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Promotion of Ovarian Maturation Accompanied with Ovulation and Changes of Pituitary Gonadotrophs after Ovulation in the Loach, *Misgurnus anguillicaudatus*, Treated with Clomiphene Citrate

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Abstract

Clomiphene citrate injected intraperitoneally into adult female loaches, *Misgurnus anguillicaudatus*, of the pre-spawning period daily or every third day at a dose of 1 μg/g body weight caused a notable promotion of the maturation of ovaries followed by ovulation in all of the treated fish. None of saline-injected control fish revealed the full ovarian maturation and ovulation during the experimental periods. No significant differences were found in the degree of the response between the fish treated daily and those treated every third day.

In the proximal pars distalis of the pituitary gland of the loach, two types of basophilic cells, which were regarded as gonadotropic cells, were observed to display characteristic morphological changes following ovulation in response to clomiphene treatment. Most prominent alterations in these cells were a decrease in the amount of small, possibly secretory granules and a remarkable dilatation of cisternae of the rough endoplasmic reticulum. The changes were well consistent in ultrastructural aspects with those described for pituitary gonadotropic cells of some teleost fishes after ovulation. Thus it seems highly likely that clomiphene can induce gonadal maturation and ovulation through an activation of secretory functions of gonadotrophs of the pituitary gland in the loach.

Introduction

Clomiphene citrate, a potent ovulation stimulator for mammalian animals, has been shown to be effective in inducing ovulation in the goldfish, *Carassius auratus*[1] and in the loach, *Misgurnus anguillicaudatus*[2]. Moreover it has been evidenced in the carp, *Cyprinus carpio*, that a significant liberation of pituitary gonadotropin occurs following the injection of clomiphene irrespective of the state of maturity and sex of the carp treated[3]. These findings, especially those by Breton *et al.*[3], enabled the present writers to consider the possibility that clomiphene may be potent to promote the gonadal maturation followed by precocious ovulation in maturing female fishes through a stimulated release of gonadotropin from the pituitary gland.

The aim of the present study is to examine whether or not clomiphene can promote the maturation of ovaries accompanied by ovulation in maturing female loaches, *Misgurnus anguillicaudatus*, in the pre-spawning period, and whether the effect of clomiphene is mediated by an activation of pituitary gonadotropic cells in the loach as well as in the carp[3].

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Material and Methods

The loach, *Misgurnus anguillicaudatus*, ranging from 9.85 to 17.34 cm in total length and from 4.52 to 18.12 g in body weight, were collected in the suburbs of Hakodate in May. They were subsequently kept in an aquarium set in the laboratory, and were fed on commercial pellets once a day. Experiments were commenced for these fish within a week after the collection. At the start of experiment, 10 females were sacrificed and served as initial controls. Other 26 maturing females were divided into 4 groups of 6 to 7 fish each, and were kept in separate glass aquaria containing about 30 liters of well-aerated water under the natural light condition. Water temperature was not regulated and ranged from 13 to 15°C during the experiment. The fish were not fed during the experimental period.

Two series of experiment, each with females of an experimental and a control group, were carried out in the present study. In the first series, clomiphene citrate, which was previously dissolved in 0.6% saline at the concentration of 1 μg/0.01 ml, was injected at a dose of 1 μg/g body weight intraperitoneally into females of the experimental group once a day. In the second series, the same drug at the same dose was given to experimental females every third day. Control fish of the two series were injected with 0.6% saline only.

All of the experimental and control fish were examined for the occurrence of ovulation by stripping them every day following the first injection. Those that had been ovulated were autopsied, and pituitary glands and ovaries were preserved for histological and cytological observations. Fish of the control groups were sacrificed at the end of the experiments lasting for 10 and 15 days. Ovulated eggs were weighed for the estimation of the ovulation index (weight of ovulated eggs/weight of ovulated eggs+unovulated portion of the ovary $\times 100$).

For light microscopic observations, pieces of the ovaries were fixed in Bouin's fluid or in Bouin-Hollande solution. Serial paraffin sections of the specimens were cut at 8 μ in thickness, and stained with Delafield's hematoxylin and eosin. The pituitary glands were fixed in Susa or Zenker-formol solution, cut sagittally at 6 μ in thickness, and stained with aldehyde fuchsin-light green-orange G (AF) or with the periodic acid-Schiff (PAS) stain.

