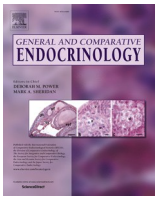
General and Comparative Endocrinology 346 (2024) 114418

Contents lists available at ScienceDirect

General and Comparative Endocrinology

journal homepage: www.elsevier.com/locate/ygcen



Research paper

Effects of testosterone on urogenital tract morphology and androgen receptor expression in immature Eastern Fence lizards (*Sceloporus undulatus*)

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ARTICLE INFO

Keywords:
Androgen
Androgen Receptor
Postnatal Development
Reptiles
Sexual Differentiation

ABSTRACT

In non-avian reptiles, the onset of sexual dimorphism of the major structures of the urogenital tract varies temporally relative to gonadal differentiation, more so than in other amniote lineages. In the current study, we used tonic-release implants to investigate the effects of exogenous testosterone (T) on postnatal development of the urogenital tract in juvenile Eastern Fence Lizards (*Sceloporus undulatus*) to better understand the mechanisms underlying the ontogeny of sexual differentiation in reptiles. We examined gonads, mesonephric kidneys and ducts (male reproductive tract primordia), paramesonephric ducts (oviduct primordia), sexual segments of the kidneys (SSKs), and hemiphalluses to determine which structures were sexually dimorphic independent of T treatment and which structures exhibited sexually dimorphic responses to T. To better understand tissue-level responsiveness to T treatment, we also characterized androgen receptor (AR) expression by immunohistochemistry. At approximately 4 months after hatching in control animals, gonads were well differentiated but quiescent; paramesonephric ducts had fully degenerated in males; mesonephric kidneys, mesonephric ducts, and SSKs remained sexually undifferentiated; and hemiphalluses could not be everted in either sex. Exogenous T caused enlargement, regionalization, and secretory activity of the mesonephric ducts and SSKs in both sexes; enlargement and regionalization of the oviducts in females; and enlargement of male hemipenes. The most responsive tissues exhibited moderate but diffuse staining for AR in control lizards and intense nuclear staining in T-treated lizards, suggestive of autoregulation of AR. The similarity between sexes in the responsiveness of the mesonephric ducts and SSK to T indicates an absence of sexually dimorphic organizational effects in these structures prior to treatment, which was initiated approximately 2 months after hatching. In contrast, the sex-specific responses in oviducts and hemipenes indicate that significant organization and/or differentiation had taken place prior to treatment.

1. Introduction

Sexual differentiation is a protracted process that becomes most evident after differentiation of the bipotential gonads and sexually dimorphic secretion of hormones. In amniotes, testicular differentiation leads to secretion of androgens and anti-Müllerian hormone (AMH) from Leydig cells and Sertoli cells, respectively. Elevated androgens in males stimulate the differentiation of mesonephric (Wolffian) ducts into epididymal ducts and ductus deferentia (Renfree et al., 2009; Murashima et al., 2015a). Elevated AMH promotes degradation of the paramesonephric (Müllerian) ducts, the anlagen to the oviduct (Josso et al., 1993; Behringer et al., 1994). The absence of pronounced androgen signaling in females is permissive of regression of the mesonephric ducts and stabilization of the paramesonephric ducts (Zhao and Yao, 2019). In

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https://doi.org/10.1016/j.ygcen.2023.114418
Received 13 September 2023; Received in revised form 14 November 2023; Accepted 26 November 2023
Available online 28 November 2023

mammals and birds, sexual differentiation of the reproductive tract is temporally associated with gonadal differentiation. That is, stabilization of the future reproductive tract anlagen and degeneration of the reproductive tract anlagen of the opposite sex occurs within days or weeks after differentiation of the gonad (Renfree et al., 1996; Kobayashi and Behringer, 2003; Zhao and Yao, 2019; Vizcarra et al., 2022). However, observations of urogenital tract development in non-avian reptiles (hereafter "reptiles") are not entirely congruent with this model.

In reptiles, there is considerable variation in the ontogeny of urogenital tract differentiation relative to gonadal differentiation. Most conspicuously, the mesonephric kidneys and ducts persist in a sexually undifferentiated state beyond hatching or birth, long after gonadal differentiation has taken place (Hartley, 1945; Forbes, 1940, 1956; Risley, 1941; Raynaud and Pieau, 1985; Austin, 1988; Neaves et al., 2006; Antonio-Rubio et al., 2015; Rheubert et al., 2015; Delssin et al., 2019). As they do in eutherian mammals, the paramesonephric ducts differentiate to become the oviducts in female reptiles and degenerate in the period between gonadal differentiation and hatching or birth in males of most reptile species examined (Hartley, 1945; Austin, 1988; Wibbels et al., 1999; Doddamani, 2006; Antonio-Rubio et al., 2015; Delssin et al., 2019). However, persistent paramesonephric ducts have been reported in postnatal males of at least two species of lizards (Forbes, 1941, 1956) and two species of turtles (Risley, 1941; Sari and Kaska, 2016). Similarly, the ontogeny of external genitalia with respect to gonadal differentiation varies in squamate reptiles (lizards and snakes). Hemiphallus differentiation to hemipenes in males and hemiclitores in females begins shortly after gonadal differentiation in many squamates (Raynaud and Pieau, 1985; Holmes and Wade, 2005; Gredler et al., 2015), yet the females of at least three species of lizards develop male-like hemipenes that are retained for an extended period of embryonic or postnatal development before involution and formation of hemiclitores (Neaves et al., 2006; Martínez-Torres et al., 2015; Whiteley et al., 2018). Lastly, squamate urogenital systems are unique in the formation of accessory reproductive structures in the renal tubules and/or collecting ducts of the metanephric kidneys, termed the sexual segments of the kidney (SSK; also referred to as the renal sexual segments). The SSK is responsive to androgens and more conspicuous in adult males than in females (Aldridge et al., 2011; Rheubert et al., 2015). To our knowledge, there are no studies detailing the initial differentiation of the SSK in squamates, but enlargement of the tubules and increased epithelial cell height and secretory activity are associated with sexual maturity and reproductive activity in males (Rheubert et al., 2020).

The ontogeny of sexually dimorphic trait expression in relation to gonadal differentiation is determined by the timing of sexually divergent signals for differentiation of the trait, and by the timing of sensitivity to these and other signals. Organizational, but morphologically cryptic, differences between the sexes may contribute to sexually divergent responsiveness to a signal. For example, androgens promote the masculinization of the male reproductive tract in two distinct phases in eutherian mammals. An initial period of elevated T secretion following testicular differentiation prevents degradation of the mesonephric kidneys and ducts, which is referred to as the stabilization phase (Welsh et al., 2009; Murashima et al., 2011). Stabilization of the mesonephric duct in embryonic males is dependent upon hormonal signaling through the androgen receptor (AR). Interruption of AR signaling in males during the stabilization phase results in organizational changes that preclude complete masculinization of the mesonephric derivatives, regardless of subsequent androgen exposure (Welsh et al., 2007). A second period of elevated T secretion stimulates elongation, coiling, and complete regionalization of the male reproductive tract. This phase of differentiation begins perinatally in rodents that reach sexual maturity within weeks to months after parturition (Murashima et al., 2015b), or years after parturition in species that take longer to mature, such as humans (Rey, 2021). Given the key roles of androgens in each of these phases, characterizing effects of androgens on urogenital tract development is expected to shed light on the decoupling of prenatal gonadal differentiation and postnatal sexual differentiation that occurs in some reptiles.

