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Histochemical and immunocytochemical study of gonadotropic pituitary cells of the killifish, *Fundulus heteroclitus* during annual reproductive cycle*

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SUMMARY: A selection of various conventional histochemical techniques, lectins and antibodies were used to identify the gonadotropic and somatotropic cells in the killifish, *Fundulus heteroclitus* pituitary. Distribution and variation of the gonadotropic cells and some histochemical reactions (PAS and Alcian Blue) largely parallel the cyclic changes in the gonads (GSI). Pituitary cells located in the ventral proximal pars distalis (PPD) and pars intermedia (PI) of the pituitary of the killifish, contain basophilic granules, which were positive to Alcian Blue and PAS reactions. These cells showed reactivity to different lectins, indicating the presence of Man (mannose) and/or Glc (glucose); GlcNAc (N-acetylglucosamine) and NANA (sialic acids); Gal (galactose) as well as GalNAc (N-acetylgalactosamine) sugar residues of glycoconjugates. These pituitary basophilic cells were stained with anti carp α,β GTH, and specially with anti carp β GTH; the maximal and minimal immunostaining, against the first antisera used, were observed during maturation/spawning period and during postspawning/resting phase, respectively. On the other hand, acidophilic cells located in the dorsal proximal pars distalis (PPD) of the pituitary gland of the killifish, were selectively stained with eosin, light green and/or orange G, when Haematoxylin-eosin, Haematoxylin-V.O.F and/or Alcian Blue-PAS-Orange G techniques were performed, respectively. These acidophilic cells were negative to PAS, Alcian Blue and lectins reactions; they contain proteins rich in different aminoacids and showed specific immunostaining against anti recombinant seabream growth hormone (anti rsbGH).

Key words: Histochemistry, immunocytochemistry, pituitary, gonadotropic cells, somatotropic cells, GSI, *Fundulus heteroclitus*.

RESUMEN: ESTUDIO HISTOQUÍMICO E INMUNOHISTOQUÍMICO DE LAS CÉLULAS GONADOTROPAS DE LA PITUITARIA DE *FUNDULUS HETEROCLITUS,* DURANTE EL CILCO REPRODUCTIVO ANUAL. – Un grupo de diferentes técnicas histoquímicas convencionales, lectinas y anticuerpos han sido utilizadas para identificar las células gonadotropas y somatotropas de la pituitaria del fundulus, *Fundulus heteroclitus*. La distribución y variación de las células gonadotropas y algunas reacciones histoquímicas (PAS y Azul Alcián) se producen de forma paralela a los cambios cíclicos anuales que experimentan las gonadas (GSI). Las células localizadas en la porción ventral de la proximal pars distalis (PPD) y en la pars intermedia (PI) de la pituitaria de *Fundulus heteroclitus* contienen gránulos basófilos y positivos a las reacciones de Azul Alcián y PAS. Estas células fueron reactivas con diferentes lectinas, indicando la presencia de glicoconjugados con residuos de Man (manosa) y/o Glc (glucosa); GlcNAc (N-acetilglucosamina) and NANA (ácidos siálicos); Gal (galactosa), así como GalNAc (N-acetilgalactosamina). Estas células basófilas, positivas con el anticuerpo anti carpa α,β GTH y especialmente con anti carp β GTH,

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mostraron las máximas y mínimas inmunotinciones, con el primer anticuerpo usado, durante el periodo de maduración/puesta y durante el periodo de postpuesta-reposo, respectivamente. Por otra parte, las células acidófilas localizadas en la porción dorsal de la proximal pars distalis (PPD) de la pituitaria de *Fundulus heteroclitus,* fueron selectivamente teñidas con eosina/verde-luz y/u orange G (Hematoxilina-eosina/ Hematoxilina-V.O.F. y/o Azul Alcián-PAS-Orange G). Estas células acidófilas que contienen proteinas ricas en diferentes aminoácidos, fueron negativas a las reacciones del PAS, Azul Alcián y lectinas y mostraron inmunotinción específica frente al antisuero, anti hormona de crecimiento recombinante de dorada (anti rsbGH).

Palabras clave: Histoquímica, inmuno citoquímica, pituitaria, células gonadotropas, células somatotropas, IGS, *Fundulus heteroclitus*.