For electron microscopy, pituitary glands were immersed in 2.5% glutaraldehyde in 0.05 M phosphate buffer (pH 7.4) for 2 hours at 4°C and then postfixed in Millonig's OsO_4 solution for 2 hours at 4°C. After the dehydration through a graded ethanol series, the organs were embedded in an Epon-epoxy resin mixture. Ultrathin sections were stained doubly with uranyl acetate and Reynolds' lead citrate, and examined with a Hitachi HS-12 electron microscope. Thick sections cut at about 1 μ were stained with methylene blue, and examined light microscopically for a comparison with electron microscopic aspects of the pituitary gland.

Results

Acceleration of gonadal maturation and ovulation by clomiphene citrate

In the present study, maturing female loaches were used as material in May

Table 1. *Ovulation-inducing effect of clomiphene citrate (Cl.C.) injected into female loaches every day.*

Dose of Cl.C. (μg/g body wt.)	No. of fish	No. of ovulating fish each day									Mean body weight at autopsy (range) g	Mean gonad weight at autopsy (range) g	Mean GSI (range)	Mean ovulation index (range)
		1	2	3	4	5	6	7	8	9				
(Initial control)	10	-	-	-	-	-	-	-	-	-	7.92 (5.42-13.49)	0.66 (0.20-1.90)	7.35 (3.32-14.08)	
0	7	0	0	0	0	0	0	0	0	0	7.90 (5.34-17.72)	0.65 (0.20-1.79)	7.60 (3.51-11.68)	
1	6	0	0	0	0	0	(3)	(3)	5(1)	1	8.90 (4.62-17.53)	0.43 (0.06-1.06)	4.34 (1.21- 7.60)	62.09 (41.83-81.12)

Table 2. *Ovulation-inducing effect of clomiphene citrate (Cl.C.) injected into female loaches every third day.*

Dose of Cl.C. (μg/g body wt.)	No. of fish	No. of ovulating fish each day											Mean body weight at autopsy (range) g	Mean gonad weight at autopsy (range) g	Mean GSI (range)	Mean ovulation index (range)
		5	6*	7	8	9*	10	11	12*	13	14	15				
(Initial control)	10	-	-	-	-	-	-	-	-	-	-	-	7.92 (5.42-13.49)	0.66 (0.20-1.90)	7.35 (3.32-14.08)	
0	6	0	0	0	0	0	0	0	0	0	0	0	7.21 (5.99-11.27)	0.53 (0.27-1.16)	7.17 (3.86-10.31)	
1	7	0	(1)	(1)	(2)	2(5)	(5)	(5)	4(1)	(1)	(1)	1	7.13 (5.02-10.08)	0.33 (0.10-0.81)	3.68 (1.17- 8.07)	64.15 (28.53-81.45)

* The 1st injection of clomiphene was done on the day 0, the 2nd on the day 3, the 3rd on the day 6, the 4th on the day 9, and the 5th on the day 12.

when they were at a later phase of the pre-spawning period. Although the gonadosomatic index (GSI: gonad weight/body weight $\times 100$) for the control groups varied to some extent in different specimens examined, it averaged about 7% in the initial control group and remained little changed at the termination of experiment. In the ovaries of these fish, the most advanced oocytes were observed to be in the secondary yolk stage.

