The model species: Blackheaded munia *(Lonchura malacca malacca)*

Experiments were performed on adult blackheaded munia (*Lonchura malacca malacca*), locally called as Chestnut Mannikin, Chestnut Munia, tri-colored mannkin or nun, three colored munia, mannikins or Nun, southern blackheaded munia, blackheaded munia, blackheaded nun, blackheaded manikin and other scientific name is *Lonchura malacca malacca* (Ali and Ripley, 1974).

Southern black-headed munia is a small passerine resident breeding bird. This finch is found in Bangladesh, Brunei, Cambodia, China, India, Indonesia, Laos, Malaysia, Burma, Nepal, Philippine, Singapore, Taiwan, Thailand, Vietnam and Hawaii. The blackheaded munia, *Lonchura* (formerly considered as a subspecies of the tri-coloured munia, *Lonchura malacca malacca*) also known as Chestnut munia. These birds live in pairs or small flocks when breeding and large social groups outside of the breeding season; they gather in rushes and use tall grasses to roost in flocks. Breeding birds and recent fledglings roost in nests. Non-breeding birds may mingle with spotted munia. The natural habitat of this species is grassy areas, especially swampy grasslands and tending to favour wet or marshy areas where the grass is rich and long, and where seeding heads at various stages of development and plentiful like reeds, rice fields, forest edge, mangrove edge, and scrub brush. So this species primarily feed and affects open cultivation, often near paddy fields, considered a pest on rice paddy fields, seeding grasses grassland and secondary scrub dotted with babool trees, date and palmyra palms and its favourite foods is large grain millet, sprouted, seed and grains.

This blackheaded munia is a small gregarious bird and its length is 11-12 cm. This is a medium sized munia with a black head and chest nut body, contrasting white lower breast and flanks, with a black belly and under tailed coverts. The adult is unlikely to be confused with any other munia or finch. These adults has a stubby pale grey-blue bill, black head, and brown body, with a brick red patch on the lower back, visible only when it flies. Some races also have a black belly. The sexes are similar, but immature birds have uniform pale brown upperparts, lack the dark head and have white to pale buff under parts. In captivity, juvenile blackheaded munia can commence breeding even when they are only part-way through attaining adult coloration during their first molt. In these cases, the molt ceases until the end of the breeding season, leading to offspring which obtain their full adult coloration before their parents.

The breeding season of these manikins is May to November in the Indian parts of its range, June to September in Myanmar (Burma), and December to October in Malaysia. Birds aged one to four years are best suited for breeding. The breeding diet should be initiated one month prior to breeding, and should include: ample sprouted seed, egg food, greens, and possibly live food. For better results, keep a group of these birds together to allow the birds to choose their own partners. Males will court females with the use of a song and courtship dance which includes carrying a piece of grass and hopping on the perch beside the hen. Receptive hens crouch and tail-quiver, inviting copulation. Mating is often followed by bill-fencing and mutual allow-preening.

The nests constructed of dried grass and fine twigs are built in dense reeds in large domed grass, tufts of grass, palm trees, or thick bushes usually between 1 and 2

meters from the ground. Pairs may also accept half-open nest boxes. Both parents share the tasks of nest building, incubation, and chick rearing. The clutch size of this species found 4-7 eggs, hatching takes place after incubation of 12-15 days, new nets become start fledge in 22-28 days of age. The young hatch naked pairs seem to tolerate nest checks and exercise nest hygiene where they carry faeces from the chicks out of the nest. Parents stop brooding the chicks at around 10 days of age, so it is important to keep the enclosure sufficiently warm at this time to prevent chicks from becoming chilled at night.

Parents may withhold food and call from outside of the nest to encourage fledging. If premature fledging occurs, do not attempt to replace the birds in the nest, but rather ensure the chicks stay warm at night by bringing them indoors or providing a brooder on the aviary floor. After the young fledge, they may continue to sleep in the nest for 1-3 weeks. It is possible to leave the youngones with the breeding pair, however, if spaces limitations require removing the youngones to their own enclosure, wait until 4 weeks after they fledged to assure independence. Ideally, the old nest should be removed so the parents build a fresh one for the next brood. Birds should be transitioned to an austerity diet when not breeding.

Procurement and maintenance

Birds were procured locally (Meerut, 29^0 01' N, 77° 45' E) brought to the laboratory and kept in outdoor aviary (size = 2.7 × 2.2 × 1.9 m) for a week. The aviary for these birds was enriched by green plants, fresh grass in the soil at the surface, nest boxes and several wooden perches and received natural light-dark cycle, temperature and humidity. In the captivity, birds were acclimatized for one or two weeks and then subjected to an artificial photoperiodic condition providing short-day

conditions (8 h light: 16 h darkness; 8L:16D). Food and water were provided *ad libitum.* Food mainly consisted of seeds of foxtail millet (*Setaria italica*) and paddy (*Oryza sativa).* A supplementary food was also given on alternate days which was rich in protein and vitamins [It was prepared by mixing bread crumbs, boiled eggs, cottage cheese, multivitamin (Vimeral containing vitamin A, D3, E, and B12, marketed by Virbac Animal Health India Pvt. Ltd, Mumbai)]. Vimeral in water for two consecutive days was given once-a-month.