INTRODUCTION

The advances in immunocytochemical and cytochemical techniques have been particularly useful in order to identify different pituitary hormones and their location in segregated pituitary cells. Generally, the basophilic gonadotropic (GTH) and thyrotropic (TSH) cells, and the acidophilic somatotropic (GH) cells are located in the dorsal and ventral part of proximal pars distalis (RPD) of fish pituitaries. These and other hormonal pituitary cells have been identified by using different piscine and/or mammal antisera (Van Putten *et al*., 1981; Quesada *et al*., 1988; García-Ayala *et al*., 1989; Nozaki *et al*., 1990 a,b; Toubeau *et al*., 1991; Yan and Thomas, 1991; Power, 1992; García-García *et al*., 1994; Rodríguez-Gómez *et al*., 1997 between other). Teleosts pituitary secretes gonadotrophins which control gametogenesis and gonadal hormone synthesis. They are glycoproteins consisting of two noncovalently bound polypeptide chains (α and β subunits). Both subunits contain multiple intramolecular cross-linked disulfide bonds and are glycosylated at specific sites (Pierce and Parsons, 1981). Con A is a lectin which has been used for the purification and isolation of GTHs (Idler and NG, 1983; García-García, 1995). A dual system based on Concanavalin A-Sepharose separation, consisting of maturational or "carbohydrate-rich" GTH (CR-GTH) and vitellogenic or "carbohydrate-poor" GTH (CP-GTH) was described by Idler and Ng (1983). Presumably, the CR-GTH corresponds to a tetrapod GTH, such as LS o FSH. However, the CP-GTH has no comparable counterpart, since GTHs isolated thus far have all been glycosylated. However, neither salmon GTH I nor GTH II bear chemical similarity to vitellogenic GTH (Itoh *et al*., 1988). Chemical duality of GTHs with different β-subunits primary structures, designated GTH I and GTH II, have been isolated and characterizated in different fish species (Kawauchi *et al*., 1991). Recently, cDNAs encoding the α and β- subunits of GTH I and GTH II of *Fundulus heteroclitus* have been cloned and sequenced. In this species, both β-subunits have well conserved cysteine positions when aligned with other members of the glycoprotein family (Lin *et al*., 1992). Burton *et al*. (1981) reported the general topographic distribution of immunoreactive cells to Con AI GTH, Con AII GTH, ConAI TSH and Con AII TSH in the winder flounder pituitary. Lectins are proteins or glycoproteins of non-inmune origin with the ability to recognize specific saccharides, such as : N-acetylneuraminic acid or sialic acid (NANA), mannose (Man), glucose (Glc), galactose (Gal), fucose (Fuc), N-acetyl-D-galactosamine (GalNAc), β-D-glucose N-acetylglucosamine (β-D-GlcNAc) residues, etc. The two main families of glycoproteins are those having carbohydrate side chains linked N-glycosidically and O-glycosidically. The only N-glycosidic bond found in glycoproteins is N-acetyl-glycosaminyl-asparagine. The O-glycosidic bond presents a variety of links, the most important being the mucin type in which the oligosaccharide chain is linked from N-acetyl-D-galactosamine to the hydroxyl groups of L-serin and/or L-threonine. Some lectins may preferentially bind to either O-or N-linked glycoproteins. Thus, DBA (Horse gram) and PNA (Peanut) identify glycoproteins with O-linked carbohydrate chains whereas Con A (Jack bean) and others lectins identify N-linked oligosaccharide chains (Pajak and Danguy, 1993). Annual changes in the reproductive cycle of teleosts fish appear to be largely under control of pituitary gonadotropic cells. Seasonnal changes of the gonadotropic cells have been described in different teleosts (Carrillo, 1977; Peute *et al*., 1978; Van Oordt *et al*., 1987; Power, 1992; Nozaki *et al*., 1990 a,b). *Fundulus heteroclitus* presents an asynchronous reproductive cycle (Wallace and Selman, 1981; Drake *et al*., 1987), with highest plasmatic oestradiol levels during maturation-spawning phase (García-García, 1995). These events have been related with synthesis and secretion of gonadotropins and with the presence of vitellogenic and mature oocytes (Lin *et al*., 1992; García-García, 1995). Olivereau (1976, 1978) pointed that in salmon captured from the sea and possesing a low

GSI, on type of putative GTH cell with few glycoprotein granules was numerous and active, whereas in sexually mature salmon, another type of putative GTH cell with glycoprotidic granules predominated in the proximal pars distalis (PPD). On the other hand, growth hormone (GH) is a polypeptide of about 22 KD produced in the pituitary gland that plays an essential role in the stimulation of somatic growth and development in vertebrates. Although natural GHs are difficult to obtain in large quantity, DNA technology has provided a means of mass producing GHs, and thus recombinant GHs may be of practical use as growth enhancers in aquaculture (Moriyama *et al*., 1993). Chum salmon GH was the first teleosts to be expressed in *Escherichia coli,* and the recombinant salmon growth hormone (rsGH) has since been demostrated to have growth-promoting activity equivalent to that the natural hormone. In *Fundulus heteroclitus* rsGH present clearly somatotropic activity. It is suggested that rsGH may play a subsidiary synergistic role to other pituitary hormones in killifish gonadal development (Oliveira *et al*., 1993). Recently, the cloning and expression of recombinant seabream growrh hormone (rsbGH) and its specific antisera were performed by Martínez-Barbera (1995); this antisera -anti rsbGH- was used with excellent results in *Solea senegalensis* pituitary (Rodríguez-Gómez *et al*., 1997) and in the present study. According to Batten (1986), the use of piscine-anti somatotropins (GH) seems to be necessary for the immunocytochemical observation of GH cells on teleosts pituitary. The pituitary gland of teleosts has been subject of research for many years; special consideration have been given to the gonadotropic and somatotropic cells, because of their importance for the control of the reproduction and growth, respectively. These pituitary cells have been studied by using a combination of classical cytochemical and immunocytochemical tests. To our knowledge, lectins are not so far largely employed as cytochemical markers of these cells, and however some lectins can be useful to differentiate some pituitary hormone cells, such as the glycoprotein hormones segregated by GTH and TSH cells and/or the polypeptidic hormones synthesized by GH cells (Rodríguez-Gómez *et al*., 1997). The purpose of this paper was, by using different histochemical (PAS, Alcian Blue, Bromophenol Blue, lectins) and immunocytochemical techniques, to identify some pituitary cells (GTH, TSH and GH), as well as the annual variation of GTH cells during reproductive cycle of killifish, *Fundulus heteroclitus* females.

TABLE 1.– Histochemical techniques used for characterization of carbohydrates and proteins in *Fundulus heteroclitus* pituitary cells.

