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Hydroxysteroid Dehydrogenase Activity Associated with Sexual Differentiation in Embryos of the Turtle *Trachemys scripta*[1]

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ABSTRACT

Many turtles exhibit temperature-dependent sex determination. We have examined the hypothesis that incubation temperature causes a differential expression of steroidogenic enzymes in embryonic turtles. The activities of three steroidogenic enzymes were studied histochemically in turtle (*Trachemys scripta*) embryos at different developmental stages: sexually undifferentiated (Stage 15), differentiating (Stage 17), and differentiated (Stage 26; i.e., hatchling). Steroidogenic enzymes were detected in several tissues prior to, during, and after gonadal differentiation in embryos incubated at both male-producing and female-producing temperatures. In all embryos, ene-5-3β-hydroxysteroid dehydrogenase (HSDH) was detected only in adrenal tissue. 3α-HSDH was localized in adrenal tissue, as well as in the mesonephros and liver. 17β-HSDH was evident in mesonephric, hepatic, and gut tissues. In hatchlings, ene-5-3β-HSDH and 3α-HSDH were evident in the adrenal gland, whereas 3α-HSDH and 17β-HSDH were present in the mesonephros and liver. While there was some variation in the activities of these enzymes during development, no temperature-specific pattern was apparent. At no stage were the enzymes observed in genital ridge/gonad. Our results show that *T. scripta* embryos possess enzymes necessary for steroid hormone synthesis. The segregated distributions of the enzymes suggest that a multi-organ regulatory system may mediate embryonic steroidogenesis. Our results do not indicate the genital ridge/gonad the principle site of steroid synthesis, although it may possess other enzymes that influence steroidogenesis.

INTRODUCTION

In many reptiles, sex is determined by the temperature at which the eggs are incubated [1–3]. More specifically, sex determination is sensitive to temperature during the approximate middle-third of embryonic development [4–11], a period during which the gonads begin to sexually differentiate [11]. Further, studies have shown that the effects of temperature can be overridden by administration of exogenous steroid hormones to the egg. Treatment of eggs with estrogen, and to a lesser extent testosterone, causes embryos incubating at male-producing temperatures to develop as females [2, 12–16]. It has been hypothesized that temperature-dependent sex determination (TSD) may be chemically mediated in nature by influencing the concentration(s) of specific sex steroid hormones [2, 15, 17]. For example, temperature could affect the abundance of specific steroid-metabolizing enzymes present in the egg yolk, thereby affecting the de novo production of steroid hormones, and the conversion of maternally derived inactive hormones into active hormones. In other oviparous vertebrates, it has been established that maternal sources may contribute significant amounts of hormones to the egg [18, 19].

The ontogeny of steroidogenic hydroxysteroid dehydrogenase (HSDH) enzymes has been studied in some vertebrates. HSDH activity has been demonstrated at relatively early developmental stages in a variety of animals [20]. However, this enzyme activity is believed to develop after gonadal sex determination in species with genetic sex determination [21]. For TSD to work via steroidogenic enzymes, enzyme activity would have to be present before or during the developmental period of sex determination.

The present study investigates steroidogenic enzyme activity in embryonic tissues prior to, during, and after gonadal sex determination. The activities of three steroidogenic HSDH enzymes were studied histochemically in red-eared slider turtle (*Trachemys scripta*) embryos to determine if these enzymes demonstrate activities that vary with incubation temperature. Two of the enzymes localized in the present study (ene-5-3-β-HSDH and 17β-HSDH) are involved in testosterone and estrogen biosynthesis; 3α-HSDH is involved in the synthesis of other androgens [20].

MATERIALS AND METHODS

Animals

Trachemys scripta eggs were obtained commercially from R. Kliebert (Hammond, LA) and incubated on moist vermiculite (2 parts water: 1 part vermiculite) at either 26°C (male-producing temperatures) or 31°C (female-producing temperatures) as previously described [16].

