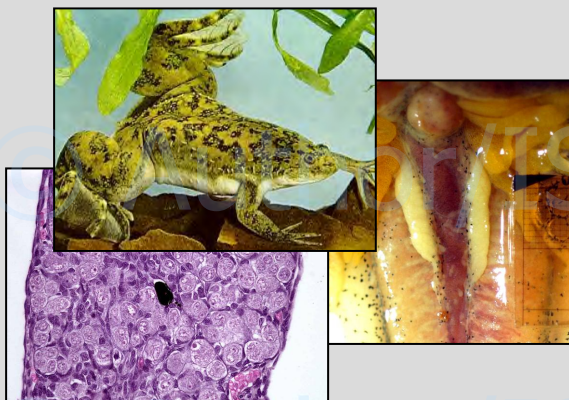


Histopathologic Effects of 17 β -estradiol on the Gonads of Larval *Xenopus laevis*



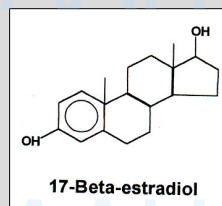
Jeff Wolf*
Ilka Lutz
Werner Kloas
Tim Springer
Hank Krueger
Larry Holden
Alan Hosmer



Introduction

17 β -estradiol (E2)

- A natural estrogen that has access to the environment via municipal effluents and runoff from livestock production
- Studied extensively in a wide range of species
- Frequently employed as a positive control substance for experiments in which estrogenic activity or effects are anticipated



Xenopus laevis

- Traditional anuran research representative
- Biology and reproductive physiology are well-characterized
- The reported histopathologic effects of E2 exposure on the gonads of larval frogs have been inconsistent and confusing



Reported Effects of E2 in Anurans

- Altered sex ratios
- Hermaphroditism
- Intersex
- Mixed sex
- Necrosis or apoptosis of spermatogonia
- Ovotestes
- Polygonadism
- Retarded gonadal development
- Testicular oocytes

Many terms are
synonymous,
ambiguous, and/or
poorly-defined

Research Goals

- Determine EC₅₀ for feminization of gonads in E2-exposed *X. laevis* sacrificed at Nieuwkoop and Faber (NF) Stage 66
- Standardize histopathologic diagnostic criteria and terminology
- Refine clinical, histopathological, and statistical methodology
- Fully characterize histopathologic findings



Stage 61



Stage 62



Stage 63



Stage 64



Stage 65



Stage 66

Methods: Experimental Design

- Three experiments performed at two different laboratories

Methods: Experimental Design

- Three experiments performed at two different laboratories
- **Experiment 1:** the pilot study designed to determine an EC₅₀ for complete gonad feminization (= 75% phenotypic females)

Experiment	Laboratory	E2 Nominal Conc. µg/L	n
1	Wildlife International	0.0 (reference control)	184
		0.0 (negative control)	165
		0.2	184
		1.5	184
		6.0	165

Methods: Experimental Design

- Three experiments performed at two different laboratories
- **Experiment 1:** the pilot study designed to determine an EC_{50} for complete gonad feminization (= 75% phenotypic females)
- **Experiments 2A and 2B:** consisted of positive and negative controls from a study involving atrazine

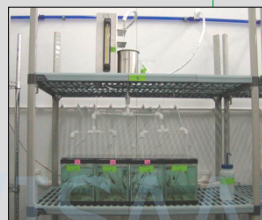
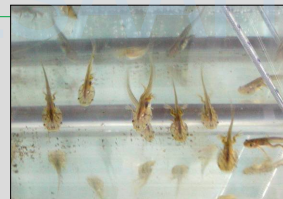
Experiment	Laboratory	E2 Nominal Conc. $\mu\text{g/L}$	n
1	Wildlife International	0.0 (reference control)	184
		0.0 (negative control)	165
		0.2	184
		1.5	184
		6.0	165
2A	Wildlife International	0.0 (negative control)	187
		0.2	193
2B	IGB	0.0 (negative control)	388
		0.2	192

Methods: Test Subjects and Housing

- ***Xenopus laevis* embryos**
 - XENOPUS I, Dexter, Michigan, USA
 - Obtained at 3 days post-fertilization (dpf)
 - Exposures initiated at 8 dpf
- **Housing**
 - 8 tanks (in 4 clusters) per E2 treatment group
 - 25 tadpoles / tank
 - Flow-through systems, E2 dosed by dilution in tank water
 - Biological loading rate < 1g / L / day
- **Terminated at either:**
 - Nieuwkoop and Faber (NF) Stage 66 complete tail resorption, end of metamorphosis

