) and measured the exact water temperature in containers of each position of the tray, plus the temperature of the circulated tap water. We replicated measurements ten times altogether on two consecutive days with a Greisinger digital thermometer (GTH175/PT). After the termination of the experiment, we repeated these measurements five times. In order to record incidental temperature fluctuations during treatment periods, we daily checked the temperature of tap water in the trays with the digital thermometer. Furthermore, automated data loggers (Onset HOBO Pendant Temperature/Light 8K Data Logger) also recorded water temperature in each tray every 30 minutes during the whole experiment. Accidental temperature fluctuations were not detected in the trays, and measurements in the same positions were very similar before and after the experiment. Therefore, we estimated the temperature likely experienced by individuals during the experiment from daily measured tray water temperature, correcting it with the average difference we measured between the water in each container (in each position in the trays) and the mean temperature of the water in the respective tray. This method minimized the disturbance caused to the animals by daily measurements, but delivered

sufficient data to draw conclusions about the temperature experienced by the animals during treatment periods, and validated the operability of the applied heating setup.

Feeding during the heating treatments

During the treatment period tadpoles were fed as follows. We homogenized 80 g slightly boiled chopped spinach in 235 mL RSW with a hand blender, and added five drops in the first, seven drops in the second, or nine drops in the third treatment period to the containers (ca. 0.06 mL per drop) after every water change. We used 5 ml Pasteur pipettes, of which we cut off the last 2.8 cm from the tips.

Histological analysis

Histological sections were prepared at the Department of Pathology, University of Veterinary Medicine Budapest. The fixed 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). The 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 Zeiss Axioskop 2 microscope equipped with an AxioCam ICc5 camera. For each individual, we examined 5-6 sections; ovaries were identified by the presence of diplotene oocytes, and testes by seminiferous tubules and spermatogonia (Fig. S4).

Table S1. Number of animals that died or survived, percentage of animals that had fat bodies, genetic and phenotypic sex ratio (% males in sexable animals), and female-to-male sex-reversal rate (% of phenotypic males in genetic females) in each treatment group. Asterisks mark sex ratios that differ significantly from 1:1 according to binomial tests.

Treatment group	Died before treatment	Died during treatment	Died after treatment	Died during metamorphosis	Died after metamorphosis	Dissected	Fat body %	Genetic sex ratio (% male)	Phenotypic sex ratio (% male)	Female-to-male sex reversal (%)
Period 1										
Control	0	1	3	2	2	40	86.8	55.0	55.0	0
Heat	1	26	1	0	0	19	52.6	63.2	89.5*	71.4
EE2	0	1	0	3	0	44	95.5	50.0	52.3	4.5
Heat + EE2	3	21	2	1	0	21	61.9	78.9*	94.7*	75.0
Period 2										
Control	0	1	1	1	0	45	93.2	48.9	48.9	0
Heat	4	4	1	6	1	32	71.9	56.7	83.3*	61.5
EE2	2	0	0	3	0	43	95.4	53.5	55.8	5.0
Heat + EE2	0	5	5	3	1	34	64.7	61.8	73.5*	30.8
Period 3										
Control	1	0	1	5	0	41	92.7	46.3	48.8	4.5
Heat	0	4	1	5	1	37	78.4	58.3	100*	100
EE2	4	0	0	2	1	41	92.7	47.5	47.5	0
Heat + EE2	1	5	1	3	0	38	73.7	38.2	97.1*	95.2

Table S2. Type-2 analysis-of-deviance tables of the statistical models. Significant effects ($P < 0.05$) are highlighted in bold.

