Chapter 2

Material and Method
MATERIALS AND METHODS:

2.0 GENERAL INFORMATION OF THE EXPERIMENTAL ANIMAL

2.0.1 TAXONOMIC POSITION

The animal selected for the present study is Common Garden lizard, *Calotes versicolor*. The systematic position of the animals in animal's kingdom is as follows:

Phylum -- Chordata
Sub-phylum - Vertebrata
Super class -- Gnathostomata
Class-- Reptiles, (Lepidosauria)
Sub-class -- Diapsids
Super order-- Lepidosuria
Order-- Squamata
Sub Order-- Lacertalia
Family-- Agamidae
Genus-- *Calotes*
Species-- *versicolor*

The common garden lizard, *Calotes versicolor* were collected from Amravati Lat. 20°56' North and Long. 77°45' East and for Washim district it is Lat. 20° 42 North and Long. 77°02 East.

2.0.2 COLLECTION AND MAINTAINANCE

The Lizard *Calotes versicolor* is a one of reptiles, which is a cosmopolitan in distribution it is commonly found in India in the region of Amravati the garden lizard, *Calotes versicolor* is found in mainly in crops and fields and even also in forest of Satapura range.

Biological control of pests involve the deliberate attempt to use natural enemies either by introducing new species into the environment of a pest or by increasing the new species into the environment of a pest or by increasing the effectiveness of those already present. Parasitoids, predators and pathogens can control the insect pests. The predators are generally used for controlling the pests on crop to increase the yield of crops. This method is very useful because this
method is very chief and very effective to control the pest. The pollution with this is method is nil and no any charges spented on this method. The garden lizard, *Calotes versicolor* is a familiar to all farmers because these is found in crops and it is a common killer of many of the insect. Pest of crops which is harm full to the crops. Lizards becomes active throughout the day to kill the insect pest of the crops. The collection of the Lizards *Calotes versicolor* is done in this work period by two methods. In the month of January 2002 and February 2002 the lizards are purchased by from local dealer of Amravati.

1) From the month of the July 2001 to December 2001 and March 2002 to the June 2002 it was collected by the many of local boys in Tq. Mangrulpur.

2) The local collector boys capture the fresh Calotes from fields with the help of stick and thread. From backward side the thread thrown in the neck of Calotes and it fits in neck of Calotes. Boys bring these Calotes to me and I bring them to the laboratory, which transfer directly to the Cage where it put up to next experimental work. They were captured in the suburbs of Amravati and Mangrulpur and were brought to the laboratory within 24 hours of their capture in the fields.

Freshly caught adult lizards during the period of sexual inactivity were purchased from the local dealer in month of January and February and brought within 24 hours many times they were acclimatized to the laboratory condition for 3-4 days. As it was difficult to determine the sex of the animals externally they were randomly selected for experimental work with complete disregard to the sex. However, when the experiments terminated it was realized that the females were always insufficient in
number for arriving at any conclusion in a given sample and this widespread Asian native can easily be found in parks and gardens, where it feeds on insects and their prey. Its colour ranges from brownish-buff to grayish, and in the breeding season the throat of the male becomes red and black, as in the photo at left. The species is identified by the short crest above the neck, the presence of small spines above the tympanum and by the lack of shoulder fold. The male has swollen cheeks.

These lizards, Calotes were always kept in a wooden cage of 2½ X 2 X 2 Cu. Ft.

A portion measuring 7" from the bottom was covered by wire gauge on all the four sizes of the cage and the upper portion on the sides as well as top was made of glass. Water was provided ad libitum to all and they were maintained at laboratory. During the experiment cage numbers are to be increased up to 6 with same size. And two of them are provided with the light and electricity connections and a 20Watt Day light fluorescent lamp (Phillips).

During the experiment, the experimental animals were force fed with small pieces of meats. And even also many of the insects are collected with the help of insect-net and these collected insects are provided as food to lizards Calotes versicolor.

During the many of the experiments, collected lizards are grouped in number of cages which is labeled by capital letters like A, B, C, D etc. or as general number like 1, 2, 3, 4, 5, etc.

Disposables siring and disposable needles of 26 no. are used for many of the injections which are injected peritonealy

2.1 REPRODUCTIVE BIOLOGY:
2.1.1 POPULATION STUDIES:

Every month fresh Calotes
were collected from local collector and local dealer. The animals were captured once in the third week of every month. But in month of April, they were caught twice i.e. in the beginning of second week and again in the middle of third week. They were captured in area of Mangulpir and Amravati and brought to the laboratory within 24 hours of their capture in the fields.

