

## Development of the renal sexual segment in immature snakes: effect of sex steroid hormones

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### Abstract

The renal sexual segment (RSS) of immature Northern and Diamondback Water Snakes and Red-Sided Garter Snakes exhibited varying responses to testosterone or 17 $\beta$ -estradiol. In both male and female water snakes, kidney mass was not a reliable indicator of hormone treatment, whereas tubule diameter, epithelial height and number of sexual granules responded to hormone treatment. In male water snakes, either hormone initiated granule development by day 16; by day 23, only testosterone increased granule density. Female water snakes receiving either hormone exhibited a small number of granules by day 16; by day 23, granules increased only in Diamondback Water Snakes receiving testosterone. Hormones did not initiate RSS hypertrophy in female Red-Sided Garter Snakes. Tubule diameter and epithelial height of testosterone-treated males exhibited significant hypertrophy, while 17 $\beta$ -estradiol initiated significant increases in tubule diameter. Garter snakes initiated sexual granule development in response to hormone treatment with males exhibiting a greater response than females and testosterone stimulating a greater response than 17 $\beta$ -estradiol. Sex steroids appear to mimic sexual maturity in immature snakes initiating RSS development. Whereas the RSS of adult males respond to testosterone, our data suggest specific changes in the RSS of females during maturation effectively negates the effect of 17 $\beta$ -estradiol evident in immature female RSS.

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### 1. Introduction

The renal sexual segment (RSS) of the reptilian kidney is a hypertrophied region of the nephron, described initially by Gampert (1866) and was subsequently found to occur only in male squamate reptiles (Reguad and Policard, 1903). Reiss (1923) was first to report that the RSS exhibited a seasonal cycle that has been confirmed in numerous studies on both lizards and snakes (Kehl, 1944; Volsøe, 1944; Forbes, 1941; Fox, 1965; Arvy, 1969; Krohmer and Aldridge, 1985; Krohmer et al., 1987). Fox (1977) described the RSS as a single layered epithelium consisting of columnar cells with basally located nuclei. A character-

istic feature of these cells is the presence of large sexual granules scattered throughout the cytoplasm. The number and density of sexual granules contained in the cytoplasm can vary significantly depending on seasonal fluctuations and species (Fox, 1977; Krohmer et al., 1987).

Snakes, unlike lizards, do not demonstrate complete involution of the RSS. Studies on snakes have reported modest or no regression of the RSS during sexual quiescence (Volsøe, 1944; Pandha and Thapliyal, 1964; Krohmer and Aldridge, 1985; Krohmer et al., 1987; Clesson et al., 2002). The RSS of *Thamnophis* sp. has been found to be three to five times larger than the diameter of adjacent tubular regions (Fox, 1952; Bishop, 1959). However, once enlarged, the RSS exhibits only modest regression during reproductive quiescence (Krohmer et al., 1987; Clesson et al., 2002).

In snakes, the abundance and staining characteristics of the sexual granules provides a much more obvious seasonal

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cycle compared to lizards (Krohmer et al., 1987; Clesson et al., 2002). During maximum hypertrophy, the cytoplasm in the cells of the RSS is completely occluded by large, deeply staining granules. During quiescence the granules are distributed throughout the cytoplasm in small, condensed groups (Kühnel and Krisch, 1974).

The influence of sex steroids (specifically testosterone) on the control and regulation of the RSS in snakes was first

demonstrated by Bishop (1959). The RSS of bilaterally castrated males was reduced to approximately the same size as that of a normal female. This decrease was associated with a reduction in cell height and a marked reduction in sexual granules. When given exogenous testosterone (T), the RSS hypertrophied and was no longer significantly different from control animals (Bishop, 1959). Also in this study, the RSS of adult female garter snakes receiving

