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Early gonad development in zebrafish (*Danio rerio*)

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Gonadogenesis in zebrafish goes through an initial ovarian phase then subsequently into either ovarian or testicular phases. How germ cells choose to commit to an oogenic fate and enter meiosis or alternatively not enter meiosis and commit to a spermatogenetic fate remains a key question. This study investigated events of early gonadogenesis in zebrafish with the aim of unraveling the events surrounding the mitotic/meiotic transition in juvenile ovaries. Primordial germ cells were identified at eight days post fertilization (dpf). Mitotic divisions were apparent at 15 dpf, and meiosis initiated in some gonads after 22 dpf. After 40 dpf, female gonads contained various germ cells including oogonia, post-pachytene and early pre-vitellogenic oocytes, whereas in some gonads, degenerative post-pachytene oocytes and proliferating germ were observed. The occurrence degenerating oocytes as well as a relatively larger proportion of proliferating "gonial" germ cells in the latter gonads was considered the first indication of spermatogenic activity, marking the onset of secondary (testicular) gonadogenesis. It could not be determined here whether primordial germ cells were involved in secondary gonadogenesis. While the mechanisms of this phenomenon in zebrafish ovaries are not well addressed, here it can be seen in the context of an apoptotic regulation.

Key words: *Danio rerio*, gonad, mitotic/meiotic transition, development, sex inversion.

INTRODUCTION

Zebrafish (*Danio rerio*) is a member of the family *Cyprinid*, and can grow up to about 3 to 5 cm total length in adulthood, although females are a little larger. This fish has become a popular tool in studying development and can be easily stimulated to produce throughout the year (Hisaoka and Firlit, 1962). A single female can spawn up to 400 eggs and fertilization success is generally over 70-80% (Eaton and Farley, 1974). It has a very short generation time and reaches sexual maturity within three months.

Many aspects of zebrafish development have been described in the literature, including early embryonic patterning (Kimmel, 1993), early development of the nervous system (Kimmel et al., 1994), and aspects of cell fate and lineage determination (Kimmel and Law, 1985; Kimmel and Warga, 1987). Embryonic development and biology of zebrafish have also been described(Streisinger et al., 1981; Warga and Kimmel, 1990; Westerfield,

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1993). Other aspects of zebrafish oocyte growth, egg formation including gene expression patterns, have been reported fairly extensively in the literature (Hisaoka and Firlit, 1962; Takahashi, 1977; Selman et al., 1993; Howley and Ho, 2000; Pelegri, 2003; Lessman, 2009). Although methods used to classify stages of oocyte growth by these early authors differ from those of Selman et al. (1993), all of the above studies have proved useful in understanding morphological events of oocyte growth in zebrafish.

Previous studies undertaken on early differentiation of zebrafish germ cells are those reported by Takahashi (1977), which described events of juvenile inter-sexuality. In this study, stages of oocyte growth in juvenile gonads are not described in detail. Hisaoka and Firlit (1962) described stages of oocyte development in immature and mature zebrafish gonads based on morphological observation with respect to the staining pattern of nuclear structures with nucleic acid stains. In this study, transitional stages of gonad development in juvenile fish are not described in detail. Selman et al. (1993) used a modified class scheme to describe stages of oocyte growth in an adult ovary based on physiological, biochemical and morphological observations. Consequently, the transformation of oogonia into primary (meiotic) oocytes is not described. Staging series of zebrafish oocytes presented by Hisaoka and Firlit (1962) precedes that of Selman et al. (1993) by 31 years. Although, all of the above studies have proved useful in understanding morphological events of oocyte development in zebrafish, overall, pictorial or graphic representations of these previous reports on the reproductive characteristics of early zebrafish gonads are scattered. Oogenesis is typically studied in adult ovary. Consequently, these studies do not help a reader to follow in detail the transitional events occurring during early ontogeny.

Since a comprehensive study on oocyte development in the adult zebrafish ovary had been described by Selman et al. (1993) and partially by Hisaoka and Firlit (1962), it was the aim of this study to follow germ cell differentiation in carefully staged phases of gonad ontogeny, prior to those covered by these previous workers. While the present study takes into account the events surrounding the mitotic/meiotic transformation during early gonadogenesis, it was important to follow the process of oogenesis through the mitotic/meiotic transition and beyond.

MATERIALS AND METHODS

Sample collection and processing

Six fish at each developmental stage were selected from hatchery tanks at hatch [4 days post fertilization (dpf)], every three days from 8 to 31 dpf and weekly thereafter up to 55 dpf. Larvae and juvenile fish were killed by anaesthetizing with MS222 (4.2 ml tricaine stock solution in 100 ml tank water) as described by Westerfield (1993). They were individually measured for total length, and the trunk region of bigger fish being cut out before fixation and smaller fish processed whole. Tissue samples were fixed in Bouin's solution overnight (Humason, 1979), dehydrated through alcohol series, cleared in methyl benzoate (Sigma–Aldrich, St. Louis, MO, USA) and embedded in Paraplast® (Merck, Darmstadt, Germany). Gonadal regions were also fixed in 2% paraformaldehyde and 2.5% glutaraldehyde (Karnovsky, 1969) in 0.1 M phosphate buffer, (0.1 M NaH_2PO4, 25 ml; 0.1 M Na_2HPO4, 81 ml; dH_2O, 250 ml; adjusted to pH 7.4 and topped up to 500 ml with dH_2O) for 6 h at room temperature and then washed in phosphate buffer overnight at room temperature, and post-fixed for 1 h in 1% osmium tetroxide in phosphate buffer and embedded in araldite. Tissue section (5–7 µm thick) of the trunk region of each fish were cut and mounted on slides coated with 3-amino-propyl-triethoxy saline (Sigma–Aldrich, St. Louis, MO, USA), and stained with Mayer's modified haematoxylin and eosin.

