

# Germ Cell Depletion Does Not Alter the Morphogenesis of the Fetal Testis or Ovary in the Red-Eared Slider Turtle (*Trachemys scripta*)

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**ABSTRACT** In the red-eared slider turtle, *Trachemys scripta*, both prospective male and female gonads contain primitive cord structures at the time when the gonad first forms. Primordial germ cells arrive in the gonad and accumulate on the coelomic surface. If testis development is initiated, these cords develop further at the same time that germ cells migrate from the coelomic surface and become sequestered in the interior of the cords. In contrast, in the developing ovary germ cells proliferate in a defined cortical domain, while the primitive cords regress and form flattened lacunae in the medulla. Because of their intimate association with these developmental processes, we investigated whether germ cells were required in turtles to establish the morphology of the fetal testis and ovary. We present evidence that normal morphological development of the fetal gonad occurs in both sexes in *T. scripta* after germ cell depletion, suggesting a conservation of developmental mechanisms across vertebrates. *J. Exp. Zool. (Mol. Dev. Evol.)* 308B:236–241, 2007. © 2006 Wiley-Liss, Inc.

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Many reptiles do not have identifiable sex chromosomes and rely on temperature-based sex determination (TSD) instead (Bull, '80; Janzen and Paukstis, '91; Pieau, '96). In TSD, the temperature at which embryos are incubated determines the sex (Bull, '80; Pieau et al., '99). There is a critical window of development, termed the thermo-sensitive period (TSP), during which temperature exerts its effects on sex. After the TSP, a change in incubation temperature does not alter the sexual fate of the animal. The red-eared slider turtle, *Trachemys scripta*, utilizes a system of TSD where a 26°C incubation temperature produces males and 31°C produces females (Bull and Vogt, '81). Incubation at temperatures between these two optima yield varying percentages of males and females (Crews et al., '91; Mrosovsky and Pieau, '91).

As in other reptiles, *T. scripta* fetal gonads incubated at the male- or female-producing temperatures have identical morphology at the beginning of the TSP (Pieau, '74; Crews et al., '91; Smith and Joss, '93; Pieau et al., '99). At this point, the gonads of embryos incubated at male-

and female-producing temperatures contain primitive cords (sex cords). During testis differentiation, these cords grow and elaborate into the future seminiferous tubules, whereas during ovary differentiation the primitive cords flatten, regress, and form the medullary lacunae of the adult ovary (Pieau et al., '99; Yao et al., 2002). This is notably different from the development of the mouse gonad where cord structures form only in the male after sex determination has occurred. In turtles, the cords are formed from contributions from two cell populations: the mesonephros and the coelomic epithelium (Pieau et al., '99; Yao et al., 2004). The epithelium of the Malphigian corpuscles in the mesonephros and the lateral coelomic epithelium of the gonad proliferate and add to the cords from the "bottom up" while the

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distal coelomic epithelium proliferates and works “top down” to generate these structures.

In the beginning of the TSP, the germ cells are located at the surface of the gonad in the coelomic epithelium. At male-producing temperatures, the germ cells become enclosed inside the cords at the same time that the cords are expanding and separating from the surface of the gonad. In contrast, at female-producing temperatures the germ cells remain in the cortex, while the cords flatten and regress to the medulla which becomes separated from the cortex by a well-defined basement membrane. In mammals, loss of germ cells does not disrupt testis development, although delays in the organization of testis cords have been reported (reviewed in McLaren, '91). However, loss of germ cells is associated with a failure of ovarian follicle formation and complete disruption of the morphology of the ovary after birth in mammals (Merchant-Larios and Centeno, '81; Guigon et al., 2005). In zebrafish, loss of germ cells has recently been shown to lead to female-to-male sex reversal of adult fish (Slanchev et al., 2005).

Because of the intimate association between the expansion of the primitive cords and the envelopment of germ cells into these cords in *T. scripta*, we hypothesized that maturation of the cords in the testis depended on the presence of germ cells. Additionally, we hypothesized that germ cells were required in the developing *T. scripta* ovary to establish the primary architecture of the ovary, represented by the morphological boundary between the cortex and the medulla. To investigate this model, we introduced busulfan into *T. scripta* embryos to ablate germ cells, and examined the gonads during the TSP and just prior to hatching. We found that germ cell depletion does not alter the morphological development of the fetal testis or ovary in *T. scripta*. Despite significant differences in the mechanisms of fetal testis and ovary morphogenesis between reptiles and mammals, these processes appear to be independent of the presence of germ cells in both vertebrates.

