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Article

Seasonal cycle of leydig cells in the Japanese Jungle Crow (*Corvus macrorhynchos*)

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Abstract: The investigation was conducted to elucidate seasonal histomorphological changes of Leydig cells in Japanese Jungle crows (*Corvus macrorhynchos*). All adult crows (n=12) were killed for H&E staining. Histologic data of Leydig cells regarding their locations in the interstitium, appearance and disappearance of lipoidal materials in their cytoplasm, precursor cells and fibroblasts are studied. In the nonbreeding season, in the interstitium, clusters of Leydig cells were found in abundant number. They looked foamy, white and easily visible. Their nuclei were found small and are located eccentrically. During recrudescence, the Leydig cell became less foamy and less white. In peak breeding season, the Leydig cells showed bigger in size and the nuclei became prominent. Cytoplasmic lipoidal material was lowest and cytoplasm was found dense pink. At the end of breeding period, heavy accumulation of lipid materials occurred in the Leydig cell. The clusters of Leydig cells were visible and looked white and foamy again. During this transition, disintegration of Leydig cells occurred after pycnotic nuclei of Leydig cells were found. During involution, new generation of fibroblast like cells appeared in the interstitium. Within the fibroblast like cells, some of them became lipoidal by accumulating lipid materials. Thus a new generation of Leydig cells appeared in the interstitium. These lipoidal cells are thought to be a new generation of Leydig cells for the next breeding season. This study suggests that in seasonally breeding birds, new generation of Leydig cell is derived from fibroblast like cells of the interstitium.

Keywords: crows; leydig cell; seasonal; cycle

1. Introduction

In the most seasonally breeding birds, males show a seasonal testicular development during increasing of daylight in spring and a postnuptial regression in prolonged daylight (Loft, 1964). Avian seasonal breeders such as penguins (Williams, 1992), mallards (Donham, 1979), ostriches (Degan *et al*., 1994), geese (Dittami, 1981), grey partridges (Fraissinet, 1987) and Japanese common pheasants (Sakai and Ishii, 1986) show seasonal changes in the serum testosterone and LH levels during reproductive cycle. It has been reported that testosterone levels changed in parallel with spermatogenic activity in the testis. In the seasonally breeding birds, testicular activity is cyclic and in some species there is evidence that these cyclic changes may, in part at least endogenous. Thus, the testes of most wild birds become spermatogenitically active and produce spermatozoa (Marshall and Serventy, 1958). Similarly, the interstices between the convoluting seminiferous tubules are packed with areolar connective containing blood capillaries, lymph vessels and interstitial Leydig cells, which secrete testosterone in the breeding season. A very recent study suggests that expression of esr genes help in

growth and differentiation for Leydig cell in testis throughout the annual reproductive cycle in Atlantic cod (Nagasawa *et al.* 2014). On the contrary, interstitial tissue of the regressed testes of the nonbreeding season appears more prominent (Loft and Murton, 1973).

Many experiments have been conducted on the interstitial cells. So, a good numbers of reports are available regarding general histomorphological changes of Leydig cells at various periods of sexual activity. The evolution of such glandular cells is in direct proximity to recrudescence of spermatogenic cells in upcoming breeding period. In fact, androgens are concerned with spermatogenesis (Dodd, 1955). It is believed that Leydig cells become lipoidal and are said to be cholesterol-positive prior to onset of breeding season. Cytological observations indicate that the cells secrete, cholesterol-positive lipids are gradually utilized, probably by conversion of androgens in the breeding season. Then the cells pass into a nonlipoidal and finally vacuolated. Ultimately, most of the Leydig cells disintegrate after breeding season (Marshall, 1955). However, in another opinion, the Leydig cells give rise to another generation of juvenile Leydig cells at the termination of the breeding season (Sluiter and van Oordt, 1949). So, it is necessary to clarify the controversy whether they disintegrate or give rise to next generation of juvenile cells.

