MATERIALS AND METHODS
Materials and Methods

Procurement and Maintenance of Animals:

Adult female zebrafish (wild) were obtained from commercial suppliers (Aquastar Aquarists, Chennai; Pet Bonding, Bengaluru and Valarius, Belagavi) and were maintained in the laboratory (permission to work on zebrafish was obtained from Animal Ethical Committee CPCSEA under Institutional Registration # 639/GO/02/a/CPCSEA) under natural temperature (26±1°C) and photoperiod (11.30L: 12.30D) for one week prior to the experimentation (Rajapurohit and Pancharatna, 2007). They were fed twice/day on commercial pellets and or Artemia naupli ad libitum to get adult and gravid females (David and Pancharatna, 2009ab) for the morphological, histological and histochemical studies and experiments.

Study Protocol

Section I

Morphology, Histology and Histochemistry of Adult Ovary

Histological examination of oogonial proliferation and their differentiation into oocytes in the adult ovary, follicular kinetics (recruitment and growth of follicles), vitellogenesis (origin and distribution of yolk granules and histochemical analysis of yolk), development and differentiation of follicular envelopes were carried out under this sub title.
Section II
Experiments

Seven experiments were conducted using in vivo and in vitro systems to elucidate factors influencing and mechanisms controlling oogonial proliferation, their differentiation into oocytes, folliculogenesis, development and growth of follicles, and oocyte maturation in the adult ovary of zebrafish.

Experiment 1: Nutritional regulation of oogonial proliferation and differentiation, oocyte recruitment and growth in the adult ovary in vivo

Adult fishes (body mass: 156±17.0 mg and body size: 3.0±0.1 cm) were used. Fishes were divided into five groups and maintained under laboratory conditions with different feeding regimes: Group-I fed daily four times; Group-II fed daily twice; Group-III fed daily once; Group-IV fed every alternate day and Group-V fed every fourth day. This experiment lasted for two months. For the first month fishes were fed with commercial pellets ad libitum and for the second month, commercial pellets were supplemented with newly hatched Artemia nauplii along with pellets. At the end of the second month all experimental fishes were euthanized by immersing in ice water. Body weights and body lengths were recorded; ovaries were excised and weighed, fixed in Bouin’s fluid and processed for paraffin embedding.
**Experiment 2: Effects of Insulin, Galactose individually and in combination on progression of germ line development in adult ovary in vitro**

Ovaries from adult fishes (body mass: 139±9 mg and body size: 2.7±0.1 cm) were used for *in vitro* cultures. Zebrafish ringer solution was used to dissolve the hormones and energy substrates. The experiment consisted following groups: (i) Control (Culture medium alone), (ii) Insulin (1ng/ml), (iii) Galactose (1ng/ml) and (iv) Insulin (1ng/ml) + Galactose (1ng/ml). This experiment lasted for 15 days.

**Experiment 3: Effects of hormones [Insulin, GH, ACTH, Thyroxine (T4), Prolactin, FSH] and Somatostatin on progression of germ line development in vitro**

Ovaries from adult female fishes (body mass: 132±5 mg and body size: 2.5±0.2 cm) were used for *in vitro* cultures in this study. Zebrafish ringer solution was used to dissolve the hormones. The experimental design consisted following groups: (i) Control (Culture medium alone), (ii) Insulin (1ng/ml), (iii) Growth hormone (Somatotropin) (1ng/ml), (iv) Somatostatin (1ng/ml), (v) FSH (1ng/ml), (vi) ACTH (1ng/ml), (vii) Thyroxine (1ng/ml) and (viii) Prolactin (1ng/ml). Cultures were terminated on 16th day.

