# *2. Materials and methods:*

## 2 .1 Animals and their maintenance:

Three months old healthy virgin adult female Wistar albino rats weighing 160-162 g obtained from inbred population of the central animal facility, University of Mysore were housed in polypropylene cages (3 rats/cage) with husk as the bedding material under 12:12 h light-dark schedule (lights on 7 AM to 7 PM) at 27 ± 2 °C and 70 % humidity. The animals were supplied with standard chow pellets and water *ad libitum* during the period of the experimentation. The adult male rats were also obtained for breeding purpose from the same inbred population of the central animal facility and were maintained under similar conditions. The protocols were approved by Institutional Animal Ethics Committee and the guide lines of CPCSEA, Govt. of India were followed for care and maintenance of animals.

## 2.2 Normal diet and high calorie diet:

Normal diet (ND) was the pallet feed (commercial name, "Amruth Animal feeds"), supplied by M/S Pranav agro industries limited, Maharashtra, India. The ND consisted of carbohydrate (63 %), protein (18.45 %) and fat (3.62 %). The high calorie diet (HCD) consisted of carbohydrate (33.63%), protein (12.78%) and fat (43.35 %) and prepared by mixing casein, starch, egg yolk, raagi powder, rice powder, sugar, vanaspathi, soya powder and groundnut powder.

**2.3 Biometric parameters:**

**i) Body and organs weight:**

Body weight (BW) was recorded to the nearest gram using top loading electronic balance. The weight of the testes, epididymis, vas deferens and seminal vesicle were recorded at the time of autopsy using electronic balance. The weight of testes and accessory organs were converted into relative weight (weight/100 g body weight) by using the formula:

$$\text{Relative weight of the organ} = \frac{\text{weight of the organ}}{\text{body weight}} \times 100$$

**ii) Body mass index:**

Body mass index (BMI) was determined by dividing body weight of a rat in grams by square of body length (nose–anus length in centimeter) according to the procedure of Novelli *et al.* (2006).

$$\text{Body mass index} = \frac{\text{Body weight (g)}}{(\text{Body length in cm})^2}$$

**iii) Thoracic circumference:**

Thoracic circumference (TC) was the circumference of an animal immediately behind the foreleg and measured in each rat according to the procedure of Novelli *et al.* (2006) using measuring tape.

**iv) Abdominal circumference:**

The abdominal circumference (AC) was the circumference of an animal immediately anterior to the hind leg. The AC was measured in each rat according to the procedure of Novelli *et al.* (2006) using measuring tape.

**2.4. Serum biochemical parameters:**

Blood was collected from the tail vein of adult female rats and from the ventricle of the heart at the time of autopsy of male and female offspring. The collected blood was centrifuged for 10 minutes at 1,500 rpm to collect serum. The serum was stored in -20$^0$C until used for analyses. The serum samples were used for the estimation of concentrations of glucose, cholesterol, triglyceride, LDL and HDL. The serum samples were also used to estimate concentration of hormones (section 2.5).

**i) Glucose**

The concentration of glucose was estimated by GOD-POD method (Tenscher and Richterich, 1971; Barham and Trinder, 1972) (sensitivity, 20 µg/ml) using kits. The serum (10 µl) was added to 1ml reagent (phosphate buffer, glucose oxidase, 4-aminoantipyrine, phenol, peroxidase) and incubated at 37º C for 10 minutes. Optical density was read at 505 nm. Sample was replaced by distilled water in blank. The concentration of glucose was expressed as mg/dl.

**ii) Cholesterol:**

Concentration of Cholesterol was estimated by CHOD-PAP (cholesterol oxidase- peroxidase) end point assay method (Herbert, 1984) (sensitivity 0.14µmol/l) using kits. The serum (10 µl) was added to 1ml reaction mixture (Good's buffer-pH

6.7, cholesterol esterase, cholesterol oxidase, peroxidase, 4-aminoantipyrine, stabilisers) and incubated at $37^0$C for 10 minutes. Optical density was read at 505 nm against reagent blank. The concentration of cholesterol was expressed as mg/100ml.