Dependent variable	Predictors	χ^2	df	*P*
Phenotypic sex ratio	Heat	36.34	1	**<0.001**
	EE2	0.01	1	0.941
	Period	1.06	2	0.588
	Heat × EE2	0.20	1	0.652
	Heat × Period	9.67	2	**0.008**
	EE2 × Period	0.10	2	0.951
	Heat × EE2 × Period	1.16	2	0.560
Survival	Heat	59.96	1	**<0.001**
	EE2	0.85	1	0.355
	Period	46.06	2	**<0.001**
	Heat × EE2	0.59	1	0.444
	Heat × Period	3.81	2	0.149
	EE2 × Period	0.33	2	0.849
	Heat × EE2 × Period	0.23	2	0.892
Time to metamorphosis	Heat	299.51	1	**<0.001**
	EE2	0.34	1	0.557
	Period	2.16	2	0.340
	Heat × EE2	0.02	1	0.880
	Heat × Period	7.90	2	**0.019**
	EE2 × Period	3.37	2	0.185
	Heat × EE2 × Period	1.26	2	0.532
Mass at metamorphosis	Heat	27.58	1	**<0.001**
	EE2	0.07	1	0.789
	Period	13.75	2	**0.001**
	Heat × EE2	0.04	1	0.840
	Heat × Period	1.60	2	0.449
	EE2 × Period	2.63	2	0.268
	Heat × EE2 × Period	2.41	2	0.300
Fat bodies	Heat	28.39	1	**<0.001**
	EE2	0.08	1	0.773
	Period	2.69	2	0.260
	Heat × EE2	0.74	1	0.389
	Heat × Period	1.03	2	0.599
	EE2 × Period	2.03	2	0.363
	Heat × EE2 × Period	0.09	2	0.953

	Sex	1.04	2	0.596
	Age at dissection	1.38	1	0.239
Mass at dissection	Heat	41.89	1	**<0.001**
	EE2	0.09	1	0.761
	Period	4.15	2	0.126
	Heat × EE2	2.07	1	0.150
	Heat × Period	2.20	2	0.333
	EE2 × Period	0.40	2	0.818
	Heat × EE2 × Period	20.52	2	**<0.001**
	Sex	6.21	2	**0.045**
	Age at dissection	124.23	1	**<0.001**

Table S3. Results of pre-planned comparisons from the models in Table 2. Linear contrasts (*c*) with standard errors (SE) are reported with non-adjusted *P*-values; asterisks indicate if the results remain significant after FDR correction performed separately for each dependent variable ($^{*}P$ = 0.01 – 0.05, $^{**}P$ = 0.001 – 0.01, $^{***}P$ < 0.001).

Dependent variable	Period	Treatment effect	*c*	SE	t	*P*
Phenotypic sex ratio	1	Heat	-1.89	0.54	-3.53	**<0.001****
	2	Heat	-1.14	0.37	-3.08	**0.002****
	3	Heat	-3.69	0.76	-4.87	**<0.001*****
	1	EE2	-0.19	0.54	-0.36	0.720
	2	EE2	0.09	0.37	0.25	0.805
	3	EE2	0.06	0.76	0.08	0.936
	1	Heat × EE2	0.61	1.07	0.57	0.568
	2	Heat × EE2	-0.74	0.74	-0.99	0.319
	3	Heat × EE2	0.06	1.51	0.04	0.967
Survival	1	Heat	-2.36	0.36	-6.64	**<0.001*****
	2	Heat	-2.31	0.65	-3.57	**<0.001****
	3	Heat	-1.29	0.49	-2.63	**0.009***
	1	EE2	0.33	0.35	0.95	0.345
	2	EE2	0.29	0.65	0.46	0.648
	3	EE2	0.32	0.49	0.66	0.509
	1	Heat × EE2	0.18	0.71	0.25	0.801
	2	Heat × EE2	0.77	1.29	0.60	0.550
	3	Heat × EE2	0.62	0.98	0.63	0.530
Time to metamorphosis	1	Heat	-10.61	1.36	-7.81	**<0.001*****
	2	Heat	-8.04	0.88	-9.17	**<0.001*****
	3	Heat	-6.60	0.55	-11.98	**<0.001*****
	1	EE2	1.59	1.36	1.17	0.242
	2	EE2	-0.41	0.88	-0.46	0.644
	3	EE2	-0.26	0.55	-0.47	0.635
	1	Heat × EE2	-2.02	2.71	-0.74	0.458
	2	Heat × EE2	-1.17	1.75	-0.67	0.504
	3	Heat × EE2	0.59	1.10	0.53	0.594
Mass at metamorphosis	1	Heat	26.18	15.73	1.66	0.097
	2	Heat	35.89	10.07	3.56	**<0.001****
	3	Heat	37.35	9.43	3.96	**<0.001*****
	1	EE2	21.44	15.70	1.37	0.173
	2	EE2	8.27	10.07	0.82	0.412
	3	EE2	-10.63	9.44	-1.13	0.261

