ACADEMIC PRESS

Available online at www.sciencedirect.com

SCIENCE DIRECT®

General and Comparative Endocrinology 132 (2003) 454–464

GENERAL AND COMPARATIVE ENDOCRINOLOGY

www.elsevier.com/locate/ygcen

Ontogeny of adenohypophyseal cells in the pituitary of the American shad (*Alosa sapidissima*)

Raul Laiz-Carrión, María del Mar Segura-Noguera, María del Pilar Martín del Río, and Juan Miguel Mancera*

Departamento de Biología, Facultad de Ciencias del Mar y Ambientales, Universidad de Cádiz, Puerto Real, Cádiz 11510, Spain

Accepted 11 March 2003

Abstract

The distribution and ontogeny of adenohypophyseal cells have been studied in the pituitary gland of embryos, larvae, and juveniles of the clupeid American shad (*Alosa sapidissima*) using immunocytochemical techniques. In juvenile specimens, adenohypophysis was composed of *rostral pars distalis* (RPD), formed by cavities lined by prolactin (PRL), adrenocorticotropic hormone (ACTH), and gonadotropic hormone (GTH) cells; *proximal pars distalis* (PPD), containing growth hormone (GH), GTH, and putative thyroid stimulating hormone (TSH) cells; and *pars intermedia* (PI) with somatolactin (SL) and melanophore stimulating hormone (MSH) cells. At 3 days post-fertilization (3 days pre-hatching) the pituitary of embryos consisted of an oval mass of cells, close to the ventral margin of the diencephalon, divided in rostral and caudal regions. At this time PRL and ACTH cells appeared in the rostral region of the adenohypophysis, while SL cells were observed in the caudal region where MSH cells showed reactivity 1 day before hatching. At variance, GH cells showed a weak immunoreactivity in the rostral portion at hatching that increased 2 days latter. GTH cells also showed weak immunoreactivity in the rostral region of the adenohypophysis at hatching time. Two days later GTH cells were located in the rostral and central regions of the adenohypophysis. At hatching, the neurohypophysis was very small and no nerve processes were seen to penetrate the adenohypophysis tissue. After hatching, the pituitary gland elongated and in 7 days old larvae, the RPD showed a small lumen surrounded by a palisade of PRL, ACTH, and GHT cells; the PPD showed GH and GTH cells while the PI contained SL and MSH cells. The adenohypophysis and neural lobe increased in size with development and, in 42 days old larvae, they were similar to those of juvenile specimens.

Keywords: Fish; Adenohypophyseal cells; Ontogeny; Embryo; Larva; Immunocytochemistry; *Alosa sapidissima*

1. Introduction

The adenohypophyseal endocrine cells of teleost synthesize eight different hormones: prolactin (PRL), adrenocorticotropic hormone (ACTH), growth hormone (GH), thyrotropin (TSH), gonadotrophins (GTH I and GTH II), melanotropin (MSH), and somatolactin (SL). In non-clupeid teleosts, the morphological characterization and distribution of each cell type have been studied by histochemical, ultrastructural, and immunocytochemical techniques (Ball and Baker, 1969; Follénius et al., 1978). In clupeid fishes, adult pituitary gland was studied using histochemical and electronmicroscopic procedures (Cook et al., 1973; Sathyanesan, 1963) but no immunocytochemical studing has yet been done.

It has been shown that pituitary hormones play important roles in fish embryonic development and larval growth (Majumdar and Elsholtz, 1994; Tanaka et al., 1995). Ontogenetic studies of pituitary cells in freshwater and seawater teleost species revealed that the onset of their activity varied among species and cell types (see Section 4 for references). In a given species, the chronological appearance of the different adenohypophyseal cell types may reflect the involvement of the respective hormone during each developmental stage. Thus according to Balon (1981), it is of great interest to know the time course of appearance of each endocrine cell

* Corresponding author. Fax: +34-56-016040.
E-mail address: juanmiguel.mancera@uca.es (J. Miguel Mancera).

0016-6480/03/$ - see front matter © 2003 Elsevier Science (USA). All rights reserved.
doi:10.1016/S0016-6480(03)00118-7

type. To our knowledge there is no information about the ontogeny of the pituitary of any clupeid fish.

The American shad, *Alosa sapidissima*, is an important commercial clupeid from western North Atlantic coastal waters. The overall morphology and development of hatchery-cultured American shad have been studied (Johnson and Loesch, 1983; Shardo, 1995). Also, physiological studies dealing with aspects such as osmoregulation, metabolism, etc. have been reported (Leonard and McCormick, 1999; Leach and Houde, 1999; Zydlewski and McCormick, 1997).

