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Title	Histology and Fine Structure of the Ultimobranchial Gland in the Zebrafish, <i>Brachydanio rerio</i>
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Citation	北海道大學水産學部研究彙報, 29(3), 213-221
Issue Date	1978-09
Doc URL	https://hdl.handle.net/2115/23648
Type	departmental bulletin paper
File Information	29(3)_P213-221.pdf



Histology and Fine Structure of the Ultimobranchial Gland in the Zebrafish, *Brachydanio rerio*

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Abstract

The ultimobranchial gland of the zebrafish (*Brachydanio rerio*) was examined by light and electron microscopy. The gland was located beneath the ventral wall of the esophagus and composed of many follicles consisting of two types of cells, a few agranular cells and a dominant number of granular cells. The glandular epithelium of immature fish was low in height, and the fine structure of the granular cells suggested their low activity. In mature males, the height of the epithelium was as low as in immature fish, and no mitotic figures were found. The gland of mature females showed hyperactive features, that is, increased height of the epithelium, a larger accumulation of secretory granules, a higher mitotic activity of the cells and a more voluminous gland. The granular cells eight hours after spawning had more secretory granules than those two days after spawning, while the agranular cells showed an increase in number two days after spawning. No difference was observed between the granular cells of mature males and females as to the development of cell organelles. These results seemed to support a hypothesis that the ultimobranchial gland in fishes plays a sex-related role at least during the maturation of females.

Introduction

The thyroid C-cells in mammals or the ultimobranchial glands in other vertebrates are known to produce and secrete a peptide hormone called calcitonin¹⁾⁻⁴⁾. In mammals, the release of calcitonin is generally under the control of blood calcium. The hypocalcemic effect is essential for the homeostasis of the blood calcium concentration together with a hypercalcemic effect of parathormone secreted by the parathyroid glands.

In teleosts, the physiological role of the ultimobranchial gland has not been well clarified yet. The partial ultimobranchialectomy of goldfish caused a significant increase to the extent of increasing the plasma calcium concentration following an acute transfer of the fish into diluted sea water⁵⁾. The ultimobranchialectomy in the silver eel resulted in a rise of serum calcium concentration with demineralization and inhibition of the osteoblastic apposition in bone⁶⁾. The effect of the injection of calcitonin on fish blood calcium, however, is variable and controversial⁷⁾. Some evidence is available in the fact that calcitonin is involved in sexual maturation of female fish. A histological hyperactivation of the ultimobranchial gland in mature females has been observed in goldfish⁸⁾, European eel⁹⁾, Japanese eel¹⁰⁾, and *masu* salmon¹¹⁾. The blood calcitonin concentrations of coho, chinook, and sockeye

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salmons and the Japanese eel were remarkably higher in mature females than in mature males¹²⁾⁻¹⁴⁾.

The present study was aimed to confirm sexual differences in the morphology of the ultimobranchial glands of mature zebrafish. In addition, the female glands before and after spawning and the glands of immature fish were compared. The zebrafish was chosen for the convenient observation of the glands in relation to the spawning because they spawn regularly within a short period.

Materials and Methods

Mature zebrafish, *Brachydanio rerio*, were obtained from a commercial dealer and acclimated in glass aquaria aerated at $27 \pm 1^\circ\text{C}$ for at least one month. They were fed on pellets for tropical fish twice a day. The fish (32-36 mm in total-length) were then grouped into ten couples, and each was kept separately in a small aquarium at the same temperature. They spawned every three or four days. After checking the spawning interval, these fish were killed eight hours or two days after the final spawning, and their ultimobranchial glands were subjected to histological examinations. Eight immature fish (9-28 mm in total-length) raised from the spawned eggs were also used as material. All fish were sacrificed at midday to avoid fluctuations of mitotic activity.

After anesthetizing the fish with MS-222, the ultimobranchial glands were removed together with the surrounding tissues and immediately fixed with Bouin's fluid for 24 hrs, or with a mixture of 2.5% glutaraldehyde and 2% paraformaldehyde in 0.1M phosphate buffer at pH 7.4 for 1.5 hrs. After washing in the buffer solution, the aldehyde-fixed specimens were post-fixed with 1% osmium tetroxide in the phosphate buffer for 1 hr. The tissues were dehydrated in ethanol and embedded in paraffin or Epon-812. Paraffin sections cut at 7μ were stained with Delafield's hematoxylin and eosin. Ultrathin Epon-sections were obtained by the use of a Porter-Blum ultramicrotome, doubly stained with uranyl acetate and lead citrate and examined with a Hitachi HS-12 electron microscope.

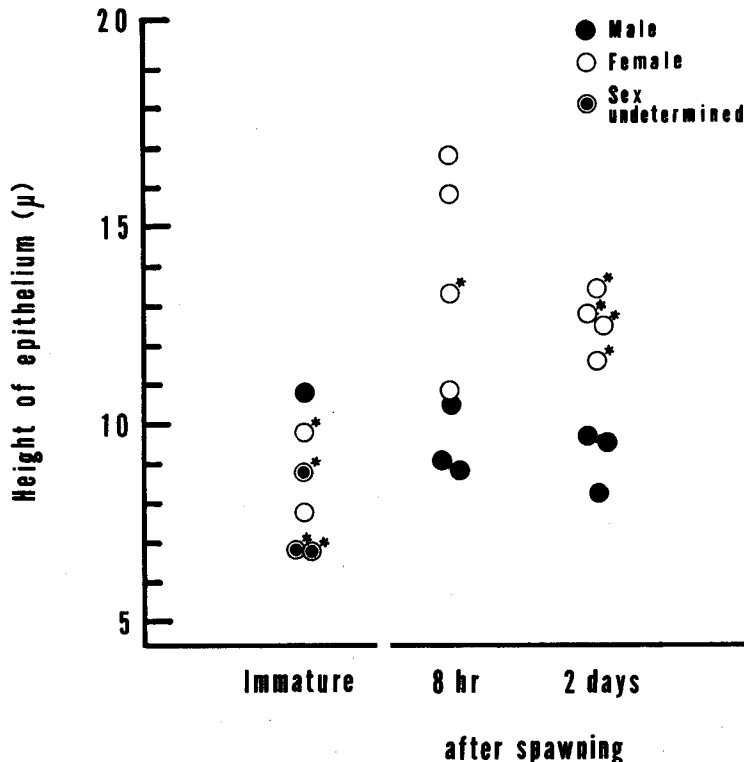
The height of the ultimobranchial cells was determined by measuring those in follicular arrangement with an eye-piece micrometer. To figure out the total volume of the glands of mature males and females, the outlines of the sectioned glands were drawn on graph papers with every three serial sections by the use of a camera lucida, and the figures were cut off and weighed. Two of the series were reconstructed into three-dimensional paraffin models and the volume of these models was measured. After calculating the ratio of the volume of the models to the total weight of the figures, the volume of the other glands was calculated from the weight of their figures multiplied by the ratio.