**Procedure for in vitro Cultures**

All the glass ware and instruments (i.e. scissors, forceps and needles) used for the experiments were thoroughly cleaned and then autoclaved at 15lbs
for 20 minutes, prior to use. Adult female zebrafish were euthanized by immersing in ice water and ovaries were removed surgically under a stereozoom in clean chamber and were placed in zebrafish Ringer solution (116mM NaCl, 2.9mM KCl, 1.8mM CaCl₂, 5mM HEPES, pH 7.2) (Tokumoto et al., 2004). Ovaries were placed in culture vessels containing 1ml of medium (Leibovitz 15) (L-15, 1.7mM Proline, 0.1mM Aspartic acid, 0.1mM Glutamic acid, 0.5% Bovine Serum Albumin fraction-V, 50μg/L retinol, 10mM HEPES, pH 7.4) (Miura et al., 1991), with or without hormones. All cultures were replaced with fresh culture medium daily and maintained for 15 days at 22°C on clean bench of Laminar air flow. On 16th day ovaries were fixed in Bouin’s fluid and processed for paraffin embedding.

**Experiment 4: Effects of insulin, Insulin like Growth Factor1 (IGF1) and Galactose individually and in combination on in vitro induction of GVBD**

Preovulatory oocytes from gravid female fishes (body mass: 615±7 mg and body size: 4.2±0.1 cm) were used. The experimental design consisted following groups: (i) Control (Ringer solution only), (ii) DES (2μM/ml), (iii) Insulin (1ng/ml), (iv) IGF1 (1ng/ml), (v) Galactose (1ng/ml), (vi) Insulin (1ng/ml) + IGF1 (1ng/ml), (vii) Insulin (1ng/ml) + Galactose (1ng/ml), (viii) IGF1 (1ng/ml) + Galactose (1ng/ml) and (ix) Insulin (1ng/ml) + IGF1 (1ng/ml) + Galactose (1ng/ml).
Experiment 5: Effects of differential concentrations of Insulin like growth Factor (IGF1) on the in vitro induction of GVBD

Preovulatory oocytes from adult fishes (body mass: 567±20 mg and body size: 4.5±0.01 cm) were used. The experimental design consisted following groups: (i) Control (Ringer solution only), (ii) DES (2μM/ml), (iii) IGF1 (0.01ng/ml), (iv) IGF1 (0.1ng/ml), (v) IGF1 (1ng/ml), (vi) IGF1 (5ng/ml) and (vii) IGF1 (10ng/ml).

Experiment 6: Effects of hormones [Insulin, GH, ACTH, Thyroxine (T4), Prolactin, FSH] and hormone releasing factor (Somatostatin) on in vitro induction of GVBD in fully grown preovulatory oocytes

Oocytes manually separated from adult gravid females (body mass: 533±25 mg and body size: 4.5±0.02 cm) were used. The experimental design consisted of following groups: (i) Control (Ringer solution only), (ii) DES (2μM/ml), (iii) Insulin (1ng/ml), (iv) Thyroxine (1ng/ml), (v) Growth Hormone (Somatotropin) (1ng/ml), (vi) Somatostatin (1ng/ml), (vi) FSH (1ng/ml), (vii) ACTH (1ng/ml) and (ix) Prolactin (1ng/ml).

Experiment 7: Effect of xenoestrogens on the in vitro induction of GVBD.

Preovulatory oocytes from adult fishes (body mass: 588±9 mg and body size: 4.3±0.1 cm) were used. Oocytes were exposed to different concentrations of xenoestrogens (Fenvalerate and Dimethoate). Experimental design consisted of following groups: (i) Control (Ringer solution alone), (ii) DES (2μM/ml), (iii)
Fenvalerate (5µg/ml), (iv) Fenvalerate (10µg/ml), (v) Fenvalerate (15µg/ml), (vi) Fenvalerate (20µg/ml), (vii) Dimethoate (5µg/ml), (viii) Dimethoate (10µg/ml), (ix) Dimethoate (15µg/ml) and (x) Dimethoate (20µg/ml).

Procedure for in vitro GVBD Assay

All glass wares/instruments used for the in vitro experiments were thoroughly cleaned, sterilized and autoclaved. Ovaries dissected from gravid females were placed in a petri dish containing Zebrafish Ringer Solution (Tokumoto et al., 2004). Stage III oocytes (500-700 µm in diameter) were isolated manually with the help of a pair of fine watchmaker's forceps under a dissecting microscope in clean chamber. Isolated oocytes (n = 10) were transferred to petri plates containing either 5 ml of ZRS alone (control) or DES (2 µM/ml in ZRS) or hormone(s) (1ng/ml) or growth factor IGF1 or Fenvalerate (5, 10, 15 and 20µg/ml) or Dimethoate (5, 10, 15 and 20µg/ml). Each hormone/growth factor/test chemical concentration was tested in triplicate sets (n=30). The assay was set on clean bench at room temperature (26 ±1° C). Occurrence of GVBD was scored for every hour for 6.00 hours.

