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Melamed, P, Gur, G, Rosenfeld, H, Elizur, A, Yaron, Z (1999) Possible Interactions between Gonadotrophs and Somatotrophs in the Pituitary of Tilapia: Apparent Roles for Insulin-Like Growth Factor I and Estradiol. *Endocrinology*, Vol. 140, No. 3, pp.1183-1191.

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# Possible Interactions between Gonadotrophs and Somatotrophs in the Pituitary of Tilapia: Apparent Roles for Insulin-Like Growth Factor I and Estradiol\*

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## ABSTRACT

The unique organization of the teleost pituitary, in which cells are grouped according to their characteristic hormone, makes this a suitable model for studying pituitary paracrine interactions. In a number of fish, including tilapia, there are variations in the circulating levels of the gonadotropins and GH, which are elevated during the reproductive season, suggesting interactions between the reproductive and growth axes. The aim of this study was to investigate paracrine interactions between the gonadotrophs and somatotrophs in the tilapia pituitary. Initially, dispersed pituitary cells were separated on a density gradient in which the gonadotrophs were found in the least dense fractions, and the somatotrophs were concentrated in the densest fraction. After 4 days in culture, cells in the least dense fractions showed characteristic cytoplasmic extensions not seen in the somatotrophs, which appeared small and failed to form aggregates; somatotrophs were found, however, attached to other non-GH cells. Staining of the nuclei with 4,6-diaminidino-2-phenyl-dihydrochloride

revealed that the isolated somatotrophs had undergone nuclear condensation and fragmentation typical of apoptosis. Addition of either estradiol or human recombinant insulin-like growth factor I (IGF-I; 10 nM) to the somatotroph cultures increased the number of cell aggregates and reduced the number of condensed or fragmented nuclei. Immunocytochemical studies on pituitary sections revealed IGF-I immunoreactivity in regions of the proximal pars distalis that stain with gonadotropin II $\beta$  antisera and also in regions of the rostral pars distalis characteristic of corticotrophs; immunoreactive IGF-I was never seen in the region of the somatotrophs. Incubation of cells from the different fractions with testosterone (10 nM; 24 h) revealed that cells of the least dense fractions, which were rich in gonadotrophs, possessed aromatizing ability, which was absent in the somatotroph-enriched fraction. These results suggest that estradiol and IGF-I, both generated from cells other than the somatotrophs, may exert antiapoptotic effects and thus possibly control the size of this population of cells. (*Endocrinology* 140: 1183–1191, 1999)

THE CELLS of the teleost pituitary pars distalis, unlike those of mammals, are segregated into distinct regions according to the characteristic hormone that they secrete. In tilapia, the somatotrophs are located in the proximal pars distalis (PPD) forming a palisade around the nerve ramifications; the gonadotropin (GtH) I (FSH-like) gonadotrophs are adjacent but slightly peripheral to them, whereas the GtH II (LH-like) gonadotrophs outlay these cells, and the lactotrophs are found in a separate location in the rostral pars distalis (RPD) (1–3). The hypothalamic regulatory factors, which in the absence of a portal system reach their target cells via nerve fibers, appear to show somewhat less specificity than in mammals. For example, GnRH stimulates the release of both GtH and GH in a number of teleosts, and *in vitro* studies in tilapia have suggested that PRL is similarly affected (2, 4, 5). In contrast, dopamine inhibits both GtH and PRL release while stimulating that of GH (2, 6, 7).

Studies of the circulating levels of these hormones also suggest some correlation in their regulation; GH levels appear to increase with GtH II at the time of ovulation and spawning, whereas levels of GtH I, which is predominant during vitellogenesis, decrease at this time (8–12). Apart from these changes in circulating hormone levels, changes in the sizes of these cell populations have been noted in rainbow trout in which the GtH I gonadotrophs are found in greater number in immature fish, whereas in mature fish the GtH II gonadotrophs predominate (13, 14). At the time of spermiation, the somatotroph population is also notably larger in these fish (15).

Studies in tilapia suggest that the direct effects of steroids on the expression of the GtH  $\beta$ -subunits may explain some of the changing patterns in their circulatory levels. No direct effects of the gonadal steroids were seen on expression of the tilapia GH gene, although they did appear to increase the sensitivity of the somatotrophs to some of the hypothalamic GH-releasing hormones. The effects of testosterone could be mimicked by estradiol (E<sub>2</sub>), but were not mimicked by the nonaromatizable 11-ketotestosterone, suggesting that the testosterone is aromatized before eliciting these effects (3, 16, 17).

Despite the apparent overlap in the regulation and levels of activity of gonadotrophs and somatotrophs, little attention has been paid to the possible interactions between them. In mammals, locally produced paracrine factors have been re-

Received June 23, 1998.

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\* This work was supported by the Israel Science Foundation founded by the Israel Academy of Sciences and Humanities.

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ported to regulate the synthesis and release of gonadotropins, GH and PRL, and also the differentiation of the cells. These paracrine factors include locally produced cytokines and growth factors, neurohormones produced in pituitary cells such as GnRH, and also the pituitary hormones themselves or parts thereof, such as the glycoprotein  $\alpha$ -subunit (18–22). In the goldfish pituitary, activin and inhibin subunits have also been implicated, as these are found in the somatotrophs and stimulate the release of both GtH and GH (23, 24). Little information is available regarding the location or actions of other cytokines and growth factors in the teleost pituitary. However, after the finding that insulin-like growth factor I (IGF-I) is produced locally in multiple organs in tilapia, it has been hypothesized that it may be involved in autocrine or paracrine actions in organ-specific functions (25). The effects of IGF-I in the teleost pituitary, depressing GH release and messenger RNA (mRNA) levels, have been shown (3, 26).

The aim of this study was to examine possible paracrine interactions operating between the gonadotrophs and somatotrophs in the pituitary of tilapia and to investigate the roles of  $E_2$  and IGF-I. This required the initial establishment of a technique for separation of the pituitary cell populations.

## Materials and Methods

### Fish

The fish used in the study were tilapia hybrids (*Oreochromis aureus* × *O. niloticus*) collected from the ponds of local fish farms. At water temperatures above 22 C, these fish will start gonadal development at a body weight of 30 g. As temperatures fall below this, fish that have already reached sexual maturity will undergo gonadal regression. Cells from fish at various reproductive stages were used for validation of the cell separation technique, whereas for other experiments cells from fish at specific reproductive stages were employed, as stated in *Results*. The reproductive stage of the fish was assessed by measurement of their gonadosomatic index (percent gonadal weight/body weight) taken together with their absolute weight and the season when they died.