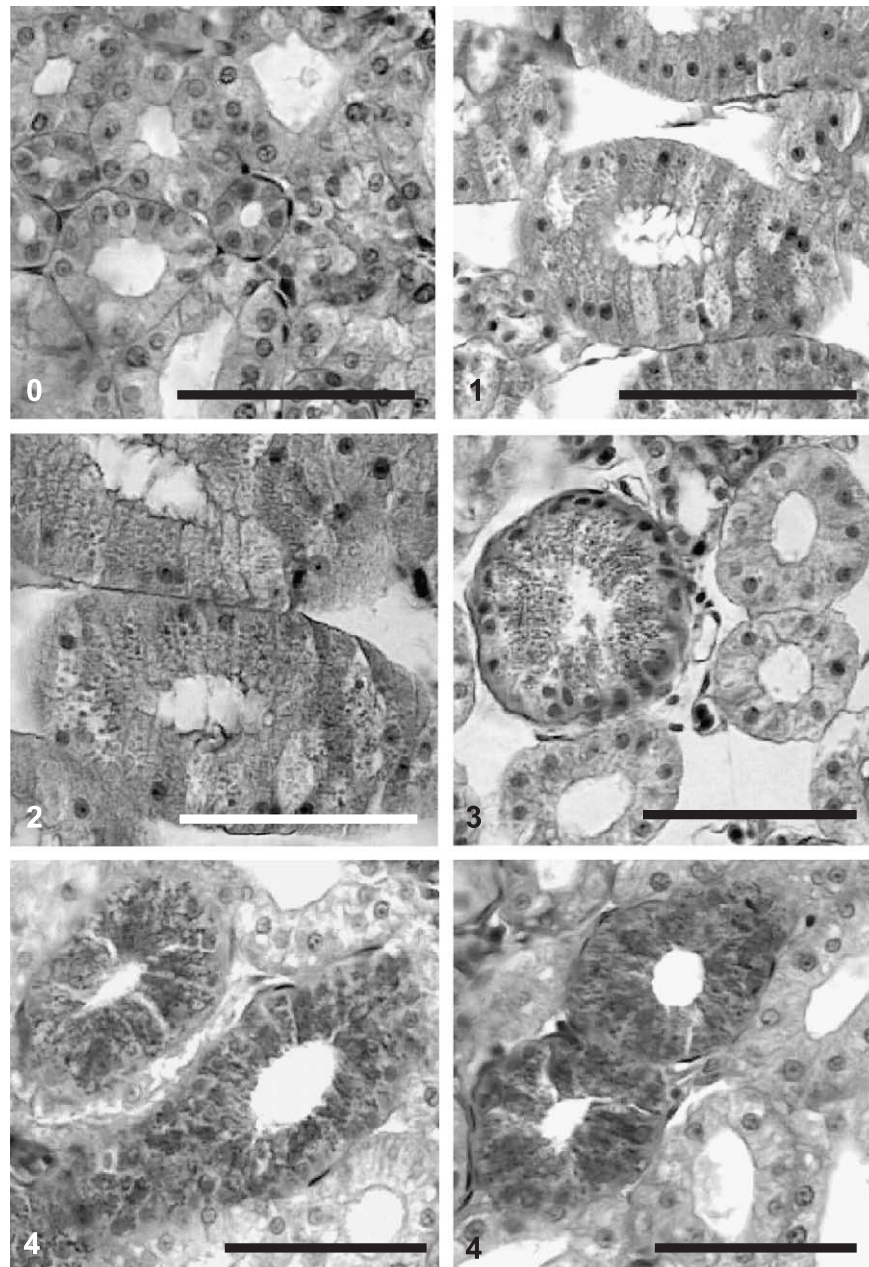


Fig. 1. This grouping of photomicrographs demonstrates the visual differences used to categorize sexual granule density (from 0 to 4) in our experimental tissues. Density of 0: Although the renal sexual segment (RSS) may be identifiable as in immature males, no observable hypertrophy is evident. Density of 1: RSS are noticeably hypertrophied and a few sexual granules maybe observed within the cytoplasm of a few epithelial cells. Density of 2: Hypertrophy of the RSS continues and sexual granules are now evident throughout the cytoplasm of all epithelial cells. Density of 3: Sexual granules have become numerous and are quite visible in the apical region of the cytoplasm, forcing the nucleus of epithelial cells towards the basal lamina. Density of 4: These last two photomicrographs demonstrate the maximum density of sexual granules within the cytoplasm. At this stage of development, sexual segment granules cause the cells to distend apically into the lumen and completely occlude the interior of the cells.

injections of T over a 60-day period underwent hypertrophy similar to that reported in males. Whereas the diameter of the RSS of T-injected females was not as large as the RSS of intact males, the female RSS was significantly larger than control females and contained a number of sexual granules. The RSS of male lizards that had been hypophysectomized regressed similar to individuals that had been castrated (Nobel and Greenberg, 1940; Reynolds, 1943; Pandha and Thapliyal, 1964). As in the castrated individuals, administration of T in hypophysectomized animals stimulated RSS hypertrophy.

The age at which functional differentiation of the RSS is initiated has been correlated with the time to reach sexual maturity. Examination of near-term male embryos and 2-day-old male *Thamnophis sirtalis* revealed that the RSS could be discerned, but was not fully developed and no sexual granules were present in either group (Bishop, 1959). In addition, the epididymis appeared to be immature and the testes pre-spermatogenic suggesting that steroidogenesis had not yet been initiated (Bishop, 1959). In the Lined Snake (*Tropidoclonion lineatum*), sexual granules could be identified in the RSS just 1 month after birth. An examination of the testes of these same animals revealed spermatogenesis had also been initiated (Krohmer and Aldridge, 1985). In the Queen Snake (*Regina septemvittata*), development of the RSS occurred in patches as the animals matured. By the end of the first full year, 10–15% of the RSS tubules contained a few sexual granules scattered evenly throughout the cytoplasm (J.J. Minesky, personal communication).

Although the RSS has been studied for more than 130 years, very little is known about the initial hypertrophy of the RSS tubules or development of sexual granules as a snake reaches sexual maturity. Therefore, this study utilized as its model system, immature animals from several snake

species to assess changes in morphology and development of the RSS during attainment of sexual maturity.

## 2. Methods

### 2.1. Animals

Immature Red-Sided Garter Snakes (*Thamnophis sirtalis parietalis*), Northern Water Snakes (*Nerodia sipedon sipedon*), and Diamondback Water Snakes (*Nerodia rhombifera rhombifera*) were born in captivity and housed in glass aquaria under a 12L:12D photic cycle and an ambient temperature of  $25 \pm 3$  °C. All animals were supplied with fresh water for drinking and soaking and fed twice weekly with a mixture of chopped earthworms and fish, supplemented with vitamins and minerals (Rep-Cal, Calcium and Vitamin D<sub>3</sub>, Rep-Cal Research Labs, Los Gatos, CA, USA). All animals were approximately 40–50 days of age at the beginning of the study.

### 2.2. Treatment and tissue collection

Thirty-six male and 36 female immature *N. sipedon sipedon* ( $n=18/\text{sex}$ ) and *N. rhombifera rhombifera* ( $n=18/\text{sex}$ ) were randomly assigned to one of three treatment groups. Animals were anesthetized with 1% Brevital Sodium (Wang et al., 1977) and implanted with a time-release tablet containing 5 mg testosterone (T), 5 mg 17 $\beta$ -estradiol (E<sub>2</sub>), or 5 mg cholesterol as control (Innovative Research of America). For each treatment/gender group,  $n=6$ . Following implantation, animals were maintained under the environmental conditions described previously. On day 16 and day 23, three males and three females of each species and treatment group were given a lethal dose of

Table 1

Comparison of hormone treatment on physiological parameters of the kidney and renal sexual segment of immature water snakes, *Nerodia s. sipedon* and *Nerodia r. rhombifera*