Results

Light microscopy

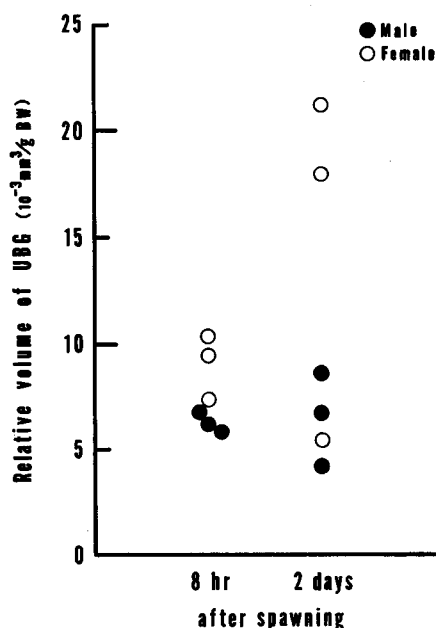
The ultimobranchial gland of immature zebrafish was located beneath the ventral wall of the esophagus, concealed in the thin connective tissue capsule. It was composed of two separate follicles in small immature fish (PL. I-2), whereas

that of large immature fish was a single mass of several follicles (PL. I-3). In immature fish, the glandular epithelium showed frequent mitotic figures, ranging from 6.8 to 10.8 μ (mean 8.5 μ) in height (Text-fig. 1). Their ovoid nuclei were generally in the basal part of the cells.



Text-fig. 1. Comparison of the height of the ultimobranchial epithelium of immature and mature fish. The epithelia of mature males and females were measured with specimens fixed eight hours and two days after spawning, respectively. Asterisks indicate the glands in which mitotic figures were observed.

The gross structure of the ultimobranchial gland of mature zebrafish as revealed by three-dimensional models was a lump of tissues extending along the ventral wall of the esophagus (PL. I-1). It was composed of the right and the left lobes interconnected by a medial part. The gland of mature females was found more bulky than that of mature males. This was reflected in the sexual difference of the relative volume of the gland (gland volume/body weight), as shown in Text-fig. 2. The glands of mature males were composed of many follicles (PL. I-4). Colloidal substances were sometimes in the lumen of the follicles. The height of the glandular epithelium ranged from 8.3 to 10.5 μ (mean 9.3 μ) in height and no mitotic figures were found (Text-fig. 1). Abundant blood capillaries were distributed in the gland. The glandular epithelium of mature females showed a



Text-fig. 2. Change of relative volume of the ultimobranchial gland ($10^{-3} \text{ mm}^3/\text{g}$ body weight) in mature males and females eight hours and two days after spawning.

marked increase in height ranging from 10.9 to 16.8 μ (mean 13.4 μ). The mitoses of the gland cells were very active particularly in the gland two days after spawning. There seemed to be no correlation between the occurrence of mitoses and the total length of fish. The follicular arrangement of the gland cells was more obvious in mature females than in mature males, but no sexual difference was found as to the distribution of blood capillaries (PL. I-5).

Electron microscopy

The follicles of the ultimobranchial gland in general were enclosed by a thin layer of the basement membrane usually accompanied by blood capillaries lined with the fenestrated endothelium (PL. II-6). The endothelial cells had many pinocytotic vesicles and small pinocytotic invaginations along the luminal and basal surfaces (PL. II-7).

The perivascular space containing a few collagen fibrils was between the capillary endothelium and the basement membrane. The follicles consisted of two types of cells, a few agranular cells and a dominant number of granular cells, distinguished on the basis of their fine structure. The lumen of the follicle was bordered by the apical surfaces of both types of cells of which the nuclei were generally situated at the basal part. The apical surface of the cells frequently showed cilia and microvilli (PL. III-8). Conspicuous desmosomes were found near the luminal surface often associated with extensive cellular interdigitations of the lateral cell surfaces. In the lumen were some colloidal substances and membranous structure.

The agranular cells were generally small in size and showed no secretory granules (PL. III-9). The nuclei showed highly irregular contours with marginal indentations. The rough surfaced-endoplasmic reticulum (r-ER) was poorly developed. The mitochondria tended to lie in the basal part of the cells and those in the supranuclear region often elongated in the direction of the follicular lumen. Glycogen particles were often distributed throughout the cytoplasm (PL. IV-10). Many pinocytotic invaginations of the plasma membrane and a few pinocytotic vesicles were seen along the basal or lateral cell surface. Some cells very similar to the agranular cells in fine structure but contained a few, very small granules (100 nm in diameter) were observed near the agranular cells (PL. IV-11).

The granular cells were characterized by an accumulation of a number of round, membrane-bound secretory granules in the supranuclear region (PL. V-12). The granules varied in size from cell to cell, usually ranging from 150 to 250 nm in diameter. They had a dense or faint heterogeneous core separated from the limiting membrane by a thin electron-lucent region. Immature electron-dense granules were often found near Golgi apparatuses the elements of which contained materials of similar density (PL. V-13). This suggests that the secretory granules are produced by the Golgi apparatus. The nuclei, irregular in shape, were similar to those of the agranular cells. Mitochondria, rich in the basal part of the cell, were generally round or spherical and contained prominent intramitochondrial granules among tubular cristae. The r-ER developed around the nucleus. Long cisternae were often observed along the lateral plasma membrane of the cells which contained a large number of secretory granules. Free ribosomes were sparsely scattered throughout the cytoplasm. There were some secondary lysosomes of irregular shapes that contained lamellae and fibrillar materials (PL. V-14). Few pinocytotic invaginations of the plasma membrane were seen at the basal or lateral surface.

