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## Promotion by Clomiphene Citrate of Gonadal Development and Body Growth in Immature and Maturing Goldfish, *Carassius auratus*

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### Abstract

Maturing male and female goldfish, *Carassius auratus*, treated with clomiphene citrate at a dose of 1  $\mu\text{g/g}$  body weight every third day for 30 days showed significant advance in gonadal development. Their pituitary gonadotrophs displayed evident degranulation accompanied by extensive dilatation of cisternae of the rough endoplasmic reticulum, indicating a stimulated release of gonadotropin which was responsible for the precocious development of the gonads. Sexually immature goldfish did not respond to clomiphene with acceleration of gonadal maturation, but their pituitary gonadotrophs revealed cytological signs of activation.

Treatment with clomiphene appeared to promote body growth of goldfish irrespective of sex and the degree of maturity. In pituitary somatotrophs of clomiphene-treated fish, degranulation and a dilatation of the rough endoplasmic reticulum were encountered. This suggests that clomiphene citrate can accelerate body growth as well as stimulate gonadal development in maturing goldfish through augmentation of somatotropic as well as gonadotropic activities of the pituitary gland.

Since Pandey and Hoar<sup>1)</sup> reported the ovulation-inducing effect of clomiphene citrate, a known ovulation stimulant of mammals, in goldfish, *Carassius auratus*, several authors have studied the action of the drug in teleosts. Clomiphene has been shown to be effective in causing ovulation in loach, *Misgurnus anguillicaudatus*<sup>2)</sup>, and catfish, *Heteropneustes fossilis*<sup>3)</sup>, though it was reported to be without effect in the cyprinid, *Labeo rohita*<sup>4)</sup>. Clomiphene exerts the effect through stimulation of pituitary gonadotropic activity, for it failed to induce ovulation in hypophysectomized females of the goldfish<sup>5)</sup> and catfish<sup>3)</sup> and evoked prominent ultrastructural changes of pituitary gonadotropic cells in the loach<sup>6)</sup>. Moreover, Breton et al.<sup>7)</sup> revealed that, in the carp, *Cyprinus carpio*, clomiphene brings about a significant rise in plasma gonadotropin content following a single intraperitoneal injection.

The work by Breton et al.<sup>7)</sup> is of particular interest since they disclosed the action of clomiphene to occur irrespective of sex and maturity of treated fish. Ovarian maturation followed by precocious ovulation was induced successfully by repeated injections of clomiphene in maturing female loaches, *Misgurnus anguillicaudatus*, in the pre-spawning period<sup>6)</sup>. This suggests the possibility that clomiphene citrate can accelerate the development of gonads of fish in early stages of maturation by stimulating precocious discharge of pituitary gonadotropic hormone(s). A series of experiments was planned to test these possibilities with

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maturing or immature goldfish, *Carassius auratus*, of both sexes. The present paper deals with the results of these experiments together with ultrastructural changes of glandular cells of the pituitary gland affected by clomiphene. Some comments will be made also on a possible growth-promoting action of clomiphene disclosed for the first time by the present study in the goldfish.

### Material and Methods

Goldfish, *Carassius auratus*, of the Wakin variety, bred and reared in the laboratory, were used in the present study. Maturing males and females, and immature fish of both sexes were divided into control and experimental groups and marked individually by fin clipping. They were kept in separate glass aquaria with approximately 50 liters of constantly aerated water at 23–25°C under an artificial photoperiod of 14-hour light and 10-hour darkness throughout the experiments, and fed on commercial pellets once a day.

Clomiphene citrate was dissolved in 0.6% saline at a concentration of 2  $\mu\text{g}$ /0.01 ml, and injected at a dose of 1  $\mu\text{g}$ /g body weight intraperitoneally into lightly anesthetized fish of experimental groups every third day for 30 days. Control groups received saline at a dose of 0.005 ml/g body weight. Immediately before each injection, the fish to be treated were measured for body weight and body length. They were killed by decapitation 3 days after the last injection, and their pituitary gland and gonads were rapidly removed for histological and cytological examinations. Ten initial controls were sacrificed for evaluation of gonadal condition at the start of each experiment.

For light microscopic observations, pieces of gonads were fixed in Bouin's fluid. Serial paraffin sections of the specimens were cut at 8  $\mu\text{m}$  in thickness, and stained with Delafield's hematoxylin and eosin. For electron microscopy, pituitary glands were immersed in 2.5% glutaraldehyde in 0.05M phosphate buffer (pH 7.4) for 2 hours at 4°C, and embedded in an epon-epoxy resin mixture. Ultra-thin sections were stained with uranyl acetate and Reynolds' lead citrate, and examined with a Hitachi HS-7 or HU-12 electron microscope. Thick sections cut at about 1  $\mu\text{m}$  were stained with methylene blue, and examined with light microscopy to compare with electron microscopic features of the pituitary gland.