OR

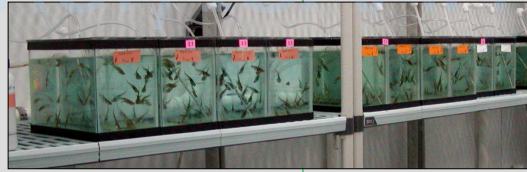
 - 81 (or 83) days post-fertilization (dpf), whichever came first



Methods: Endpoints Assessed

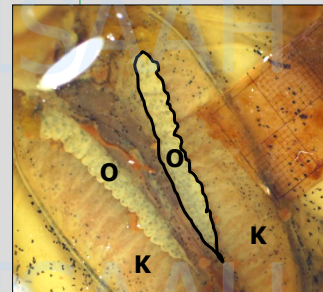
- **In life**

- General health
- Feeding
- Swimming behavior
- Survival
- Age at completion of metamorphosis
- Tank E2 concentrations (1 – 2 X / week)



- **Post-mortem**

- Snout-to-vent length
- Body weight
- Gross gonadal morphology
- Gonad area (digital gross photographs)
- Gonad histopathology



Gonad Area Measurement

- **Randomized, blinded, GLP**

Methods: Endpoints Assessed

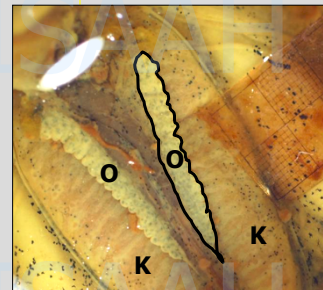
- **In life**

- General health
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Gonad Area Measurement

- **Gonad histopathology**

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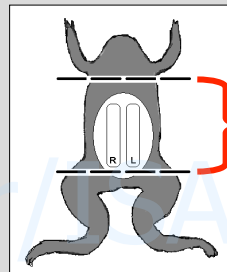


Methods: Necropsy and Gross Trimming



Necropsy Performed Using Dissecting Microscope and Microdissection Instruments

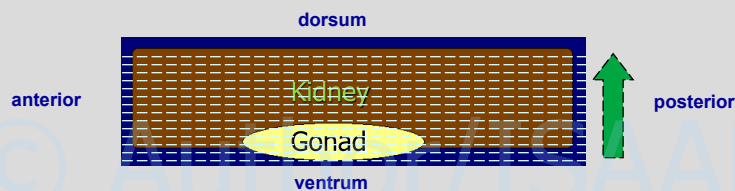
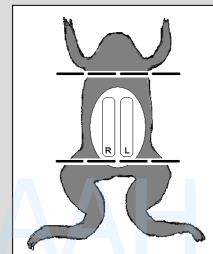
- Removed ventral body wall and abdominal viscera
- Fixed in Bouin's, rinsed → 10% NBF (formalin)
- Removed anterior and posterior segments



only utilized the trunk segment

Methods: Embedding and Microtomy

- Trunk segment embedded in paraffin
- Positioned for horizontal sectioning from ventral to dorsal surface
- 4-6 micron sections cut at 12 micron intervals
- Provided complete assessment of gonads and kidneys



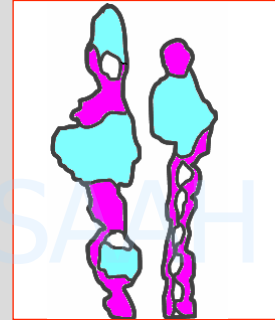
Visual correlation of gross and histologic findings