### Separation of pituitary cells

Pituitaries were collected aseptically, and the cells were dispersed by trypsinization as described previously (27). After the addition of FCS (final concentration, 20%) to stop the reaction, the cells were counted, and the cell suspension (5 ml) was loaded onto the density gradient (10 ml). The gradient was made up of four concentrations (35%, 45%, 55%, and 65%) of Universal Separation Media (Sigma Chemical Co., St. Louis, MO) with densities of 1.0544–1.0985 g/ml. The cells were centrifuged through the gradient at  $1200 \times g$  for 15 min at 18–20 C. The majority of cells were found to segregate into three clear fractions (fractions 1–3), whereas some cells failed to penetrate the gradient, and a few (mostly red blood cells) were found below the densest part of the gradient. Each fraction was collected by gentle aspiration and rinsed in 10 ml Hanks' Balanced Salt Solution (5 min,  $800 \times g$ , 18–20 C). The cells of each fraction were then resuspended in 1 ml Hanks' Balanced Salt Solution and counted. The viability of the cells in each of three major fractions was greater than 95%. Repeated experiments showed that an average of  $59.14 \pm 6.57\%$  of the initial number of cells were recovered ( $n = 8$ ), with  $49.47 \pm 2.47\%$  of these found in the least dense fraction 1,  $29.24 \pm 2.31\%$  in fraction 2, and  $20.3 \pm 0.97\%$  in the densest fraction 3 ( $n = 9$ ). The validation of this technique was performed on numerous occasions, using fish at different reproductive stages.

### Culture of separated cells

After separation, cells were plated in Corning 24- or 96-well tissue culture plates (Corning, Corning, NY), at a density of  $2.5 \times 10^5$  cells/well in 1 ml or a density of  $6.25 \times 10^4$  cells/well in 200  $\mu$ l medium [medium

199, 10% FCS, 10 mM HEPES, and 1% antibiotic suspension (Pen-strep-nystatin suspension, Biological Industries, Bet HaEmek, Israel)]. Previous experiments on GH and GtH release have shown these to be optimal densities for hormone release. Additional preliminary experiments were carried out in the present study in which the cells from fraction 3 were plated at as much as twice this density. On finding that the increased density had no effect on cell morphology, subsequent experiments employed the cell densities stated above. The cells were incubated for 4 days at 28 C under 5%  $CO_2$ .

On the fourth day, cultured cells were photographed using an inverted microscope (Olympus Corp., Melville, NY) and phase contrast optics. In various experiments, the cells were exposed to recombinant human IGF-I (rhIGF-I; dissolved in 0.1 M acetic acid; Life Technologies, Grand Island, NY), recombinant salmon IGF-I (provided by GroPep, Adelaide, Australia; dissolved in 10 nM HCl),  $E_2$ , or testosterone (Sigma Chemical Co., dissolved in ethanol). The final concentration of the solvents comprised less than 0.1% of the culture medium. The undiluted FCS contained 7.2 nM IGF-I determined as human IGF-I (Silbergeld, A., personal communication).

### Immunocytochemistry (ICC)

ICC was carried out on paraffin-embedded pituitary sections, as described previously (2), after initial blocking with 10% normal goat serum. The primary antisera used were anti salmon IGF-I (1:2500; a gift from GroPep, Adelaide, Australia), antirecombinant tilapia GH (1:8000) (2), and antiserum produced against the recombinant  $\beta$ -subunit of tilapia GtH II (1:4000; Zmora, N., and A. Elizur, unpublished). The second antibody (goat antirabbit) was used at a dilution of 1:600, and peroxidase-antiperoxidase (Sigma Chemical Co.) was used at a dilution of 1:200. The reaction was detected using diaminobenzidine (DAB) and peroxidase.

Cells were fixed in the culture wells (after plating as described above) overnight by the addition of formaldehyde to the incubation medium to a final concentration of 4%. The ICC reactions employed the GH and GtH II  $\beta$  antisera as described above, and the technique was essentially the same.

### 4,6-Diaminidino-2-phenyl-dihydrochloride (DAPI) staining of fixed cells

The cells were fixed as described above and rinsed in phosphate buffer solution (1 M PBS, pH 7.4) before the addition of DAPI (1  $\mu$ g/ml PBS; Sigma Chemical Co.) for 5 min. After the removal of DAPI, the cells were rinsed again in PBS before being examined under an inverted microscope fitted with a U-MNU filter (Olympus Corp.) and photographed using Fuji Photo Film Co. Ltd. TMX 400 film (Tokyo, Japan).

In the case of double staining, nuclei were stained first with DAPI, and consequently, ICC reactions were performed as described above. The cells were photographed with and without the U-MNU filter, using Fuji Photo Film Co. Ltd. TMX 400 film.

### RIAs and measurement of aromatase activity

The cellular hormone content of the different fractions was measured using RIAs specific for tilapia GtH or GH. The RIA for GH, using the recombinant hormone, has been described previously (2). The RIA for GtH was based on native GtH that was purified from tilapia pituitaries harvested during the spawning season (28).

For the measurement of aromatase activity in cells of the various fractions, the level of  $E_2$  secreted into the incubation medium was measured after 24-h exposure to graded concentrations of testosterone (Sigma Chemical Co.). This RIA employed a previously validated antiserum and an iodinated standard (Diagnostics Products Corp., Los Angeles, CA). The cross-reaction of the assay was 0.79% with estrone, 0.34% with estriol, and less than 0.0001% with testosterone,  $17\alpha,20\beta$ -dihydroxyprogesterone and cortisol (29). The sensitivity of this assay was 0.66 pg/tube.

### Measurement of mRNA levels

Comparative levels of GH, GtH II  $\beta$ , and GtH II  $\beta$  mRNA were measured in  $1 \times 10^6$  cells from each of the fractions. Total RNA was extracted

from the cells using a modification of the guanidinium-phenol-chloroform method, as previously described (27). The samples were run on a 1.2% agarose gel and transferred to nylon membranes (GeneScreen Plus, New England Nuclear Research Products, Boston, MA) by Northern blotting. RNA on the membranes was hybridized with complementary DNA probes for GH and GtH I $\beta$  and a DNA probe for GtH II $\beta$ , as described previously (17). After rinsing, the membranes were exposed to the image plate of a phosphorimager (BAS 1000, Fuji Photo Film Co., Ltd.) for 1 h.