In immature fish, the granular cells were very tightly packed showing accumulations of numerous large secretory granules. Immature granules with densely stained contents were seen near Golgi apparatuses. Two phases of Golgi apparatuses were distinguished on the basis of their components; one consisted of many small vesicles and well-developed lamellae which often contained dense materials and the other being in majority consisted of many small vesicles only (PL. VI-15). Both of the two phases were of large dimensions. The r-ER was extended around the nucleus and along the lateral plasma membrane. Well-developed cisternae in swollen profile were noted in some of the cells. Lysosomes were poorly developed. Most of the mitochondria seemed to be under degeneration showing a disappearance of cristae or an appearance of concentric lamellar bodies (PL. VI-16).

In mature males, the granular cells showed the poorly developed r-ER but contained many secretory granules in the supranuclear region (PL. VI-17). The Golgi apparatuses of relatively small dimensions and the terminal sacs often contained materials of a density similar to that of the granules. Well-developed lysosomes were common among the granules.

In the granular cells of mature females, the secretory granules were more abundant than in those of mature males. This increase in the number of granules was accompanied by cellular hypertrophy as observed by light microscopy. Moreover, the granular cells eight hours after spawning were packed with a larger accumulation of granules than those two days after spawning (PL. VII-18 and -19). No difference was observed between mature males and females as to the development of the organelles of the granular cells. An increased number of the agranular cells were observed occasionally in clusters in the female glands two days after spawning (PL. VIII-20). The intercellular spaces of these agranular cells were often expanded, and desmosomes interconnected their apical parts. Occasionally, dense osmiophilic inclusions composed of many needle-shaped substances of varied length and thickness occurred in the lumen of the follicle which was predominantly composed of these agranular cells. Some cells which contained a

few, very small granules were sometimes located near the clustered agranular cells.

Discussion

The results of the present study offered evidence to support a hypothesis that the ultimobranchial gland in fishes plays a sex-related role at least during the maturation of females. Morphological characteristics which indicate a hyperactivity of the gland were predominantly observable in mature females; an increase in height of the glandular epithelium accompanied by a larger accumulation of secretory granules, higher mitotic activities of the cells and a larger relative volume of the gland are all suggestive of the hyperactivity of the gland. The hyperactivation of the female gland at maturation has also been reported in goldfish⁸⁾, European eel⁹⁾, Japanese eel¹⁰⁾, and *masu* salmon¹¹⁾. These morphological findings agree with the radioimmunological studies of coho, chinook and sockeye salmon¹²⁾, sockeye salmon¹³⁾, and Japanese eel¹⁴⁾, in which much higher concentrations of blood calcitonin were shown in mature females than in mature males.

No significant difference was observed as to the development of the r-ER in the granular cells between mature male and female zebrafish, although the r-ER in large concentric or stratified lamellar configurations was observed only in mature females of *masu* salmon¹¹⁾ and Japanese eel¹⁵⁾. The less-developed r-ER in mature female zebrafish may suggest a relatively low rate of production of calcitonin in this species.

It is of particular interest that a high mitotic activity was observed in the female glands two days after spawning but not in the male glands. High mitotic activities of the thyroid C-cells accompanied with their degranulation were reported in dog¹⁶⁾ and Indian grey mongoose¹⁷⁾ when experimental hypercalcemia was induced in these animals. If the enhanced mitotic activity indicates a sign of hyperactivation of the gland, the gland may be involved in a physiological role at the interspawning period of the female. On the other hand, Watts et al.¹³⁾ observed in female sockeye salmon a marked increase of plasma calcitonin at pre-spawning and a sharp decrease of it after spawning. Yamane and Yamada¹¹⁾ observed that the degeneration of epithelial cells occurs characteristically in ovulated *masu* salmon. These findings suggest that calcitonin may be released at spawning in regard to females. In the zebrafish, however, no degeneration of cells nor degranulation of the granular cells were found in female glands eight hours after spawning. A larger accumulation of calcitonin granules was observed in these cells. Therefore, the problem whether or not a large amount of calcitonin is released in mature females at interspawning periods requires further study.

Lopez et al.⁶⁾ suggested that calcitonin may have a role in the protection of skeletal calcium in females until spawning. They showed that calcitonin injected into female eels inhibited acute osteoclastic resorption and bone demineralization caused by experimental maturation of the fish. In female ruminants a physiological role of calcitonin is known to protect the skeleton from excessive resorption during pregnancy and lactation¹⁸⁾⁻²⁰⁾. The opinion of Lopez et al. is suggestive of functional similarities between fish and mammalian calcitonins.

Most of the granular cells in immature zebrafish had numerous large granules, vesiculated Golgi apparatuses and degenerating mitochondria. These features suggest low synthetic and secretory activities in the immature fish.

Two types of cells were always distinguished by electron microscopy in the gland of zebrafish. A few reports have mentioned about cell types of fish ultimobranchial glands. Kitoh²¹⁾ described two cell types in two species of elasmobranchs (*Mustelus manazo* and *Dasyatis akajei*), barrel cells having secretory granules and finger cells thinly elongated between the barrel cells. On the other hand, Takagi and Yamada²²⁾ distinguished three cell types in the gland of *Carassius carassius*; light cells having granules, granule-less dark cells bordering the follicular lumen or penetrating deeply between the light cells, and semi-dark cells having more granules than the light cells. Since the semi-dark cells are much the same as the light cells in the appearance of granules and cell organelles, this type of cells probably represents a different functional stage of the light cells. In the gland of *Anoptichthys jordani*, also, light cells and dark cells were reported light-microscopically²³⁾. The presence of two cell types was also recognized in the gland of the Japanese eel¹⁵⁾; the granular cells having secretory granules, prominent Golgi apparatuses and well-developed r-ER, and the agranular cells having no granules and poor organelles, bordering the follicular lumen. These two cell types were distinguished also by a different distribution of chromatin in the nucleus. In the zebrafish, however, a distinctive distribution of chromatin was not detected between the granular and agranular cells. Moreover, the granular cells of the zebrafish occasionally faced the follicular lumen, but this was not the case in Japanese eel.