Reactions	Fuctional groups and/or components demonstrated.
Periodic acid-Schiff (PAS)	Neutral mucins, sialomucins (without side-chain substituent of with O-acyl substituents at C7 or C9)
Diastase-PAS	Glycogen and/or Neutral and/or acid sialomucins
Alcian Blue pH 2.5/ 1, 0.5	Acidic groups (carboxylated and/or sulphated)
- Sialidase-Alcian Blue pH 2.5 - Chlrorhydric hydrolisis - Alcian Blue pH 2.5	Sialic acid
Mild periodate oxidation-Schiff	Sialomucins
Saponification-mild periodate oxidation-Schiff	Sialic acid and/or sialic acid with O-acyl substituent at C8 or di or tri substituted
Desulphation (methylation and saponification) and Alcian Blue pH 2.5, 0.5,1/PAS	Blockage of acidic groups and Sulphatolisis (Hydrolisis of sulphated groups) and reactivity of hydroxyl groups acetylated and of carboxyl groups methylated
Bromophenol Blue-Hg	Proteins in general
Ferric Ferricyanide (Fe III)	Proteins rich in SH
N-ethylmaleimide-Thioglycollate-Ferric Ferricyanide (Fe III)	Proteins rich in SH and S-S and reductor groups
Ninhydrin-Schiff	Proteins rich in Lysine
1,2 Naphtoquinone-4-sulphonic acid	Proteins rich in Arginine
Hg sulphate-sulphuric acid-sodic nitrate	Proteins rich in Tyrosine
p-dimethylamino-benzaldehyde	Proteins rich in Tryptophan

Histochemical techniques are taken of Pearse (1985) and Bancroft *et al*. (1990).

TABLE 2. – Peroxidase conjugated lectins (HPR-lectin) used for histochemical characterization of carbohydrate residues of glycoconjugates.

Lectin	Concentration (μg/ml)	Carbohydrate binding sugar
Group 1. Affinity for glucose and mannose		
Con A (*Canavalia ensiformes*)	25	α-D-Man>α-D- Glc
Group 2. Affinity for N-acetylglucosamine		
WGA (*Triticum vulgaris*)	20	(β-D-GlcNAc)n, sialic acids
Group 3. Affinity for galactose and N-acetylgalactosamine		
PNA (*Arachis hypogea*)	25	β-D-Gal(1-3)-GalNac
DBA (*Dolichus biflorus*)	25	α-D-GalNAc
RCA (*Ricinus communis*)	20	β-D-Gal >α-D-Gal >>GalNAc
SBA (*Glycine maximus*)	20	α-D-GalNAc >β-D-GalNAc
Group 4. Affinity for L-Fucose		
UEA (*Ulex europaeus*).	25	α-L-Fuc

Abbreviations: Fuc: fucose, Gal: galactose, GalNAc: N-acetylgalactosamine, Glc: glucose, GlcNAC: N-acetylglucosamine, Man: mannose. Lectin techniques are taken from the monographs of Pearse (1985) and Bancroft *et al*. (1990).

MATERIAL AND METHODS

Adult females of the killifish, *Fundulus heteroclitus,* were collected from March to February in the salt-marshes surrounding the Bay of Cádiz (SW Spain) and kept in the laboratory in running seawater until used. Specimens were stunned in ice-water, killed by decapitation and the head fixed in 10% v/v buffered formaldehyde (pH 7.2) for two hours at room temperature. Brains with the pituitary attached were then carefully removed and further fixed overnight in the same fixation. Gonadosomatic index (GSI) (n=12-14 females/month) was also determined in these specimens (weight gonad x 100/body weight). Stadistical differences were determinated by one-way analysis of variance. After fixation, tissues were washed for one hour in running tap-water and embedded in paraffin. Parasaggital sections (5-6 μm) were mounted on gelatin-coated glass-slides and deparaffinized through xylene-ethanol-water. Haematoxylin-eosin and Haematoxylin-Gutierrez V.O.F (light green-orange G-acid fuchsin) morphological techniques were performed according to Gutíerrez *et al*. (1985) and Sarasquete *et al*. (1993). Histochemical conventional techniques of carbohydrates and proteins and histochemistry of lectins, used in this paper, are summarized in Table 1 and 2, respectively. All histochemical tests were carried out according to the directions given in monographs of Pearse (1985) and Bancroft *et al*. (1990). For histochemistry of lectins, endogenous peroxidase activity was blocked with 1% hydrogen peroxide in methanol during 30 minutes. Sections were washed in Tris Buffer Saline (TBS) (50 mM Tris-HCl, 0.5 M NaCl, pH 7.2-7.5) and incubated for 2 h at room temperature in horseradish-peroxidase-conjugated lectins (Sigma, St Louis, MO) at the appropiate dilution (Table 2). After washing in TBS, peroxidase was developed in TBS containing 0.05% 3,3 diaminobenzidine tetrahydrochloride (Sigma, St Louis, MO) and 0.05% hydrogen peroxide. When developed, sections were dehydrated, cleared and mounted in Eukitt. Substitution of lectin-HRP conjugated for TBS was used as control. For immunohistochemistry, endogenous peroxidase activity was also blocked. Before immnunostaining, sections were transferred for 1-2 hours to 3% bovine serum albumine (BSA) in TBS and washed in TBS (2 x 5 min). Sections were incubated overnight in a moist chamber at 4ºC with different primary antibodies, such as: anti carp α,β gonadotropin (GTH) (donated by Dr. Peute), anti