Other detailed designs of the experiments were described in corresponding sections of the present paper.

### Results

In a preliminary series of experiments carried out from July through September, male and female goldfish were injected with 1  $\mu\text{g}$  clomiphene citrate/g body weight every third day for a period of 30 and 60 days. At the end of the treatment, the treated fish of both sexes showed considerably greater increases than the controls in gonadosomatic indices (GSI: gonad weight/body weight  $\times 100$ ) and in their body weight and body length. Since the effects of clomiphene treatment was apparent after 10 injections of the drug, the duration of treatment was shortened to 30 days in the following three experiments.

*Gonadal development*

In experiment 1, a group of 9 goldfish, averaging 9.9 g in body weight and 6.5 cm in body length, received the clomiphene treatment starting in November. A group of 9 fish of a similar size served as controls. The sex was identified 3 days after the last injection when the fish of both groups were sacrificed and their gonads were examined histologically. Females and males of the initial control group had ovaries with many oocytes in early vitellogenic phases (GSI 1.39%) and testes with germ cell cysts in various spermatogenetic stages (GSI 1.05%), apparently in the early process of gonadal maturation.

No significant difference was found in GSI values between the initial control fish and saline-injected controls of both sexes sacrificed at the end of the 30-day treatment (Table 1 a). Ovaries of the saline-injected fish were observed, histologically, to have regressed in development. Clomiphene injections were obviously effective in causing a notable development of the gonad in both sexes, since GSI values were significantly higher in treated fish than in controls (Table 1 a). Histologically, ovaries of the treated females were filled with many oocytes advancing to the secondary and tertiary yolk stages. Testes of clomiphene-treated fish also revealed accelerated spermatogenesis: sperm content in affected testes was considerably larger than in controls.

In experiment 2 carried out during the months from February to March, clomiphene treatment was made on a group of 11 fish of 14.1 g in mean body weight and 7.0 cm in mean body length. The experimental fish, and controls of the same number were laparotomized and their gonads were biopsied prior to treatment in order to follow individually the gonadal development influenced by clomiphene. The degree of ovarian maturity at the start of treatment appeared to vary to some extent in different specimens: oocytes in the primary yolk stage were conspicuous in many ovaries, whereas those in the late peri-nucleolus stage were predominant in others. In biopsied testes, germ cell cysts in various spermatogenetic stages were present, but only a small amount of spermatozoa existed in lobule lumina.

Following 10 injections of clomiphene, GSI values in treated males and females were significantly higher than those in controls (Table 1 b). Ovaries of control fish showed little advance in development when compared with those biopsied at the start as the most advanced oocytes were in the primary yolk stage in these ovaries. In contrast, the ovaries of clomiphene-treated fish, whose oocytes had been in the primary yolk stage at the start of treatment, developed into the tertiary yolk stage at the end. In ovaries in which a majority of oocytes had been in the peri-nucleolus stage, oocytes of the yolk vesicle stage increased in number following the treatment. In control males, testes examined at the end of treatment were not different in histological aspects from those observed at the start. In testes of treated males, on the contrary, a large amount of spermatozoa had accumulated in widely expanded lumina of testicular lobules.

Experiment 3 began in May with young goldfish of 4.8 g in mean body weight and 4.9 cm in mean body length. Two groups of 10 fish each were used as an experimental and a control group, but only 6 survived 10 repeated injections of clomiphene. Observations of the gonads of initial controls showed that their gonads were very small in size and were quite immature: the most advanced stage

Table 1. *Changes in body weight, body length and gonadosomatic index (GSI) in females (F) and males (M) of the goldfish treated with clomiphene citrate (Cl. C.).*