The aim of the present investigation was to show the anatomical distribution of the different adenohypophyseal cells in juvenile *A. sapidissima* as well as the time course of appearance of the different adenohypophyseal cell types in the pituitary of embryos and larvae. This information will be very useful for understanding the role of pituitary hormones in developmental processes in the American shad larvae. In addition, the results will be discussed in relation to those in other teleost groups in order to infer the possible phylogenetic relationship.

2. Material and methods

Adult American shad (*A. sapidissima*) were captured in the Connecticut River by gill netting on June 1996. Eggs from seven females were fertilized with milt from three males and transported to the laboratory of S.O. Conte Anadromous Fish Research Center (Turners Falls, MA). Eggs and larvae were reared according to Zydlewski and McCormick (1997). *A. sapidissima* larvae hatched from eggs 6 days after fertilization (on June, 12, 1996) (day 0). Larvae were sampled at 1, 2, 3, 4, 5, 6, 7, 11, 14, 17, 21, 24, 28, and 42 days after hatching. On June 18, 1996 another batch of eggs was fertilized and carried to the laboratory. Larvae hatched 6 days after fertilization (on June, 24, 1996) (day 0). Embryos and larvae were taken at −4, −3, −2, −1, 0 (hatching), 1, and 2 days. The temperature of water during the egg incubation and larval development was 17–18 °C. Ten specimens of each group from both batches were placed in Bouinás fluid (24 h for embryos and 48 h for larvae), decalcified with EDTA when necessary, dehydrated, and embedded in paraffin.

Ten juvenile (7.5–9.5 g body weight; 7.2–9.4 cm body length) migrant American shad were captured in the Connecticut River at Turners Falls, at a by-pass structure at Cabot Station hydroelectric facility operated by Northeast Utilities. Fish were captured on October 8 during the migratory season from river to sea (see Zydlewski and McCormick, 1997). Fish were anesthetized with MS-222 (100 mg/l; pH 7.0) dissolved in the water and killed by decapitation. The brains were dissected out placed in Bouinás fluid for 48 h, then dehydrated, and embedded in paraffin.

Sagittal and transverse (8 μm thick) sections of embryos, larvae, and brains of juvenile specimens were obtained. The sections were stained with hematoxylin–eosin, periodic acid-Schiff technique (PAS, McManus and Mowry, 1968), and Alcian blue-PAS-Orange G (AB-PAS-OG, Adams and Swettenham, 1958) for histochemical study. For the immunocytochemical study the tissue sections were immunostained according to the unlabelled enzyme method of Sternberger (1986). The following primary rabbit antisera were used in this study: (i) anti-chum salmon PRL (1:8000) and anti-chum salmon SL (1:8000) (Kaneko et al., 1993; Kawauchi et al., 1983), (ii) anti-recombinant sea bream GH (1:1000) and anti-sbSL (1:1000) (Astola et al., 1996; Martínez-Barberá et al., 1994), (iii) anti-human ACTH (1:3000), (iv) anti-bovine mono-acetyl α–MSH (1:2000) (van Zoest et al., 1989), and (v) anti-carp α, β-GTH II (1:1000), and anti-carp β-GTH II (1:3000) (Dubourg et al., 1985).

All sections were incubated for 18 h at 22 °C in the primary antiserum. The second antiserum (anti-rabbit IgG, raised in goat and kindly provided by Dr. P. Fernández-Llebrez, Málaga, Spain) was used at a dilution of 1:25 for 30 min at 22 °C and the PAP complex (1:150) (Sigma) for 30 min at 22 °C. 3,3-diaminobenzidine tetrahydrochloride (DAB, Sigma) was used as electron donor. All antisera and the PAP complex were diluted in TRIS buffer, pH 7.8, containing 0.7% non-gelling seaweed gelatin, lambda carrageenan (Sigma), 0,5% Triton X-100 (Sigma), and 0.02% sodium azide. Coplin jars were used for incubation in the first and the second antisera, whereas PAP incubation was carried out in a moist chamber. To enhance immunoreaction, 0.04% DAB plus 0.04% ammonium nickel sulfate hexahydrate (Fluka) and/or the bridge PAP method (Vacca, 1982) were used. In order to control the immunoreactive procedure, contiguous sections were stained according to the above-described protocol but incubation in the primary antisera was omitted. In addition, normal rabbit serum was used instead of primary antiserum. No positive structures or cells were found in these sections.