Assessment of GVBD

Oocyte maturation process was assessed by observing the oocytes under a stereozoom, after placing them in clearing solution (4% acetic acid + 5% paraformaldehyde in ringer) and the GVBD was ascertained by dissolution of GV (Lessman and Kavumpurath, 1984; Tokumoto et al., 2004). The occurrence of
GVBD was further confirmed by staining oocytes with 5µg/ml acridine orange (AO) dissolved in E3 medium (5mM NaCl, 0.17mM KCl, 0.33mM CaCl$_2$ and 0.33mM MgSO$_4$) at room temperature for 15 minutes. They were then observed under a fluorescent inverted microscope. DNA intercalated AO fluoresces green at 525nm (Darzynkiewicz, 1990).

**Hormones, Growth Factors, Culture media and Xenoestrogens**

All the hormones (Insulin, ACTH, Thyroxine, Prolactin, Somatotrophin, FSH, DES), releasing hormone (Somatostatin) and Growth Factor IGF1 were procured from Sigma, St Louis, USA. Galactose, HEPES, Aspartic acid, Glutamic acid, Retinol, Acridine Orange were purchased from Himedia, India. Insecticides/Xenoestrogens (dimethoate and fenvalerate) were purchased locally.

**Preparation of hormonal solutions:**

Control:

5ml of zebrafish Ringer solution alone was used as control without addition of any hormones.

**DES (2µM/ml):**

1350µl of DES was dissolved in 3650µl of culture medium to prepare a solution of volume 5ml of concentration 2µM/ml.
**Insulin: Stock Solution**

0.0005gm (0.5mg or 500µg) of Insulin was dissolved in 1ml of distilled water to make a solution of volume 1ml of concentration 0.5mg/ml or 500µg/ml (I stock solution).

10µl from I stock solution (I S.S) was dissolved in 990µl of distilled water to make a solution of volume 1ml of concentration 5µg/ml (II S.S).

15µl from II S.S was dissolved in 1485µl of distilled water to make a solution of volume 1.5ml of concentration 0.05µg/ml or 50ng/ml (III S.S).

**Insulin (1ng/ml):**

100µl from III S.S was dissolved in 4900µl of culture medium in petri plate to make a solution of volume 5ml of concentration 1ng/ml.

**Galactose: Stock Solution**

0.0005gm (0.5mg or 500µg) of Galactose was dissolved in 1ml of distilled water to make a solution of volume 1ml of concentration 0.5mg/ml or 500µg/ml (I stock solution).

10µl from I stock solution (I S.S) was dissolved in 990µl of distilled water to make a solution of volume 1ml of concentration 5µg/ml (II S.S).

15µl from II S.S was dissolved in 1485µl of distilled water to make a solution of volume 1.5ml of concentration 0.05µg/ml or 50ng/ml (III S.S).
Galactose (1ng/ml):

100μl of Galactose from III S.S was dissolved in 4900μl of culture medium to prepare solution of volume 5ml of concentration 1ng/ml.

Insulin (1ng/ml) + Galactose (1ng/ml):

100μl of Insulin from III S.S and 100μl of Galactose from III S.S were dissolved in 4800μl of culture medium to prepare solution of volume 5ml of concentration 1ng/ml.

20μl of Insulin from III S.S and 20μl of Galactose from III S.S were dissolved in 960μl of culture medium in culture vessel to prepare solution of volume 1ml of concentration 1ng/ml.

Somatotropin: Stock Solution

0.0005gm (0.5mg or 500μg) of Somatotropin was dissolved in 1ml of distilled water to make a solution of volume 1ml of concentration 0.5mg/ml or 500μg/ml (I stock solution).

