CHAPTER 1

Experimental protocol and methodology of evaluation

The present investigation is divided into three major phases, each of which is elaborated separately.

Phase I

Day old pullets were subjected to photoperiodic and/or adrenocortical manipulations for the first trimester of post-hatch development and, maintained thereafter under LD 12:12 (NLD), to assess their laying performance in the first cycle. Also, adult hens (72 weeks) of age were subjected to the same experimental manipulations for 30 days to assess the effect on second cycle of egg laying.

Phase II

The eggs of the hens (collected from phase I) were analysed during their initial, mid and late phases of lay, to study the changes in their physical features and biochemical composition.

Phase III

Day old pullets were subjected to the experimental schedule (same as in phase I), and were sacrificed during the experimental period (at 30, 60, and 90 days) . The changes in organ growth kinetics, serum hormone

profile and histomorphology were studied to have a better understanding of the photo-endocrine interactions and ovarian development.

Procurement and maintenance of chicks :

One day old pullets of the domestic fowl *Gallus gallus domesticus* of Indian RIR breed were procured from the Model Poultry farm, Baroda, Gujarat. The Indian RIR breed of domestic fowl is obtained by cross breeding the American Rhode Island red and the Kalinga Brown of Bhuvaneshwar, for better survival in the tropical climatic conditions. The Indian RIR is considered to be a dual purpose breed, as it is used for egg laying and table purpose. The adult RIR hens towards the end of their lay (72 weeks) were procured from the Government poultry, Dahod, Gujarat.

Day old pullets were housed in cages (4x2.5x2ft) and placed in light proof enclosures for a period of 90 days. Thereafter, they were shifted from the light proof enclosures to laying cages partitioned into enclosures of 2 ft^2 for single caging and maintained under a constant photoperiod of LD 12:12 (NLD). The control group and each of the experimental groups had 12 birds each. The pullets were vaccinated for Ranikhet disease on the 4^{th} day of hatch and after 2 months. The vaccines against fowl pox and fowl cholera were also given. Debeaking was done at 4 months. Each phase of investigation was carried out in duplicates for the confirmation of results and, the data was analysed for statistical significance by student's 't' test and ANOVA.

Feeds and feeding regimens :

The feed and water was provided to all the chicks through feeding trays fitted at the sides of the cages. All the birds were fed with a rationed diet and water *ad libitum.* From day 1 till day 56, chicks were fed with chick mash twice a day with an average of 30 gm/ chick. After 57 days till lay, they were fed on grower mash provided twice a day at an average of 90 gm/bird. After the initiation of egg laying, the birds were fed on the layer mash provided twice a day at an average of 110 gm/bird. The feeds were purchased from Model Poultry farm, Baroda. The composition of the chick, grower and layer mash is shown in table 1. Calcium supplement in the form of shell grit was provided whenever required. The feeding schedule of Government poultry given table 2. and compared to it, the feeding schedule employed in the present investigation is that of uniform restricted diet.

Lighting regimens and photoperiodic manipulations :

The lighting of the cages was done by four fluorescent tubes fitted atop the cages. The light intensity was maintained at 250 lux and checked with a lux meter.

One day old chicks were maintained under a long photoperiod of LD 18:6 (LP) for a period of 90 days. After 90 days, they were transferred to a constant photoperiod of LD 12:12 (NLD), and maintained in it thereafter. This shifting involved a decrease of 6 hrs of light from 18 hrs of light to 12 hrs. Hence, this photoperiodic manipulation has been referred to as a step-down photic schedule. Another set of birds maintained under NLD, since the day of hatch, served as the control. The lights were on at 08 00hrs in all the cages and, switched off at 2000hrs for control birds, and at 0200hrs for the LP birds by an automatic timer.

Adrenocortical manipulations :

One day old chicks were subjected to mild hypercorticalism (HPR) or hypocorticalism (HPO) by implantation of bees wax pellets.

Pure bees wax was procured from the local market and was heated to a molten state, so that the impurities would settle down. Pure bees wax was separated from the upper surface and pellets of 19 mg and 40 mg were made out of it. Corticosterone and Metyrapone were procured from Sigma Chemical Co. USA. Metyrapone (Metopirone SU 4885) is a competitive 11β-hydroxylase inhibitor of adrenocortical steroidogenesis in mammals (Liddle, et. al., 1959) and inhibit corticosterone synthesis thereby inducing hypocorticalism.

