MATERIAL AND METHODS

PROCUREMENT AND MAINTENANCE OF PULLETS FOR Ist LAYING CYCLE

Freshly hatched, female RIR breed of chicks, procured from the Government Poultry, Baroda, India, were used for the present experimental study. The chicks were brought into the experimental design as soon they were brought to the animal house. From day 1 till day90, the chicks were housed in wire matted cages of dimension 4 × 2.5 × 2" ft. , not exceeding 12 chicks per cage. During the feeding hours, each cage was partitioned into three subset chambers by vertical sliding partitions, ensuring a uniform number of four chicks/subchamber and, at the end of feeding, the partitions were removed. At the end of 90 days, the chicks were shifted to laying cages, partitioned into an enclosure of 2ft^2 for single caging.

PROCUREMENT AND MAINTENANCE OF ADULT HENS FOR IInd LAYING CYCLE

Adult hens, of RIR breed, towards the end of egg laying (72 weeks of age) were procured from the Government Poultry Dahod, Gujarat, and were brought into the experimental design as soon as they were brought into the animal house. The hens were housed in laying cages of 2 ft.^{2} dimension for single caging.

FEED AND FEEDING REGIMEN

Feed was provided twice in a day, in the morning (08.30 A.M) and, in the evening (04.30 P.M.). Rationed diet was provided on a chronological basis, ensuring that the birds are were not underfed during the feeding programme. The allocation of diet was based on the feeding regimen provided by the Government Poultry Baroda. From d1 to d56, the chicks were fed on chick mash,at an average of 30 gm chick mash /chick/day, from 59 days till initiation of lay, they were fed on grower mash at an average of 90 gm/bird/day and from the day of initiation of lay, the birds were fed on layer mash, at an average of 110 gm/bird/day. Feed was purchased from the Government Poultry Baroda, India. The composition of feed and feeding regimen are depicted in tables 1 and 2 .Fresh water was provided *ad libitum* in water trays.

LIGHTING SCHEDULE FOR PULLETS AND ADULT HENS

The lighting of the cages was done by four fluorescent tubes/ cage fitted atop the cages and light intensity at the level of the chick was 250 lux as measured by a digital lux meter.

Exposure to Short Photoperiod (SP: LD 6:18)

From day 1 till day90, chicks were exposed to a short photoperiod of 6hrs. of light followed by 18 hrs. of darkness, with lights on at 08.00 hrs in the morning and lights off at 14.00 hrs controlled by an automatic timer.
Adult hens, of 72 weeks of age were exposed to a similar photoperiod for 30 days.

Exposure to Normal light and dark photoperiod (NLD: LD 12:12)

From day1 till day90, chicks were exposed to a normal light and dark photoperiod of 12hrs of light and 12hrs of darkness with lights on at 08.00 hrs in the morning and off at 20.00 hrs, controlled by an automatic timer. Adult hens of 72 weeks of age were exposed to NLD for 30 days in a similar manner.

Shifting of pullets/hens from a short photoperiod to Normal light and dark photoperiod

At the end of 90 days, pullets reared irrespective of short photoperiod or normal light and dark photoperiod, were shifted into laying cages of 2 ft^2 for single caging and were maintained under normal photoperiod (NLD). At the end of 30 days, adult hens, reared under SP or NLD, were reared under normal photoperiod (NLD).

INDUCTION OF HYPER//HYPOCORTICALISM

Hypercorticalism (HPR)

In day old chicks or adult hens, implant of pellets weighing 20mg, consisting of a homogenous mixture of bees wax (19mg) and **Corticosterone** (Sigma Chemical Co. U.S.A.) (1mg) were prepared and implanted subcutaneously in the thigh as per the technique described by Davison *et al.* (1985b).

Hypocorticalism (HPO)

In day old chicks or adult hens, implant pellets weighing 50 mg, consisting of a homogenous mixture of bees wax (40 mg) and **Metapyrone** (Sigma

Chemical Co. U.S.A) (10 mg) , were prepared and implanted subcutaneously in the thigh as per the technique described by Davison *et al.* (1985).

The rate of diffusion of corticosterone or Metapyrone was calculated empirically, by weighing the pellets at intervals of 15 days and based on the proportion of corticosterone or metapyrone and the change in weight of the pellets (Table 4).

EXPERIMENTAL SET-UP

Two sets of experiments in duplicate for confirmation of results were carried out in pullets and adult hens

The *pullets (from d1 till d90)* and *adult hens (72 weeks of age till 76 weeks)*, reared under NLD or SP were divided into 5 groups of 12 birds each under each lighting schedules. At the end of treatment schedule, shifting of pullets or adult hens was carried out as mentioned earlier.

