Chapter 3 Materials and Methods

3.1. Animals

Adult, healthy and reproductively active male Red Munia (*Amandava amandava* or *Estrilda amandava*) were captured in and around Allahabad (25°27'N 81°44'E), UP, India, in quiescent (April-May), preparatory/recrudescent (July-August), courtship/mating (September-October) and regressive (November-December) stages of the gonadal cycle. Birds were housed for 10 days to acclimatize in open air aviaries under natural conditions of temperature, humidity and photoperiod in each stage of the cycle. Average body weight/bw of adult and healthy male birds was 8.5 ± 0.5 gm. Food and water was given *ad libitum*. Crushed wheat grains (grown organically/available in the market) were provided twice in each day (06:30-08:30 A.M. and 04:00-06:00 P.M.).

Experimental protocols were approved by Institutional Animal Ethical Committee (IAEC) of the Department of Zoology, University of Allahabad. Guidelines of Committee for the Purpose of Control and Supervision of Experimental Animals (CPCSEA), Ministry of Environment and Forests, Government of India were followed for maintenance and termination of the bird. Permission was taken for use of wild birds from Principal Chief Conservator of Forests, Wildlife, U. P., India, by vide Letter No. 3564/23-2-12(9).

3.2 Experimental Design

To evaluate the neuroendocrine regulation during reproductive cycle and interrelationship of HPT and HPG axes, the investigation was carried in two parts.

1. First, we evaluated the seasonality of HPT and HPG neuroendocrine axes as well as their potential interrelationship during quiescent, preparatory, breeding and
regressive stages of the reproductive cycle of *A. amandava*.

ii. Second, we examined the impact of neuroendocrine disruption on seasonality of reproduction as well as interrelationship of HPT and HPG axes using endocrine disrupting pesticides/EDPs.

In second part of investigation, to evaluate the EDPs-induced neuroendocrine disruption, two experimental set ups were maintained:

**Experiment I: Evaluation of MCZ and IMI as Neuroendocrine Disruptors of Pituitary-Thyroid Axis during Gonadal Cycle**

Two experimental set ups were maintained (one in each stage) to evaluate the neuroendocrine disruptive potential both the pesticides using environmentally equivalent dose.

In both preparatory (stage of testicular recrudescence; mid July-mid August) and breeding/courtship (stage of active mating/courtship with fully grown testes; mid September-mid October) stages, acclimatized male birds (bw 8.5 ± 0.5 gm) were divided randomly and maintained in three groups ($n = 8$/group): MCZ-exposed group, IMI-exposed-group and control. Birds were exposed to environmentally equivalent low dose of commercial pesticides MCZ and IMI through diet using soy oil as vehicle. Control birds were given food with vehicle. The doses were selected taking reference of established chronic no observable adverse effect level (NOAEL) of respective technical compound (MCZ and IMI) in birds.
Mancozeb/Uthane M45 (75% wt/wt MCZ; United Phosphorous Ltd., India)

Dietary route LD_{50} of technical compound in birds: 860 mg/kg BW (HCPDG, EC, 2009).

Dose Selected: 0.5% LD_{50} (0.028 mg: 5.5 ml soy oil/3 gm food)

Imidacloprid/Confidor (17.8% wt/wt IMI; Beyer Crop Science Ltd.)

Dietary route LD_{50} of technical compound in Japanese quail: 31 mg/kg BW (Lopez-Antia, 2013).

Dose Selected: 0.5% LD_{50} (5.5 ml: 5.497 ml soy oil/3 gm food)

Food was mixed with pesticides using vehicle and kept overnight. Two sets of the experiment were executed in preparatory and courtship stages respectively and were exposed for 30 d in both sets. All the birds (pesticides-exposed as well as control) in each set of experiment were euthanized at the end of experiment. Body weight was recorded every alternate day. Precision of pesticide-dose intake by each bird was maintained by exposing them to the decided dose through calculated amount of food taken by birds during first 2 h of feeding (7:00–9:00 A.M.) each day. The test dose (0.5% of LD_{50}) was considered as environmentally relevant. Studies have reported the environmental concentrations of IMI (Blacquie’re et al., 2012) and MCZ (Koppad and Umarbhadsha, 2006 and Adamski et al., 2009) in invertebrates, seeds/grains and crop fields which is equivalent to our test dose, however, the precise biomonitoring data on test compounds are not available for birds.