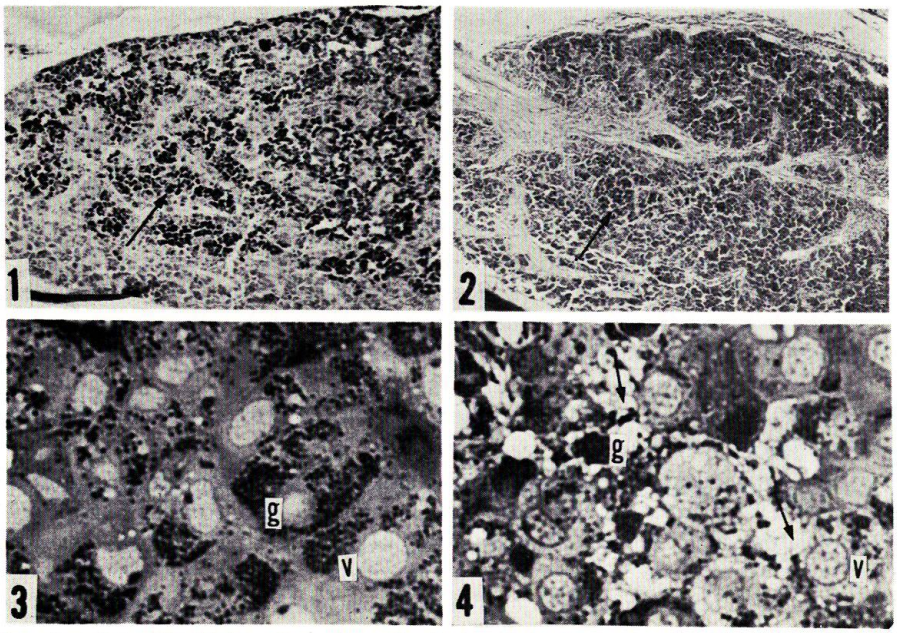
In the first series of experiments in which clomiphene was given daily, none of the control fish ovulated during the experimental period lasting for 10 days. By contrast, all of the treated fish responded with definite ovulation during the same period (Table 1). However, at least 6 successive injections were needed to induce a discharge of the least amount of eggs in these fish. Prominent ovulatory responses of the treated fish occurred on the 8th day: 5 out of 6 fish injected 8 times with clomiphene ovulated a large amount of ripe eggs. The remaining fish discharged 30–50 eggs after the 8th injection, and showed clear advancement of ovulation after the 9th injection.

In the second series of experiments clomiphene was injected into maturing females every third day. In this case, too, none of the 6 control fish exhibited any sign of ovulation even 15 days after the start of treatment. By contrast, 1 out of 7 fish receiving clomiphene came to show a sign of ovulation on the 6th day, 3 days following the 2nd injection. Definite ovulation was noticed to occur in 6 out of 7 fish on the 9th and 12th day, 3 days following the 4th and 5th injection, respectively (Table 2). No difference was observed in the mean ovulation index between the two groups of fish treated with clomiphene daily and at intervals of 2 days (Tables 1 and 2), 62 to 64% in weight being ovulated in both groups.

Histological comparison of ovaries of both the initial and the saline-injected control fish scarcely showed progress in oocyte development during the experimental periods. On the other hand, ovaries of the clomiphene-treated fish had a number of postovulatory follicles along with many yolkless and yolkladen oocytes. It was noticed, however, that the number of unovulated ripe eggs was more abundant in ovaries of the clomiphene-treated fish than in those of the fish spawned spontaneously.

Changes in pituitary basophilic cells of the loach influenced by clomiphene citrate

In the loach pituitaries examined, there were prominent basophilic cells, which were stained intensely with aldehyde fuchsin, in the proximal pars distalis (PPD) of the pituitary gland of control fish (Fig. 1). In Epon-embedded $1\,\mu$ sections stained with methylene blue, two types of basophilic cells were discernible in that region of the gland. Those of one type were characterized by having several large, lightly stained globules mounted in numerous small, deeply stained granules in the cytoplasm. The nucleus of the cell was small in size, with indistinct nucleolus, and the nuclear membrane was seen to be concaved or undulated frequently. Basophilic cells of the other type were large in size and had small granules in the cytoplasm. In addition, they were provided characteristically with small, clear vacuoles scattered in the cytoplasm. The nucleus of the cells was large in size, round or oval in shape, with a fairly prominent nucleolus (Fig. 3).



Figs. 1 and 2. Sagittal sections through pituitary gland of a saline-injected (Fig. 1) and a clomiphene-treated female loach (Fig. 2), showing aldehyde fuchsin (AF)-positive, basophilic cells (arrows) in the proximal pars distalis. Note a decrease in the stainability to AF of the cells after clomiphene treatment. × 135.

Figs. 3 and 4. Sections of Epon-embedded pituitary glands of a saline-injected (Fig. 3) and a clomiphene-treated loach (Fig. 4), demonstrating the two types of presumed gonadotrophs, namely globular (*g*) and vesicular (*v*) ones, in the gland. Arrows in Fig. 4 reveal a vacuolation of the cytoplasm of these cells resulting from clomiphene treatment. Methylene blue stain. × 1350.