The gland of female zebrafish two days after spawning showed an increased number of the agranular cells accompanied by a high mitotic activity. Cells having a few, very small granules with a faint core and showing a fine structure similar to the agranular cells were observed to be close to the agranular cells. These cells may be interpreted as transitional from the immature to mature granular cells. However, it is not clear whether or not the agranular cells commonly seen in the gland are precursors of the granular cells.

Acknowledgement

I am indebted to Professor Jurô Yamada of the Faculty of Fisheries, Hokkaido University, for his kind guidance and criticism in the course of the present study.

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EXPLANATION OF PLATES

Figures 2-5 in Plate I are photomicrographs of transverse sections of the ultimobranchial glands of immature and mature zebrafish to show their general histological characteristics. All stained with Delafield's hematoxylin and eosin.

Figures 6-14 in Plates II-V are electron micrographs of the ultimobranchial gland to show the fine structural characteristics commonly observed irrespective of sex and maturation. All stained with uranyl acetate and lead citrate.

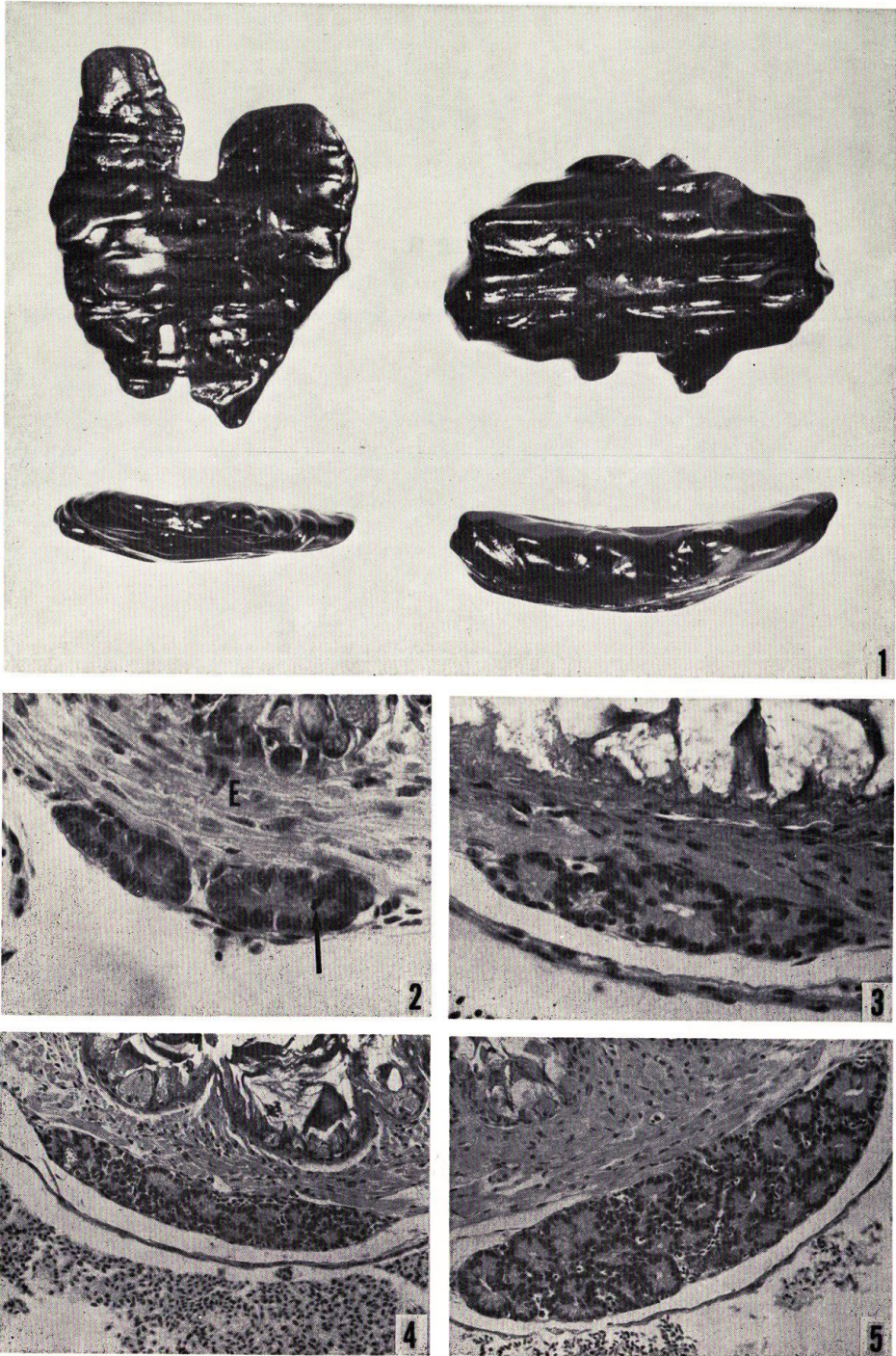
Figures 15-20 in Plates VI-VIII are electron-micrographs of the ultimobranchial gland to show the fine structural differences of the gland cells with sex and maturation. All stained with uranyl acetate and lead citrate.

ABBREVIATIONS

AC: agranular cell; BC: blood corpuscle; BM: basement membrane; C: cilia; CF: collagen fibrils; CL: capillary lumen; D: desmosome; E: esophagus; EC: endothelial cell; ER: rough surfaced-endoplasmic reticulum; FL: follicular lumen; GA: Golgi apparatus; GC: granular cell; I: interdigitated plasma membrane; IS: intercellular space; L: lysosome; M: mitochondria; MS: membranous structure; MV: microvilli; OI: osmiophilic inclusions; PS: perivascular space; SG: secretory granules

PLATE I

- Fig. 1. Three-dimensional models of the ultimobranchial glands of zebrafish. Upper (ventral view) and lower (horizontal view) left: from a mature male (35 mm in total-length). Upper and lower right: from a mature female (35 mm in total-length). The volumes of the original glands were 2.00×10^{-3} mm³ (male) and 3.22×10^{-3} mm³ (female), respectively. $\times 150$.
- Fig. 2. From an immature fish (12 mm in total-length). The mitotic figure is indicated by an arrow. $\times 430$.
- Fig. 3. From an immature fish (22 mm in total-length). $\times 370$.
- Fig. 4. From a mature male (35 mm in total-length). $\times 190$.
- Fig. 5. From a mature female (32 mm in total-length). The follicular arrangement is more obvious because of the cellular hypertrophy. $\times 190$.

