Chapter-III

Materials and Methods

III.1. To study the effect of photoperiod in the breeding biology of the animal in the laboratory under natural lighting (NDL) and constant photoperiod (LL) in different season of the year in Imphal valley.

Quail eggs were procured from Central Avian Research Institute, Izatnagar (U.P.) and hatching was done in Imphal. After hatching the pullets were brought to Departmental Laboratory, Manipur University and divided into groups (12 birds each) having almost equal body weight using a Single digital top pan balance. Then they were transferred into photoperiod chambers. The birds were fed with known feed stuff and reared together upto the end of 3rd week after hatch. From the 4th week onwards the birds were divided into pairs (one male and one female) upto the end of the experiment in both NDL and LL.
The feed and water were provided ad-libitum. The sizes of the cages were the dimension of 40” x 30” x 25” for housing group of birds and 20” x 15” x 12” for rearing the pairs. The experiment comprised a duration of ninety (90) days during which observations of food intake, body weight, maturity and egg laying were made. The commencement of shedding of feathers, crowing sound of the quails was also recorded. Total number of eggs and volume of the eggs were also recorded up to the end of the experiment. Volume of the eggs was recorded by using Bissonet formula, $\frac{4}{3}\pi ab^2$ given by Thapliyal, 1961. The body weight and food consumption values were expressed in terms of mean ±S.E. Statistical analysis was done by using ANOVA test followed by Tukeys test. The level of significance was considered at a value where P<0.05. The same experiment with all the necessary observations and data analysis was repeated during early summer, summer & winter season of a year.
Plate I

Fig. 3.1 Rearing of quails in the laboratory
Plate: II

Fig 3.2 Pairing of quails in the cage
III.2 To analyse the available marketed feed sold by the suppliers and study of the development and growth of the pullet fed with the market feed.

Collection of Samples: Samples of Samrat starter (S.S.), Samrat grower (SG), Samrat layer Mash (SLM), Samarat broiler finisher, (SBM), Maharaja starter (MS), Maharaja layer mash (MLM) and Amrit crumble finisher (ACF) were collected randomly from different shops of Imphal. They were brought into laboratory and oven dried and ground into powders. The samples were kept in air tight containers for use in the biochemical analysis. The six (6) main compositional components in proximate analysis are protein, lipid, fibre, carbohydrate and ash (A.O.A.C., 2000) and analysis of these components were done.

III.2.1 Estimation of Protein

The micro kjeldahl method (A.O.A.C., 1970) is the most reliable method for nitrogen estimation. It is used in a variety of forms. The percentage of protein can be calculated by multiplying with kirk’s conversion co-efficient i.e., 6.25 for animal and 5.7 for plant protein (Tshinyangu and Hennebert, 1996) to the total nitrogen value.

Principle

The nitrogen in the protein or any other organic material is determined by digesting the samples in concentrated sulphuric acid at high temperature with catalyst to convert it into ammonium sulphate. This salt when steam distilled with excess of NaOH liberates ammonia which is collected in boric
acid solution. The ammonium borate formed is then titrated with standard hydrochloric acid.

**Equipment and glassware**

1. Micro-kjeldahl digestion unit
2. Micro-kjeldahl distillation unit
3. Digestion tubes/flasks
4. Measuring cylinder
5. Volumetric flask

**Reagent**

1. Sulphuric acid (Sp.Gr. 1.84, nitrogen free)
2. Catalyst mixture – 99.0 of K$_2$SO$_4$, 4.1g of HgO and 0.8g of CuSO$_4$, well mixed
3. Sodium hydroxide and sodium thiosulphate solution – dissolved 50g of NaOH and 5g of Na$_2$S$_2$O$_3$ in 50 ml distilled water and diluted to 100ml.
4. Boric acid solution - Dissolved 4 g of boric acid in warm distilled water and diluted to 100ml.
5. Standard Hydrochloric acid solution-0.02N.
6. Mixed indicator - one part of 0.2% bromocresol green in ethanol with 5 parts of 0.2% bromocresol green in ethanol were mixed.

**Procedure**

1. Accurately weighed 100mg of sample, placed in the digestion tube/flask. Added a pinch of catalyst mixture and 10ml of concentrated sulphuric acid.
2. Digested the sample using digestion unit until the solution becomes colourless, cooled and added minimum quantity of water to dissolve the solid.