However, data on seasonal cycle of Leydig cells in passerine birds specially in the jungle crow are rare and need to conduct more research on how the Leydig cell involutes during the breeding and nonbreeding season. Therefore, the piece of work elucidated about seasonal histomophological changes of Leydig cells in the jungle crow (*Corvus macrorhynchos*).

2. Materials and Methods

The adult male Jungle crows (n=12) used in this investigation were captured from Niza city of Saitama Prefecture and Moka City of Tochigi Prefecture, Japan from January to June during 2008-2009. January to June in Japan covers the nonbreeding period, breeding period and post breeding period of jungle crows (Islam *et al.* 2010; Kuroda, 1990). The catching of crows was permitted by Niza City (Permit nos. 01 and 02) and Tochigi Prefecture (Permit no.0010). All of the Jungle crows were cared for according to guidelines suggested in the Care and Use of Laboratory Animals at Utsunomiya University, Japan. All of the crows were killed on the day following capture. Crows were considered to be adult by visual assessment of the rudimentary size of the bursa of Fabricius and the black color of the upper palate of the beak.

All crows were anesthetized with an overdose of pentobarbital sodium (30 mg/body weight). The abdomen was opened through an incision on the ventral surface. The birds were then perfused through the left ventricle by injecting 1 ml of heparin and 50 ml of Ringer solution, followed by 500 ml of Zamboni's solution. After evisceration, the testes were immediately removed and measured for the length and the width. Next, the testes were post-fixed in 10% formalin containing 0.1M phosphate buffer (pH 7.24) for 7 days, dehydrated in a graded alcohol series over 48 h, cleared in xylene, embedded in paraffin, and cut at 4-µm-thick by sliding microtome (REM-710, Yamato Kokhi Industrial Co. Ltd., Japan). The sections were mounted on the glass-slides. A total of four cross-sections taken from across the testes for H&E staining, were analyzed for each assessment.

Histologic data for each of the testis regarding Leydig cells, their locations in the interstitium, appearance and disappearance of lipoidal materials in their cytoplasm, number of Leydig cells in between the seminiferous tubules, spermatogenesis state of seminiferous tubules, appearance of precursor cells and fibroblasts, development of blood vessel close to Leydig cells, development and regression of seminiferous tubules, etc. are studied and documented.

Photomicrographs were obtained from testes sections using a photomicroscope (Olympus Digital Camera DP12, Japan). The photomicrographs were processed with Adobe Photoshop CS5 for expected size and dpi and saved as JPG format. Finally, the photomicrographs were numbered and placed in the manuscript.

3. Results and Discussion

In our present study, seasonal histomorphological changes of the Leydig cells were examined in the testis of the jungle crow during the nonbreeding, the main breeding and the post breeding seasons. Grossly in the nonbreeding season, the testes of the jungle crow remained very small and grew gradually towards breeding season and became larger. After breeding having been completed, the testes became gradually smaller to those of the nonbreeding season (Figure 1). The cyclical waxing and waning of the cellular lipids in the Leydig cells is a useful index of the functional activity of the testicular tissues of the seasonally breeding avian species. There are some literatures concerning the seasonal changes observable in the avian Leydig cells and interstitial

tissue, but a few of the early reports are contradictory, often being based on unsatisfactory and now outmoded histological procedures. Because of their dispersal by seasonal seminiferous tubule expansion, the Leydig cells have sometimes been stated to be absent from the gonad at certain times of the year (Rowan and Batrawi, 1939) and inverse relationship between sexuality and the Leydig cell activity has sometimes been claimed (Oslund, 1928). This is not true and more recent histochemical and electron microscopic observations have clearly established the close relationship between these cells and the androgen-dependent sexual structure. During the sexually quiescent period, the cells are small and contain little lipoidal material.