The animals were sacrificed under ether anesthesia within the next 48 hours, after recording their body weights. Both the testes were carefully dissected and blotted on a filter paper and weighted on electronics balance to the nearest milligram. When the gonads were in regressed condition the entire testes were fixed in Bouin’s Picroformal. At other times, when the testes showed maximum development, it was cut into 2 to 3 pieces and then fixed in Bouin’s fluid. Along with the testes the ductus epididymis was also studied. For histological study further process was carried out. Bouin’s fixed tissues were routinely processed for paraffin embedding at 56 - 58° C; Block with tissue material are trimmed properly. Then fix the block to the block holder. Cutting the sections at 5 to 7μ formed the ribbon of the wax paraffin in thickness through Rotary and rocking microtome with the help of knife of microtome. Ribbon formed is cut in pieces of 1.5 -2' inch. The piece fixes on each slide with the help of Mayer’s albumin.

This slide is now prepared for the process of staining. For histological studies Double staining method is used. The stains are used in the study of gonadal parts are Haematoxylin and Eosin. Before starting this step dewaxing of the sections of the slide is done. Xylene is used for this purpose. Pass the individual slide through two changes of
Xylene (keep the slide in Xylene for 30min. in first Xylene staining jar and 30min. for second Xylene staining jars), so that the wax is totally dissolved and removed and only sections of material remains fixed to the slide.

Now pass the slide in descending series of alcohols: Absolute or 100% ----- 90% , --- 70% , --50% , ----- 30% , -------distilled water. Time recommended for keeping the slides in these in these series are 10 min. up to 90% and 5min. for other graded alcohols. Give the two changes in water of each 5 min.

Then stain the section in aqueous Haematoxylin for 5 min. After staining deep the slide in distilled water and in tap-water. Examine the slide under microscope. If stain appears to be dark, then deep the slide in acid water. By doing this excess of stain from the sections is removed.

Then dehydrate the sections through ascending order of series of alcohols: as 30%, --50%, ---70%, for 10 min. in each grade respectively then stain the slide with the help of Eosin for about one min. Then again transfer the slide to 70% alcohol grade for 5 min. then transfer the slide to 90% & 100% for 10 min. each. Then transfer the slide in staining jar that contains the Xylene.

After this, place a few drops of D.P.X. on the center of slide with the help of glass rod. Place a rectangular or circular cover slip on the slide in such a way that air bubbles do not get trapped below it. Keep such slide for drying and then store in slide box. Labeled the slide with marking the date of preparation then name of part name of animal etc. For the calculations of population of male in founding animals that varies according to the seasons number of Calotes versicolor per month is to be noted.
And then the number of male and female is to be calculated with percentage of occurrence in population is calculated. But in non-breeding season it is very difficult to distinguish the male and female externally but only after dissecting the animal it identified. But in the breeding season the throat of the male becomes red and black, as in the photo. The species is identified by the short crest above the neck, the presence of small spines above the tympanum and by the lack of a shoulder fold. The male has swollen cheeks. And all these marks help in identification externally. In the month of September and October the percentage of female is higher, at this time the egg become mature and egg may be laid on ground in this month. The data to be calculated and maintained with notes.

2.1.2 GONADO-SOMATIC INDEX

Every time the lizard *Calotes versicolor* is captured by local collector or local dealer which are transfer to cage and bring to the laboratory in next 24 hours. First of all when it bring it was measure the size and weight the was taken in two types i.e. 1) from snout to anal aperture 2) from snout to end of tail. The all measurements were taken in centimeters.

Then the weight of *Calotes versicolor* was taken in the electronic balance upto the accurate point in grams and after the dissection the gonads were removed carefully and dried with the help of blotting paper to remove the excess of water from surface of testes. The seasonal variation was seen in data of weight.

In many times there may be small sized animals are to collected which change the size of animal.

Therefore it was noted that in small animals the gonad may not appear mean, it shows that the
maturity also depends on size which may determine the stage of maturity in the animal.

In the month of September and October local collector that show the egg with them also collects female. Weight of these egg lying female is also done in electronics balance even also the size of the female is to be noted corrected. When they are in cage they are provided with the small insect and pieces of meat. With this the water in petridish was provided.

After some period in the entry of animal, the animals are sacrificed. The female Calotes was sacrificed under ether Anesthesia, the egg from the body off female is to be separated from the body and carefully measured in electronic balance. The weight of eggs in clusters is also to be noted and even also the average weight of egg is to be calculated. The size (length and diameter) and weight of eggs is to noted.