Group mean	Kidney Mass (g)		Tubular Diameter ( $\mu\text{m}$ )		Epithelial Height ( $\mu\text{m}$ )	
	♀	♂	♀	♂	♀	♂
<i>Treatment/Day</i>						
C/16 ( <i>N. s. sipedon</i> )	0.011 $\pm$ 0.001	0.012 $\pm$ 0.001	16.0 $\pm$ 1.11 <sup>1</sup>	19.3 $\pm$ 1.74 <sup>1,2</sup>	6.2 $\pm$ 0.4 <sup>1,2</sup>	8.3 $\pm$ 0.31 <sup>1,2</sup>
E <sub>2</sub> /16 ( <i>N. s. sipedon</i> )	0.013 $\pm$ 0.004	0.011 $\pm$ 0.001	22.2 $\pm$ 0.43 <sup>2</sup>	28.3 $\pm$ 0.84 <sup>1</sup>	10.3 $\pm$ 0.76 <sup>1,3</sup>	14.1 $\pm$ 0.92 <sup>1</sup>
T/16 ( <i>N. s. sipedon</i> )	0.011 $\pm$ 0.001	0.015 $\pm$ 0.001	27.5 $\pm$ 0.32 <sup>1,2</sup>	29.2 $\pm$ 0.33 <sup>2</sup>	14.1 $\pm$ 0.8 <sup>2,3</sup>	17.3 $\pm$ 0.67 <sup>2</sup>
C/23 ( <i>N. s. sipedon</i> )	0.011 $\pm$ 0.001	0.012 $\pm$ 0.001	19.4 $\pm$ 1.31 <sup>a</sup>	21.4 $\pm$ 0.95 <sup>a,b</sup>	9.4 $\pm$ 0.42 <sup>a,b</sup>	11.2 $\pm$ 0.78 <sup>a,b</sup>
E <sub>2</sub> /23 ( <i>N. s. sipedon</i> )	0.013 $\pm$ 0.001	0.013 $\pm$ 0.001	22.9 $\pm$ 1.00 <sup>b</sup>	35.2 $\pm$ 0.81 <sup>a</sup>	14.9 $\pm$ 0.54 <sup>a</sup>	18.0 $\pm$ 0.86 <sup>a</sup>
T/23 ( <i>N. s. sipedon</i> )	0.012 $\pm$ 0.001	0.011 $\pm$ 0.001	31.5 $\pm$ 0.67 <sup>a,b</sup>	38.8 $\pm$ 0.92 <sup>b</sup>	14.7 $\pm$ 0.39 <sup>b</sup>	17.9 $\pm$ 0.64 <sup>b</sup>
C/16 ( <i>N. r. rhombifera</i> )	0.011 $\pm$ 0.001 <sup>1</sup>	0.012 $\pm$ 0.001 <sup>1,2</sup>	20.1 $\pm$ 1.00 <sup>1</sup>	24.3 $\pm$ 1.81 <sup>1,2</sup>	9.0 $\pm$ 0.38 <sup>1</sup>	10.4 $\pm$ 0.55 <sup>1,2</sup>
E <sub>2</sub> /16 ( <i>N. r. rhombifera</i> )	0.012 $\pm$ 0.002 <sup>2</sup>	0.029 $\pm$ 0.001 <sup>1,3</sup>	20.3 $\pm$ 0.27 <sup>2</sup>	35.7 $\pm$ 1.70 <sup>1</sup>	10.4 $\pm$ 0.69 <sup>2</sup>	17.9 $\pm$ 0.88 <sup>1</sup>
T/16 ( <i>N. r. rhombifera</i> )	0.023 $\pm$ 0.001 <sup>1,2</sup>	0.023 $\pm$ 0.001 <sup>2,3</sup>	35.3 $\pm$ 0.70 <sup>1,2</sup>	36.8 $\pm$ 0.95 <sup>2</sup>	18.7 $\pm$ 0.55 <sup>1,2</sup>	17.6 $\pm$ 0.52 <sup>2</sup>
C/23 ( <i>N. r. rhombifera</i> )	0.014 $\pm$ 0.001 <sup>a,b</sup>	0.015 $\pm$ 0.001 <sup>a,b</sup>	20.7 $\pm$ 0.46 <sup>a</sup>	23.8 $\pm$ 1.70 <sup>a,b</sup>	10.5 $\pm$ 0.47	13.0 $\pm$ 0.24 <sup>a,b</sup>
E <sub>2</sub> /23 ( <i>N. r. rhombifera</i> )	0.023 $\pm$ 0.002 <sup>a</sup>	0.027 $\pm$ 0.002 <sup>a</sup>	20.3 $\pm$ 0.33 <sup>b</sup>	35.0 $\pm$ 1.80 <sup>a,c</sup>	10.3 $\pm$ 0.80	14.4 $\pm$ 0.38 <sup>a,c</sup>
T/23 ( <i>N. r. rhombifera</i> )	0.025 $\pm$ 0.003 <sup>b</sup>	0.026 $\pm$ 0.001 <sup>b</sup>	38.1 $\pm$ 0.92 <sup>a,b</sup>	42.5 $\pm$ 1.20 <sup>b,c</sup>	11.9 $\pm$ 0.19	21.3 $\pm$ 1.30 <sup>b,c</sup>

Post\_hoc Analysis: Significant differences between treatments are indicated by corresponding numbers (Day 16) or letters (Day 23).

For all significant differences,  $P \leq 0.04$ .

All group data were analyzed by ANOVA with  $\alpha=0.05$ . Data presented as mean $\pm$ 1 SEM.

Brevital Sodium after which their kidneys were collected, weighed, and prepared for paraffin histology.

Twenty-four immature *T. sirtalis parietalis* (12 male, 12 female) received a subcutaneous time-release implant of 4 mg T, 4 mg E<sub>2</sub> or a placebo tablet as control ( $n=4/\text{treatment/sex}$ ). Animals were housed under the conditions described above. Twenty-one days after implantation, all animals were given a lethal dose of Brevital Sodium; their kidneys were collected, weighed, and prepared for paraffin histology.

### 2.3. Light microscopy

The left kidney from each animal was rinsed in water, dehydrated in progressive alcohols, cleared in toluene, and embedded in paraffin. All tissues were sectioned on a rotary microtome at a thickness of 8–10  $\mu\text{m}$ , floated in a warm water bath and collected on gelatin-coated slides. Tissues were stained using a modified trichrome procedure of hematoxylin, Biebrich scarlet-orange II and fast green (Krohmer and Aldridge, 1985), dehydrated to xylene, and covered using Permount<sup>®</sup> mounting medium (Fisher Scientific).

Tubule diameter and epithelial height of 15 sexual segment tubules per animal were measured using an ocular

micrometer. Only tubules that appeared to be circular in cross-section were measured.

### 2.4. Sexual granules and RSS development

Analysis of sexual granule numbers and density was assessed by visible comparison (Krohmer et al., 1987). Briefly, with no knowledge of treatment, two individuals evaluated kidney sections from each study animal, rating the number and density of sexual granules on a scale of 0 to 4 (Fig. 1). If a major discrepancy in interpretation occurred, a third evaluator examined the tissues in question.