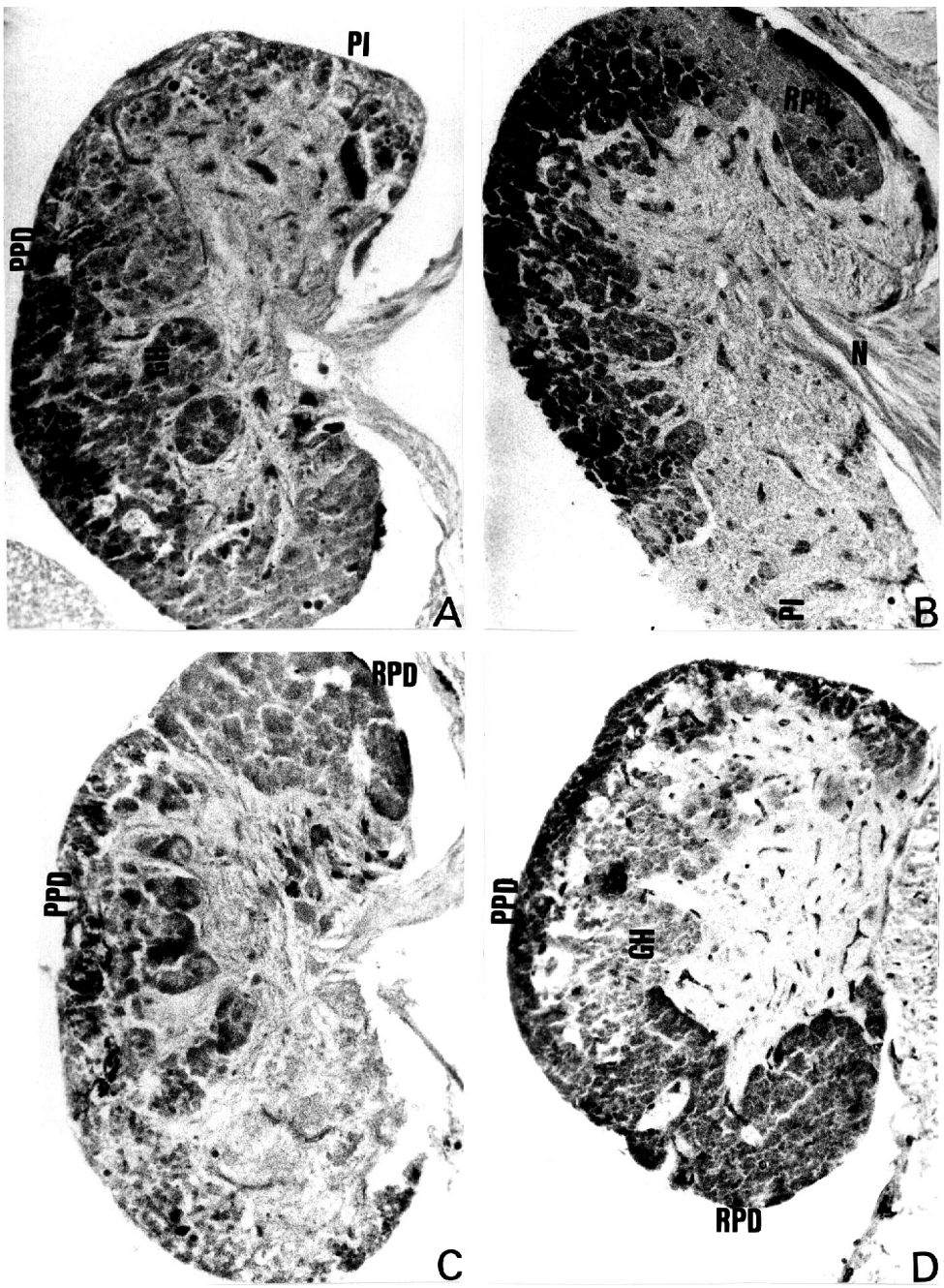

FIG. 1. – (A). Pituitary gland of *Fundulus heteroclitus* in March. Positivity to PAS reaction in the basophilic cells located in the ventral part of the proximal pars distalis (PPD) and in the pars intermedia (PI). Negativity to PAS reaction in the somatotropic cells with affinity to light green. PAS-V.O.F. X250 (B). Pituitary gland of *Fundulus heteroclitus* in May. Strong reactivity to PAS reaction in the gonadotropic cells. Somatotropic cells are PAS negative. PAS-V.O.F reaction. X250 (C). Pituitary gland of *Fundulus heteroclitus* in September. Weak PAS reactivity in the gonadotropic cells (GTH). Somatotropic cells (GH) are stained with light green of the polychrome V.O.F and they are PAS unreactive. PAS-V.O.F. reaction. X250 (D). GTH cells of the pituitary gland of *Fundulus heteroclitus* stained with WGA. GH cells cells are WGA unreactive with this and other lectins. WGA reaction and counterstaining with Haematoxylin. X250 GH: Growth hormone or somatotropic cells; N: neurohypophysis; PI: pars intermedia; PPD: proximal pars distalis; RPD: rostral pars distalis.

carp β GTH (donated by Dr. Burzawa-Gerard) as well as anti human growth hormone hGH (purchased from Sigma, St Louis, MO and from UCD Bioproducts, Brussels, Belgium) and anti recombinant seabream growth hormone (rsbGH) (donated by Dr. Valdivia). Antibodies were diluted 1:500 or 1:5000 in TBS containing 1% BSA (bovine serum albumin). Protein G-horseradish peroxidase and/or PAP immunohistochemical methods were performed according to García-García *et al*. (1994).