In the current study, we investigate the effects of exogenous testosterone (T) on postnatal development of the urogenital tract in Eastern Fence Lizards (*Sceloporus undulatus*) to explore the mechanisms underlying the ontogeny of sexual differentiation in reptiles. *Sceloporus* lizards are a relatively well-studied squamate lineage regarding associations between androgens and sexually dimorphic coloration (Kimball and Erpino, 1971; Abell, 1998; Quinn and Hews, 2003; Cox et al., 2005a; Cox et al., 2008; Robinson et al., 2023), sexual size dimorphism (Cox and John-Alder, 2005; Cox et al., 2005b; Pollock et al., 2017), reproductive and territorial behavior (Klukowski and Nelson, 1998; Smith and John-Alder, 1999; Haenel et al., 2003; Hews et al., 2012), and reproductive seasonality (McKinney and Marion, 1985; John-Alder et al., 2009). Moreover, two of the most detailed histological studies of the embryonic development in the urogenital system of any squamate were conducted on species in this genus (Austin, 1988; Antonio-Rubio et al., 2015). These studies provide a foundation for testing two potential hypotheses for the asynchrony of sexual differentiation between the gonads and urogenital tract structures in reptiles. First, a sexually dimorphic stabilization phase, such as what has been described in mammalian mesonephric ducts (Welsh et al., 2007), may be absent. Under this hypothesis, the onset of gonadal maturation and increasingly sexually dimorphic secretion of T should be sufficient to drive the differentiation of the male urogenital tract in the absence of a prior organizing signal. Alternatively, sexually dimorphic signaling during embryonic or perinatal development could result in organizational changes, such as dimorphic hormone receptor expression, that are only morphologically discernable after the onset of adult-like hormone signaling during sexual maturation. To test these two hypotheses, we exposed postnatal *S. undulatus* juveniles to exogenous T using tonic release implants and examined the urogenital system at both gross anatomical and histological levels to assess the effects of T on development. We examined gonads, reproductive tracts, SSKs, and hemiphalluses to determine which structures were sexually dimorphic at this stage of development independent of treatment and which structures exhibited sexually dimorphic responses to T. In the absence of prior sexually dimorphic organization, we predicted that male and female urogenital tracts would respond similarly. Conversely, if prior organization events shape responsiveness to elevated T, we predicted that male and female urogenital tracts would respond differently. Lastly, we characterized AR expression using immunohistochemistry to better interpret any morphological changes we observed in response to T treatment.

2. Materials and methods

2.1. Lizard collection and maintenance

The University of Virginia Animal Care and Use Committee approved all animal protocols for this study (protocol 3896). We collected 30 Eastern Fence Lizards (*Sceloporus undulatus*) of approximately 1 month in age from Colliers Mills Wildlife Management Area, New Jersey, USA (latitude 40.07889, longitude −74.43736; New Jersey Fish and Wildlife collecting permit SC 2020070) on September 7, 2019. After transport to the University of Virginia (Charlottesville, VA, USA), we housed lizards individually in 18 x 35 x 27 cm terraria furnished with a brick for basking under a 45 W incandescent reflector bulb (Bulbrite Industries, Moonachie, NJ, USA) and two 13 W UVB bulbs (Repti-Sun 10.0, Zoo Med Laboratories, Inc., CA, USA). We maintained photoperiod at 12 h light: 12 h dark throughout the duration of the study, and rotated terraria weekly to prevent shelf effects. Lizards received fresh drinking water daily and 5–7 crickets (*Gryllodes sigillatus*) thrice weekly, dusted twice per week with calcium and once per week with vitamin supplements (Fluker's Cricket Farms, Port Allen, LA).

2.2. Testosterone implants

After a four-week acclimation period, we assigned eight lizards per sex to the T-implant group and seven lizards per sex to the control group. We prepared tonic-release implants as previously described (Cox et al., 2015; Cox et al., 2017; Wittman et al., 2021). We cut Silastic® tubing (1.47 mm I.D. x 1.96 mm O.D.) to 4-mm lengths and sealed one end of each tube with waterproof silicone gel before filling each tube with either 100 μg T (Sigma T1500) dissolved in 1 μl dimethyl sulfoxide (DMSO) or 1 μl of pure DMSO. We sealed the open end of each tube and allowed implants to cure for several days, during which time the DMSO diffused through the tubing and evaporated, leaving crystalline T (T-treatment group) or an empty implant (control group). Prior to surgery, we anesthetized each lizard with a 1 μl subdermal injection of 0.25% bupivacaine HCl (Sensoricaine®) in its right lower quadrant before briefly cooling it at –20 °C for 3–5 min until it failed to exhibit a righting response. We then immediately returned the lizard to ambient room temperature and secured it with surgical tape to a slightly thawed chemical ice pack enclosed in plastic. Surgeries were conducted at room temperature atop this cool surface. We placed the implant into the pleuroperitoneal cavity through a 5-mm ventrolateral incision, closed the incision with cyanoacrylate surgical adhesive (Vetclose®), and placed each lizard into a sanitized recovery container overnight prior to returning it to its terrarium.

2.3. Tissue collection and testosterone assays

Six weeks after surgery, we measured the width of the tail base to the nearest 0.1 mm with calipers as an indirect measure of hemipene development. Two weeks later, we recorded snout-vent-length (SVL) and body mass immediately prior to euthanasia via rapid decapitation. After collecting whole blood in heparinized capillary tubes, we centrifuged the capillary tubes for separation of plasma, and stored the plasma at −20 °C until assayed for T. We selected three individuals of each sex within each treatment group for examination of urogenital morphology, histology, and AR expression, and fixed the lower trunk and cloacal region of those animals in 10% neutral-buffered formalin. After 24 h in fixative, we washed the tissues in sterile PBS and transferred them to 70% ethanol for storage at 4 °C.

We measured plasma T using radioimmunoassay (RIA) as previously described (Smith and John-Alder, 1999; Cox and John-Alder, 2005; Cox et al., 2005b). Briefly, we extracted samples twice in diethyl ether, evaporated the diethyl ether under a stream of ultra-filtered air, and reconstituted the extract in phosphate buffered saline with gelatin. Samples were assayed with ^{3}H-T (PerkinElmer Life Sciences) and T antiserum (diluted 1:18000) developed in rabbits by A.L. Johnson (The University of Notre Dame, IN, USA). Intra-assay variation was 4.8 ± 0.7% (mean ± 1SD), inter-assay coefficient of variation was 6.6%, and the limit of detection was 6.5 pg.