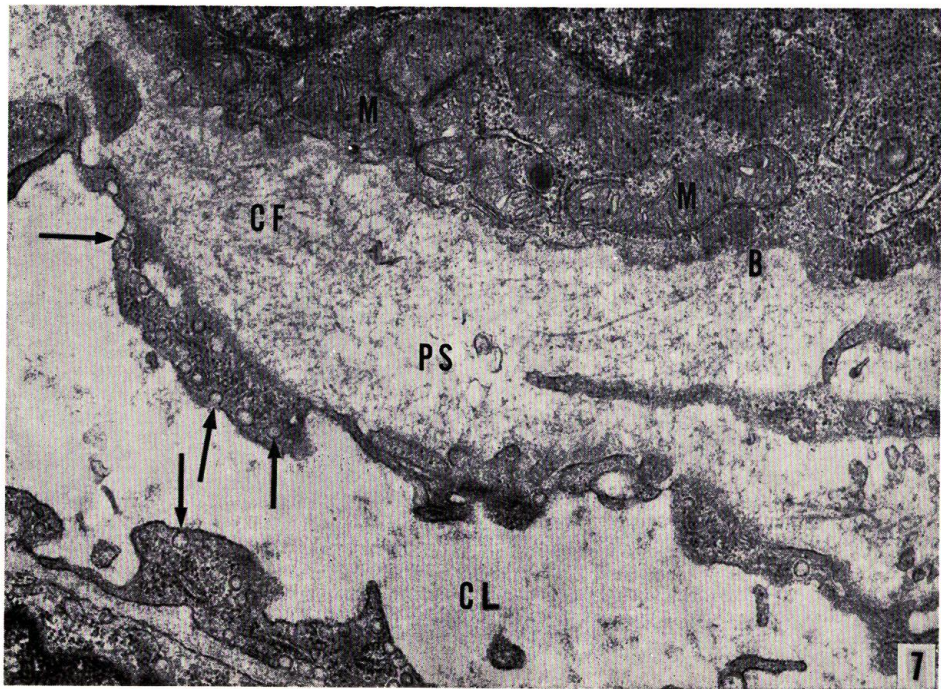
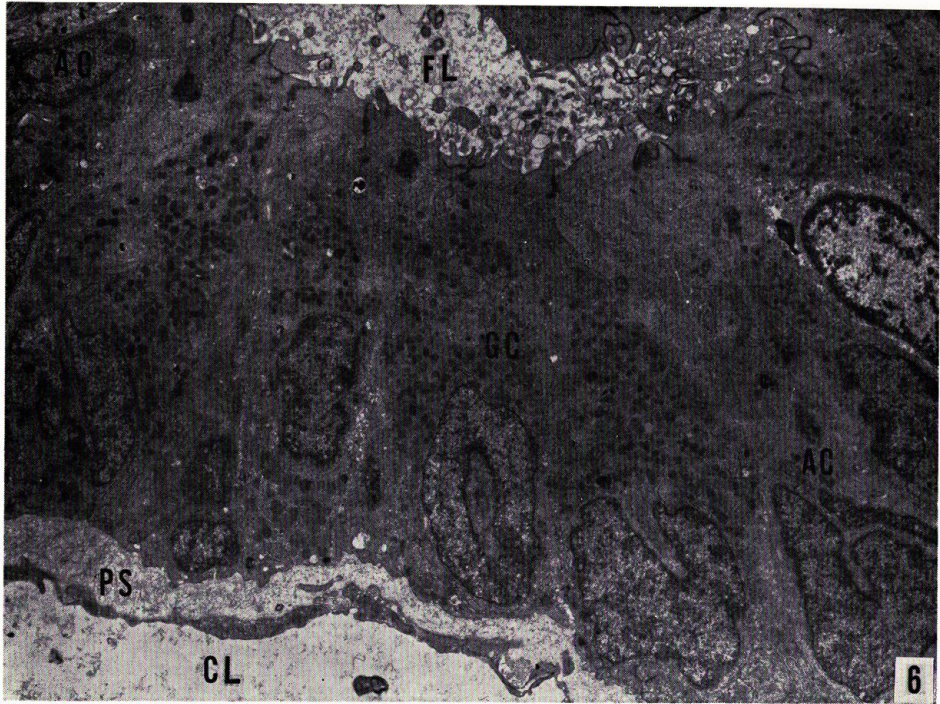


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PLATE II

Fig. 6. Typical follicle. $\times 5100$.

Fig. 7. Basal part of the follicle. Arrows indicate pinocytotic invaginations and vesicles.
 $\times 20000$.

