Relationship between seasonal changes in spermatogenesis in the juvenile ostrich (Struthio camelus) and the presence of the LH receptor and 3β-hydroxysteroid dehydrogenase

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The immunohistochemical localization of the LH receptor and 3β-hydroxysteroid dehydrogenase (3β-HSD) was studied in the testis of the juvenile ostrich (Struthio camelus) throughout a 1 year period. Spermatogenic activity of juvenile birds changed throughout the year, as has been reported previously for sexually mature birds. During the active stage of the testicular cycle, from September to January, spermatogenesis progressed up to the stage of formation of spermatozoa, although spermatozoa could not be detected in the epididymis. Leydig cells stained intensely with antibodies against the LH receptor and 3β-HSD during the quiescent, recrudescent and active phases of the testicular cycle. During the regressive phase, there was a slight decrease in immunostaining for 3β-HSD in these cells. These results indicate that Leydig cells in the testis of the juvenile ostrich are able to respond to LH and are capable of steroid synthesis. Furthermore, in juvenile (prepubertal) ostriches, spermatogenic activity can be observed and, as in mature birds, spermatogenesis undergoes seasonal changes.

Introduction

The ostrich (Struthio camelus) is considered to be a seasonal breeder. Several studies have indicated that the breeding season of this ratite bird depends on daylength and that testicular activity is restricted to a specific period of the year when daylength is long (Hicks, 1992; Mellet, 1993; Degen et al., 1994; Soley and Groenewald, 1999). The photo-dependency of the reproductive cycle of the ostrich was also observed under experimental conditions in which exposure to artificial light initiated the growth of the testes in adult ostriches (Jensen et al., 1992). However, it is necessary to consider that with an increase in daylength, there may also be an increase in food intake, which may also influence the onset of the breeding season (Sauer, 1972; Degen et al., 1994), as has been shown in another ratite species, the emu (Dromaius novaehollandiae; Blanche and Martin, 1999). Hence, the ostrich can be considered to be a photoperiod-dependent seasonal breeder, although it is possible that other factors, such as increased food intake as a result of increased daylength, also contribute to the initiation of the breeding season.

In avians, as in mammals, the testes are responsible for the production of spermatozoa and the secretion of androgens. Functional studies have identified the Leydig cells as the site of androgen production in a variety of birds, including the domestic fowl, Gallus domesticus (Woods and Domm, 1966; Galli et al., 1973; Rosenstrauch et al., 1998), the spur-winged goose, Plectropterus gambensis (Halse, 1985), the brown-headed cowbird, Molothrus ater (Dufty and Wingfield, 1986), the Japanese quail, Coturnix coturnix japonica (Nicholls and Graham, 1972) and the king penguin, Aptenodytes patagonicus (Maquet et al., 1994). In mammals, the production of the androgens, androstenedione and testosterone, from dehydroepiandrostosterone and androstenediol, respectively, is catalysed by 3β-hydroxysteroid dehydrogenase (3β-HSD), a marker enzyme for the Leydig cells (Lorence et al., 1990; Majdic et al., 1998). In birds, very little is known about the localization of 3β-HSD in the testis. Freking et al. (2000) demonstrated the expression of this steroidogenic enzyme in the interstitial compartment of the zebra finch testis, but they were unable to identify the positive cells.

Steroidogenesis in Leydig cells is regulated by LH, secreted by the pituitary gland (Brown et al., 1975). In birds, as in mammals, it has been demonstrated that changes in plasma concentrations of LH coincide with changes in testosterone concentrations (Schmidt et al., 1991; Degen et al., 1994; Fowler et al., 1994; Jacquet and Sauveur, 1995). These functional studies indicate that the Leydig cells in the avian testis possess LH receptors and 3β-HSD activity, although this finding has not been confirmed at the protein level. Hence, the objective of the present study was to investigate the immunohistochemical localization of the LH receptor and 3β-HSD in relation to seasonal changes in testicular morphology in the juvenile ostrich.

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Materials and Methods

Animals

Juvenile male ostriches used in this study originated from commercial farms in the Mashonaland province of Zimbabwe (18°S). This is a sub-tropical region with day-lengths varying from 13.01 h of light at the summer solstice (from the end of December to March) to 10.80 h of light at the winter solstice (from the end of June to September). The eggs from which these birds hatched were laid between July and February. The birds had been fed commercially produced food throughout the rearing period. In addition, lucerne and water had been provided ad libitum.

Testes were obtained from juvenile ostriches at an abattoir. Samples were collected from 15 birds each month for 1 year. The birds were 9–12 months of age when they were killed and their body weights were 90–100 kg.