Electron microscopically, the cells of the former type were much conspicuous due to the occurrence of large globules along with small granules in their cytoplasm, thus designated as globular cells in the following description. The globular cells were present in clusters of various numbers and were distributed throughout the PPD (Fig. 5). The globules in these cells were roundish in form, measuring from 550 to 1500 mμ in diameter, and contained somewhat electron-translucent material. The granules were small in size, ranging from 150 to 350 mμ in diameter, and were highly electron-dense. Well-developed rough endoplasmic reticulum was lamellar or vesicular in configuration. Golgi apparatus were sparse and were distributed in the perinuclear region. Mitochondria were few in number and were mostly elongate in shape.

The cells of the other type, which were termed vesicular cells in this paper, were found intermingled among the globular cells (Figs. 5 and 8). The cells also contained a few large globules of varying electron-densities, 400–1000 mμ in size, together with numerous small electron-dense granules of 150–300 mμ, being similar in this respect to the globular cells. They were, however, characterized by the presence of rough endoplasmic reticulum consiting of numerous small vesicles

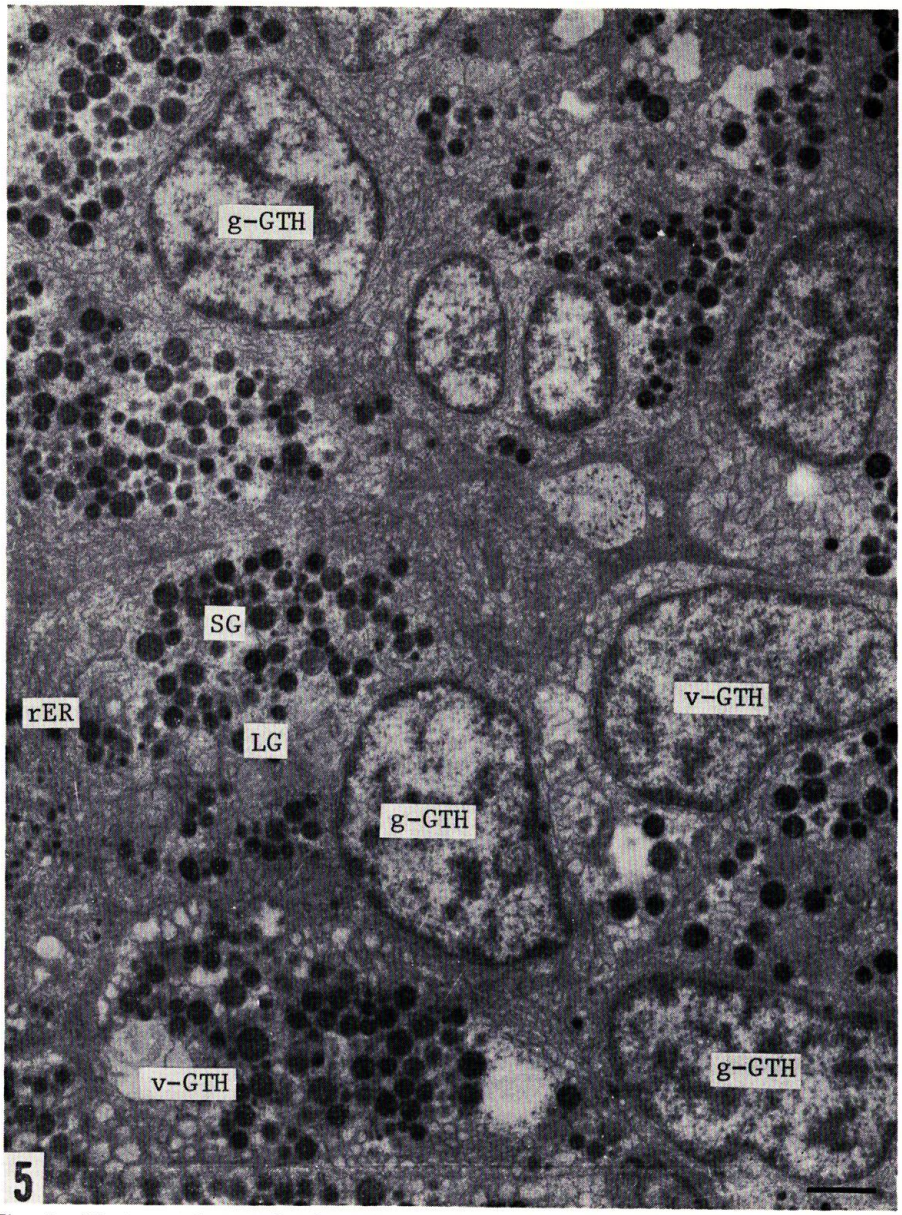
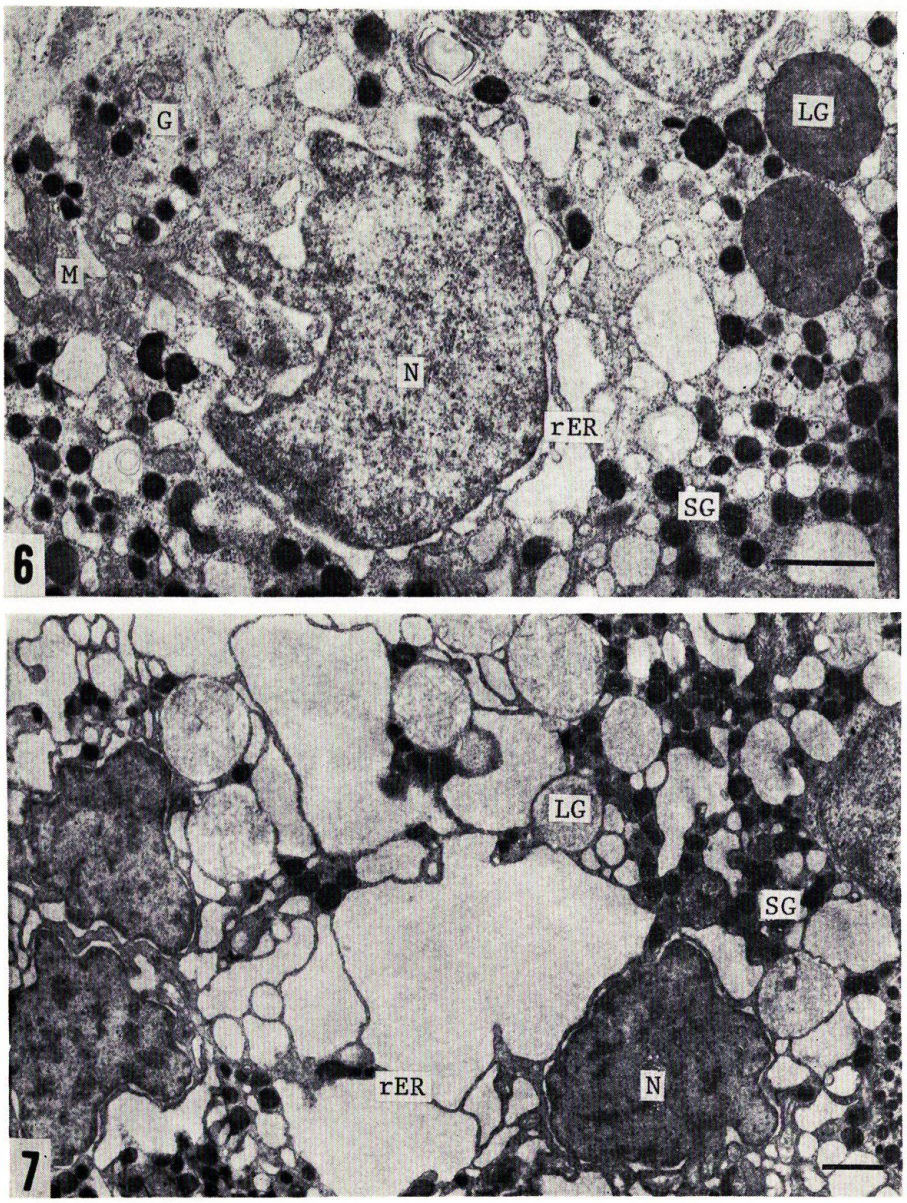


Fig. 5. Electron micrograph of pituitary gonadotrophs of a saline-injected control loach, demonstrating globular gonadotrophs (*g-GTH*) and vesicular ones (*v-GTH*). *LG*, large globule; *rER*, rough endoplasmic reticulum; *SG*, small granule. Scale, 1 μm.

with material of low electron density. In addition, the nucleus of the vesicular cells was of round or oval shape, and was scarcely concaved or undulated in contour. Mitochondria were roundish in shape and were rarely elongated.