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Tissue Preparation

Whole embryos (n = 5 for each combination of temperature and developmental stage) from both male-producing and female-producing temperatures were harvested at Yntema [22] developmental Stages 15 and 17 and frozen immediately on dry ice. Additionally, the liver, gonads, adrenals, and mesonephroi of 10 hatchling (Stage 26) turtles (5 at each incubation temperature) were dissected and prepared as above. Tissues were stored at −80°C for no more than 5 mo prior to use (storage had no apparent effect on histochemical results). Frozen embryos and tissues were embedded in Tissue Tek O.C.T. compound (Miles, Elkhart, IN), then serial 14-μm sections were cut at −16°C using a Minotome cryostat (Damon/IEC, Needham Heights, MA). Frozen sections were mounted on subbed coverslips, air-dried for 20–30 min, then rinsed in 0.1 M sodium phosphate buffer (PB, pH 7.4) at 5°C for 10 min prior to incubation.

Chemicals

All chemicals were from Sigma (St. Louis, MO) unless otherwise noted.

Enzyme Histochemistry

NADH-diaphorase activity was evaluated using a method modified from Kiernan [23]: Paired serial sections were incubated at 29°C (a temperature intermediate between the male-producing and female-producing temperatures experienced by the eggs; see *Discussion*) for 10 min in 4.4 ml freshly prepared incubation buffer: 0.068 M PB, pH 7.4, containing 9% *N,N*-dimethylformamide, NADH (0.9 mg/ml), and nitroblue tetrazolium (Grade III, 0.5 mg/ml). After incubation, sections were rinsed in distilled water, fixed in neutral buffered formalin (5 min), and rinsed again in distilled water. Slide covers were mounted with glycerine gelatin.

The activities of three HSDH enzymes were evaluated; the enzymes and the substrates used for their detection were as follows: 3α-HSDH, 4-androsten-3α-ol-17-one (androsterone); 17β-HSDH, 4-androsten-17β-ol-3-one (testosterone); and ene-5-3β-HSDH, 5-androsten-3β-ol-17-one (dehydroepiandrosterone).

HSDH-enzyme histochemistry was conducted essentially according to Baillie et al. [20]: Serial sections were incubated in duplicate at 29°C for 3 h in 4.4 ml freshly prepared incubation buffer: 0.068 M PB, pH 7.4, containing 9% *N,N*-dimethylformamide, steroid substrate (0.25 mg/ml), NAD (2.0 mg/ml), and nitro blue tetrazolium (Grade III, 0.5 mg/ml). Control sections were incubated in buffer lacking steroid substrates. After incubation, sections were rinsed in distilled water, fixed in neutral buffered formalin (5 min), and rinsed again in distilled water. Some sections were counterstained with 0.1% Safranin O to demonstrate nuclei. Slide covers were mounted with glycerine gelatin.

Analysis of Data

Enzyme reactions were evaluated qualitatively immediately after termination of the incubation. Sections were examined visually to determine the distribution of enzyme reaction products and the relative intensity of the reactions. Reaction intensity was described as (−), no reaction observed; (+), weak reaction, including weak pink color and few formazan granules formed; (++), moderate reaction, with pink or purple color and many dark formazan granules formed; (+++), strong reaction, with intense purple color and many dark formazan granules, forming zones of cells covered purple-black.

RESULTS

Figure 1 shows the distribution of NADH-diaphorase activity in a cross section of a Stage 17 embryo raised at the male-producing temperature (26°C). Diaphorase activity is strong in mesonephric, hepatic, and gut tissues, relative to adrenal and genital tissues (Fig. 1B). The distribution of this enzyme was similar in embryos from both male-producing and female-producing temperatures. Ene-5-3β-HSDH, 3α-HSDH, and 17β-HSDH enzymes were each demonstrable in turtles at all developmental ages (Table 1). The temperature of embryonic development did not appear to influence the activities of the enzymes at any developmental stage. Surprisingly, each of the three enzymes possessed a segregated, distinctive distribution among the developing organs.