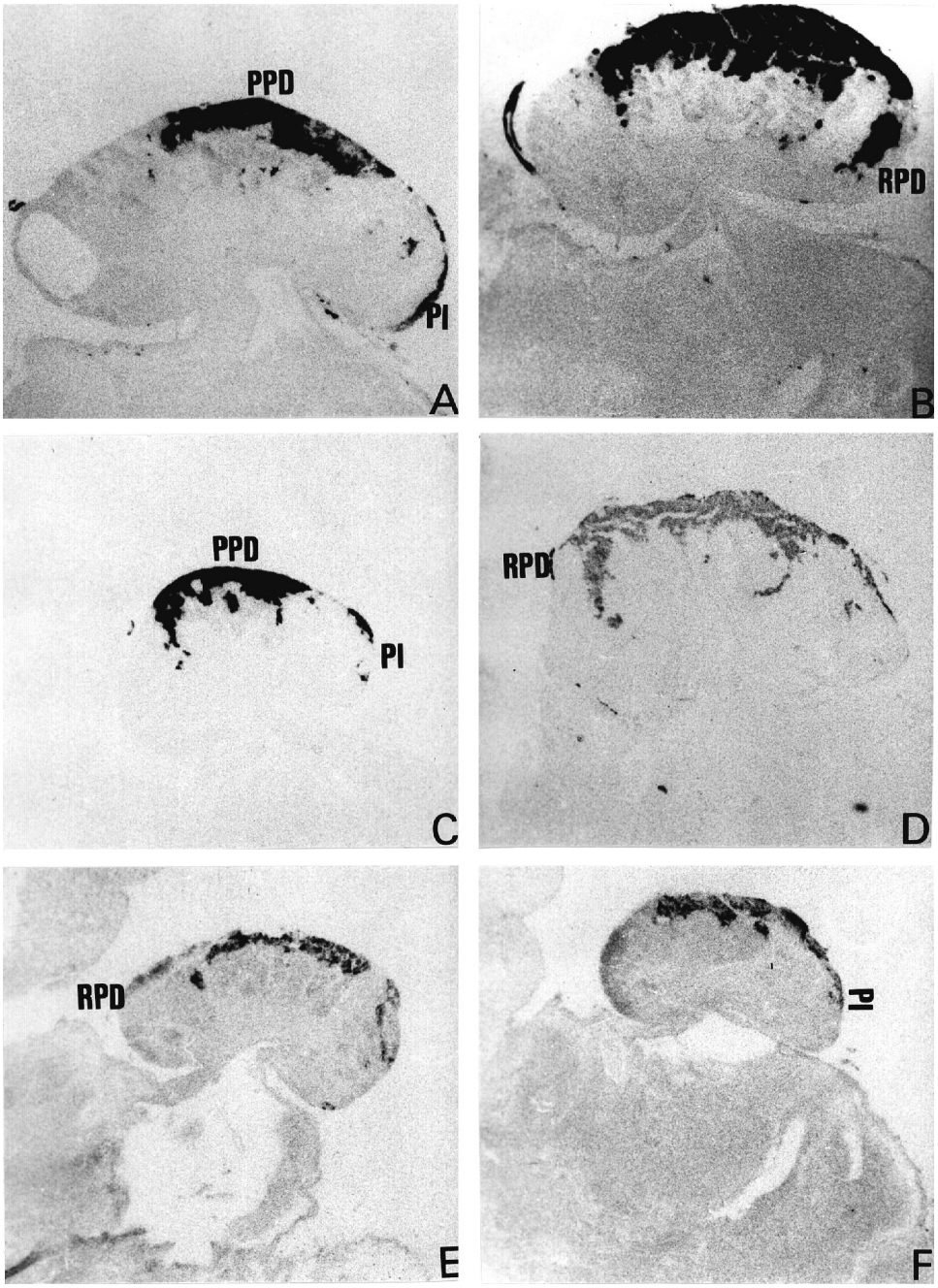

FIG. 2. – Parasaggital sections of the *Fundulus heteroclitus* pituitary gland stained with anti carp α, β gonadotrophin (GTH). Presence of gonadotropic cells in the ventral part of the proximal pars distalis (PPD) and in pars intermedia (PI). (A): GTH cells in March; (B): May; (C): June; (D): August ;(E): October and (F): December. Peroxidase-antiperoxidase technique (PAP). X125. PI: pars intermedia; PPD: proximal pars distalis; RPD: rostral pars distalis.

RESULTS

The cells located in the ventral and dorsal part of the proximal pars distalis (PPD) of the killifish, *Fundulus heteroclitus* pituitary gland (Fig. 1, 2 and 3A) contain basophilic granules and were stained with PAS (Fig. 1A, 1B and 1C), diastasa-PAS and Alcian Blue pH 2.5, suggesting the absence of glycogen and the presence of glycoproteins (Fig. 1D). These reactions, as well as previous treatments, such as: mild periodate oxidation-Schiff, sialidase, chloridric hidrolisis, methylation, saponification, etc. (Table 1), indicate the presence, in these cells, of glycoproteins containing sialic acid. These pituitary cells also contain a weak presence of sulphated (Alcian Blue pH 0.5 and 1), as well as abundant SH and S-S