	1	Heat × EE2	-47.13	31.40	-1.50	0.134
	2	Heat × EE2	5.31	20.10	0.26	0.792
	3	Heat × EE2	6.64	18.90	0.35	0.725
Fat bodies	1	Heat	2.22	0.59	3.77	**<0.001****
	2	Heat	1.99	0.57	3.51	**<0.001****
	3	Heat	1.47	0.58	2.54	**0.012***
	1	EE2	-0.82	0.56	-1.46	0.146
	2	EE2	-0.02	0.56	-0.03	0.974
	3	EE2	0.16	0.52	0.31	0.759
	1	Heat × EE2	-0.49	1.12	-0.43	0.666
	2	Heat × EE2	-0.79	1.12	-0.72	0.475
	3	Heat × EE2	-0.34	1.03	-0.33	0.742
Mass at dissection	1	Heat	-0.15	0.03	-5.02	**<0.001*****
	2	Heat	-0.09	0.03	-3.95	**<0.001*****
	3	Heat	-0.12	0.03	-4.09	**<0.001*****
	1	EE2	-0.003	0.03	-0.11	0.910
	2	EE2	0.005	0.02	0.20	0.842
	3	EE2	0.005	0.02	0.22	0.829
	1	Heat × EE2	0.06	0.06	0.99	0.322
	2	Heat × EE2	-0.12	0.05	-2.49	**0.013***
	3	Heat × EE2	0.19	0.05	3.92	**<0.001*****

Table S4. Results of Firth's bias-reduced logistic regression models on female-to-male sex reversal. Parameter estimates (*b*) with standard errors (SE) are given on logit scale. In each period, the first parameter refers to the mean of the control group (19 °C, no EE2), whereas the second and third parameters refer to the effect of heat without EE2 and the effect of EE2 without heat, respectively. The fourth parameter refers to the interaction, i.e. the effect of EE2 on the effect of heat. We report *P* values only for parameters that refer to differences between treatments.

Period	Model parameter	*b*	SE	χ^2	*P*
1	Control	-3.61	1.47	21.72	–
1	Heat	4.40	1.68	14.04	**<0.001**
1	EE2	0.95	1.71	0.37	0.545
1	Heat × EE2	-0.89	2.18	0.19	0.661
2	Control	-3.85	1.46	28.41	–
2	Heat	4.29	1.57	17.98	**<0.001**
2	EE2	1.29	1.70	0.70	0.404
2	Heat × EE2	-2.47	1.89	2.09	0.148
3	Control	-2.66	0.86	20.8	–
3	Heat	6.10	1.72	37.88	**<0.001**
3	EE2	-1.10	1.70	0.50	0.479
3	Heat × EE2	0.28	2.42	0.01	0.905

Table S5. Differences in body mass (g) at dissection between female-to-male sex-reversed individuals, concordant males, and concordant females. Linear contrasts (*c*) with standard errors (SE) are reported with non-adjusted *P*-values, calculated from the model in Table 2. Asterisk indicates if the result remained significant after FDR correction ($^{*}P = 0.01 – 0.05$).

Linear contrasts	*c*	SE	t	*P*
Concordant female vs. concordant male	0.03	0.02	1.69	0.09
Sex-reversed female vs. concordant female	-0.07	0.03	-2.43	**0.02***
Sex-reversed female vs. concordant male	-0.04	0.03	-1.68	0.09

Fig. S1. Schematic illustration of the 12 treatments, each horizontal bar representing a treatment group. Treatment periods are symbolized with horizontally striped bars; vertical stripes symbolize hormone treatment, and red bars symbolize high-temperature treatment.