At Stage 15, prior to the period of temperature-sensitive gonadal development, and at Stage 17, during gonadal sex determination, the specific distributions and activities of each enzyme were qualitatively similar, and will be discussed together (e.g., Fig. 2). A moderate 3α-HSDH reaction was localized in adrenal tissue. A weaker 3α-HSDH reaction was present in mesonephric tubules and hepatic tissue of all embryos, as well as in cross sections of the gastrointestinal tract of some embryos (Fig. 2A). Ene-5-3β-HSDH formed a strong reaction evident only in the area of the adrenal tissue (Fig. 2B). A weak 17β-HSDH reaction was co-localized with 3α-HSDH in mesonephric tubules and hepatic tissue; a weak-to-moderate 17β-HSDH reaction was also present in all cross sections of the gastrointestinal tract observed (Fig. 2C). No reaction was evident in control sections (Fig. 2D). In the Stage 26 (hatchling) turtle, moderate-to-strong reactions for 3α-HSDH and ene-5-3β-HSDH were evident in the adrenal gland. Weak-to-moderate reactions were also noted for 3α-HSDH and 17β-HSDH in hepatic tubules and liver. No enzymes were evident in the testes or ovaries of hatchlings; intestinal tissues of hatchling turtles were not tested.

DISCUSSION

Whether gonadal steroid hormones are involved in the process of sexual differentiation in turtles has been debated