3. 100 ml conical flask containing 20ml of boric acid was taken and added 2 to 4 drops of mixed indicator dye and placed the flask beneath the condenser with the delivery tip immersed in the solution.

4. Transferred the digest to distillation apparatus and added 40ml of sodium hydroxide sodium thiosulphate solution to the digest and steamed until about 20ml of distillate is collected in the conical flask.

5. Rinsed the tip with water and titrated the distillate against the standard acid solution until the first appearance of violet colour, as the end point.

6. A blank was also run containing the same quantities of all the quantities without the sample.

Calculation: Percentage of nitrogen (N) has been calculated as following:

\[
\%N = \frac{V_1 - V_2 \times \text{Normality of HCl} \times 14 \times 100}{\text{Weight of sample (mg)}}
\]

Where \(V_1\) = Volume of HCl used in the titration with sample

\(V_2\) = Volume of HCl used in the titration without sample

\(\% = \% \) Nitrogen x factor for particular sample.
III.2.2 Estimation of Lipid

The lipid of the feed samples were determined by soxhlet method as described by Folch et al., (1957). One gram of each powdered samples were weighed in a porous thimble of a soxhlet apparatus, with its mouth plugged with a cotton wool. The thimble was placed in an extraction chamber which was suspended above a pre-weighed receiving flask containing petroleum ether (b.p. 40-60°C). The flask was heated on a heating mantle for 6-8 hours to extract the crude lipid.

After the extraction the thimble was removed from the soxhlet apparatus and the solvent distilled off. The flask containing the crude lipid was heated in the oven at 100°C for 30 minutes to evaporate the solvent, then cooled in a dessicator and reweighed. The difference in weight was expressed as percentage crude lipid content.

III.2.3 Estimation of crude fibre

Crude fibre was estimated by acid base digestion method (1.25% H₂SO₄ and 1.25% NaOH solutions). The residue after crude lipid extraction was put into a 600 ml beaker and 200ml of boiling 1.25% H₂SO₄ was added. The contents were boiled for 30 minutes, cooled and filtered through a whatman no. 1 filter paper and the residue is washed three times with 50ml aliquots of boiling water. The washed residue was returned to the original beaker and further digested by boiling in 200ml of 1.25% NaOH for 30 minutes. The digest was filtered to obtain the residue. This was washed three times with 50ml aliquots of boiling water and finally with 25ml ethanol. The washed
residue was dried in an oven at 130°C to constant weight and cooled in a dessicator. The residue was scraped into a pre-weighed porcelain crucible, weighed, reweighed. Crude fibre content was expressed as percentage loss in weight on ignition (AOAC, 2000).

\[
\text{% Crude fibre} = \frac{\text{Loss in weight noted}}{\text{Weight of sample taken}} \times 100
\]

III.2.4 Estimation of total carbohydrates

The basic units of carbohydrates are monosaccharides which cannot be split by hydrolysis into simpler sugars. The carbohydrate content can be measured by hydrolysing the polysaccharides into simple sugars by acid hydrolysis and estimating the resultant monosaccharides.

Carbohydrates are measured by anthrone method of Dubois et al., (1956) using D-Glucose (Sigma) as a standard. For the estimation of total carbohydrate 50mg of dry powdered samples were extracted with 80% ethanol using pestle and mortar. The supernatant collected after centrifugation was then evaporated until it was free from alcohol. The extract was then made aqueous with a few ml of distilled water. A known volume of the extract was heated with anthrone reagent prepared in concentrated H₂SO₄ for 10 minutes in boiling water bath and then allowed to cool at room temperature in a water bath. The absorbance of blue green colour developed is measured at 625mm in a spectrophotometer. The values were calculated by a standard curve prepared from glucose.
III.2.5 Ash Content

The ash of a biological material is the inorganic content residue remained after the organic matter has been burnt away. Total ash content of the samples were estimated by igniting moisture free sample at 550 ± 20°C for 6 hrs in a muffle furnace as described by ISI, (1982). Percent of ash was determined gravimetrically of the following formula

\[
\% \text{ ash} = \frac{\text{Crucible with ash} - \text{Crucible}}{\text{Weight of the sample taken}} \times 100
\]

III.2.6 Estimation of minerals

Mineral contents of the samples were determined by atomic absorption spectrophotometry, flame photometry and spectrophotometry.