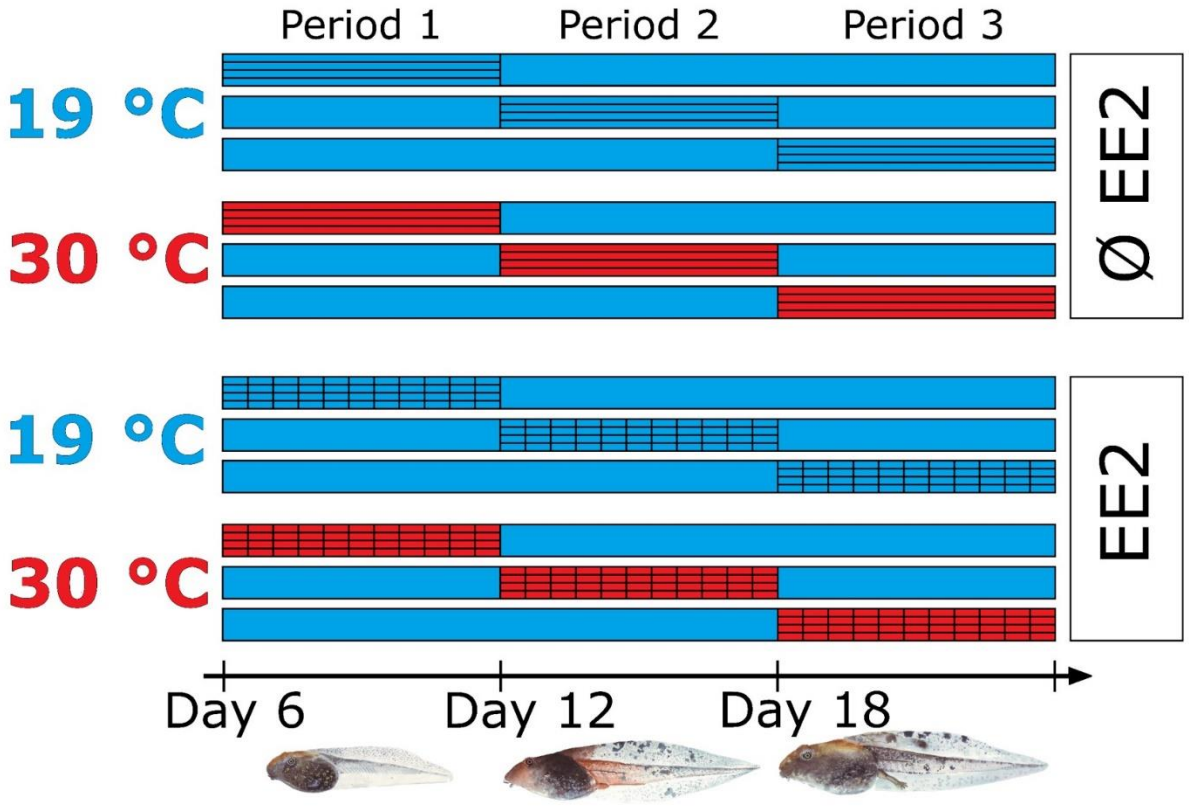

Fig. S2. Experimental setting during the heating treatment. Individual tadpole containers (18 × 13 × 12.5 cm, white rectangles) were placed in an 80 × 60 × 12 cm tray (gray rectangle) filled with ca. 18 L tap water, which was circulated using a Tetra WP 300 aquarium water pump and heated using a Tetra HT 300 aquarium heater (black rectangles).