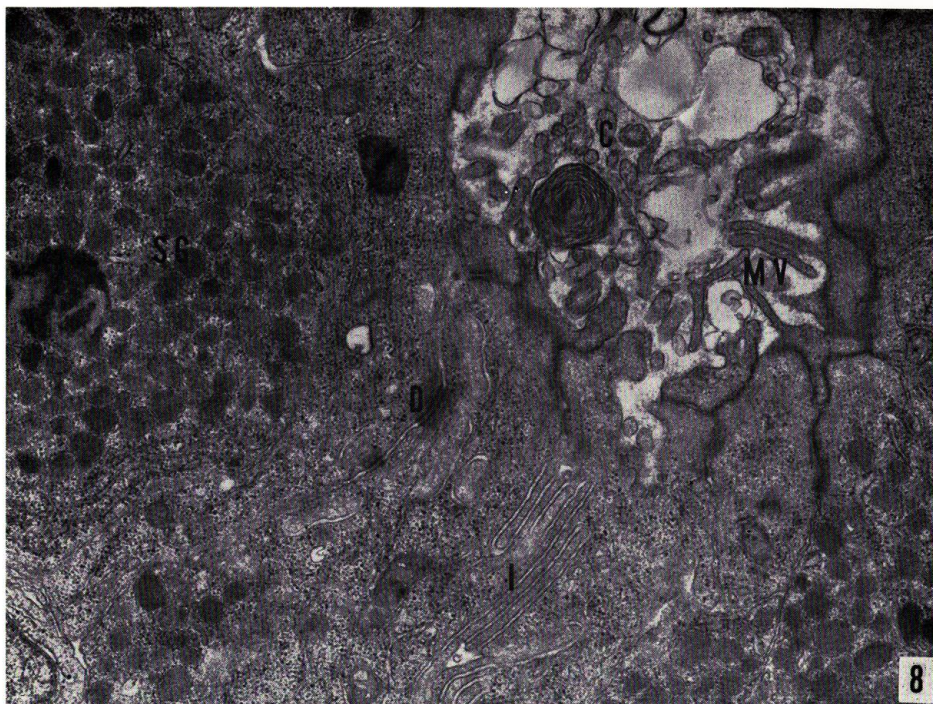


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PLATE III

Fig. 8. Apical part of a follicle. $\times 16000$.

Fig. 9. Agranular cells showing many pinocytotic invaginations (arrows) along the basal and lateral cell surface. $\times 22000$.

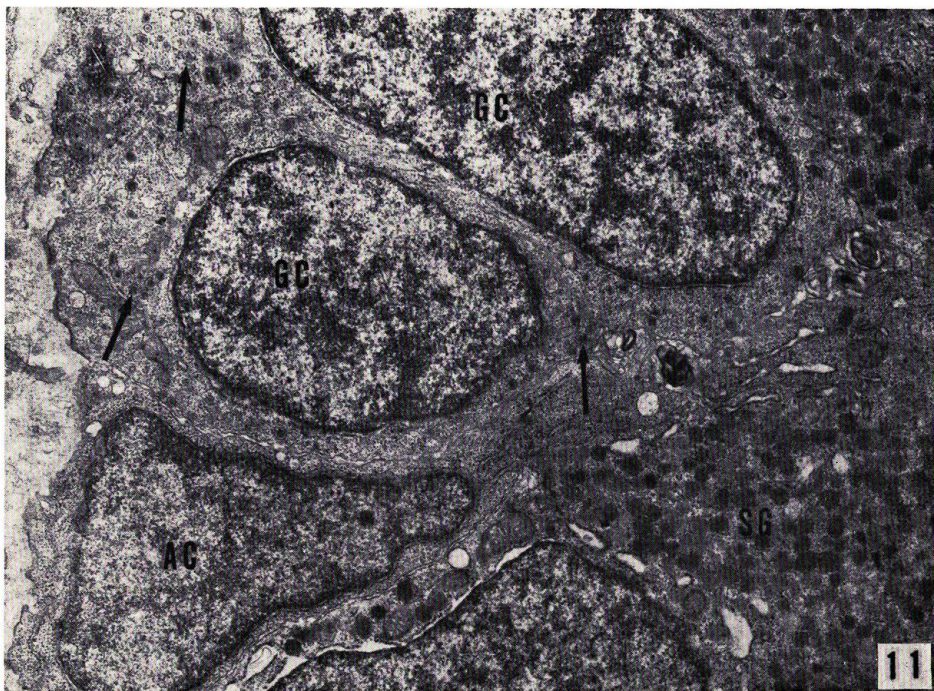
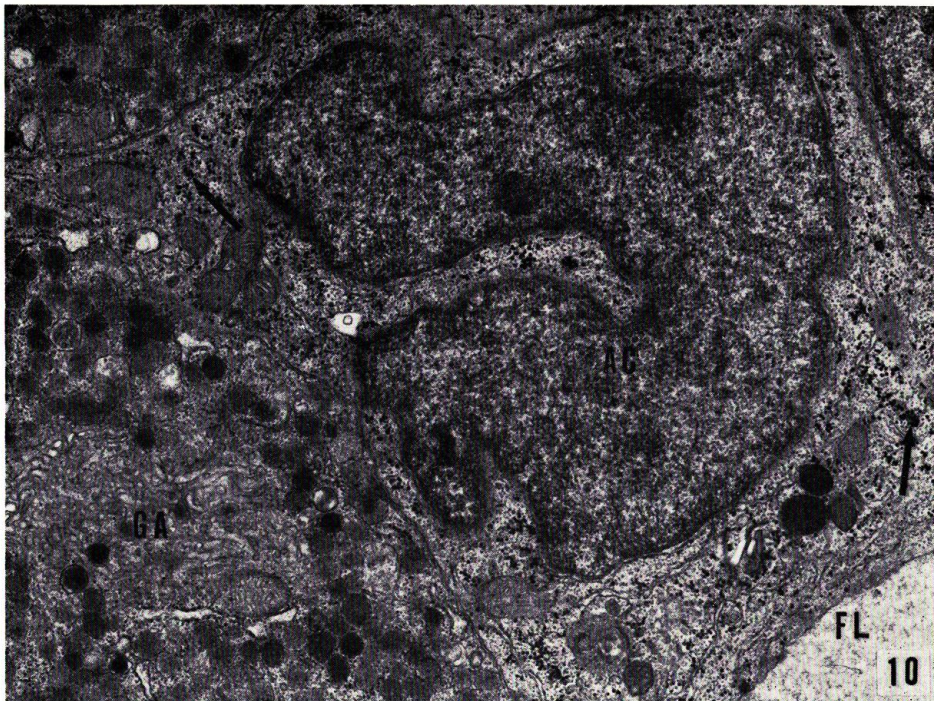


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PLATE IV

Fig. 10. Agranular cell containing glycogen particles (arrows). $\times 15000$.

