Fish Physiology and Biochemistry vol. 11 no. 1–6 pp 255-263 (1993)
Kugler Publications, Amsterdam/New York

GTH-cells in the pituitary of the African catfish, *Clarias gariepinus*, during gonadal maturation: an immuno-electron microscopical study

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Keywords: catfish, pituitary, ontogeny, gonadotrops, ultrastructure, stereological analysis, immunocytochemistry, GTH subunits, plasma androgen

Abstract

In an ultrastructural immunocytochemical study we investigated the development of the gonadotropic cells in the pituitary of two to six months old male African catfish in relation to testicular development. In this period, pituitary and testicular tissue samples were collected on five occasions (groups I–V). Blood samples could only be taken from the fish in groups III–V. The testicular development was divided in three stages *i.e.*, *immature* (only spermatogonia, group I), *early* (spermatogonia and spermatocytes, groups II and III) and *advanced* (all germ cell stages including spermatozoa, groups IV and V) spermatogenesis. 11-Ketotestosterone blood levels were low, except for the last group. Antisera were raised against the complete catfish α,βGTH-II, as well as to the separate α- and β-subunits of catfish GTH-II. In the proximal pars distalis of immature fish, undifferentiated cells, somatotrops, putative thyrotrops (pTSH) and putative gonadotrops (pGTH) were found. In the two latter, secretory granules were labeled with anti-αGTH, but not with anti-βGTH-II. pTSH- and pGTH-cells were distinguished on the basis of the size of their secretory granules. During early spermatogenesis, two classes of putative gonadotrops could be distinguished. One type had the same immunocytochemical and ultrastructural characteristics as in immature fish; the secretory granules in the second cell type, which was more abundant, were also immunopositive for anti-βGTH-II. The mean volume of the secretory granules in these GTH-II cells was three times larger than that in the early appearing pGTH-cells. In addition, the later appearing GTH-II cells contained large inclusions, known as globules. These structures labeled with anti-$\alpha\beta$GTH-II and with anti-βGTH-II, but not with anti-αGTH. It is assumed that the globules are involved in a differential storage and/or breakdown of the GTH-II subunits. During advanced spermatogenesis the two gonadotropic cell types could still be distinguished, but the early appearing pGTH-cell type was scarce. The present observations permit the conclusion that the early appearing cells may be GTH-I cells. However, definitive proof about their identity depends on the availability of antibodies or cDNA probes specific for GTH-I.

Résumé

A l'aide d'une étude d'immunocytochimie ultrastructurale, nous avons suivi chez le poisson chat africain mâle âgé de 2 à 6 mois le développement des cellules gonadotropes hypophysaires en relation avec celui des testicules. Pendant cette période, 5 prélèvements hypophysaires et testiculaires ont été effectués (groupe I–V) alors que les prélèvements sanguins n'ont pu être pratiqués que dans les groupes III–V. Le développement

testiculaire a été divisé en 3 stades c'est à dire *immature* (spermatogonies seulement, groupe I), *début de spermatogenèse* (spermatogonies et spermatocytes, groupe II et III), *spermatogenèse avancée* (tous les stades de cellules germinales incluant les spermatozoïdes, groupe IV et V). Les niveaux sanguins en 11-ketotestostèrone étaient bas excepté dans le dernier groupe. Des anticorps ont été fabriqués contre l'α,βGtH-II et les sous-unités α et β de GtH-II de poisson chat. Dans la partie proximale distale de l'hypophyse du poisson immature, des cellules indifférenciées, des cellules somatotropes, des cellules putatives thyrotropes (pTSH) et gonadotropes (pGtH) ont été trouvées. Dans les deux derniers types cellulaires, des granules de sécrétion ont été marqués avec l'anti-αGtH mais pas avec l'antiβ-GtHII. Les cellules présumées à TSH et GtH ont été distinguées par la taille des granules de sécrétion. Pendant le début de la spermatogenèse, 2 classes de cellules gonadotropes putatives ont pu être distinguées. Un type a les mêmes caractéristiques immunocytochimiques et ultrastructurales que chez les immatures; les granules de sécrétion dans le second type cellulaire, qui était plus abondant, répondaient aussi immunopositivement à l'anti-βGtHII. Le volume moyen des granules de sécrétion de ces cellules à GtHII était 3 fois supérieur à celui des cellules putatives à GtH apparaissant tôt. De plus, les cellules à GtHII apparaissant tardivement contiennent de grandes inclusions, connues sous le nom de globules. Ces structures étaient marquées avec l'anti-$\alpha\beta$GtHII et avec l'anti-βGtHII, mais pas avec l'anti-αGtH. Il a été supposé que les globules sont impliqués dans un stockage différentiel et/ou destruction des sous-unités de GthII. Pendant les stades plus avancés de la spermatogenèse les 2 types gonadotropes pouvaient encore être distingués, mais les cellules putatives à GtH apparaissant tôt étaient rares. Les présentes observations permettent de supposer que les cellules à GtH apparaissant tôt peuvent être des cellules à GtHI. Cependant, la preuve définitive de leur identité dépend de la disponibilité d'anti-GtHI ou de cDNAs spécifiques de la GtHI.