In every month the male Calotes versicolor are collected and bring in the laboratory. The size and shape is to be noted. The weight of the animals is also calculated. The male Calotes were sacrificed under ether anesthesia in dissection tray carefully and gonads are carefully separated from the body, then it was dried with the help of blotting paper and weight of gonads are calculated in electronics balance. Formula for calculation of gonado-somatic index is as follows

\[
\text{Gonado-somatic index} = \frac{\text{Weight of gonads}}{\text{Weight of the Calotes}} \times 100
\]

2.1.3 HISTOLOGICAL STUDIES

From 2001 to 2002 a total number of the 225 male lizards were studied. The animals were captured once in the third week of every month. In the month of April, they were caught twice i.e. in the beginning of second week (9th April) and again in the
middle of third week (19th April). They were captured in the suburbs of Mangrulpur and were brought to the laboratory within 24 hours of their capture in the fields.

The animals were sacrificed under ether anesthesia within the nest 48 hours, after recording their body weights. Both the testes were carefully dissected, blotted on filter paper and weighed on a torsion balance to the nearest milligram. When the gonads were in a regressed condition the entire right testis was fixed in Bouin's Picroformal. At other times, when the testes showed maximum development, it was cut into 2 to 3 pieces and then fixed in Bouin's 'fluid'. Either the entire left testes or part of it depending of its size was fixed in cold Baker's fixative for 3 days. Along with the testis the ductus epididymis was also studied Histologically every month. Bouin's fixed tissues were routinely processed for paraffin embedding at 56-58°C, sectioned at 5 to 7 μ and stained with Harris' or Delafield Haematoxylin and eosin. The histological observations are based on the tests from five animals studied every month.

The tissues fixed in Baker's fixative were washed in running tap water for 4 to 5 hours and embedded in 25% gelatin at 37°C. The gelatins embedded tissues were cut at 15 μ on a freezing microtome. The section were washed in distilled water and then stained with Sudan black B for total lipids and Sudan red for natural lipids. For histochemical demonstration of cholesterol positive lipids the following two methods were used (1) Schultz method using ferric ammonium sulphate and Sulphuric acid and (Pearse, 1960). The sections from the middle region of the testis were examined under the microscope. The diameter of ten more or less transversely cut seminiferous tubules

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were measured with the help of an ocular micrometer (1 cm x100). The total numbers of cells per seminiferous tubule were counted and the predominant cell type in each sample was noted.

The activity of the epididymis was determined by measuring the cell height of its epithelium.

2.3 EFFECT OF ENVIRONMENTAL PARAMETERS ON TESTES:

2.3.1 PHOTOPERIOD:

Freshly caught adult lizards during the period of sexual inactivity were purchased from the local dealer. They were brought to the laboratory within 24 hours after their capture from fields. They were acclimatized to the laboratory conditions for 3 to 4 days. As it was difficult to determine the sex of the animals. Externally, they randomly selected for experimental work with completed disregard to sex. However when the experiments were terminated it was realized that the females were always insufficient in number for arriving at any conclusion in a given sample and therefore, in the following account observations on female lizard are excluded. The lizards weighting 30 to 60 grams were divided into two groups viz. control (8 animals) and experimental (12 animals) as shown in the table. The animals from the experimental group were kept in a wooden cage (2 ½ x 2 x 2 cu.ft). A portion measuring 7 " from the bottom was covered by wire gauge on all the four sides of the cage the upper portion on the sides as well as the top was made of glass. A 20-watt Daylight fluorescent lamp (Phillips) was fixed on the top of the cage. An automatic timer controlled the duration of the light. The controls were kept in a cage of similar dimension. Both control and experimental animals were force fed with small pieces of meat. Water was provided ad-libitum. During the
day, both the groups were exposed to the natural daylight. However, after the sunset the experimental group was exposed to artificial illumination as described above. The daily period of artificial illumination was increased by ½ hour per day until a maximum of 7 hours was reached, Which was then kept constant till the end of the experiment. The control group received only the mature daylight and was kept in the dark during the night. In this way two sets of experiments were carried out. Experiment ' A ' was started on the 8 th December 2001 and was terminated on 12 th January 2002. Experiment ' B ' was started on 12 th January 2002 and was terminated on 14 th February 2002.

2.2.2 Temperature

Sexually quiescent animals were purchased from the local dealer and local boys were acclimatized to the laboratory conditions for 3 to 4 days and then divided into two groups. The animals under group 1 derived as controls and were kept in the described above and were maintained at laboratory conditions of temperature and daylight. The animals belonging to group 2 were kept in a cage, which was placed in a thermostatically controlled chamber except at the time feeding, when they were taken out for feeding at laboratory temperature. In this process, they were exposed to the laboratory temperature daily for half an hour. the temperature inside the cage was maintained at 39±1 C .The experimental animals were kept in complete darkness. Water was provided “ad-libitum” to both control and experimental animals and they were force feed with small pieces of meat. Three sets of experiments were carried out for this purpose as detailed below.