### Statistical analysis

Quantitative analysis of apoptotic cells was performed by counting the number of normal nuclei in a field ( $6.4 \times 0.95$  mm) that spanned the diameter of the well; this was then compared with the situation in control (untreated) cells. In addition, the degree of aggregation of the cells was assessed by counting the number of aggregates with a diameter greater than 0.15 mm in a similar field. These measurements were repeated in three or four wells in three separate experiments.

Statistical analysis employed ANOVA followed by the least significant difference test. All experiments were carried out numerous times.

## Results

### Validation of cell separation technique

**Contents of GtH and GH in cells of the different fractions.** Pituitary cells were dispersed and separated as described above, and 250,000 cells from each fraction were homogenized in Triton X-100 (0.1%). The GtH and GH contents were measured in an aliquot from each fraction of cells. In fraction 1, there was 8.4 times more GtH than GH, whereas in fraction 2 the GH was 2.5 times the quantity of GtH, and in fraction 3 GH was found at over 20 times the amount of GtH. This distribution of hormone over the gradient was characteristic regardless of the reproductive stage of the fish.

**GH, GtH I $\beta$ , and GtH II $\beta$  mRNA levels in cells of the different fractions.** Pituitary cells from sexually regressed fish were dispersed and separated as described above. RNA was extracted from  $1 \times 10^6$  cells in each fraction and hybridized with probes for GH, GtH I $\beta$ , and GtH II $\beta$  mRNAs. GtH I $\beta$  and GtH II $\beta$  transcripts were present in fractions 1 and 2 and also in cells of the top fraction that did not penetrate the gradient. In this particular experiment, there appeared to be a greater proportion of GtH I cells in the uppermost fractions compared with cells producing GtH II, although such differences were not noted in cells from fish at other reproductive stages (not shown). Neither GtH  $\beta$ -subunit mRNA was found in fraction 3. In contrast, GH mRNA was found primarily in the densest fractions, whereas levels in the least dense fraction were considerably lower than those in the unsorted cells (Fig. 1).

**ICC on separated cells.** Four days after dispersion and separation, cells from the different fractions were fixed and reacted with antiserum specific to tilapia GH or GtH II $\beta$ . This confirmed that immunoreactive (ir-) GtH II-type gonadotrophs numbered among the cells in fractions 1 and 2 (Fig. 2, A and B), whereas only isolated cells stained with the GH antiserum in this fraction (not shown). In contrast, cells in the third fraction stained primarily with GH antiserum (Fig. 2C), and although a few unstained cells were also seen, these had notably different morphology and the ir-somatotrophs appeared to attach to and intertwine with them (Fig. 2D). Dif-

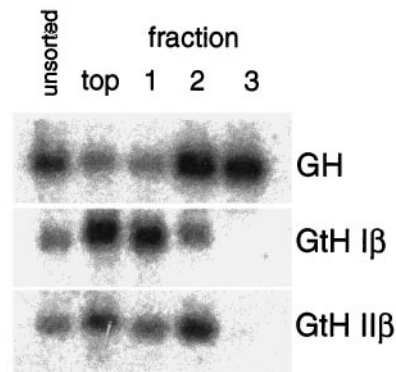


FIG. 1. Levels of GH, GtH I $\beta$ , and GtH II $\beta$  mRNA in tilapia pituitary cells after separation on a density gradient. After collective dispersion, pituitary cells were centrifuged through a column of Universal Separation Media (Sigma Chemical Co.; 1.054–1.098 g/ml) and collected in three main fractions (fractions 1–3); some cells were also found above the column (*top*). RNA was extracted from  $1 \times 10^6$  cells in each fraction and from unsorted cells and was run on agarose gel before Northern blotting. The RNA on the membrane was hybridized with DNA probes for GH, GtH I $\beta$ , and GtH II $\beta$ .

ferences in the morphology of the cells were noted, as in the live cells (see below).

### Morphology of separated live cells

Live cells from the different fractions were examined microscopically on the fourth day of culture, and their morphology was compared with that of unsorted cells. Cells in the first and second fractions, like unsorted cells, showed characteristic spreading and numerous cytoplasmic extensions (Fig. 3, A–C). In contrast, the majority of cells in fraction 3 appeared to be smaller, lacked cytoplasmic extensions, and showed considerably less aggregation than the other fractions or the unsorted cells (Fig. 3D).

### DAPI nuclear staining of separated cells

Four days after dispersion and separation, cells from the different fractions were fixed, and their nuclei were stained with DAPI. In unsorted control cells and those found in fractions 1 and 2, most of the cells had normal, ovoid nuclei staining dimly with the nuclear stain (Fig. 4, A–C). In contrast, most of the cells in the third fraction had smaller nuclei that were round in shape and stained brightly with DAPI. Some of these contained multiple nuclear fragments within a single cell (Fig. 4D).

### Cells in fraction 3 after the addition of IGF-I and/or E<sub>2</sub>

Fraction 3 cells (from sexually regressed fish) were cultured for 3 days in the presence of rhIGF-I or E<sub>2</sub> (10 nM). Both treatments were followed by an increase in the number of cell aggregates in the cultures compared with that in the untreated cells. In addition, staining of the nuclei with DAPI revealed that many of nuclei of the treated cells were larger and less dense, as in the unsorted control cells, as opposed to the smaller condensed nuclei in the untreated cells from fraction 3. The combined treatment of IGF-I and E<sub>2</sub> led to an additive effect on the number of cell aggregates and the “normal” appearing nuclei (Fig. 5). ICC studies on these