3. Results

3.1. Juvenile specimens

The adenohypophysis of juvenile *A. sapidissima* showed three distinct areas: *rostral pars distalis* (RPD) displaying the orohypophyseal duct and several cavities lined by prolactin (PRL), adrenocorticotropic hormone (ACTH), and gonadotropic hormone (GTH) cells; *proximal pars distalis* (PPD), containing growth hormone (GH), GTH, and putative thyroid stimulating hormone (TSH) cells; and *pars intermedia* (PI) with

somatolactin (SL) and melanophore stimulating hormone (MSH) cells. The neurohypophysis consisted of the pituitary stalk and the *pars nervosa* with several processes penetrating the different adenohypophyseal areas (Fig. 1).

Those cells that cross-reacted with anti-sPRL were identified as PRL cells and were restricted to the RPD (Fig. 2A). Using the AB-PAS-OG method, cells in this location were orange-stained. PRL cells formed a pseudostratified columnar epithelium lining cavities. Among cavities, neurophypophyseal processes penetrated (Fig. 2B). Strong anti-hACTH-immunoreactive cells were observed in the dorsal and ventral parts of RPD and were considered ACTH cells. At variance with PRL cells, they did not line the cavities (Fig. 2C). These cells stained purple with the AB-PAS-OG method and had round shape. In the dorsal RPD, ACTH cells contacted neurophypophyseal tissue while in the ventral RPD they were isolated or clustered among the basal region of PRL cells (Fig. 2D).

Anti sbGH-ir cells were considered GH cells and were restricted to the PPD, in the central portion of the pituitary gland. They were never found in other locations (Fig. 2E). Cells of the PPD stained orange with the AB-PAS-OG method and showed oval shape with a large central nucleus. Those GH cells located more dorsally contact branches of neurohypophyseal tissue that penetrated the PPD (Fig. 2F).

GTH cells were found at the RPD (Fig. 3A) and PPD (Fig. 3B) and were identified as those cells showing immunoreactivity to anti-carp α,β-GTH II, and anti-carp β-GTH II. Cells in the same locations were PAS+ and stained blue with the AB-PAS-OG method. At RPD, GTH cells were fusiform, intermingled with PRL cells, and contacted the neurophypophyseal tissue and cavities by both poles (Fig. 3C). At PPD they occupied dorsal and ventral areas, and had small size and round shape.

The use of α,β-cGTH II and β-cGTH II antisera on consecutive sections was utilized to identy putative TSH cells. We consider TSH cells as those exhibiting immunoreactivity to α,β-cGTH II but not to β-cGTH II antisera. TSH cells occupied the posterior dorsal area of the PPD (Figs. 3D and E). Using the AB-PAS-OG method cells, purple-stained cells of small size and round shape were found.

SL and MSH cells were identified by using anti-sSL or anti-sbSL and anti-α-bMSH, respectively, and were found only in the PI. In the same location PAS-negative cells and orange-stained cells using AB-PAS-OG method were found. Strongly SL immunoreactive cells (Fig. 4A) showed columnar shape and contacted neurohypophyseal branches that penetrated the *pars intermedia* (Fig. 4B). MSH cells also showed immunoreactivity to anti-hACTH$_{(1-24)}$ antiserum. These cells displayed elongated shape and surrounded the neurohypophyseal processes intermingled with SL cells (Fig. 4C).

3.2. Embryonic and larval development

The time course of the onset of immunoreactivity to different antisera in pituitary cells of *A. sapidissima* embryos and larvae is shown in Table 1. The number and immunoreactivity of the different adenohypophyseal cells increased with development (Fig. 5).

The pituitary of 3 days post-fertilized embryos (stage 21 according to Shardo, 1995) is a small mass of cells adhered to the brain floor. At this stage, a weak immunoreactivity to anti-sPRL, anti-hACTH$_{(1-24)}$, and anti-sSL antisera was seen after the use of the bridge PAP method in some specimens. One day later (stage 24), embryos have partly separated the head from yolk sac, a small mouth appeared, eye pigmented, and the thin pigmented longitudinal lines appeared along the body. The pituitary was an oval mass of cells where rostral and caudal adenohypophyseal regions can be distinguished. PRL cells (Fig. 6A) and ACTH cells (Fig. 6B) occupied the rostral region while SL-ir cells were found more caudally (Fig. 6C). Putative MSH cells were observed in this caudal region 1 day before hatching using anti-hACTH$_{(1-24)}$ antiserum (Fig. 6D), and 1 day after hatching using anti-α-bMSH antiserum.

