MATERIALS AND METHODS

Materials :

The model species :

1. The Bank Myna, *Acridotheris ginginianus* (Latham): Family: Sturnidae. Measures about 9 inches in length and is a well-groomed bluish grey coloured bird with black head with orange beak. A patch of orange naked skin surrounds the brick-red eyes. The primary feathers have a white wing patch. Sexes are alike and they flock in open country and in the vicinity of human inhabitations. It is a colonial hole nester (Ali, 1993).

2. The Brahminy Myna or Black headed Myna, *Sturnus pagodarum* (Gmelin) Family: Sturnidae : It is a smaller bird as compared to Bank Myna and measures about 8 inches in length. Head has a prominent glossy black crown with long recumbent crest, which is longer in males during breeding season. Prominent light blue skin covers the base of beak, which has a yellow tip. Body feathers are grayish above and fawn coloured on the dorsal side. Secondary wing feathers black in colour and tail feathers brown with white endings. Legs are yellow. Sexes alike. Observed in small flocks or in pairs in wooded country. It is an individual hole nester (Ali, 1993).

Methods :

Field Observations :

The field observations were made from March 1997 to July 1997 during morning (06:00hrs. to 11:00hrs. IST) and evening hours (15:00hrs. to 19:00hrs IST.) on a breeding Bank Myna and Brahminy Myna pair. Pair bond formation, nest building, nest-defense and

parental activities including territorial behaviour of both the Mynas were noted down all through the breeding cycle.

Laboratory Work :

The seasonal changes in the histochemical activities of hydroxysteroid dehydrogenases (3β-HSDH, 17β-HSDH and 3α-HSDH) and lipids in the gonads (ovaries/testes) and the liver, intestine and kidneys of the Bank Myna and Brahminy Myna were carried out for three consecutive years from September 1997 to September 2000. The birds were obtained from a local bird supplier and kept in the Zoology Department aviary (10x15 feet) till sacrificed. Food and water were supplied *ad libitum.* The birds were sacrificed early in the morning between 05:30 hrs. and 7:00 hrs IST. On an average, at least six birds, each of males and females, were used per phase of the reproductive cycles *i.e.,* Pre-Breeding, Breeding, Post-Breeding and Non-Breeding seasons.

Individual birds were anesthetized and blood was collected from heart (ventricle) before sacrificing them. Body weights were duly recorded. The gonads, left part of the liver, proximal part of the ileum and left kidney were dissected out, freed from the adhering tissue as quickly as possible and blotted free of tissue fluids. Gonads were weighed accurately on single pan Mettler balance. These tissues were then fixed with 10% gelatin on chucks of Cryocut E cryostat microtome maintained at -20° C. Fresh frozen sections of 16μm thickness were taken on glass slide and processed for histochemical studies. For the histological studies, the tissues were fixed in Bouin's fluid.

Histochemical Studies : (Summer, 1988)

[A] : Hydroxysteroid dehydrogenase :

1. Δ^5 3β-hydroxysteroid dehydrogenase (3β-HSDH): Localization of 3β-HSDH in tissue sections was carried out by using the method of Wattenberg (1958). Pregnenolone (5-Pregnen-3β-ol-20-one) and Dehydroepiandrosterone (DHEA) were used as substrates. Nicotinamide Adenine Dinucleotide (NAD) was used as a cofactor and Nitro Blue Tetrazolium (NBT) a tetrazolium salt was used as electron acceptor. Magnesium Chloride was added to the incubation medium. The substrates were dissolved in Dimethylformamide (DMF). The incubation medium was prepared in tris (HCl) buffer with pH 8.3. After incubating the sections in the medium for 25 minutes at 37°C, the slides were rinsed with distilled water and fixed with 10% neutral formalin for half an hour. Sections were rinsed again with distilled water to remove fixative and then mounted with glycerine jelly.

2. 17β-hydroxysteroid dehydrogenase (17β-HSDH): Localization of 17β-HSDH in tissue sections was carried out by using the method of Kellog and Glenner (1960). Testosterone and β-estradiol were used as substrates, NAD as cofactor, NBT as electron acceptor and BA (Bovine Albumin) was added to maintain viscosity. Substrates were dissolved in DMF and the incubation medium was prepared in tris buffer maintained at pH 8.3. Sections were incubated at 37°C for 20 minutes, thereafter rinsed with distilled water and later fixed with 10% neutral formalin for half an hour. Sections were rinsed again with distilled water and then mounted with glycerine jelly.