**iii) Triglyceride (TG):**

Concentration of TG was estimated by GPO-PAP (glycerol-3- phosphate oxidase- peroxidase) end point assay method (Herbert, 1984) (sensitivity 0.4mmol/l) using kits. The serum (10 µl) was added to 1ml reaction mixture (pipes buffer, 4-cholorophenol, magnesium ion, ATP, lipase, peroxidase, glycerol kinase, 4-aminoantipyrine, glycerol 3-phosphate oxidase, detergents, preservative, stabiliser) and incubated at $37^0$C for 10 minutes. Optical density was read at 505 nm against reagent blank. The concentration of TG was expressed as mg/100ml.

**iv) Low density lipoprotein (LDL):**

The concentration of serum LDL was determined by using kits, supplied by Span diagnostics Ltd, Surat, India. The serum (10 µl) was added to 750 µl of reaction mixture-1 (Buffer pH-6.3, detergent-1, cholesterol esterase, cholesterol oxidase, peroxidase, 4-AAP, ascorbic acid, oxidase, preservative) and incubated at $37^0$C for 5 minutes and reading was taken at 550 nm against reagent blank. Then 250 µl of reaction mixture-2 (buffer pH-6.3, deretgent-2, DSBmT, preservative) was added and incubated at $37^0$C for 5 minutes. Optical density was read at 660 nm against reagent blank. The concentration of LDL was expressed as mg/100ml.

**v) High density lipoprotein (HDL):**

The concentration of serum HDL was determined by using kits (Span diagnostics Ltd, Surat, India). The serum (10µl) was added to 750 µl of reaction mixture-1 (Good's buffer, cholesterol oxidase, peroxidase, DSBmT, ascorbic acid oxidase, accelerator, preservative) and incubated at $37^0$C for 5 minutes and reading was taken at 550nm against reagent blank. Then added 250 µl of reaction mixture-2 (Good's buffer, cholesterol esterase, 4-AAP, detergent, preservative) and incubated at $37^0$C for 5 minutes. Optical density was read at 660 nm against reagent blank. The concentration of HDL was expressed as mg/100ml.

**2.5. Hormone estimations:**

**i) Serum concentration of insulin:**

The concentration of serum insulin was estimated by ELISA using DRG diagnostic kit manufactured by DRG instruments GmbH, Germany. The concentration was expressed as µIU/ml serum. Microtiter wells were coated with insulin monoclonal antibodies. Each calibrator (25 µl), control and sample was pipetted out into different wells. To this, 100 µl of insulin enzyme conjugate was added and incubated at room temperature for 60 minutes. Plates were washed thrice by wash solution (300 µl) and wells were aspirated. Then 3, 3', 5, 5'-tetramethylbenzidine (TMB) substrate (100 µl) was added into each well and again incubated for 15 minutes at room temperature. Reaction was stopped by adding 50 µl of stop solution into each well. Absorbance was read at 450 nm within 15 min and concentrations of serum sample were calculated using semi log graph in which

absorbance value of each calibrator was plotted against the corresponding concentration of insulin.

**ii) Serum concentration of leptin:**

The serum concentration of leptin was estimated by ELISA using DRG diagnostic kit manufactured by DRG instruments GmbH, Germany. The concentration was expressed as ng/ml serum. Microtiter wells were coated with leptin monoclonal antibodies. Each calibrator (15 µl), control and sample was pipetted out into different wells. To this, 100 µl of assay buffer was added and incubated at room temperature for 120 minutes. Plates were washed thrice by wash solution (300 µl) and wells were aspirated to remove the residual droplets. Then 100 µl of antiserum was added to each well and incubated for 30 minutes at room temperature. Plates were washed thrice by wash solution (300 µl) and wells were aspirated to remove the residual droplets. Then 100 µl of enzyme complex was added to each well and incubated for 30 minutes. Plates were washed thrice by wash solution (300 µl) and wells were aspirated to remove the residual droplets. Then 100 µl of substrate solution was added to each well and incubated for 15 minutes. Reaction was stopped by adding 50 µl of stop solution into each well. Absorbance was read at 450 nm within 10 min and concentrations were calculated using semi log graph in which absorbance value of each calibrator was plotted against the corresponding concentration of leptin.