Semi-thick sections (1-2 µm) of araldite embedded tissues were cut with a glass knife on a Reichert Ultracut S and stained on a 90°C hot plate with toluidine blue and thereafter examined and photographed under a Leica DM750 microscope attached to a DFX 310 FX digital camera. Images were possessed using LAS imaging software Version 3.5. To measure oocyte sizes, slides were examined under low power. Gonads of juvenile fish were sexed on the basis of tissue configuration and germ cell composition into different developmental stages. Gonads of inverting individuals were categorized according to the extent of degeneration of female germinal cells.

Experimental protocols for the study were approved by the Animal Ethics Screening Committee (No. 2000/98/1), University of the Witwatersrand.

RESULTS

Gonads of larval zebrafish

At hatch, no distinct primordial germ cells (PGCs) were found in sections due to distortion of the larvae due to tissue processing. At 8 days post fertilization (dpf), the yolk sac was present but smaller in size. In some individuals' yolk, globules were assimilated and disappeared with the transition of larvae to active feeding. Primordial germ cells (PGCs) were attached to the dorsal walls of the coelomic cavity on both sides of the mesentery (Figure 1a) often in isolation or in pairs. A group of PGCs were observed at the intestinal level. The PGCs were large (4-5 µm) in diameter, ovoid or rounded shaped.

Their large irregular shaped nuclei contained loose network of thin chromatin threads and did not contain a nucleolus. There were no signs of mitotic activity at this stage of development.

At 13 days post fertilization (dpf), the number of germ cells had increased from two to six per cross section; signs of mitotic activity of the PGCs were evident in some gonads (Figure 1b). Germ cells were attached to the wall of the ceolomic wall by a narrow band of peritoneal cells, in the region of the posterior half of the swim bladder. The nucleoli of the germ cells observed at this stage of development were solitary, intensively stained with haematoxilyn and eosin (H&E) and occupied a central

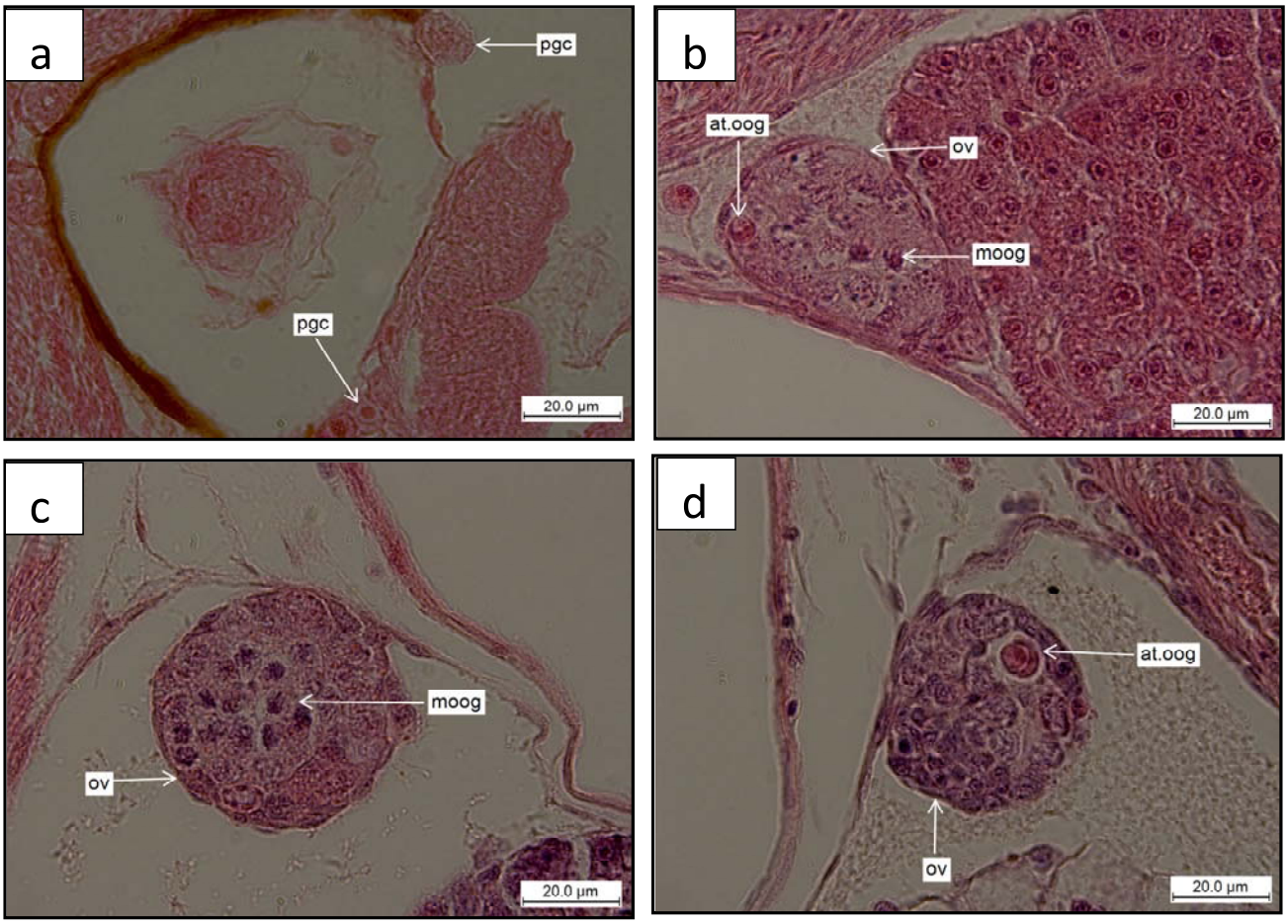

Figure 1. Selected histological sections of larval zebrafish gonads stained with H & E. **a**, Primordial| germ cells (pgc) attached to the body wall on both sides of the mesentery (Scale bar = 20.0 µm). **b,** Gonads of fish at 13 dpf. **c** and **d**, Mitotic activity evident in sections of juvenile fish at 16 dpf. pgc, primordial germ cell; oog, oogonia; moog, mitotic oogonia; at.oog, atretic oogonia. n.oog, nest of oogonia.

position. The scant cytoplasm of the germ cells stained weakly with H&E. Germ cells observed at this stage of development had morphological characteristics of either oogonia or spermatogonia and measured 4-10 µm in diameter. Although germ cells had morphological characteristics of oogonia or spermatogonia, implying onset of morphological sex differentiation, sex differences could not be distinguished with certainty. Therefore, gonads were interpreted as of the indifferent type.

