DISCUSSION
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Morphology, histology and histochemistry of adult ovary

Zebrafish (*Danio rerio*) is a small tropical freshwater riverine fish originating from northern India. The zebrafish has become one of the most important and attractive model organism to study biological processes within a living body. As a vertebrate, this fish has many of the strengths of invertebrate model systems; it offers numerous advantages to researchers interested in many aspects of embryonic development, physiology and disease (Nusslein-Volhard and Dahm, 2002). An adult female lays large sized (~750 μm) eggs year round (Westerfield, 1995). A fertilized egg completes development within 72 hours and hatches into a larva that attains sexual maturity by 3 months (Maack and Segner, 2003). Under the laboratory conditions a female oviposits 200-300 eggs on an average once in 2-3 weeks when fed regularly (Rajapurohit and Pancharatna, 2007; David and Pancharatna, 2009ab). The longevity of this fish varies from 2-3 years.

Ovarian morphology, classification of stages of oocyte development, ultrastructure of the ovary are studied in this fish previously (Selman *et al.* 1993; Orn *et al.*, 2003; Cakici and Ucuncu, 2007; Yon *et al.* 2008). Reports on hormonal control of reproduction, development of gonads and primordial germ
cells are also available (Maack, 1964; Van Ree, 1977; Nagahama, 1994; Garg, 1998; Weber et al. 2002; Orn et al. 2003; Raz, 2003; Fenske and Segner, 2004; Nagahama and Yamashita, 2008). In the present study we observed that adult ovary had possessed oogonial nests, dividing oogonia and newly formed oocytes indicating oogonial proliferation and oogenesis are persistent and prevalent in the adult ovaries (Figs 1.1A-C). In an earlier study Selman et al (1993) have reported the presence of oogonia but without the evidence for dividing oogonia suggesting that oogonial proliferation is not apparent in adult ovary. Further, we observed in the present study, germ cells were clearly distinguishable from the somatic/follicular cells by their larger size, spherical shape and greater nucleo-cytoplasmic ratio. Binucleate oocytes were also observed although, not very frequently.

Our observations of the present investigation confirm that oocyte/follicular development in the adult ovary of this fish is continuous and asynchronous and all the classes of growing oocytes (stage I - stage V) reported by Selman et al (1993) were observed. Primary oocytes exhibited ooplasm that stained positive to proteins, mucopolysaccharides and negative to lipids (Fig. 1.3). With the onset of vitellogenesis the yolk granules accumulated in the ooplasm; vitellogenesis proceeded in two directions (i) lipid yolk particularly neutral lipids originated from nucleus and their distribution was extended to peripheral ooplasm (Figs. 1.3 & 1.4). (ii) Protein yolk, carbohydrate yolk and acidic lipids originated from the
peripheral ooplasm and were distributed interior (Figs. 1.3 & 1.5). The size of these lipid droplets increased from the site of their incorporation to the site of storage in the oocyte (Fig. 1.4). At the end of the vitellogenesis the size of the oocytes increased exponentially i.e. nearly 15-17 folds.

Follicle cells which were very few at the initial stage of their investment at the beginning of the folliculogenesis increased in their number and surrounded the growing follicle as a single layer in large previtellogenic follicles. With the growth of the follicle, zona radiata a non-cellular layer became apparent in between the follicle envelope and vitelline membrane. In vitellogenic follicles an outer thecal layer was also added. In a fully grown preovulatory follicle four distinct layers vitelline membrane, zona radiata, follicle cell layer and thecal layer were observed (Fig. 1.2D).

In summary this part of the study clearly suggests that (i) in adult zebrafish, oogonia (ovarian a stem cells) are persistent (ii) oogonial mitosis (proliferation) and their transformation (differentiation) into oocytes (oogenesis) are prevalent. Thus this fish forms suitable animal to study the regulatory mechanisms underlying proliferation and differentiation of germ line stem cells, as the adult ovary of the fish has persistent germ line stem cells/oogonia unlike adult mammalian ovary (iii) yolk originates from two sources from nucleus and from the exterior (iv) yolk granules are composed of protein yolk, lipid (neutral and acidic lipids) and carbohydrate yolk (v) Folliculogenesis begins with the investment of
single or few follicle cells and in the fully grown preovulatory follicles, follicular envelope comprises two distinct cell layers theca and follicle layer external to vitelline membrane and zona radiata.