A percent of tissue exhibiting RSS development in the kidneys of Red-Sided Garter Snakes was determined by examining tissue on four slides selected at random from slides containing sections of the middle one-third of the tissue. A percentage for each animal was then calculated by counting the number of tubules exhibiting hypertrophy and/or granule development, divided by the total number of tubules  $\times 100$ .

### 2.5. Photomicrographs

Images were captured directly with a Moticam 350 digital camera. Images were downloaded to Photoshop 7.0

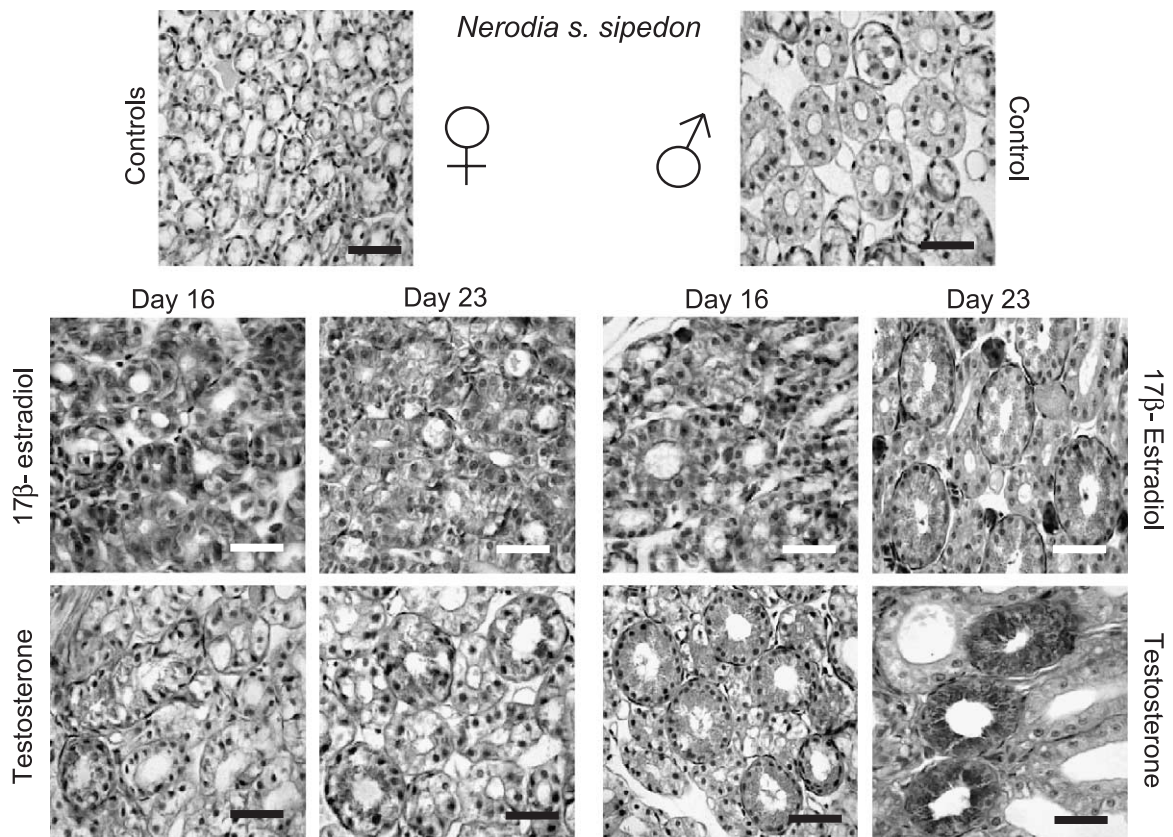


Fig. 2. This compilation of micrographs demonstrates changes in the renal sexual segment (RSS) of both male and female *Nerodia s. sipedon* in response to hormone treatment and time after implant. These photos demonstrate a gender difference in the RSS to implants of sex steroids in which males appear to develop to a much greater extent than females. While both T and E<sub>2</sub> are seen to stimulate RSS and sexual granule development, implants of T appear to have a greater effect on RSS and sexual granule development than E<sub>2</sub> in both males and females. Bars=50  $\mu\text{m}$ .

(Adobe Systems, Mt. View, CA, USA) to crop and adjust brightness and contrast. Figures were constructed by arranging and lettering the images with Microsoft Power-Point and saved as a jpeg.

### 2.6. Statistical analysis

A one-way analysis of variance (ANOVA) was used to examine differences among means. Significant effects were further delineated by the Fisher PLSD post hoc procedure. All group data were reported as mean  $\pm$  S.E.M. with the rejection level for all statistics  $p < 0.05$ .

## 3. Results

### 3.1. *N. sipedon sipedon*

Kidney mass of immature *N. sipedon sipedon* receiving implants of T, E<sub>2</sub> or a placebo (Table 1) was not significantly different in males on day 16 ( $F(2,6)=5.19$ ,  $p=0.06$ ) or day 23 ( $F(2,6)=1.08$ ,  $p=0.39$ ) or females on day 16 ( $F(2,6)=2.48$ ,  $p=0.17$ ) or day 23 ( $F(2,6)=3.70$ ,  $p=0.09$ ).

There was, however, a significant increase in RSS tubule diameter in both males and females receiving T or E<sub>2</sub> compared to control animals on day 16 ( $F(2,6)=23.65$ ,  $p < 0.001$ , ♂;  $F(2,6)=40.86$ ,  $p < 0.001$  ♀) and day 23 ( $F(2,6)=118.61$ ,  $p < 0.001$  ♂;  $F(2,6)=24.51$ ,  $p=0.002$  ♀; Table 1, Fig. 2). Epithelial height was also significantly greater than controls in T or E<sub>2</sub> implanted animals on day 16 ( $F(2,6)=46.49$ ,  $p < 0.001$  ♂;  $F(2,6)=15.04$ ,  $p=0.005$  ♀) and day 23 ( $F(2,6)=25.98$ ,  $p=0.003$  ♂;  $F(2,6)=31.54$ ,  $p < 0.001$  ♀; Table 1, Fig. 2).