Introduction

It is now firmly established that in several salmonid species, in carp and in tuna two chemically distinct GTHs, GTH-I and GTH-II, are synthetized in the pituitary (Kawauchi *et al.* 1986; Ando and Ishii 1988; Suzuki *et al.* 1988 a,b; Chang *et al.* 1990). The question is raised whether these two hormones are synthetized and secreted by two gonadotropic cell types. With specific antibodies to the β subunit of GTH-I and GTH-II respectively, two GTH-cell types were localized in the salmonid pituitary at the light microscopic level Nozaki *et al.* 1990a; Naito *et al.* 1991).

In a review on the cellular origin of pituitary gonadotropins, Van Oordt and Peute (1983) discussed that, at least in adult teleosts, only one GTH-cell type may be responsible for the production of gonadotropin. As for adult African catfish, *Clarias gariepinus*, in fact only one GTH-cell type was and is observed in ultrastructural and immunocytochemical studies (Peute *et al.* 1984, 1986). Recently, however, the separate α- and β-subunits of catfish GTH-II have become available (Koide *et al.* 1992) and were used to generate antisera.

The aim of the present study was the immunocytochemical re-examination of the GTH-cells in the catfish pituitary, using these GTH-II subunit specific antisera. A stereological analysis of the secretory granules in the GTH-cells is included. In order to account for possible changes in the gonadotropes' population, pituitaries were examined at different stages of testicular development during ontogenesis. Testis development was monitored by histological analysis of the spermatogenetic stages and by quantification of a major androgen, 11-ketotestosterone, in the blood samples.

Materials and methods

Experimental animals

African catfish, *Clarias gariepinus*, were reared in the laboratory. For the present study, males were sampled on five occasions (between two and six months after hatching). The grouping was based on

the testicular histology: immature (group I: only spermatogonia), early spermatogenesis (groups II and III: spermatogonia and spermatocytes) and advanced spermatogenesis (groups IV and V: all stages, including spermatozoa; in group V the number of spermatozoa was higher). The spermatogenetic stages were determined at the light microscopical level and based on a study by Van den Hurk *et al.* (1989).

Fixation and immunocytochemistry

The fish were decapitated and the pituitaries were removed, fixed and Epon-embedded (Peute *et al.* 1986). Ultrathin sections were treated with 10% hydrogen peroxide for 10 min and rinsed in distilled water. Subsequently, the grids were placed for 10 min on droplets containing a mixture of Tris-buffer (20 mM Tris and 130 mM NaCl, pH 8.2) and 50 mM glycine, to block free aldehyde groups in the tissue. The grids were then transferred to droplets of Tris-buffer + 0.2% teleostean gelatin (Sigma, St. Louis, Mo. USA) for 3 × 5 min and incubated for 2h on droplets of primary antiserum. The primary antisera, raised in rabbit, were used in the following dilutions: anti-catfish somatotropin (1:2000); anti-catfish α,β GTH-II (1:1000 or 1:5000), anti-catfish αGTH (1:1000 or 1:2000), anti-catfish βGTH-II (1:1000 or 1:4000). Primary antibodies were visualized using goat anti-rabbit (GAR) gold 10 nm (Aurion, Wageningen, The Netherlands), as described by Zandbergen *et al.* (1992). The sections were stained with uranylacetate and leadcitrate and examined in a Zeiss EM 109.