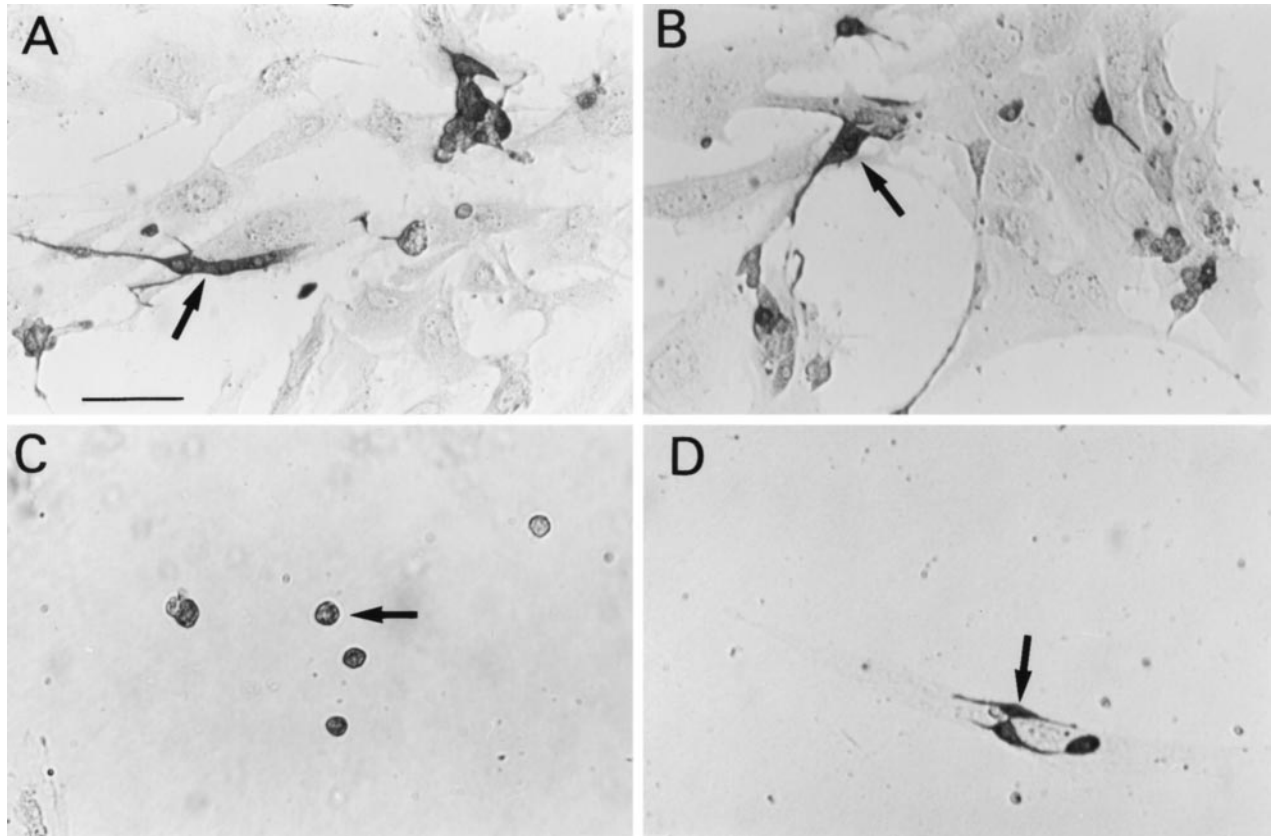


FIG. 2. ICC of tilapia GH and GtH on cells from fractions 1, 2, and 3. Pituitary cells were dispersed and separated as described in Fig. 1 and were cultured for 4 days before fixation. ICC studies employed antiserum specific to tilapia GtH II $\beta$  (1:4000) or GH (1:8000), with goat antirabbit antiserum (1:600) and peroxidase-antiperoxidase complex (1:200); detection was performed using DAB. Fraction 1 (A) and fraction 2 (B) cells after reaction with GtH II $\beta$  antiserum. C and D, Cells from fraction 3 after reaction with GH antiserum. Arrows indicate immunoreactive cells. Scale bar, 50  $\mu$ m.

same treated cells confirmed that they were indeed somatotrophs (Fig. 6, A and B). Similar results were obtained when salmon IGF-I was used in place of the recombinant human peptide (not shown).

#### Cells from fraction 3 after addition of conditioned medium from fraction 1 or 2

Conditioned medium was collected from fraction 1 and 2 cells (from sexually regressed fish) after 4 days in culture. This medium was added, either undiluted or after dilution (1:1) with freshly prepared medium, to cells from fraction 3 immediately after their separation. After 2–4 days the cells were fixed, and their nuclei were stained with DAPI. No change was noted in either the morphology of the live cells or in the size or shape of the nuclei, and the cells remained similar in appearance to untreated cells from this fraction (not shown).

#### ICC localization of IGF-I in the tilapia pituitary

Numerous ICC reactions on sections of tilapia pituitaries failed to show ir-IGF-I in the region of the somatotrophs (e.g. Figs. 7 and 8). In some, but not all, pituitaries from mature fish, IGF-I immunoreactivity was seen in areas of the proximal pars distalis corresponding to those also reacting with antiserum to GtH II $\beta$  (Fig. 7, A and C). In another pituitary,

ir-IGF-I was also apparent in the rostral pars distalis in a thin layer of epithelial cells lining the junction of the RPD and the neurohypophysis (Fig. 8A).

#### Aromatase activity in separated pituitary cells

Pituitaries were collected from sexually mature male and female tilapia at the height of the spawning season (gonadosomatic index of  $0.4 \pm 0.09$  for males or  $1.91 \pm 0.35$  for females;  $n = 13$ ); the cells were dispersed, separated, and cultured at  $6.25 \times 10^4$  cells/well as described above. On the third day of culture, testosterone (1–100 nM) was added for 24 h, after which the medium was collected for the measurement of  $E_2$ . In cultures of both male (Fig. 9A) and female (Fig. 9B) pituitary cells, the highest levels of  $E_2$  were found in cells of fraction 1. Cultures of fraction 2 cells contained lower levels of  $E_2$ , similar to the levels in unsorted cells. In cells not exposed to testosterone and those from fraction 3 after all levels of testosterone exposure,  $E_2$  was undetectable (Fig. 9, A and B).