Gross Photograph



Histologic Section



Schematic Drawing

**Mixed Sex Gonads From a Frog
Exposed to 17 β -estradiol (E2)**

■ = testis

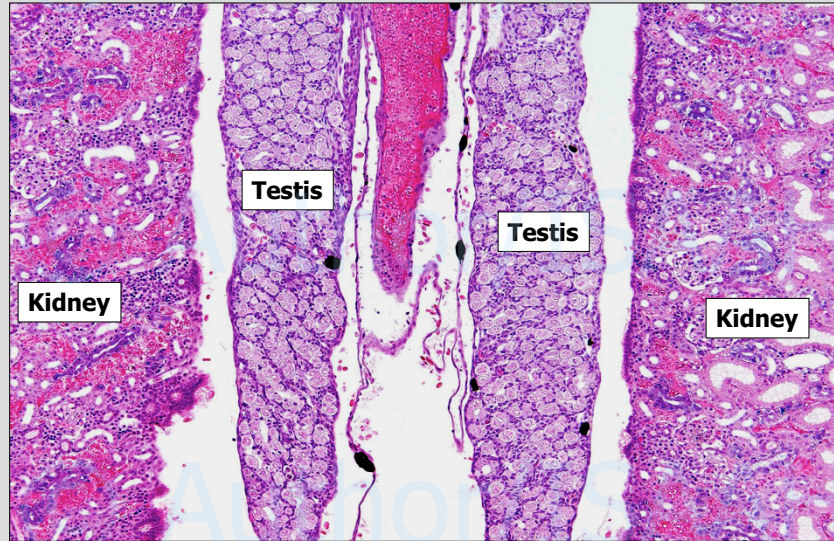
■ = ovary

Methods: Gonad Pathology

- Established diagnostic criteria and terminology

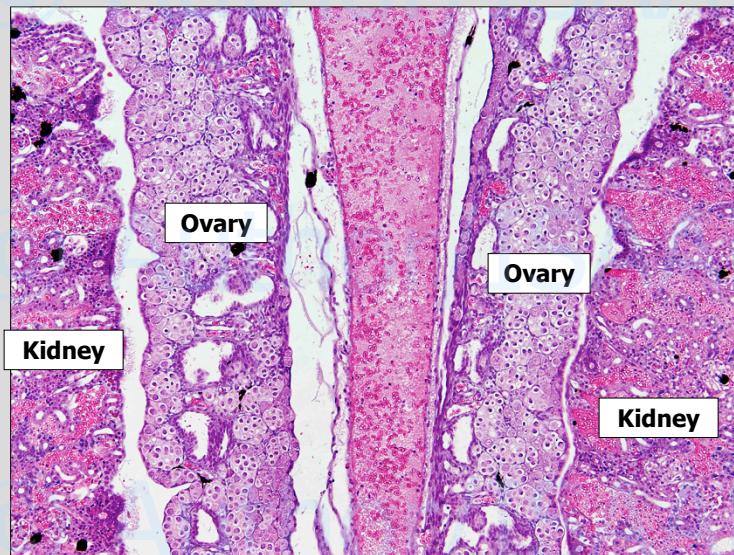
Gonad Phenotype	Histopathologic Diagnosis	Terminology and Criteria
Testis, Ovary	Angular Deformity	A malformation or deformation of the gonad that creates an angle in the axial plane.
Ovary	Atresia, Increased	An increase in the number of degenerating oocytes as compared to expected.
Testis, Ovary	Bifurcation	A malformation characterized by the non-artifactual splitting of one pole of a gonad into two distinct branches, creating a "Y"-shaped appearance. This change should not be confused with the single lateral protuberance that is normally present at the proximal 1/3 of the post-metamorphic testis.
Testis, Ovary	Decreased Germ Cells	A relative decrease in the density of germ cells as compared to a gonad that is packed with germ cells, this change is often observed as bare areas in, and thinning of, the fibrous scaffolding of the post-metamorphic gonad.
Ovary	Decreased Melanophores	A decrease in the density of dark brown pigment-laden cells as compared to what would normally be expected. Because this change is subtle and often difficult to appreciate among multiple histologic sections, the diagnosis of decreased melanophores is often prompted and/or confirmed by necropsy findings and gross photographs.
Testis	Dilated Tubules	The presence of multiple, irregularly enlarged tubules within a testis.
Testis	Internal Melanophores	The presence of one or more small oval or polygonal cells with solid dark brown cytoplasmic staining, or large stellate cells with long cytoplasmic processes that contain medium brown granular pigment. By convention, internal melanophores are those that are located within the testis parenchyma at least one spermatogonial cell's width from the surface.
Testis, Ovary	Mixed Sex	The presence of opposite sex tissue in the gonad.
Ovary	Decreased Ovarian Cavity Size	A substantial reduction in the size of the central ovarian cavity, among all examined sections, as compared to expected.
Ovary	Increased Ovarian Cavity Size	A substantial enlargement in the size of the ovarian cavity, among all examined sections as compared to expected. In addition to being enlarged, an affected ovarian cavity may have a "ragged" irregular contour.