By 16 dpf, gonads were larger in size but still showed a simple anatomical organization. Germ cell mitotic activity had intensified (Figure 1c). Although the total number of germ cells was unknown, germ cells had increased in number compared with the previous stage of development. Gonads of fish at 16 dpf were considered to be at the initial stage of germ cell proliferation.

Gonads of Juvenile zebrafish

Mitotic germ cells had increased in number in gonads of fish by 19 dpf. Gonads of some fish contained large numbers of proliferating germ cells (Figure 2a and b). At three weeks, a prominent cavity had formed (Figure 2b) in some gonads. At this developmental period, an increase in number of proliferating germ cells was observed. Between days 22 and 35 dpf, gonads were composed of “transforming oogonia” (oogonia that were no longer dividing), early meiotic prophase and post-pachytene oocytes (Figure 2c).

"Transforming" oogonia were morphologically similar to those germ cells observed at 16 dpf, but were larger in size (7-10 µm) and showed an increased cytoplasmic-nuclear ratio. It was not possible to detect any changes in the nature of chromatin in "transforming" oogonia in Paraplast embedded sections but in 1 µm sections (Figure 2c) fine sparse chromatin granules were seen scattered throughout the nucleoplasm. The centrally located nucleolus was still present in these germ cells. The nuclei of these germ cells stained only lightly with H&E and toluidine blue

Early meiotic oocytes were round and had become

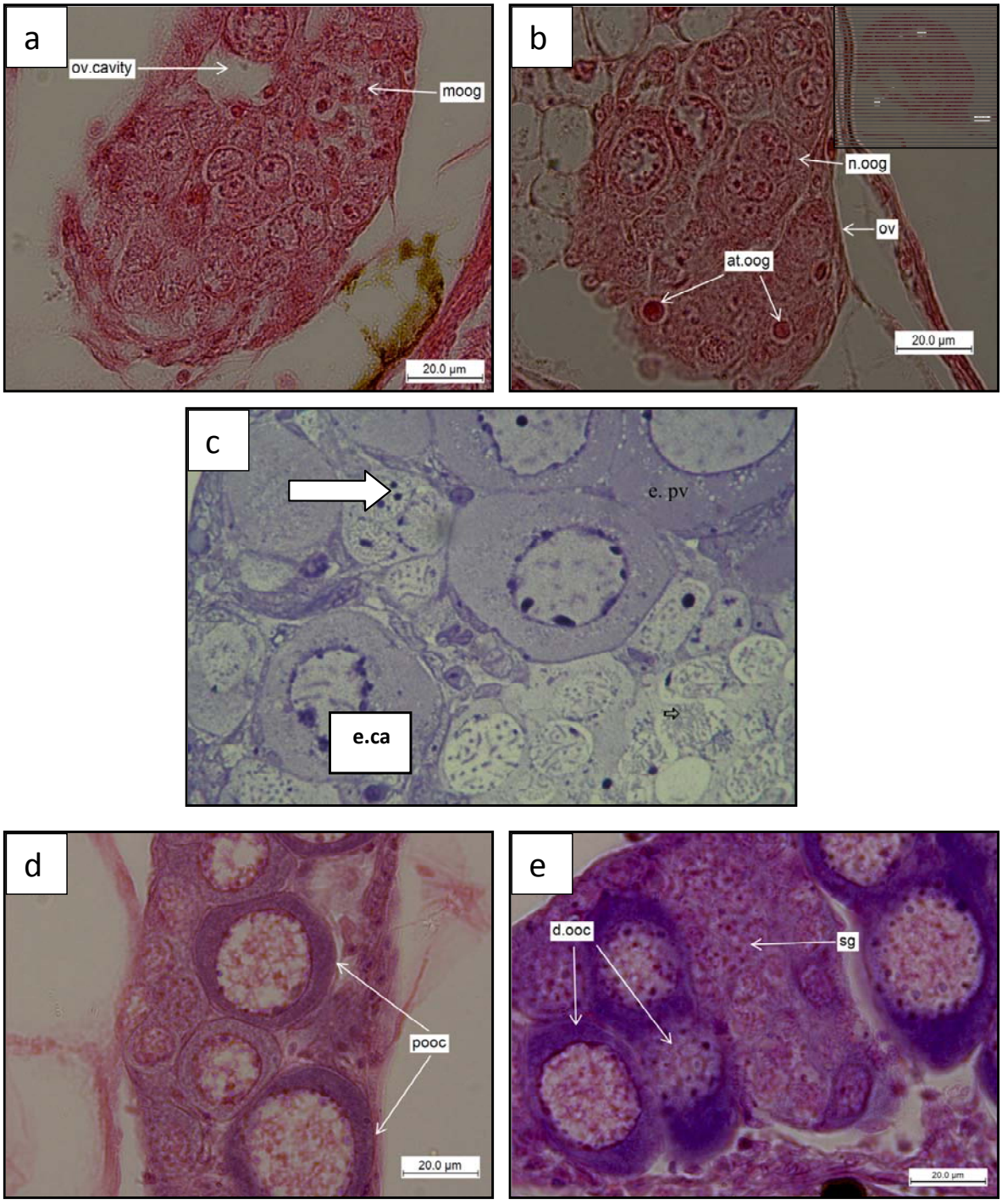

Figure 2. Selected histological sections of Juvenile zebrafish gonads stained with H&E. **a** and **b**, shows mitotic oogonia, atretic oogonia, and an ovarian cavity at days 19 and 22 post fertilization. **c**, shows transforming oogonia (long thick arrow) and early meiotic oocytes (short thick arrows). d and e, shows gonads in early stages of sex inversion at 45-49 dpf. m.oog, mitotic oogonia; at.oog, atretic oogonia; d.ooc, degenerating post pachytene oocytes; sg, spermatogonial proliferation; e.ca, early cortical alveoli stage oocytes; ov, ovary; ov. cavity, ovarian cavity; ca, cortical alveoli; pooc, post pachytene oocytes.