**iii) Serum concentration of testosterone:**

The concentration of serum testosterone was estimated in male rats by ELISA using DRG diagnostic kit manufactured by DRG instruments GmbH, Germany. The concentration was expressed as ng/ml serum. Microtiter wells were coated with

testosterone antibody. Each calibrator (25 µl), control and sample was pipetted out into different wells. To this, 100 µl of testosterone-enzyme conjugate was added and incubated at room temperature for 60 minutes. Plates were washed thrice by wash solution (300 µl) and wells were aspirated. Then TMB substrate (100 µl) was added into each well and again incubated for 15 minutes at room temperature. Reaction was stopped by adding 50µl of stop solution into each well. Absorbance was read at 450 nm within 15 min and concentrations were calculated using semi log graph in which absorbance value of each calibrator was plotted against the corresponding concentration of testosterone.

**iv) Serum concentration of estradiol:**

Serum concentration of 17β-estradiol was determined in female rats by enzyme immunoassay using kits (Estradiol EIA-2693) purchased from DRG Instruments GmbH, Germany and the assay was conducted following the procedure of the manufacturer. The concentration was expressed as pg/ml serum. Microtiter wells were coated with estrogen antibody. Each calibrator (25 µl), control and sample was pipetted out into different wells. To this, 100 µl of estradiol-enzyme conjugate was added and incubated at room temperature for 60 minutes. Plates were washed thrice by wash solution (300 µl) and wells were aspirated. Then TMB substrate (100 µl) was added into each well and again incubated for 30 minutes at room temperature. Reaction was stopped by adding 50 µl of stop solution into each well. Absorbance was read at 450 nm within 15 min and concentrations were calculated using semi log graph in which optical density value of each calibrator was plotted against the corresponding concentration of estadiol.

**2.6. Day of preputial separation:**

The separation of the prepuce from the glans penis is called "preputial separation" which is an external sign of onset of puberty in male rats. The penis of the pre-pubertal rats was examined every day from post natal day (PND) 40 until the preputial separation occurred to record the PND of preputial separation (Korenbrot *et al.,* 1977).

**2.7. Total epididymal sperm count:**

The cauda epididymis of one side of each rat was minced in 1 ml phosphate buffer saline (pH 7.2) to obtain suspension and the suspension was filtered through muslin cloth. The filtered sperm suspension was mixed with a drop of 1 % aqueous eosin for the staining of the spermatozoa and kept for 30 minutes. An aliquot of stained filtrate was taken in a WBC pipette up to the 0.5 mark and diluted further up to the mark 11 with PBS, and mixed well and charged into Neubauer's counting chamber. The number of spermatozoa present in 8 outer squares of 1 $mm^2$ area except the central erythrocyte counting area was counted. The aggregate of counts of 8 squares was multiplied by $5\times10^4$ factor to obtain the total sperm count/epididymis (Vega *et al*., 1988; Narayana *et al.,* 2002).

**2.8. Count of abnormal spermatozoa:**

A drop of stained filtrate as described above was put on a clean glass slide and a uniform smear was made. One thousand spermatozoa per epididymis were observed under higher magnification (40X) in randomly selected areas of smear and number of spermatozoa showing head shape and tail abnormalities viz. amorphous head, pin head, hammer head, curved tail, hook less head, double head was counted. The

aggregate of different types of spermatozoa showing abnormal shapes was considered to compute percentage of spermatozoa with abnormal morphology/epididymis (Vega *et al*., 1988; Narayana *et al*., 2002).

**2.9. Day of vaginal opening and study of estrous cyclicity:**

**i) Day of vaginal opening:**

The vaginal opening is the external sign of puberty in female rats. The vaginal region of the pre-pubertal rats was examined every day from PND 21 until the day of vaginal opening. On the day of vaginal opening a light yellow colored watery liquid emanating a characteristic smell appeared in the vaginal region and a pasture pipette could be inserted in the vagina to obtain exfoliated cells.

**ii) Estrous cyclicity:**

A pasture pipette containing normal saline was inserted into the vagina, the saline was flushed and exfoliated cells were collected by pipeting out the fluid. A smear of this fluid was prepared on a clean glass slide and observed under light microscope every day (10 AM) to determine the stage of the cycle i.e. proestrus, estrus, metestrus and diestrus based on the presence of types of exfoliated cells (Cooper *et al.,* 1993).

**2.10. Histology of the testis and ovary:**

**i) Histology of the testis and study of spermatogenesis:**

The testis fixed in Bouin's solution was dehydrated with different grades of alcohol, cleared in chloroform, infiltrated with molten paraffin wax and was embedded in paraffin wax. Five micron thick paraffin sections of the testis were cut

using Erma microtome. Testis sections were mounted on glass slides and stained with Mayer's haematoxylin and eosin to study the spermatogenesis. The stained sections were observed under high magnification and some sections were photographed using Olympus digital camera under appropriate magnification.