Histology

Testicular and epididymal tissues were immersed in Bouin’s fixative for 48 h. After the tissues were fixed, they were dehydrated through graded concentrations of ethanol and embedded in paraffin wax. For each animal, five testicular tissue blocks were chosen at random and investigated in more detail. From each block of tissue, ten sections of 5 μm in thickness were cut, at least 200 μm apart, and studied after staining with haematoxylin and eosin. In each tissue section, ten cross-sections through the ductus epididymidis were investigated for the presence of spermatozoa.

Statistical analysis

Statistical differences were evaluated by ANOVA and the Turkey–Kramer multiple comparisons test. Values were considered to be significantly different at P < 0.05.

Antibodies

The LH receptor monoclonal antibody (P1B4) was a gift from J. Wimalasena (University of Tennessee, Knoxville, TN). This antibody was raised against the purified rat LH receptor, as described by Indrapichate et al. (1992), and has been shown to bind specifically to LH receptors in rat gonads (Bukovsky et al., 1993; Teerds and Dorrington, 1995; Majdic et al., 1998). A biotinylated goat anti-mouse IgG was used as a secondary antibody (Vector stain kit elite, Vector Laboratories, Burlingame, CA).

The 3β-HSD polyclonal antibody was a gift from V. Luu-The (Laval University, Quebec, PQ). This antibody was obtained after immunization of rabbits with 3β-HSD purified from human placenta (Luu-The et al., 1989). This antibody has been used for the localization of 3β-HSD in the human placenta and ovary, as well as in guinea-pig and rat gonads and adrenal glands (Luu-The et al., 1989; Dupont et al., 1990a,b, 1992; Teerds and Dorrington, 1993; Majdjc et al., 1998). A biotinylated goat anti-rabbit IgG was used as a secondary antibody (Vector Labs).

Immunohistochemical staining

The immunostaining technique was performed on sections of 5 μm thickness according to methods described previously (Teerds and Dorrington, 1993, 1995; Majdjc et al., 1998; Teerds et al., 1999). Sections from ten birds for each group were used for the immunohistochemical detection of the LH receptor and 3β-HSD. Briefly, paraffin wax was removed from the sections and endogenous peroxidase activity was blocked with 3% (v/v) H2O2 in methanol for 30 min. The slides were then washed in a 0.01mol Tris-buffered saline (TBS) solution l–1 (pH 7.4), incubated with 0.1 mol glycine l–1 in TBS for 30 min and then rinsed in TBS. This was followed by a blocking step with 5% normal goat serum for 30 min. The slides were incubated overnight at 4°C in a humidified chamber with antibodies against the LH receptor and 3β-HSD, at a dilution of 1:5000 and 1:300, respectively, in TBS with 0.05% acetylated BSA (Aurion, Wageningen) and then rinsed in TBS. The slides treated with the LH receptor antibody were incubated for 60 min with a biotinylated goat anti-mouse IgG, diluted 1:200 in TBS containing 0.05% acetylated BSA. The slides treated with the 3β-HSD antibody were incubated for 60 min with a biotinylated goat anti-rabbit IgG diluted 1:100 in TBS containing 0.05% acetylated BSA. Both incubations were carried out at room temperature.
temperature. The slides were then rinsed in TBS and incubated for 60 min at room temperature with the components avidin (A) and biotin (B) from the ABC staining kit. Both components (A and B) were diluted (1:3000) and prepared at least 15 min before addition to the sections. The slides were then rinsed in TBS and 0.05 mol Tris–HCl l–1 (pH 7.6). Bound antibody was visualized by the addition of a 0.06 mg ml–1 solution of 3,3’-diaminobenzidine tetra-chloride (Sigma-Aldrich, Deisenhofen) in 0.05 mol Tris–HCl l–1, to which 0.03% H2O2 was added. Slides were counterstained with Mayer’s haematoxylin.

Two types of controls were included during the immunohistochemical incubation procedures. In the first type of control incubation, normal rabbit and mouse sera were used instead of the 3β-HSD and LH receptor antibodies, respectively. In the second type of control incubation, rat testicular sections were incubated with 3β-HSD and LH receptor antibodies to demonstrate that the same type of cell was recognized by the antibodies in the ostrich testis as in the rat testis.