## MATERIALS AND METHODS

### *Study species*

The red-eared slider turtle, *T. scripta*, is an aquatic turtle which is widely distributed throughout North and South America. Females lay 2–3 clutches per year between April and July. Clutches contain approximately 7–10 eggs each. Eggs incubated at 26°C hatch as males while eggs

incubated at 31°C hatch as females (Bull and Vogt, '81; Crews et al., '91). Although not endangered, these animals are regulated by local law. Therefore, permission was obtained from the Louisiana Department of Agriculture and Forestry before collection. *T. scripta* eggs laid within a 36 hr period were obtained within 2 days from Kliebert's Turtle and Alligator Tours (Hammond, LA). Eggs were immediately divided into two groups; one group incubated at 26°C and the other at 31°C. Developmental staging was determined by dissecting 2 eggs per group every other day and compared to embryonic stages established by Yntema ('68). Eggs were opened and embryos were immediately decapitated and placed into phosphate buffered saline (PBS). Shell length was measured from the longest point rostral to caudal and compared using the students *t*-test.

### *Treatments*

Experimental eggs were individually weighed to determine mass and candled using a 100 W bulb to identify the location of the blood ring at stage 15. Busulfan-treated eggs were given a 20 mg/kg dose in 20 µl of ethanol. Control eggs were mock treated with 20 µl of ethanol. Treatments were applied to the surface of the eggs tracing the location of the blood ring at stage 15 (Table 1).

### *Immunohistochemistry*

Gonads were processed for immunocytochemistry as previously described (Yao et al., 2004). Briefly, embryos were dissected in PBS and

TABLE 1. Incubation temperature, stage examined, shell length, and germ cell numbers of *Trachemys scripta* eggs treated with or without busulfan

Temp. (°C)	Stage	n	Busulfan?	Shell length (mm)	GC# <sup>1</sup>
26♂	17	8	N	7.8±1.1	69.4±12.4
31♀	17	8	N	8.1±1.3	84±19.4
26♂	17	8	Y	6.7±1.6	4.1±1.3
31♀	17	9	Y	7.5±0.8	8.8±3.9
26♂	18	8	N	8.3±2.5	78±25.6
31♀	18	8	N	8.6±1.9	89.4±42.2
26♂	18	8	Y	7.8±3	1.6±2.4
31♀	18	8	Y	8.2±1.4	2±1.7
26♂	23	8	N	31.5±3.5	431.6±21.3
31♀	23	7	N	30.8±2.8	585.4±64
26♂	23	8	Y	28.5±4.1	2.7±1.5
31♀	23	8	Y	29.2±5.3	1.5±2.8

Shell length and germ cell number expressed as mean±SE (range).

<sup>1</sup>Total germ cells per 10 optical sections per gonad.

gonads were removed and fixed in 4% paraformaldehyde overnight at 4°C. The next day they were washed in PBS and placed into blocking solution (3% BSA, 10% goat serum, 0.1% Triton X-100) for 1 hr at room temperature. Gonads were incubated with either rabbit anti-mouse VASA homolog (1:50, Abcam) or rabbit anti-laminin-1 antibody (1:200, the kind gift of Harold Erikson) in blocking solution for 12 hr at 4°C. Gonads were rinsed three times for 30 min in washing solution, and incubated for 12 hr at 4°C in Cy5-conjugated anti-rabbit secondary antibody (1:500, Jackson Immunochemicals) and Syto-13 DNA dye (1:2,000, Invitrogen) in blocking solution. Gonads were rinsed in PBS, cleared, and mounted in 2.5% DABCO in glycerol, and imaged using the Zeiss LSM 410 confocal microscope. Cord circumference was measured by tracing the basement membrane in optical sections. Germ cells were identified by nuclear morphology and by VASA antibody staining. Total germ cell numbers per gonad were counted via optical sectioning and compared using the students *t*-test. At stage 23, presumptive Sertoli cells were identified by their compact nuclei and localization to the Sertolian epithelium of the testis cords. Total numbers of presumptive Sertoli cells were counted in all testis cords in 10 optical sections per testis. Somatic cells in the cortex of the ovary at stage 23, which were identified by their compact nuclei and localization above the basement membrane, were counted in 10 optical sections per ovary. Numbers of somatic cells in testes and ovaries were counted in optical sections and compared using the students *t*-test.