In the present study, testis section showed smaller seminiferous tubule and larger amount of interstitium in the nonbreeding season. In this season, clusters of Leydig cells were found in abundant number (Figure 2a). This observation is similar to those in mammals (Connell, 1972; Rothwell 1973). As in mammals undifferentiated and differentiated forms of the interstitial cells occur in all ages of birds except the very young. In addition, The Leydig cells looked foamy, white and easily visible prior to onset of breeding season. Accumulated lipid and cholesterol materials in their cytoplasm were found in our present study. Some cells were found to form syncytium and cell outline was less visible. The cells also distributed singly in the triangle of seminiferous tubules. Their nuclei were found small and were located eccentrically in the cell (Figure 2a). Like our findings, Lake (1981) found that mature interstitial cells are polygonal. He also characterized the Leydig cells by the presence of mitochondria with tubular cristae, smooth endoplasmic reticulum and lipid droplets. Since the lipids are cholesterol-positive and strongly birefringent, it seems probable that they represent precursor materials in the production of androgenic steroids. Lipids in the Leydig cells are cholesterol-positive and strongly birefringent, reactions that are probably indicative of precursor materials involved in androgen biosynthesis. The prenuptial buildup in birds often precedes the hypertrophy of the accessory sexual apparatus and behavioral sexual apparatus and behavioral activities thought to be dependent on androgen secretion (Loft and Murton, 1973; Loft, 1978).

At the beginning of breeding season or recrudescence period, the Leydig cell became less foamy and less white. Nuclear size showed larger with dense cytoplasm in this period. Seminiferous tubules also became larger (Figures 2b, c). Our findings are in agreement with the reports of Murton *et al.* (1969) in which they stated that prior to the acceleration phase, the cells rapidly increase in size and there is a buildup of the lipoidal inclusions, so that the interstitial tissue is seen to consist of compressed aggregations of heavily lipoidal and cholesterol-rich cells. They also react positively to tests for 3β-HSDH (Murton *et al.*, 1969). Prior to peak breeding period, seminiferous tubules gradually became larger and interstitium became gradually showed thinner and blood vessels were apparent close to Leydig cells. Leydig cells in the triangle of the seminiferous tubules were found fewer in number (Figure 2c). In our present study, the Leydig cells became larger sparse in the interstitium in the Jungle crow. In peak breeding season, seminiferous tubules had largest diameter and interstitium became scanty. Seminiferous epithelium also became thickest with all stages of spermatogenic cells and sperms (Figure 2d). The Leydig cells showed bigger in size and the nuclei became also prominent. Cytoplasmic lipoidal material was found lowest and the Leydig cells didn't look foamy and white; rather cytoplasm was found dense pink. In most of the cases, the Leydig cell was found singly in the interstitium. The lumen contained bundles of spermatozoa of the seminiferous tubules (Figure 2d).

Figure 1. Showing paired testes of the Jungle crow collected during April, May and Late June in Japan. The left paired was collected during April in breeding season. The middle pair was collected during in May whereas the right pair collected during late June. Small graduated scale indicates 1 cm.