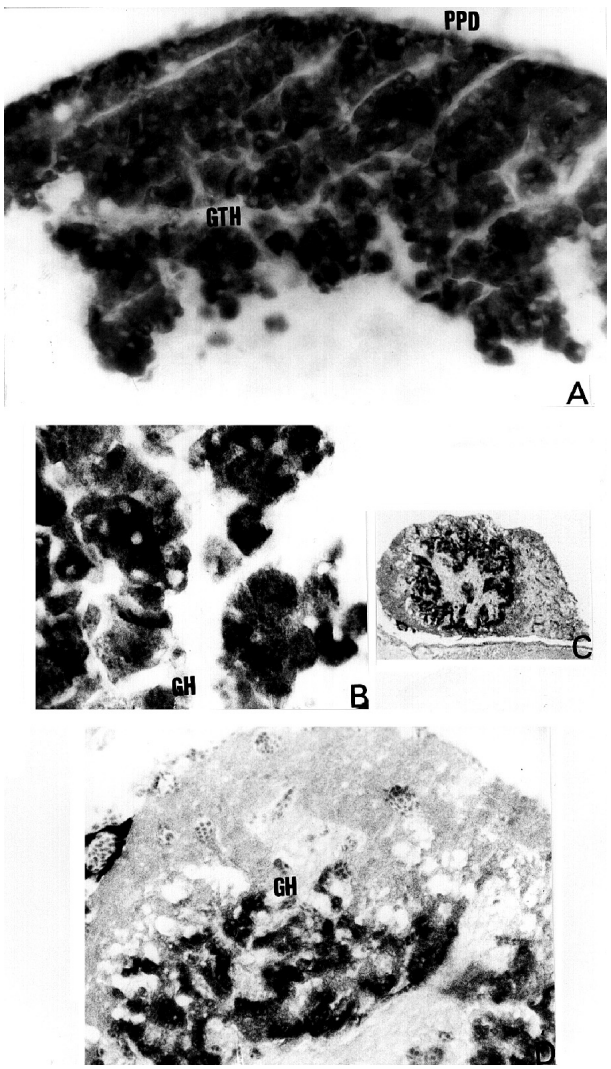

FIG. 3. – (A). GTH cells located in the ventral part of the proximal pars distalis (PPD) stained with anti carp a,b gonadotrophin (GTH). Protein G-Horseradish Peroxidase method (PG-HRP). X 400 (B). GH cells strongly stained with Bromophenol Blue reaction that evidence general proteins. X400 (C). Anti-recombinant seabream growth hormone (rsbGH) immunoreactive cells located in the dorsal part of the proximal pars distalis (PPD) of the *Fundulus heteroclitus* pituitary. Peroxidase-antiperoxidase method (PAP). X125 (D). GH cells stained with anti-recombinant seabream growth hormone(rsbGH). Protein G-Horseradish Peroxidase method (PG-HRP). X400GH: growth hormone or somatotropic cells; GTH: gonadotropic cells; PPD: proximal pars distalis.

groups. Different sugar residues of glycoconjugates (Table 2) were detected in some basophilic cells of *Fundulus heteroclitus* pituitary. These cells reacted with ConA (α-D-Man and/or α-D-Glc) and RCA (β-D-Gal and /or α-D-Gal and/or GalNAc), showing these cells (Fig. 1D) higher affinity for WGA (β-D-Glc NAc and sialic acid) than other pituitary cells. Negative results were observed with other lectins, such as UEA (α-L Fuc), DBA (α-D-GalNAc) and PNA (β-D-Gal(1-3)-D- GalNAc). However, after desulphation technique (methylation and saponification processes), negativity to Alcian Blue pH 0.5 and 1, positivity to Alcian Blue pH 2.5, as well as moderate PNA reactivity were detected in these pituitary cells. Moreover, after sialidase treatment, the alcianophilia of these cells stained with Alcian Blue at pH 2.5 decreased and weak and moderate staining apperared for WGA and PNA lectins. These results suggest again the presence of sialic acids, β-D-Glc NAc, as well as the presence of β-D-Gal (1-3)-D-GalNAc sugar residues. The basophilic cells, located in the ventral and dorsal areas of the PPD of the killifish pituitary, react against anti carp a,b GTH (Fig. 2 and 3A) and anti carp β GTH (dilution 1:5000). A weakly immunostained cell group, in the anterior and medial dorsal parts of the PPD, was also detected by using anti carp α, β GTH (dilution 1: 500) but negative results were observed with higher dilutions (1: 5000) and with anti carp β GTH (diluted 1:500). These "putative TSH cells" were also positive with Alcian blue pH 2.5, but the PAS reaction and the reactivity to WGA appeared more weak in these than in GTH cells. Moreover, binding to the PNA lectin was not detected in these basophilic cells. *Fundulus heteroclitus* presents an asynchronous gonadal development. The highest values of the GSI were observed during maturation-spawning period and the minimal values were observed after spawning and during resting phase (Fig. 4). Gonadosomatic index (GSI) increased from January (3.86) to peak in April (11.91). From May to September, the GSI decreased until 1.48, indicating the gonadal regression after spawning. From September to December, GSI values were low (<2) (Fig. 4). The annual changes of the GSI, the PAS, Alcian Blue reactions and the immunocytochemical staining of the gonadotropic cells showed paralell results. Thus, during the maturation-phase, from April to June, we can observe the maximal immunoreactivity against anti carp α, β GTH (Fig. 2); the presence of glycoproteins and PAS reactivity (Fig. 1A, 1B and 1C) were also higher in this than in other periods of the reproductive cycle of *Fundulus heteroclitus*. During this time, reactive gonadotropic cells were also observed in the limit between PDP and RPD and in the pars intermedia (PI) (Fig.2) During the postspawning-resting period (August-October), and specially in September, the immunoreactivity against anti carp α, β GTH (Fig. 2), and the glycoprotein content of these cells was very weak. Finally, anti carp α,β GTH (dilution 1:500) immunoreactive cells, located in the anterior and medial dorsal