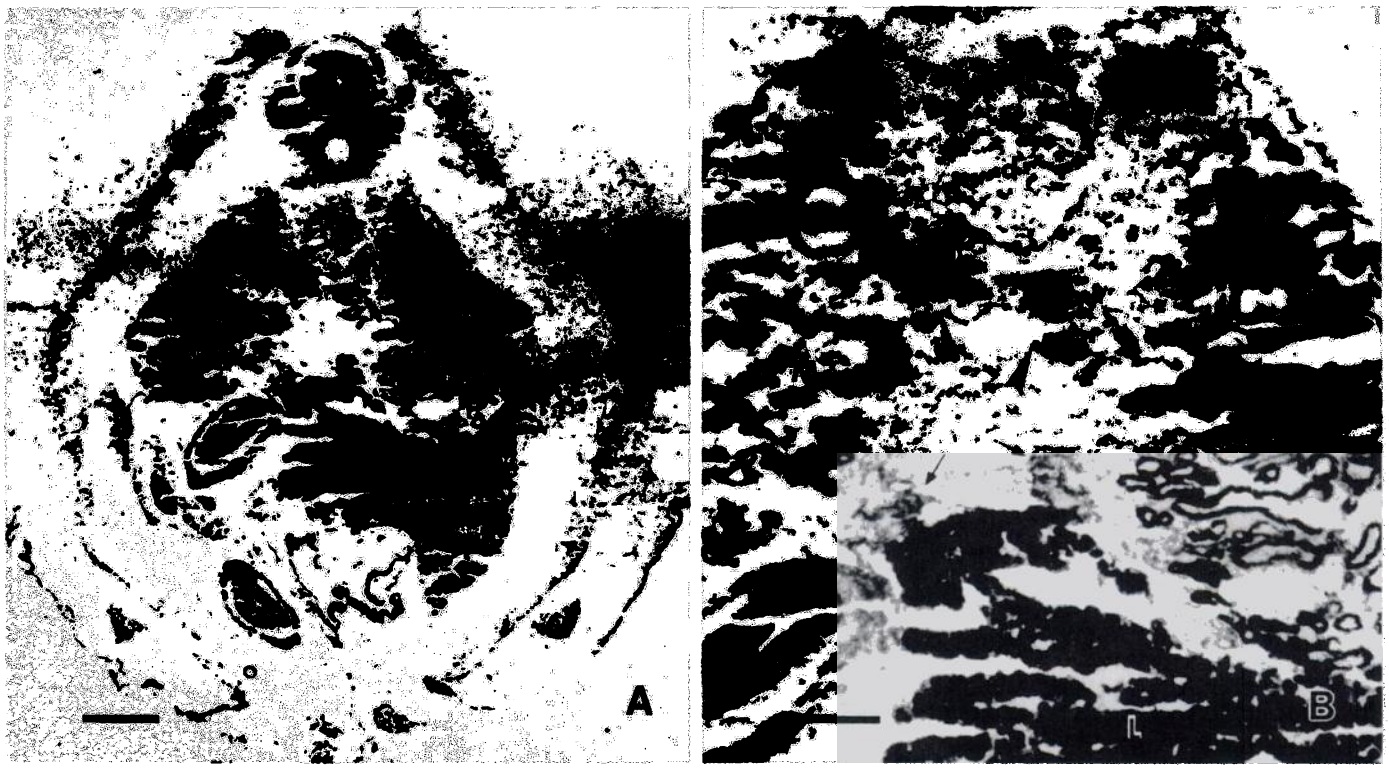

FIG. 1. NADH-diaphorase activity in a Stage 17 embryo raised at 26°C. d, dorsal aorta; G, gut; L, liver; M, mesonephros; arrows, genital ridge; arrowheads, adrenal tissue. (A) Bar = 500 μm. (B) Bar = 200 μm.

TABLE 1. HSDH activity in turtle (*Trachemys scripta*) embryos at different incubation temperatures.

	3α-HSDH		ene-5-3β-HSDH		17β-HSDH	
Stage and tissue	26°C	31°C	26°C	31°C	26°C	31°C
Stage 15						
Mesonephros	+[a]	+	−	−	+	+
Liver	+	+	−	−	+	+
Adrenal	++	++	+++	+++	−	−
Gut	−	−	−	−	+	+
Genital ridge	−	−	−	−	−	−
Stage 17						
Mesonephros	+	+	−	−	+	+
Liver	+	+	−	−	+	+
Adrenal	++	++	+++	+++	−	−
Gut	−	−	−	−	+	+
Genital ridge	−	−	−	−	−	−
Stage 26						
Mesonephros	+	+	−	−	+	+
Liver	+	+	−	−	+	+
Adrenal	++	++	+++	+++	−	−
Gonad	−	−	−	−	−	−

[a] + to +++, increasing intensity of enzyme reaction product formed; −, no reaction.

for over three decades. There is now no question that gonadal hormones (estrogens and androgens) are capable of experimentally influencing the sexual determination and differentiation of turtle embryos [12–17, 24]. Further, a recent study indicates that estrogen and temperature exert a synergistic effect on sex determination [25]. However, whether exogenous steroids are superseding a natural process involving similar chemical signals or are acting at other regulatory levels is still unknown.

One unresolved issue is whether incubation temperature naturally mediates sex determination by inducing the metabolism or production of endogenous steroid hormones. Further, if steroidogenesis occurs during the temperature-sensitive window of sex determination, does it occur in the genital ridge? The steroidogenic enzyme ene-5-3β-HSDH has been demonstrated in the embryonic genital ridge of the European pond turtle *Emys orbicularis*; the activity of this enzyme has been observed to vary between developmental stages and with incubation temperature [12, 26]. Pieau et al. [17, 26] also found measurable (but low) levels of both estrogens and androgens in pooled embryonic gonads of *E. orbicularis*, but other sites of steroid production and/or localization were not identified. However, there is also evidence of extragonadal steroidogenesis. As in the present study, Pieau [12, 27] described an intense ene-5-3β-HSDH activity in the adrenal tissue of female *E. orbicularis* embryos, without further comment on potential sexual dimorphism or the ontogenesis and possible significance of this activity. Gaitonde and Gouder [28] detected the presence of 3β-HSDH in the adrenals of the lizard *Calotes versicolor* (a lizard with TSD) prior to the sexual differentia-