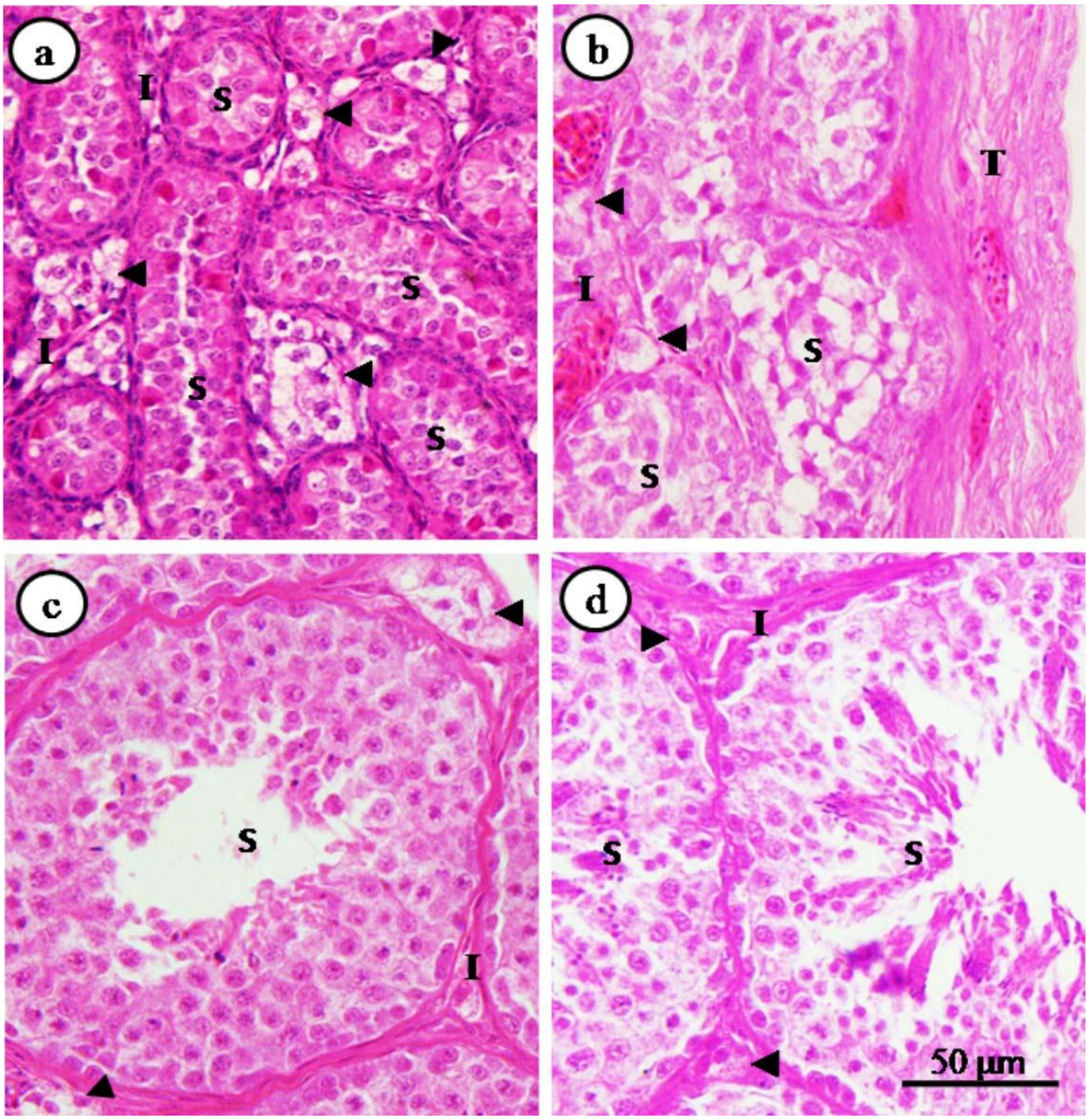

Figure 2. Showing sections of testis of the Jungle crow. In micrograph a, Leydig cells accumulated heavily lipid materials in their cytoplasm (Solid arrow head) collected sample in January. In micrograph b, nucleus looked larger with moderate lipid materials left in their cytoplasm with expanding seminiferous tubule in February. In micrograph c, lumen appeared in the seminiferous tubule in March. In micrograph d, little or no lipoidal material was present in the cytoplasm of the Leydig cells of large nucleus in April. S, Seminifeorus tubule; Solid arrow head, Leydig cells; T, Tunical albuginea; I, Interstitum. H&E staining.