**Nutritional regulation of oogonial proliferation and oogenesis in adult ovary (in vivo)**

Ovaries of lower non-mammalian vertebrates especially fishes and amphibians are characterized by persistent oogonial stem cells which undergo proliferation and differentiation periodically (Tokarz, 1978; Norris and Jones, 1987). The regulatory mechanisms operative in controlling oogonial mitosis are seldom reported for these vertebrates. However, in the invertebrate animal models studied nutritional condition is known to play an important role and the germline stem cell divisions are delayed when nutritional sources become limiting (Narbonne and Roy, 2006; Michaelson et al., 2010). For example, when cholesterol levels are insufficient, the brood size of *C. elegans* is markedly reduced due to a defect in germline proliferation and differentiation (Shim et al., 2002). Likewise, in female Drosophila GSCs adjust their proliferation rates in response to nutrition (Drummond-Barbosa and Spradling, 2001). Mammalian germ cells are also known to depend for survival and proliferation on specific growth factors and other undetermined compounds (Farini et al., 2005).

In the present study we observed that frequency of feeding has a significant influence on body mass. Overfeeding (daily four times) lead to
increase in body mass alone and the effects were not manifested in body size and also did not alter the rate of oogonial mitosis. While, in regularly daily once or twice fed fishes oogonia increased numerically ($P < 0.05$) compared to start controls. Conversely, in underfed (every alternate day and every fourth day) fishes body weights and oogonial number remained significantly lower. These results lead us to conclude that nutrition regulates germ line stem cell mitosis optimally, and over feeding do not increase proliferation of oogonia but underfeeding curtails it (Tables. 2A, 2B; Fig. 2.2). Interestingly in over fed fishes the number of primary oocytes was greater and cortical alveolar oocytes were maximal compared to all other groups giving a clue that excess of feeding may stimulate oocyte recruitment and enhance growth of primary oocytes to their next stage. This is also observed in our routine practice that under laboratory conditions when the female fishes were fed 3-4 times/day *ad libitum* they attain gravid state and lay eggs within two weeks after their previous spawning episode. Whether this nutritive regulatory mechanism is mediated through any signalling molecule had required further *in vitro* experimentation.

**Hormonal and nutritional regulation of oogonial proliferation and oogenesis in adult ovary (in vitro)**

In *in vitro* experiments designed to understand the nutritional regulatory mechanism, it was observed that there was no significant change in the rate of oogonial mitosis or oocyte production in insulin or galactose treated ovaries
(either individually or in combination) indicating that insulin action may require further mediation by a growth factor (IGF?) which was not included in the present set of series of experiments as there was a delay in the supply of the chemical and the inclusion of this group forms an immediate priority in our next series of in vitro experiments for the elucidation of IGF1 mediation.

When ovaries were cultured in presence of different hormones, maximal number oogonia were recorded in somatotropin/GH treated group indicating the mitogenic effects of growth hormone on oogonia. Conversely, somatostatin or growth hormone releasing inhibiting hormone treated ovaries exhibited minimal (P < 0.05) number oogonia confirming the positive action of GH on oogonial mitosis (Table. 3B; Figs. 3.2 & 3.3). Somatostatin also caused a significant decrease in the number of primary oocytes indicating its inhibitory action on oogenesis (Table. 3B).

Among other hormone treated groups, FSH and insulin did not cause increase in oogonial number but oocyte number was slightly higher compared to controls (Table. 3B). In ACTH, Thyroxine and Prolactin treated ovarian cultures neither oogonia nor oocytes were significantly differed from controls suggesting that although these hormones are required to optimize metabolism they may not be directly involved in germ cell proliferation and differentiation in vivo.
In vitro Germinal vesicle break down: Role of hormones, Growth factors and endocrine disrupters

In the present study Diethylstilbestrol (DES) a synthetic estrogen elicited maximal (>70%) GVBD response in all the four experiments confirming the results reported by Tokumoto et al (2005) on the same species. Therefore, we treated inclusion of this (DES) group in all our GVBD experiments as positive control to compare the potency of other hormones/IGF1/xenoestrogens tested for the induction of in vitro GVBD.

Of all the hormones used Insulin induced GVBD in 57% of oocytes next to DES (77%) (Table. 4C). In other hormone treated groups (Thyroxine, Somatotropin, FSH, ACTH, Prolactin) and Somatostatin groups the GVBD elicited was less than 13% (Table. 4C; Fig. 4.5) suggesting these hormones may be essential to maintain general metabolism and may not be involved precisely in the induction of GVBD in vivo.