## RESULTS AND DISCUSSION

To determine whether germ cells are required to establish the morphology of fetal testes and ovaries in *T. scripta*, we treated embryonic stage-15 eggs with busulfan, a well-characterized germ cell toxin. We tested a range of busulfan doses between 10 and 80 mg/kg, and determined that a dose of 20 mg/kg was the maximum dose which effectively depleted germ cells, yet showed minimal effects on embryo survival.

Eggs were divided into two groups, either treated with busulfan dissolved in ethanol, or mock treated with ethanol alone as a control. In order to establish that busulfan did not cause confounding developmental deformities by the end of the experiment, eggs were allowed to progress until they exhibited signs of hatching, then were immediately sacrificed and examined (Fig. 1).

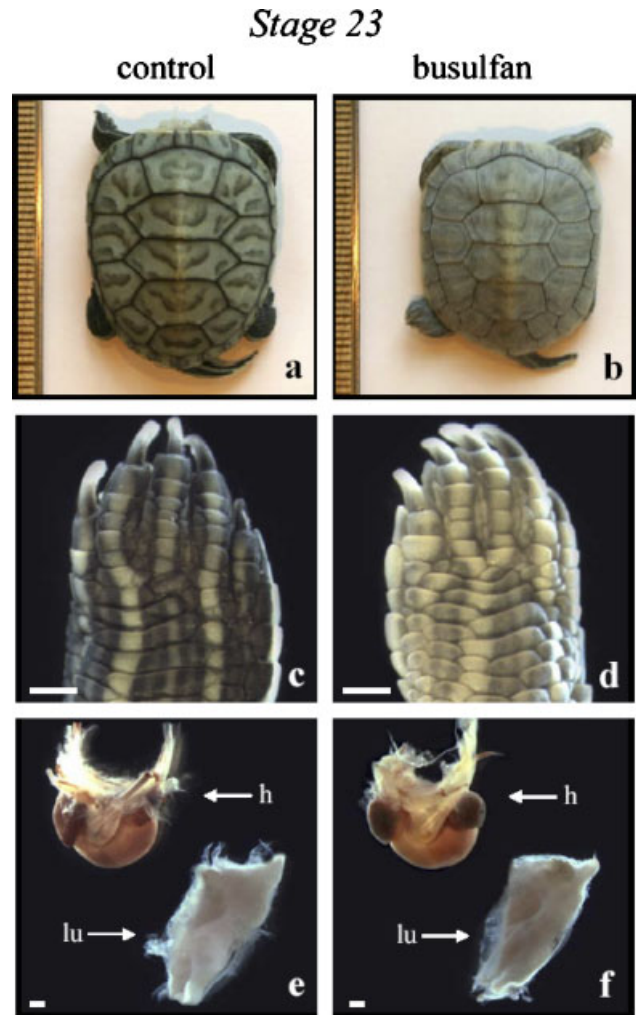


Fig. 1. Gross anatomy of *Trachemys scripta* embryos at stage 23. (a, c, e) Control treatments and (b, d, f) busulfan treatments (20 mg/kg). (a, b) Bodies of embryos next to ruler measuring millimeters. (c, d) Foreclaws and (e, f) internal organs of mock or busulfan-treated embryos. Images representative of the  $n = 8$  for each panel. Scale bars (c-f) = 1 mm; hr—heart; lu—lung.

All embryos treated with busulfan appeared smaller in size (Fig. 1a and b) but this difference was not significant compared with controls (Table 1). There was no observable difference in general morphology of the body, limbs, and internal organs between control and busulfan-treated embryos (Fig. 1). Importantly, the gross morphology of the gonads and mesonephroi was not different between the two groups (Fig. 2). From these data, we concluded that this regimen of busulfan treatment did not affect the overall development or morphology of the embryos.

We also found that embryos treated with busulfan were uniformly less pigmented than

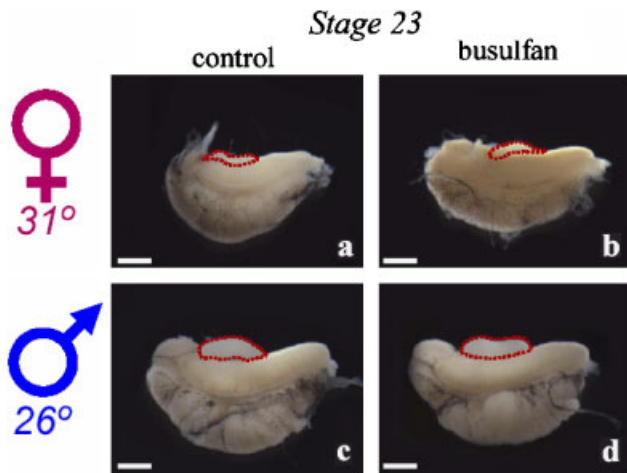


Fig. 2. Busulfan treatment did not affect gross morphology of the gonads or mesonephroi of *Trachemys scripta*. Gonads and mesonephroi of stage 23 embryos from (a, c) control and (b, d) busulfan treated. Gonads are outlined with a red dotted line. Images representative of the  $n = 8$  for each panel. Scale bars = 1 mm.