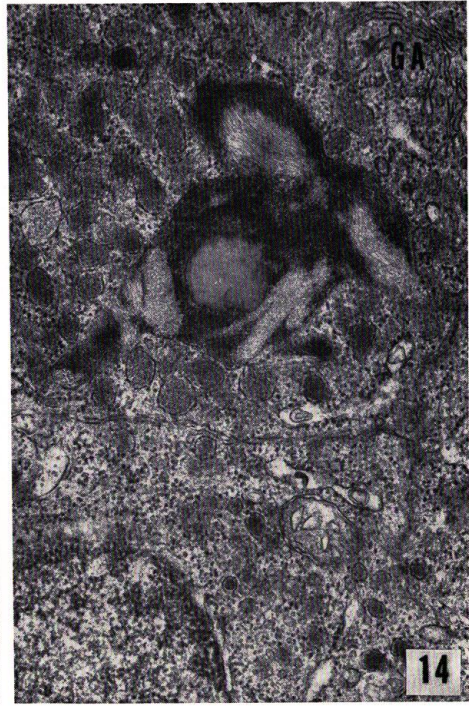
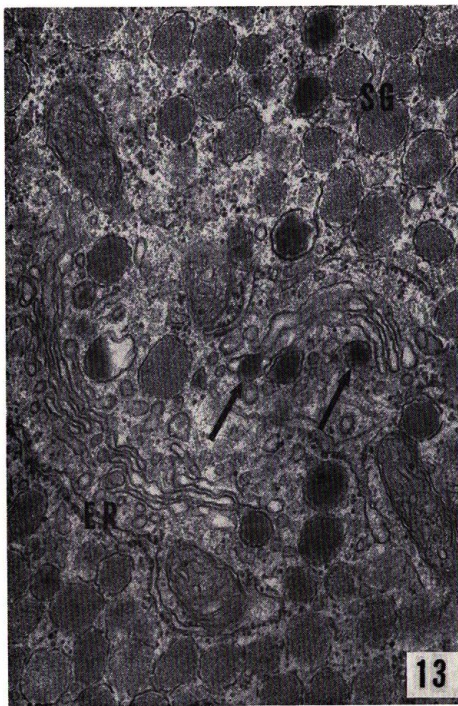
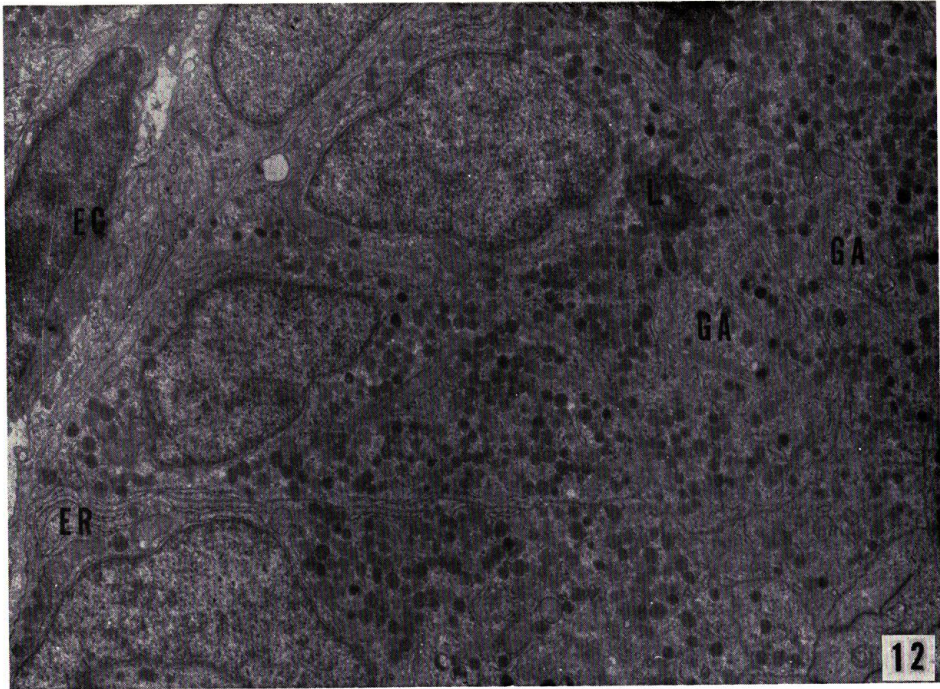
Fig. 11. Immature granular cells containing a few, very small secretory granules (arrows).
 $\times 11000$.