Figs. 6 and 7. Electron micrographs of globular gonadotrophs in the pituitary gland of clomiphene-treated loaches. In Fig. 7, an extreme expansion of the rough endoplasmic reticulum and a pycnotic change of the nuclei of the cells associated with pronounced ovulation are shown. *G*, Golgi apparatus; *LG*, large globule; *M*, mitochondrion; *N*, nucleus; *rER*, rough endoplasmic reticulum; *SG*, small granule. Scales, 1 μm.

Golgi apparatus was poorly developed.

The two types of cells displayed some characteristic changes in the pituitary gland of all the fish induced to ovulate by clomiphene treatment. Histologically, basophilic cells in the PPD showed a remarkable decrease in stainability to AF in these fish (Fig. 2). In Epon-embedded 1 μ sections, the globular cells were observed to be highly vacuolated in their cytoplasm, and came to have AF-positive granules which were clearly diminished in amount as compared with those in the cells of control fish. Moreover, their nucleus became extraordinarily dark and was quite irregular in shape (Fig. 4). The vesicular cells were also seen to be prominently vacuolated, and their AF-positive inclusions were also decreased in amount. However, the nuclei of these cells exhibited little change in the treated fish (Fig. 4).

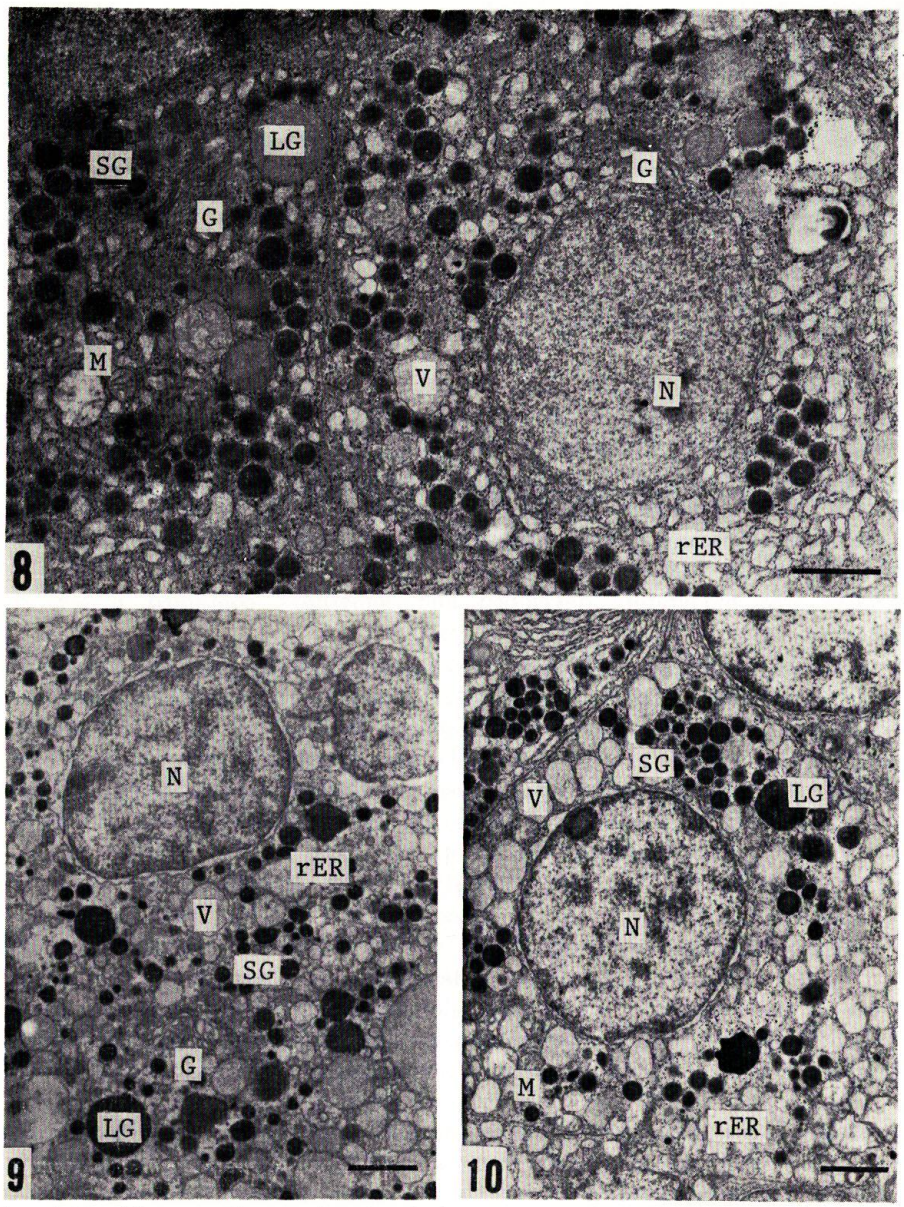
Ultrastructurally, too, the globular cells in the pituitary gland of clomiphene-treated fish showed a distinct decrease in number of small granules of 150–350 mμ in size. Although large globules in these cells appeared to remain unchanged in number, they were observed to become more electron-dense on many occasions (Fig. 6). Most important change was encountered in the rough endoplasmic reticulum, which became to be composed of numerous cisternae dilated conspicuously to various degrees (Fig. 6). Mitochondria were seen to be much elongated. Golgi apparatus was moderately developed and sometimes contained a few small granules or material in the associated vesicles.