2.4. Gross anatomy imaging and measurements

We photographed the urogenital anatomy in ventral view at 2.5× (males) or 3.5× magnification (females) using a Canon EOS 5DSR camera and MP-E 65 mm macro lens. We photographed each specimen at increasing depths along the ventral–dorsal axis and combined the images in Zerene Stacker (Zerene Systems, LLC., version 1.04) to achieve sharp focus of the urogenital system in a single image. To obtain gross anatomical measurements of urogenital structures, we used the Fiji image processing package (Schindelin et al., 2012). We measured gonadal cross-sectional area by tracing the perimeter of each ovary and testis as photographed in ventral view. We recorded ovarian follicle diameter as the longest straight-line distance passing through the center of any follicle that appeared > 75% visible in ventral view of the ovary, which was typically 5–6 follicles per ovary. We obtained oviduct and posterior mesonephric duct (ductus deferens) diameter by measuring the width of each duct in 4–6 different locations as equally spaced as possible along the anterior–posterior axis. The oviducts were oval in cross section and coiled to varying degrees along their length. To standardize the measurement of oviduct diameter, we restricted the location of measurements to regions where the longest dimension of the oviduct that is perpendicular to the anterior–posterior axis of the duct was parallel to the plane of view. For each morphological variable that involved multiple measurements of paired organs, we used the average of the estimated means from each of the paired organs to estimate the overall mean for each individual.

2.5. Histology and immunohistochemistry

We removed the left urogenital tract of each lizard as a single unit consisting of the gonad, reproductive tract, and kidney before dehydrating the tissues in an ascending series of 70–100% ethyl alcohol baths. After clearing the urogenital tracts in CitriSolv, we embedded them in paraffin wax and serially sectioned each tissue at a thickness of 6 μm along the transverse plane from the anterior end of the gonad to the posterior end of the kidney. We mounted alternating series of tissue sections on either plain glass slides, which were stained with Masson's trichrome (Bancroft and Layton, 2019) for general observation and morphological measurements, or on positively charged slides (Colorfrost Plus, Fisher Scientific) for immunohistochemistry. Using a calibrated ocular micrometer, we measured the diameters of 30 seminiferous tubule cross-sections distributed as evenly as possible throughout the testis of each animal. We measured SSK epithelial cell height and SSK cross-sectional diameter similarly using a total of 20 SSK cross-sections per kidney.

In preparation for immunohistochemistry, we performed antigen retrieval by heating deparaffinized slides at 110 °C in Tris-EDTA buffer (pH 9.0) under pressure for 15 min. After cooling and three washes in assay buffer (Tris buffered saline with 0.025% Triton X-100 (TBS-TX)), we incubated tissue sections at room temperature for 1 h in TBS supplemented with 0.2% Triton X-100, 10% normal goat serum, and 1% bovine serum albumin (BSA) to block non-specific binding and permeabilize the tissues. We added primary antibody (rabbit polyclonal anti-AR, MilliporeSigma PG-21), diluted 1:2000 in TBS-TX supplemented with 1% BSA, to tissue sections prior to overnight incubation at 4 °C. We removed unbound primary antibody with three washes in TBS-TX, and then blocked endogenous peroxidase activity with 0.3% H_2O_2 for 10 min. To visualize binding of our primary antibody, we used the Abcam Mouse and Rabbit Specific HRP/DAB IHC Detection Kit (Ab236466) according to manufacturer's protocol and hematoxylin counter staining. In every immunohistochemistry run, we included control sections in which we carried out all procedures with the exclusion of primary antibody to serve as a reference for detection of non-specific binding of the secondary antibody and/or endogenous peroxidase activity. Antigen retrieval of ovarian tissue sections resulted in significant tissue detachment and artifacts in the vicinity of previtellogenic follicles, the dominant feature of the ovaries, therefor we excluded ovaries from AR immunohistochemistry. Moga et al. (2000) and Hews et al. (2012) previously demonstrated the specificity of PG-21 for *Sceloporus* AR by pre-adsorption of antibody with the immunizing peptide (amino acids 1–21 of rat AR) and unrelated partial AR peptides in its use to detect AR in various brain regions of *S. undulatus* and *S. virgatus*. We observed and photographed sectioned tissues on a Zeiss AxioLab A1 compound microscope equipped with an Axiocam ERc 5s digital camera. In instances where a larger field of view was desirable for figures, we stitched together adjacent images with Adobe Photoshop Lightroom Classic (release 11.4).

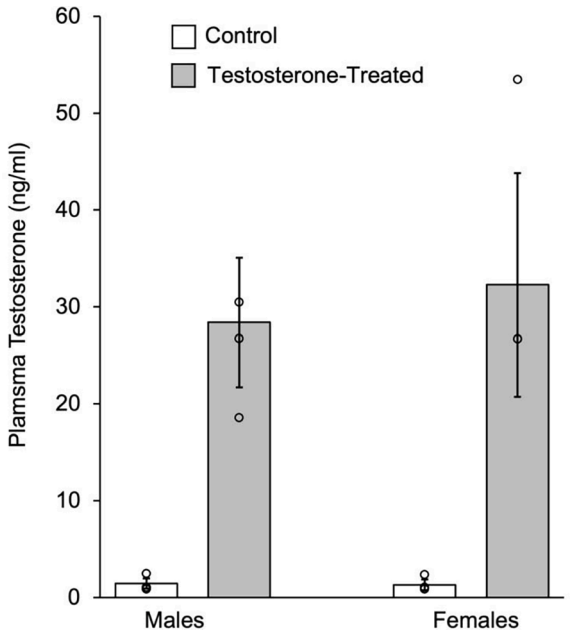

Fig. 1. Plasms testosterone (T) concentrations (means ± 95% CI) for control and testosterone-treated immature *Sceloporus undulatus* males and females. N = 6 for each group except T-treated females (N = 5). Circles represent plasma T concentrations for the subset of individuals included in gross anatomy and histological analyses.

3. Results

3.1. Plasma testosterone and body size

Mean plasma T concentration was approximately 20 times greater in T-treated lizards in comparison to control lizards (Fig. 1). We did not observe overlap in individual plasma T concentrations between T-treated and control animals, and plasma T concentrations appeared equivalent between sexes within each treatment group. We did not observe any differences in SVL or body mass between sexes or treatment groups (Fig. S1).

3.2. Gross anatomy

Examples of the gross anatomy of the urogenital system of control and T-treated males are shown in Fig. 2A and 2B, respectively. The morphology of the urogenital system of control males was indicative of an immature state. The testes were medial to the spermatic duct anlage and ventromedial to the adrenal glands. The semi-transparent mesonephric kidneys extended caudally beyond the anterior edge of the metanephric kidneys, and coiled mesonephric tubules were visible through the stroma of each mesonephros. The mesonephric ducts paralleled the lateral edges of the mesonephric kidneys and the ventral midline of the metanephric kidneys, which have a distinct ventral-lateral lobe in the caudal half. There were no observable remnants of the paramesonephric ducts in males, indicating their complete regression prior to the time of dissection. In control males, the SSK was not grossly distinguishable and the hemipenes were not manually eversible, indicating only rudimentary development.