#### Discussion

This study has demonstrated that tilapia somatotrophs and gonadotrophs can be separated on a density gradient and remain viable. Consistently, regardless of the reproductive state of the fish, the gonadotrophs were found in the least

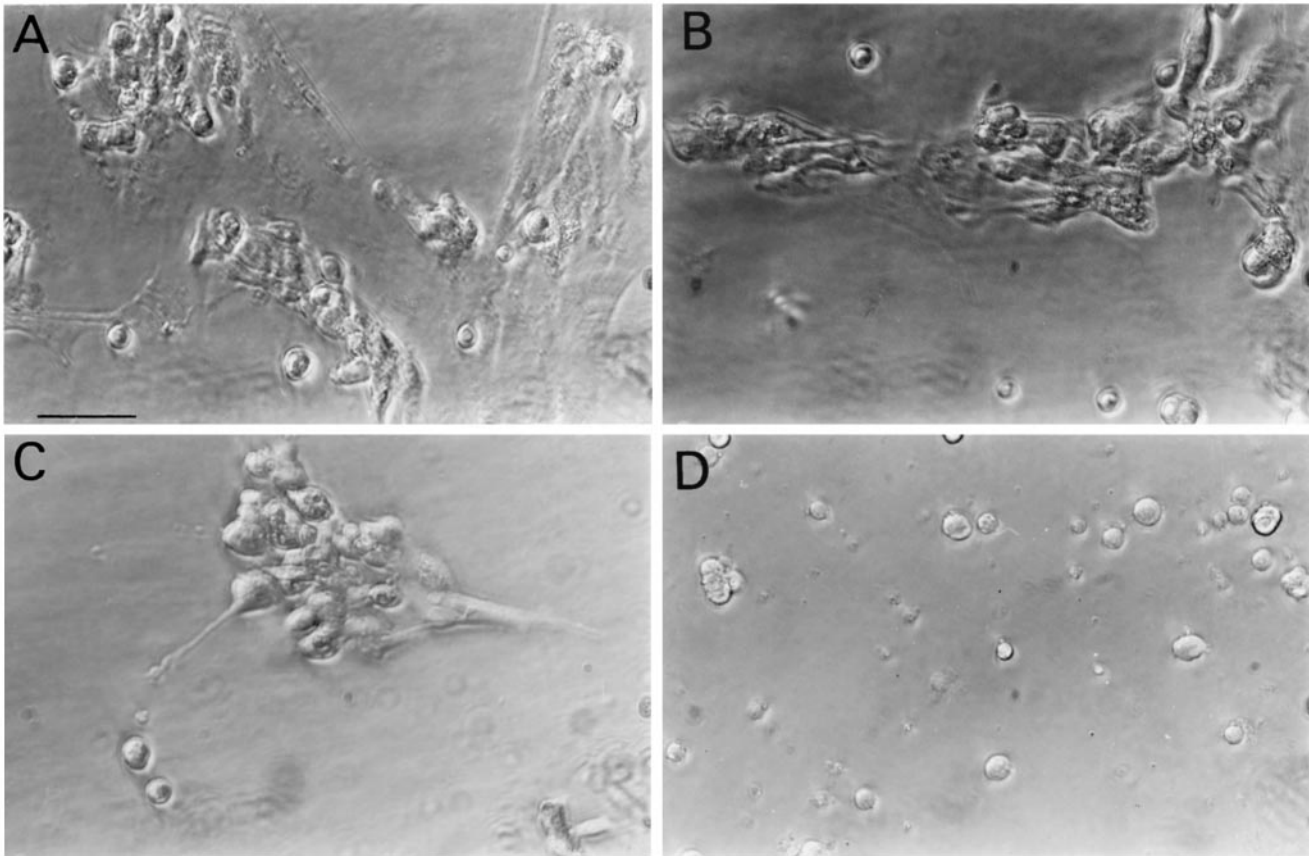


FIG. 3. Live pituitary cells 4 days after separation. Pituitary cells were dispersed and separated as described in Fig. 1 and cultured for 4 days. A, Unsorted cells; B, fraction 1 cells; C, fraction 2 cells; D, fraction 3 cells. Phase contrast. Scale bar, 50  $\mu$ m.

dense fractions, although in sexually regressed fish, slight differences were noted between the two gonadotroph populations; the somatotrophs were invariably found in the densest fractions. The densities of these pituitary cells appear to differ in various teleosts; for example, in the African catfish, gonadotrophs separated on a Percoll gradient were found below the densest fraction (1.095 g/ml), whereas the acidophils were mostly in the least dense (1.049–1.083 g/ml) fractions (30). Goldfish gonadotrophs were found in Percoll density fractions similar to those in the catfish (1.095 g/ml), but the somatotrophs were largely in the 1.083 g/ml fraction (31). In contrast, but similar to the current findings in tilapia, rainbow trout somatotrophs were found in the densest Percoll fractions (1.066–1.102 g/ml), although the GtH II gonadotrophs were almost equally distributed among the remaining fractions (1.027–1.096) (15).

In the separated tilapia pituitary cell cultures, marked differences in morphology were noted in cells of the various fractions. Notably, cells in the somatotroph-enriched fraction had a peculiar appearance and failed to aggregate. Unsorted cells or those from the least dense fractions examined after 4 days in culture show numerous cytoplasmic extensions. ICC studies revealed that the extensions were present in gonadotrophs as well as in other unidentified cells in these fractions. However, the ir-somatotrophs in fraction 3 were devoid of such extensions. This contrasts with the situation in goldfish, where small cytoplasmic extensions were seen on

pituitary cells identified as somatotrophs (31). In fact, when cultured with other cell types, the tilapia somatotrophs appeared to be attached to the spreading cells.

After staining the nuclei with DAPI, a reason for the peculiar morphology of the separated somatotrophs became evident. The nuclei of isolated somatotrophs had undergone condensation and fragmentation typical of apoptosis. Also typical of this kind of cell death was a decrease in the cytoplasmic volume of the cells leading to a shrunken appearance. In contrast, tilapia somatotrophs in mixed culture, as shown in previous studies, retained their viability without showing signs of nuclear damage or cell death (17, 27) (our unpublished observations).

To test the hypothesis that substances secreted from other cell types prevent the peculiar appearance of the isolated somatotrophs, the somatotroph-enriched culture was incubated with conditioned medium from the uppermost fraction, but the appearance of the cells was not altered. However, the addition of either rhIGF-I or  $E_2$  to the somatotroph-enriched cultures increased the numbers of normal appearing nuclei and increased the degree of cell aggregation. Previous studies on tilapia pituitary cells have shown that somatotrophs respond to treatment with rhIGF-I by reducing the GH mRNA levels (3), whereas studies in mammals have demonstrated the presence of IGF-I receptors on these cells (32). The present study has demonstrated that in the tilapia pituitary, ir-IGF-I is not found in the somatotrophs