controls. Given that the busulfan was administered at a stage before pigmentation begins, we suggest two explanations. First, the precursors of the pigment cells, melanoblasts, may be present at the time of the application (stage 15) and may be affected by busulfan. In fact, busulfan treatment may affect an array of stem cell populations. For example, we speculate that these turtles are also anemic. Alternatively, there may be residual busulfan in the eggs at the stage when pigmentation begins (stage 21) and could affect melanin production at that stage.

To ascertain that the busulfan treatments had indeed eliminated germ cells, we counted the number of germ cells in gonads at two stages during the TSP (stages 17 and 18) and after the TSP (stage 23) immediately prior to hatching. Germ cells were identified by their distinct nuclear morphology, which is large and round with distinguishable nucleoli (e.g., Fig. 5a and c; arrows). There was a significant reduction in numbers of germ cells at stages 17 and 18 in the busulfan-treated gonads at both male- and female-producing temperatures (Table 1). By stage 18, germ cells were almost entirely ablated (>95%) and did not recover through stage 23 (Table 1). It remained formally possible that germ cell nuclear morphology was altered by the busulfan treatment. To exclude this possibility, we used a molecular marker of germ cell identity, VASA, to confirm germ cell loss in our treated samples (Fig. 3). From these data, we concluded that the

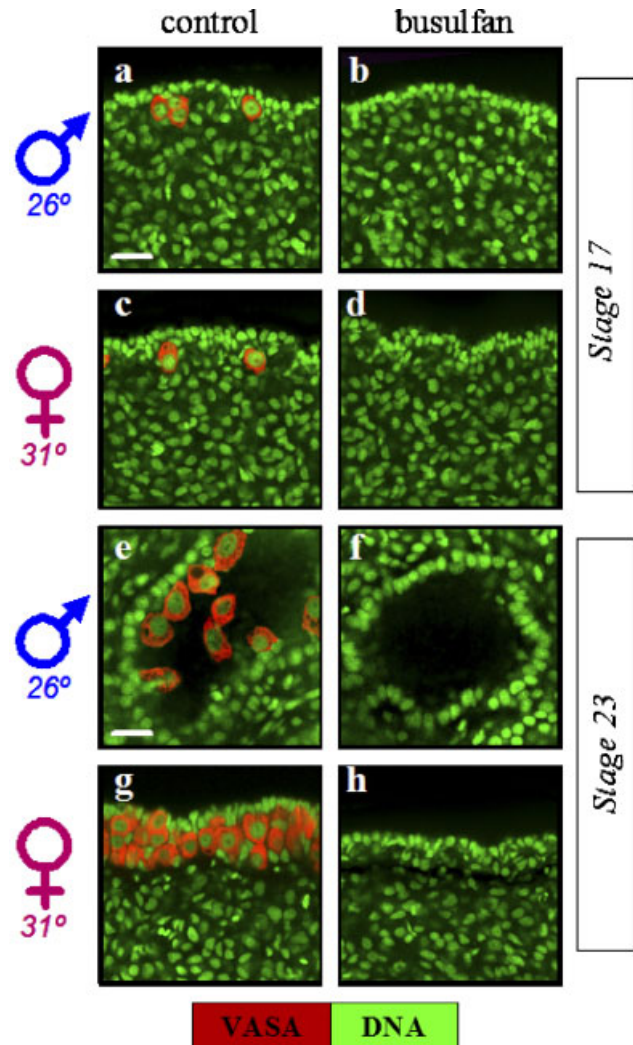


Fig. 3. Germ cells are depleted by busulfan treatment. Gonads from (a, c, e, g) control and (b, d, f, h) busulfan-treated embryos at (a–d) stage 17 and (e–g) stage 23. Germ cells, labeled with anti-VASA antibodies (red), are absent from busulfan-treated gonads. Images representative of the  $n \geq 6$  for each panel. Scale bars = 100  $\mu\text{m}$ .

dose of busulfan was sufficient to eliminate enough germ cells from the early gonads to test our hypothesis.