Fig. 1. Immunohistochemical localization of (a,c) 3β-hydroxysteroid dehydrogenase (3β-HSD) and (b,d) the LH receptor in the testis of the juvenile ostrich. During the quiescent phase (a,b) of the reproductive cycle, brown stained (immunopositive) Leydig cells with round to oval nuclei (large arrows) were located in the wide interstitial areas between the seminiferous tubules. Within the seminiferous tubules, only Sertoli cells (large asterisks) and spermatogonia (small asterisks) were present. The recrudescent stage (c,d) was marked by the presence of primary spermatocytes (large arrowheads) and round spermatids (small arrowheads) in the seminiferous tubules. Fibroblast-like Leydig precursor cells are indicated by small arrows. Scale bar represents 23 μm.

Results

On the basis of seasonal changes in testicular morphology, the reproductive cycle of the juvenile ostriches could be divided into four phases: (i) a quiescent phase from April to June when daylength is decreasing; (ii) a recrudescent phase in July and August during the winter when daylength is short; (iii) an active phase from September to January when daylength increases; and (iv) a regressive phase in February and March when daylength starts to decrease again. Testicular morphology changed markedly throughout the reproductive cycle, with only a single layer of Sertoli cells and spermatogonia present at the quiescent phase and a full complement of germ cells during the active phase. In the quiescent period, positive immunohistochemical staining for the LH receptor (Fig. 1a) and for 3β-HSD (Fig. 1b) was evident in the Leydig cells located in the wide interstitial areas between the seminiferous tubules. These positively stained Leydig cells were recognized by their oval to spherical nuclei and the presence of one or two prominent,
round nucleoli. The same type of cell stained positively with both antibodies in the rat testis (data not shown; Majdic et al., 1998, Teerds et al., 1999).

Recrudescence of the testes in July and August was marked by the appearance of primary spermatocytes and round spermatids within the seminiferous tubules. Leydig cells that stained positively with the antibodies against the LH receptor (Fig. 1c) and \(3\beta\)-HSD (Fig. 1d) were present within the interstitial compartment.

During the active phase, from September to January, and the early regressive phase, which started in February, the diameter of the seminiferous tubules increased progressively (Fig. 2). The thickness of the seminiferous epithelium had a tendency to increase during the active phase and was significantly increased during the early regressive phase. The active phase was characterized by the presence of a full complement of spermatogenic cells, ranging from spermatogonia, spermatocytes, spermatids to spermatozoa. As a consequence of the increase in diameter of the seminiferous tubules, the interstitial space seemed to be reduced in size.

Leydig cells that stained positively with the antibodies against the LH receptor (Fig. 3a) and \(3\beta\)-HSD (Fig. 3b) were distributed sparsely between the enlarged seminiferous tubules.

The presence of degenerating spermatogenic cells was characteristic of the regressive phase in February and March. During this period the cytoplasmic bridges between the degenerating germ cells enlarged, resulting in the formation of multi-nucleated giant cells. Apoptotic spermatid nuclei were also observed (Fig. 3c,d). Healthy and slightly apoptotic Leydig cells stained positively with the antibodies against the LH receptor (Fig. 3c) and \(3\beta\)-HSD (Fig. 3d). Nevertheless, it appeared that not all Leydig cells expressed immunoreactivity. The intensity of the staining for \(3\beta\)-HSD was slightly less than that observed during the other phases of the reproductive cycle. In addition to the Leydig cells, numerous fibroblast-like cells with elongated nuclei, which are presumed to be the precursors of the Leydig cells, were present in the interstitium (Fig. 3c,d). Background staining was negligible in the control slides that were incubated with normal serum instead of the primary antibody. Representative sections are shown (Fig. 3e,f).

**Epididymis**

Throughout the year, the epididymal tubules were lined with a simple cuboidal epithelium. Although spermatozoa were observed within the seminiferous tubules during the active and early regressive phases of the reproductive cycle, spermatozoa were not observed in the epididymis of any of these juvenile birds, regardless of the part of the epididymis studied (data not shown).

**Correlation between daylength and histological characteristics**

There was a positive correlation between daylength and the diameter of the seminiferous tubule \((r = 0.92; P < 0.0001)\), and between daylength and the thickness of the seminiferous epithelium \((r = 0.75; P < 0.005)\). There was a negative correlation between daylength and the size of the interstitial areas located between the tubules \((r = -0.70; P < 0.01)\).
Discussion

The present study has established that the testes of the juvenile ostrich undergo seasonal changes. There are indications that these changes in spermatogenic activity are related to variations in photoperiod, although at this stage other factors, such as increased food intake as a consequence of increasing daylength, may also positively influence the initiation of reproductive activity. The quiescent phase of the testicular cycle in the juvenile...
ostiches extended from April to June, a period marked by short daylengths. In the inactive testes, the seminiferous tubules contained only Sertoli cells and spermatogonia. A period of testicular inactivity has also been observed in adults of other avian species, such as the Japanese quail, Coturnix coturnix japonica (Follett and Maung, 1978; Baraldi Artoni et al., 1997, 1999) and the Canadian goose, Branta canadensis (John et al., 1983). In these two species, testicular inactivity was related to low circulating concentrations of gonadotrophins, due to reduced stimulation of the hypothalamic–pituitary–gonadal axis.