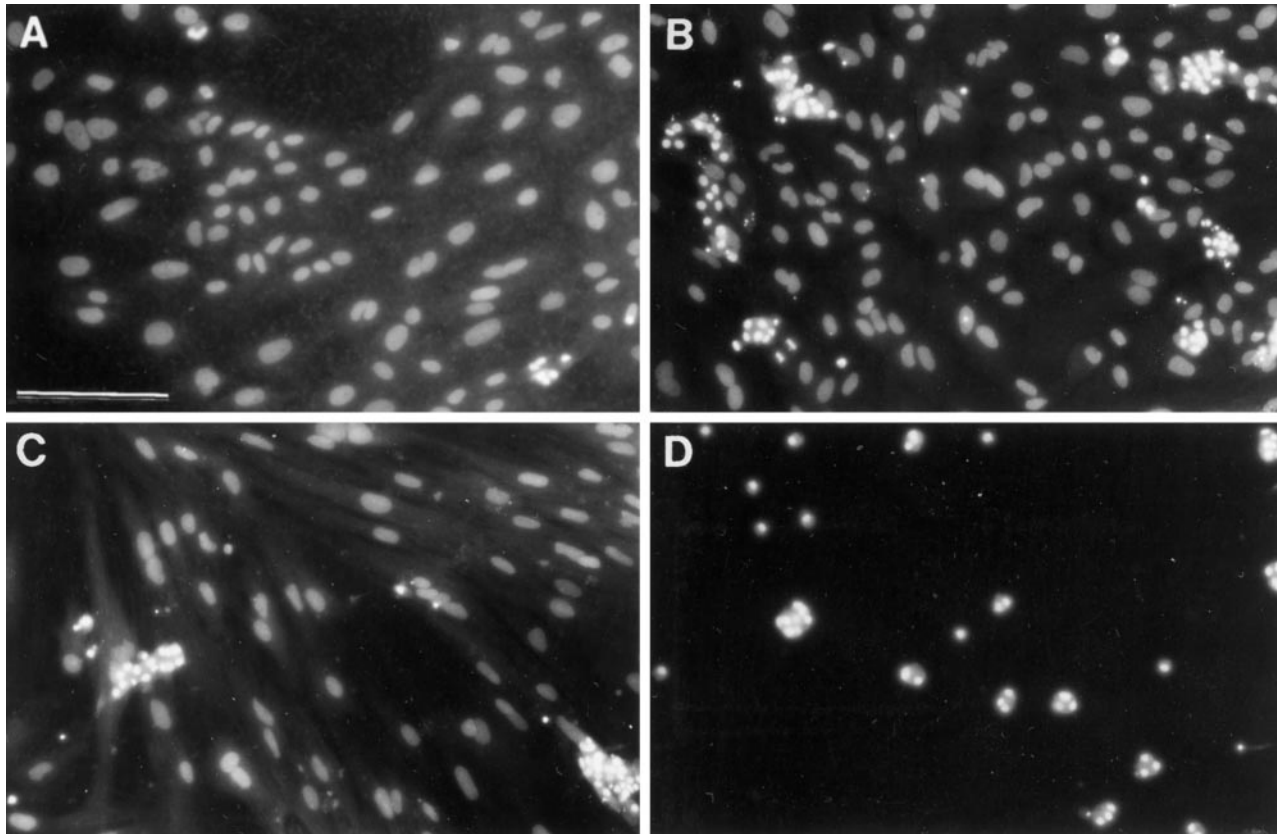


FIG. 4. DAPI staining of nuclei in cells from fractions 1–3. Cells were dispersed and separated as described in Fig. 1 and were cultured for 4 days before fixation and staining of the nuclei with DAPI. Control cells (A) and those in fraction 1 (B) and fraction 2 (C) have mostly large ovoid nuclei that stain dimly with the nuclear stain. D, Cells in fraction 3 have smaller, dense nuclei that stain brightly with DAPI, and some cells show multiple nuclear fragments. Scale bar, 100  $\mu$ m.

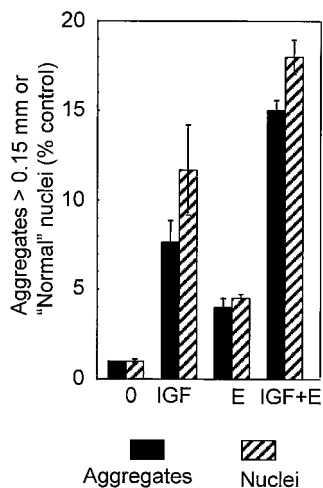


FIG. 5. Addition of IGF-I or  $E_2$  to cells from fraction 3. After dispersion and separation (as described in Fig. 1), pituitary cells were cultured for 3 days in the presence of IGF-I and/or  $E_2$  (10 nM) before fixation and staining of the nuclei with DAPI. The number of cell aggregates with a diameter of at least 0.15 mm was counted in a field spanning the diameter of the well ( $6.4 \times 0.95$  mm) and was compared with that for untreated fraction 3 cells. The same procedure was used to evaluate the number of normal appearing nuclei. Means of all treatment groups differed from those of controls ( $P < 0.05$ ). Values are the mean  $\pm$  SEM ( $n = 3$ ).

but was seen in the same region of the PPD reacting with antisera to GtH II $\beta$  in some, but not all, pituitaries. The reason for these variations is not yet apparent. In addition, cells in the RPD, in a location typical of the corticotrophs (33), also reacted with the IGF-I antiserum. Although the type of pituitary cells producing IGF-I in mammals remains in dispute, most evidence points to the folliculostellate cells, with other types, including the somatotrophs, possibly also being involved (20, 21, 32).

Aromatase activity was found only in the least dense fractions of separated cells where the majority of gonadotrophs are located. It is suggested, therefore, that the source of pituitary  $E_2$  in tilapia is the gonadotrophs, although the possibility that it originates from other unidentified cells in this fraction cannot be excluded. This is opposed to previous studies of the *Oreochromis mossambicus*, in which it was suggested that aromatase activity, predominant in the PPD, originates from the somatotrophs (34). Also in another teleost, the longhorn sculpin, and in mammals, somatotrophs have been implicated as the location of this enzyme (35, 36). Aromatase activity was seen in separated gonadotrophs of the African catfish, although it was also present in other cell types (37). It appears that in teleosts, brain and pituitary aromatase levels far exceed those in mammals and also exceed those found in the gonads, suggesting that *in situ* synthesis may be the major source of estrogen within these tissues (38). In the