Group	Sex	No. of fish	Weight change* (%)	Length change* (%)	GSI*
<b>a. Experiment 1</b>					
Control	F	3	23.70±8.37	6.12±1.15	1.28±0.23
	M	6	14.19±3.75	5.44±1.07	1.22±0.09
	F	4	40.16±5.40***	10.04±0.74***	4.85±1.02**
	M	5	46.23±7.10**	12.77±0.98**	3.02±0.35**
<b>b. Experiment 2</b>					
Control	F	7	-2.94±5.60	3.49±1.45	1.78±0.55
	M	4	-6.59±3.22	2.77±1.08	2.30±0.79
Cl.C.	F	5	5.68±5.17	6.39±1.24	5.23±0.90**
	M	6	-1.07±5.28	2.71±2.36	4.08±0.62***
<b>c. Experiment 3</b>					
Control	F	5	13.68±5.43	8.75±1.41	0.96±0.07
	M	4	11.58±9.28	4.17±2.80	0.49±0.07
Cl.C.	F	4	32.01±3.48**	9.75±3.20	0.72±0.04
	M	2	88.21, 29.01	17.87, 9.72	1.15, 0.96

\* Mean±SE    \*\* Significantly greater than control ( $P<0.01$ ).

\*\*\* Significantly greater than control ( $P<0.05$ ).

of germ cell development was the early peri-nucleolus stage in ovaries and the spermatogonial stage in testes at the start of treatment.

Clomiphene treatment did not accelerate gonad weight in the treated fish following the 30-day treatment (Table 1 c). Signs of stimulated development of germ cells could scarcely be detected both in ovaries and in testes. In treated ovaries, oocytes still remained in the early peri-nucleolus stage as in those of saline-injected controls. In treated testes as well as in control ones, germ cells did not develop beyond the spermatogonial stage.

### *Body growth*

In accord with the results obtained by the preliminary experiments, it was observed in experiments 1 and 3 that clomiphene-injected goldfish grew faster than saline-injected controls irrespective of their sex. In experiment 1 in particular, males and females subjected to 10 injections of clomiphene showed significant gains not only in body weight but in body length in comparison with controls (Table 1 a). The increase in body weight of the treated fish was significant even with regard to the weight increment of their gonads. Moreover, clomiphene seemed to exert its growth-promoting effect also on the immature goldfish of experiment 3 in which it failed to stimulate the development of their gonads, though the number of the fish examined was insufficient to insure the result (Table 1 c).

In contrast, the weight change of laparotomized fish was not pronounced by the treatment but appeared to be inhibited during the treatment (Table 1 b). Control fish also had suppressed growth in weight in the same experiment. It

seems likely that the young fish which had been laparotomized prior to treatment for determining the initial developmental state of their gonads could not recover from the ensuing physiological stress throughout the treatment and thus could not respond duly with a promotion of growth to the clomiphene treatment.

#### *Changes of the pituitary gland*

In general, basophils with conspicuous globules in the cytoplasm, which are comparatively large in number and are distributed mainly in the ventral part of the proximal pars distalis (PPD) of the goldfish pituitary, are easily identified as gonadotrophs. The cells are varied in shape, though round or elliptical in most cases, and are largest among glandular cells in the PPD, measuring 10–15  $\mu\text{m}$  in diameter. In epon-embedded 1  $\mu\text{m}$  sections, the cytoplasm of gonadotrophs was packed with fine granules intensely stained with methylene blue. Moreover these cells were characterized by having several large, lightly stained globules buried among the fine granules. Both the small granules and large globules of gonadotrophs of maturing fish (Fig. 1) were found to be larger in size and number than those of immature ones (Fig. 3).