The testes of T-treated males were similar in appearance and size to those of control males (Fig. 3A). In contrast, we observed hypertrophy and virilization throughout the remainder of the urogenital tract of T-treated males in comparison to control males. T-treated males exhibited slightly enlarged mesonephric tubules (nascent efferent ductules) within relatively compact mesonephric kidneys that abutted the anterior edge of the metanephric kidney. The mesonephric duct of T-treated males was greatly enlarged relative to controls (Fig. 3B) and consisted of a convoluted anterior portion (nascent epididymal duct) located lateral to each mesonephros and testis, and a comparatively straight posterior portion (nascent ductus deferens). Testosterone treatment stimulated secretory activity in the SSK, which was distinguishable by the white secretions filling the collecting ducts and ureter of each metanephric kidney. Relative to control males, we observed hypertrophied hemipenes in T-treated males, as indicated by increased width of the tail base (Fig. 3C) and the ease of everting the hemipenes with slight lateral pressure just caudal to the vent. We were unable to evert hemiphalluses in any group of lizards except T-treated males.

Examples of the gross anatomy of the urogenital system of control and T-treated females are shown in Fig. 2C and 2D, respectively. Control

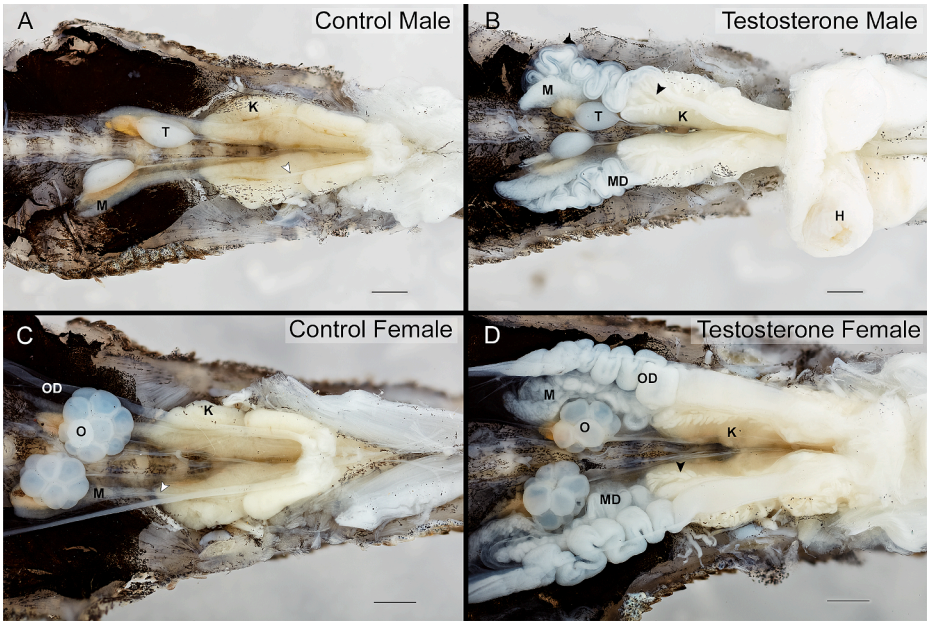

Fig. 2. Gross anatomy of the urogenital system (ventral view) of immature *Sceloporus undulatus* (A) control males, (B) testosterone-treated males, (C) control females, and (D) testosterone-treated females. H, hemipenis; K, kidney; M, mesonephric kidney; MD and white arrowheads, mesonephric duct; O, ovary; OD, oviduct; T, testis; black arrowheads, sexual segment of the kidney; scale bars, 0.5 mm.

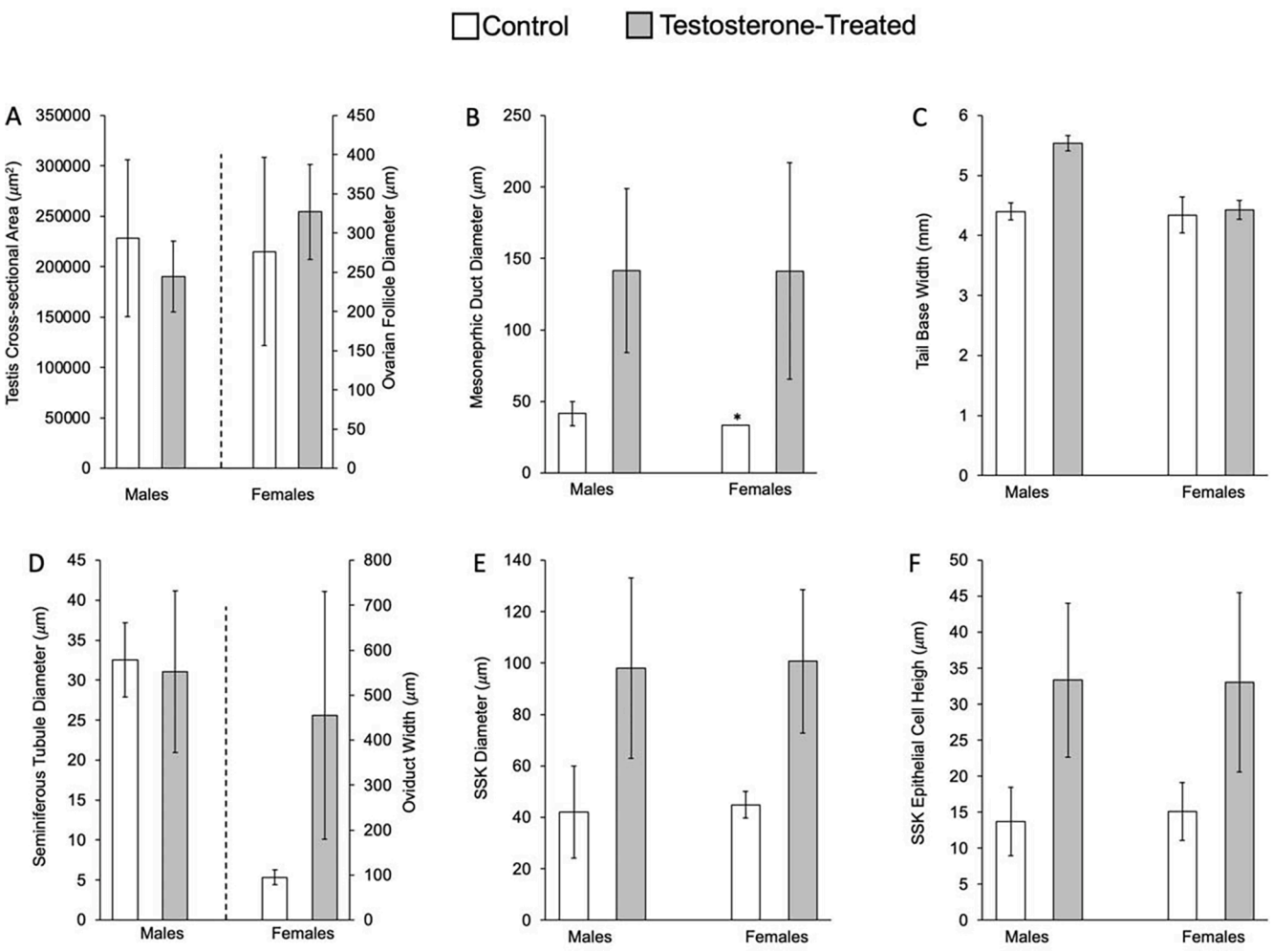

Fig. 3. Morphological comparisons (means ± 95% CI) between control and testosterone-treated immature *Sceloporus undulatus* males and females. (A) testis ventral cross-sectional area and ovarian follicle diameter, (B) posterior mesonephric duct (ductus deferens) diameter, (C) tail base width, (D) seminiferous tubule diameter and oviduct width, (E) sexual segment of the kidney (SSK) diameter, (F) SSK / collecting duct epithelial cell height. N = 3 for each group except *, where N = 1 because the oviducts concealed and prevented measurement of the mesonephric ducts in 2 control females.