In a loach which had ovulated a large quantity of eggs following daily injections of clomiphene, drastic changes were detected in the ultrastructure of the globular cells (Fig. 7). Besides a prominent decrease of small electron-dense granules in the cytoplasm, large globules became increased in size, attaining to 600–3000 mμ in diameter, and became to be filled with finely granulated material of rather low electron-density. Cisternae of the rough endoplasmic reticulum were dilated extensively to occupy most of the cytoplasm. The nucleus of these cells was quite irregular in shape and was unusually dense in electron density.

Ultrastructural changes in the vesicular cells in the pituitary gland of clomiphene-treated fish were essentially similar to those found in the globular cells (Figs. 9 and 10). Small electron-dense granules were clearly diminished in number in the cytoplasm, while large globules remained unchanged in size but appeared to become high in electron density and irregular in shape in some cases. Vesicular cisternae of the rough endoplasmic reticulum with contents of somewhat increased electron-density were evidently larger in size than those in the cells of control fish, but the change was less prominent than that in the globular cells in most cases. No notable change was detectable in mitochondria and Golgi apparatus, except for a possible increase in size of the mitochondria in the vesicular cells observed.

Discussion

It has previously been confirmed that clomiphene citrate is effective, when given intraperitoneally at a dose of 1 μg/g body weight, in accelerating ovulation of mature female loaches in the spawning period[2]. Female fish used in the present



Figs. 8–10. Electron micrographs of vesicular gonadotrophs in the pituitary gland of a saline-injected control (Fig. 8) and clomiphene-treated loaches (Figs. 9 and 10). *G*, Golgi apparatus; *LG*, large glouble; *M*, mitochondrion; *N*, nucleus; *rER*, rough endoplasmic reticulum; *SG*, small granule; *V*, vesicular cisterna of the rough endoplasmic reticulum. Scales, 1 μm.

study were in the pre-spawning conditions as evidenced by the developmental state of their ovaries in which the most advanced oocytes were at the secondary yolk stage. Repeated administrations of clomiphene at a dose of 1 μg to such maturing female loaches could successfully cause full maturation of ovaries accompanied by ovulation whether the injections were done every day or every third day.

Breton *et al.*[3] reported that clomiphene given to the carp, *Cyprinus carpio*, can induce a significant release of pituitary gonadotropin (GTH) irrespective of the state of maturity and sex of the fish treated. There is much evidence indicating that ovarian maturation and ovulation in fishes are achieved primarily by the pituitary gonadotropic activity[4]. Accordingly, it is highly possible that the promotion of gonadal maturation in the loach may be ascribed to an accelerated and continual release of GTH from the pituitary gland stimulated by repeated administrations of clomiphene. In other words, the pituitary gland of maturing females as well as mature ones of the loach may be capable of responding to clomiphene with the discharge of GTH to favour the gonadal maturation leading eventually to ovulation.