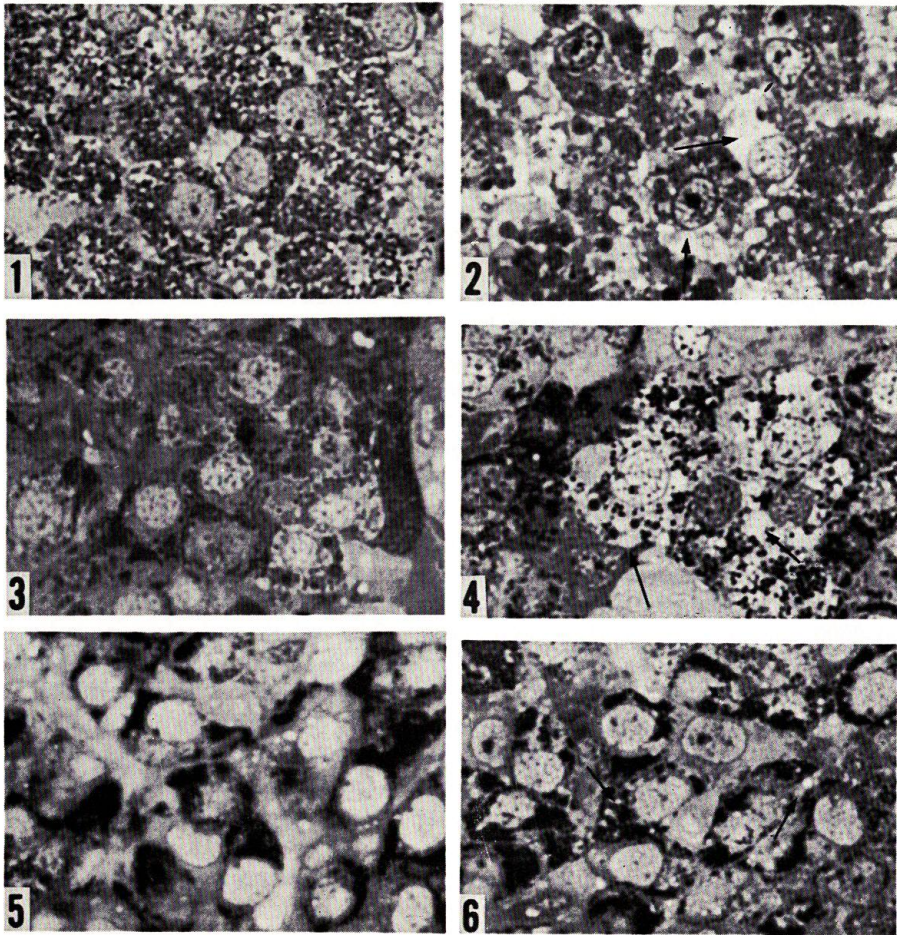
Electron microscopically, gonadotrophs of maturing goldfish were also characterized by the presence of numerous small, electron-dense granules of about 150–250 nm in diameter and a few larger, less electron-dense globules of about 1000–2500 nm in size in their cytoplasm. The rough endoplasmic reticulum consisted of slightly dilated cisternae, and Golgi apparatus developed moderately with only a few small vesicles (Fig. 7). In gonadotrophs of immature fish, the two kinds of cytoplasmic inclusions were found to be smaller in size and number than those of maturing fish: small granules were 100–200 nm and globules about 800–1200 nm in size. The rough endoplasmic reticulum of these cells was composed of flat or vesicular cisternae (Fig. 9) and Golgi apparatus and mitochondria were poorly developed (Fig. 10).

Gonadotrophs in the pituitary gland of clomiphene-treated goldfish displayed some characteristic changes, regardless of sex and the degree of maturity of the fish. Histologically, gonadotrophs of clomiphene-treated fish were observed to have many small vacuoles in the cytoplasm, and their small granules were clearly decreased in amount (Figs. 2 and 4).

Ultrastructurally, gonadotrophs of clomiphene-treated maturing fish showed a distinct decrease in number of small granules of 150–250 nm in size. Although large globules in these cells appeared to remain unchanged in number, they were observed to become irregular in shape in some cases. The rough endoplasmic reticulum was composed of numerous cisternae which were conspicuously dilated to various degrees. Mitochondria were much enlarged and Golgi apparatus was increased in volume with many vacuoles and small vesicles (Fig. 8).

Similar ultrastructural changes occurred in gonadotrophs of clomiphene-treated immature fish that failed to show gonadal development. Small cytoplasmic granules in these cells were clearly diminished in number, while large globules remained unchanged in number but appeared to increase in electron density and decreased in size to about 600–800 nm. The rough endoplasmic reticulum showed a marked dilatation, but the change was less prominent than in the cells of