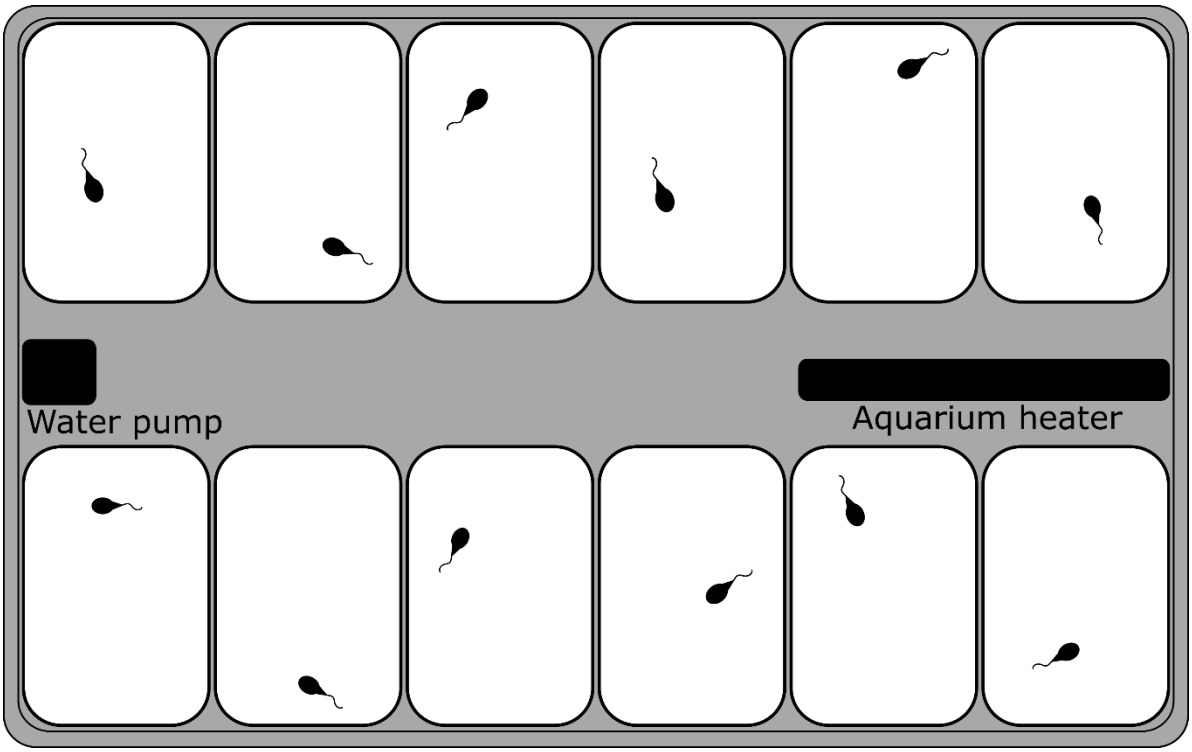

Fig. S3. Gonads in juvenile agile frogs at 16× magnification: A) normal testes (t), B) normal ovaries (o), C) intersex gonads (ovotestes; ot); and varying amounts of fat bodies (f).

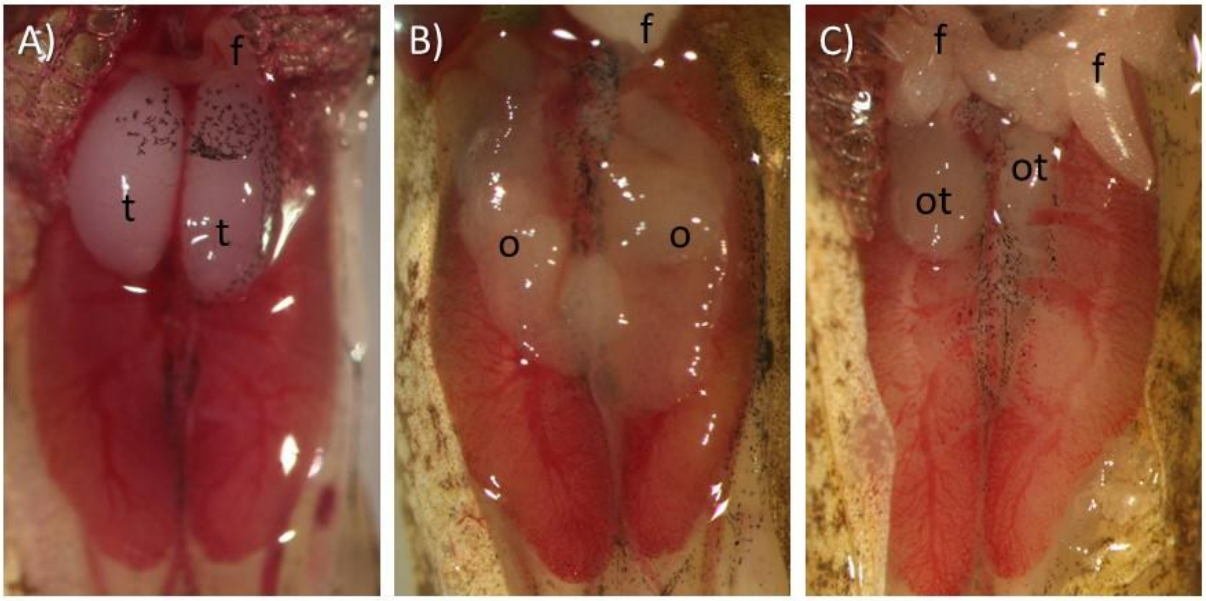

Fig. S4. Histological sections of agile frog gonads (10×): A) normal testis with seminiferous tubules (black arrowheads) and somatic cells forming the precursor of rete testis (yellow arrow), B) normal ovary with previtellogenic diplotene oocytes about 120-200 μm in diameter, C) ovotestis with seminiferous tubules, previtellogenic diplotene oocytes (o), and degenerating oocytes (red arrows). D) Larger magnification (20×) of an ovotestis, with somatic tissue forming a seminiferous tubule (encircled yellow) between normal and degenerating oocytes.