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PLATE V

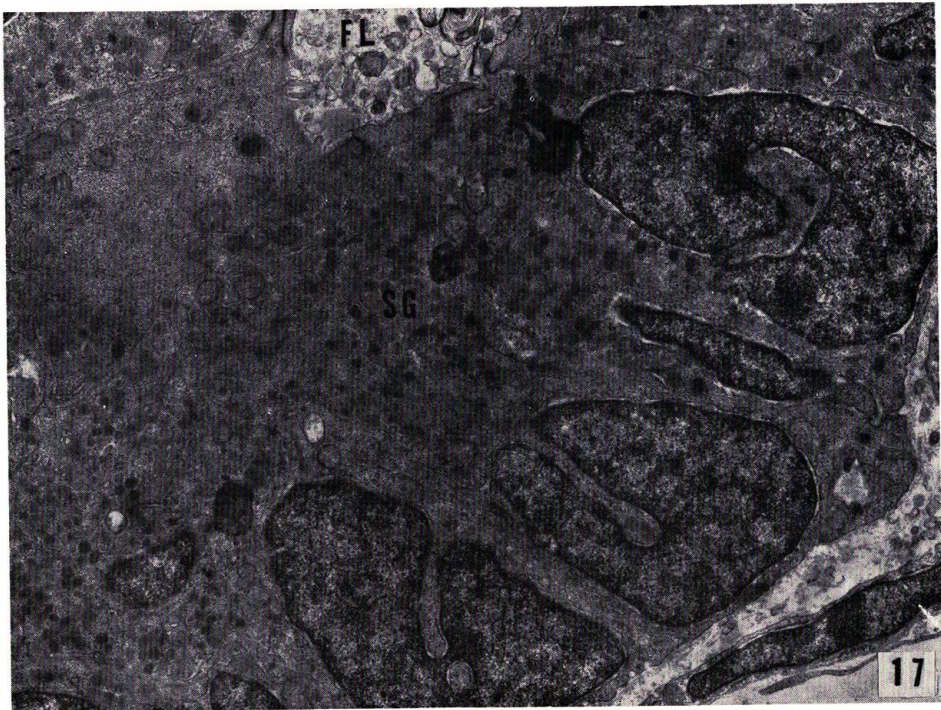
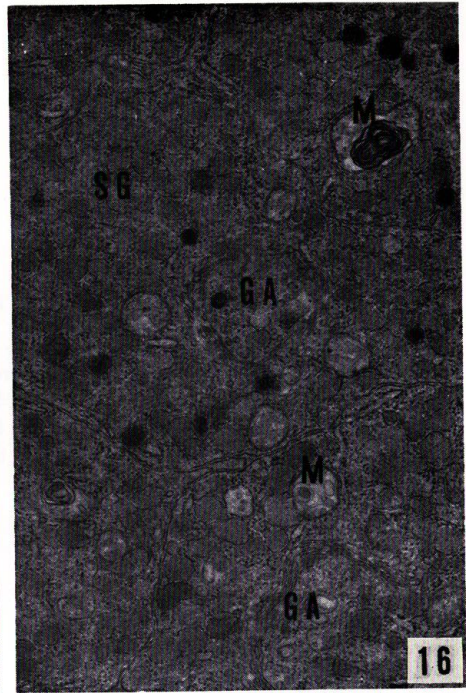
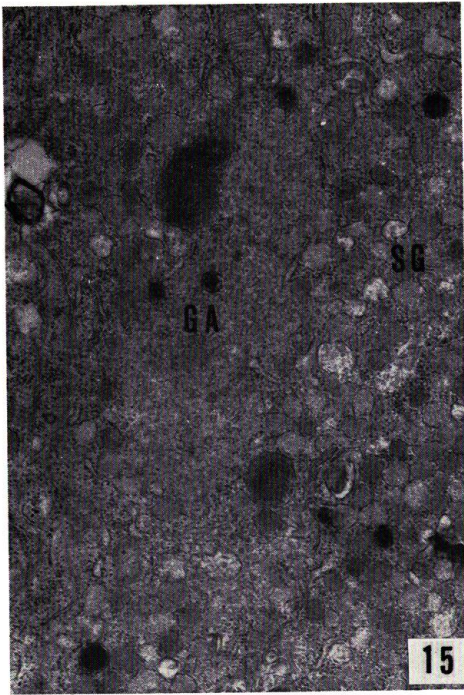
- Fig. 12. Granular cells containing a large accumulation of secretory granules. The r-ER are seen around the nucleus and along the lateral plasma membrane. $\times 7900$.
- Fig. 13. Well-developed Golgi apparatus in a granular cell. The Golgi elements contain dense materials (arrows). $\times 28000$.
- Fig. 14. Secondary lysosome near Golgi apparatus in a granular cell. $\times 23000$.



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PLATE VI

- Fig. 15. Golgi apparatus composed of many small vesicles in a granular cell. Immature fish. $\times 16000$.
- Fig. 16. Degenerating mitochondria showing the disappearance of cristae or the appearance of lamellar bodies in the granular cells. Immature fish. $\times 16000$.
- Fig. 17. Follicle of a mature male. The epithelium is low in height and the granular cells contain many secretory granules. $\times 9800$.

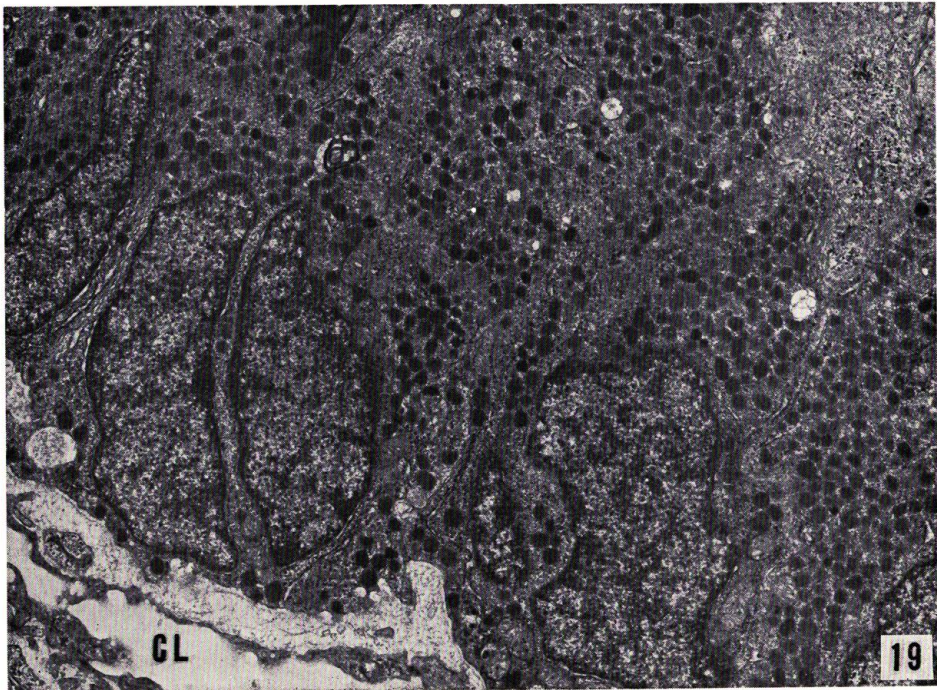


YAMANE: Ultimobranchial gland of zebrafish

PLATE VII

Fig. 18. Granular cells of a mature female eight hours after spawning. The granular cells possess an accumulation of numerous secretory granules. The r-ER is seen along the lateral plasma membrane . $\times 10000$.

Fig. 19. Granular cells of a mature female two days after spawning. Fewer secretory granules are noted in comparison with those eight hours after spawning (Fig. 18). $\times 10000$.



YAMANE: Ultimobranchial gland of zebrafish

PLATE VIII

Fig. 20. Follicle composed predominantly of agranular cells in a mature female two days after spawning. The intercellular spaces are expanded. Osmiophilic inclusions of needle-shaped substances occur in the follicular lumen. A granular cell which contains a few, very small granules (arrows) is seen between agranular cells. $\times 12000$.



YAMANE: Ultimobranchial gland of zebrafish