increasingly larger (10-19 µm) and their nuclei, which were also relatively large and filling the majority of the cell, stained only lightly with H&E (Figure 2c). These oocytes were distinguished from "transforming" oogonia because of the changes in chromosome structure. The most distinctive difference among the various stages of

early meiotic stages was the pattern of chromatin condensation. In the leptotene stages of the meiotic prophase, chromosomes had thickened and could be recognized in sections.

The zygotene oocytes were approximately the same size as the leptotene oocytes, but characterized by a more clearly defined and localized chromatin. A distinguishing feature of the zygotene oocytes being the increase in size and density of chromatin strands. Oocytes of the zygotene stage had prominent nucleoli on the opposite side of the conspicuous chromatin within the nuclei.

Post-pachytene oocytes were surrounded by a monolayer of somatic (granulosa) cells, indicating the onset of ovarian follicle organization (Figure 2d). These oocytes had increased cytoplasmic volume, were larger than early meiotic oocytes (30 - 117 μm) with their nuclei being central and contained two to four relatively large basophilic nucleoli. Cytoplasmic RNA in post-pachytene oocytes was basophilic when stained with haematoxylin, and deep blue when stained with toluidine blue (Figure 2c), while a less basophilic cytoplasm and an increased number of nucleoli in the nucleus characterized late post-pachytene oocytes. Accumulation of cytoplasmic RNA was coincident with the formation of multiple nucleoli in the oocyte nucleus.

Gonads with altered morphology (inverting gonads)

At 45 to 49 dpf, two types of gonads were identified. Typical gonads were common and exhibited asynchronous oocyte development similar to those observed in the previous stage of development. The other type of gonads contained only post-pachytene oocytes, (Figure 2d and e) and mitotically dividing "gonial" germ cells (Figure 2f). Oocytes with unusual shapes showed morphological features similar to degenerating cells and lacked the clear structure of a normal growing oocyte. It appeared that many of these oocytes begun to degenerate accompanied by various degrees of abnormalities.

The most prominent feature observed in degenerating oocytes was a marked distortion in the overall appearance of the oocytes accompanied by the disintegration of cytoplasm. An interesting feature observed in some gonads at 45 days of development was the occurrence of empty follicles (spaces) in histological sections. The area around the empty follicles or degenerating oocytes was characterized by the presence of densely packed stromal (epithelial) cells believed to be either blood cells or phagocytes. Post pachytene oocytes in inverting gonads had unusual shapes and their cytoplasm stained intensively with Haematoxylin.

In contrast to the appearance of degenerating oocytes in these gonads, numerous small "islets" of quiescent and clusters of proliferating “gonial" germ cells were seen in parts of the gonads in some fish at 49 dpf. Quiescent and proliferating germ cells were seen along the slit-like openings (ovarian lamellae) within gonad sections (Figure 2e). The formation of these slits was a characteristic feature of these gonads at this developmental stage. The number of mitotically dividing germ cells observed at this stage of development outnumbered those observed during the initial ovarian differentiation stages of development.

At 49 dpf, typical ovaries exhibited asynchronous oocyte development wherein ovaries possessed oocytes in the post-pachytene and pre-vitelogenic stages of development. Inverting gonads remained smaller in size possessing "gonial" germ cells and fewer growing oocytes.

Gonads of immature zebrafish

In the subsequent stages of development (55 to 60 dpf), normal ovaries showed typical patterns of sexual development for asynchronously spawning fish (Tyler and Sumpter, 1996), where gonads contained oocytes at various stages of development. From 55 dpf onwards, male and female gonads could be distinguished clearly as maturing testes or ovaries. In Immature testis the number of germ cells, now termed spermatogonia, spermatocytes and spermatids had increased in number. In some individuals, large cysts of spermatocytes at the various stages of the meiotic prophase could be recognized in sections as well as spermatids (Figure 3c and d). In addition, testicular tissues were arranged almost completely in lobules.

Ovaries contained numerous large post-pachytene and a few early pre-vitellogenic oocytes (Figure 3a and b). An increase in the number of cortical alveoli was noted in pre-vitellogenic oocytes. The nuclei of the later oocytes contained numerous small nucleoli. Cortical alveoli were in a thin ring between the germinal vesicle or seen all over the cytoplasm of the pre-vitellogenic oocytes. There were no signs of testicular tissues in all gonads.

Maturing ovaries were formed of rows of post-pachytene, early as well as late pre-vitellogenic and early and late vitellogenic oocytes. Late pre-vitellogenic oocytes were similar to early pre-vitellogenic oocytes but were larger measuring 98 - 264 μm in diameter with multiple cortical alveoli situated in a thick ring between the germinal vesicle and the vitelline membrane or filling the whole cytoplasm. The nuclei of the later germ cells were lobed and contained numerous nucleoli. Early vitellogenic oocytes were large (117-314 μm).