### 3.2. *N. rhombifera rhombifera*

Male and female *N. rhombifera rhombifera*, receiving implants of T or E<sub>2</sub> had significantly greater kidney mass than controls on day 16 ( $F(2,6)=95.57$ ,  $p < 0.001$  ♂;  $F(2,6)=23.84$ ,  $p=0.002$  ♀) and day 23 ( $F(2,6)=37.37$ ,  $p < 0.001$  ♂;  $F(2,6)=17.36$ ,  $p=0.003$  ♀). The RSS tubule diameter of animals implanted with either T or E<sub>2</sub> was also significantly larger than control animals on day 16 ( $F(2,6)=21.40$ ,  $p=0.002$  ♂;  $F(2,6)=135.7$ ,  $p < 0.001$  ♀) and day 23 ( $F(2,6)=35.25$ ,  $p < 0.001$  ♂;  $F(2,6)=163.64$ ,  $p < 0.001$  ♀, Table 1, Fig. 3). The epithelial height of male *N.*

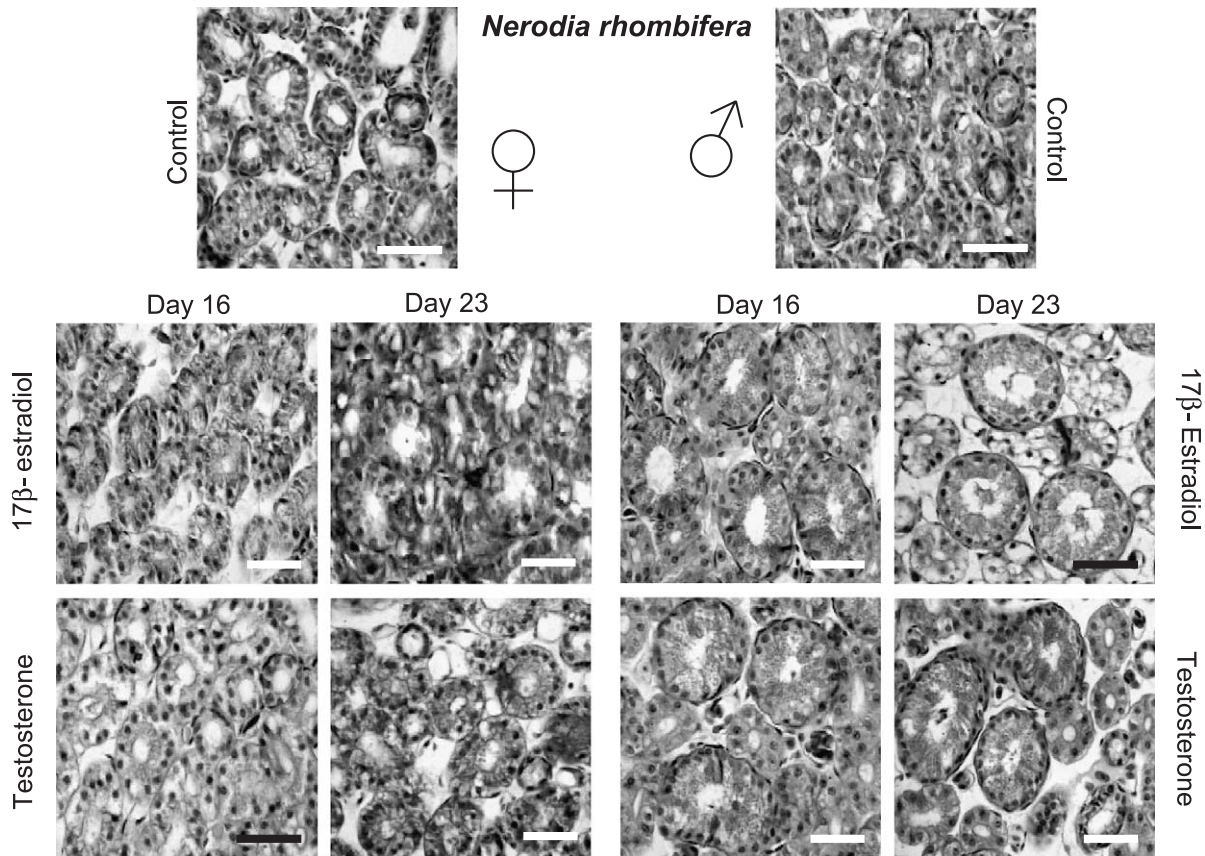


Fig. 3. This compilation of micrographs demonstrates changes in the renal sexual segment (RSS) of both male and female *Nerodia r. rhombifera* in response to hormone treatment and time after implant. These photos demonstrate a gender difference in the RSS to implants of sex steroids in which males appear to develop to a much greater extent than females. While both T and E<sub>2</sub> are seen to stimulate RSS and sexual granule development; implants of T appear to have a greater effect on RSS and sexual granule development than E<sub>2</sub> in both males and females. Bars=50  $\mu$ m.

Table 2

Density of sexual granules in the renal sexual segment of hormone treated immature water snakes (*Nerodia s. sipedon* and *Nerodia r. rhombifera*) and Red-sided Garter Snakes (*Thamnophis sirtalis parietalis*)

Species	Gender	Treatment	Days post-implant	
			16	23
<i>N. s. sipedon</i>	<b>male</b>	control	0	0
		17 $\beta$ estradiol	++	++
		testosterone	++	+++
	<b>female</b>	control	0	0
		17 $\beta$ estradiol	+	+
		testosterone	+	+
<i>N. r. rhombifera</i>	<b>male</b>	control	0	0
		17 $\beta$ estradiol	++	+++
		testosterone	++	++++
	<b>female</b>	control	0	0
		17 $\beta$ estradiol	+	+
		testosterone	+	++
21 Days post-implant				
<i>T. s parietalis</i>	<b>male</b>	control	0	
		17 $\beta$ estradiol	+	
		testosterone	+++	
	<b>female</b>	control	0	
		17 $\beta$ estradiol	+	
		testosterone	++	

Number and density of sexual granules represented by +.

*rhombifera rhombifera* was significantly different from controls on day 16 ( $F(2,6)=39.82$ ,  $p<0.001$ ) and 23 ( $F(2,6)=54.63$ ,  $p<0.001$ ), however, females receiving T or E<sub>2</sub> exhibited a significant increase in the RSS epithelial height on day 16 ( $F(2,6)=81.67$ ,  $p<0.001$ ) but not on day 23 ( $F(2,6)=2.38$ ,  $p=0.17$ ; Table 1, Fig. 3).