Expt. C - Commenced on 9 th March 2002 Terminated on 8 th April 2002
Expt. α - Commenced on 20 th January
2002. Terminated on 21st February 2002


At the end of the experiments both control and experimental animals were sacrificed on the date of termination of experiments by an overdose of ether anesthesia / their body weights were recorded at the commencement of the experiments and again prior to autopsy.

The gonads were carefully dissected, blotted on a filter paper and weighed to the nearest milligram on a electronic balance and then fixed in Bouin's fluid. They were routinely processed for paraffin embedding. The sections were cut at 5 μ and were stained with Harris' Haematoxylin and Eosin. The sections from the middle region of the testes were examined under the microscope. The diameters of them more or less transversely cut seminiferous tubules were measured with the help of an ocullometer (1 mm x 100). The total number of cells per seminiferous tubule was counted.

2.3 EFFECTS OF HORMONES:

2.3.1 EFFECT OF TESTOSTERONE PROPIONATE:

In the month of November, the male lizards were purchased from the local dealer and acclimatized to the laboratory conditions for 4 days. The animals were matched as far as possible with respect to body weight and length and divided into two groups. Groups A served as an experimental group. Each animals from the experimental group was injected intramuscularly with 1mg. of testosterone propionate (Perandren, Ciba) in 0.1ml. of oil daily for 10 days. The animals from the control group (group B) received equivalent amount of oil only. Both experimental and control animals were maintained under identical conditions of light and
temperature. Water was provided "ad-libitum" and they were force fed every day with minced meat.

At the end of the two experiments (30 days in temperature experiment and ten days in TP administration experiment), animals from both control and experimental groups were sacrificed by an overdose of ether anesthesia. The thyroid glands were removed along with the surrounding adipose tissue and were fixed in Bouin's fluid, routinely processed through alcohol grade, embedded in paraffin and cut at 5. The sections were then stained with either Heidenhain's Azan or haematoxylin and eosin. The following measurements were made on 20 follicles from the thyroids of each animal with the help of an ocular micrometer (1mm x 100), (1) the epithelial cell height and (2) the follicular diameter was then expressed on percentage basis (Wilhoff, 1958).

2.3.2 EFFECT OF COMPOUND ICI 33,828:

The male lizards were collected from the fields during their breeding period (July). Animals weighting 35 to 55 gms. were divided into groups as shown in the table 15 & 16. The experimental animals in the two groups were given two different doses 200 ug. And 800 ug. of 1-Methylallythiocarbanoyl-2 Methylthiocarbamoylhydrazine (ICI compound 33,828), commercially known as Methallibure daily, for a period of twenty days. The substance was taken day 21 th and testes from both sides from each animal were quickly removed and weighted on a torsion balance. The testis on the right side of the animal was used for cholesterol and ascorbic acid estimations. The cholesterol content of testis was determined by the method of Knobil et al. as described by Zarrow et al.,
(1964) and ascorbic acid was estimated by the method of Dasgupta et al. (1962). Histological findings on the left testis and thyroid were based on the sections cut from routine Bouin’s fixed paraffin embedded material. The testis sections (5-6 μ) were stained haematoxylin eosin and those of the thyroid (5-6 μ) with Heidenhein’s Azan.

The diameters of 25 seminiferous tubules were measured and the number of cells within each tubule was counted for each animal. In this way observations were made on the gonads of five animals from each group.

Measurements were taken on the epithelial cell height, follicular diameter and the number of cells per follicle from twenty thyroid follicles from each animal. In this way six animals each from experimental and control groups were examined.

2.4 EFFECT OF ENDOCINE GLANDS:
2.4.1 PITUTARY GLAND AND TESTES:
2.4.1.1 EFFECT OF PITUTARY HORMONES (FSH, TP):
In November and December the adult male lizard were purchased from the local dealer. During this period their gonads were completely regressed. After acclimatizing them to the laboratory conditions for 4 days, they were divided into various groups. Care was taken to match the animals under the different experimental groups and those under corresponding control groups in respect of body weight and length. The animals from group a and c were injected intraperitoneally with 0.1 mg. bovine FSH (Nutritional Biochemical Corp. International, Canada) in 0.1 ml. Saline (0.6) daily for a period of 8 days and 15 days respectively. The animals from group
received the same dose but on alternate days and for a period of 30 days. The animals from group b, d and f served as controls and received 0.1 ml of saline in the same manner as their experimental counterparts. All the control animals were housed in the same cage, while the experimental animals from different groups were kept in different cages. All the animals belonging to both experimental and control groups were maintained at the laboratory conditions of light and temperature water was provided 'ad libitum' and the animals were force fed daily with small pieces of fresh meat.