Late vitellogenic oocytes were very large (151 - 361 μm) with yolk granules distributed homogeneously in the cytoplasm. The nuclei of oocytes in the advanced stages of vitellogenic stages of development were slightly displaced from the center to the oocyte membrane. A thin, but distinguishable granulosa and thecal layers

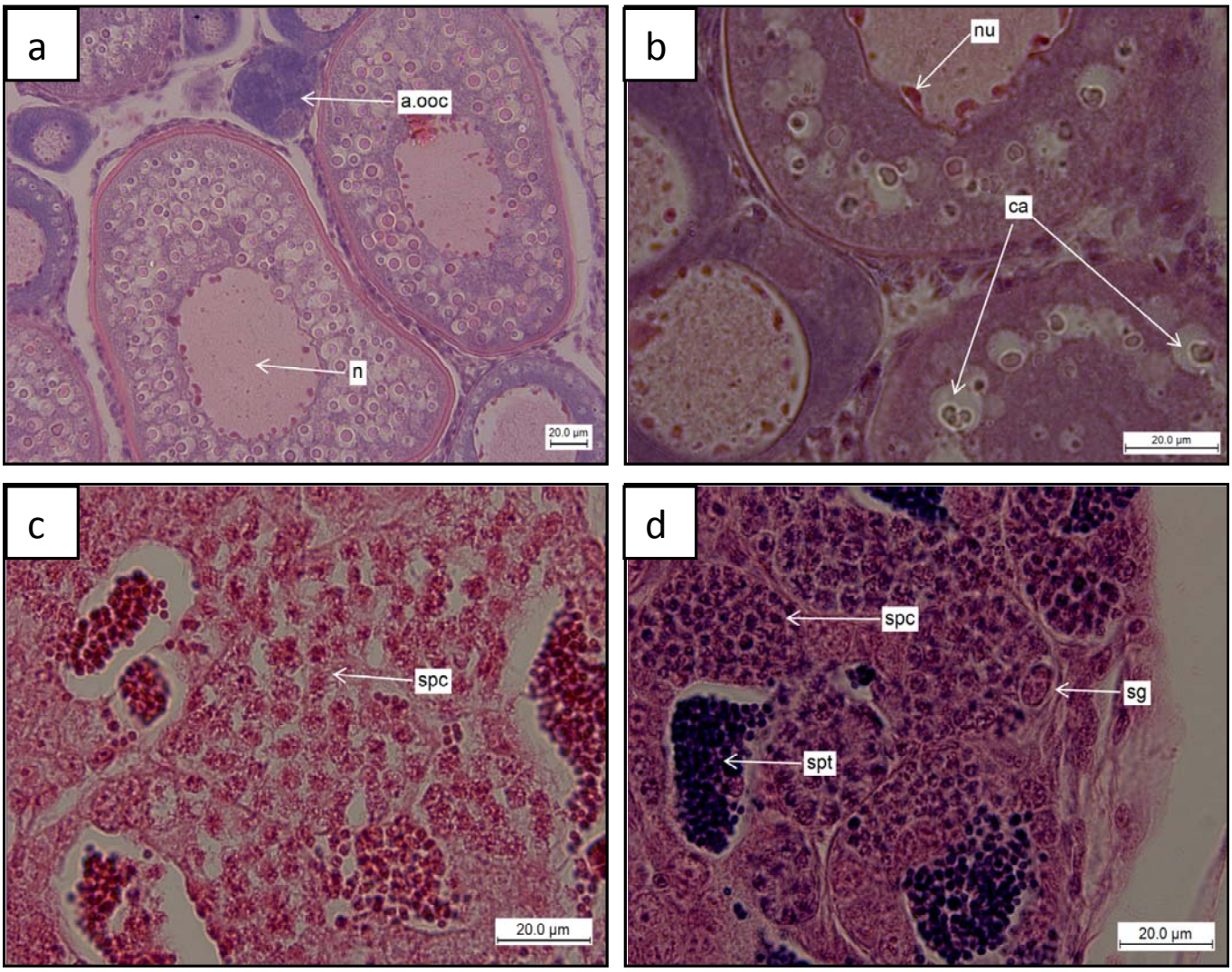

Figure 3. Selected histological sections of maturing zebrafish gonads stained with H & E. **a** and **b,** shows oocytes at the cortical alveoli stage of growth at 55 dpf. **c** and **d,** shows maturing testis in fish at 55 and 60 dpf. a.ooc, atretic oocyte; n, nucleus; nu, nucleoli; ca, cortical alveoli; spc, spermatocyte; sg, spermatogonia; spt, spermatids.

surrounded these oocytes. Vitellogenic oocytes were the most advanced stage of oocyte development observed therefore no mature stage oocytes has been described here.

DISCUSSION

This study, examined the sequence of events leading to the formation of oocytes including secondary gonado-genesis (testicular differentiation) in zebrafish. It has been confirmed here that gonad development follows the general pattern described by Takahashi, (1977), but there are other aspects that differ, specifically the timing of events.

It is believed that differences are related to rearing conditions, and differences in studied strains rather than differences in gonad development. Primordial germ cells (PGCs) were identified with certainty at 8 dpf. Although not labeled with lineage markers, the PGCs were identified by their common morphological characteristics: Irregular outlines, a high nucleus to cytoplasm ratio, a relatively large cell in relation to surrounding somatic cells, by their location and age of fish. The appearance of some germ cells at the intestinal level in the present study led to the assumption that the PGCs in zebrafish settle first at the gut (intestinal) level before spreading out to other parts of the gonadal anlage. Some investigators consider PGCs segregation at the sub-intestinal yolk part as their initial site of colonization of the genital ridge prior to their settlement to other parts of gonadal anlage (Mezhnin, 1978). PGCs observed at the intestinal level in the present study, were possibly still in the process of migration to the dorsal mesentery. In teleost fish studied

to date, primordial germ cells migrate to the genital ridges before hatching (Satoh and Egami, 1972; Hamaguchi, 1982; Parmentier and Timmermans, 1985). In the present study, and at 13 days post fertilization, the initial development of gonadal anlage had commenced, suggesting that the PGCs in zebrafish migrated to the gonadal ridges prior to its formation. This would be in accordance with the findings of Parmentier and Timmermans (1985) and Timmermans and Taverne (1987) in other cyprinid fish. In contrast, in other vertebrates, PGCs migrate to the previously formed genital ridges (Nieuwkoop and Sutasurya, 1979).