X. laevis Normal Testis*



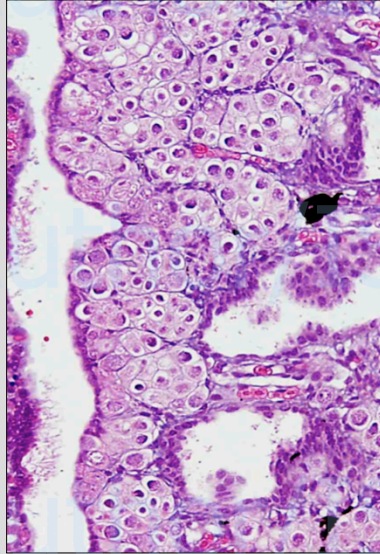
***Post-metamorphosis, Stage 66**

X. laevis Normal Ovary*, "Juvenile"



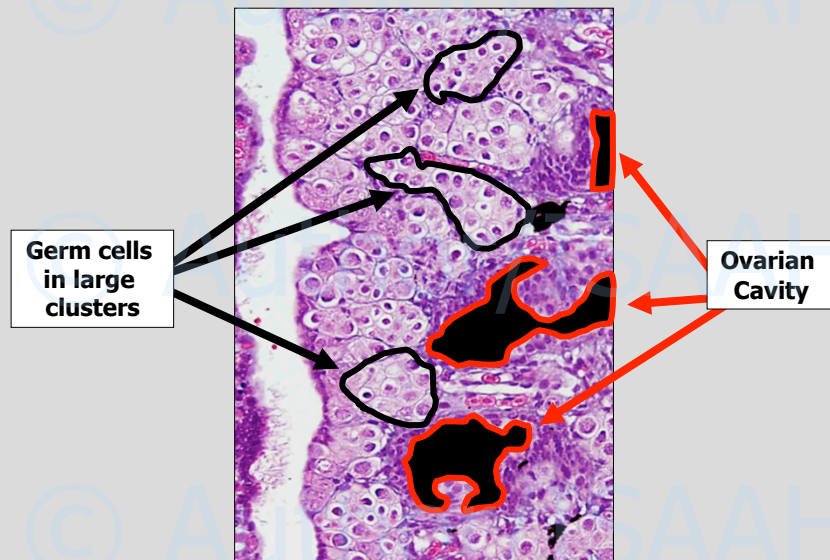
***Post-metamorphosis, Stage 66**

X. laevis Normal Ovary*, “Juvenile”



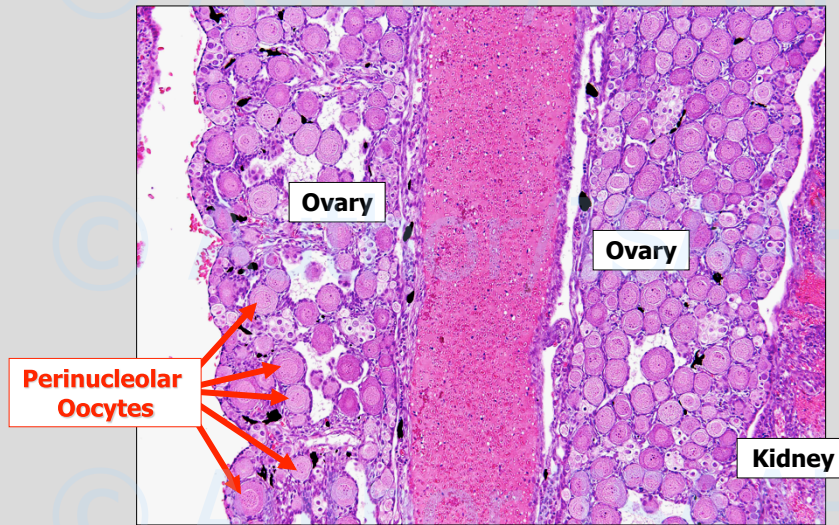
***Post-metamorphosis, Stage 66**

X. laevis Normal Ovary*, “Juvenile”



***Post-metamorphosis, Stage 66**

X. laevis Normal Ovary*, “Immature”



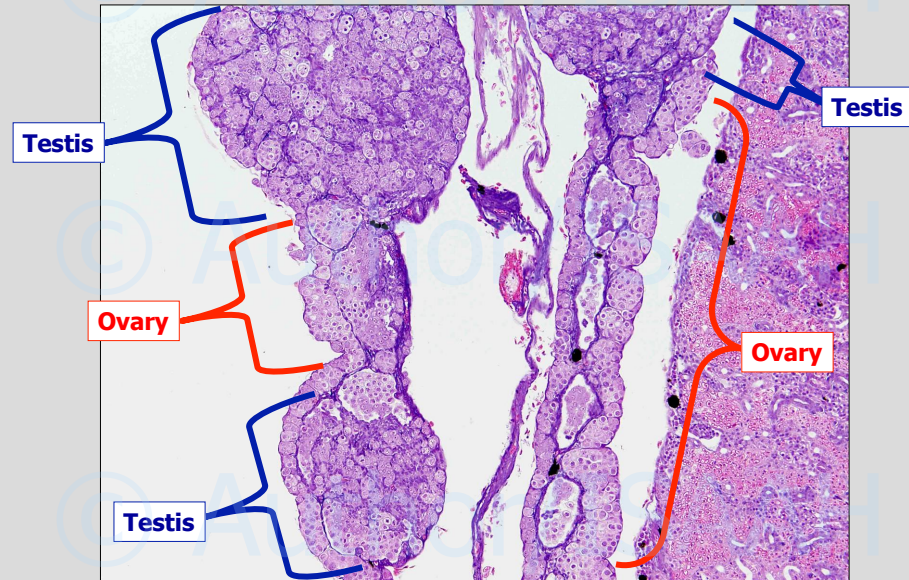
*Post-metamorphosis, Stage 66

Results: Sex Distribution and Mixed Sex

Experiment	E2 Nominal Conc., µg/L	Total Number	Histologic Gonadal Phenotype Frequency, % of Total			p-value
			Males	Mixed Sex	Females	
1	0	165	41.2	0.0	58.8	
	0.2	184	↓ 4.4 ***	↑ 9.8 ***	↑ 85.9 ***	<0.0001
	1.5	184	↓ 2.7 ***	↑ 4.9 **	↑ 92.4 ***	<0.0001
	6.0	165	↓ 0.0 ***	↑ 1.8 ns	↑ 98.2 ***	<0.0001
2A	0	187	49.2	0.0	50.8	
	0.2	193	↓ 18.1 ***	↑ 6.2 ***	↑ 75.7 ***	<0.0001
2B	0	388	46.9	0.0	53.1	
	0.2	192	↓ 21.9 ***	↑ 18.2 ***	↑ 59.9 ns	<0.0001

p-values: * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, ns = $p > 0.05$

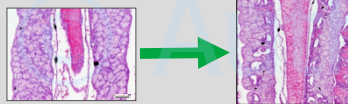
Results: $0.2 \mu\text{g/L E2} \rightarrow$ **Mixed Sex**



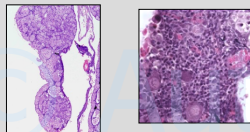
Results Interpretation – E2 Effects

Morphologic Finding

Altered sex distribution



Mixed sex gonads



Interpretation

Complete feminization of genotypic males

Partial feminization of genotypic males

Accuracy of gross observations of mixed sex

Experiment	Histologic Gonadal Phenotype	Total Number	Gross Gonadal Phenotypes		
			Male	Mixed Sex	Female
1	Male	81	78	2	1
	Mixed Sex	30	1	14	13
	Female	587	0	1	585

○ = false positive
○ = false negative

Gross observations not very accurate!

	Female	241	0	0	241
2B	Male	224	224	0	0
	Mixed Sex	35	16	8	11
	Female	321	0	0	321

Results: Other E2-Associated Histopath Findings

Phenotypic Males

- Dilated testis tubules
- “Dividing gonocytes”
- Segmental hypoplasia
- Internal melanophores