The germ cells of different categories of spermatogenic cells, viz, spermatogonia, spermatocytes, round spermatids and elaongated spermatids were indentified as per the description of Clermont (1972). The number of germ cells belonging to different stages of spermatogenesis/seminiferous tubule were counted in 50 randomly selected cross sections of the seminiferous tubules/testis and expressed as mean number of germ cells/category/tubule cross section. Though, testis sections are stained with PAS-haematoxylin for the study of spermatogenesis, in this study haematoxylin-eosin stain was used, because majority of study pertained to pre-pubertal testis, wherein full pledged spermatogenesis is not established. The PAS-haematoxylin technique is useful in identifying different steps of spermatid development, which enables to determine the stage of seminiferous epithelial cycle, which is established in adult testis.

**ii) Histology of the ovary and study of follicular development and follicle counts:**

The left and right ovaries were fixed separately in Bouin's fluid for 24 h at room temperature and then washed in water and transferred through three washes of 70% alcohol, dehydrated through different grades of alcohol, cleared in chloroform, infiltrated with paraffin wax (melting point 58-60°C) and embedded in paraffin wax. Serial sections of 5µm thickness of the left ovary were cut using Erma microtome. The sections were mounted on glass micro slides. The sections were stained in

Mayer's haematoxylin and eosin and examined under the light microscope (Olympus BX 60F5) for histological changes. Major histological alterations were photographed using Olympus DP11 digital camera under appropriate magnification. In the serial sections of the ovary, the large follicles were counted under 10X magnification, whereas small follicles were counted from serial cross sections of the ovary under 40X magnification. The ovarian follicles were identified and classified into different developmental stages (types) i.e., 1 to 8 according to the method of Pedersen and Peters (1968). According to this classification (Pedersen and Peters, 1968) characteristics of each stage are as follows.

Stage I: Naked oocytes (type 1).

Stage II: Primordial follicle (type 2) i.e., oocytes surrounded by 3-4 flat granulosa cells and 23- 27μm in diameter

Stage III: Primary follicle (type 3a) i.e., oocytes surrounded by a single layer of granulosa cells, number of granulosa cells ≤20 and diameter 55-80μm.

Stage IV: Primary follicle (type 3b), oocytes surrounded by two layers of granulosa cells, number of granular cells >20 and ≤60 and diameter 85-160μm.

Stage V: Pre-antral follicle (type 4), oocytes surrounded by several layers of granulosa cells, number of granulosa cells >61 and <100 and diameter 170-200μm.

Stage VI: Pre-antral follicle (type 5a), i.e. oocytes surrounded by multilayered

granulosa cell number of granulosa cells > 101 and < 200 and diameter 210-260μm.

Stage VII: Large pre-antral follicle (type 5b), i.e. oocytes surrounded by multilayered granulosa cells, number of granulosa cells >201 and < 400 and diameter 270-300μm and signs of antrum formation visible.

Stage VIII: Antral follicle (type 6), i.e. oocytes surrounded by granulosa cells with a visible antrum, number of granulosa cells > 401 and < 600 and diameter 310-385μm.

Stage IX: Large antral follicles (type 7), antrum enlarged and oocytes pushed towards follicular wall, number of granulosa cells >600 and diameter 400-500μm.

Stage X: Pre-ovulatory follicles (type 8), consisting of oocytes surrounded by corona radiata, very thin layer of granulosa with large antrum, granulose cells >600, and diameter >500μm.

Stage XI: Corpus luteum, identified based on presence of hypertrophied cells with rich blood supply giving appearance of a gland.