In most teleosts fish, the number of PGCs at hatching is small. The average number in carp for example, is 23 per larvae (Parmentier and Timmermans, 1985). The number remains low for six weeks after which a rapid proliferation occurs, resulting in a mean number of 130 at seven weeks and 250 at nine weeks. Proliferating germ cells were seen for the first time in the present study at 16 dpf. Initially the germ cell numbers remained low for up to two weeks, after which a rapid increase in the number of germ cells was noticed. However, their exact number was not known and no information was found in the literature about their exact number at this stage in development.

The increase in the proliferative activity of germ cells at 19 dpf signified the transition from gonadal primordia to differentiating gonads. The beginning of this developmental period corresponds with the transition from larval to juvenile life stages in the zebrafish (Brown, 1997). Day 19, therefore, marked the end of the indifferent period of gonad development.

At three weeks of age, a cavity was recognized and identified as the future ovarian cavity in females. In some teleosts, the development of associated somatic cells, such as the ovarian cavity is helpful in the following ovarian differentiation besides the morphology of the cells. The proliferative activity of germ cells at 19 dpf appears to be the strongest indicator of ovarian differentiation in zebrafish followed by the presence of an ovarian cavity at 22 dpf. Based on this finding from this stage onwards, gonads were called ovaries and the period marked the end of the indifferent period of gonad development and the onset of morphological sex differentiation.

Ovarian differentiation (transformation of an oogonium into an oocyte) and the commencement of meiosis in zebrafish as in other teleost fish are characterized by changes of nuclear structure. One of the ultrastructural characteristics of oocytes at the early meiotic prophase is the formation of synaptonemal complexes, which appear in meiotic germ cells of animals and plants generally. These structures have been identified in oocytes at zygotene and pachytene stages ovarian development in the medaka (Hamaguchi, 1982), the amago salmon (Nakamura and Nagahama, 1993) and in the channel catfish (Patino et al., 1996). According to Selman and Wallace (1989), oogenesis commences when oogonia transform into leptotene oocytes at the initiation of meiosis. The process is preceded by DNA replication and begins with the pairing and condensation of homologous chromosomes in the first meiotic division (leptotene-zygotene). At pachytene, the homologous condensing chromosomes form synaptonemal complexes. Sooner or later, oocytes arrest in the lamp brush (diplotene) stage of growth. According to Takahashi (1977) oogenesis is said to begin when cysts of germ cells originating from the mitoses of pre-existing PGCs appear to be in initial phases of the meiotic prophase as evidenced by the formation of "auxocytes" (Stage IB oocytes). In the work by Takahashi (1977) no mention is made of the criteria used in identifying the various stages of germ cell development making comparisons difficult.

Examination of the zebrafish larval gonads during the mitotic/meiotic transition in the present study led to the identification of four cell types. "Transforming" oogonia being the smallest germ cells (7 - 10 µm), which give rise to primary oocytes, which eventually pass through the leptotene, zygotene, pachytene and the diplotene stages of the meiotic prophase, diplotene oocytes being the largest germ cells (30-117 µm). The most distinctive difference among the first three stages of the primary oocyte is the pattern of chromatin condensation. mitotic/meiotic transition is a gradual process and is distinguished by active gene transcription that results in the production of vast amounts of RNA species. Accumulation of cytoplasmic RNA is coincident with the formation of multiple nucleoli in the oocyte nucleus or germinal vesicle. In the current study, cytoplasmic RNA in post-pachytene oocytes was basophilic when stained with haematoxylin, and deep blue when stained with toluidine blue.

The presence of synaptonemal complexes, which represents the condensed, homologous chromosomes in synapses, identifies oocytes in the pachytene stage of the first meiotic prophase (Grier, 2000). This implies that during this developmental period, germ cells were in the meiotic phase of development and that germ cells identified in some gonads prior to this stage of development were definitely oogonia. Thus ovarian differentiation in some zebrafish had begun. Initially, clusters of early meiotic oocytes formed a smaller proportion of germ cell composition in gonads.

By 31 to 35 dpf, clusters occupied extended areas of the gonads still enveloped by follicle cell processes. In adult ovaries (Selman et al., 1993), the number of oogonia and early meiotic oocytes appear to be relatively low compared to those observed in juveniles in the present study; moreover, transformation of oogonia into meiotic oocytes is rare event in adult ovarian sections (Selman et al., 1993). The low number of early meiotic oocytes, in adult ovarian sections implies a fast development from oogonia via oocytes in chromatin nucleolus stage to oocytes in early post-pachytene phase

in adult females. In most teleosts, oogenesis (Bromage and Cumaranatunga, 1982) appear to occur cyclically with an accelerated development just after spawning making it difficult to observe oogonia and early meiotic oocytes in adult ovarian sections.

As oocytes of the early meiotic prophase continue to develop, they become completely enveloped by pre-follicle cells disrupting the organization of the cells nests. The mechanisms leading to the separation of germ cells from the "nests" could not be established from the observations of the present study. However, it has been suggested elsewhere that the thin cytoplasmic digitations that stromal cells develop around the clustered oocytes play a role in this process (Andreucceti, et al., 1990). The newly formed oocytes continue to differentiate independently and asynchronously, enveloped by a monolayer of squamous follicle cells and eventually transform themselves into growing post-pachytene (diplotene) oocytes.

This is the last stage at which the last events of meiotic prophase are completed, after which the oocyte enters the diplotene stage.