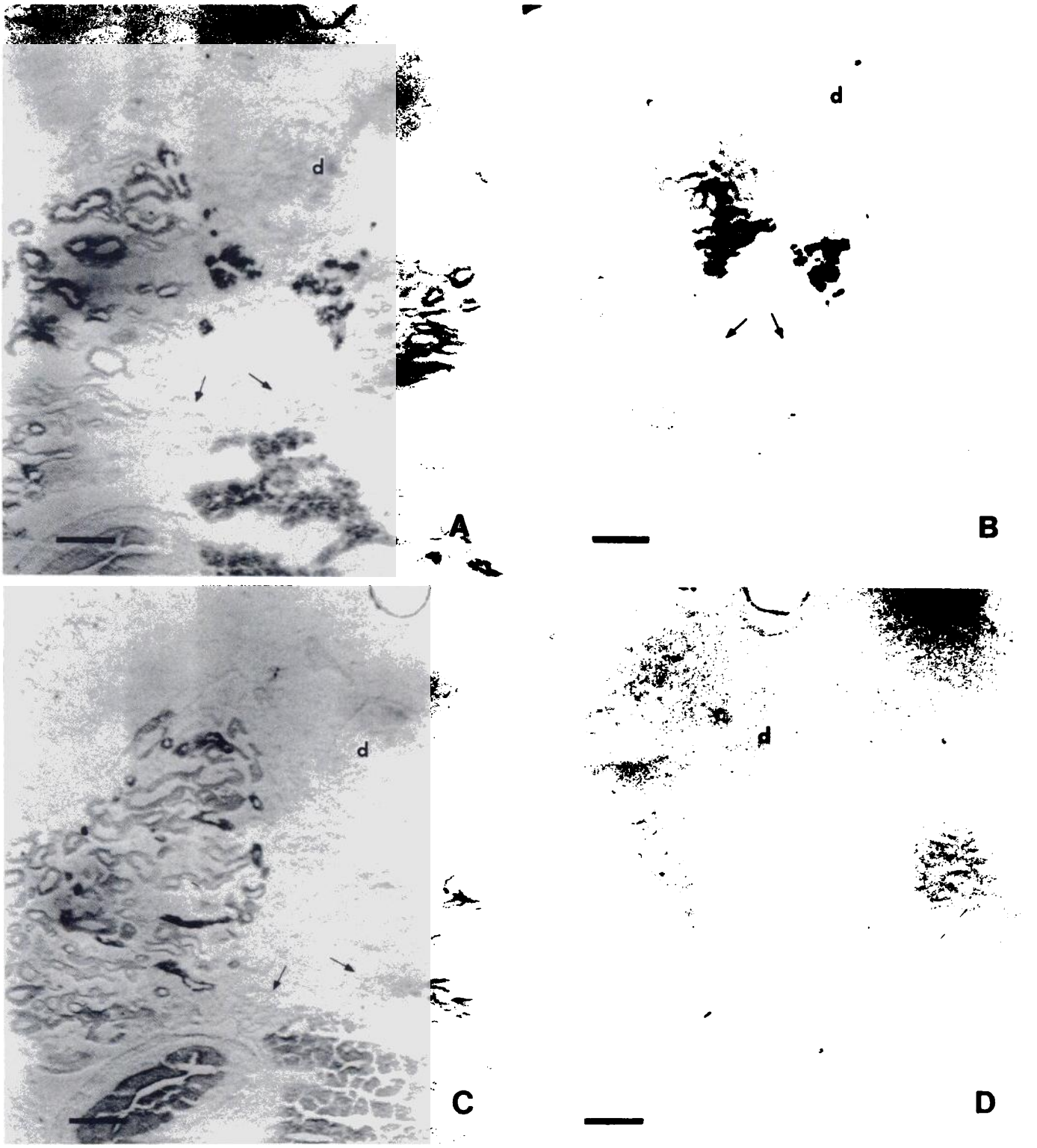

FIG. 2. HSDH activity in a Stage 17 embryo raised at 26°C. (A) 3α-HSDH. (B) 3β-HSDH. (C) 17β-HSDH; note absence of reactions in genital ridge (arrows). (D) Control section of Stage 17 embryo raised at 26°C incubated in the absence of steroids. d, dorsal aorta. Bars = 200 μm.

tion of the gonads. However, they found no such enzyme activity in the embryonic gonads. Additionally, White and Thomas [29] found no evidence of steroid secretion by isolated gonads of *T. scripta* in response to gonadotropin challenge. The adrenals and mesonephros, however, were shown to secrete steroids in vitro.

The present results for *Trachemys scripta* are only partly consistent with previous results for embryonic ene-5-3β-HSDH enzyme activity in *E. orbicularis* [12, 26]. Histochemically active HSDH enzymes are present in embryos of both species. Unlike ene-5-3β-HSDH activity in *E. orbicularis* [27], differences in HSDH activity in *T. scripta* were not apparent between developmental stages or incubation temperatures.