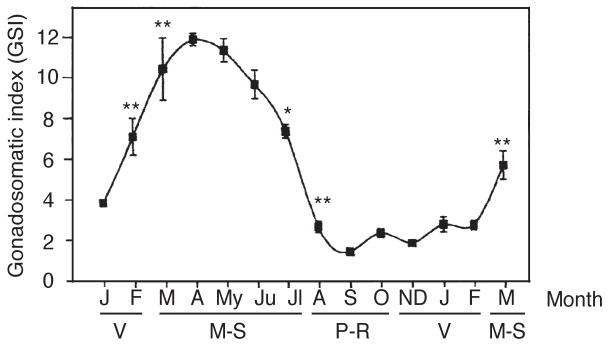

FIG. 4. – Annual variation (monthly mean values) of the gonadosomatic index (GSI) in *Fundulus heteroclitus* females. Phases of the reproductive cycle: M-S: Maturation-Spawning; P-R: Postspawning-Resting, V: vitellogenesis. Abbreviation of months: J: January; F: February; M: March; A: April; My:May; Ju: June; Jl: July; A:August; S:September; O: October; N: November; D: December. (* P< 0.05; ** P<0.01).

pars of the PPD, did not appear vary during reproductive cycle. On the other hand, the acidophilic cells (Fig. 1A, 1B and 1C) present in the dorsal part of the proximal pars distalis (PPD) showed a strong affinity to orange G (Alcian Blue pH 2.5-PAS-Orange G), eosin (Haematoxylin-eosin) and light-green (Haematoxylin-V.O.F.). These cells were positive to general protein reaction (Fig. 3B) and for those reactions that evidence proteins rich in lysine, arginine, tryptophan, tyrosine and specially in SH and -S-S- groups. However, these pituitary cells were negative to PAS, Alcian Blue and lectin reactions (Fig. 1). Specific binding to the anti recombinant sebream growth hormone (rsbGH) was observed in these acidophilic cells (Fig. 3C and 3D), but negative results were obtained with antisera to human GH (data no presented).

DISCUSSION

In killifish, *Fundulus heteroclitus,* the pituitary cells located in the ventral and dorsal area of the proximal pars distalis (PPD) and pars intermedia (PI) contained basophilic granules and were positive to PAS, diastase-PAS and Alcian Blue pH 2.5 reactions, suggesting the absence of glycogen and the presence of glycoproteins. Some similar histochemical results were observed in other species (Carrillo, 1977; Peute *et al*., 1978; Olivereau and Nagaham, 1983; Cambre *et al*., 1986; García-Ayala *et al*., 1989; Fabridge *et al*., 1990; Yan and Thomas, 1991). In *Fundulus heteroclitus,* as in *Solea senegalensis* pituitary (Rodríguez-Gómez *et al*., 1997), these pituitary cells showed reactivity to different lectins, indicating the presence of glycoproteins containing β-D-Gal and/or α-D-Gal and/or GalNAc; α-D- Man and/or α-D- Glc; sialic acids and β-D-GlcNAc, as well as β-D-Gal(1-3)-D-GalNAc sugar residues. According to Roberts (1977), the "mild" periodate oxidation, also performed in this paper, selectively oxidizes sialic acid residues while other sugar components remain unreactive to Schiff s reagent. Taking in account our results, we can suggest that sialic acids present in these pituitary cells of *Fundulus heteroclitus* were not C8, C7-8, C7-9 or C7-8-9 acetylsialic acids, because in this case the PAS reaction should be negative; gonadotrophic cells of the killifish could contain unsubstituted sialic acid and/or C7 or even C9 substituted sialic acids (in Pearse, 1985). In *Fundulus heteroclitus,* oestradiol synthesis was not stimulated by deglycosilated GTHs (García-García, 1995). In *Fundulus heteroclitus,* these basophilic pituitary cells also contain sulphated groups (Alcian Blue pH 0.5 and 1) and only reacted with PNA when a desulphation process (hydrolisis of sulphated groups) was previously performed. According to Martínez-Menárguez *et al.* (1992), the binding of PNA to its specific sugar acceptor is challenged by sulphation of galactose. The sulphated groups occur in glycoproteins mainly in ester linkages with GlcNAc and Gal (Carter *et al*., 1988). On the other hand, after sialidase treatment, reactivity with WGA was slightly depressed and PNA reactivity was detected on the gonatotrophic cells, thus demostrating the presence of sialic acid and β-D-GlcNAc, as well as β-D-Gal (1-3)-GalNAc. According to Gueri *et al.* (1993) when the substract bound to PNA after sialidase treatment, as in our study, sialic acid was present linked to penultimate D-galactose-(β1-3)-N-acetyl-D-galactosamine residue.The well-known inhibitory action of terminal residues of sialic acid over the binding of PNA might be caused as Lotan *et al.* (1995) suggested by the strong negative charge and/or steric hindrance. The location of the gonadotropic cells in the pituitary of the killifish, *Fundulus heteroclitus,* was previously studied by García-García *et al.* (1994), using anti human LH, anti human CG antisera and different immunohistochemical methods. In this study, a similar distribution was observed using different piscine antibodies (anti carp α,β-GTH and anti carp β-GTH). It might be expected that antisera raised against a teleost α,β GTH reacts with the thyrotropic cells (TSH-cells). In *Fundulus heteroclitus,*