females exhibited follicular organization within the ovaries, which consisted of 8–12 pre-vitellogenic follicles per ovary. Paired oviducts appeared in control females as thin, semi-transparent tubes along the dorsal-lateral pleuroperitoneal cavity. Modest corrugations were present in the anterior region of the oviducts corresponding to posterior infundibula and/or anterior uteri. The mesonephric kidneys and associated ducts persisted in untreated females, similar in appearance to homologous structures of control males.

The ovaries of T-treated females were similar in appearance and average follicular diameter to those of control females (Fig. 3A). In contrast, we observed hypertrophy and greater regional differentiation of the oviducts of T-treated females in comparison to control females (Fig. 3D). Anteriorly, the oviducts of T-treated females consisted of a distinctly pleated infundibulum, followed by a deeply corrugated anterior uterus and relatively straight posterior uterus. We observed a thin, longitudinal band of smooth muscle lying along the antimesometrial surface of each oviduct, as described by Blackburn (1998). T-treated females exhibited virilized mesonephric derivatives similar in appearance and size to the nascent efferent ductules, epididymides, and ductus deferentia of T-treated males. Likewise, T treatment stimulated secretory activity in the SSK of females.

3.3. General histology

The testes of control and T-treated males exhibited distinct seminiferous tubules populated by Sertoli cells and spermatogonia; however, there was no indication of lumen expansion or spermatogenic activity in either group (Fig. 3D, 4A-B). The cuboidal epithelia of the mesonephric tubules of control males had few cilia, whereas cilia were abundant, and adjacent tubules were further separated by connective tissue and smooth muscle in the incipient efferent ductules of T-treated males. A non-secretory and simple columnar epithelium characterized the mesonephric ducts of control males. In contrast, the mesonephric ducts of T-treated males exhibited pseudostratified columnar epithelial cells with an abundance of apical secretory granules, which were also plentiful among the luminal debris throughout the future epididymal and anterior ductus deferentia regions. Sexual segments of the kidney were not discernable in control males but were evident in the collecting ducts and ureters of T-treated males, as indicated by greater duct diameter, epithelial cell height, and secretory activity (Fig. 3E-F, 4C-D).

The ovaries of control and T-treated females exhibited similar histological characteristics (Fig. 5A-B). Previtellogenic follicles were composed of a well-defined theca surrounding a follicular epithelium comprised of small, intermediate, and pyriform cells, and a prominent zona pellucida. In cross-section, control oviducts (Fig. 5A and 5D) varied little along the anterior to posterior axis, consisting of a low columnar

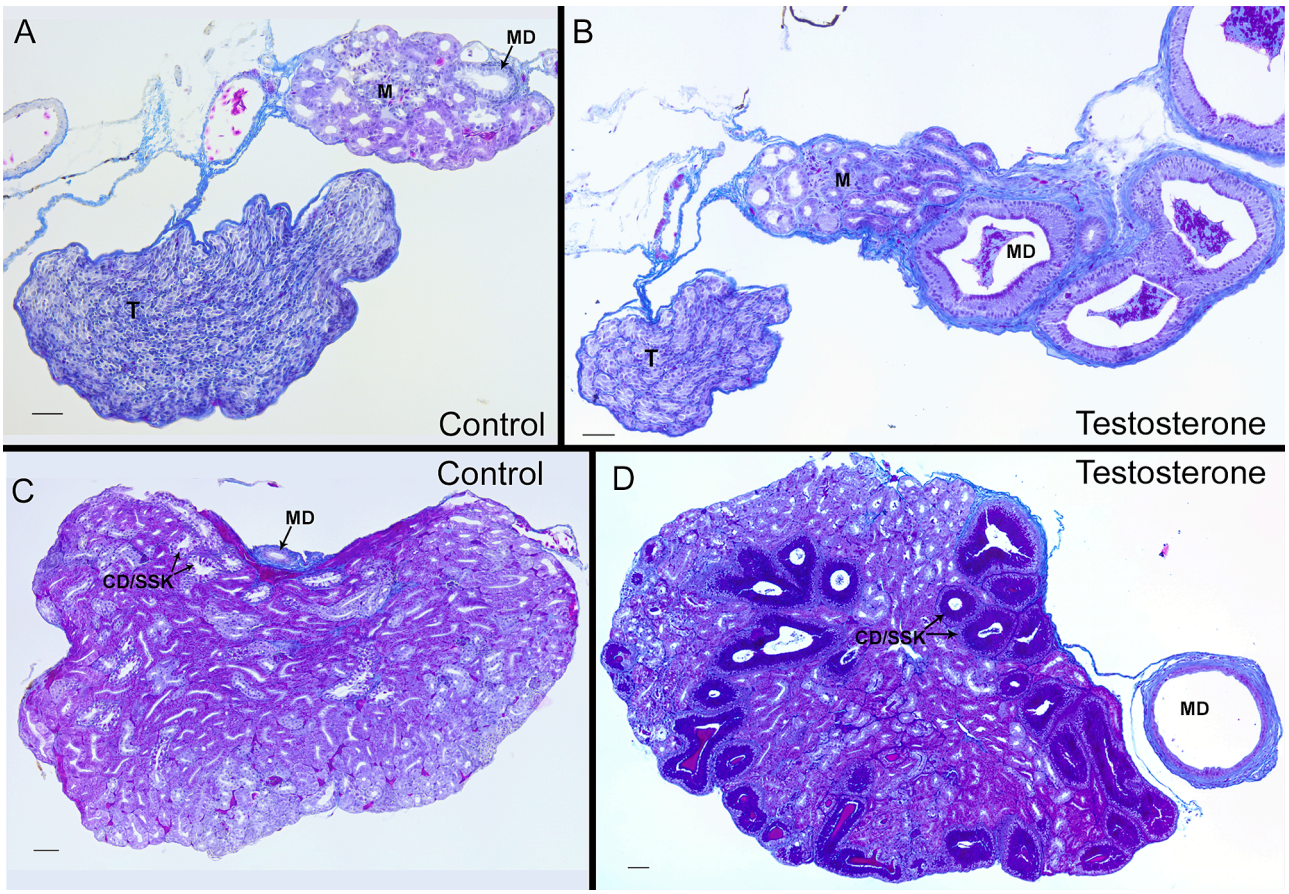

Fig. 4. Masson's trichrome stained sections from (A, C) control and (B, D) testosterone-treated immature *Sceloporus undulatus* males. CD/SSK, collecting duct / sexual segment of the kidney; K, kidney; M, mesonephric kidney; MD, mesonephric duct; scale bars, 50 μm.

epithelium separated from the surrounding serosa by a thin mesenchyme layer. The posterior infundibula and anterior uteri of T-treated lizards (Fig. 5C) had ciliated pseudostratified columnar epithelia, infrequent simple tubular glands, and a distinct layer of dense connective tissue surrounded by a multi-layered muscularis. The more posterior and glandular regions of the uteri (Fig. 5E) exhibited an abundance of simple tubular and acinar glands in the mucosa, which was surrounded by layers of dense connective tissue and smooth muscle. Similar to observations at the gross anatomical level, the mesonephric derivatives and the SSK of control and T-treated females were strikingly similar at the histological level to the homologous structures of control and T-treated males, respectively.