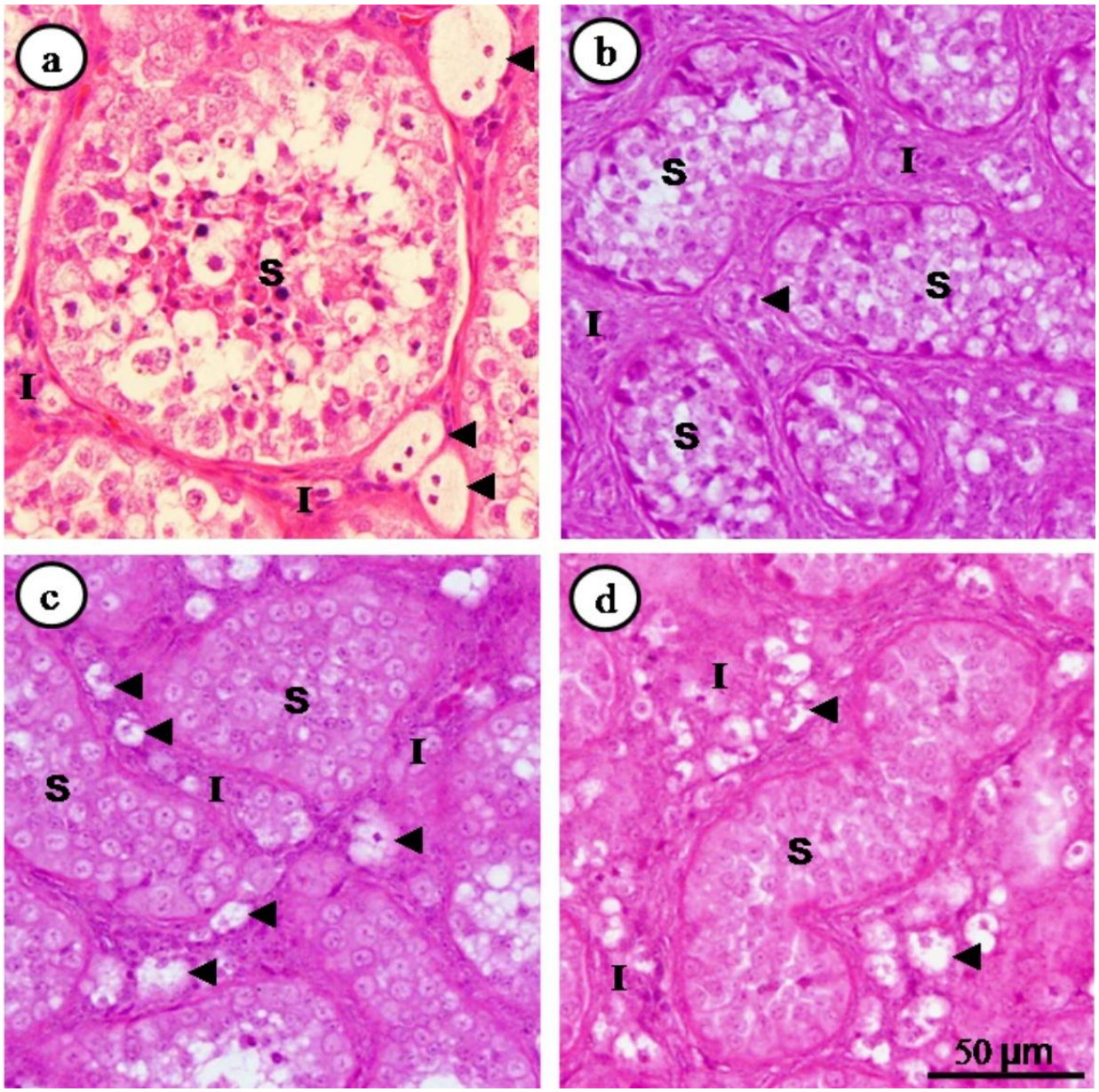

Figure 3. Showing sections of testis of the Jungle crow. In micrograph a, Leydig cells again accumulated heavily lipid materials in their cytoplasm (Solid arrow head) collected sample in early May. Note that seminiferous tubule was collapsing with degenerating cells. In micrograph b, Leydig cells looked disintegrated and interstitial tissue increased in late May. In micrograph c, a new generation of fibroblast like cells appeared in the interstitium, some of which accumulated lipid materials in early June. In micrograph d, more lipoidal material appeared in the cytoplasm of the Leydig cells in late June. S, Seminifeorus tubule; Solid arrow head, Leydig cells; T, Tunical albuginea; I, Interstitum. HE staining.