Stereological analysis

Electron micrographs (from GTH-cells of animals during early and advanced spermatogenesis) were digitized and analysed in a quantitative image analyser (IBAS, Kontron). Profiles of secretion granules were automatically discriminated and identified based on their grey value in the digitized image. An user edit step allowed the removal of erroneously identified structures such as mitochondria. Subsequently, the volume-weighted mean volume of the secretory granules was determined by point-sampling of linear intercept lengths (Gundersen and Jensen 1985). The advantage of this method, compared to classical morphometric techniques (Weibel 1979), is the complete absence of assumptions about the shape of the particles. A regular grid of test points was randomly projected on each photograph. For profiles hit by a test point the length (L_o) of the intercept through the point was measured (Fig. 1). Since all profiles were convex, an unbiased estimate of the volume weighted mean volume of the granules is given by

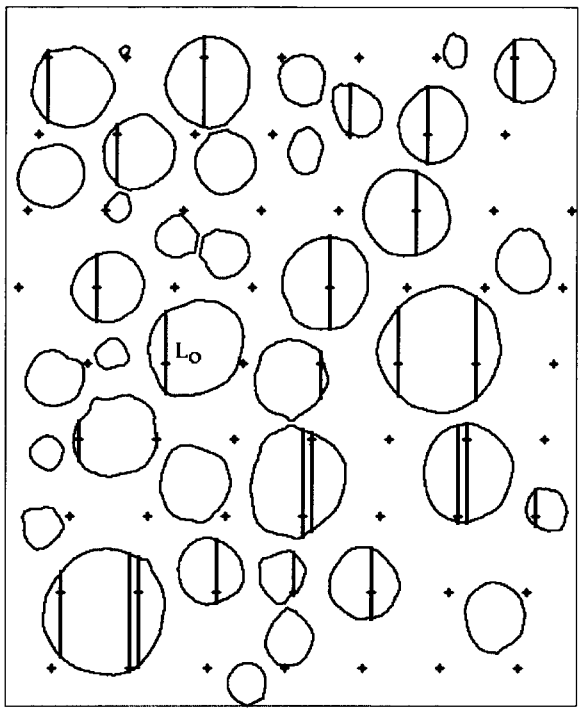

Fig. 1. Diagram showing the point-sampling of linear intercept lengths (L_o). Only intercepts through random points hitting a granule profile are measured. This diagram is derived from Fig. 7.

$$\bar{V}_v = \frac{\pi}{3} * \bar{L}_o^3$$

(Gundersen and Jensen 1985), resulting in a volume estimate per photograph. Each electron micrograph was measured 10 times, with random positions of the test grid; the mean of the estimates per