In the current study, post-pachytene oocytes were seen for the first time between days 31 and 35 post fertilization. In the study of Takahashi (1977) oocytes of the first phase of growth or auxocytes were observed by 14 days post hatch (dph). In this study, characteristic features of germ cells were not provided making comparisons difficult. The present view is that germ cells labeled as auxocytes by Takahashi (1977) at 14 dph may have been oogonia. Consequently, his previous report of occurrence of meiotic oocytes at 10 dph is doubtful. Oogonia may have been erroneously described as stage I ocytes, since there are strong similarities between oogonia and oocytes when they first enter diplotene especially before the onset of cytoplasmic RNA accumulation so that basophilic cytoplasm would confer unequivocal similarity.

According to the findings of the current study, gonads of nearly all fish examined between days 45 to 49 post fertilization contained degenerating or atretic oocytes. This may have been indicative of impending sex reversal. In various mammalian (Byskov, 1978) as well as fish species (Coward and Bromage, 1998), atresia (resorption) is a common phenomenon. Atresia can be found throughout the reproductive cycle in fish, but has only been clearly shown to affect vitellogenic oocytes at the post-spawning periods. According to Takahashi (1977), "the atretic disintegration of auxocytes is the most general and legible feature representing the initiation of sex inversion in gonads. Histological observations of sex inversion of zebrafish juveniles in the present study reveal that sex inversion from ovaries to testes begins with a degeneration of oocytes as stated above. Here, the presence of slit like openings (ovarian lamellae) was one of the features associated with the onset of oocyte degeneration and eventual sex inversion. "Islets" of "gonial" germ cells seen along gonad periphery and along slits within the gonad wall were considered to be spermatogonia although it was not possible to distinguish resting oogonia from spermatogonia, as both are of similar size and appearance.

The occurrence of numerous degenerating oocytes as well as a relatively larger proportion of "islets" of proliferating "gonial" germ cells was considered the first outstanding indication of spermatogenic activity in these ovaries. The formation of the future testis was thought to start from these peripheral “male nests", when the first indications of ovarian degeneration appeared. Gonads with numerous "islets" of "gonial", degenerating as well as mitotic germ cells, were termed inverting gonads (presumptive testes).

The process of oocyte degeneration and resorption in juvenile zebrafish ovaries appear to depend on the size, differentiation level and specific features of the individual germ cells. Lysis of the post-pachytene oocytes is preceded by an intensification of cytoplasmic staining and change in shape of the oocytes probably indicating degeneration (Kobayashi and Hishida, 1985). The resorption of larger pre-vitellogenic oocytes that have cortical alveoli is preceded by active participation of cells of the follicular epithelium.

The invasion of the shrunken degenerating oocytes by aggregations of phagocytes or blood cells as observed in the present study may indicate phagocytic mechanisms, which could lead to the removal of the nuclear and ooplasmic contents with the phagocytes being probably derived from the follicle cells. Whether the migrating cells absorbed residual degenerating cells phagocytically or not could not be determined with certainty. Interestingly, disintegration of cytoplasm of germ cells and follicular atresia also occurs in normal adult female fish (Guraya, 1986).

The first indication of the sex inversion as observed in the present study was the degeneration of female germ cells (post-pachytene oocytes). As degeneration of female germ cells proceeded, stromal somatic cells invade the gonad. Sooner or later, spermatogonial proliferation ensued. The presence of spermatogonia was the first indication of spermatogenic activity in the gonad, whether PGCs were involved in spermatogonial proliferation or not could not be detected in histological preparations. Shortly afterwards, the gonads became testes; female germ cells having disappeared.

It can be said here that the strongest indicators of pre-maturational sex change in juvenile zebrafish was the presence of transitional individuals whose gonads contain degenerative ovarian tissues and developing testicular tissues. The presence of degenerating ovarian tissues simultaneously with a large number of developing spermatogonia in the same gonad strongly supports protandrous hermphroditism in juvenile zebrafish. Juvenile inter-sexuality has also been reported in male and female yellow eels of *Anguilla anguilla* by Kuhlmann

(1957), cited in Colombo and Grandi (1995). The coexistence of both germinal tissues in zebrafish juveniles is probably exclusive to the juvenile period, since functional female germ cells were not seen in active male gonads confirming previous indications of protogyn in juvenile zebrafish (Takahashi, 1977).

The duration of sex change in zebrafish juveniles could not be determined definitely from the results of the present study, but in specimen examined, sex change occurred in fish at 16 to 22 mm total length (TL), a quite different size range from the 12 to 26 mm (TL) range reported by Takahashi (1977). This difference could be attributed to differences in growth rate of individuals or other social and/or genetic variation among individuals (Wang et al., 2007). Molecular factors initiating and regulating the processes of oocyte degeneration or atresia and sex inversion in zebrafish could not be determined in the present study. While mechanisms of this phenomenon in normal ovaries are also not well addressed (Guraya, 1986), here, it can be seen in the context of an apoptotic regulation.

In conclusion, the present work presents a description of the morphology of early gonad differentiation in zebrafish. The exhaustive stage analysis conducted in this study has integrated and interconnected the findings of Selman et al. (1993) for the adult ovary and has also extended the study of Takahashi (1977). From results presented in this study, the possible involvement of primordial germ cells in secondary gonadogenesis (male testicular differentiation) could not be determined. The mitotic/meiotic transition was a gradual process. This work was necessary for other works to follow.

Conflict of Interests

The author(s) have not declared any conflict of interests.

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REFERENCES

Andreucceti P, Motta C, Filosa S (1990). Regulation of oocyte numbers during oocyte differentiation in the lizard (*Podarcis sicula*). Cell Differ. Dev. 29:129-134.

Bromage N, Cumaranatunga R (1982). Egg production in the rainbow trout, In: Recent Advances in Aquaculture. (Muir JF, Roberts RJ (Eds.). 62-38. London: Croom Helm.