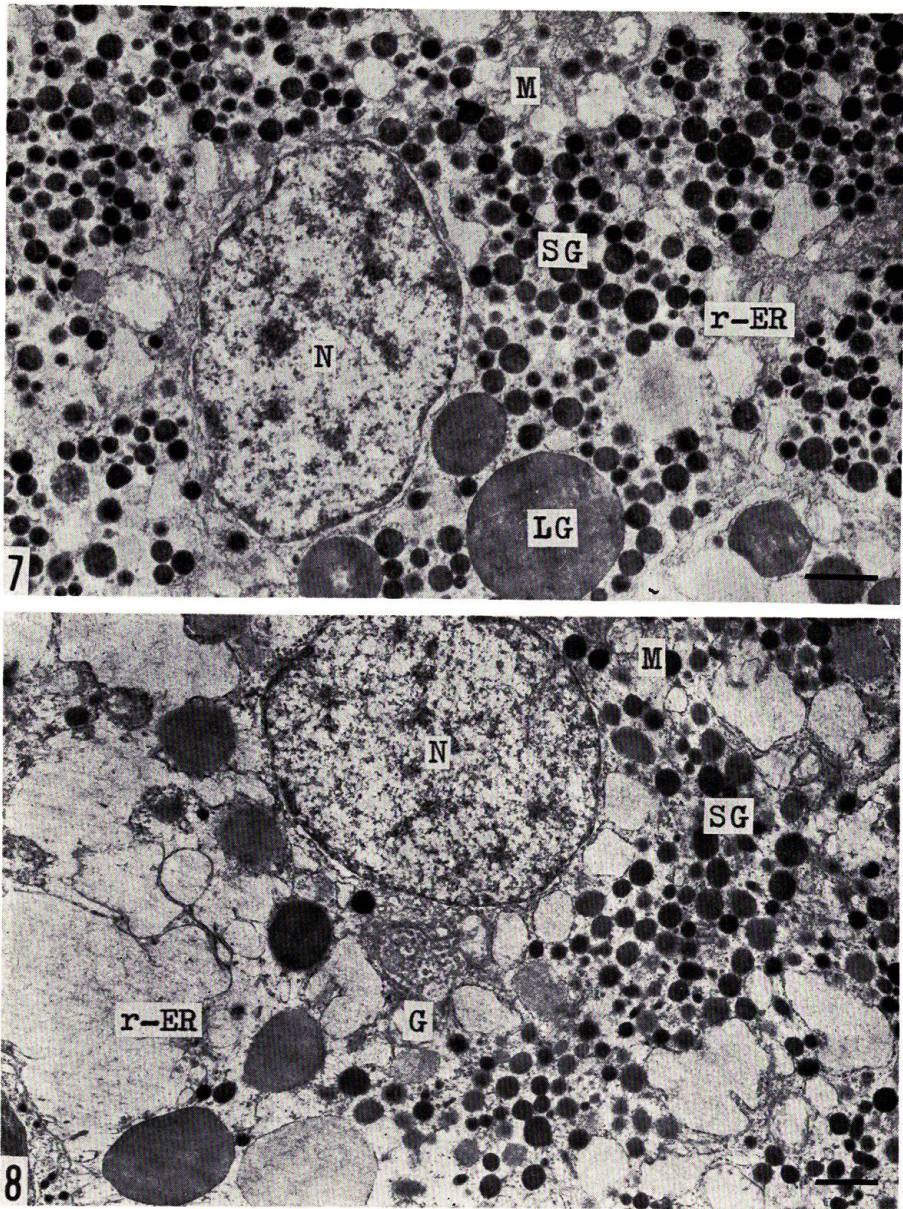


Figs. 1-6. Sections of epon-embedded pituitary glands of saline-injected (Figs. 1, 3 and 5) and clomiphene-treated goldfish (Figs. 2, 4 and 6). Figs. 1 and 2 demonstrate gonadotropic cells of maturing fish, Figs. 3 and 4 those of immature ones, and Figs. 5 and 6 somatotrophic cells of immature ones. Arrows indicate cytoplasmic vacuolization in these cells influenced by clomiphene treatment. Methylene blue stain.  $\times 850$ .

maturing fish. Mitochondria increased in size, and Golgi apparatus also increased in volume and contained a few small vesicles (Fig. 11).