Experiment II: Experimental Manipulation of Interrelationship between Pituitary-Thyroid and Pituitary-Testicular Axes using EDPs

Two experimental set ups were maintained (one in each stage) to evaluate the disruption of interrelationship between pituitary-thyroid and pituitary-testicular axes using low dose
individual as well as their combinatorial doses.

In both the preparatory and courtship stages, acclimatized male birds (bw 8.5±0.5 gm) were maintained in five groups (n=8/group): MCZ-exposed group, IMI-exposed group, MIX-I exposed group, MIX-II exposed group and control. Commercial formulations of pesticides were given through food using soy oil as vehicle in every morning 06:00-07:00 A.M. The doses and precision of dose-intake were maintained as in Experiment-I.

**Mancozeb/Uthane M45**
Dose Selected: 0.25% LD$_{50}$ (0.014mg: 2.5ml soy oil/3gm food)

**Imidacloprid/Confidor**
Dose Selected: 0.25% LD$_{50}$ (2.75µl:5.495ml soy oil/3gm food)

Effects of combinatorial exposures were assessed using two different doses of each pesticide in mixture through same diet and vehicle. In one group (MIX-I), the doses of combinatorial exposure were decided keeping in view the dose of individual exposure of each of MCZ and IMI (0.25% LD$_{50}$). In another group (MIX-II), the individual pesticide dose was increased (0.5% LD$_{50}$ of each) to make a mixture dose double to the individual dose.

**MIX-I (1:1LD$_{50}$ of MCZ and IMI)**
Dose Selected: 0.25% LD$_{50}$ of both MCZ and IMI (0.014mg MCZ+2.75µl IMI) in 5.495ml soy oil/6gm food

**MIX-II (1:1LD$_{50}$ of MCZ and IMI)**
Dose Selected: 0.5% LD$_{50}$ of both MCZ and IMI (0.028mg MCZ+5.5µl IMI in 5.495ml soy oil/6gm food)
Birds were exposed for 30 days; BW recorded every alternate day and terminated by decapitation.

3.3 Behavioral Observations

Development of secondary sexual characteristics (plumage pigmentation and beak/toe color) and sex-related behaviors (singing patterns and pairing attempts) of individual males was reported as per the identification tag. Sexual behavior was observed twice a day for 2 hr (07:00-09:00 A.M. and 05:00-07:00 P.M.) from the start of courtship in each group when courtship rates were typically at maxima. Visual observations were made from a distance of 3 meter from each cage edge after a habituation period of 15 min. Data for each male was averaged for sex-related behaviors recorded during the experiment. Notes of song tunes and numbers of mating attempts were taken as the parameters for sex-related behaviors of males.

3.4 Tissue Sampling

3.4.1 Plasma Sampling and Hormonal Assays

Blood was collected in 0.1% EDTA treated vials, centrifuged at 3000 r. p. m. for 20 minutes, pooled and separated for hormonal assays. ELISA kits were used for measuring plasma conc. of TSH (SmarTest Diagnostic, Israel), LH (Diagnostics Systems Ltd., Russia), FSH, PRL, testosterone, estradiol, T4 and T3 (LDN GmbH & Co. KG, Germany). Inter-assay and intra-assay coefficient of variations (%) were <10% for TSH (7.6 & 4.6), LH (4.36 & 4.06), FSH (4.75 & 3.4), PRL (7.6 & 6.9), testosterone (7.3 & 6.6), estradiol (6.2 & 4.6), T4 (10.7 & 6.4) and T3 (7.6 & 7.0) respectively. The samples were run in duplicate and optical density was measured by 3.4.2 Bio-Rad iMark microplate reader (USA).
3.4.2 Histopathology of Testis, Thyroid and Brain

Testes and thyroids were quickly dissected out, blotted, weighed before fixation in Bouin’s fixatives for overnight followed by washing, dehydration in upgrade ethanol series and paraffin embedding. Brains were dissected out and fixed in formol solution (9:1, mercuric chloride: formaldehyde) overnight followed by washing and paraffin embedding. Paraffin embedded testes, thyroids and brains were cut serially (5-6μm). Sections were stretched on sterilized glass slides and incubated overnight at 45°C.