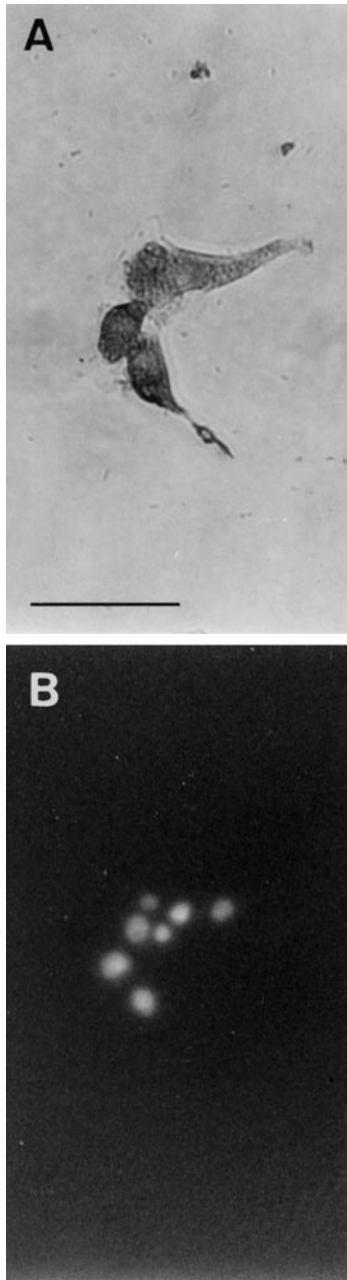


FIG. 6. After combined IGF-I and  $E_2$  treatment, cells from fraction 3 were stained with DAPI and then reacted with antiserum to GH as described in Fig. 2. A, Cluster of cells showing immunoreactivity to GH; B, the same cells after staining with DAPI. Scale bar, 10  $\mu$ m.

present study, the location of the aromatizing ability in tilapia pituitary cells was similar in cells from sexually mature males and females, although it was more potent in cells from the male fish. This difference presumably arose because of slight differences in the reproductive state of the fish.

The additive effects of IGF-I and  $E_2$  treatment suggest that these factors activate different mechanisms to preserve the somatotroph population. In mammals, endogenously produced IGF-I prevents apoptosis of the ovarian follicles and also slightly increased the production of  $E_2$  by cultured pre-ovulatory follicles (39, 40). In granulosa cells of early or

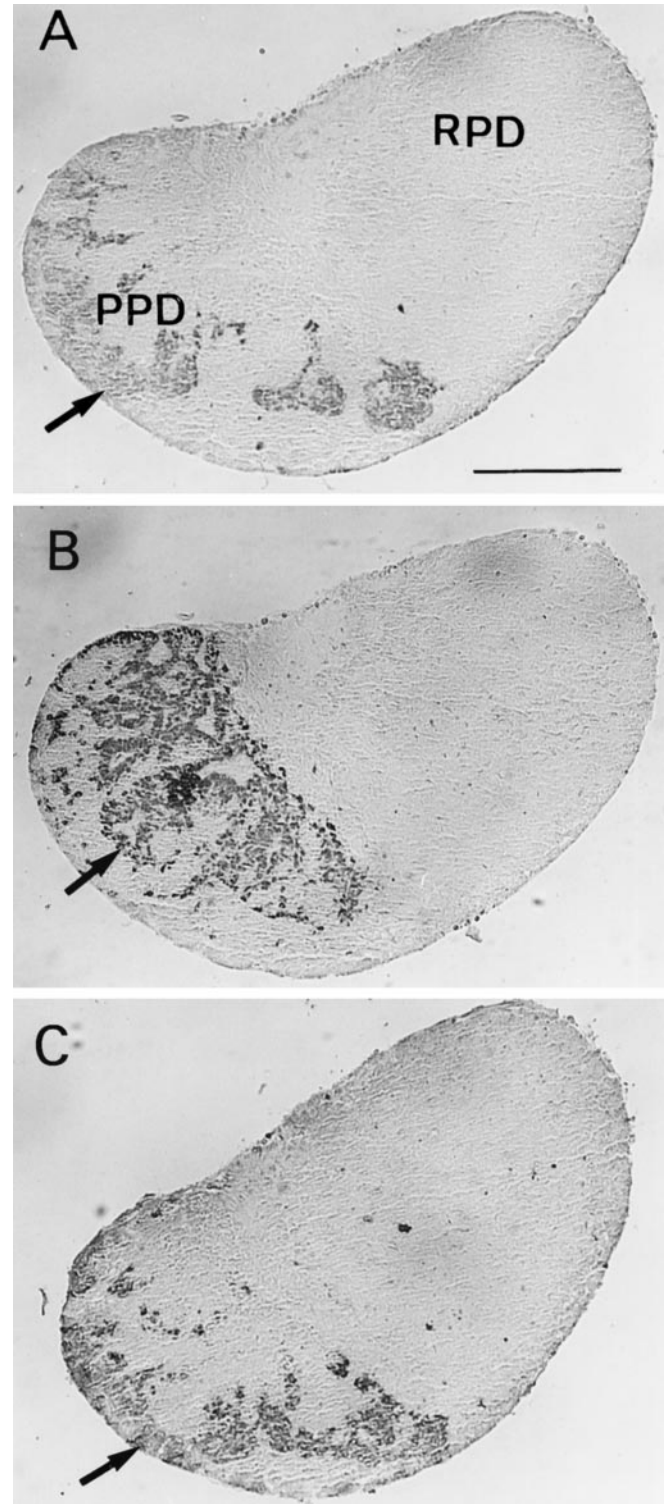


FIG. 7. IGF-I immunoreactivity in the proximal pars distalis of the tilapia pituitary. Paraffin sections were reacted with antisera to salmon IGF-I (A; 1:2500), tilapia GH (B; 1:8000), or tilapia GtH II $\beta$  (C; 1:4000), which were detected using goat antirabbit antiserum (1:600) and peroxidase-antiperoxidase complex (1:200). PPD, Proximal pars distalis. The DAB reaction product is marked by arrows. Scale bar, 40  $\mu$ m.



