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# Juvenile Hermaphroditism in the Zebrafish, *Brachydanio rerio*

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## Abstract

The gonads of all juveniles of the zebrafish, *Brachydanio rerio*, start differentiating as ovaries about 10-12 days after hatching irrespective of their definitive sex. In about half of the fish, ovaries continue to grow yielding mature ova, while in the other half, ovaries begin to be transformed into testes when the fish reach about 23-25 days of age. The structural changes from ovaries to testes begin with the disintegration of auxocytes followed by a prominent increase in the amount of somatic cells of the gonadal stroma along the periphery of the gonad. Subsequent infiltration of proliferating somatic cells inside the gonad leads to a rapid sequential occurrence of the multiplication of spermatogonia, the formation of seminal lobules and the active spermatogenesis in intersexual gonads. The whole process of the sex reversal appears in most cases to be completed within 40 days after hatching, and thus gonochorism in the zebrafish is established in subadult and adult stages.

Among the gonochoristic species of teleosts, there are some which reveal transitory hermaphroditism during a juvenile period. For example, developing gonads of the European eel, *Anguilla anguilla*, pass through an intersexual phase in a certain period of the elver stage[1]. Another characteristic example of juvenile hermaphroditism is known in some anabantid fishes such as *Macropodus concolor* and *M. opercularis*, in which the gonads of the male members differentiate initially as non-functional ovaries and are then transformed, through intersexual phases, into definite testes during the juvenile stage[2].

The natural juvenile intersexuality found in teleost fishes is certainly an interesting subject of research for elucidating the mechanism of sex differentiation of the gonad, as exactly as it was for promoting the study on sex differentiation in amphibians[3]. However, a rather scanty amount of information has been gained so far about the phenomenon in teleost fishes. In the present paper, histological observations on the process of gonadal sex differentiation accompanied with obvious juvenile hermaphroditism in the zebrafish are dealt with.

## Material and Methods

The zebrafish, *Brachydanio rerio*, which had been obtained from a commercial dealer and subsequently bred in the laboratory, were used as material in the present study carried out from June to November. Mature females were mated each with two mature males in a glass aquarium with a breeding trap. The first spawn was usually discarded because of its high mortality. After being isolated from

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males for the subsequent 4 or 5 days, the same females were kept again with males. Soon after spawning they were removed, together with the breeding trap, from the aquarium in which the eggs and fry thus obtained were kept successively. The aquarium water was aerated constantly and regulated at 25–26°C under the natural day length. The water was not changed for 10 days or more following hatching in order to minimize the disturbance for normal development of feeble fry, but was completely changed thereafter with temperature-regulated and well-aerated water generally once a week.

Fry in an early growth period of about 10 days after hatching were raised on a sufficient amount of boiled egg yolk suspended daily in the rearing water. Live freshwater copepods were also introduced in the water beginning on the 3rd or 4th day after hatching. Later the yolk was gradually replaced by an appropriate amount of finely powdered commercial diet for carp larvae, which was given generally once a day.

The offspring of 9 spawns, 302 individuals in total, were employed for the present study. Samplings for histological studies on the differentiation and development of the gonad were started with the fry 2 days after hatching and continued, in varying numbers and at chosen intervals of days, up to 60 days after hatching. The sampled fish were fixed *in toto* in Bouin's fluid. The total length of the fish was measured on fixed specimens. Serial paraffin sections of their trunks with the gonads and their associated structures in position were cut frontally or sagittally at 7–10 $\mu$m in thickness, and stained with Delafield's hematoxylin and eosin.

## Observations

Notwithstanding the careful feeding of the newly hatched fry, there were notable differences in the rate of body growth during the first 60 days following hatching among some different broods of the zebrafish examined in the present study. Four broods representative of the variation in body growth are dealt with in Fig. 1. The broods A and B in the figure followed the growth curve of a sigmoid pattern while the broods C and D grew in a linear pattern, the former representing a better condition as to the increase in body length at least during their initial growth period as shown in the work by Eaton and Farley[4]. In spite of such a variation in body growth, fish in each brood displayed a similar mode and time-course of the occurrence of intersexuality in their juvenile period.