### 3.3. Sexual granule development in *Nerodia*

Implantation of T or E<sub>2</sub> initiated sexual granule development in both sexes of all species examined in this study (Table 2). In general, RSS development initiated by implantation of sex steroids appeared initially in areas of

the tissue associated with the greatest blood supply. In addition, development and density of sexual granules varied depending on treatment, sex, and time post-implant. The RSS in males of both species of water snake receiving implants of T or E<sub>2</sub> appeared to exhibit a greater number of sexual granules than female water snakes receiving the same treatment. While implantation of either T or E<sub>2</sub> in *N. sipedon sipedon* and *N. rhombifera rhombifera* appeared to initiate a similar response in sexual granule development on day 16, by day 23, implantation of T elicited a much greater response with increased numbers and density of sexual granules within the RSS epithelium (Table 2). In contrast, the number and density of sex granules in T implanted females of both species did not appear to increase between days 16 and 23.

### 3.4. *T. sirtalis parietalis*

No significant difference in kidney mass was observed in either male or female Red-Sided Garter Snakes implanted with T, E<sub>2</sub> or a placebo (Table 3). Female *T. sirtalis parietalis* implanted with either T or E<sub>2</sub> demonstrated quantifiable hypertrophy of the RSS tubule diameter and epithelial height, but ultimately were not significantly different from the control group (Table 3, Fig. 4). In contrast, male *T. sirtalis parietalis* exhibited significant hypertrophy in both tubule diameter and epithelial height. However, post hoc analysis revealed that males receiving T implants had a significantly larger tubule diameter and epithelial height, while animals receiving E<sub>2</sub> exhibited only significantly larger tubule diameters (Table 3, Fig. 4).

Renal sexual segment granules were found to develop in both male and female Red-Sided Garter Snakes implanted with either T or E<sub>2</sub>. However, the effect of T appeared to be greater than E<sub>2</sub> evident by the relative number and density of sexual granules (Table 2). In females, the percent of kidney tissue involved by the developing RSS was significantly greater in animals receiving T compared to

Table 3

Comparison of hormone treatment on physiological parameters in the kidney and renal sexual segment of immature Red-sided Garter Snakes (*Thamnophis sirtalis parietalis*)

Gender	Kidney Mass (mg)		Tubular Diameter ( $\mu$ m)		Epithelial Height ( $\mu$ m)		% Tissue Involved		
	♀	♂	♀	♂	♀	♂	♀	♂	
♀	$F(2,9)=2.82$ ; $p=0.11$		$F(2,9)=0.63$ ; $p=0.56$		$F(2,9)=1.0$ ; $p=0.39$		$F(2,9)=5.3$ ; $p=0.03$		
♂	$F(2,9)=1.33$ ; $p=0.31$		$F(2,9)=53.5$ ; $p<0.0001$		$F(2,9)=39.4$ ; $p<0.0001$		$F(2,9)=6.9$ ; $p=0.02$		
	Control	7.0 $\pm$ 1.0	9.0 $\pm$ 1.0	17.9 $\pm$ 1.19	25.7 $\pm$ 1.2 <sup>1,3</sup>	6.1 $\pm$ 0.41	9.8 $\pm$ 0.92 <sup>1</sup>	0.75 $\pm$ .48 <sup>1</sup>	3.8 $\pm$ 1.9 <sup>1</sup>
	17 $\beta$ Estradiol	9.0 $\pm$ 1.0	10.0 $\pm$ 1.0	24.7 $\pm$ 0.72	32.3 $\pm$ 0.31 <sup>1,2,3</sup>	10.6 $\pm$ 1.1	11.1 $\pm$ .11 <sup>2</sup>	8.3 $\pm$ 7.3	15.0 $\pm$ 2.6 <sup>2</sup>
	Testosterone	10.0 $\pm$ 1.0	12.0 $\pm$ 1.0	26.0 $\pm$ 0.91	43.5 $\pm$ 1.7 <sup>1,2</sup>	10.3 $\pm$ 0.85	16.5 $\pm$ .32 <sup>1,2</sup>	20.8 $\pm$ 2.2 <sup>1</sup>	36.3 $\pm$ 6.6 <sup>1,2</sup>

Post-hoc Analysis. Corresponding numbers indicate significant differences between treatments and also correspond to the level of significance identified here: 1,  $p\leq 0.03$ ; 2,  $p\leq 0.006$ ; 3,  $p=0.004$ .

All group data were analyzed by ANOVA with  $\alpha=0.05$ . Data presented as mean $\pm$ 1 SEM.