At hatching (6 days post-fertilization), the pituitary gland became more elongated in shape and neurohypophysis was recognized although, no nerve processes were found to penetrate the adenohypophyseal tissue. At this time, a very weak GH-immunoreactivity was detected, using the bridge PAP method, in the anterior part of adenohypophysis. GH cells were more patent 1 day post-hatching (Fig. 6E). At hatching GTH cells occupied the central region of the adenohypophysis, while 2 days after hatching, they were located in the

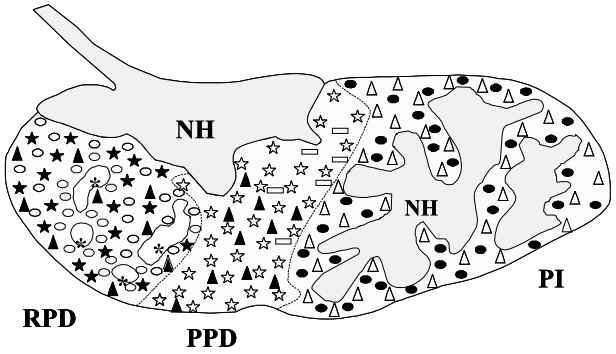

Fig. 1. Schematic sagittal representation of the pituitary of *Alosa sapidissima*, showing the distribution of adenohypophyseal cells in juveniles. RPD, *rostral pars distalis*; PPD, *proximal pars distalis*; PI, *pars intermedia*; and NH, neurohypophysis. Cavities (*), PRL (★), ACTH (○), GH (☆), GTH (▲), TSH (▭), SL (●), and MSH (△) cells.