The sex distribution for 3 representative broods of the fish sampled at various intervals of days after hatching is shown in Table 1. During the first several days following hatching, primordial germ cells with indistinct contour are localized singly or in clusters of a few cells in the dorsolateral corners of the body cavity in the proximity of the caudal end of the swim bladder. Some of them are seen to be undergoing mitotic divisions at the age of 8 days, but the gonad anlagen are still very small in size at that time. The first sign of gonadal sex differentiation occurs by the age of 10 days, when a few cysts of germ cells originating from the active mitoses of preexisting primordial germ cells appear to be in initial phases of the meiotic prophase denoting the initiation of oogenesis (Fig. 2). The gonads

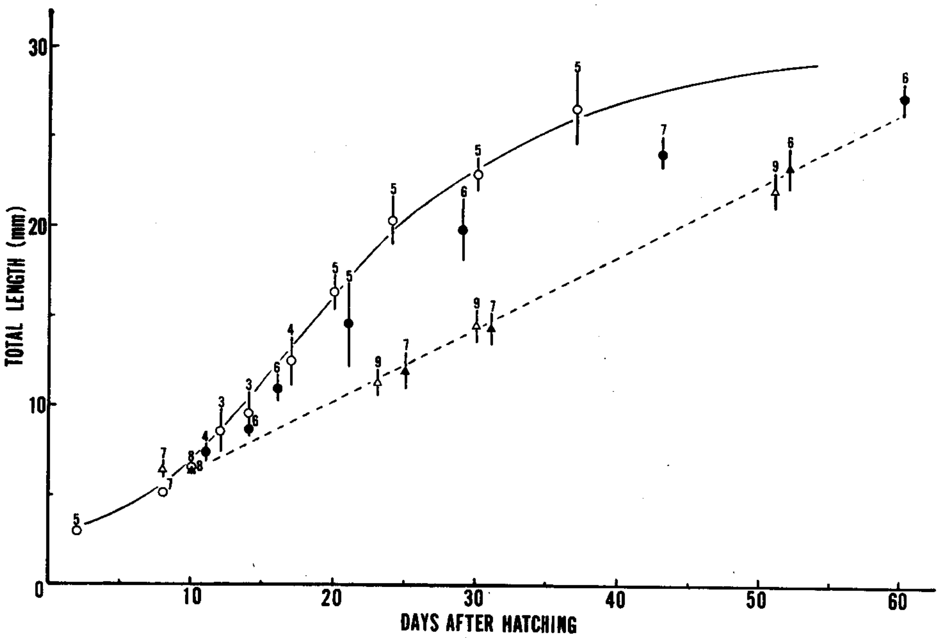



Fig. 1. Body growth observed in four different broods (A, ○: B, ●: C, △: D, ▲) of the zebrafish. Each mark with bars and affixed figure represent the mean ± 95% confidence interval and the size of the samples, respectively.

of all juvenile fish are directed to begin the oogenesis sooner or later, becoming provided with a few oocytes of the first growth phase (auxocytes) in them by 14 days of age (Fig. 3).

Thus all juvenile fish are recorded as females with structurally well-defined ovaries at least until they arrive at about 20 days of age (Table 1). Flat ovarian cavities are formed dorsal to the ovaries around 14 to 20 days of age by the fusion of the distal edge of the ovarian ridges with dorsolateral parts of the coelomic wall. There is, however, a tendency in these ovaries that the development of oocytes is delayed in the fish growing slower as compared with that in the fish keeping a better growth.