3.4.2.1 Haematoxyline-Eosin Staining

Testis and thyroid sections were deparaffinized in xylene, hydrated in downgrade ethanol series, co-stained with Haematoxyline-Eosin stains according to standardized protocol of our laboratory (Verma and Mohanty, 2009) and produced for light microphotography using Leica DM 2500 (Germany) light microscope.

3.4.2.2 Acridine Orange and Ethidium Bromide (AO/EB) Staining

AO and EB (Sigma, U.S.A.) staining was used to differentiate normal/healthy and apoptotic cells in testes. Paraffin embedded testis sections were first incubated at 45-50°C for 30 minutes on a hot plate, deparaffinized in xylene, hydrated and washed repeatedly in phosphate buffered saline (PBS). Slides were then incubated in permeabilization solution (1: 1 of 0.1% triton X-100 and 0.1% sodium citrate in d. H₂O) for 10 minutes and washed in PBS followed by incubation in 3% H₂O₂ in methanol. After repeated washing in PBS, each section was then mixed with aqueous AO/EB solution (1: 1; AO: EB in PBS) and fluorescence microscopy was performed immediately using Leica DM
2500 (Germany) fluorescence microscope. Normal/healthy cells stained only by AO were bright green with intact structure whereas apoptotic and necrotic cells stained by EB were bright orange/red respectively with condensation of chromatin as dense orange areas and reduced cell size.

3.4.2.3 Cresyl Violet Staining

Neuronal density was studied in ME, PVN and PeVN of hypothalamus using cresyl violet staining method staining method. Paraffin embedded brains were cut serially (8-10 μm) and sections having ME, PVN and POA were deparaffinized in xylene, hydrated in downgrade ethanol series and washed in PBS followed by staining with cresyl-violet stain (Wang et al., 2002).

3.4.4 Immunohistochemistry

Paraffin wax embedded testis and brains (8-10 μm) having ME, PVN and PeVN in hypothalamus were processed for GnRH, GnIH and AR immunohistochemistry. ABC method of Hsu et al., (1981) was used for immunohistochemistry following the protocol of our laboratory (Verma and Mohanty, 2009). Slides were deparaffinized, hydrated and washed repeatedly in PBS followed by a treatment with 3% H₂O₂ (in PBS) and incubation in normal goat serum (Genei, Bangalore, India) for 30 minutes. Tissues were then incubated with primary antibodies anti-GnRH (Sigma, 1:500), anti-GnIH (prepared in the laboratory of Kazuyoshi Tsutsui, 1:500) and anti-ARs (1:100) at 4°C for 20 hours. Slides were washed in PBS and incubated with goat anti- rabbit IgG biotin conjugate (Genei, Bangalore, India) followed by incubation with biotinylated horse radish peroxidase and streptavidin (Genei, Bangalore, India). Immunoreactivity was visualized
by staining slides using chromogen 3, 3’-DAB (diaminobenzidine, Genei, Bangalore, India) in 0.1% H₂O₂ and photomicrographs were produced.

3.5 Morphometric Analysis

Every 5th section out of total (120-150 in thyroid, 200-250 in brain and 400-450 sections in testis) were analyzed for morphometric analysis using ImageJ 1.32j (Image analysis software package, NIH, Bethesda, MD).

3.5.1 Morphometry of Testis

In testis, total volume of testis, width of testicular capsule, width of interstitial space, diameters of seminiferous tubules, densities of Leydig cells, Sertoli cells and cells of seminiferous cycle (spermatogonia, primary and secondary spermatocytes, spermatids and mature spermatids) were measured morphometrically. Densities of apoptotic and necrotic cells (/mm²) were measured in interstitial space and seminiferous tubules.

(a) Total Volume of Testis

Total volume of testis was calculated according to principle of prolate spheroid (Wingfield et al., 1996). Sections were investigated at magnification 10×. Major (a) and minor (b) axes were measured and the volume was then calculated according to formula:

\[ V = \frac{4}{3\pi} a b^2 \]

Where \( V \) is volume of testis, \( a \) is the radius of the testis at its widest point and \( b \) is half the long axis.