In another experiment the animals from experimental groups were injected with crude extracts made from the pituitaries of the same species in the following way. Pituitaries were removed from the male animals, when they were in full breeding conditions and were preserved in cold acetone (at freezing temperature) for 24 hours. Acetone was then allowed to evaporate room temperature and the dried pituitaries were then preserved in a tightly corked bottle in deep freeze till required for use) 0.1 ml of the extract was then injected intra peritoneal to each of the experimental animals from group 'g'.

An extract five pituitaries was made by grinding them in a tissue homogenize in 0, 5 ml of saline (0.6) The animals from group ‘h’ received a similar does of crude pituitary extract made from the fresh pituitaries removed from adult animals captured in received 0.1 ml. saline.

In this way, animals from groups ‘g’ and ‘h’ received the extracts in a dose equivalent to 1 pituitary daily, for a period of 10 days. All the other conditions were similar to those used for animals receiving FSH injections.

Twenty-four hours after the last
injection the animals from all experimental and control groups were sacrificed by an overdose of ether anesthesia. Their body weights were recorded prior to autopsy. Both the testes were carefully dissected, blotted on a filter paper and weighed on a torsion balance to a nearest milligram. Depending upon the size of the testis either the entire right testis or part of it was fixed in Bouin's fixative, routinely processed for paraffin embedding at 56°-58°, sanctioned at 5 μ and stained with Harris haematoxylin and eosin.

The sections from the middle region of the testes were examined under the microscope. The diameter of ten or more of less transversely cut seminiferous tubules was measured with the help of an ocular micrometer (1 mm x 100). The total number of cells per seminiferous tubule was counted and the predominant cell type in each group was noted.

Adult male lizards were purchased from the local dealer during the month and November. After acclimatizing them to the laboratory conditions for 4 days the animals were divided into 4 groups. Animals from group B received 1 mg. of testosterone propionate in 0.1 ml. of oil, daily for 10 days, while those under group D were given 2.5 mg. of testosterone in 0.1 ml. of oil thrice a week for 5 weeks.

The animals from control groups 'A' and 'C' received 0.1 ml. of the vehicle over the same period as their experimental counterparts. All the injections were given intramuscularly in the left and right thigh regions alternately. Thus, the animals under group 'B', received a total dose of 10 mg. whereas those under group 'D', 37.5 mg. All the animals from the experimental and control groups were sacrificed 48 hours, after the last
injection by an overdose of ether anesthesia.

The tests from both the sides were carefully dissected, blotted on a filter paper and quickly weighed on a torsion balance to the nearest milligram. They were then fixed in Bouin's fluid for about 20-24 hours, routinely processed for paraffin embedding at 56° C. The serial transverse section cut at u was stained with Harris's Haematoxylin. The section of the ductus epididymis was similarly prepared for microscopic examination.

The section from the middle region of the testes was examined under the microscope. The diameter of ten more or less transversely cut seminiferous tubule was measured with the help of an ocularimeter (1 mm x 100). The total number of cells per seminiferous tubules was also counted. The height of the epithelial cells of the ductus epididymis was also measured. The results obtained are given in table No. 14.

2.4.1.2 CYCLIC CHANGES IN ADENOHYPOPHYSIS AND NEUROSECRETORY SYSTEM:

The adult male lizards were captured once in the third week of every month in and around the suburbs of Amravati and Mangrulpir

were brought to the laboratory within 24 hours of their capture from the fields. All the lizards were sacrificed within the next 48 hours, in order to avoid any possible changes, which might occur by initiation. The animals were killed by an overdose of ether anesthesia and the pituitaries were carefully exposed. The entire brains, along with the pituitaries were removed and fixed either in aqueous Bouin's fluid or mercury formal. Helly's alcoholic Bouin's and Glevland Wolfe's fixatives tried, mercury formal gave the best results, while those obtained with aqueous Bouin's were

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fairly satisfactory. The remaining fixatives did not give good results. Therefore, the tissues were fixed mostly in mercury formal and aqueous Bouin's for 20 to 24 hours.