3. 3α-hydroxysteroid dehydrogenase (3α-HSDH): Localization of 3α-HSDH in tissue sections was carried out by using the method of Balough (1966). Androsterone was used as substrate, NAD as cofactor, NBT salt as electron acceptor and EDTA as chelating agent.

Polyvinylpyrrolidone was used to increase the osmolarity of the incubation medium and to control enzyme diffusion. As for previous incubation media, here also substrates were dissolved in DMF and incubation medium was prepared in tris HCl buffer maintained at pH 8.3. Sections were incubated at 37°C for 1hour, then rinsed with distilled water and fixed with 10% neutral formalin for half an hour. Sections were rinsed again with distilled water and then mounted with glycerine jelly.

Control sections were incubated in media devoid of substrates for all the three steroid dehydrogenases studied.

The methods were standardized for avian tissues before starting the actual work. Not much significant variation could be noted with pregnenolone (5-Pregnen-3β-ol-20-one) and dehydroepiandrosterone (DHEA) as substrates in the localization of 3β-HSDH and also in the localization of 17β-HSDH with testosterone and β-estradiol as substrates. Hence, the discussion will not include any variations based on the different substrates used.

[B] : Lipids : (Pearse, 1968; High, 1975)

1. Neutral fat localization with Fettrot-7B :

Fresh frozen sections were first fixed with 10% calcium formal for half an hour and then stained with the saturated solution of 70% Fettrot-7B for 15 minutes, after a quick dip in 70% alcohol the sections were rinsed in distilled water. The sections were then mounted with glycerine jelly.

2. Total lipid localization with Sudan Black B :

Fresh frozen sections were first fixed in 10% calcium formal for half an hour, and then stained in 70% Sudan Black B for 30 minutes. After a quick rinse in 70% alcohol the sections were rinsed in distilled water and then mounted in glycerine jelly.

3. Phospholipid localization with Nile Blue Sulphate:

The fresh frozen sections were stained in Nile Blue Sulphate for 90 minutes at 60°C. The sections were rinsed in distilled water to remove excess stains and then placed in acetone, which was preheated at 50^0 C, for 30 minutes. The sections were again rinsed in distilled water and differentiated in 0.5% HCl for 3 minutes. The sections were again rinsed in distilled water to remove excess HCl and mounted in glycerine jelly.

Enzyme Linked Immunosorbant Assay (ELISA) :

The blood samples were collected from the ventricle of the heart of anesthetized birds and later centrifuged at 3000rpm for 60minutes to obtain the plasma. ELISA for progesterone (P) and testosterone (T) were carried out in ELISA kits purchased from Bio-Chem Immunosystems Italia S.P.A. The plasma progesterone and testosterone levels were expressed as ng/ml of blood plasma.

Histological Techniques : (Gurr, 1956 ; Gordon and Bardbury, 1977 ; Stevens, 1977)

The fresh tissues were fixed in Bouin's fluid for 24 hours washed several times in 70% alcohol to remove excess Bouin's fluid; dehydrated in alcohol grades of 70%, 90%, and 100%, cleared in toludine and then embedded in wax to prepare wax blocks. Microtome sections of 5μm thickness were taken and stained with haematoxylin and eosin (to stain the nuclei and cytoplasm respectively) and mounted with DPX.

Testes and ovaries were cytometrically analyzed to validate the changes observed during the reproductive cycle. The histochemical as well as histological sections of gonads and the extra-gonadal tissues of

the Mynas were photographed on Leica Photomicroscope (Leitz Periplan) at 25X or 40X magnifications.

Ovary :

1. The ovary of each bird was weighed on Mettler before it was processed for histochemical or histological techniques.
2. From the 5μm thick sections of the ovaries, healthy as well as atretic follicles were counted and diameter of healthy follicles measured with the help of occulometer. Follicles were categorized as: <50μm; 51-100μm; 101-200μm; 201-300μm; 301-500μm and >500μm (1 div.= 19.5μm; Objective=3.2X).

Testes :

1. The right and left testes were weighed nearest to 0.1mg on mettler prior to tissue processing.
2. Tubule diameter : The diameter of the seminiferous tubules was estimated by measuring 25 tubules from each section with the help of an ocular micrometer. Perpendicular diameter of each tubule were measured, averaged and expressed as mean tubular diameter.

Body Weight :

The body weights of both sexes of Bank Myna and the Brahminy Myna were measured prior to decapitation on a single pan balance.

Statistical Analysis :

All the results were statistically analyzed and are reported as Mean ± Standard Deviation (SD). The data were also subjected to students 't' test with a 95% confidence limit.