

Supplementary Material

Using reproductive technologies to assess the development of secondary sexual characteristics, ovarian senescence and hermaphroditism in the endangered mountain yellow-legged frog *Rana muscosa*

Leah E. Jacobs^{A,D}, Talisin T. Hammond^A, Patricia M. Gaffney^A, Michelle J. Curtis^A, Debra M. Shier^A, Barbara S. Durrant^A, Alison Righton^B and Candace L. Williams^A and Natalie E. Calatayud^{A,C}

^ASan Diego Zoo Wildlife Alliance, Beckman Center for Conservation Research, 15600 San Pasqual Valley Road, Escondido, CA 92025, USA.

^BOmaha's Henry Doorly Zoo and Aquarium, 3701 S 10th Street Omaha, NE 68107, USA.

^CConservation Science Network, 24 Thomas Street, Mayfield, NSW 2304, Australia.

^DCorresponding author. Email: ljacobs@sandiegozoo.org

Table S1. Summary of study animals.

Individuals are shaded by status: NPF females (purple), females (pink) and males (blue) and include the last breeding records for the individual, with U referring to missing or incomplete records. Data availability for hormone analysis (■, yes; □, no) or evidence of hermaphroditism: yes (■), and normal necropsy (□).

Sex	ID	Last recorded breeding	Avg. # Ova produced	Year of nuptial pad emergence	Hormone analysis	Hermaphrodite	Pathological examination
NPF	906738	2011	190	2012	■	■	Testes & mature oviducts
NPF	907078	2015	376	2015	■	□	
NPF	907080	2015	312	2015	■	■	Testes & mature oviducts
NPF	907352	2015	321	2015	■	■	Ovotestes & mature oviducts
NPF	906697	U	N/A	2012	□	■	Testes & mature oviducts
NPF	907026	U	N/A	2012	□	■	Testes & mature oviducts
NPF	907186	2011	215	2014	□	■	Testes & mature oviducts
F	906695	2014	500	N/A	■	□	
F	906700	2016	351.8	N/A	■	□	
F	906712	2017	363.2	N/A	■	□	
F	907009	2016	485	N/A	■	□	
F	907037	2017	426.6	N/A	■	□	
F	907077	2016	347	N/A	■	□	
F	907213	2016	438.2	N/A	■	□	
F	907330	2019	389.3	N/A	■	□	
F	907331	2016	549	N/A	■	□	
F	907360	2016	324	N/A	■	□	
M	906711	2015	140	N/A	■	□	
M	906736	2015	523	N/A	■	□	
M	907006	2016	447	N/A	■	□	

M	907011	2016	722	N/A	■	<input type="checkbox"/>
M	907024	2016	504	N/A	■	<input type="checkbox"/>
M	907034	2017	500	N/A	■	<input type="checkbox"/>
M	907046	2017	214	N/A	■	<input type="checkbox"/>
M	907051	2017	794	N/A	■	<input type="checkbox"/>
M	907057	2016	396	N/A	■	<input type="checkbox"/>
M	907081	2015	65	N/A	■	<input type="checkbox"/>
M	907092	2016	13	N/A	■	<input type="checkbox"/>
M	907190	2016	390	N/A	■	<input type="checkbox"/>
M	909243	N/A	N/A	N/A	■	<input type="checkbox"/>
M	908136	2017	550	N/A	■	<input type="checkbox"/>

Table S2. Hormone standards and their optimized MRM conditions for LC-MS/MS.

Hormones	CAS	Source	LC-MS/MS MRM condition				
			Precursor Ion (m/z)	Product Ion (m/z)	Fragmentor	Collision Energy (eV)	Polarity
Testosterone	58-22-0	Sigma	289.2	109	80	28	+
				97.1		21	
β -estradiol	50-28-2	Sigma	506.2	171.1	71	40	+
				156.1		41	
Estriol	50-27-1	Sigma	522.2	171.1	80	36	+
				156		41	

Table S3. GLMM results testing for differences between nuptial pad females, phenotypically normal females, and males in testosterone across sampling times. Significant terms are shown in bold.

	Estimate	S.E.	df	t	p
(Intercept)	0.13	0.14	56	0.92	0.36
Sex (Females vs. Thumbpads Females)	0.45	0.15	56	3.08	0.003
Sex (Males vs. Thumbpads Females)	-0.04	0.14	56	-0.28	0.78
Month (Jul vs. May)	-0.25	0.12	56	-2.00	0.05
Month (Nov vs. May)	0.21	0.14	56	1.45	0.15
Random effects:	Variance	SD			
Individual Identity	0	0			
Residual	0.18	0.42			

Table S4. GLMM results testing for differences between nuptial pad females, phenotypically normal females, and males in β -estradiol across sampling times. Significant terms are shown in bold.

	Estimate	S.E.	df	t	p
(Intercept)	1.65	0.20	52	8.40	<0.001
Sex (Females vs. Thumbpads Females)	-0.25	0.25	52	-0.99	0.32
Sex (Males vs. Thumbpads Females)	0.12	0.23	52	0.51	0.61
Month (Jul vs. May)	0.12	0.26	52	0.44	0.66
Month (Nov vs. May)	-0.11	0.26	52	-0.42	0.68
Sex (Female) x Month (Jul)	0.73	0.33	52	2.17	0.03
Sex (Male) x Month (Jul)	0.11	0.31	52	0.34	0.73
Sex (Female) x Month (Nov)	0.58	0.35	52	1.65	0.11
Sex (Male) x Month (Nov)	0.20	0.34	52	0.59	0.56
Random effects:	Variance	SD			
Individual Identity	0	0			
Residual	0.15	0.39			

Table S5. GLMM results testing for differences between nuptial pad females, phenotypically normal females, and males in estriol across sampling times. Significant terms are shown in bold.

	Estimate	S.E.	df	t	p
(Intercept)	0.92	0.14	52	6.79	<0.001
Sex (Females vs. Thumbpads Females)	-0.13	0.17	52	-0.76	0.45
Sex (Males vs. Thumbpads Females)	-0.07	0.16	52	-0.42	0.68
Month (Jul vs. May)	-0.04	0.18	52	-0.23	0.82
Month (Nov vs. May)	-0.08	0.18	52	-0.44	0.66
Sex (Female) x Month (Jul)	0.50	0.23	52	2.17	0.03
Sex (Male) x Month (Jul)	0.22	0.22	52	1.02	0.31
Sex (Female) x Month (Nov)	0.26	0.24	52	1.06	0.29
Sex (Male) x Month (Nov)	0.18	0.23	52	0.79	0.43
Random effects:	Variance	SD			
Individual Identity	0	0			
Residual	0.07	0.27			