The tissues fixed in mercury formal were washed in running tap water for 16 to 24 min and then routinely processed through alcohol grades and embedded in paraaffin at 56 °C. Tissues fixed in Bouin's fluid were similarly processed for paraaffin embedding. All the tissues were horizontally cut in 5 μ. Those investigations, where differential cell counts on the pituitary were desired, the sections were serially mounted and were stained with periodic acid Schiff's Orange G Methylene blue method (PAS-Or G, Methyl. Bl.). With the following modifications. After staining with PAS, the section were treated with 1% aqueous orange G for 3 to 4 minutes. After staining in orange G, the section were directly passed to 5% phosphotungstic acid for 2 minutes; thoroughly rinsed in distilled water and counter-stained with 0.1% Aqueous methylene blue (BDH) for about 15 seconds, washed in distilled water, rapidly dehydrated in 90% alcohol and then absolute alcohol, cleared in xylene and finally mounted in Canada balsam. For the identification of different cell types in the adenohypophysis the following staining methods were used:

(1) Periodic acid schiff's method counter stained with 1% aqueous orange G (PAS-Or-G)
(2) Alain blue, periodic schiff's orange G (AB-PAS Or G) after preliminary oxidation with formic acid (Adams and Swetterham, 1958).
(3) Periodic acid Schiff's orange G, counterstained with methylene blue PAS-Or-Methyl.bl.) as described above.
(4) Heidenhain's Azan.
(5) Gomori's Chrom-alum.
haematoxylin phloxin (CHP)

Gomori's (1950) paraldehyde-fuchsin method was also tried but the results obtained were not consistent and were not reproducible.

For differential counts, all the cells from a mid sagittal and one parasagittal section of each pituitary were counted and the percentages of different cell types were calculated. In this way counts were made on the pituitaries from 4 to 5 animals every month.

The male lizards were collected from the fields during their breeding period (July). Animals weighting 35 to 55 gms. were divided into groups. The experimental animals in the two groups were given two different doses 200 ug. And 600 ug. of 1-Methylallythiocarboxyl-2Methylthiocarbamoylhydrazin (ICI compound 33,828 commercially known as Methallibure) daily, for a period of twenty days. The substance was taken in distilled water to watch Tween-80(approximately 1 drops/5ml. of distilled water) was previously added. All injections were given intraperitoneally in the form of suspension.

The control group of animals received 0.1ml. of vehicle. The animals from experimental and control groups were maintained in separate wired cages under similar conditions of food, light temperature, water etc. All the animals were sacrificed under ether anesthesia on day 21 and testes from both sides from each animal were quickly removed and weighted on a torsion balance. The testis on the right side of the animal was used for cholesterol and ascorbic acid estimations. The cholesterol content of testis was determined by the method and ascorbic acid was estimated by the method of Dasgupta et. al. (1962).

Histological findings on the left testis and thyroid were based on the sections cut from routine Bouin's fixed
paraffin embedded material. The testis sections (5-6 μ) were stained haematoxylin eosin and those of the thyroid (5-6 μ) with Heidenhain's Azan.

The diameters of 25 seminiferous tubules were measured and the number of cells within each tubule was counted for each animal. In this way observations were made on the gonads of five animals from each group. Measurements were taken on the epithelial cell height, follicular diameter and the number of cells per follicle from twenty thyroid follicles from each animal. In this way six animals each from experimental and control groups were examined.

2.4.2 THYROID GLAND AND TESTES:

The lizards were purchased from a local dealer and were brought to the laboratory alive within 24 hours after their capture from the fields. They were weighed and then sacrificed by an overdose of ether anesthesia within 24 hours after they were brought to the laboratory. In this way, at least a minimum of five animals was sacrificed once in every month. The data presented in the following account is based on the observations made only in males.

After careful dissection, the thyroid, from the right side along with the adhering adipose tissue was fixed in Bouin's fluid, routinely processed through alcohol grades, embedded in paraffin at 56°C. And serially cut at 5 μ. The sections were stained either with Heidenhain's Azan or with Haematoxylin eosin and examined under a microscope. The following measurements were made either on 25 or the maximum number of follicles available in the broadest section of the gland with the help of an ocular micrometer (1mm x 100). These measurements included the diameter of the follicles and the cell height of the follicular diameter was then
expressed on percentage basis

2.4.3 ADRENAL GLAND AND TESTES:

The present study was carried out on the fifty acclimatized Calotes, the lizard were scarified by decapitation which shows the Adreno-somatic indices of male *Calotes versicolor*.

The adrenal gland of above 10 calotes from each experimental set were dissected out after the time interval like as 24, 48, 72, 96 hrs. weight the adrenal tissues and total body weight of the animals. The adreno-somatic index were calculated on wet weight basis was used for calculating index by following formula.