Lighting condition

The experimental light dark condition were provided artificially by fluorescent tubes or CFL lamps (5 & 14-watt cool compact fluorescent lamp; model B22 BC from Philips India ltd.) at required intensity. Timing of lights ON and OFF was regulated by Automatic time switches (Müller clock, Germany). The dark phase of an LD cycle was invariably had dim illumination (0.3 lux) so that birds could probably see around in the cage. Dim constant light (LL_{dim}) condition was produced by 3.8V-0.30A bulb (Eveready Industries India Ltd., Calcutta). Light intensity was measured by the radiometer (Model no. Q203 from Macam Photometric Ltd., Scotland).

Experimental details

Several experiments were performed to answer specific questions and all experimental details are mentioned in respective studies. Initially birds were kept in NDL, or exposed to short day lengths (8L:16D) for at least 2 weeks before being subjected to experimental protocol.

Data collection

Wide array of parameters as indicated in the respective experimental designs, were recorded at regular intervals. In general, observations were made on food, blood sampling, molt, body mass (body weight) and gonadal size. Data from these measurements were collected at the beginning, at end of the experiment and at appropriate intervals during the experiment.

(i) Body mass **-** Body mass was recorded to examine the effects weight gain or loss. Birds were individually wrapped in a cotton bag and weighed on a top pan balance to the accuracy of 0.1g. Before weighing a bird, the weight of the cotton bag was tared to zero. According to (King and Farner, 1965; Helms *et al.*, 1967; Wade and Bartness, 1984), in most cases, the change in body mass was considered to account for the gain and loss in weight due to fat deposition and fat depletion.

(ii) Food Intake **-** Food intake was recorded in cages which were lined with opaque white polythene sheets on sides up to the 7cm above the perch level, thus making them isolated visually but they can hear each other, placed them in metal trays to facilitate spillage collection. A quantity of food was weighed on a top pan balance to the accuracy of 0.1g. Before weighing a food, the weight of the empty cup was tarred to zero then was food dispensed into cup. After a specified period as experimental protocol mentioned the food cup was removed and feces sorted out. The difference between the initial and final weight of food cups gave the amount of food consumed by bird in the specified period.

(iii) Molt **-** The pattern of molt cycle was recorded by observations on body molt (Regeneration of whole body feathers) and primary wing feathers (called primary molt).

Body molt - Body molt was recorded as: The body part was categorized into 12 different regions as follows: (1) head, (2) neck, (3) shoulder, (4) back, (5) pelvic, (6) caudal, (7) throat, (8) chest, (9) abdomen, (10) flank, (11) shank, (12) sub-caudal. The body molt score was 0, if these regions had old feathers but when new feathers emerged, they were scored as 1. Thus, for body molt the maximum score could be up to 12, and the minimum score could be as low as 0. This was scored according to Saxena and Thapliyal (1962), Trivedi *et al.* (2006a), Rani *et al.* (2007) and Dixit and Sougrakpam (2013).

Primary molt -The pattern of molt cycle was recorded by observations on primary wing feathers (10 to 1 called primaries) and body feathers. We followed scoring of primaries pattern as outlined by Boswell (1991), Trivedi (2004), Trivedi *et al.* (2006a) and Dixit and Sougrakpam (2013) and briefly, this score pattern was as follows:

0- Worn or old feather

1- Missing feather (just dropped)

2- From a new feather papilla emerging up to one-third growth

3- New feather has attained two-third growth

4- New feather grown, but still growth is incomplete

5- New feather fully grown

Thus, each primaries feather could have a maximum score of 5. Because there are nine primaries on each wing, the maximum score for one wing can be 45 (9 x 5 = 45), and hence for each bird the feather score will total to 90 (2 x 45 = 90). Minimum score could be as low as 0.

(iv) ***<u>Testicular volume and follicular diameter</u>*** - The size of gonads was considered as an index of gonadal development and regression. The development in size of gonads over a period of days can be considered as a measure of the photoperiodic effect on the summation of gametogenic changes involving at least LH, FSH and androgens (Lofts and Murton, 1973; Lofts *et al.*, 1973; Lofts, 1975). For recording change in ovarian follicle (diameter of largest follicle) and testicular volume (mm^3) was done by unilateral laparotomy Kumar *et al.* (2001). A small incision was made between the last two ribs on the left flank, the gonads were located within the abdominal cavity with the help of a spatula and the size of left gonads was measured using a calliper with reference to markings on a graph sheet (1 cm × 1 cm with 100 squares; each square is 0.01 mm^2). This procedure was done quickly and incision was stitched by the surgical thread. An antibacterial skin ointment (Soframycin skin cream, Aventis Pharma Ltd.) was applied on the wound. Healing was rapid; post-operative infections are usually absent from this procedure. Testis volume and ovary follicular size (largest follicle) was calculated using formula $4/3\pi ab^2$, where *a* and *b* denote half of the long (length) and short (width) axes, respectively.