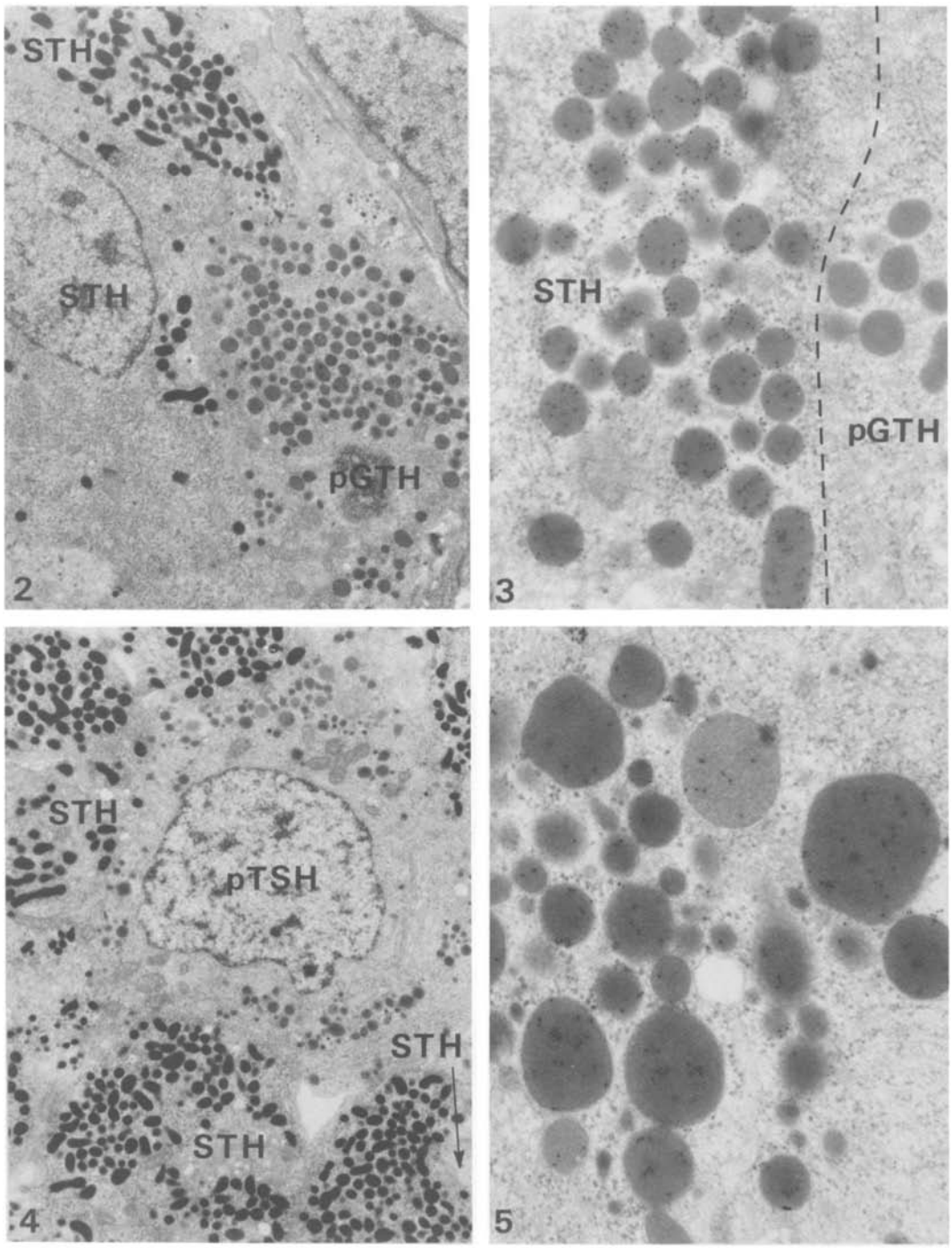

Fig. 2. Cells in the proximal pars distalis (PPD) from immature male. STH somatotrop, pGTH putative gonadotrop (× 10,200).
Fig. 3. PPD from immature male: secretory granules of somatotrop labeled with anti-STH; the granules of the putative gonadotrop (pGTH) are negative (× 44,200).
Fig. 4. PPD from immature male: a putative thyrotrop (pTSH), surrounded by STH-cells (× 10,200).
Fig. 5. Detail from Fig. 4: heterogeneous population of secretory granules in pTSH-cell, which are immunoreactive for the α-subunit of GTH (× 44,200).

photo were used to calculate the mean volume of the granules per GTH-cell type. The coefficient of variation for the mean volume estimate over the ten replications per micrograph was on the average 15%. A further increase in the number of replications did not reduce this variation.

Statistics

Differences in mean granule volume for three tissue blocks (*i.e.*, three animals) of GTH-II cells (group V of advanced spermatogenesis) were tested with one-way analysis of variance. Since no statistically significant differences were found between these blocks, the data of different blocks were pooled in further analysis. Differences in the mean granule volume between the two GTH-cell types and between the stages of spermatogenesis were tested with a two-way analysis of variance. Statistical significance was accepted at $p < 0.05$.

Steroid measurements

11-Ketotestosterone (11-KT) levels in blood serum were quantified by a specific radioimmunoassay (RIA). Zero point two to zero point five ml of blood serum was extracted twice with 4–5 ml of ether. The combined ether phases were evaporated. The dry residues were taken up in RIA-buffer and were used for 11-KT quantification (Schulz 1984). The assay procedure was evaluated for catfish serum as described by Schulz *et al.* (1993).