10μl from I stock solution (I S.S) was dissolved in 990μl of distilled water to make a solution of volume 1ml of concentration 5μg/ml (II S.S).

15μl from II S.S was dissolved in 1485μl of distilled water to make a solution of volume 1.5ml of concentration 0.05μg/ml or 50ng/ml (III S.S).

Somatotropin (1ng/ml):

100μl of somatotropin from III S.S was dissolved in 4900μl of culture medium to prepare solution of volume 5ml of concentration 1ng/ml.
Somatostatin: Stock Solution

0.0001gm (0.1mg or 100µg) of Somatostatin was dissolved in 1ml of distilled water to make a solution of volume 1ml of concentration 0.5mg/ml or 500µg/ml (I stock solution).

10µl from I stock solution (I S.S) was dissolved in 990µl of distilled water to make a solution of volume 1ml of concentration 1µg/ml (II S.S).

15µl from II S.S was dissolved in 1485µl of distilled water to make a solution of volume 1.5ml of concentration 0.05µg/ml or 50ng/ml (III S.S).

Somatostatin (1ng/ml):

50µl of somatostatin from III S.S was dissolved in 4950µl of culture medium to prepare solution of volume 5ml of concentration 1ng/ml.

Follicle Stimulating Hormone (FSH): Stock Solution

0.0005gm (0.5mg or 500µg) of FSH was dissolved in 1ml of distilled water to make a solution of volume 1ml of concentration 0.5mg/ml or 500µg/ml (I stock solution).

10µl from I stock solution (I S.S) was dissolved in 990µl of distilled water to make a solution of volume 1ml of concentration 5µg/ml (II S.S).

15µl from II S.S was dissolved in 1485µl of distilled water to make a solution of volume 1.5ml of concentration 0.05µg/ml or 50ng/ml (III S.S).
**FSH (1ng/ml):**

100µl of FSH from III S.S was dissolved in 4900µl of culture medium to prepare solution of volume 5ml of concentration 1ng/ml.

**Adrenocorticotropic hormone (ACTH): Stock Solution**

0.0005gm (0.5mg or 500µg) of ACTH was dissolved in 1ml of distilled water to make a solution of volume 1ml of concentration 0.5mg/ml or 500µg/ml (I stock solution).

10µl from I stock solution (I S.S) was dissolved in 990µl of distilled water to make a solution of volume 1ml of concentration 5µg/ml (II S.S).

15µl from II S.S was dissolved in 1485µl of distilled water to make a solution of volume 1.5ml of concentration 0.05µg/ml or 50ng/ml (III S.S).

**ACTH (1ng/ml):**

100µl of ACTH from III S.S was dissolved in 4900µl of culture medium to prepare solution of volume 5ml of concentration 1ng/ml.

**Thyroxine: Stock Solution**

0.0005gm (0.5mg or 500µg) of Thyroxine was dissolved in 1ml of distilled water to make a solution of volume 1ml of concentration 0.5mg/ml or 500µg/ml (I stock solution) (I S.S).

10µl from I stock solution (I S.S) was dissolved in 990µl of distilled water to make a solution of volume 1ml of concentration 5µg/ml (II S.S).
15µl from II S.S was dissolved in 1485µl of distilled water to make a solution of volume 1.5ml of concentration 0.05µg/ml or 50ng/ml (III S.S).

Thyroxine (1ng/ml):

100µl of thyroxine from III S.S was dissolved in 4900µl of culture medium to prepare solution of volume 5ml of concentration 1ng/ml.

Prolactin: Stock Solution

0.0005gm (0.5mg or 500µg) of Prolactin was dissolved in 1ml of distilled water to make a solution of volume 1ml of concentration 0.5mg/ml or 500µg/ml (I stock solution).

10µl from I stock solution (I S.S) was dissolved in 990µl of distilled water to make a solution of volume 1ml of concentration 5µg/ml (II S.S).

15µl from II S.S was dissolved in 1485µl of distilled water to make a solution of volume 1.5ml of concentration 0.05µg/ml or 50ng/ml (III S.S).

Prolactin (1ng/ml):

100µl of prolactin from III S.S was dissolved in 4900µl of culture medium to prepare solution of volume 5ml of concentration 1ng/ml.