During the period subsequent to about 20 days of age, the ovaries of some females continue to develop further showing an increase in the number and size of auxocytes and progressive lamination of the ovarian tissue leading to the formation of ovigerous lamellae (Fig. 4). On the other hand, the gonads of others remain smaller in size and begin to show some changes characteristic of the transformation from ovaries into testes. The structural change is shown first by progressive degeneration of oocytes that have been growing in these ovaries. The earliest occurrence of oocyte degeneration among juvenile fish of the 9 broods observed was noticed at the age of 23 days. There appeared to be no wide variation among the 9 broods in the age when the sexual transformation of the gonad began, and it seemed to range generally from 20 to 25 days after hatching. The

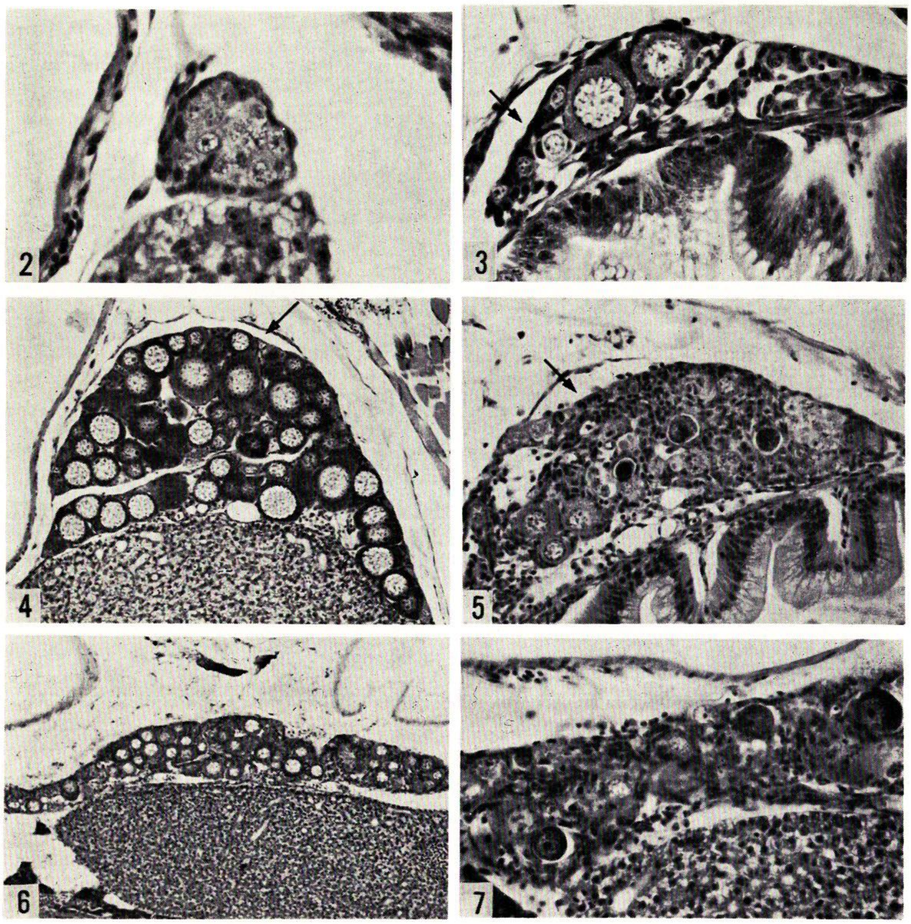
Table 1. *Sex distribution in three different broods of the zebrafish at various ages after hatching.*

| Age (days) | Total length (mm) | Sex distribution | | | |
|---|---|---|---|---|---|
| | | ♀ | I* | ⚥ | ♂ |
| **A** | | | | | |
| 2 | 3.02 | | 5 | | |
| 8 | 5.24 | | 7 | | |
| 10 | 6.62 | 3 | 5 | | |
| 12 | 8.60 | 3 | | | |
| 14 | 9.66 | 3 | | | |
| 17 | 12.52 | 4 | | | |
| 20 | 16.42 | 5 | | | |
| 24 | 20.44 | 3 | | 2 | |
| 30 | 23.06 | 2 | | 3 | |
| 37 | 26.72 | 2 | | 1 | 2 |
| (Total) | | (50) | | | |
| **B** | | | | | |
| 11 | 7.45 | | 4 | | |
| 14 | 8.68 | 5 | 1 | | |
| 16 | 11.01 | 6 | | | |
| 21 | 14.62 | 5 | | | |
| 29 | 19.91 | 3 | | 3 | |
| 43 | 24.22 | 4 | | | 3 |
| 60 | 27.33 | 4 | | | 2 |
| (Total) | | (40) | | | |
| **C** | | | | | |
| 10 | 6.41 | | 8 | | |
| 25 | 12.07 | 5 | | 2 | |
| 31 | 14.37 | 5 | | 2 | |
| 52 | 23.38 | 2 | | 4 | 1 |
| (Total) | | (29) | | | |