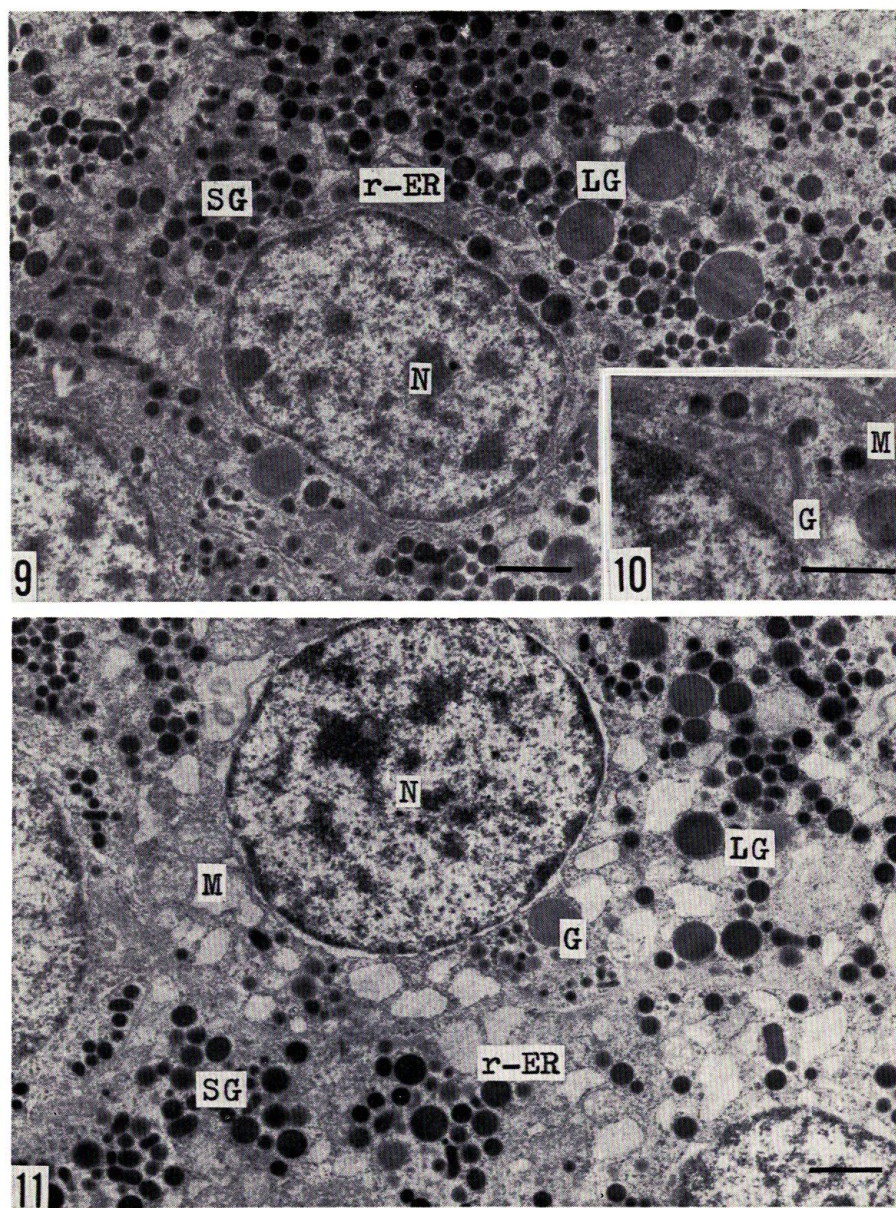
Among other glandular cell types of the adenohypophysis, only somatotrophs displayed some cytological changes denoting their functional activation following clomiphene treatment. Acidophilic somatotrophs are generally found in the central and dorsal regions of the PPD, and measure  $8-13\mu\text{m}$  in diameter. Most of the cells are round or elongate in shape, with the nucleus of oval or elongate shape (Fig. 5). Their cytoplasm was stained relatively deeply with methylene blue. Ultrastructurally, somatotrophs in the PPD of immature fish had one kind of