Next, we examined the cellular morphology of *T. scripta* gonads at the beginning of the TSP, stage 17 (Fig. 4). The structure of the primitive cords in gonads at male- and female-producing temperatures was indistinguishable between controls and busulfan-treated samples (Fig. 4b and d; compare to a and c). The coelomic surface domain was clearly distinguishable from the medulla, which contained primitive cords, in all samples. The control gonads contained germ cells in the cortical domain as expected, but these were absent

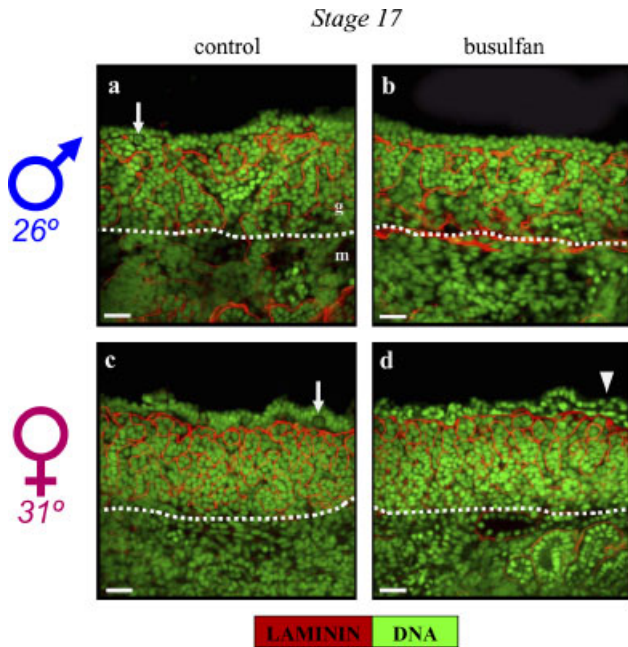


Fig. 4. Busulfan treatment does not disrupt stage-17 gonad organization at male- or female-producing incubation temperatures. Gonads and mesonephroi from (a, c) control and (b, d) busulfan-treated stage-17 embryos. Germ cells (arrows) are located at the coelomic surface of control gonads. Dotted line separates gonad (g) above from mesonephros (m) below. Coelomic epithelium is disrupted in busulfan-treated gonad at the female-producing temperature (arrowhead). Images representative of the  $n \geq 8$  for each panel. Scale bars = 100  $\mu\text{m}$ .

from busulfan-treated samples (Fig. 4b and d and Table 1). The cortical domain of busulfan-treated ovaries often showed gaps between somatic nuclei (Fig. 4d, arrowhead), which may represent positions where germ cells were recently lost.

At stage 23, the stage just prior to hatching, mock-treated control gonads exhibited normal morphology (Fig. 5a and c). The testis cords, the future seminiferous tubules, were fully elaborated and contained sequestered germ cells (Fig. 5a, arrow) within the characteristic Sertolien epithelium. In the busulfan-treated testis, cords with normal morphology formed in the absence of germ cells (Fig. 5b). Interestingly, the testis cord cross-sectional area was not reduced in busulfan treatments relative to controls (data not shown). The only observable difference between controls and busulfan-treated testes was the number of somatic cells, the presumptive pre-Sertoli cells, within the cords (Fig. 5b asterisks). Control testis cords contained a mean  $\pm$  SE of  $4 \pm 1.8$  Sertoli cells per 100  $\mu\text{m}$  of cord circumference in sections, while busulfan-treated testes contained  $10 \pm 3.2$  Sertoli cells per 100  $\mu\text{m}$  across 10 optical sections.