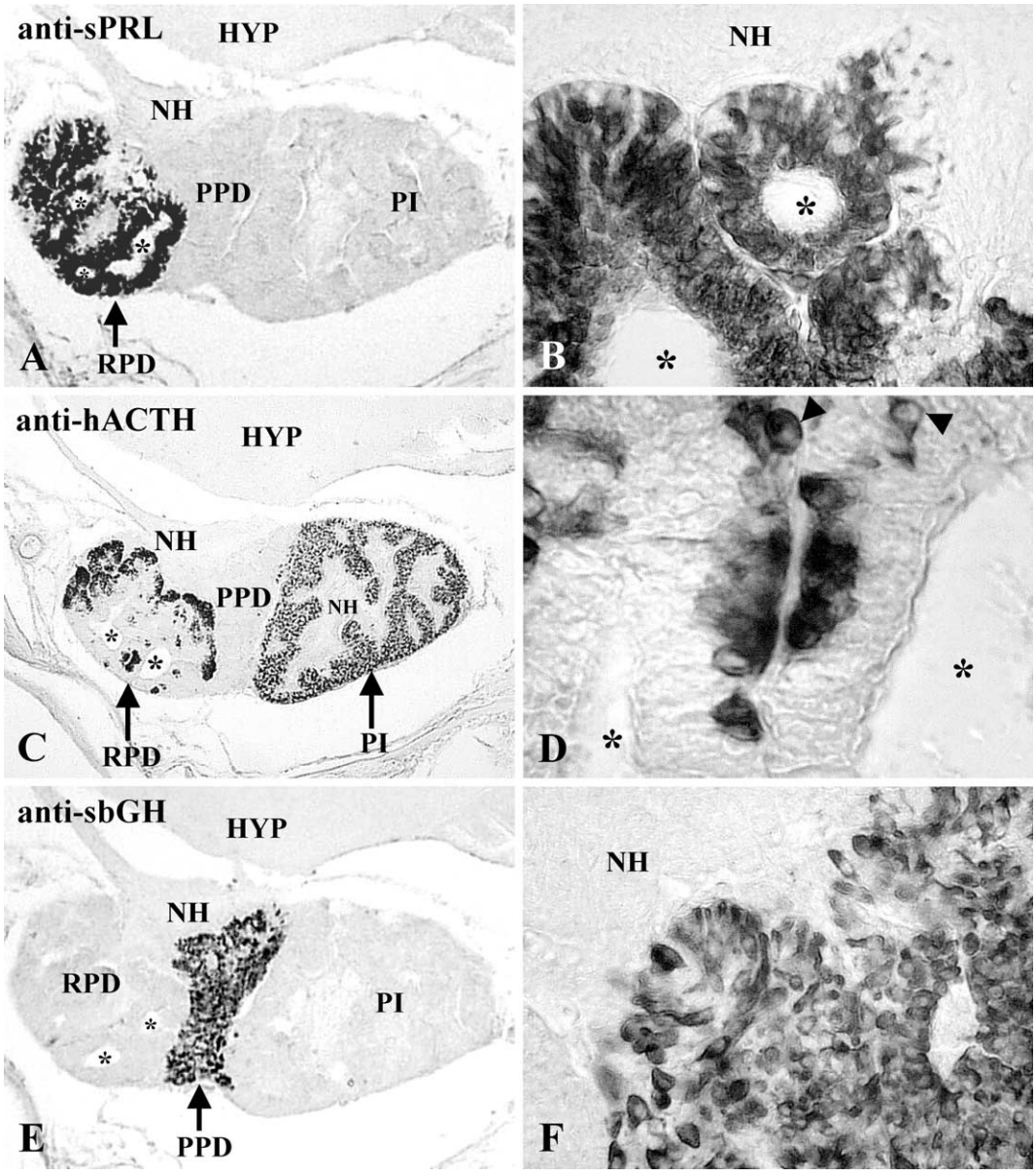

Fig. 2. Sagittal sections through the pituitary of juvenile *Alosa sapidissima* showing the localization of PRL- (A) and ACTH-ir cells (C) in RPD and GH-ir cells (E) in PPD. Using the antiserum anti-hACTH$_{(1–24)}$ putative MSH cells were observed at PI, 195×. (B) Detail of PRL-ir cells around cavities of RPD, 700×. (D) Details of RPD showing clusters of ACTH-ir cells (arrowheads) between PRL cells and neurohypophyseal tissue, 600×. (F) GH-ir cells arranged in clusters or around neurohypophyseal tissue, 700×. Asterisks, cavities; HYP, hypothalamus; NH, neurohypophysis; PI, *pars intermedia*; PPD, *proximal pars distalis*; and RPD, *rostral pars distalis*.

rostral portion and in the middle of the adenohypophysis (Fig. 6F).

At day 3 post-hatching the characteristic divisions of the pituitary can be distinguished (Fig. 5C), and it became patent between 7 and 14 days post-hatching (Fig. 5D). The size of the neural lobe increased during the first post-hatching week. In 7 days old larvae, the RPD showed a small lumen surrounded by a palisade of PRL, ACTH, and GHT cells; the PPD showed GH and GTH cells while the PI contained SL and MSH cells. The adenohypophysis and neural lobe increased in size with development and, in 42 days old larvae, they were similar to those of juvenile specimens. The time of appearance of TSH immunoreactivity could not be studied due to the the small size of pituitary in embryos and larvae of *Alosa sapidissima* that preclude the use of consecutive paraffin sections.

4. Discussion

The adenohypophysis of *A. sapidissima* was divided, similarly to those described in other teleost fishes, into three different regions: RPD, with PRL, ACTH, and