**Procedure for follicle count:**

Only the left ovary was considered for follicular counts as it was evident from the pilot studies that counts of follicles in right and left ovary did not markedly differ. In the serial sections of each ovary number of naked oocytes (type 1) and follicles of

each category, i.e primordial (type 2), primary (type 3a and 3b), pre-antral (type 4, 5a and 5b), antral (type 6 and 7) and pre-ovulatory (type 8) categories were counted. The number of follicles in each category in entire left ovary per rat was counted using hematoxylin-eosin stained serial sections. The number of oocytes present in each cross section of the ovary was recorded and sum of these counts was the count of oocytes/ovary. The number of primordial follicles present in every fourth section of the ovary and number of primary follicles in every $6^{th}$ section of the ovary were counted from first section of the ovary to the last section and sum of these counts was taken as count of each category per rat. A different procedure was followed for pre-antral, antral and pre-ovulatory follicles. Each section of the ovary was observed and the numbers of follicles showing full size oocytes/ category of follicle were recorded and sum of these in all the sections of the ovary was considered as the count for the given category per rat ovary. The counting was carried out carefully to avoid counting of the same follicle more than once. In each category, counts of healthy and atretic follicles were separately recorded.

**Follicular atresia**

Atretic follicles were identified according to morphological criteria described by Greenwald and Roy (1994) in hematoxylin-eosin stained serial sections of the ovary. A follicle was considered atretic when the oocyte showed signs of degeneration such as fragmentation, loss of nuclear membrane or thinning of cumulus oophorus. In addition the earliest sign of atresia was the presence of 5% pyknotic granulosa cells in the largest cross section of the follicle. While counting the follicles

of each category as mentioned above, healthy and atretic follicles were separately recorded.

**2.11. Fertility test and fertility indices:**

A proestrus female rat was allowed to mate with a male rat in a cage. The female was examined for the presence of vaginal plug or spermatozoa in the vaginal smear next day and presence of vaginal plug or spermatozoa in the smear confirmed the mating. Percentage of fertility was calculated by considering number of females bred and number of females conceived. The pregnant females were allowed to deliver the pups to find out differences, if any in the litter size and litter weight between control and obese animals. Number of females conceived, litter size, and litter weight of each group were recorded and expressed as mean values of each group.

Other fertility parameters were determined according to the procedure of Kennedy *et al.* (1973), Adilaxmamma *et al.* (1994) and Narayana *et al.* (2005) as follows:

i) Fertility index of male = number of fertile males/number of males used in the test x100,

ii) Fertility index of female = number of pregnant rats/number of females mated x100,

iii) Parturition index= number of females delivered/ number of pregnant rats x100,

iv) Gestation index= number of pups born alive/ total number of pups born x 100.

v) The mortality of pups was expressed as percentage of dead pups at parturition.

**2.12. Experimental design:**

This is a two generation study, i.e. $F_0$ and $F_1$. The study consisted of a single experiment wherein 30 randomly selected adult female rats were initially used to induce obesity. These females ($F_0$) after induction of obesity were mated with healthy non-obese male rats ($F_0$) to get $F_1$ male and female offspring. The impact of obesity on gametogenetic activity was studied by sacrificing these $F_1$ male and female rats at different age intervals corresponding with landmarks of spermatogenic and oogenetic activity. Since, the age at which different stages of gametogenesis appear in males and females differ, the $F_1$ males and females were autopsied at different age intervals. Some of $F_1$ male and females were used to assess reproductive performance after PND 100. The details of the experiment are given under 4 sub divisions for clarity and convenience.

**i) Induction of obesity in adult female rats ($F_0$ generation):**

Adult female rats weighing 160-162 gm were randomly divided into two groups, viz ND and HCD groups. The rats in ND group were fed with normal diet i.e laboratory chow (Pranav Agro industries limited, Maharashtra, India) *ad libitum* and those in HCD group were fed with high calorie diet (10gm/rat/day), in addition to normal laboratory chow (ND) for 8 weeks. The rats had free access for clean drinking water. The biometric parameters (section 2.3 i, ii, iii and iv) were recorded at weekly intervals for each rat in both groups. The serum concentrations of glucose, cholesterol and triglyceride (section 2.4 i, ii, iii) were determined at the end of $8^{th}$ week.

Each HCD rat ($F_0$ generation) after confirmation of obesity as determined by biometric and biochemical parameters (sections 2.3 and 2.4) was mated (section 2.11) with normal healthy non-obese male of proven fertility. These HCD females were fed with HCD during pregnancy and lactation. The fertility indices (section 2.11) of these females ($F_0$) were recorded. The pups ($F_1$ generation) born to mother rats in ND and HCD groups were used for studies on impact of obesity during pre-natal (*in-utero*) and post-natal (pre-pubertal and pubertal) periods on gametogenic activity and reproductive performance.