(v) ***<u>Locomotor activity</u>*** - The activity pattern was considered as an index of the response of the circadian system to light conditions as described in Malik *et al.* (2004). Each activity cage was furnished with two perches and mounted with a passive Infrared Motion Sensor (12 m range; C & K Systems [Intellisense XJ 413T] Conrad Electronic, Germany, Haustier PIR, Melder). The sensor continuously detected, counted and recorded the movement of the bird within the cage on to a separate channel and the computerized recording system. The Chronobiology Kit software from Stanford Software Systems, Stanford, California, USA, (Malik *et al.*,

2004) was used for the activity recording and analysis. Activity records (actograms) were double-plotted, where in two subsequent days laid on each horizontal line and all days of the experiment are sequentially shown along the vertical axis in increasing order. Chi-square periodogram analysis was used to determine the presence and absence of circadian rhythmicity. This was calculated by selecting a 10-day (day 5-14) segment in each light condition. From this, the mean (±SE) for the group was obtained. Similarly, using the same 10-day segment, the activity profile over 24 hours, and the total activity during the day and night as well as for 2-h period in the morning and evening were calculated, using the 'The ACTCNT' program of 'The Chronobiology Kit'. For this, the counts recorded at hourly or during the specified period of 12-h (hour 0-12 or hour 12-24; hour 0 = time of the light on at the beginning) or 2-h (hour 0 to 2 or hour 12 to 14) were averaged for 10 days for each bird and then the mean (±SE) for the group was calculated.

(vi) ***Histology*** - Histology was performed in some experiments which provided information with regard to structure of testis and its status. The growth-development of gonads was done periodically and birds were castrated and the gonads were fixed in 4% paraformaldehyde in 0.1 M phosphate buffer, (pH 7.4) for overnight. They were then cryoprotected by immersing gonads at 40^{o} C for 2-h each in 10%, 20% and 30% sucrose (Merck) solutions in overnight (Dixit and Singh, 2014).

The gonads were embedded into 15% poly vinylpyrrolidone (PVP; PVP40T, Sigma) blocks and serially sectioned in the coronal plane at 10-15 µm thickness using a Leica CM 1850 cryostat. The PVP-embedded testes were serially sectioned and placed on poly-L-lysine-coated slides (Sigma co.). The sections on the slides were stained through double staining technique using Harris haemotoxylin (for nuclei) and

eosin (for cytoplasmic). For this procedure, the sections were passed through xylene and then descending grades of alcohol and finally transferred to distilled water. Sections were then stained in haemotoxylin solution differentiated in 70% alcohol, dehydrated in ascending grades of alcohol. Sections were then stained in alcoholic eosin solution, cleared in xylene and mounted in DPX (Prod. No. 18404; Qualigen) and then examined the sections under light microscope.

Microscopy- The images of the sections were taken from a microscope (Olympus BX 51) equipped with a digital camera (Prog Res C5). The dimension of the image was 2592 x 1944 pixel RGB 8 bit. The magnification/ numerical aperture (NA) used were as follows: 20x/0.25 or 100x/0.65. The desired fields from the sections were photographed using standardized illumination. The minor contrast and brightness adjustment, if needed, were done in Adobe Photoshop 7.0 (San Jose, CA). The images were labeled and panels were prepared using the Corel Draw X3 (Toronto, Canada).

(vii) Collection of blood samples and Biomolecule analysis- The blood was collected from groups of all birds (male and female) on the start of experiment, pre–reproductive phase, reproductive phase and post reproductive phase. For blood sampling of bird, feathers were removed over the puncture site by using cotton dipped with distilled water. Swabbing the site with a small amount of 70% alcohol may help visualize the vein. Insert the needle slightly anterior to the vein and in the direction of the tip of the wing (i.e. against the flow of blood). Re-direct the needle to puncture the vein. Thus brachial vein was pricked with a needle and about 5-10 µl of blood was collected in micro centrifuged tubes, with the help of heparinized capillary tube. The heparin prevents blood clotting in capillary tube. The blood samples placed

for centrifugation for 10 minutes at 2,000-3,000 rpm using a refrigerated (40c) centrifuge. And for deplete platelets in the plasma sample centrifugation for 15 minutes at 2,000 rpm. The isolated plasma was collected and stored at –800 C for future testing of proteins, carbohydrate and cholesterol measurements.