The corticosterone pellets were prepared by uniformly mixing of 1 mg of corticosterone in 19 mg bees wax (1+19) and, the metyrapone pellets were prepared by mixing 10 mg of metyrapone in 40 mg of bees wax (10+40). The preparation of pellets was done as per the method described by Davison *et al.* (1985).

The corticosterone or metyrapone pellets were implanted subcutaneously in the inner part of the right thigh surgically. The birds were sacrifices at 30, 60 and 90 days and, the pellets were removed and weighed on an electronic mettler with 1µg sensitivity. The difference of pellet weight, before and after implantation was noted and the empirical rate of diffusion was calculated (Table 3). An empty pellet (bees wax only) of 20 or 50 mg was also implanted in three different groups which served as the sham operated groups.

Parameters assessed and methodologies of evaluation:

Each phase had 6 experimental groups :

1. NLD : Day old pullets reared under LD 12:12 photic schedule.
2. LP : Day old pullets reared under LD 18:6 (long photoperiod;LP) upto 90 days and then shifted to NLD.
3. NLD+HPR : Day old pullets rendered hypercorticalic and reared under NLD for 90 days.
4. NLD+HPO : Day old pullets rendered hypocorticalic and reared under NLD for 90 days.
5. LP+HPR : Day old pullets rendered hypercorticalic and reared under LP for 90 days.
6. LP+HPO : Day old pullets rendered hypocorticalic and reared under LP for 90 days.
7. Groups of pullets containing empty bees wax pellet (20 or 50 mg) served as the sham group.

The photoperiodic and endocrine manipulations were carried out as mentioned earlier.

Phase I

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.

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 allrepresented in the results section of respective chapters.

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.

Setup 2 : Adult RIR hens towards the end of their first cycle of lay (72 weeks) were subjected to photoperiodic and/or endocrine manipulations for 30 days and the effects on the second cycle of lay were assessed.

Phase II

A study of changes in physical features and biochemical composition of eggs of pullets subjected to photoperiodic and/or endocrine manipulations during the first trimester of post-hatch development was carried out during the initial phase (35-40 days after IL), Mid phase (5-6 months after IL) and late phase (10-12 months after IL). A total of 12 eggs were selected by random sampling for each group and their analysis was carried out. The physical features of the eggs of control and experimental groups of birds analysed were the egg weight, yolk, albumen and shell

Table.1: Composition (Kg/ton) of chick, grower,and layer mash.

CONSTITUENTS		CHICK	GROWER	LAYER
1.	Corn	481	420	385
2.	Groundnut cake	180	110	120
3.	Rice bran	150	200	200
4.	Wheat bran	76	100	-
5.	Rice polish	-	90	130
6.	Fish meal	50	30	40
7.	Proto Liv.	30	15	40
8.	Mineral mixture	25	25	26
9.	Dicalcium phosphate	4	5	15
10.	Coxidot	0.5	-	-
11.	Ventrimix (A,B,2D,3K)	0.1	0.1	0.1
12.	Salt	4	5	4
13.	Calside/Shell Grit	-	-	40
14	Neftine-200	-	0.25	-

Table 2. Amount of feed consumed at different period of rear in Government poultry *(ad libitum* schedule).

Age (in days).	Number of days.	feed/day (gms.)	Total feed consumed (Kg.)
1-- 28	28	37.5	1.050
29 -- 56	28	57.5	1.610
57 -- 84	28	80.0	2.240
85 -- 112	28	102.5	2.870
113 -- 140	28	120.0	3.360
141 -- 178	38	135.0	4.996
179 -- 530	352	135.0	47.380
Total no. of days.	530	Overall --->	63.500

Values : Mean

Table 3. Rate of diffusion of corticosterone or metyrapone from the continuous release implants (bees wax pellets).

	treatment	total dosage	duration of dose	amount released/day
NLD	HPR	1 mg corticosterone in 19 mg beeswax	1-30 days	0.15
			30-60days	0.20
			60-90 days	25-30
	HPO	10 mg metyrapone in 40 mg beeswax	1-30 days	0.40
			30-60days	0.66
			60-90 days	2.26
LP	HPR	1 mg corticosterone in 19 mg beeswax	1-30 days	0.20
			30-60days	0.32
			60-90 days	30-32
	HPO	10 mg metyrapone in 40 mg beeswax	1-30 days	0.50
			30-60days	0.65-0.70
			60-90 days	2.5

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

weight measured on a pan balance. The width and height of eggs was measured using a vernier calliper and, the shell thickness was measured using a micrometer screw gauge. The egg volume was measured by water displacement method.