3.4. Androgen receptor immunoreactivity

Androgen receptor immunoreactivity was absent or weak in most control male (Fig. 6) and control female (Fig. 7) tissues. The cytoplasm and some nuclei of the mesonephric tubule, mesonephric duct epithelia, and oviductal epithelia of control lizards exhibited modest AR-immunopositive staining in comparison to negative control sections (Fig. S2). Peritubular myoid cells and interstitial cells in the testes of T-treated males presented moderate nuclear staining for AR (Fig. 6B), as did the mesonephric tubule epithelia of T-treated males and females (Fig. 6D and 7D). The mesonephric duct derivatives (epididymides and ductus deferentia) and SSK of T-treated males exhibited intense nuclear staining for AR (Fig. 6F and 6H). We observed similarly intense nuclear staining in the oviductal mucosa and muscularis (Fig. 7B and 7H), mesonephric duct epithelium (Fig. 7G), and SSK (Fig. 7F) of T-treated females.

4. Discussion

We characterized the gross anatomy and histology of the urogenital systems of immature *S. undulatus*, as well as the phenotypic changes in the urogenital system resulting from precocious exposure to elevated T. The morphology of mesonephric tubules, mesonephric ducts and SSKs did not differ between control males and females, and these tissues exhibited similar degrees of enlargement and increased secretory activity in both sexes in response to elevated T. These results support the hypothesis that the asynchrony between gonadal differentiation and sexual differentiation of the mesonephric tubules, mesonephric ducts, and SSKs occurs in the absence of sexually dimorphic organizational changes temporally associated with gonadal differentiation, and virilization of these structures is primarily dependent on sexually dimorphic secretion of androgens. Additionally, we observed proliferation and regional differentiation of the oviduct only in T-treated females, and enlargement of the hemipenes only in T-treated males. The sex-specific responses in these tissues indicate that significant organization and/or differentiation had taken place prior to our treatment, which was initiated approximately 2 months after hatching.

The morphology of the urogenital systems of our control lizards, which were 4 months of age upon examination, was consistent with previous anatomical descriptions at hatching or birth in this genus (Austin, 1988; Antonio-Rubio et al., 2015) and in immature lizards in general (Rheubert et al., 2015; Siegel et al., 2015). Use of immature lizards enabled us to examine the response of the urogenital system to elevated androgens after gonadal differentiation, but without the confounding issue of significant and sexually dimorphic endogenous androgen production, as confirmed by low plasma T concentrations in control lizards of both sexes. Plasma T concentrations in T-treated

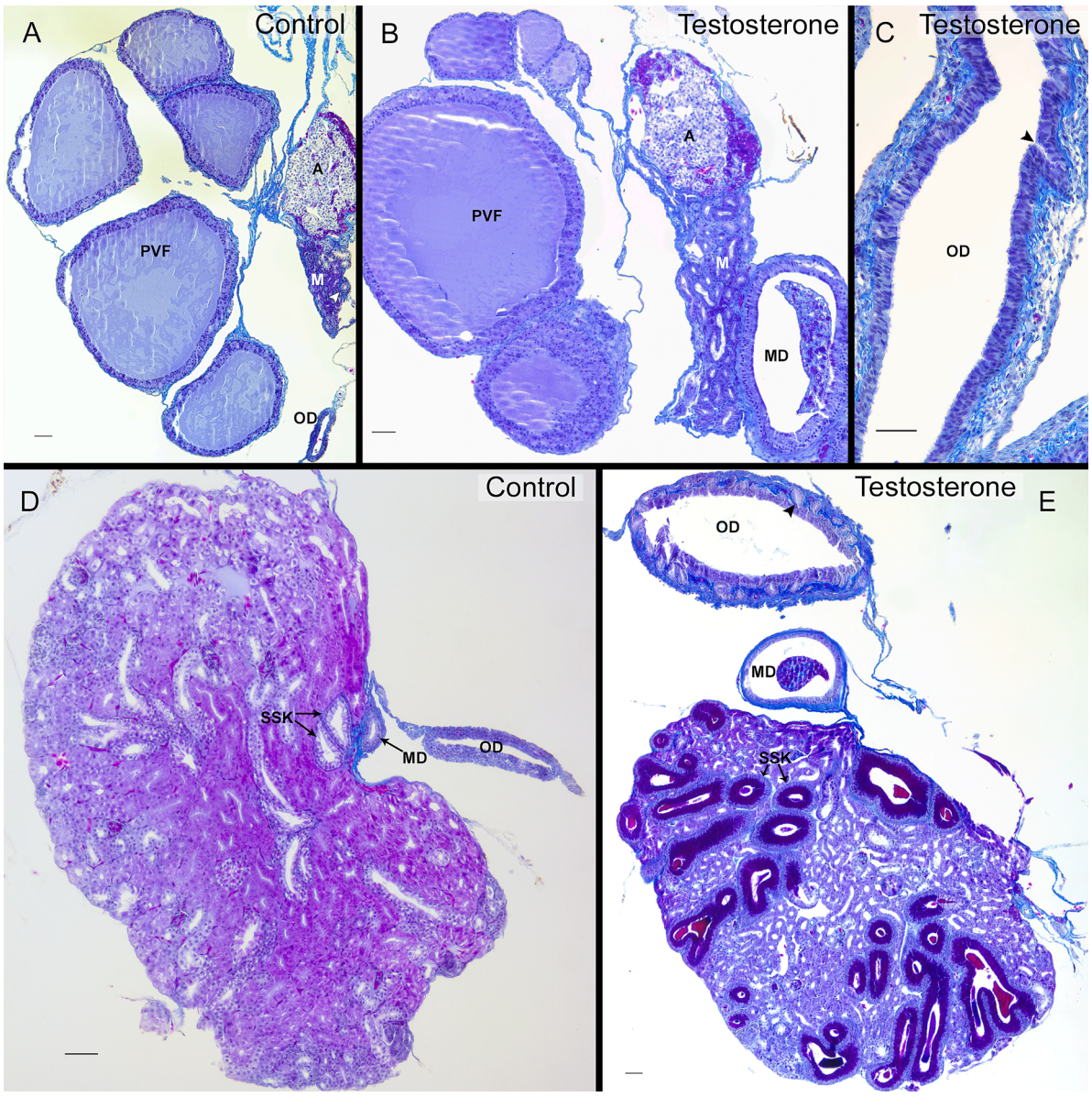

Fig. 5. Masson's trichrome stained sections from (A, D) control and (B, C, E) testosterone-treated immature *Sceloporus undulatus* females. A, adrenal gland; CD/SSK, collecting duct / sexual segment of the kidney; K, kidney; M, mesonephric kidney; MD, mesonephric duct; PVF, previtellogenic follicle; OD, oviduct; black arrowheads, oviductal gland; scale bars, 50 μm.

lizards were well within the range previously reported for free-living 9- to 12-month-old males from the same source population (Cox et al., 2005a), thus the concentrations achieved by the implants were physiologically relevant.