*I; fish with sexually indifferent gonads.

atretic disintegration of auxocytes is the most general and legible feature representing the initiation of sexual transformation of the ovaries (Fig. 5). However, the auxocytes do not undergo degeneration simultaneously but asynchronously and sporadically at first, the degree of the change varying sometimes in different regions of the same gonad (Figs. 6 and 7). Some auxocytes seem to survive long in these transforming gonads, which may account for the frequent appearance of testis-ova in maturing testes of the zebrafish (Fig. 12).

Concomitantly with the disintegration of oocytes, the somatic cells of the stroma in transforming gonads begin to increase in number along the peripheral region of the gonad (Fig. 8). The change is most prominent along the ventral border of the gonad, and the somatic cells in that region are frequently seen to be

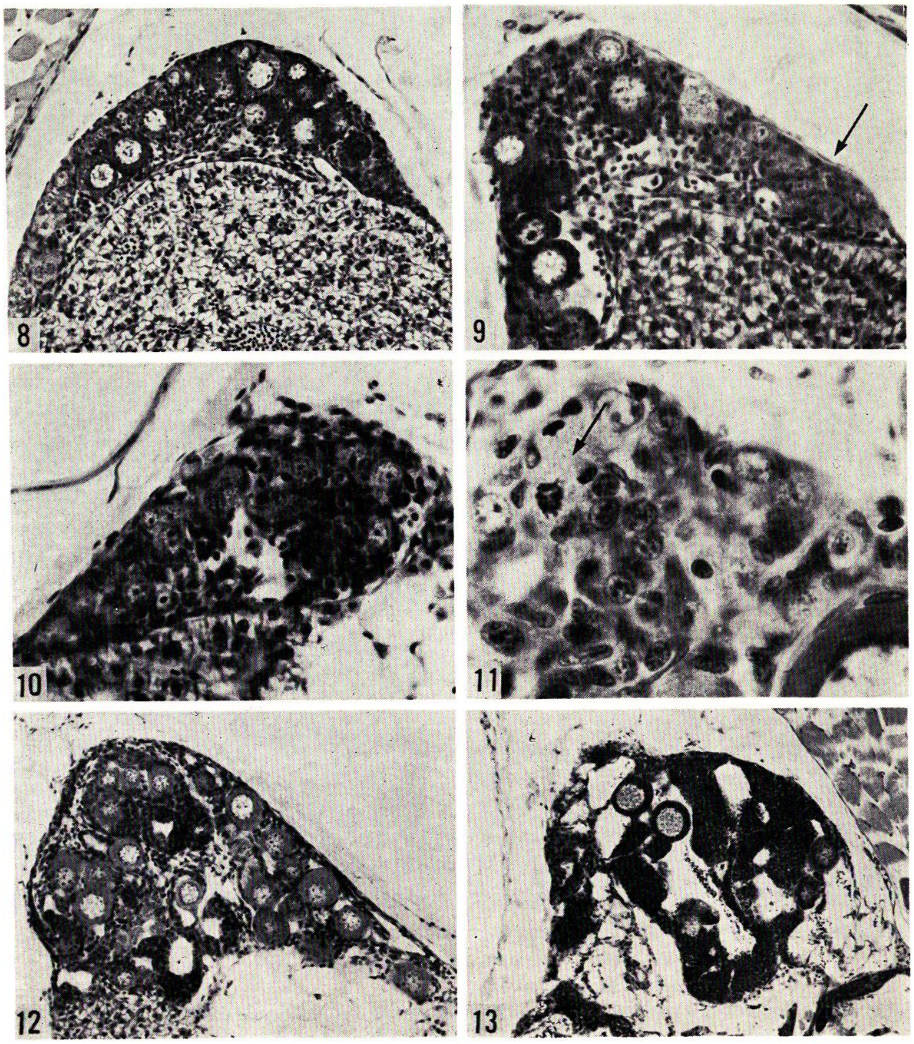



Figs. 2–4. Cross sections through ovaries of juvenile zebrafish, 10 (Fig. 2), 14 (Fig. 3) and 24 days (Fig. 4) after hatching. Arrows indicate ovarian cavities. Fig. 2, ×340; Fig. 3, ×280; Fig. 4, ×110.