Phenotypic Females

- Enlarged ovarian cavity
- Decreased ovarian cavity



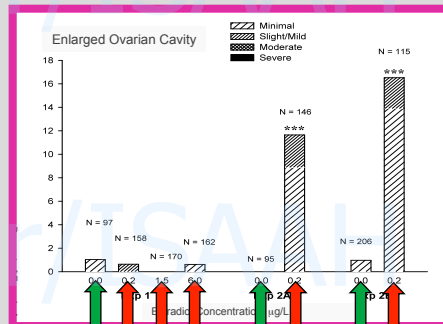
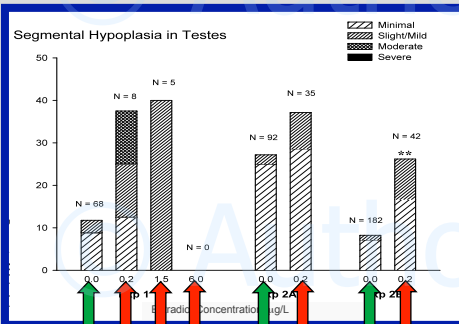
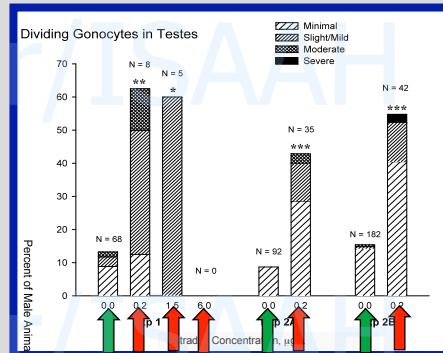
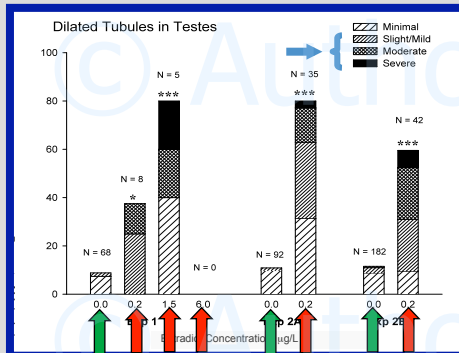
Results: Other E2-Associated Histopath Findings

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Phenotypic Females

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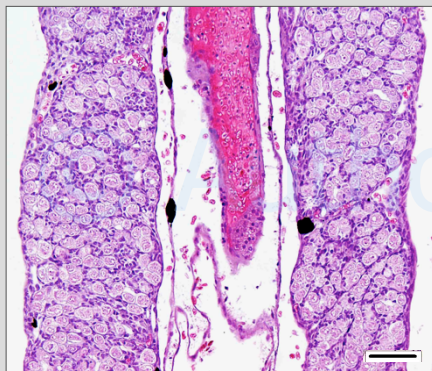


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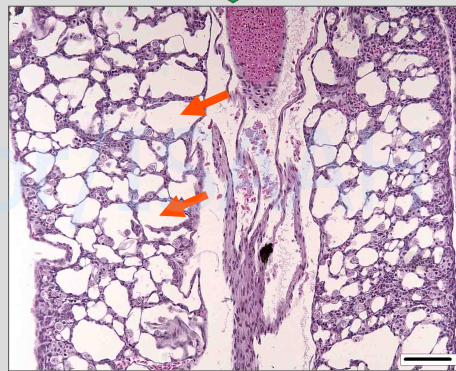
Results: Findings in Phenotypic Males

© Author/ISAAH

Results: 0.2 μ g/L E2 → Dilated Testis Tubules

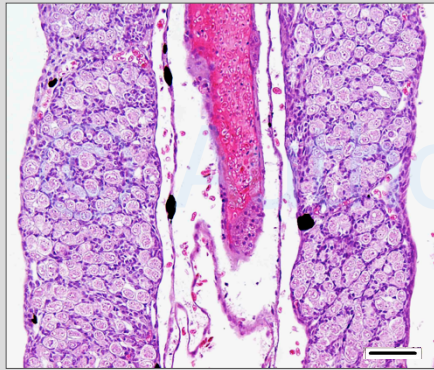


Negative Control Testis

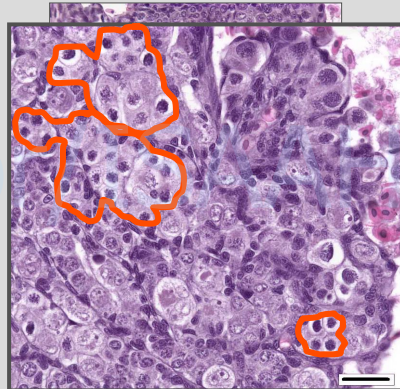


E2-Exposed

Results: 0.2 $\mu\text{g/L}$ E2 \rightarrow “Dividing Gonocytes”