Production of putative intraovarian regulators and various growth factors by the ovarian cells indirectly suggests their local actions and possible paracrine/autocrine role(s) in oogenesis, folliculogenesis, development, growth, selection of follicles and oocyte maturation (Sirotkin et al., 2000). Although, various hormones (steroids, gonadotropins and growth hormone), activin, synthetic estrogens and xenoestrogens are known to elicit GVBD in zebrafish,
evidences are also accumulating on the indispensable role of different growth factors e.g. insulin like growth factors (IGF1, IGF2, IGF3), epidermal growth factor (EGF) in the in vitro induction or inhibition of oocyte maturation in different species of fishes. IGF1 and IGF2 are known to enhance oocyte maturational competence in teleost fishes such as seabream Pagrus major, white bass Morone chrysops and spotted seatrout Cynoscion nebulosus through up regulation of gap junctions and expression of membrane receptors of maturation inducing steroids (Kagawa et al., 1994; Thomas and Das, 1997; Patino and Kagawa, 1999; Weber and Sullivan, 2005). In vitro effects of insulin like growth factors and insulin in oocyte maturation and maturation inducing steroid production by ovarian follicles are also reported earlier for common carp (Dasgupta et al., 2001; Mukherjee et al., 2005). Dynamics of insulin and IGF1 ovarian receptors during oocyte maturation are studied in detail in brown trout, Salmo trutta (Maestro et al., 1999).

In the present study, when the fully grown preovulatory oocytes of zebrafish were incubated with energy substrate galactose, hormones (DES and insulin) and growth factor (IGF1), highest percentage of GVBD was found elicited in oocytes exposed to DES (73 %), followed by those oocytes exposed to the combination of Insulin+IGF1+galactose (70 ± 11 %) (Fig. 4.3). IGF1 elicited GVBD in the first hour itself. The lowest percentage of GVBD was observed in oocytes exposed galactose alone (Fig. 4.3). No significant difference was
observed in the incidence of GVBD in the oocytes treated with insulin and energy source galactose, when tested either individually or in combination. The results of another experiment reveal that IGF1 induced GVBD in zebrafish oocytes, both in dose and time dependent manner. Highest percentage of GVBD (83 ± 3%) was observed at the higher concentration (10 ng/ml) used, followed by positive control DES (73 ± 7%) and remaining concentrations of IGF1 in dose dependent manner (Fig. 4.4). In 5ng and 10ng/ml treated oocytes the rate of incidence of GVBD induced was significantly (P < 0.05) higher compared to corresponding controls (Fig. 4.4).

The results of these experiments clearly suggest that presence of energy source (galactose) or its uptake molecule (insulin) may not be adequate and IGF1 seems to be essential for potentiating the effects of both, which is evidenced by the highest percentage of occurrence of GVBD in galactose+insulin+IGF1 exposed oocytes compared to that elicited by galactose or insulin individually or in combination. Further, IGF1 elicited GVBD in a dose-dependent manner even in absence of either galactose or insulin or both suggests IGF1 is a potential inducer of GVBD in zebrafish. This indirectly suggests that in zebrafish, IGF system is operative during the process of induction of GVBD. The expression of IGF3 gene in the ovary and its role in oocyte maturation is already elucidated in this fish (Li et al., 2010). Similarly signalling by epidermal growth factor (EGF) involved in the inhibition of oocyte
maturation is also studied in this fish (Kraak and Lister, 2011). The localization and expression pattern of IGF1 gene, its receptor and binding protein in the ovary at the time of initiation of GVBD and their dynamics need to be elucidated for a clear understanding of regulation of oocyte maturation in this fish.

Recent research based on the experiments employing animal models indicate that exposure to xenoestrogens is linked with perturbation in reproductive processes. The present study reveals that organophosphorous and pyrethroid insecticides (with estrogenic activity) tested induced GVBD in vitro in the fully grown oocytes of zebrafish. Other estrogenic compounds such as, DES and ethynyl estradiol are reported to induce GVBD in this fish (Tokumoto et al., 2005). Organophosphorous compounds, malathion, mevinphos, chlorovinphos, tetravinphos and organochlorine endosulphan are also known to induce GVBD in common carp (Haider and Upadhyaya, 1986; Inbaraj and Haider, 1988). Ghosh et al., (1999) demonstrated that final oocyte maturation was induced in fresh water perch Anabas testudineus by metacid-50. On the contrary, several endocrine disrupting chemicals (EDCs), such as Kepon and o, p-DDD, have been reported to antagonize induction of meiotic maturation of fish oocytes in vitro (Thomas, 1999). EDCs such as methoxychlor and ethynyl estradiol also antagonize frog oocyte maturation (Pickford and Morris, 1999).

Although the adverse effects of pesticides on fish reproduction have been documented previously, studies involving analysis at cellular and molecular level
are virtually lacking. The present study uses *in vitro* system and shows that the xenoestrogens (Fenvalerate and Dimethoate) tested have induced GVBD preternaturally and thus interfere with normal reproduction of fish fauna. In conclusion, the study emphasizes and cautions on the impact of excessive use of agro-chemicals on the reproductive health of aquatic fauna.