(b) Width of Testicular Capsule, Width of Interstitial Space and Diameter of Seminiferous Tubules
Width (µm) of testicular capsule, interstitial space seminiferous tubule was measured by using ImageJ at magnification 40×.

(c) Cell Densities

Densities of Leydig cells, Sertoli cells, Cells of Seminiferous cycle and apoptotic & necrotic cells were calculated at the magnification 40×.

3.5.2 Morphometry of Thyroid

In thyroid, volume of thyroid gland, number of follicles filled with colloids, total volume of colloids, epithelial cell height and nucleus size of follicular epithelial cells and N/C ratio in epithelial and stromal cells were measured.

(a) Volume of Thyroid Gland

Volume of thyroid gland was determined according to the principle of prolate spheroid (Kendeigh et al., 1966). Sections were investigated at magnification 10×. Major and minor axes were measured and the volume was then calculated according to the formula:

\[ V_g = \frac{4}{3} \pi a b^2 \]

Where \( V_g \) is volume of the gland, \( a \) is radius of major axis and \( b \) is radius of minor axis passing through centre of the thyroid gland.

(b) Density of Colloid-filled Follicles

Density of colloid-filled follicles was determined according to Hartoft-Neilsen et al. (2005). A counting frame of 500×500 µm² was created and then photomicrographs were...
analyzed for no. of follicles and colloids within the frame. Sections were investigated at magnification 40×.

(c) **Total Volume of Colloids**

Total volume of colloid in thyroid gland was determined as illustrated by Kot et.al. (2013). In each selected follicle major and minor axes of colloids (perpendicular to each other) were measured by Image J and the diameter of colloid (d) was obtained by using the formula:

\[ d = \sqrt{ab} \]

(\(a\) and \(b\) are the major and minor axes of the colloid respectively). To compensate for the effects of sectioning a sphere, the measured diameter (d), was corrected to provide an estimate of the mean diameter \([D_{(m)}]\) by using formula:

\[ D(m) = \frac{d^4}{\pi} \]

Volume of the colloid \([V_{(col)}]\) was calculated using formula:

\[ V(col) = \frac{\pi \times D(m)^3}{6} \]

(d) **Epithelial Cell Height and Nucleus Size**

Epithelial cell height and nucleus size was measured at magnification 100×. Cell height and nucleus size were determined by analyzing at least 100 cells in each section.

(e) **Nucleus-to-Cytoplasm (N/C) Ratio**

N/C ratio in epithelial and stromal cells was also determined at magnification 100× in each section (Kot et al., 2013). In each selected cell, area of the cytoplasm with nuclei
was determined by measuring total cell area (Anc). Similarly, area of each individual nucleus within the cytoplasm was also traced. The areas of all the nuclei (An) were summed up and were subtracted from the total cell area (Anc) and obtained the resultant cytoplasmic area (Ac). The nuclear-to-cytoplasmic (N/C) ratio was then determined by the equation:

\[
\frac{N}{C} = \frac{An}{Anc - An}
\]

3.5.3 Morphometry of Hypothalamus

In hypothalamus, total neuronal density (/mm²) was assessed in ME, PVN and PeVN by selecting 10 random locations at magnification 10×.

3.5.4 Immunointensity and Cellular Densities of GnRH-ir, GnIH-ir and AR-ir in Hypothalamus and Testis

Density of AR, GnRH and GnIH active cells was determined by after measuring the immunointensity of AR-ir, GnRH-ir and GnIH-ir cells as described earlier (Verma and Mohanty, 2009). The immunointensity of immunoreactive cells was quantified by measurement of the mean optical density/OD of 100 immunoreactive cells and then corrected by subtraction of the background OD of the corresponding section. The Kodak photographic No. 3 step tablet was used for the calibration of OD.

3.6 Statistical Analysis

GraphPad Prism 5 (GraphPad Software Inc., USA) statistical software was used for all analyses. Results with a p-value of less than 0.05, 0.01 and 0.001 were considered
significant. Data with normal distribution and homogeneity of variance were analyzed using one way analyses of variance (ANOVA), represented as mean ± S. D. (standard deviation) followed by Tukey’s posthoc test. Statistical analyses of data were done using Fisher’s exact test.