Results

In the proximal pars distalis (PPD) of pituitaries from immature animals (group I), undifferentiated cells and three morphologically distinct granulated cell types were observed. The secretory granules in one cell type labeled with anti-catfish somatotropin and not with the antisera raised against the GTH-subunits (Fig. 3). These granules were strongly electron dense and mostly round or elongated (Figs. 2 and 3). This cell type is identified as the somatotropic cells.

The secretory granules in the two other cell types were less electron dense and labeled with anti-αGTH (1:1000) and anti-αβGTH-II (1:1000), but not with anti-βGTH-II (1:1000). One of these αGTH-positive cell types contained a heterogeneous granule population (Figs. 4 and 5) and will be referred to as putative TSH cells. The secretory granules in the other αGTH-positive cell type were uniform (Fig. 6). This latter cell type will be referred to as the putative GTH-I cells.

During early spermatogenesis (groups II and III) a fourth granulated cell type appeared in the PPD, in which the secretory granules were also less electron dense and uniform. The granules were immunopositive for anti-αGTH (1:2000), anti-αβGTH-II (1:5000) *and* anti-βGTH-II (1:4000, Fig. 7) and can therefore be identified as GTH-II cells. In addition, these cells occasionally contained large globular inclusions. The GTH-II cells were more abundant than the pGTH-I cells.

During advanced spermatogenesis (groups IV and V) the PPD contained the same four secretory cell types as described above. However, the pGTH-I cells became progressively scarce. Globular inclusions were now regularly found in the GTH-II cells. These globules labeled with anti-αβGTH-II and with anti-βGTH-II (Fig. 8), but they were immunonegative for anti-αGTH (Fig. 9).

The results of the stereological analysis of the mean volume of the secretory granules in the putative GTH-I cells and the GTH-II cells are summarized in Table 1. Two-way analysis of variance of mean volume between GTH cell types and between spermatogenetic stages showed a statistically significant difference between GTH cell types ($p = 0.022$), but not between spermatogenetic stages. The mean volume of the secretory granules in the GTH-II cells is approximately three times larger than that in the putative GTH-I cells. When the volume equivalent diameter is calculated from the mean volume per electron micrograph, the mean granule diameter in pGTH-I and GTH-II cells is 2.61 and 3.69 nm, respectively (two-way ANOVA, $p = 0.001$).