A. Wet Diacid Digestion

Wet diacid digestion method of Capar et.al., (1978) was adopted for different mineral analysis. The samples were air dried and ground to powder forms and sieved the powders. From the ground samples, 100mg were weighed and added in a 100ml volumetric flask. In each volumetric flask, 20ml of conc. nitric acid was added and kept in a sand bath for around 2-3 hrs until brown fume ceased. The samples were cooled at room temperature 10ml of 70% perchloric acid was added in each volumetric flask and heated again for 1 hr. then the digested samples were kept overnight. The volumes of the digest were made upto 50ml with distilled water and the extracts were filtered in whatman no.42 by using gravity. These digest were used for different mineral analysis except for Nitrogen.
B. Estimation of Macro elements and Micro elements by Atomic absorption

The macro elements Ca and Mg with five micro elements Mn, Fe, Cu, Co and Zn were analysed by using Perkin Elmer absorption spectrophotometer, Analyst AA-200.

Principle

In this technique the atoms of an element are vapourized and atomized in the flame. The atoms then absorb the light at a characteristic wavelength. The source of the light is a hollow cathode lamp, which is made up of the same element, which has to be determined. The lamp produces radiation of an appropriate wavelength, which while passing through the flame is absorbed by the free atoms of the sample. The absorbed energy is measured by a photo-detector read-out system. The amount of energy absorbed is proportional to the concentration of the element in the sample.

Method

For this analysis, respective standards are prepared using 1000ppm stock for each element. For the macro elements, 2ppm, 4ppm and 6ppm standards were used. Micro elements required lower concentration for making the standard, so 1ppm, 2ppm and 3ppm were prepared. For estimation of the elements, the instrument was set by using their respective standards to prepare a standard curve for calibration of samples. Then the digest were analysed using suitable lamps of each element.
III.2.7 Estimation of Potassium

The diacid digest samples were used for the estimation of potassium by using systronics 105 flame photometer.

Principle

Potassium in solution was atomized to flame and the flame excites atom of potassium causing them to emit radiation at specific wavelength proportional to concentration of the solution and it is measured in a flame photometer with suitable filter which forms only potassium at wavelength of 768nm (Gupta, 2006).

Preparation of Standard stock solution: Previously dried KCl for 2 hrs at 105°C was weighed (1.907g) accurately and dissolved in deionised water and volume is made upto 1000ml. This solution gives 1000ppm K. From this 1000ppm K solution, 100ml is pipetted out into 1000ml volumetric flask and volume is made upto the mark by diluting with distilled water. This solution gives 100ppm K. From this 100ppm K solution, final standard solutions of 5.0 and 10ppm K were prepared.

For determination of total K, the instrument was first set with standard solution and standard curve was prepared. The instrument was set out to full scale with highest concentration of 5ppm using a standard filter. The digest was diluted to the suitable concentration range so that final concentration lies between 0 to 5mg/kg. The samples were then read in flame photometer at 548nm or using filter for K.
Calculation

Percent K in the sample was calculated as follows which was later converted to mg/100.

\[ \% \text{ K} = \frac{C \text{ (ppm)} \times \text{Volume of digest}}{\text{wt of sample (g)} \times 100/100000 \times df} \]

Where \( C \) = flame photometer reading

III.2.8 Estimation of phosphorus

Total phosphorus of sample extracts were estimated by Vanado phosphomolybdate yellow colour method (Gupta, 2006) from the digest prepared for K.

Principle

Phosphorus is converted to orthophosphate during digestion. These orthophosphates react with molybdate and give yellow coloured nonreducing vanadomolybdo-phosphoric heteropoly-complex in acid medium. The yellow colour is attributed to a substitution of oxyvanadium and oxymolybdenum radicals for the oxygen or phosphate. The intensity of this colour is directly proportional to the concentration of phosphate present in the sample which can be read on spectrophotometer. The colour is developed in about 30 minutes and is stable for 2-8 weeks.

Barton’s Reagent

For preparation of Barton’s reagent, 25 gm of AR grade ammonium molybdate in about 400ml of warm water was dissolved. In another beaker,
1.25 gm of AR grade ammonium metavanadate was dissolved in 200ml of boiling water and it was cooled. After cooling, 250 ml of conc. nitric acid was added and mixed. In that solution, ammonium metavanadate solution was added and made up the volume to 1 litre. The solution was mixed well and stored in amber colour bottle.