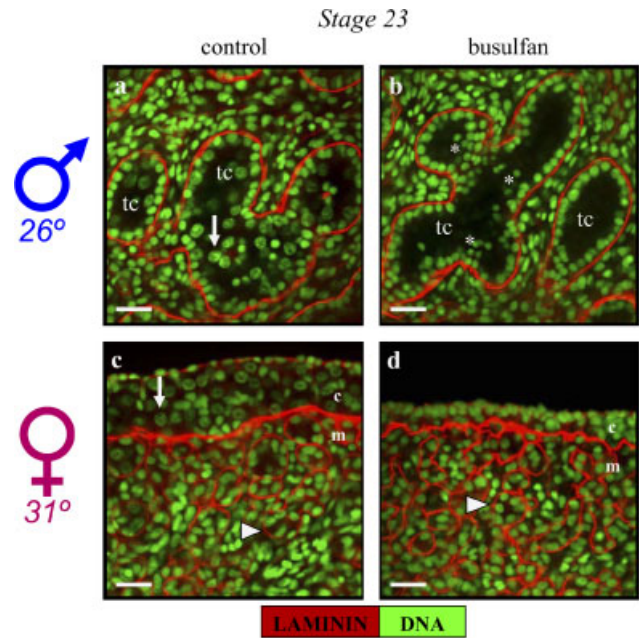


Fig. 5. Busulfan treatment did not disrupt stage-23 testis or ovary morphology. Gonads and mesonephroi of stage 23 embryos from (a, c) control and (b, d) busulfan treated. (b) Testis cords form without germ cells and are grossly normal. Ovarian cortical domain is established in the absence of germ cells. Arrows indicate germ cells; arrowheads indicate lacunae. Images representative of the  $n \geq 7$  for each panel. Scale bars = 100  $\mu\text{m}$ . tc—testis cord; c—cortex; m—medulla.

Given that cord size was not different between control and experimental testes, this implies a hyper-proliferation of Sertoli cells in the absence of germ cells.

In the ovary, the primitive sex cords became discontinuous and flattened to form the future lacunae of the adult ovary (Fig. 5c and d arrowheads; compare to testis cords in a and b). The germ cells were localized to the cortical domain which was separated from the medulla by a basement membrane. Ovaries from busulfan-treated animals lacked germ cells, but the morphological landmarks of the ovary were not altered (Fig. 5c and d). The basal laminae were well formed and clearly separated the cortex from the medulla. The outer layer of the cortex was continuous and more coherent than the earlier stage examined (compare Fig. 5d to Fig. 4d). This possibly reflects later stage repair of earlier gaps created by dying germ cells. The distance between the basal lamina that separated the cortex and medulla and the surface of the ovary was reduced in busulfan-treated gonads. This likely reflects the loss of germ cells that would normally occupy space in this domain. In point of fact, there was

no difference across 10 optical sections in the mean number of somatic cells in the cortex ( $69 \pm 8.2$  in control vs.  $76 \pm 14.5$  in busulfan-treated ovaries per section). This is consistent with the idea that the primary reason for the difference in size of the cortical domain is the absence of germ cells and not loss of the somatic cell population.

Because not all germ cells were ablated in our busulfan treatments (Table 1), we cannot exclude the possibility that the remaining germ cells exert an effect on gonad morphology. However, the majority (90%) are lost by stage 17 and >95% by stage 18 (Table 1). Notably, this is during the TSP when gonads remain bipotential and can alter their fate. We believe it is unlikely that the remaining germ cells could coordinate the morphology of the entire gonad, especially in the regions that are completely devoid of germ cells. Therefore, we propose that germ cells are not required for morphogenesis of the testis or ovary during fetal stages in the red-eared slider turtle, *T. scripta*.

In mice, testis cord morphogenesis occurs during fetal life in the absence of germ cells (reviewed in McLaren, '91). Furthermore, the structure of testis cords in the adult is not disrupted in the absence of germ cells, although spermatogenesis, of course, does not occur. In contrast, it is widely known that follicle formation, which occurs near birth in the female gonad, depends on the presence of germ cells. In the absence of germ cells, the XX gonad fails to form follicles and undergoes ovarian degeneration often associated with partial sex reversal, and expression of testis markers (Merchant-Larios and Centeno, '81; Burgoyne and Baker, '85; Guigon et al., 2005). Adult female-to-male sex reversal also occurs in zebrafish in the absence of germ cells (Slanchev et al., 2005).

These findings have led to the idea that post-natal ovarian development in mammals requires the presence of germ cells. However, it is important to make the distinction that the initial morphogenetic program in the fetal ovary does not require germ cells. Fetal mouse gonads from XX embryos lacking germ cells form "embryonic ovigerous cords" in the cortex near the time of birth (Nagamine and Carlisle, '96; Nagamine et al., '98), but fail thereafter. The present findings in *T. scripta* are consistent with earlier results in mice. The establishment of the initial phases of ovary morphogenesis in both of these vertebrates strongly suggests that the molecular

program for fetal ovary development does not depend on germ cells.

As the time to sexual maturity is 4–6 years in *T. scripta*, more long-term experiments will be required to determine if the adult turtle testis or ovary degenerates in the absence of germ cells.

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