The androgen-producing Leydig cells were located in the wide interstitial spaces between the inactive seminiferous tubules, an observation comparable to that reported in the Canadian goose (John et al., 1983) and the Japanese quail (Aire, 1997). In ostriches, the Leydig cells specifically bound antibodies raised against the LH receptor and 3β-HSD. This finding indicates that even in the inactive testis of the juvenile ostrich, Leydig cells have the capacity to produce steroids.

Spermatogenesis in the juvenile ostrich was initiated in July and August, as daylength started to increase. In the starling, Sturnus vulgaris (Williams et al., 1987a,b; Dawson and Goldsmith, 1989; McNaughton et al., 1992), the Muscovy duck, Cairina moschata (Jacquet and Sauveur, 1995) and the great tit, Parus major (Silverin and Sharp, 1996), photoperiod-refractoriness was dissipated by short daylengths. As a result, the hypothalamic–pituitary–gonadal axis was able to respond to increasing daylengths with the progression of the season, resulting in the initiation of spermatogenesis. Whether photoperiod-refractoriness also plays a role in juvenile ostriches is not clear.

Testicular recrudescence in the juvenile ostrich was marked by the appearance of primary spermatocytes and round spermatids in the seminiferous tubules. As the seminiferous tubules enlarged progressively from the end of the recrudescence phase to the active phase, the interstitial space became reduced in size. The Leydig cells, which stained positively for 3β-HSD and the LH receptor, became distributed sparsely in the narrow interstitial areas between the tubules. Similar morphological changes were observed in the testes of the white-crowned sparrow, Zonotrichia leucophrys gambelii (Lam and Farner, 1976) and the Japanese quail (Aire, 1997; Baraldi Artoni et al., 1997, 1999). In birds, as in mammals, the steroidogenic activity of Leydig cells is stimulated by LH, resulting in an increase in LH and testosterone production (Degen et al., 1994) may be responsible for the cessation of spermatogenesis. Nevertheless, additional experiments need to be carried out in which plasma concentrations of LH and testosterone are measured in these birds throughout the year. Similar effects of decreased gonadotrophin production and subsequent reduction in testosterone synthesis have been observed in rats (Bartlett et al., 1986; Kerr et al., 1993; Woolveridge et al., 1999).

The slight decrease in staining intensity for 3β-HSD in Leydig cells during the regressive phase was possibly due to degeneration of the Leydig cell population. Apoptotic Leydig cells were observed occasionally. Degeneration of the Leydig cell population has also been observed during the regressive phase in the testis of the white-crowned sparrow (Lam and Farner, 1976).

Generally, during the recrudescence phase, degenerated Leydig cells were replaced by immature Leydig cells that had differentiated from fibroblast-like, precursor Leydig cells (Nicholls and Graham, 1972; Teerds et al., 1987, 1989a, 1992). Indeed, cells with a fibroblast-like morphology, assumed to be Leydig cell precursors, were observed in the juvenile ostrich testes during the recrudescence, quiescent and regressive phases of the testicular cycle. On the basis of data available for rats (Hardy et al., 1989; Teerds et al., 1989b), it is probable that in the juvenile ostrich these precursor cells are also able to proliferate and differentiate into Leydig cells in the presence of low LH concentrations, characteristic of the regressive and quiescent phase of the reproductive cycle (Degen et al., 1994).

In conclusion, the results of the present study indicate that spermatogenesis is already initiated in juvenile ostriches. Moreover, spermatogenic activity seems to undergo seasonal changes, which is presumably related to changes in daylength. This annual reproductive cycle...
results in the production of semen containing spermatozoa, although transport of these sperm cells may be affected, as indicated by the lack of spermatozoa in the epididymis. In addition, the present study has shown that in the juvenile ostrich testis, Leydig cells possess LH receptors and the steroidogenic enzyme 3β-HSD during the quiescent, recrudescent, active and regressive phases of the testicular cycle, indicating that these cells are capable of steroid production. It may be possible that even in juvenile birds, the hypothalamic–pituitary–gonadal axis is responsive to photoperiod stimulation. However, before firm conclusions can be drawn, results of studies currently investigating plasma concentrations of gonadotrophins and testosterone throughout the annual testicular cycle are required.

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