Placing our findings in the context of comparable studies in other species is complicated by variation in ontogenetic timing, mode of delivery, dose, and duration of androgen treatment. Nevertheless, our results are generally consistent with pioneering studies on the influence of androgens on urogenital tract development in amniotes following gonadal differentiation. For instance, opossum (*Didelphis virginiana*) pouch young exposed topically or interperitoneally to exogenous T or testosterone proprionate (TP) developed normally organized but slightly smaller gonads and exhibited significant growth throughout the male and female reproductive tracts and external genitalia (Moore, 1941; Burns, 1939). Similarly, T or TP injections over a period of weeks had no effect on gonad organization in immature alligators (*Alligator mississippiensis*), but caused enlargement and precocial maturation of the oviducts and clitoris in females and penis enlargement in males (Forbes, 1938; 1939). Exogenous androgens did not alter mesonephric kidney or duct morphology in 17-month-old alligators of either sex, indicating a lack of androgen sensitivity in the mesonephric kidney and duct at this stage of development in a species that may take a decade or longer to reach maturity (Wilkinson et al., 2016). In *Anolis carolinensis*, crystallin TP pellets implanted in gonadectomized immature lizards increased the size of the oviducts in females and increased the size and secretory activity in the mesonephric derivatives and SSKs of both sexes (Noble and Greenberg, 1940).

The morphology and secretory activity of the luminal epithelium of mesonephric ducts and SSK of T-treated lizards of both sexes was similar to that of adult *S. undulatus* males during the active periods of the annual reproductive cycle (Rheubert et al., 2020). Likewise, the oviducts of T-treated females exhibited regionalization and morphology expected in adult oviparous squamates (Girling, 2002). Enlargement of the oviducts was also observed in adult ovariectomized anoles (*A. carolinensis*) given T or estradiol benzoate (EB) implants in comparison to ovariectomized controls (Winkler and Wade, 1998). The oviducts of EB-treated anoles

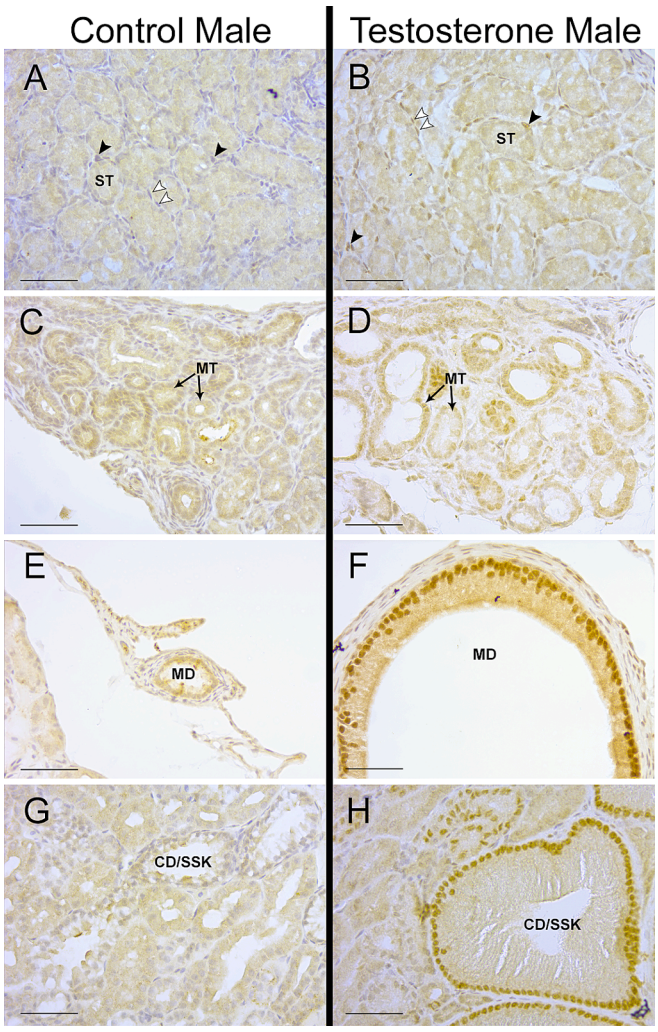

Fig. 6. Immunohistochemistry staining (brown signal, DAB) for androgen receptor in the urogenital system of (A,C,E,G) control and (B,D,F,H) testosterone-treated immature *Sceloporus undulatus* males. (A,B) testis; (C,D) mesonephric kidney; (E,F) kidney; (G,H) kidney; black arrowheads, interstitial cell; white arrowheads, peritubular myoid cell; CD/SSK, collecting duct / sexual segment of the kidney; MD, mesonephric duct; MT, mesonephric tubule; ST, seminiferous tubule; scale bars, 50 μm.

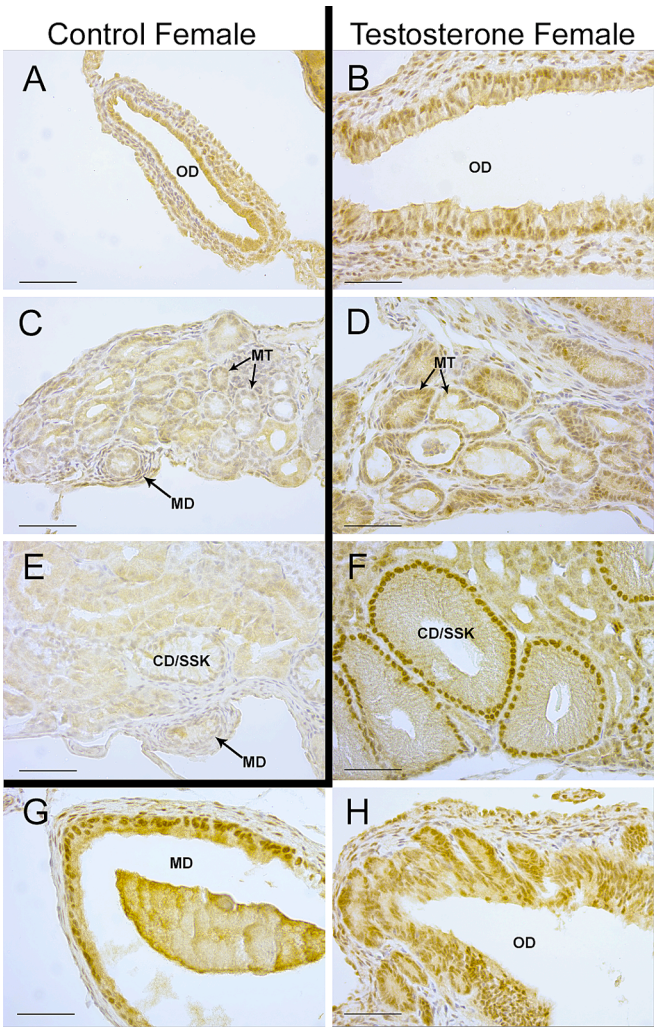

Fig. 7. Immunohistochemistry staining (brown signal, DAB) for androgen receptor in the urogenital system of (A,C,E) control and (B,D,F,G,H) testosterone-treated immature female *Sceloporus undulatus*; (A,B) anterior oviduct; (C,D) mesonephric kidney; (E,F) kidney; (G) posterior mesonephric duct; (H) posterior oviduct; CD/SSK, collecting duct / sexual segment of the kidney; MD, mesonephric duct; MT, mesonephric tubules; OD, oviduct; scale bars, 50 μm.