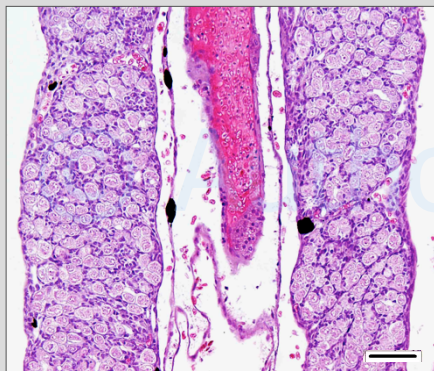


Negative Control Testis

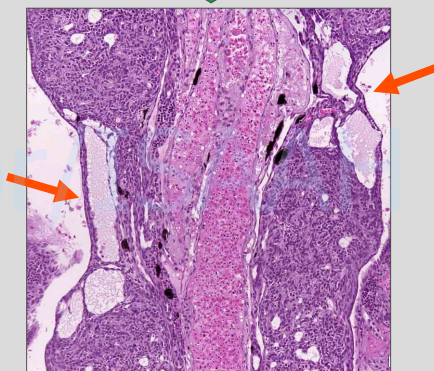


E2-Exposed

Results: 0.2 $\mu\text{g/L}$ E2 \rightarrow Segmental Hypoplasia



Negative Control Testis



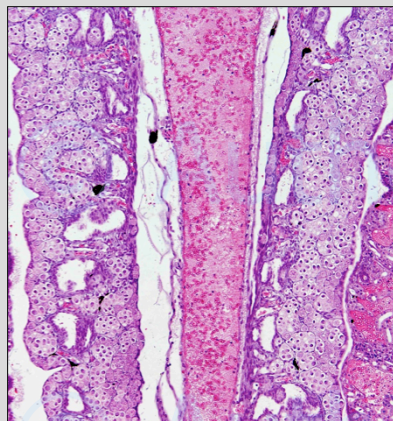
E2-Exposed

© Author/ISAAH

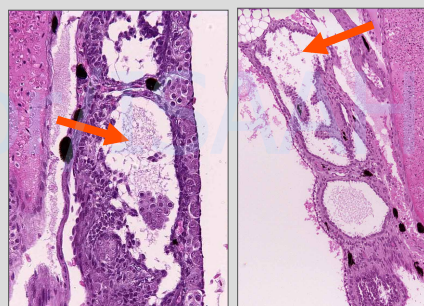
Results: Findings in Phenotypic Females

© Author/ISAAH

Results: 0.2 μ g/L E2 → Enlarged Ovarian Cavity



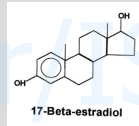
Negative Control Ovary



E2-Exposed (2 examples)

Morphologic Effects of E2

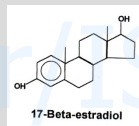
**Genotypic
Gender**



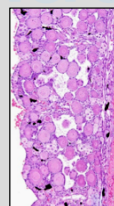
**Gonad
Phenotype**

Morphologic Effects of E2

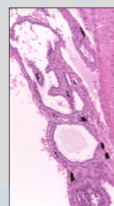
**Genotypic
Gender**



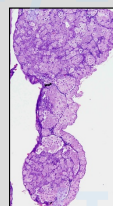
**Gonad
Phenotype**



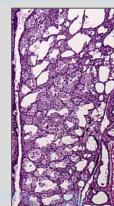
**Normal
Ovary**



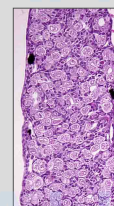
**Altered
Ovary**



**Mixed Sex
Gonad**



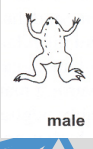
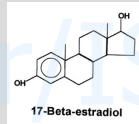
**Altered
Testis**



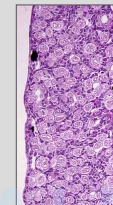
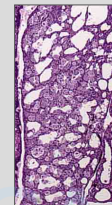
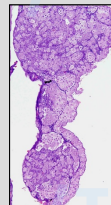
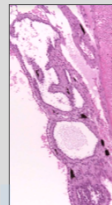
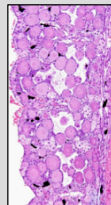
**Normal
Testis**