Adreno-somatic index (ASI) 

\[
ASI = \frac{\text{Weight of the adrenal}}{\text{Weight of the calotes}} \times 100
\]

<table>
<thead>
<tr>
<th>Gr</th>
<th>Expt. Condition</th>
<th>Condition of Adrenal</th>
<th>Tissue Fixation in hrs</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Control 1</td>
<td>Normal</td>
<td>24, 48, 72 &amp; 96</td>
</tr>
<tr>
<td>2</td>
<td>Expt. 1</td>
<td>Unilateral Adrenal Lactomy</td>
<td>24, 48, 72 &amp; 96</td>
</tr>
<tr>
<td>3</td>
<td>Expt. 2</td>
<td>Bilateral Adrenal Lactomy</td>
<td>24, 48, 72 &amp; 96</td>
</tr>
<tr>
<td>4</td>
<td>Expt 3</td>
<td>Injection of Adrenal extract</td>
<td>24, 48, 72 &amp; 96</td>
</tr>
<tr>
<td>5</td>
<td>Expt 4</td>
<td>Injection of Unilateral Adrenal Lactomy</td>
<td>24, 48, 72 &amp; 96</td>
</tr>
</tbody>
</table>

For each experimental set of 10 male *Calotes versicolor* were used and were subdivided into 5 groups of each experiment period.

**Group I - Control.**

*Calotes versicolor* male without injection or adrenal ablation.

**Group II - Experiment 1.**

Unilateral adrenalectomy after 24, 48, 72 and 96 hrs. of period. testes of Calotes were fixed.
**Group III - Experiment 2.**

Bilateral adrenalectomy after 24, 48, 72 & 96 hrs. of period. Testes of Calotes were removed.

**Group IV - Experiment 3.**

Injection of adrenaline (0.2 ml/body weight) to the unilateral adrenalectomized Calotes after 24, 48, 72 & 96 hrs. of period. At this respective period the testes were fixed.

**Group V - Experiment 4.**

Injection of adrenaline (0.2 ml/body weight) to unilateral adrenalectomized calotes after 24, 48, 75 & 96 hrs. At respective treatment period testes were fixed for studies.

With all the above experiments male Calotes versicolor were used. After experimentation, water content of gonad, testes was studied at different hours. Behavioral pattern was observed. Histological studies of gonad, Testes and adrenal of different injected and adrenal ablated tissues. ---

**Experiment No. 1. --**

Unilateral adrenalectomy:

For this unilateral adrenalectomy experiment the 4 group were made like 24, 48, 72 & 96 hrs. of period. To remove the adrenal gland on the one side, weight and fix it for histological studies. Immediately after the removal of the above adrenal gland. To inject the 0.2 ml/per body weight of adrenaline to the Calotes for the experiment of bilateral adrenalectomy.

**Experiment No. 2.**

Bilateral adrenalectomy after unilateral adrenalectomy experiments the Calotes of 4 groups like 24, 48, 72 & 96 hrs. of period, to inject 0.2 ml/body weight of the adrenaline in the body of Calotes. In first group of 24 hrs. single dose of adrenaline is sufficient. But in the group of 48, 72 & 96 hrs. period, such group of Calotes versicolor required 2, 3 & 4 doses of (0.2 ml/body weight of adrenaline of
each) injection after 24 hrs.

After the particular interval of time hours to dissect out adrenal gland on other side weight and fix it for histological studies.

**Experiment No. 3.**

**Unilateral gonadectomy—**

For this unilateral gonadectomy experiment 4 groups were made like as 24, 48, 72 & 96 hrs. of period. To remove the gonads on one side, weight and fix it for the histological observations.

Immediately after the removal of one side gonad, to inject 0.2 ml/body weight of adrenaline to the Calotes, for the purpose of the bilateral gonadectomy.