Figs. 5–7. Cross sections through gonads of juvenile zebrafish at early phases of sexual transformation, 24 days after hatching. Figs. 6 and 7 show the two different regions of the same gonad. Fig. 5, ×205; Fig. 6, ×80; Fig. 7, ×250.

undergoing mitotic division. No sign of a new invasion of somatic cells from the peritoneal wall into the gonad is noticeable either in the mesogonium or in the hilar region of the gonad.

In general, gonial germ cells are more numerous in ovaries destined to sex reversal than in those of "true" females. In the former, most of the static gonia occur in clusters of varying numbers mainly along the dorsal border facing the ovarian lumen (Fig. 9). As proliferated stroma cells infiltrate inside through gonads, gonial germ cells come to be surrounded by these cells and separated from each other (Fig. 10). The germ cells appear to develop very rapidly toward testicular morphogenesis after they have once come in contact with the stroma cells.




Figs. 8–11. Cross sections through intersexual gonads of juvenile zebrafish, 29 (Figs. 8–10) and 30 days (Fig. 11) after hatching. Arrows in Figs. 9 and 11 reveal a cluster of static gonia and that of dividing spermatogonia, respectively. Fig. 8, ×170; Fig. 9, ×275; Fig. 10, ×365; Fig. 11, ×715.

Figs. 12 and 13. Cross sections through developing testes with frequent testis-ova found in juvenile zebrafish, 30 (Fig. 12) and 37 days (Fig. 13) after hatching. Fig. 12, ×135; Fig. 13, ×105.

The isolated gonia, which are now to be called spermatogonia, begin to multiplicate quite actively (Fig. 11), and yield many spermatogonial cysts which are arranged in clusters with a narrow empty space in the center of the cluster, thus originating the anlagen of seminal lobules.

The gonads of some of the fish examined at 30 days of age were evidently in the state of an intersexual phase characterized by the formation of immature seminal lobules containing intact and degenerating auxocytes together with male germ cells in early stages of spermatogenesis (Fig. 12). The active proliferation of male germ cells in the seminal lobules is followed by a notable enlargement of the gonad associated with a prompt succession of spermatogenesis. Males with definite testes become thus easily distinguishable from ordinary females somewhat later than 35 days after hatching (Table 1), though some of them bear the testes in which intact and degenerating testis-ova are still encountered dispersedly on the wall of the seminal lobules (Fig. 13).

The whole process of transformation of the ovaries into testes seems to have been completed by 40 days of age, but it tends to be protracted to some extent in broods of the fish which have undergone a retarded growth in their early life (Table 1). Some variations are found as to the pace of gonadal development among individuals of even the same brood and age. Generally speaking, however, some males and females come to produce a notable amount of mature spermatozoa and vitellogenic oocytes, respectively, by 45 days of age, and are matured fully by 60 days. Nearly 1:1 sex ratio seems to be established in the zebrafish following the completion of the sex reversal.

## Discussion

As the present study indicates, all juveniles of the zebrafish 15 to 20 days after hatching have definite ovaries with developing oocytes and without any testicular characteristics. Later, whereas in about half of the fish the ovary continues to grow to have maturing ova, in the remaining half the degeneration of oocytes and the proliferation of stroma cells followed by the activation of spermatogonia in the ovaries result in the appearance of a transitory intersexual phase in these gonads. The subsequent rapid occurrence of spermatogenesis in these gonads corroborates the sex inversion from ovaries into testes. In the latter, oocytes may survive long, but they seem to disappear completely sooner or later without entering into vitellogenic stages. Gonochorism in the zebrafish of the subadult and adult stages thus ensues.