Group I	:	Served as controls.
Group II	:	Sham operated (Chicks were implanted with empty bees wax pellets weighing 20mg).
Group III	:	Sham operated (Chicks were implanted with empty bees wax pellets weighing 50 mg).
Group IV	:	Hypercorticalic (HPR; chicks were implanted with implants containing corticosterone mixed in bees wax - 19 mg bees wax : 1mg corticosterone).
Group V	:	Hypocorticalic (HPO; chicks were implanted with implants containing metapyrone mixed in bees wax - 40 mg bees wax : 10mg corticosterone).

Table :4 Rate of diffusion of corticosterone or metyrapone from the continuous release implants (bees wax pellets).

	treatment	total dosage	duration of dose	amount released in µg/day
NLD	HPR	1 mg corticosterone in 19 mg beeswax	1-14 15-30 days	0.15
			30-44 45-60 days	0.20
			60-74 75-90 days	25-30
	HPO	10 mg metyrapone in 40 mg beeswax	1-14 15-30 days	0.40
			30-44 45-60 days	0.66
			60-74 75-90 days	2.26
SP	HPR	1 mg corticosterone in 19 mg beeswax	1-14 15-30 days	0.18
			30-44 45-60 days	0.27
			60-74 75-90 days	20-25
	HPO	10 mg metyrapone in 40 mg beeswax	1-14 15-30 days	0.30
			30-44 45-60 days	0.55-0.60
			60-74 75-90 days	2.0

The impirical rate of diffusion was calculated by noting the difference in weights of implants at 15, 30, 45, 60, 75 and 90 days.

PARAMETERS AND METHODOLOGY OF EVALUATION

The respective treatments were discontinued at the end of 90 days for pullets and at the end of 30 days for adult hens. Egg laying performance in terms of quantitative as well as qualitative aspects, and gravimetric, histomorphometric changes and alterations in serum hormone levels were all assessed in the present study.

I. Egg laying performance

a. Initiation and termination of lay : The age at first egg or initiation of lay (AFE/IL) and age at last egg was noted for each bird, and the effective duration of lay for each of the group was recorded. Mean values of the same under different experimental schedules are represented in the respective chapters.

b. Number of eggs and weight of eggs : Total number of eggs laid by individual birds throughout the first year of lay was recorded. The eggs were weighed, and the total number of small eggs laid (eggs weighing < 40gms) by each bird during their course of lay was subtracted from the total number of eggs laid and effective number of eggs laid (eggs weighing >40gms) was determined. Total number of eggs laid , total number of small eggs and effective eggs, average monthly egg yield and mean monthly egg weights are all represented in the results section of respective chapters.

c. Rate of lay and Number of clutches : Oviposition interval, average number of clutches and clutch size were recorded throughout the period of lay for individual birds.

II. Egg analysis

For the sake of convenience, analysis of eggs laid by different groups of birds was carried out thrice, during the annual laying period at 10 week interval, *ie.* every three months from the time of initiation. Three eggs from each bird, during collection period, were randomly chosen, and were used for egg analyses. The eggs were analysed within 24hrs of being laid. To minimise the experimental discrepancies, the samples were taken in triplicates for analysis.

***a.** Physical features of egg :*

1. Egg height, egg width and shell thickness : The egg height and egg width were measured by a vernier callipers, and shell thickness, with the help of a micrometer screw gauge. The values are expressed in mm.
2. Egg weight and egg volume : Egg was weighed in pan balance and its volume was determined by the water displacement method (Sainz *et al.,*1983); the egg weight is expressed in gms and the egg volume in cc.
3. Yolk and albumen weights : The egg was carefully dissected and yolk and albumen were separated free from the membranes. Care was taken to keep the samples of yolk and albumen uncontaminated. Yolk and albumen were weighed separately and their values are expressed in gms.

***b.** Biochemical composition of eggs :*

Biochemical analysis was carried out in yolk and albumen separately.

1. Percentage water and solid contents : The contents of water and dry residues were determined by differential weighing of the samples before and after drying for 48hrs at 105 ± 5°C (Sainz *et al.,* 1983).

2. Protein content : Protein content in the wet samples was determined by the method of Lowry *et al.,* (1951) using folin-ciocalteau as the colour reagent. Known amount (50mg) of yolk / albumen is mixed in 5ml chilled distil water and 0.1ml of sample is taken and diluted further by adding 0.9ml of distil water so as to make the final volume of 1ml. Blank as well as standard tubes are also run simultaneously. To each of the test-tube 5ml of reagent C (freshly prepared alkaline Cu solution) is addeded. After 10 min., 0.5ml of colour reagent is added in each tube. The optical density (O.D) is read at 660nm wave length against blank tube after 30 min. and, the protein content was expressed in terms of mg percent of wet yolk / albumen.