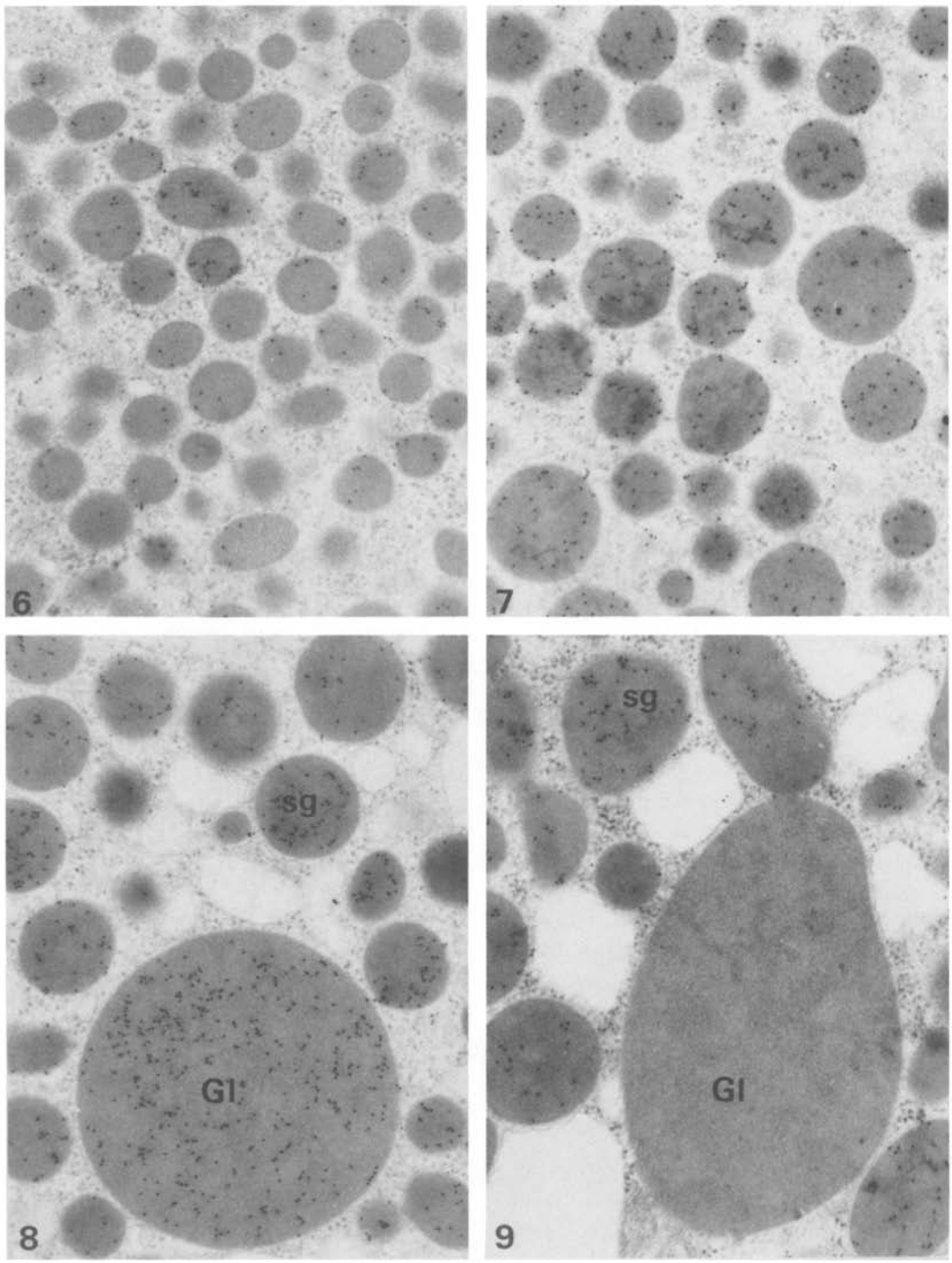

Fig. 6. Immature male: homogeneous population of secretory granules in putative GTH-1 cell, immunoreactive for αGTH (× 44,200).
Fig. 7. Early spermatogenesis: βGTH-II positive secretory granules in GTH-II cell (× 44,200).
Fig. 8. GTH-II cell at advanced spermatogenesis: both the secretory granules (sg) and the globules (Gl) are immunopositive for βGTH-II (× 44,200).
Fig. 9. GTH-II cell at advanced spermatogenesis: note that the secretory granules (sg) are immunopositive for αGTH, whereas the globule (Gl) is not labeled (× 44,200).

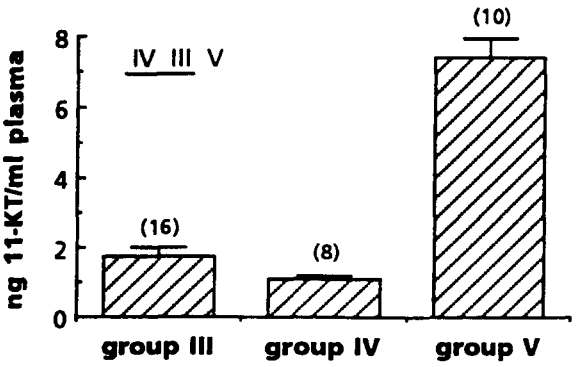

Fig. 10. Blood 11-ketotestosterone (11-KT) levels (mean ± SEM; the number of samples analysed is given in parenthesis) at different stages of testicular development. Groups marked with the same underscore are not significantly different (Student's t-test).

Table 1. Mean volume and standard deviation (SD) of secretory granules in two types of GTH-cells (in $nm^3 \times 10^6$)

	Number of photos	Mean	SD
pGTH-I	7	9.68	3.69
early	4	9.98	4.22
advanced	3	9.28	3.71
GTH-II	23	28.53	16.50
early	5	20.38	6.46
advanced	18	30.79	17.82

Steroid measurements

No blood could be collected from fish of groups I and II. Plasma 11-KT levels were below 2 ng/ml during early spermatogenesis (group III) and in group IV of advanced spermatogenesis (Fig. 10). A significant 4-fold increase was recorded in group V, which showed a higher number of spermatozoa than group IV.