IGF-1: Stock Solution

Pre lyophilized IGF-1 was first reconstituted in 1ml of sterile distilled water to make stock solution of concentration 50µg/ml (I S.S).

100µl from I S.S was dissolved in 900µl of distilled water to make a solution of concentration 5µg/ml (II S.S).
10μl from II S.S was dissolved in 990μl of distilled water to make a solution of volume 1ml of concentration 0.05μg/ml or 50ng/ml (III S.S).

IGF 1 (0.01ng/ml):

1μl from III S.S was dissolved in 4999μl of culture medium in petri plate to make a solution of volume 5ml of concentration 0.01ng/ml.

IGF 1 (0.1ng/ml):

10μl from III S.S was dissolved in 4990μl of culture medium in petri plate to make a solution of volume 5ml of concentration 0.1ng/ml.

IGF 1 (1ng/ml):

100μl from III S.S was dissolved in 4900μl of culture medium in petri plate to make a solution of volume 5ml of concentration 1.0ng/ml.

IGF (5ng/ml):

500μl from III S.S was dissolved in 4500μl of culture medium in petri plate to make a solution of volume 5ml of concentration 5.0ng/ml.

IGF (10ng/ml):

1000μl from III S.S was dissolved in 4000μl of culture medium in petri plate to make a solution of volume 5ml of concentration 10.0ng/ml.

Insulin (1ng/ml) + IGF-1 (1ng/ml):

100μl of Insulin from III S.S and 100μl of IGF-1 from III S.S were dissolved in 4800μl of culture medium to prepare solution of volume 5ml of concentration 1ng/ml.
IGF-1 (1ng/ml) + Galactose (1ng/ml):

100μl of IGF-1 from III S.S and 100μl of Galactose from III S.S were dissolved in 4800μl of culture medium to prepare solution of volume 5ml of concentration 1ng/ml.

Insulin (1ng/ml) + IGF-1 (1ng/ml) + Galactose (1ng/ml):

100μl each of insulin, IGF-1 and Galactose from their respective III S.S were dissolved in 4700μl of culture medium to prepare solution of volume 5ml of concentration 1ng/ml.

Tissue Preparation for Microscopy

After 24 hr of fixation in Bouin's fluid ovaries were processed for paraffin embedding. Serial sections (3μm thick) were cut on a semi-automated microtome (Leica RM2255) and stained with haematoxylin and eosin. Oogonia (6-10 μm), primary oocytes (10-100 μm), cortical alveolar oocytes (110-320 μm), vitellogenic oocytes (320-700 μm) and atretic follicles were quantified from serial histological sections.

Quantification of Oogonia, Primary oocytes, Vitellogenic oocytes, Cortical alveolar oocytes and Atretic oocytes

Oogonia (6-10 μm) were counted from every alternate histological sections while, primary oocytes (10-100 μm) were quantified from every third section; cortical alveolar oocytes (110-320 μm) from every 35th section and vitellogenic oocytes (320-690) from every 100th section. The value of total
number of different types of oocytes thus obtained was divided by 3 (thickness of the section) to get the actual number of that category of oocytes. Mean and standard errors were calculated. Atretic follicles were identified based on the morphological features and counted separately.

**Histochemistry**

Parallel paraffin sections were simultaneously processed for histochemical detection of proteins (mercury bromophenol blue), glycoproteins (periodic acid Schiff's - PAS), mucopolysaccharides (alcian blue), lipids (Sudan black B), neutral lipids (oil red O) and acidic lipids (Nile blue) (Pearse, 1960). Stained sections were observed and photographed with a digital camera (Progress Capture, Pro 2.5, Jenoptik, Germany).

**Statistical analysis & Graphical presentation**

The results were analyzed using One-Way Analysis of Variance (ANOVA) followed by Tukey's test or non-parametric Kruskal-Wallis/ Mann-Whitney test wherever appropriate (after checking the data for normal distribution) using SPSS version 7.5. The results were judged at 5% level of significance. For graphical presentation of the data Sigma Plot version 2 (Jandel Scientific Corporation USA/Germany) was used.