At the approach of the sexual season and consequent buildup of spermatogenic activity in the seminiferous elements, they become rapidly depleted of their lipids and cholesterol and become strongly fuchsinophilic (Marshall, 1955; Lake, 1981). From our findings, it might be plausible that once the bird enters the acceleration phase, however, the cells become much larger and cholesterol-rich lipoidal materials start to be used up for steroid hormone synthesis. Taken together the cytoplasmic features of the fully active cell in the breeding season, i.e. the pattern of cytoplasmic membrane differentiation, the build-up of lipid droplets concomitant with this differentiation, and development of mitochondria with tubular cristae followed by the subsequent apparent diminution of the lipid, are the characteristics of trends in cells in which hormone stimulation is known to be increase androgen production (Lake, 1981). But Farias *et al*. (2014) express different opinion in such a way

that body mass and diameter of Leydig cell nuclei showed no significant differences between dry and rainy seasons and stages of annual reproductive cycle of Chiroptera**.**

In young chick, for example, the increase in concentration of birefringent material in the interstitial tissue is in close agreement with the gradual hypertrophy of the comb (Kumaran and Turner, 1949), and in the house sparrow (*Passer domesticus*) the level of 17α-hydroxylase activity in the interstitial tissue has been shown to increase rapidly between February and March (Fevold and Eik-Nes, 1962), a time when the interstitial cells are losing the lipoidal material accumulated earlier in January and February. The sudden depletion of the lipids and cholesterol at the height of the breeding activity has also been noted in a number reptilian species, and it has been suggested that it is probably indicative of rapid utilization of precursor material at a time of high androgen release (Loft, 1968).These reports are in agreement with our findings in the jungle crows.

Annual reproductive cycle and cyclic variations in reproductive hormones those are associated with structural changes in the testicular tissues (Bartke *et al*., 1987; Sinha-Hikim *et al*., 1988). These cyclic changes are partially caused by alteration in the rate of androgen biosynthesis by Leydig cells. Androgen biosynthesis in the Leydig cells during seasonal cycle is regulated by the level of LH in the circulation and capacity of Leydig cells to respond to LH (Sharp *et al*., 1986). It has been reported that testosterone levels changed in parallel with spermatogenic activity in the testis. A very recent study suggests that expression of esr genes help in growth and differentiation for Leydig cell in testis throughout the annual reproductive cycle in Atlantic cod (Nagasawa *et al*. 2014).

In the species such as Northern Fulmar (*Fulmarus glacialis*), in which the young do not begin breeding until seven year old, the interstitial cells of newly hatched birds are less lipoidal but become more heavily impregnated when the birds are just over two years old (Marshall, 1949). Then, as in juvenile, the lipoidal content rapidly diminishes at a time when androgen-dependent sexual displays (Murton *et al.* 1969) reach their maximum intensity. During this period, the 3β-HSDH activity remains strong. The nuclei of the Leydig cells also attain maximum diameter at this time, reflecting an increase in secretory activity (Alfert *et al*. 1955). In migratory waders (Charadriiformes), the depletion of the interstitial lipids is often evident just before the birds leave their African wintering grounds on their north-bound migration (Loft, 1962). In breeding season, the Leydig cells showed the general characteristics of steroid-producing tissues in the jungle crow. Previous histochemical studies have shown the presence of the Δ^5-3β-hydroxysteroid dehydrogenase (3β-HSDH) enzyme system in the cell (Wood and Domm, 1966). This enzyme catalyzes the conversion of Δ^5-3β- hydroxysteroid to Δ^5-3-ketosteroids (Samuels, 1960), which is an important stage in the production of steroid. Its presence in the Leydig cells provides strong evidence of biosynthesis of testosterone. However, the 3β-HSDH activity is absent in nonbreeding season. The reports support our findings in the jungle crow.