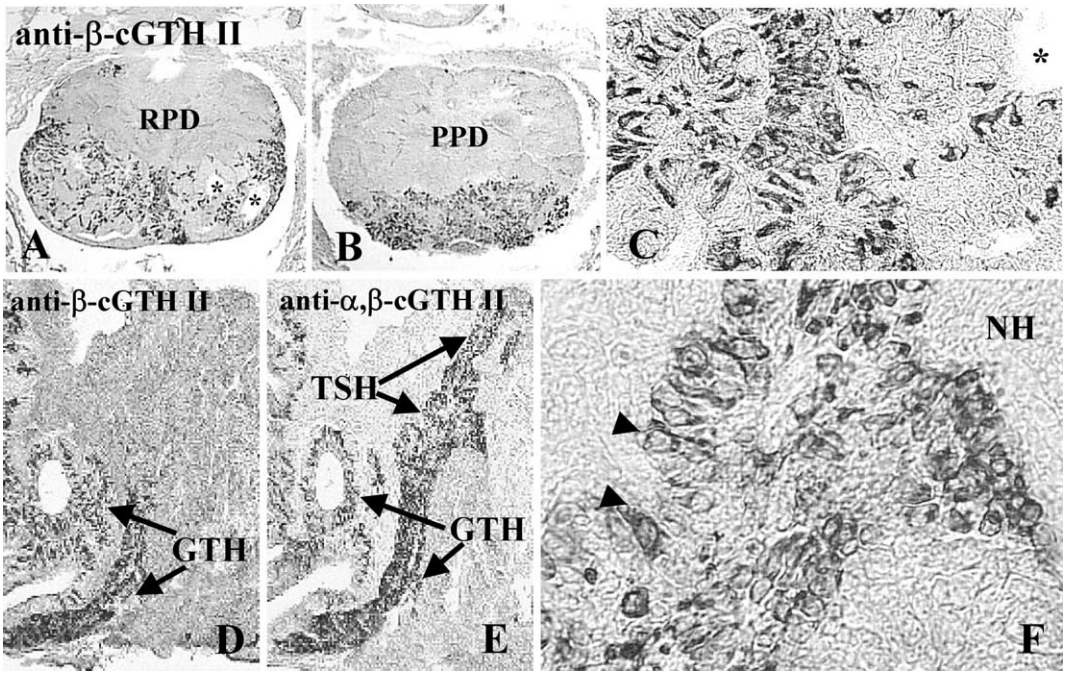

Fig. 3. Transverse sections through the pituitary of juvenile *Alosa sapidissima* showing the localization of GTH-ir cells in RPD (A) and PPD (B), 150×. (C) Details of RPD showing GTH-ir cells between PRL-cells and in contact with the lumen of cavities (asterisks), 350×. (D–E) Sagittal consecutive section immunostained with anti-β-cGTH II (D) and anti-α, β-cGTH II (E). GTH cells located at RPD and ventral part of PPD were immunostained with both antisera, while putative TSH, located at dorsal part of PPD, only were immunostained with anti-α, β-cGTH II, 550×. (F) Details of putative TSH cells (arrowheads) located in a dorsal area of PPD. They showed small size, round shape, and contacted with neurohypophysis (NH), 700×.

GTH cells; PPD, with GH and GTH cells and putative TSH cells; and PI with SL and MSH cells (Ball and Baker, 1969; Follénius et al., 1978).

The existence of an orohypophyseal duct with lining cells and a tubular lumen has been previously reported in juvenile and adult specimens of clupeid species. At RPD this duct ends in several cavities (Sathyanesan, 1963; see Cook et al., 1973 for others references). The persistence of an orohypophyseal duct is a characteristic of the pituitary of clupeid fish and is considered a primitive condition. Thus, the grade of development of the orohypophyseal duct can be used as an index of evolution of a given species (see Sathyanesan, 1963). *A. sapidissima* also has an orohypophyseal duct whose cavities are surrounded by PRL, ACTH, and GTH cells. Histochemical study in the clupeid *Alosa pseudoharengus* revealed putative PRL and ACTH cells, but not GTH cells, in the same location (Cook et al., 1973).

The existence of a releasing activity, by an apocrine or holocrine mechanism, from PRL cells into the lumen of the cavities and the orohypophyseal duct of RPD has been reported for some clupeids (Cook et al., 1973; Sathyanesan, 1963). Although we also observed chromophilic substances in the lumen of the cavities of *A. sapidissima*, we never found any kind of immunoreactivity with the antisera used in this study. Thus, either PRL release does not occur or PRL in the lumen exists in a form that is not recognized by our antibodies.

4.1. PRL cells

As for other teleosts, in *A. sapidissima* the immunoreactivity to anti-sPRL antiserum was restricted to cells in the RPD (Farbridge and Leatherland, 1986; Follénius et al., 1978). PRL cells surround the typical cavities present in the RPD of clupeid fish (see Sathyanesan, 1963).

The development of pituitary PRL cells in marine fish varied among species appearing at hatching, after hatching during the phase of yolk absorption, and even during the phase of exogenous feeding (Cambré et al., 1990; Power and Canario, 1992; see Tanaka et al., 1995; Villaplana et al., 2000). In the ayu (*Plecoglossus altivelis*), which is spawned and had rapid development in brackishwater, PRL-ir cells appeared one day before hatching (Saga et al., 1999). In several freshwater fishes, it has been reported that the PRL cells could be functional at hatching time (Naito et al., 1993; Ruijter et al., 1984; Saga et al., 1993; Schoots et al., 1983; Volckaert et al., 1999). However, in the cichlid fish *Cichlasoma dimerus*, PRL cells were detected 2 days after hatching (Pandolfi et al., 2001). In *A. sapidissima*, an anadromous clupeid species, the embryonic development lasted about 6 days (Johnson and Loesch, 1983; Shardo, 1995; present results) and PRL cells showed immunoreactivity at least 3 days before hatching.