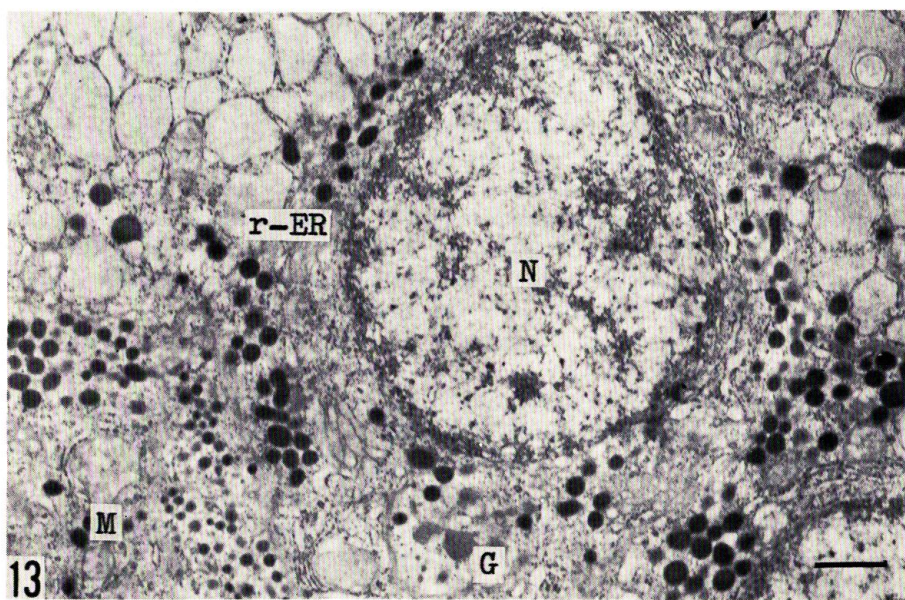
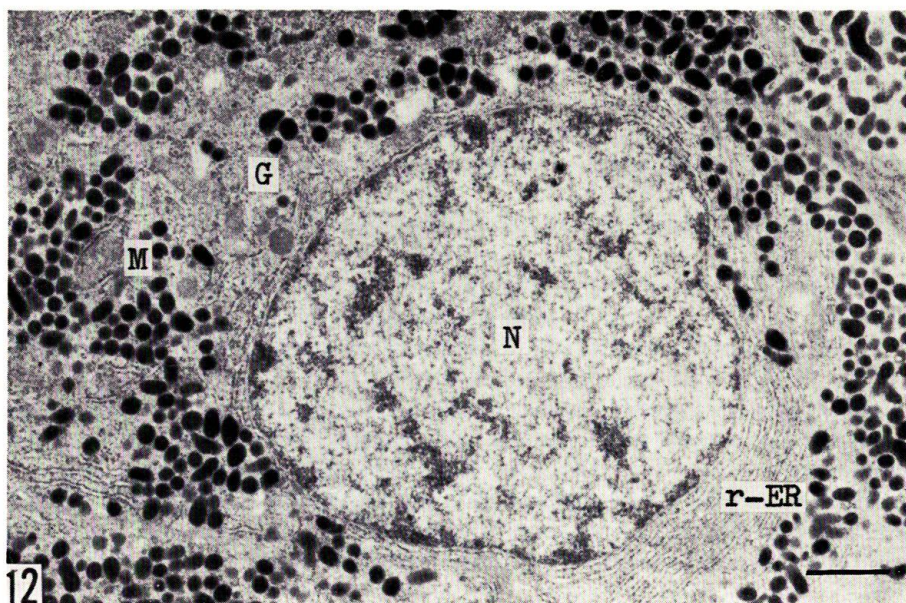
Figs. 7 and 8. Electron micrographs of gonadotrophs in the pituitary gland of a saline-injected (Fig. 7) and a clomiphene-treated maturing goldfish (Fig. 8). *G*, Golgi apparatus; *LG*, large globule; *M*, mitochondrion; *N*, nucleus; *r-ER*, rough endoplasmic reticulum; *SG*, small granule. Scales, 1  $\mu$ m.





Figs. 9-11. Electron micrographs of gonadotrophs in the pituitary gland of a saline-injected (Figs. 9 and 10) and a clomiphene-treated immature goldfish (Fig. 11). *G*, Golgi apparatus; *LG*, large globule; *M*, mitochondrion; *N*, nucleus; *r-ER*, rough endoplasmic reticulum; *SG*, small granule. Scales, 1  $\mu$ m.





Figs. 12 and 13. Electron micrographs of somatotrophs in the pituitary gland of a saline-injected (Fig. 12) and a clomiphene-treated immature goldfish (Fig. 13). *G*, Golgi apparatus; *M*, mitochondrion; *N*, nucleus; *r-ER*, rough endoplasmic reticulum. Scales, 1  $\mu$ m.

small, possibly secretory, granules of 100–250 nm in diameter packing the cytoplasm. The granules were mostly round but sometimes elongate in shape. The rough endoplasmic reticulum consisted of flat cisternae which sometimes appeared as well-developed parallel lamellae. Golgi apparatus was moderately developed, and mitochondria were roundish or elongate in shape (Fig. 12). Somatotrophs of maturing fish were almost equivalent in ultrastructure to those of immature ones.

Following the treatment with clomiphene, somatotrophs were seen to become vacuolated (Fig. 6), though the vacuolation appeared to be less conspicuous than that found in the activated gonadotrophs. It was evident by electron microscopy that, in somatotrophs of clomiphene-treated fish, a prominent decrease in number of granules of 100–250 nm in size occurred concomitantly with conspicuous dilatation of the rough endoplasmic reticulum. In these cells mitochondria were large in size and were mostly round in shape. Golgi apparatus increased in volume and consisted of several small vesicles and vacuoles; granules in various stages of formation were observed in this area (Fig. 13). The ultrastructural changes of somatotrophs affected by clomiphene were also observed in the pituitary gland of maturing fish of the 2nd experiment, in which the fish did not show any acceleration of body growth in response to clomiphene treatment.

### Discussion

It has been previously reported<sup>6)</sup> that clomiphene citrate injected into female loaches, *Misgurnus anguillicaudatus*, of the pre-maturation stage successfully caused precocious ovulation following a promotion of the development of affected ovaries. Comparable results were obtained in the present study with young goldfish with gonads in the process of their maturation. Clomiphene was apparently effective in accelerating maturation of ovaries in which oocytes had been in or beyond the yolk vesicle stage at the start of treatment. In addition, the drug stimulated the testis strikingly to increase spermatogenesis, which had yielded only a small amount of spermatozoa at the start of treatment.