In our present observations, at the end of breeding period, tetogenesis occurred i.e., heavy accumulation of lipid materials occurred in the Leydig cell as well as in the seminiferous tubules. The clusters of Leydig cells were visible and looked white and foamy in the interstitium. Cell membranes in the cluster were not found and nuclei arranged close together (Figure 3a). During this transition from breeding period to post-breeding condition, disintegration of Leydig cells occurred. As a result, no Leydig cell was found. Sometimes, pycnotic nuclei of Leydig cells were found in the interstitium (Figure 3b). Blood vessels looked disappeared close to Leydig cells. Interstitium looked thicker.

During involution, new generation of fibroblast like cells appeared in the interstitium. Among those fibroblast like cells, some of them became lipoidal by accumulating lipid materials (Figure 3c). This condition gradually continued as more number of fibroblast like cells became more lipoidal (Figure 3d). A new generation of Leydig cells appeared in the interstitium. These lipoidal cells might be a new generation of Leydig cells for the next breeding season, which appeared more pronounced in groups or clusters.

With the advent of the regeneration phase, the now exhausted interstitial cells have reached the end of their secretory cycle, and it has been reported that they disintegrate so that ultimately their total number is drastically reduced (Marshall, 1955; Jones, 1970). In gonadally inactive birds they accumulated lipid droplets, dense heterogeneous bodies, probably lysosomes, and appeared to degenerate (Aire, 1997). An increase in apoptosis and a declining in BcI-X_L are the characteristics of testicular regression in American crows *(Corvus brachyrhynchos*) (Jenkins *et al*. 2007; Islam *et al.*, 2012). It may be that massive invasion by leukocytes that takes place at this time clear the spent Leydig cells by phagocytic action. The possibility that the spent Leydig cells returning to inconspicuous fibroblast- like cells should not be excluded. Concomitant with the atrophy of the exhausted generation, a new generation of juvenile interstitial cells begins to arise, presumably by their differentiation from the fibroblast-like progenitors (Nicholls and Graham, 1972), and gradually begins to

mature. An immunohistochemical study demonstrates a similarity between fibroblasts of interstitium and the Leydig cells in such a way that vimentin is expressed both in Leydig cells and in fibroblasts during maturation of Japanese quails (*Coturnix coturnix japonica*) ((Madekurozwa, 2013).Concluding, seasonal testicular regression in seasonally breeding birds is mediated by apoptosis (Young *et al*., 2001).

The seasonal replacement by the new interstitial cells at the end of each breeding period is not unique to avian testicular cycles. A similar phenomenon has been also recorded in some snakes (Lofts *et al*. 1966) and in common frogs (Rana temporaria) (Lofts, 1965). Marshall (1961) suggests that the sequence is part of an endogenous rhythm that can occur even in the absence of any gonadotropic rhythm. Thus, even after complete removal of the adenohypothysis, Coombs and Marshall (1956) have reported that the interstitial cells of the domestic cockerels still renew themselves and develop a new generation of Leydig cells with some lipoidal and cholesterol-positive material. It is doubtful, however, whether such cells would ever become secretory in the absence of gonadotropins.

The length of time necessary for interstitial cell rehabilitation probably varies from species to species, but there is evidence that in some birds at least it may be a fairly rapid process. Thus, Lofts and Marshall (1957) have recorded that the newly arisen interstitial cells of fifteen different migratory birds were already beginning to manufacture small cholesterol positive lipid droplets in their cytoplasm when they were autopsied at the time of their departure from Britain on their southward migration.

4. Conclusions

In conclusion, the secretory cycle of the Leydig cells in the seasonal birds is well-defined. Thus during the sexually quiescent period, the cells are small and contain little lipoidal material. Prior to main breeding period, however, the cells become much larger and there is a marked increase in the cholesterol-rich lipoidal materials. At the height of the breeding season, the cells become depleted of lipids. The beginning of the regeneration phase marks the end of the secretory cycle of the Leydig cells although their ultimate fate seems to be in doubt. During this period a new generation of interstitial cells is derived from fibroblast like cells. Further studies are needed how quantitative information on histo-architectures of Leydig cells at ultrastructural level correlating with serum LH and testosterone level during breeding and nonbreeding season in wild birds.

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Conflict of interest

None to declare.

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