It could be argued that temperature-specific variations in enzyme activity would not be observed using our technique because tissues from both embryonic incubation temperatures (26°C and 31°C) were tested at a common intermediate temperature (29°C), thus equalizing enzymatic activity due to thermal effects. However, to say that developmental temperature influences enzymatic activity does not necessarily mean that changes in enzymatic activity level are strictly due to a simple Q_{10}-like effect on a similar number of enzyme molecules in embryos from both temperatures. If tissues were incubated at the same temperature as their respective embryos, Q_{10} effects would confound the results and, on that basis alone, some tissues would be predicted to have greater enzyme activity than others. Rather, temperature could have the more stable effect of enhancing or reducing the number of standing enzyme molecules per embryo. As such, although the activity per enzyme molecule would be equivalent at the intermediate tissue temperature, the number of molecules would vary between tissues and consequently the total enzyme activity would vary between tissues from embryos raised at different temperatures. Furthermore, even when embryos are raised at the intermediate temperature (in this case, approximately 29°C), some of the embryos will develop as male and others as female, indicating that if enzymatic differences are present between embryos becoming males and females, these differences are not lost at the intermediate incubation temperature. Effectively, it is the standing population of enzyme molecules that we have examined in *T. scripta* embryos.

Perhaps the most interesting results of this investigation concerned the distribution of HSDH activity among the various organs. In *T. scripta* embryos, HSDH activity was not detected within the genital ridge of either sexually undifferentiated or differentiated embryos. Rather, the HSDH activity was differentially distributed between the adrenal, mesonephric, hepatic, and gut tissues (Fig. 2; Table 1). This suggests that a multi-organ system including adrenal, mesonephric, and hepatic tissues, and possibly the gut, may regulate embryonic steroidogenesis in *T. scripta* during sexual differentiation. In some mammalian species, the embryonic adrenal gland and liver interact with the placenta to regulate fetal synthesis of androgens and estrogens during pregnancy [30].

The organ-specific distributions of 3α-HSDH, 17β-HSDH, and ene-5-3β-HSDH in *T. scripta* embryos coincide with published data for HSDH activity in other vertebrates. Baillie et al. [20] reported 17β-HSDH activity in intestinal mucosa of mammals, although they did not observe HSDH activity in the intestinal tract of a tortoise. Also, 3α-HSDH and 17β-HSDH, but not ene-5-3β-HSDH, activity have been found in tortoise hepatic and metanephric tissues [20].

Given the results for *E. orbicularis* [26], it is surprising that HSDH activity was absent from the genital ridge of *T. scripta* and that HSDH activity did not vary with incubation temperature or developmental stage. However, these findings do not exclude the genital ridge from playing a significant role in embryonic steroidogenesis, nor do they imply that steroidogenesis is identical in embryos raised at male-producing and female-producing temperatures. It is always possible that our incubation technique was not as sensitive as that used by Pieau et al. [26], or that HSDH activity in the genital ridge of *T. scripta* is present at a level not detectable by our technique. However, the strong ene-5-3β-HSDH reaction in the adrenal tissues in our study would seem to indicate that (1) our technique was adequate to detect this enzyme, and (2) physiologically, adrenal enzyme activity might potentially overwhelm any gonadal ene-5-3β-HSDH activity if the resulting steroid metabolites can move between these tissues for further metabolism.

Alternatively, the genital ridge may possess other enzymes that could serve as the critical regulators further down in the steroidogenic pathway, affecting steroids potentially produced in the other tissues and resulting in the formation of sex-specific steroids that mediate sexual differentiation. Aromatase would be a primary candidate as a key regulatory enzyme. It has been suggested that endogenous estrogens may be important in facilitating ovarian differentiation in reptiles [2, 15–17]. Another recent study indicates that exogenous dihydrotestosterone can masculinize the gonads [24], implicating the 5α-reductase as a key regulatory enzyme. It is plausible that the abundance of these steroidogenic enzymes, rather than HSDH enzymes, could affect sexual differentiation by regulating the production of androgens versus estrogens at the level of the genital ridge. These enzymes were not studied here because no histochemical tests exist for either the aromatase or 5α-reductase enzyme.

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