(viii) ***SDS Gel Electrophoresis*** **-** For the detection of photoperiodic stress in birds, performs SDS-PAGE of blood plasma protein which was collected in different photoperiodic regimes of birds. The agarose gels have been used for protein separation for many decades. Although they are particularly useful for analytical and preparative separation of large proteins and protein complexes, their main drawback has been the lack of sufficient resolution to analyze small (< 30_{kDa}) proteins and peptides. We have found a way to overcome this disadvantage using a new system of sodium dodecyl sulfate (SDS) agarose gel electrophoresis. Not only does this system resolve small proteins well, but it also has several advantages over SDS-PAGE. First, no toxic components are used in the preparation of the gel. Also, this gel has a dynamic separation range equivalent to a 4%–20% gradient polyacrylamide gel so that proteins ranging in size from 6 to over 200_{kDa} can be analyzed using a single concentration gel. Furthermore, the staining of protein bands by Coomassie Brilliant Blue is uniform and provides up to 50-fold higher detection sensitivity compared to SDS polyacrylamide gel stained under equivalent conditions. Some proteins are also better resolved by agarose than by polyacrylamide. In particular, high-molecular-weight proteins appear to transfer better from this gel than from polyacrylamide gels. Finally, the stability of agarose allows for gels to be precast and stored for a long period of time. SDS-PAGE was performed according to Laemmli, (1970). In this method following procedure are used.

Prepare 12% lower gel (Resolving gel/separating gel) by adding (1.6 ml ddH_2O, 2.0 ml 30% acrylamide mixture, 1.3 ml Tris-CL (1.5 M, pH 8.8), 0.05 ml 20% SDS, 0.05 ml 10% ammonium sulfate, 0.002 ml TEMED (add it right before pour the gel) solutions (for prepare 5 ml gel solution. To avoid polymerization, after adding TEMED, mixed well and quickly transferred the gel solution by using 1 ml pipette to the casting chamber between the glass plates and filled up to about 0.7 cm below the bottom of comb, when the comb was in place. Add a small layer of isopropanol to the top of the gel prior to polymerization to straighten the level of the gel. Once the gel has polymerized, start to prepare stacking gel (5%) by adding the following solutions (total volume= 3 ml), 2.1 ml ddH_2O, 0.5 ml 30% acrylamide mixture, 0.38 ml Tris-Cl (1 M, adjusted to pH 6.8), 0.03 ml 10% SDS, 0.03 ml 10% ammonium persulfate, 0.003 ml TEMED (add it right before the gel is poured). Remove the isopropanol layer by using the filter paper. Rinse the top layer of the gel with ddH_2O and dry off as much of the water as possible by using filter paper. Added TEMED and mix the stacking gel solution well. Quickly transferred the gel solution by using a 1 ml pipette till the space is full, and then insert the appropriate comb. Allow the top portion to solidify and then carefully remove the comb.

Sample preparation- Same amount of protein samples was prepared according to BCA (bicinchoninic acid) protein assay. Add the same volume of 2x protein sample buffer to each protein sample, mix and boiled the samples at 95 °C heating block module for 10 min. Spin the samples at the maximal speed for 1 min (samples from some tissue/cell sources may need longer spin) in tabletop centrifuge and leave the samples at room temperature until ready to load onto the gel.

Electrophoresis- Remove the gel cassette from the casting stands and placed it in the electrode assembly with the short plate on the inside. Pressed down on the electrode assembly while clamping the frame to secure the electrode assembly and put the clamping frame into the electrophoresis tank. Poured 1x electrophoresis running buffer into the opening of the casting frame between the gel cassettes and added enough buffer to fill the wells of the gel. Fill the region outside of the frame with 1x running buffer. After that same amount of protein sample loaded slowly into each well as well as load 10 µl of protein MW marker, and connect the electrophoresis tank to the power supply.

Protein detection- For protein staining in the sample typically used Coomassie blue staining. The protein bands were stained with 0.125% Coomassie Brilliant Blue R-250 in 40% methanol, 10% acetic acid for 1 h in 50°C water bath with gentle agitation. Destaining was performed in 5% methanol, 7.5% acetic acid for overnight at room temperature with gentle agitation.

Statistical Analysis

Data generated from experiments are presented as mean ± SE. One-way analysis of variance with repeated measures (One-way RM ANOVA), followed by Newman-Keuls post-hoc tests if ANOVA indicated the significance of difference, was employed to examine the effect of a photoperiodic treatment over a period of time. The means from three or more groups at one time point were compared using 1-way ANOVA with repeated measures, and the means from only two groups at one time point were compared using Student's t-test. In short-term experiments, before and after means of the same group were compared using paired t-test. Significance was always taken at $P < 0.05$.