Several different conditions seem to be distinguishable in juvenile hermaphroditism in fishes. Juvenile intersexuality in the brown trout, *Salmo trutta*, is characterized by the occurrence of occasional cysts of spermatogonia and spermatocytes in the ovaries of part of the young females[5]. In the European eel, *Anguilla anguilla*, juvenile intersexuality is found both in males and in females[1]. Recently Kuhlmann[6] confirmed that the gonads of most females and those of all males in a certain period of the elver stage come to take an intersexual structure having oogonial cysts and solitary oocytes together with spermatogonia mounted in interstitial cell cords. The predominance in the development of either male or female element accompanied by the degeneration of the opposite sex element may direct further sexual differentiation of the gonad to attain definitive sex.

Juvenile hermaphroditism of another condition is known to appear in the rainbow trout, *Salmo gairdneri*[7]. In this species, the germ cells in all gonads of the young at an early growth period develop into oocytes, giving the gonads a feature of the ovary, and later the oocytes in the gonads of future males come to degenerate and the gonial germ cells begin to act as spermatogonia. Juvenile hermaphroditism of this type appears to be the case also in some anabantid fishes such as *Macropodus concolor* and *M. opercularis*[2]. In these fishes, all of the individuals which are destined to become males pass through a female phase and then an intersexual phase during their juvenile period, though the degree of femaleness in terms of histological aspects of the gonad may vary with the species studied. The zebrafish, *Brachydanio rerio*, a cyprinid fish observed in the present study, conclusively follows this type of juvenile hermaphroditism.

Juvenile males of the goldfish, *Carassius auratus*, which belongs to the same family, Cyprinidae, as the zebrafish, also display a slight tendency to juvenile hermaphroditism during the initial course of testicular development[8][9]. In the goldfish, some germ cells in the gonads that are destined for testes develop precociously into meiotic prophase resulting in an occasional appearance of a few testis-ova in the developing testes. However, the intersexual aspects of the gonads are encountered only in some and not all male goldfish of the same spawn. Another cyprinid fish, the sumatra *Barbus tetrazona*, also exhibits juvenile hermaphroditism which is of a similar but less obvious condition when compared with that of the zebrafish (unpublished data).

The different degrees of intersexuality appearing in these cyprinid fishes are reflected not only in the behaviour of germ cells but also in the formation of the gonoduct system. As reported previously[10], while the testis of male goldfish traces, though rudimentarily, the course of formation of the ovarian cavity-oviduct system during its morphogenesis, the sperm duct system is constructed ultimately in a manner clearly different from the formation of the ovarian cavity-oviduct system. In the zebrafish, on the contrary, the ovarian cavity-oviduct system that has been shaped, even in the case of future males, during their female phase, persists after the transformation of ovaries to testes, and comes to function at least as a part of the anastomosed sperm ducts appearing in mature males. Thus juvenile hermaphroditism in cyprinid fishes is recognized more easily in the zebrafish than in the goldfish.

The sexual transformation of ovaries into testes in the zebrafish appears to begin histologically with the appearance of degenerating oocytes in the gonad. Then the active multiplication of somatic cells of the gonadal stroma follows, which is the most prominent feature signifying the progress of sex reversal. The activation of stromal cells decisively precedes the proliferation of spermatogonia and the ensuing formation of seminal loubles. The same is true for the sexual transformation of ovaries in juvenile *Macropodus opercularis*[2]. In a protogynous hermaphrodite, *Monopterus albus*, too, the development of interstitial cells occurs, during natural sex reversal, in concomitance with the proliferation of male germ cells preceding the formation of testicular lobules[11]. A previous study by the present writer also showed that, in the guppy *Poecilia reticulata*, somatic cells of the gonad take some essential parts in the differentiation and morphogenesis of the testis[12]. Possible physiological roles played by somatic cells of the gonad in the sex differentiation of teleost fishes remain to be substantiated by further studies.

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