Brown DD (1997). The role of thyroid hormone in zebrafish and axolotl development. P. Natl. Acad. Sci. USA. 94:13011-13016.

Byskov AG (1978). Follicular atresia, In: Jones, R.E. (Ed), The Vertebrate Ovary. Plenum, New York. pp. 533-562.

Colombo G, Grandi G (1995). Sex differentiation in the European eel: Histological analysis of the effects of sex steroids on the gonad. J. Fish Biol. 47:394-413.

Coward K, Bromage NR (1998). Histological classification of oocyte growth and the dynamics of ovarian recrudescence in *Tilapia zillii*. J. Fish Biol. (53):285-302.

Eaton RC, Farley RD (1974). Spawning cycle and egg production of zebrafish, *Brachydanio rerio,* in the laboratory. Copeia 1974:195-204.

Grier H (2000). Ovarian germinal epithelium and folliculogenesis in the common snook, *Centropomus undecimalis* (Teleostei: Centorpomide, J. Morphol. 243:249-321.

Guraya SS (1986). The cell and molecular biology of fish oogenesis, In: Monographs of Developmental Biology, Karger, Basel. 18:1-223.

Hamaguchi S (1982). A light and electron-microscopic study on the migration of primordial germ cells in the teleost, *Oryzias latipes.* Cell Tissue Res. 227:139-151.

Hisaoka KK, Firlit CF (1962). The localization of nucleic acids during oogenesis in the zebrafish. Am. J. Anat. 110:203-216.

Howley C, Ho RK (2000). mRNA localization patterns in zebrafish oocytes. Mech. Dev. 92:305-309.

Humason GL (1979). Animal tissue techniques, W.H. Freman, San Fransisco. p. 661.

Karnovsky MJ (1969). A formaldehyde-gluteraldehyde fixative of high osmolality, for use in electron microscopy. J. Cell Biol. 276:137-138.

Kimmel CB (1993). Patterning the brain of the zebrafish embryo. Annu. Rev. Neurosci. 16:707-732.

Kimmel CB, Law RD (1985). Cell lineage of zebrafish blastomeres. I. Cleavage pattern and cytoplasmic bridges between cells. Dev. Biol.108:78-85.

Kimmel CB, Warga RM (1987a). Cell lineages generating axial muscle in the zebrafish embryo. Nature. 327:234-237.

Kimmel CB, Warga RM (1987b). Intermediate cell lineage of the zebrafish embryo. Dev. Biol. 124:269-280.

Kimmel CB, Warga RM, Law DA (1994). Cell cycles, clone strings, and the origin of the zebrafish central nervous system. Development. 120:265-276.

Kobayashi MK, Hishida T (1985). Morphological observation on reversal processes of sex-differentiation in the female gonad of the medaka, *Oryzias latipes*, by androgen. Medaka 3:25-37.

Lessman CA (2009). Oocyte maturation: converting the zebrafish oocyte to the fertilizable egg. Gen. Comp. Endocrinol. 161:53-7.

Mezhnin, N I (1978). Development of the gonads in the early ontogeny of the common perch Perca fluviatilis L. Vopr. Ikhfiol. 18 (1): 84-101.

Nakamura M, Nagahama Y (1993). Ultrastructural study on the differentiation in the Amago salmon (*Oncorhynchus rhodurus*). Aquaculture 112:237-251.

Nieuwkoop BP, Sutasurya LA (1979). Primordial germ cells in the chordates: *Embryogenesis and phylogenesis*, Cambrige University Press, London.

Parmentier HK, Timmermans LPM (1985). The differentiation of germ cells and gonads during development of carp (*Cyprinus carpio*): a study with anti-carp sperm monoclonal antibodies. J. Embryol. Exp. Morph. 90:13-32.

Patino R, Davis KB, Scheoore JE, Urguz C, Parker NC, Simco BA, Goudie CA (1996). Sex differentiation of channel catfish gonads: normal development and effects of temperature. J. Exp. Zool. 276: 209-218.

Pelegri F (2003). Maternal factors in zebrafish development. Dev. Dyn. 2003:535-54.

Sato N, Egami N (1972). Sex differentiation of germ cells in the teleost, *Oryzias latipes*, during normal embryonic development. J. Exp. Morph. 28:385-395.

Selman K, Wallace RA (1989). Cellular aspects of oocytes growth in teleosts. Zool. Sc. 6:211-231.

Selman K, Wallace RA, Saarka A, Qi X (1993). Stages of oocyte development in the zebrafish, *Brachydanio rerio.* J. Morphol. 218:203-224.

Streisinger G, Walker C, Dower N, Knauber D, Singer F (1981). Production of clones of homozygous diploid zebrafish (*Brachydanio rerio*). Nature 291:293-296.

Takahashi H (1977). Juvenile hermaphroditism in the zebrafish

Brachydanio rerio. Bull. Fac. Fish Hokkaido Univ. 28:57-65.
Timmermans LPM, Taverne N (1987). Origin and differentiation of primordial germ cells in the rosy barb, *Barbus conchonius* (Cyprinidae, Teleostei). Acta Morphol. Neer. Sci. 21:182.
Tyler CR, Sumpter JP (1996). Oocyte growth and development in teleosts. Rev. Fish. Biol. Fish. 6:285-318.
Wang XG, Bartfai R, Sleptsova-Freidrich I, Orban L (2007). The timing and extent of 'Juvenile ovary' phase are highly variable during zebrafish testis differentiation. J. Fish. Biol. 70:33-44.
Warga RM, Kimmel CB (1990). Cell movements during epiboly and gastrulation in zebrafish. Development. 108:569-580.
Westerfield M (1993). The zebrafish book: Guide for laboratory use of zebrafish. (*Brachydanio rerio*), Eugene: Oregon Press.