were larger than those treated with T, and injections with the aromatase inhibitor Fadrozole following placement of T implants did not diminish the effect of T on anole oviduct size. That the oviducts are responsive to androgens and estrogens is consistent with previous reports of AR and estrogen receptor (ER) mRNA expression in the oviducts of leopard geckos (*Eublepharis macularius*). Androgen receptor mRNA is expressed throughout the oviduct in leopard geckos and highest in the luminal epithelium, whereas ER mRNA is primarily expressed in the myometrium (Rhen and Crews, 2001; Rhen et al., 2003). Furthermore, the development of uterine glands, which occurs between the previtellogenic and late vitellogenic stages of the leopard gecko reproductive cycle, corresponds to increasing plasma T and estradiol concentrations, suggesting a potential role for both hormones in the oviduct (Rhen et al., 2000; Rhen et al., 2003). The relative contributions of T-mediated AR activation versus the aromatization of T and the subsequent activation of ERs towards the growth and differentiation of the oviducts remain unresolved, but neither of these pathways are mutually exclusive of the other.

Our finding that only male hemipenes responded to exogenous T and our contention that prior organizational changes precluded a response in female genitalia is consistent with what has been observed in *A. carolinensis*. Specifically, embryonic exposure to androgens shortly before hemiphallus regression in female anoles results in male-like hemipenes development (Holmes and Wade, 2005), whereas exogenous androgen exposure beginning 30 days post hatching does not induce hemipenes development in females (Lovern et al., 2004). In contrast to what has been observed in anoles and in our study, female geckos from two different families (Eublepharidae and Gekkonidae) retain the capacity for hemipenes enlargement in response to exogenous T after attaining sexual maturity (Rhen et al., 1999; Golinski et al., 2014; Golinski et al., 2015). Geckos (Gekkota) form an evolutionary distinct and basal clade among the squamates (Simões and Pyron, 2021). Additional studies of a wider variety of squamate taxa would determine if the findings in geckos are unique to that lineage or if variation in the organizational development of hemiphalli is widespread.

Collectively, the changes observed in the spermatic ducts, the SSK, and the oviducts of T-treated lizards indicate that androgens play a significant role in promoting urogenital tract maturation and secretion. In addition, our results show that the mesonephric tubules and ducts of immature females retain androgen sensitivity similar to that of males for at least 2 months after hatching. How long this period of sensitivity

persists and to what extent mesonephric androgen sensitivity in postnatal females varies among squamate taxa is not known. To this end, future research examining the timing of morphological changes associated with differentiation and maturation of the reproductive tract in this species is warranted. Corresponding studies of concomitant changes in plasma steroids, AMH, and growth factor concentrations, along with expression of their respective receptors, would improve our understanding of hormonal control of urogenital tract differentiation and maturation.

In the present study, we used androgen receptor immunolocalization to determine the extent to which AR expression aligns with tissue-specific responses to increased T concentrations. In untreated lizards, AR staining was absent to moderate and tended to be diffuse throughout AR positive cells, rather than highly localized to the nuclei. Like other type-I nuclear receptors, unliganded AR resides in the cytoplasm, and translocation of AR to the nucleus is ligand-dependent (Georget et al., 1997). Our AR immunohistochemistry results in control animals suggest that the urogenital tracts of immature *S. undulatus* males and females are potentially responsive to androgens based on the presence of cytoplasmic AR, but not currently involved in significant AR-dependent transcription based on low levels of nuclear localization.

The overall pattern of AR immunolocalization in T-treated lizards was very similar to AR expression reported in reproductive tracts of male mice (Zhou et al., 2002). The tissues that were most responsive to elevated T were the mesonephric ducts, oviducts, and SSKs, and each of these tissues exhibited intense nuclear staining for AR. The increased intensity of AR staining in animals exposed to higher T concentrations suggest that AR is under positive autoregulation in portions of the urogenital tract. Our observations of positive autoregulation are consistent with the results of Cardone et al. (1998), who found AR mRNA expression to be positively autoregulated by T in primary cultures of testis cells from the lizard *Podarcis sicula*. An analysis of the composition of their primary culture revealed that peritubular myoid cells and interstitial cells made up 20 % and 1 % of the culture, respectively. In *S. undulatus,* we found that the same cell types were AR-negative in testes from control males and AR-positive in testes from T-treated males.

5. Conclusions

Our study provides a detailed examination of the effects of exogenous T on postnatal urogenital tract development in a lizard. The correspondence between the most morphologically responsive tissues to elevated T and the most intense AR-immunopositive nuclear staining reinforces the presumptive role of AR in mediating proliferation and differentiation of the male urogenital tract. The fact that T treatment stimulated male-like growth, differentiation, and secretion in the mesonephric derivatives and SSK in females suggests that sexually dimorphic organizational changes had not yet occurred, and that androgen responsiveness persists in those structures for at least several months after hatching in females. Hypertrophy of the oviducts was also observed in concurrence with AR immunoreactivity in T-treated females. Future investigations are needed to better characterize the ontogeny of post-hatching urogenital tract differentiation, and to discern the relative contributions of androgen and estrogen signaling towards the maturation of squamate oviducts.

CRediT authorship contribution statement

Matthew R. Milnes: Conceptualization, Investigation, Formal analysis, Methodology, Supervision, Visualization, Writing – original draft. **Christopher D. Robinson:** Conceptualization, Investigation, Project administration, Writing – original draft. **Alexis P. Foley:** Investigation, Methodology, Writing – original draft. **Charleigh Stepp:** Investigation, Methodology, Writing – original draft. **Matthew D. Hale:** Conceptualization, Investigation, Writing – review & editing. **Henry B. John-Alder:** Resources, Methodology, Writing – review & editing. **Robert M. Cox:** Conceptualization, Funding acquisition, Supervision, Writing – original draft.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Acknowledgements

This work was supported by a Collaborative Research award from the U.S. National Science Foundation (IOS 1754934 to H.B.J.-A. and IOS 1755026 to R.M.C.), and the Georgia College Provost Summer Research Fellowship. We thank Sarah Holder for her assistance with morphological measurements.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ygcen.2023.114418.

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