In teleost fishes, as well as in mammals, clomiphene may exert its influence on the gonad through the pituitary gland: hypophysectomy of mature females of goldfish, *Carassius auratus*<sup>5)</sup>, and catfish, *Heteropneustes fossilis*<sup>3)</sup>, was reported to repress completely the ovulation-inducing effect of clomiphene. Breton et al.<sup>7)</sup> demonstrated that clomiphene injected into carp, *Cyprinus carpio*, resulted in a significant rise in plasma gonadotropin (GTH) level independently of the state of maturity and sex of the fish treated. Since vitellogenesis and spermatogenesis in teleost fishes are known to be essentially under the control of pituitary GTH<sup>8)</sup>, it is highly likely that the gonadal maturation in females and males of clomiphene-treated goldfish is due to the augmentation of GTH release from the pituitary gland in response to clomiphene treatment. This is supported by electron microscopic studies on pituitary gonadotrophs affected by clomiphene.

In clomiphene-treated goldfish of both sexes, pituitary gonadotrophs were characterized by a decrease in amount of secretory granules accompanied with a dilatation of cisternae of the rough endoplasmic reticulum. Similar prominent changes were observed in the pituitary gonadotrophs of the loach following clomi-

phene-induced ovulation<sup>6)</sup>, denoting the occurrence of a surge of GTH release for achieving ovulation. Although pituitary gonadotrophs of maturing fishes may probably be less responsive to clomiphene than those of fully matured ones, the observed changes of the goldfish pituitary cells can be indicative of augmented release of GHT caused by clomiphene treatment.

It was evident that gonads of immature male and female goldfish were quite refractory to clomiphene treatment but that pituitary gonadotrophs of these fish appeared to respond fairly with activated discharge of their secretory products as Breton et al.<sup>7)</sup> noticed in their studies on immature carp. Hypophysectomy studies in the goldfish show that the germ cells in those stages of development are not under the control of GTH<sup>9)</sup>. However, biopsy studies carried out in the present experiments showed that oocytes of the late peri-nucleolus stage could reach the yolk vesicle stage under the influence of clomiphene. It remains uncertain at present whether the germ cells at early stages of development are unresponsive to GTH or whether pituitary gonadotrophs of immature fish are not capable of releasing a sufficient amount of GTH, even with clomiphene treatment, to stimulate gonadal development.

The results substantiate that, in the goldfish, clomiphene can bring about a precocious development of the gonad in both sexes, if their gonads have once begun to be controlled by GTH, by augmenting the release of pituitary GTH. The responsiveness of male fish to clomiphene is interesting, since it has been suggested that, for inducing ovulation in fishes<sup>7,10)</sup> as well as in rat<sup>11)</sup>, the drug acts as an antiestrogen by counteracting the GTH-suppressing action of endogenous estrogen. Although there might be a possibility of direct stimulative action of clomiphene on testicular steroidogenic tissue<sup>12)</sup> and in turn on spermatogenesis, the present results point to the maturation-promoting action of the drug through an activation of pituitary gonadotropic potency. A possible negative feedback regulation of pituitary GTH release by testicular secretions has been indicated by the result of unilateral castration in the goldfish<sup>13)</sup> and the rainbow trout, *Salmo gairdneri*<sup>14)</sup>, and by the determination of circulating GTH levels after castration in the trout<sup>15)</sup>. On the other hand, according to Breton et al.<sup>16)</sup>, a considerable amount of estradiol-17 $\beta$  is found in the circulating blood of the male trout. The mechanism involved in the action of clomiphene in male goldfish is open to question.

Finally, the present study demonstrated for the first time the possibility that clomiphene could also promote body growth of goldfish regardless of sex and the degree of their gonadal maturation. Somatotrophs of the pituitary gland of treated fish appeared to be activated with degranulation and a marked dilatation of cisternae of the rough endoplasmic reticulum, denoting the occurrence of a stimulated discharge of somatotropin from the cells under the influence of clomiphene. So far as the present writers know, no report has been concerned to date with other actions of clomiphene citrate than the ovulation-inducing and antiestrogenic ones. This is probably because the other experiments have been done during comparatively short periods of time with fully matured animals. Further studies are now in progress to establish, in teleost fishes, the growth-promoting effect of clomiphene which seems to be useful, in combination with its maturation-stimulating effect, for the practice of fish propagation.



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