3. Glycogen content : The glycogen content was estimated by the method of Siefter *et al.,* (1950). Yolk/albumen were dropped in preweighed test tubes containing 2 ml of 30% KOH and the tubes were kept in boiling water bath till the complete digestion of the samples. So as to precipiatate glycogen, 2-3 ml of 95% ethanol is added in the tubes and again kept in boiling water bath for few seconds. The samples are then centrifuged at 3000 rpm for 15 minutes and after discarding the supernatant, 2 ml of 95% ethanol is added and the same precess is repeated again. Tubes are then placed in the refrigerator to precipitate glycogen. The precipitates of glycogen is then extracted in 2 ml ethanol and the tubes are centrifuged at 3000 rpm. The supernatant is discarded and the ppts. are dissolved in known amount of distilled water. The glycogen content is estimated by adding 4 ml of anthrone (color) reagent prepared in conc. H_2SO_4 . After adding the color reagent, the tubes are transferred to boiling water bath for 4 min, and upon cooling, percentage transmission is read at 620 nm. Glycogen content is expressed as mg/100mg yolk/albumen.

4. Lipid content : Lipid content was estimated as per the method described by Folch *et al.*(1957). Known amount of yolk / albumen is taken in the test-tubes and for extraction of lipids, 2 ml of chloroform-methanol mixture and 1 ml of 1% $CaCl_2$ is added in the test-tubes. The test-tubes were left overnight so as to separate the water content from the sample. The aqueous layer thus obtained, is removed with the help of syringe without disturbing the test-tubes. Known amount of aliquot is taken from the sample in preweighed test-tubes and oven dried at 50-60^{0}c. When the sample is dried completely the tubes are weighed again and the difference between dry weights before and after extraction is expressed as lipid/100 ml yolk or albumen.

5. Cholesterol : Total cholesterol is estimated as per the method described by Crawford (1950). Lipids were extracted from the samples as mentioned in the previous method and dry residues were taken for the estimation of cholesterol, using $FeCl_3$ as color reagent. 3 ml of $FeCl_3$ solution is added and the tubes are then placed in boiling water bath for 5 minutes. After removing the tubes from water bath, 2 ml of conc. H_2SO_4 is added. When room temperature is attained, the contents of the tubes are mixed well and after 30 min. O.D. is measured at 540 nm. Total cholesterol is expressed as mg/100mg yolk/albumen.

6. Lipid Index, water index, non-lipid dry weight and calorific value:

Caolorific value was calculated by utilizing energy equivalents of 9 Kcal/g lipid and 4 Kcal/gm each for carbohydrate and protein *ie.* sum total of : weight of lipid x 9, total protein x 4, carbohydrates x 4.

The values obtained are expressed as energy per gm fresh egg / energy per 100 gm egg or energy per 100gm edible egg. (see RamaRao, 1986).

III. Body weight and gravimetric changes

At the end of 0 day, and at intervals of 30 days thereafter till 90 days, the birds were weighed and sacrificed under mild anaesthesia taking maximum care to avoid any stress during handling. The viscera was cut open and organs were quickly excised, blotted free of blood and body fluids and various organ (thyroid, adrenal, ovary, oviduct, liver, thymus, bursa and spleen) were weighed using a mettler balance. Organs were weighed accurately upto 0.01 mg. The absolute weights thus obtained wer converted to relatively weights and expressed in terms of percentage. Per day growth rate for body and organ was calculated at varuous intervals (30 days interval from 0 day till 180 day) by subtracting the absolute weight of day old chick or organ weight of day old chick from the absolute weights at respective intervals and dividing it by number of days, so as to get per day -growth rate, *ie*. growth rate between 0-30 will be

weight at 30 days - 0 day weight ÷ 30

-overall growth rate at the end of 180/90 days as

weight at 180 days - 0 day weight 180.

-Growth indices for various organs were calculated as :

Per day growth rate of organs at resp. interval ÷ per day growth rate of body at resp. interval

IV. Hormone assay (R.I.A)

Radio immuno assay :

The blood samples were collected by puncturing the right jugular vein and, later centrifuged at 4000 RPM for 40 minutes to obtain the serum. The RIA for T3, T_4, progesterone and corticosterone was carried out by RIA kits. The RIA kits for T3, T4, progesterone and corticosterone

were purchased from INSTAR corporation, Minnesota, USA and were expressed as ng/dl and, for corticosterone from Diagnostic products corporation (DPC), Los Angeles, CA and expressed as µg/dl. The ratios of these hormones with respect to each other and also with organ and body weights was calculated.

V. Histological techniques :

The thyroid, adrenal and ovary of pullets were fixed in Bouin's fluid and processed further, dehydrated and embedded in paraffin. Sections of 5 µ thickness were cut on a microtome and stained with haematoxylin-eosin and mounted in DPX. These sections were photographed on a photomicroscope.

The histometrics of the ovarian follicles was done with the help of an occulometer. A specific region in each of the sections was selected for the follicular count. Initially, a total count of the follicles was made, counting the pre-ovulatory follicles (POF) and atretic follicles (AF) separately. The POF, on the basis of their size were categorised as small (6-200), big (200-300) and large (>300) follicles and counted separately.

VI. Statistical Analysis

All the results were statistically analyzed and are depicted as Mean ± S.E. The data were also subjected to Students 't' test with 95% confidence level.