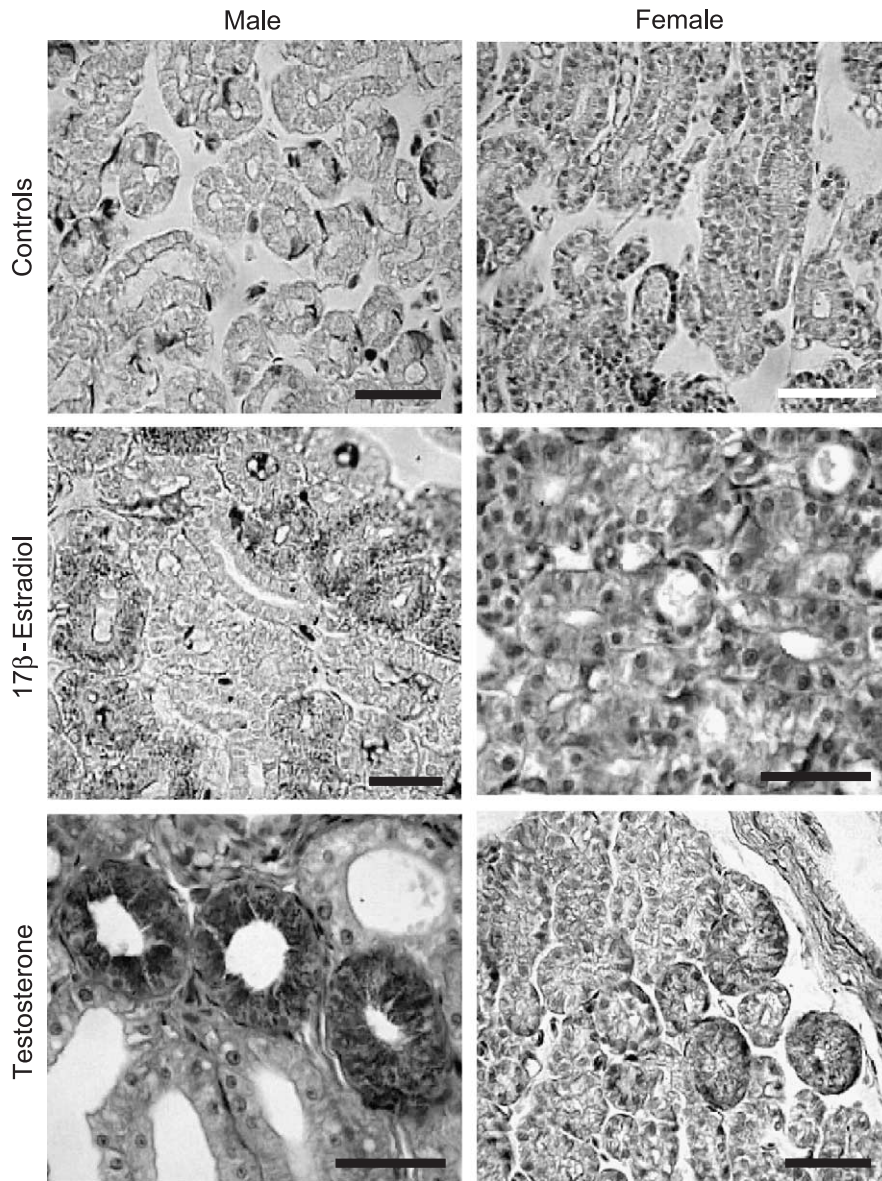


Fig. 4. This grouping of micrographs compares changes in the renal sexual segment (RSS) of male and female Red-Sided Garter Snakes (*T. sirtalis parietalis*) in response to 21 days of treatment with T, E<sub>2</sub> or a placebo. Bars=50  $\mu$ m.

control animals, whereas females receiving E<sub>2</sub> exhibited observable RSS development but were not significantly greater than controls (Table 3, Fig. 5). In males, there was a significant increase in the percent of tissue involved in RSS development in animals receiving either T or E<sub>2</sub>, however, not to the same extent (Table 3, Fig. 5).

In the current study, tubule diameter and epithelial height appeared to be greater in immature male controls compared to immature female controls. In actuality, only tubule diameter of *N. rhombifera rhombifera* was found to be significantly different between male and female controls ( $F(1,10)=16.6$ ;  $p=0.002$ ). However, in all species examined in this study, immature control males appeared to possess an identifiable, although non-functioning, RSS not evident in immature female controls (Fig. 6).

#### 4. Discussion

The RSS of squamate reptiles is a unique, sexually dimorphic structure that has been studied extensively for more than a century; however, very little has been written about the process of RSS tubule hypertrophy and the production of sexual granules as a snake reaches sexual maturity. The only reference pertaining to immature snakes found that the RSS could be identified in near-term embryos as well as 2-day-old garter snakes (Bishop, 1959).

In this study, implantation of sex steroids resulted in varying effects on the kidney and RSS depending on treatment, gender and species. Implantation of T or E<sub>2</sub> did not significantly alter kidney mass in *N. sipedon sipedon* or *T. sirtalis parietalis* of either sex. However, implantation of

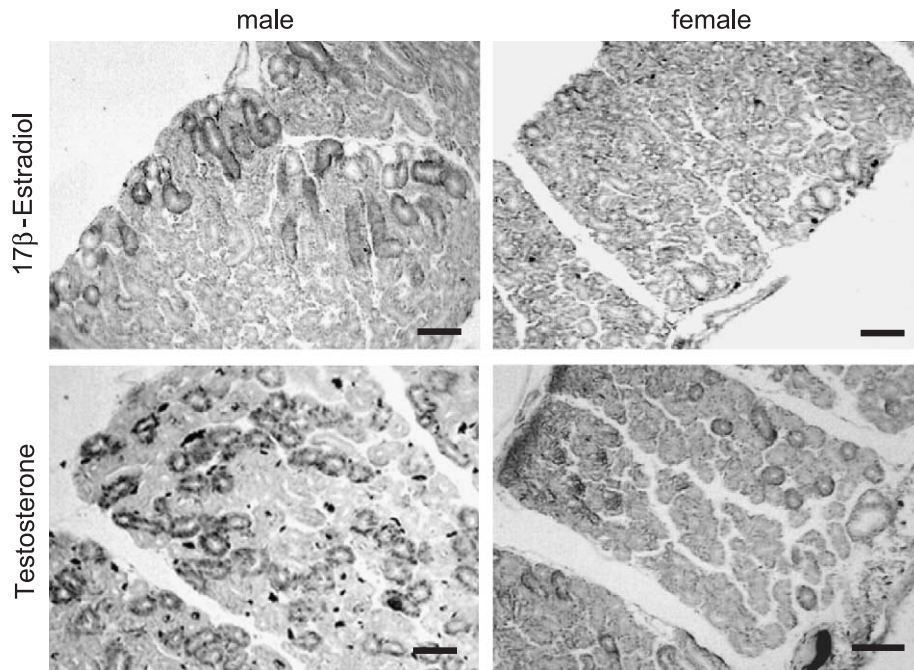


Fig. 5. These photomicrographs depict the varying response of renal sexual segment (RSS) development in the kidneys of immature Red-Sided Garter Snakes (*T. sirtalis parietalis*). At 21 days post-implant, males receiving T exhibited the largest percent of kidney tissue involved in RSS development ( $36.3 \pm 6.6\%$ ) compared to females receiving  $E_2$  exhibiting only  $8.3 \pm 7.3\%$  of the total tissue involved in RSS development. Bars=100  $\mu\text{m}$ .

T or  $E_2$  did result in significantly greater kidney mass in both male and female *N. rhombifera rhombifera*.

Seasonal changes in the RSS of squamate reptiles have been demonstrated in several studies. However, not until the work of Bishop (1959) was seasonal changes in the RSS shown to be correlated to seasonal changes in the testes. When the testes of the garter snake *T. sirtalis* were spermatogenically active and circulating levels of androgens elevated; the RSS tubule diameter was five times larger than non-RSS tubules (Bishop, 1959). In the Pacific Coast Garter Snake (*Thamnophis elegans terrestris*) tubule diameter of the RSS was reported to be three times larger than adjacent non-RSS tubules (Fox, 1952). Fox (1952) also reported that seasonal fluctuations in kidney mass were sexually dimor-

phic and that the kidneys of male snakes during spermatogenic activity were much larger than kidneys of comparable sized females. In addition, it was noted that the kidneys of females and immature males appeared red or pink whereas kidneys of an adult male at the height of tubule hypertrophy appeared pale yellow (Fox, 1952). This color difference has also been reported in *Natrix natrix* (Reguad and Policard, 1903).