**Procedure**

From the digested sample, 5ml of diacid extracts were taken in 25ml volumetric flask. In that volumetric flask, 5ml of Barton’s reagent was added and made up the volume with distil water to 25ml which were shaken well and allowed to stand for 30 minutes for the development of yellow colour. The yellow colour developed was measured at 420nm using calibration curve of Na₂HPO₄ (25ppm to 125ppm).

**III.3 Analysis of Locally available feed ingredients and feed formulation**

Determination of biochemical composition before formulation of feed is important and widely accepted for the nutritional evaluation of food whether conventional or nonconventional.

**Collection of feed ingredients**

The feed ingredients collected during the experiment were procured from in and around Imphal market, washed, dried and ground into fine particles. The feed ingredient includes fish waste, rice bran, rice, till cake, snail and maize.
Feed formulation

Feed formulation is the process of quantifying the amount of feed ingredients that need to be put together to form a single uniform mixture (diet) for poultry that supplies all of their nutrient requirements. It requires a good knowledge of poultry and feed ingredients. Feed formulation is both a science and an art, requiring knowledge of feed and some patience and innovation when using formula. The nutrient composition of the diet will indicate the adequacy of the diet for the particular class of poultry for which it is prepared (e.g., starter, egg layers, meat chicken or breeders). Diet formulation enables the poultry industry to maintain some uniformity in levels of production.

The feed ingredients of maize, rice, rice bran, til cake, fish waste and snail are collected randomly from in and outside the Imphal city. They are washed, cleared and oven dried for 2-3 days at 70°C. Then they are ground into powders and kept in air tight containers for their biochemical composition. After knowing their proximate composition the ingredients are ready for feed formulation. Feed formulation was done according to NRC Council 1994 to meet the nutrient requirements of Japanese quail for pullets and egg layers. The feeds are formulated as starter feed type I, II, III, and layer mash Type I, II and III. Fish waste, snail and til cake were the main protein source.

After feed formulation, the formulated feed was given to the different groups of animals having 12 in number. The control group of this experiment was fed with feed from the market (Samrat) to see the overall performance of development, food consumption, egg laying. Starter was given upto 23 days
and from 24\textsuperscript{th} onwards the experimental animals were provided with layer mash feed. The feed were provided in known amount. Body weight, food consumption were recorded in a weekly basis. Number of eggs collected were also recorded during the experimental, volume of eggs were calculated by using $4/3\pi ab^2$. The experiment was conducted for ninety (90) days during summer months and kept in the photoperiodic chamber.

\textbf{III.4 Quail Meat analysis}

At the end of the experiment, the animals were sacrificed by using chloroform anaesthesia. The whole body of the animals were oven dried at 75\degree C for three days. The dried up samples were ground into powder and kept in air tight container for biochemical use. Analysis of protein, lipid, carbohydrate, fibre, ash, minerals were done by using similar methods employed for analysing the feed and feed ingredient.

\textbf{III.5 Cost benefit analysis}

The cost benefit of formulating feed over the market feed were done to see if there is any benefit by feeding the formulated feed to the quails. The comparison was done taking parameters like growth and development, food consumption, sex maturation, and egg laying.

\textbf{III.6 Histology of gonads}

Some Japanese quails of different stages before breeding phase, breeding phase and regressive phase of male and female quail were taken and individual body weight was recorded using a single top pan balance. The birds
were anaesthetized by using chloroform inhalation in closed chambers and then
the laparoscopy was applied to remove the testes, vas deferens, ovary and
oviduct. Tissues were cleaned, weighed and fixed in Bouin’s fluid for 24 hours.
The tissues were washed, dehydrated and embedded in paraffin wax for
preparation of tissue sections. Deparaffinsied hydrated 5-6µ thick sections were
stained with haematoxylin. Then the sections were dehydrated through
upgraded series of ethanol upto 70%, stained in eosin, wash in two changes of
90% ethanol dehydrated in absolute alcohol, cleared in xylene and mounted in
DPX.

Measurements of testicular axes were made using fine divider. Testis
volume was calculated by the formula of Bissonnett i.e., \( \frac{4}{3} \pi ab^2 \), where ‘a’ is
the half of the long axis and ‘b’ the half of short axis as described by Thapliyal
\textit{et.al.}, 1961.