anticarp α, β GTH (diluted 1: 500) revealed the presence of a weakly immunostained cell group in the anterior and medial dorsal pars of the PPD, most likely representing the TSH-cells, as in other species (García-Ayala *et al*., 1989). Yan and Thomas (1991) pointed that the cross-reaction between gonadotropic and thyrotropic cells could be significantly reduced by diluting the antiserum (1:2500). In *Fundulus heteroclitus* pituitary, these basophilic cells - TSH cells- were positive with Alcian Blue pH=2.5, but the PAS and WGA reactivities (β-D-GlcNAc and/or sialic acid) were more weak in these pituitary cells. These TSH cells did not appear in sections treated with anti-carp β-GTH (1:500). According to Pierce and Parsons (1981), within a given species, the α -subunit is essentially identical among the glycoprotein hormones and is highly conserved, even between distantly related species, whereas the β-subunit is unique to each hormone and apparently bestows the biological specificity. Taking in consideration the annual variation of the GSI, the histological and histochemical characteristics of the ovary, as well as the plasmatic oestradiol levels, García-García (1995) divided the reproductive cycle of *Fundulus heteroclitus* in three periods: full gametogenesis (November to February), maturation-breeding period (March to July) and postpawning-resting period (August to October). In *Fundulus heteroclitus,* as in other species (Carrillo, 1977), the basophilic cells located in the PPD or gonadotrophic cells, showed secretory changes during different phases of the annual reproductive cycle. Thus, the GSI, the immunostaining of the gonadotropic cells, as well as the PAS and Alcian Blue reactions showed paralell results. The higher GSI levels and the maximal tinctorial affinity (PAS, Alcian Blue, lectins, antibodies, etc.), were observed during maturation/spawning phase. Cytochemical affinities (glycoproteins, antisera, etc.) and GSI values begin to decrease during spawning period. Although to study the annual changes of *Fundulus heteroclitus* GTH pituitary cells, we used antisera against whole carp gonadotropin, the histochemical differences observed in these pituitary cells (PAS, Alcian Blue, Con A and WGA), as well as the seasonal changes in the immunostained GTH cells, could be related with the annual variations of the GTHs (I and II) cells described in salmonids by Nozaki *et al*. (1990 α, β). During different phases of the reproductive cycle of some species (Carrillo, 1977), as in *Fundulus heteroclitus,* the cytoplasm of the gonadotropic cells presents numerous PAS granulations, as well as nuclear and cellular hypertrophy. During the breeding period, these cells progessivelly become involutioned and during postspawning phase, the PAS cytoplasmic granulations, as well as the tinctorial affinity for the morphological and histochemical stains were scarce. The high hypertrophy of these cells, and the higher content of sialoglycoproteins, can be related with a higher production of GTHs during different periods of the annual reproductive cycle (García-García, 1995). According to Peute *et al*. (1978), both vitellogenesis and the formation of sperm cells can be correlated with a decreased pituitary GTH content. In *Salmo gairdeni,* GTH I and GTH II cells were found at the time of final reproductive maturation, although the number of GTH II cells was higher that that of GTH I cells (Nozaki *et al*., 1990 b). In *Pleuronectes platessa,* the pituitary of spent male contained weakly staining GTH cells confined to the PPD (Power, 1992). In *Fundulus heteroclitus,* an increased immnunostaining of the gonadotropic cells, together with higher plasmatic oestradiol levels (García-García, 1995) were observed during maturation/spawning phase (March to June) and a progressive decrease was observed since spawning. Taking account our results, we can suggest that the strong PAS, Alcian Blue pH 2.5 and lectin reactions, as well as the high immunoreactivity against anti carp α, β GTH observed during maturation/spawning phase of *Fundulus heteroclitus,* could be related with GTH II and TSH cells. In different salmonids and according to Nozaki *et al*. (1990 a), GTH I cells were PAS unreactive, while GTH II and TSH cells were stained intensely with PAS. Moreover, these authors suggested that GTH I and GTH II were produced in distinctly different cells of the pituitary in both rainbow trout and Atlantic salmon. On the other hand, the acidophilic growth hormone cells (GH) restricted to the dorsal part of the proximal pars distalis (PPD) of the pituitary of *Fundulus heteroclitus,* were specifically bound to anti recombinant seabream growth hormone (anti rsbGH) prepared by Martínez-Barberà (1995). In different species, the PPD of the pituitary gland contain somatotropic cells in the dorsal region, which were bound specifically with anti-porcine GH (Tubeau *et al*., 1991), anti chum and coho salmon GH antisera (Yan and Thomas, 1991) and with anti-trout and anti-salmon GH antisera (Cambre *et al*., 1986; Power, 1992). In agreement with previous findings in other species (Margolis-Kazan and Schreibman, 1981; Nagahama *et al*., 1981; Siegmund *et al*., 1987; Yan and Thomas,

1991), in killifish pituitary gland, negative results were obtained with human antiGH. According to Batten (1986), the use of piscine-anti GH antisera seems to be necessary for the immunocytochemical observation of these cells on teleost pituitary. As in *Solea senegalensis* pituitary (Rodríguez-Gómez *et al*., 1997), the somatotrophic cells of the killifish pituitary, were negative to Alcian Blue, PAS and lectin reactions and were positive to general and specific protein reactions and to reactions that identify SH and -S-S- groups.

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