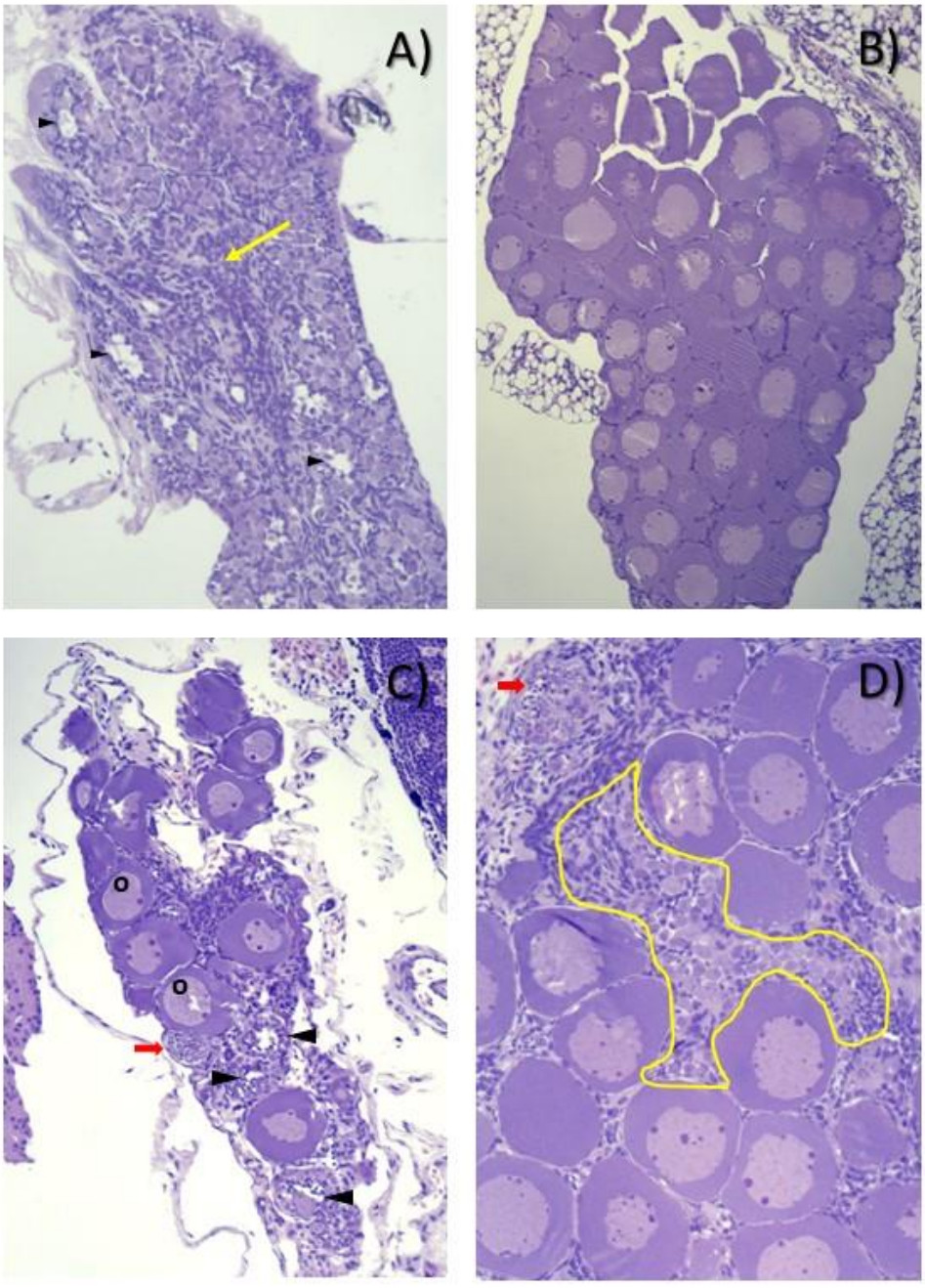

Fig. S5. Boxplots (medians, interquartile ranges, and data ranges) of juvenile body mass for concordant females, concordant males, and female-to-male sex-reversed individuals. Empty and filled circles mark phenotypic males with unknown genetic sex and intersex individuals, respectively. Treatment groups are indicated as in Figures 1-2.