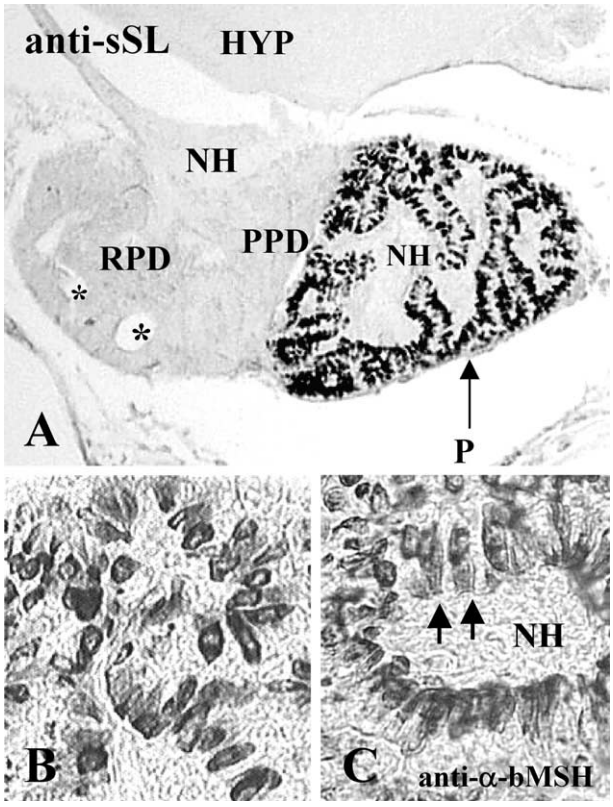

Fig. 4. (A) Sagittal section through the pituitary of juvenile *Alosa sapidissima* showing the localization of SL-ir cells in PI, 195×. (B) Details of SL-ir cells that bordering and contacting neurohypophyseal tissue. Negative cells correspond to MSH-ir cells, 600×. (C) Details of MSH-ir cells surrounded and contacted neurohypophyseal tissue (arrowheads), 600×. HYP, hypothalamus; NH, neurohypophysis; PI, *pars intermedia*; PPD, *proximal pars distalis*; RPD, *rostral pars distalis*; and asterisks, cavities of RPD.

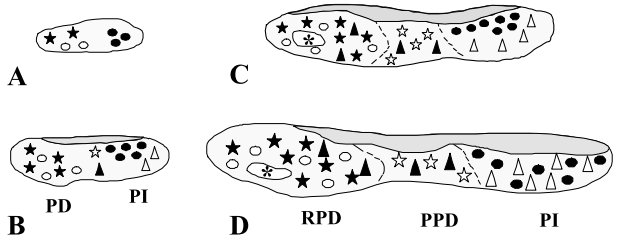

Fig. 5. Schematic diagrams of the pituitary gland of embryos and larvae of *Alosa sapidissima*. (A) Two days before hatching; (B) hatching time; (C) Three days post-hatching; (D) Seven days post-hatching. Note the characteristic distribution of each hormone producing cell. Cavity (*), PRL (★), ACTH (○), GH(☆), GTH (▲), TSH (▭), SL (●), and MSH (△) cells.

The osmoregulatory role of PRL in hypoosmotic environments is well established in adult teleost, this role being more prominent in euryhaline fishes (Manzon, 2002). In addition, several studies have also demonstrated a osmoregulatory role during embryonic and larval stages in freshwater or diluted seawater (Kimura and Tanaka, 1991; Ruijter et al., 1984). Thus, the early appearance of PRL cells in embryos of freshwater teleost, including in *A. sapidissima*, agrees with its important osmoregulatory role for the adaptation to hypoosmotic environments. In addition, it has been reported that PRL may also be involved in fish larval growth and differentiation (Majumdar and Elsholtz, 1994; Tanaka et al., 1995). In this sense, a stimulating effect of PRL on the secretion of teleostean hatching enzyme has been demonstrated (Schoots et al., 1982). So, the early appearance of PRL cells in *A. sapidissima* indicates that, in addition to its osmoregulatory role, PRL could be involved in growth and hatching.

4.2. GH cells

In juvenile *A. sapidissima*, as in other teleost fish, GH cells were restricted to the dorsal and ventral parts of PPD (Farbridge and Leatherland, 1986; Follénius et al., 1978; Segura-Noguera et al., 2000). In some teleosts, two types of GH cells have been described (*Morone saxatilis*: Huang and Specker, 1994; *Seriola dumerilii*: García-Hernández et al., 1996). In *A. sapidissima* we identified only one type of GH cell.

The development of GH cells of *A. sapidissima* was different to other hormones of the GH/PRL family (PRL and SL). Labelled GH cells first appeared clearly at 1 day post-hatching. As much, by enhancing immunoreactivity (bridge PAP method), a very weak immunoreaction could be detected at hatching in some larvae. We have not found immunoreactivity before hatching in any case.

Some marine species showed GH-immunoreactivity at hatching, while others in larvae during the phase of yolk absorption (Cambré et al., 1990; Power and Canario, 1992; see Tanaka et al., 1995; Villaplana et al., 2000). To our knowledge, there are no reports on the

Table 1
Chronological appearance of immunoreactivity in adenohypophyseal cells in embryos and larvae of *Alosa sapidissima*

Time	PRL	ACTH	GH	GTH	SL	MSH
−3 days	+/−	+/−	−−	−−	+/−	−
−2 days	+	+	−−	−−	+	−−
−1 day	+	+	−−	−−	+	+/−
Hatching	+	+	+/−	+/−	+	+
1 day	+	+	+	+	+	+
2 day	+	+	+	+	+	+

(+) Positive immunoreactivity; (−−) negative immunoreactivity; and (+/−) weakly immunoreactivity.