Morphologic Effects of E2

Genotypic
Gender

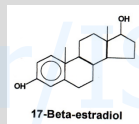


Gonad
Phenotype

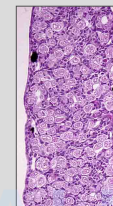
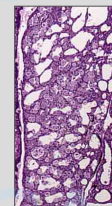
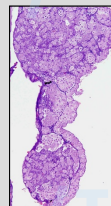
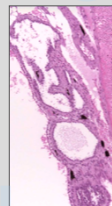
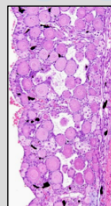


Morphologic Effects of E2

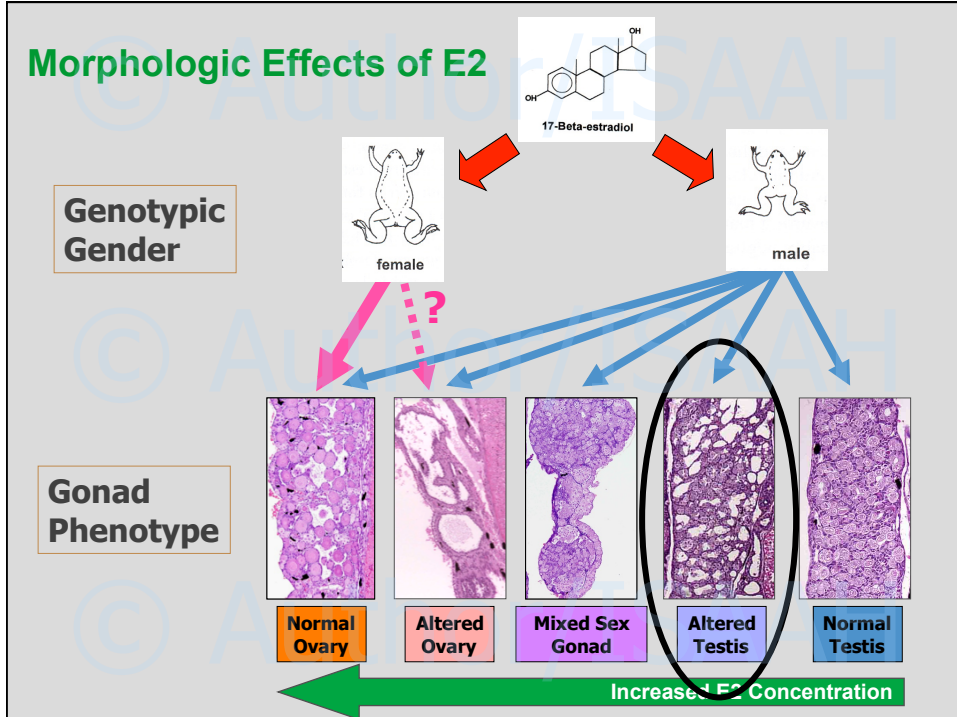
Genotypic
Gender



Gonad
Phenotype



Morphologic Effects of E2



Summary and Conclusions

- Developed standardized diagnostic criteria and terminology
- Determined useful EC_{50} for gonadal feminization
- Confirmed importance of histopathology as an endpoint for assessing E2 effects
- Examined relationship between ovarian development and age at completion of metamorphosis
- Identified novel histopath findings that are both sensitive to, and strongly associated with, E2 exposure:
 - Dilated tubules
 - Dividing gonocytes
 - Enlarged ovarian cavity



Publications

Lutz I, Kloas W, Springer TA, Holden LR, Wolf JC, Krueger HO, Hosmer AJ (2008) Development, standardization and refinement of procedures used for the evaluation of endocrine active compounds (EAC) on the development and sexual differentiation of *Xenopus laevis*. Anal Bioanal Chem 390:2031–2048.

Kloas W, Lutz I, Springer T, Krueger H, Wolf J, Holden L, Hosmer A (2009) Does atrazine influence larval development and sexual differentiation in *Xenopus laevis*? Toxicol Sci 107:376–384.

Wolf JC, Lutz I, Kloas W, Springer TA, Holden LR, Krueger HO, Hosmer AJ (2010) Effects of 17 beta-estradiol exposure on *Xenopus laevis* gonadal histopathology. Environ Toxicol Chem 29(5):1091-1105.