According to Breton *et al.*[3], a plasma GTH peak occurs in the carp 56–60 hours following a single injection of clomiphene at the dose of 1 μg/g body weight. Daily injections of the drug might promote the GTH release from the pituitary gland of treated fish more effectively, but might possibly provoke an exhaustive change of the affected pituitaries. In fact, it is suggested experimentally in the rat that clomiphene may act on the pituitary gland through the hypothalamus[5], and that a continuous infusion of luteinizing hormone-releasing hormone may result in an exhaustion of the pituitary GTH stores[6]. Results of the present study reveal that clomiphene injected into maturing loaches every third day was enough to have essentially the same effect as it had when given daily, in terms of the duration of days required for inducing ovulation and the efficiency in causing ovulation. The intermittent treatment with clomiphene may be profitable particularly in cases of a long-term treatment with a view to accelerate the maturation of gonads in fishes which should be maintained as healthy as possible during the artificial management.

It seems certain that, in teleost fishes as well as in mammals, clomiphene may act on the pituitary gland, either directly or indirectly, to release GTH, as the work by Breton *et al.*[3] in the carp demonstrated. In gravid goldfish, *Carassius auratus*, in which clomiphene was clearly effective in causing ovulation[1], hypophysectomy was reported to eliminate the action of the drug perfectly[7]. In order to ascertain whether clomiphene actually exerted its influence on the gonad through gonadotropic actions of the pituitary gland also in the loach, changes in glandular cells of the pituitary gland following ovulation were studied light and electron microscopically in the present study.

Although it was not possible in the present study to distinguish thyrotrophs from gonadotrophs decisively, prominent changes were detected to occur in globular basophilic cells which were distributed extensively through the proximal pars distalis of the pituitary gland. The cells are characterized by having numerous small granules and some large globules in their cytoplasm, resembling clearly those

identified as established gonadotrophs of the goldfish[8] and other fishes[9] in electron microscopic features.

The cells of the loaches after ovulation displayed a notable decrease in number of small granules and a prominent dilatation of cisternae of the rough endoplasmic reticulum. The changes of the cells are fairly consistent with those found in pituitary gonadotrophs of the goldfish immediately after their spontaneuos ovulation[10]. A surge of circulating GTH is known to occur at ovulation in the goldfish[11] and salmonid fishes[12], and this seems to be reflected in the noticeable decrease in amount of small secretory granules in the stimulated pituitary gonadotrophs. The fact that the changes in the possible gonadotrophs of the loach were more drastic in the fish showing more pronounced ovulation, may further support the view that the cells in question are undoubtedly the gonadotrophs in nature, and that clomiphene can bring about ovulation in the loach through augmented release of GTH from the pituitary gland.

Presumed gonadotrophs in the pituitary gland of the loach, *Misgurnus anguillicaudatus*, have been observed electron microscopically by Oota[13]. He described the occurrence of two types of gonadotrophs, but could find only one type of small, possibly secretory granules of 200–500 mμ in size in both types of cells. The size of the secretory granules is almost equal to that of the small granules of the gloublar cells mentioned above. In the loach examined in the present study, there was another type of basophilic cells, designated as vesicular cells in the present paper, in the proximal pars distalis of the pituitary gland which also exhibited the characteristic changes in association with clomiphene-induced ovulation. These cells are provided with small secretory granules and some large globules in their cytoplasm, being in common in this aspect to the gonadotrophs described above, but represented some ultrastructural differences particularly in the membrane system of the rough endoplasmic reticulum.

Ball and Baker[9] classified in their review the gonadotrophs of teleost pituitary gland into the globule-containing cells and vesicular cells. Their classification seems to be adoptable to the two types of presumed gonadotrophs found in the loach pituitary gland observed in the present study. Since the globular gonadotrophs predominate in distribution in the proximal pars distalis of the gland, and show more conspicuous changes associated with ovulation when compared with the vesicular gonadotrophs, the two types of gonadotrophs might be implicated in different gonadotropic roles in the loach, which remains to be elucidated by further studies.

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