In the present study, all animals implanted with T or  $E_2$  exhibited varying stages of RSS development. In these animals, RSS hypertrophy appeared to be initiated in highly vascular regions, eventually encompassing all RSS in the kidney. Our data appear to support the pattern of RSS development in the Queen Snake (*R. septemvittata*) where

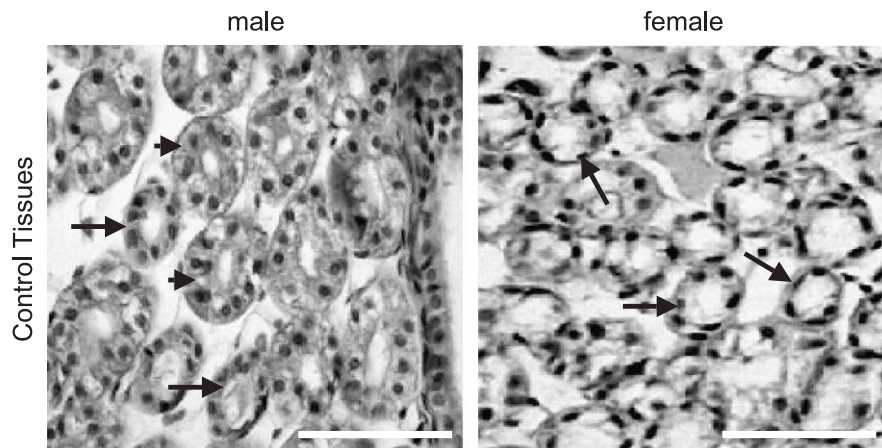


Fig. 6. Comparisons of kidney tissue from control male and female Red-Sided Garter Snakes (*T. sirtalis parietalis*). Note the presence of identifiable renal sexual segment (RSS) in control males. Arrows indicate non-RSS tubules; Arrow heads indicate the non-hypertrophied but identifiable RSS; Bars=50  $\mu\text{m}$ .



hypertrophy was reported to occur initially in isolated regions as the animals matured, with only 10–15% of the RSS hypertrophied by the end of the first year (J.J. Minesky, personal communication).

As with tubule hypertrophy, the development and density of sexual granules also varied depending on sex, treatment and time post-implant. Testosterone or E<sub>2</sub> initiated development of sexual granules in both sexes of *N. sipedon sipedon* and *N. rhombifera rhombifera*. Males, however, appeared to exhibit a greater number of sex granules than identically treated females. While T or E<sub>2</sub> initiated similar sex granule development in male *Nerodia* by day 16, animals sacrificed on day 23 exhibited an increased number and density of sex granules only in animals receiving T implants. In female *Nerodia*, T implants did not appear to increase granule numbers or density between days 16 and 23.

The age at which functional differentiation of the RSS is initiated has been associated with approaching or reaching sexual maturity. In *T. sirtalis*, the RSS can be recognized in near-term male embryos as well as 2-day-old males. However, at this early age, the RSS does not appear functional and no sexual granules are evident (Bishop, 1959). In the present study, immature males of each species examined demonstrated an apparent sexual dimorphism in tubules of the RSS. Although tubule diameter and epithelial height in control males appeared larger than female controls at the termination of this study, the only significant gender difference found was in the tubule diameter of immature *N. rhombifera rhombifera*. In addition, our data agree with Bishop (1959) and demonstrate that RSS tubules can be identified in other species of immature male snakes. However, no evidence of sexual granule development was observed in any of the control males.

In the Lined Snake (*T. lineatum*), spermatogenesis and corresponding androgenesis are initiated within the first month following birth (Krohmer and Aldridge, 1985). In response to this precocial spermatogenesis and androgen synthesis, the RSS began to develop and, prior to entering winter dormancy, a few sexual granules were observed (Krohmer and Aldridge, 1985). In the current study, our data support previous studies that have reported RSS development to be related to maturation and subsequent steroidogenic activity of the testes.

This study has examined the effects of sex steroids on the RSS of three species of immature snakes. We found that the RSS of both male and female immature snakes respond to sex steroids in a similar fashion but exhibit varying stages or intensity of development. Interestingly, if the RSS of immature female snakes hypertrophy in response to estrogen and testosterone as shown in this study, why then do adult females lack any degree of RSS hypertrophy? One answer, suggested by earlier studies, may involve the presence or absence of specific cellular receptors (Licht and Midgley, 1976a,b; Davidoff et al., 1980). Krohmer (1985) identified what appeared to be steroid receptors in the kidneys of immature *N. sipedon sipedon*. These receptors, however, did

not prove to be specific for T as suggested previously (Forbes, 1941; Bishop, 1959; Reddy and Prasad, 1970). Whereas the affinity of steroid receptors in the RSS of immature *N. sipedon sipedon* appeared greater for T and dihydrotestosterone (DHT), it was not significantly greater than the affinity for E<sub>2</sub> or progesterone. These data suggest the affinity of receptors in the RSS of immature *N. sipedon sipedon* appears relatively nonspecific to any specific steroid hormone (Krohmer, 1985).

In the current study, immature animals of both sexes exhibited RSS hypertrophy and sexual granule development following treatment with either T or E<sub>2</sub>. Therefore, our data suggests that receptors capable of binding both T and E<sub>2</sub> appear to be present and functional. However, absence of RSS hypertrophy and sexual granule development in adult females might be explained in several ways. First, the concentration of circulating sex hormones (specifically E<sub>2</sub>) in females may not be sufficiently elevated to stimulate RSS hypertrophy. Although no mechanism has been defined, presence of an intact female reproductive system appears to reduce or negate the effects of E<sub>2</sub> (D.D. Baleckaitis and R.W. Krohmer, unpublished data). Finally, although immature animals appear to exhibit nonspecific binding to sex steroids, at maturity, these same receptors in the RSS of adult snakes may become highly specific for androgens eliminating development in the adult female.

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