Discussion

In the PPD of immature male African catfish two cell types shared the immunopositive reaction of their secretory granules with anti-αGTH and anti-αβGTH-II, and the absence of labeling with anti-βGTH-II. Since the α subunits of thyrotropin (TSH) and gonadotropin are similar (Burzawa-Gérard 1974), this immunolabeling does not permit to distinguish between TSH and GTH cells. However, the morphological differences between the two cell types, mainly reflected by their size and by the type of secretory granules, allow their identification. The cells with the heterogeneous granule population represent the smallest secretory cells and resemble the small and angular TSH cells as observed in the PPD of adult African catfish (Peute *et al.* 1984). Thus, the third cell type in the PPD of immature fish is a putative GTH cell, not only since their secretory granules are immunopositive for anti-αGTH and anti-αβGTH-II, but also with regard to their general ultrastructure. Based on the size of their secretory granules, the absence of anti-βGTH-II labeling and the time of appearance, these cells in the catfish pituitary strongly resemble the GTH-I cells as described in the salmonid pituitary by Kawauchi *et al.* (1989). Functionally, this cell type has been related to the earlier stages of spermatogenesis and vitellogenesis in salmonids (Nozaki *et al.* 1990b; Naito *et al.* 1991). In addition, GTH-I was the main gonadotropin in blood plasma of juvenile coho salmon (Swanson *et al.* 1989). In the catfish pituitary these putative GTH-I cells were only observed during the spermatogenetic development in pre-adult fish.

At the time of early spermatogenesis (spermatocytes, groups II and III), a second gonadotropic cell type appeared. In contrast to the granules of the pGTH-I cells, their secretory granules were specifically labeled with anti-βGTH-II and these cells may thus be regarded as the GTH-II cells. A similar cell type was observed in the salmonid pituitary after the onset of spermatogenesis until the spawning period (Kawauchi *et al.* 1989). Both in the salmonids and in the African catfish, these cells increase in number during the course of spermatogenesis. In the adult catfish, reared under laboratory conditions, only the GTH-II cell type has been encountered (Peute *et al.* 1984). Since the hormone produced by the GTH-II cells is primarily involved in the later stages of gonadal maturation (Swanson *et al.* 1989; Nozaki *et al.* 1990b), further studies are

necessary to elucidate the fate of the GTH-I cells in fish pituitaries, not only during the first but also in following reproductive cycles.

To determine the mean volume of the secretory granules in the two GTH cell types, point sampling of linear intercept lengths was used (Gundersen and Jensen 1985). Based on this stereological analysis, it appears that the mean volume of GTH-II granules is about three times larger than the volume of the granules in the pGTH-I cells (Table 1), implying a larger storage capacity of the GTH-II granules.

Apart from the secretory granules the GTH-II cells contained some large globules. A new and interesting observation is the presence of αGTH in granules but its absence in globules, whereas βGTH-II is present both in granules and in globules (see also Naito and Nakai 1993). Since globules were shown to contain lytic enzymes as well (Peute *et al.* 1987), this phenomenon may be interpreted in terms of differential storage and/or breakdown of the GTH-II α- and β-subunits.

11-Ketotestosterone (11-KT) is produced by the catfish testis (Schoonen and Lambert 1986) and was also detected in the blood of mature male catfish (Vermeulen *et al.* 1991). 11-KT blood levels increase after GnRH-induced elevation of plasma GTH levels, and decrease after castration (Schulz *et al.* 1993). This androgen can therefore be considered as being a qualitatively and quantitatively important one, also in the African catfish. 11-KT blood levels were low during early spermatogenesis (group III) and also in the first group of advanced spermatogenesis (group IV). A significant 4-fold increase was observed in the last group. There are several possibilities to explain this increase. The steroidogenic capacity and/or the GTH sensitivity of testicular tissue may have become higher. Alternatively, GTH levels may have risen, but they could not be determined in these samples. It is clear, however, that the major changes in pituitary cytology (appearance and then predominance of GTH-II cells, groups III and IV) preceded the increase of blood 11-KT levels in group V.

Acknowledgements

The authors wish to thank Dr. T.T. Chen (Center of Marine Biotechnology, Baltimore USA) for kindly providing anti-catfish somatotropin. We are also grateful to the staff of the Dept. for Image Processing and Design for the preparation of photographs and plates.

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