The percentage water and solid contents, total protein, carbohydrate, total lipids and total cholesterol were estimated in both, yolk and albumen according to the following methods.

Percentage water content and solids :

Yolk or albumen was taken in preweighed lipid tubes and then weighed again. These tubes were oven dried at 50-60°c for 24 hrs in the case of albumen and for 72 hrs for yolk. These tubes containing dry yolk or albumen, were weighed again. The difference of the two readings gave the water content and the total dry matter left, was the total solid content. Both were expressed in terms of percentage.

Total Protein in yolk and albumen :

The protein content was estimated by the method of Lowry *et al.* (1951). Known amount (50mg) of yolk / albumen is mixed in 5ml chilled distill water and 0.1ml of sample is taken and diluted further by adding 0.9ml of distill 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.

Total carbohydrate in yolk and albumen :

The total carbohydrate content was estimated by employing the method of Seifter *et al.* (1950). Known quantity of fresh yolk (150 mg) or albumen (250 mg) was digested in KOH and precipitated with alcohol. The precipitate were dissolved in redistilled water and treated with anthrone reagent in 95% sulphuric acid which gives green colour. The intensity was measured on a spectrophotometer at 620mμ red filter and the values expressed as mg carbodydrate / 100 mg yolk or albumen.

Total lipids in yolk and albumen :

The total lipid content was measured by the method of Folch *et al.*(1957) using a chloroform-methanol mixture (2:1, v/v) as an extractant. The known quantity of fresh yolk (80-100 mg) is taken in testubes and cholroform-methanol mixture (2 ml) and 1% $CaCl_2$ (1 ml) are added to each of the tubes. The aqueous layer is seperated after 24 hrs with a syringe and a 2 ml aliquot was taken in preweighed lipid tubes and oven dried at 50°c. The tubes are weighed again and the lipid is thus measured gravimetrically and, expressed as mg lipid in 100 mg yolk/albumen.

Total cholesterol in yolk or albumen :

The total cholesterol was estimated by the method of Crawford (1950) using an alcohol ether mixture (3:1, v/v). The lipid, after extraction, is dried (as mentioned above) and then, dissolved in 2 ml of cholroform methanol mixture (2:1 v/v). Known quantity of aliquot (0.2 ml for yolk and 2 ml for albumen) was taken in testubes and oven dried at 50° C. Ferric chloride (2 ml) is added to it each of the tubes and is boiled for 5 min. in water bath and then cooled in icebath. Sulphuric acid (2.5 ml) is added along the side of the testube and mixed by swirling the tubes and the readings are taken after 30 minutes on a colorimeter at 540mμ green filter.

All these metabolites were expressed as mg/100 mg of yolk or albumen. These values were also expressed as mg in whole yolk or albumen based on the yolk and albumen weights. To decipher overall changes during the initial, mid and phases of lay, an average of the three was calculated and expressed as the overall content. The values obtained were also expressed in gms in entire yolk or albumen.

The water and lipid indices, non-lipid dry and calorific value were also calculated.

Water and lipid indices and, non-lipid dry matter : The relative component of egg was expressed in terms of water index (ratio by weight of water to non-lipid dry matter) and lipid index (ratio by lipid / non-lipid dry matter). Non-lipid dry matter was calculated by subtracting the sum of total protein and glycogen from total lipids (Ricklefs, 1980).

Calorific value : Energy / gm fresh egg was calculated utilizing energy equivalants of 9 Kal/gm lipid and 4 Kal/gm for carbohydrate and protein (See RamaRao, 1986).

Phase III

The day old chicks were subjected to photoperiodic and/or endocrine manipulations (as mentioned earlier) for a period of 90 days. The pullets were sacrificed under mild anaesthesia at 1, 30, 60 and 90 days and, following parameters were assessed :

Gravimetry

Changes in the weights of body, thyroid, adrenal, ovary oviduct, liver and lymphoid organs-thymus, bursa and spleen were noted in control and experimental groups.

The birds were quickly decapitated under mild anaesthesia to avoid stress during handling. The viscera was cut open and the organs were quickly excised, blotted free of blood and tissue fluids and gravimetric evaluations were carried out using a digital mettler. The organs were weighed upto 0.01 mg accuracy. The absolute weights thus obtained were converted into relative weights and expressed in terms of percentage of body weight. Per day growth rate, growth rate kinetics were calculated on the basis of absolute body weight of individual birds.

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.

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 seperately.

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