**Experiment No. 4.---**

**Bilateral Gonadectomy.** :

After unilateral gonadectomy experiments the Calotes of 4 groups like 24, 48, 72 & 96 hrs. of period, to inject the 0.2ml/body wt of adrenaline in the body of calotes. For first group of 24 hrs. a single dose of adrenaline is sufficient, but in group of 48, 72 & 96 hrs. Period, sub groups of calotes versicolor required 2,3 & 4 doses of (0.2 ml/body wt. Of adrenaline if each) injection after 24 hrs. After specific interval of time hrs. to dissect out gonad on other side, weight, and fix it for histological studies. Histological and histophysiological studies are carried out in the male Calotes versicolor by fixing either gonad or adrenal gland at different experimental periods. Fixed tissue were further processed up to the block preparation, then section cutting and slide preparations. Staining was made with haematoxylin and eosin. Slide was observed under the microscope. Histological correlation was made between adrenal and gonad testes. Gonads and adrenal glands which are dissected out in case of normal and experimental animals are fixed in Holland Bouin's fixative. The tissue
were wash in running tap water for about 5 to 8 minutes. It is then transferred at 70% alcohol, dehydrated in graded series of alcohol, the cleaned in xylol and finally embedded in 58 °C to 60 °C paraffin wax, according to the standard histological procedures. The sections of the gonads and adrenal gland were cut at 5 to 7 μ and mounted serially using Mayer’s albumen. The sections were stained with Enriche’s haematotoxylin and counter stained with eosin. then transferred at 70% alcohol, dehydrated in graded series of alcohol, the cleaned in xylol and finally embedded in 58 °C to 60 °C paraffin wax, According to the standard histological procedures. The sections of the gonads and adrenal gland were cut at 5 to 7 μ and mounted serially using Mayer’s albumen. The sections were stained with Enriche’s haematotoxylin and counter stained with eosin.

2.5 BIOCHEMICAL CHANGES

2.5.1 CARBOHYDRATES

The adult male lizard Calotes versicolor collected local dealer and even also from local collector of the animals in Amravati. This animals now brought in laboratory within 24 hrs per month. There were placed for few hours in cage to stand in cage. About 15 – 20 animals per month are to be collected. And dissected in laboratory by removing the soft and hard skeleton separate. Excess of water from the body is to be removed from muscles then take the body weight muscle and heptopancreas were gently blotted by filter paper to remove excess of water. They were weighted and dried in an oven adjusted at 100± 5 °C constant weight was obtained. The dried material was finally powdered from which biochemical analysis were made no distinct sex was made in the chemical analysis as the number of males in

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collection is rich.

Analytical techniques: - The object of present study was to show relation ship of carbohydrate metabolism with the reproductive cycle. For estimation of glycogen the method of Kemp et. al. (1954) was employed using colorimeter. Multiplying the amount of glucose by factor 0.927 derived the glycogen value. The adult male lizard, Calotes versicolor collected local dealer and even also from local collector of the animals in Amravati and Mangrulpir. This animals now brought in laboratory within 24 hrs per month. There were placed for few hours in cage to stand in cage About 15 – 20 animals per month are to be collected And dissected in laboratory by removing the soft and hard skeleton separate. Excess of water from the body is to be removed from muscles then take the body weight muscle and heptopancrease were gently blotted by filter paper to remove excess of water. They were weighted and dried in an oven adjusted at 100 ± 5 °C constant weight was obtained. The dried material was finally powdered from which biochemical analysis were made no distinct sex was made in the chemical analysis as the number of males in collection is rich.

2.5.2 LIPIDS:

From the month of July 2001 to the month of June, every Month the animals are to be collected from local dealer and are also to be collected from the fields and in region of grasslands The collected animals can brought in laboratory the 24 hrs. be kept under observations after word the animals are sacrificed under ether anesthesia.

The tissues fixed in Baker's fixative were washed in running tap water fro 4 to 5 hours and embedded in 25% gelatin at 37 °C. The gelatin
embedded tissues was cut at 15 μ on a freezing microtome.

The section were washed in distilled water and then stained with Sudan black 'B' for total lipids and Sudan red for neutral lipids. For histochemical demonstration of cholesterol positive lipids the following two methods were used (1) Schultz method using ferric Ammonium sulphate and (2) Sulphuric acid. The sections from the middle region of the testis were examined under the microscope. The diameter of ten more or less transversely cut seminiferous tubules were measured with the help of an ocular micrometer (1 cm x100). The total number of cells per seminiferous tubule was counted and the predominant cell type in each sample was noted.

The activity of the epididymis was determined by measuring the cell height of its epithelium.

2.5.3 PROTEINS:

The adult male lizard, *Calotes versicolor* collected local dealer and even also from local collector of the animals in Amravati. This animals now brought in laboratory within 24 hrs per month. There were placed for few hours in cage to stand in cage About 15 - 20 animals per month are to be collected And dissected in laboratory by removing the soft and hard skeleton separate Excess of water from the body is to be removed from muscles then take the body weight muscle and heptopancreas were gently blotted by filter paper to remove excess of water They were weighted and dried in an oven adjusted at 100 ± 5 °C. Constant weight was obtained. The dried material was finally powdered from which biochemical analysis were made no distinct sex was made in the chemical analysis as the number of males in collection is
Analytical techniques: - The object of present study was to show relationship of protein with the reproductive cycle. For estimation of nitrogenous end product are to be calculated by Kjeldahl method by multiplying the nitrogen value by 6.25. was employed using colorimeter.