**ii) Impact of exposure to obesogenic environment during pre-natal (*in-utero*) and post-natal (pre-pubertal and pubertal) periods on spermatogenesis and onset of puberty:**

The male offspring ($F_1$) born to control and HCD females ($F_0$) as described above (section 2.12 (i)) were used.

Each male pup in HCD group was exposed to maternal obesity before weaning period as pregnant and lactating dams ($F_0$) were fed with HCD. After weaning period, pups ($F_1$) in control group were fed with ND and those in HCD group were fed with high calorie diet (10 g/rat/day) upto the completion of experimental duration i.e. PND100. The rats were autopsied on PND 7, 13, 17, 24, 36, day of preputial separation and PND 100. At autopsy biometric parameters (section 2.3) were recorded and testis was fixed in Bouin's fluid for histological studies and counts of spermatogenic cells as described earlier (section 2.10 i). The epididymis was removed from each rat on PND 100 and processed for total and abnormal sperm count as described in sections 2.7 and 2.8. The serum biochemical parameters i.e.

concentration of gluocose, cholesterol, TG, LDL and HDL were determined (section 2.4 i, ii, iii, iv, and v) on PND of preputial separation and 100 by the collection of blood from ventricle of heart. The serum concentration of leptin, testosterone and insulin were determined on PND 13, 17, 24, 36, 100 and day of preputial separation as per the procedure described earlier (section 2.5 i, ii and iii).

**iii) Impact of exposure to obesogenic environment during pre-natal (*in-utero*) and post-natal (pre-pubertal and pubertal) periods on ovarian follicular development and onset of puberty:**

The female offspring ($F_1$) born to rats ($F_0$) in ND and HCD groups in the above experiment [Section 2.12 (i)] were used. Each female pup in HCD group was exposed to maternal obesity before weaning period, as pregnant and lactating dams ($F_0$) were fed with HCD. After weaning, the rats in ND group were fed with (normal laboratory chow) *ad libitum* whereas those in HCD group were fed with 10 g/rat/day in addition to ND. All the rats had free access to clean drinking water. The ND/HCD feeding continued upto onset of puberty (vaginal opening). The biometric parameters (section 2.3) were determined and rats were autopsied on post-natal day 1, 4, 7, 15, 21, 28 and day of vaginal opening as these age points correspond to land mark of early ovarian follicular development. At autopsy, the ovaries were fixed in Bouin's fluid for histological studies and follicular counts as per description given in section 2.10. ii. The serum biochemical parameters i.e. concentration of gluocose, cholesterol, TG, LDL and HDL were determined (section 2.4 i, ii, iii, iv, and v) on the day of vaginal opening by collection of blood from ventricle of heart. The serum concentration of leptin, estradiol and insulin were determined on PND 15, 21, 28, and

day of vaginal opening as per the procedure described earlier (section 2.5 i, ii and iv). The day of vaginal opening was recorded in HCD and ND offspring as per procedure described in the section 2.9 i.

**iv) Impact of HCD on reproductive performance of $F_1$ offspring born to obese mothers:**

Some of the male and female offspring ($F_1$) born to HCD females ($F_0$) (section 2.12. i) exposed to HCD during lactating period were fed with HCD after weaning upto PND 100. During this period estrous cycle of female offspring ($F_1$) was recorded (section 2.9. ii) for 2 months period and compared with female offspring of ND female ($F_0$). Then these rats were mated with female and male offspring of ND females ($F_0$) in the following combinations viz.,

ND♂ x ND♀,

ND♂ x HCD♀,

HCD♂ x ND♀,

HCD♂ x HCD♀

(ND-normal diet, HCD-high calorie diet)

Each pro-estrous female in each group was allowed to mate with one male and successful mating was confirmed by presence of spermatozoa in vaginal smear next day morning. After gestation and parturition, different fertility parameters (section 2.11) were recorded.

**2.13. Statistical analyses:**

The mean value of each parameter of each group was computed considering at least five animals/group. The mean value of each parameter was computed using data on at least 5 rats in each group and the mean values of ND and HCD groups were compared using Student's t-test and judged significant if $P<0.05$, as only two groups were compared. The mean values of each parameter was compared by one-way ANOVA followed by Duncan's multiple range test in experiment on reproductive performance (section 2.12 iv) as there were four groups.