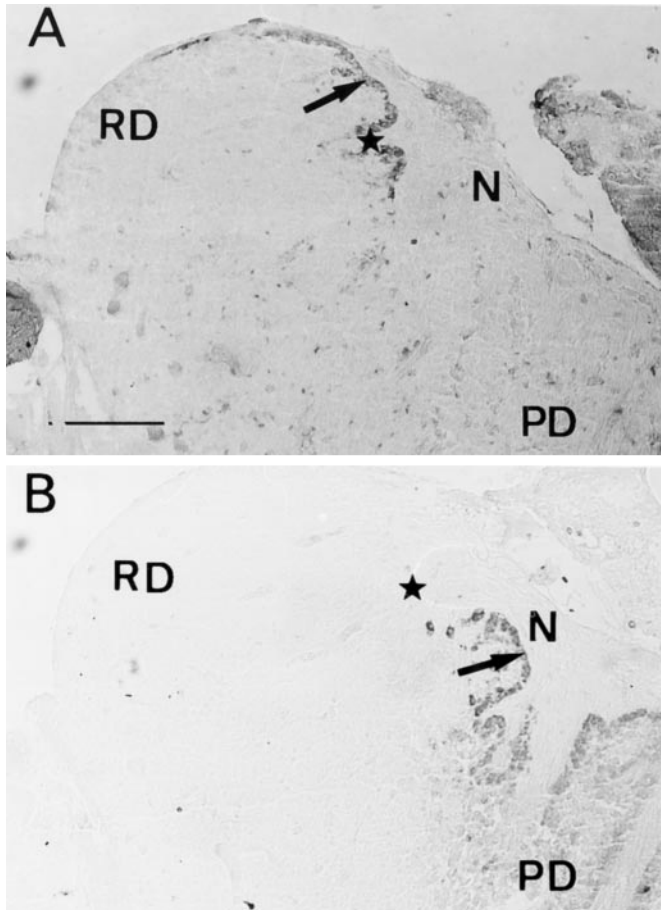


FIG. 8. A, IGF-I immunoreactivity in the RPD of the tilapia pituitary. The ir-IGF-I is seen in a thin layer of epithelial cells lining the junction of the RPD and the pars neurointermedia (N). B, GH immunoreactivity is seen in a different region of the pituitary, in the proximal pars distalis. Reaction details are given in Fig. 7. RD, RPD; PD, proximal pars distalis; N, pars neurointermedia. An asterisk marks a nerve tract in the RPD; arrows mark the DAB reaction product. Scale bar, 100  $\mu$ m.

preantral follicles, apoptosis increased after estrogen withdrawal, and this was completely prevented by replacement with diethylstilbestrol or estradiol benzoate, whereas androgens had the opposite effect (41, 42).

The reason for the failure of conditioned medium from cells of the upper fractions to prevent the morphological changes in the isolated somatotrophs is not entirely clear. It has been suggested, however, that the control of IGF-I in the pituitary is mediated by GH (21). If a similar situation prevails in tilapia, then a reduction in the number of somatotrophs in the upper fractions could have abated the release of IGF-I. It should be noted that the basal  $E_2$  levels in all fractions were below the detection limit (*i.e.* <6.6 pg/ml was secreted during 24 h) even though these cells were from sexually mature fish. It is probable, therefore, that the conditioned medium (taken from cells of sexually regressed fish) simply did not contain enough  $E_2$  or IGF-I to elicit an effect.

There is direct evidence in certain teleosts that the numbers of somatotrophs and type II gonadotrophs increase during the breeding season and are lower in sexually regressed fish (13–15). In tilapia, cyclical circulating GH levels and their

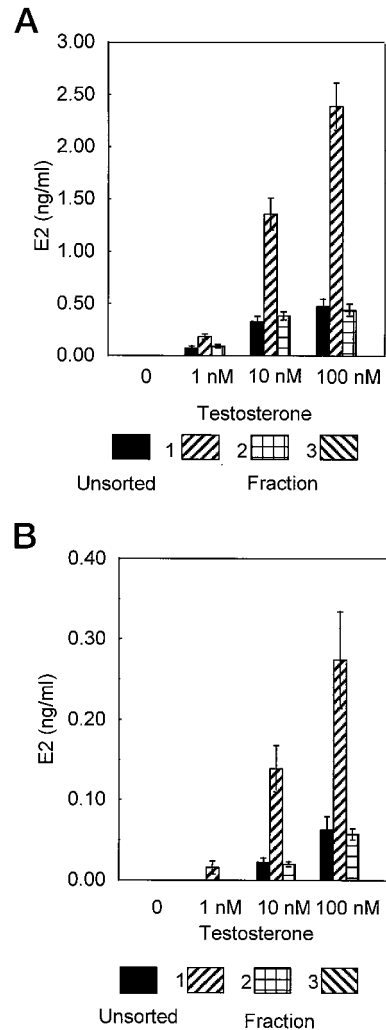


FIG. 9. Aromatase activity in separated pituitary cells. Cells were collected from sexually mature male (A) or female (B) fish, dispersed, and separated as described in Fig. 1. The separated cells were cultured, and on the third day, testosterone (1–100 nM) was added for 24 h. The level of  $E_2$  accumulated in the medium was measured. Values are the mean  $\pm$  SEM ( $n = 4-5$ ). Mean values not visible on the graph were undetectable (*i.e.* <0.66 pg  $E_2$ /tube).

response to GH-releasing hormones are greatest at the height of the reproductive season, whereas GtH II $\beta$  mRNA levels peak with maximal gonadal development (16, 17). These changes could arise from either an increase in the size of specific cell populations or simply from increased activity of the same cells. Results from the present study suggest that a decrease in the number of cells producing GtH II, for whatever reason, could lead to a reduction in both the aromatizing ability of the pituitary and the output of IGF-I. This coupled with a decrease in circulating steroid levels (occurring in the sexually regressing fish) could also lead to a decrease in the number of somatotrophs by allowing programmed cell death.

Thus, IGF-I appears to have paradoxical effects on the somatotrophs in tilapia: on the one hand, reducing GH release and synthesis (3) and, on the other, preventing a reduction in the somatotroph population. Such actions mean

that although negative feedback operates to control the effects of GH on IGF-I, the positive effects on the viability of the cells ensure the capacity of the GH-IGF-I axis to operate optimally. In contrast, the ability of E<sub>2</sub> to have similar positive effects on the condition of the somatotrophs suggests that gonadal steroids may act indirectly to increase this population of cells in reproductively active fish. There is evidence to suggest that both of these actions are of a paracrine nature.

### Acknowledgments

We thank GroPep Pty. Ltd. (Adelaide, Australia) for the gift of salmon IGF-I recombinant peptide and antisera, and Dr. Rentier-Delrue for the gift of tilapia GH complementary DNA. Thanks also to Mr. Ahikam Gissis and the team of HaMa'apil fish farm for supplying the fish, and to Dr. A. Silbergeld, Felsenstein Medical Research Center (Petah-Tiqwa, Israel), for the hIGF-I determinations.

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