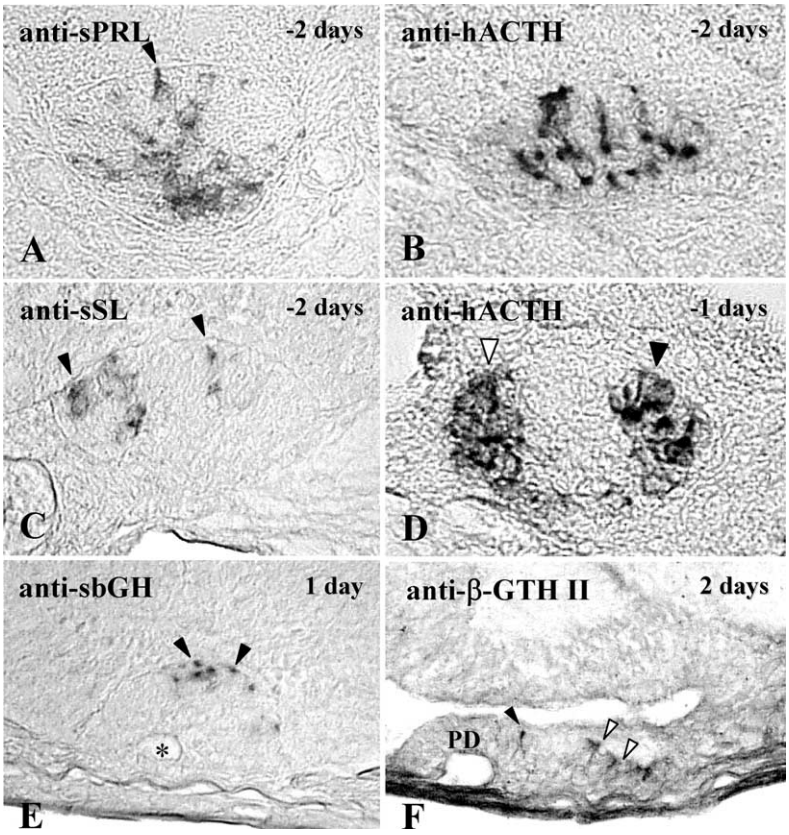

Fig. 6. Transverse (A, B, C, and E) and sagittal (D, F) sections of the pituitary of embryos and larvae of *Alosa sapidissima*. (A–C) Two days before hatching immunoreactivity was observed in PRL (A), ACTH (B), and SL cells (C). Note the processes of PRL-ir cells contacting the neural tissue (arrowheads), 750×. (D) One day before hatching, and using an antiserum anti-hACTH$_{(1-24)}$, ACTH-ir cells were observed at RPD (black arrowhead) and putative MSH cells at PI (white arrowhead). This was the first moment that ACTH-ir cells were located at PI. Using an antiserum anti-α-bMSH, MSH-ir cells were detected 1 day post-eclosion, 600×. (E) One day old larva immunostained with antiserum anti-sbGH. A weak immunoreactivity was located in the dorsal portion of PPD (arrowheads), 750×. (F) Two days after hatching immunostained with antiserum anti-β-cGTH II. GTH-ir cells were observed at the rostral portion (black arrowhead) and in the middle portion (white arrowhead) of adenohypophysis, 300×.

presence of GH-immunoreactivity before hatching in marine teleost. In embryos of ayu, *P. altivelis*, raised in brackishwater, GH cells were detected 1 day before hatching (Saga et al., 1999). Regarding freshwater fishes, GH cells were evident before hatching (Mal et al., 1989; Naito et al., 1993; Saga et al., 1993). In *C. gariepinus*, GH cells were visible from hatching time and GH is active at the very early stages of development (Volckaert et al., 1999). However, in the cichlid fish *C. dimerus*, GH cells were detected 2 days after hatching (Pandolfi et al., 2001).

The physiological role of GH as a growth-promoting hormone has been well established (Pérez-Sánchez and Le Bail, 1999). It seems likely that in freshwater teleosts GH appears early than in seawater teleost, thus indicating a putative role of this hormone in embryonic and larval development. However, the physiological meaning of the delay in the appearance of immunoreactivity in GH cells of *A. sapidissima* with respect to the other adenohypophyseal hormones of the PRL/GH family deserves future research.

4.3. SL cells

The SL is a pituitary hormone of the GH/PRL family (Kaneko, 1996). Several studies revealed a high homology among the different teleostean SL. So, it is likely that the antisera used in the study showed a specific reaction only with SL cells of *A. sapidissima*. In this specie SL appear in the PI around neurohypophysial branches, intermingled with MSH cells, similarly to those reported in other teleosts (Kaneko, 1996; Rand-Weaver et al., 1991).

In teleost, PI contains PAS positive and PAS negative cells. Immunocytochemical studies indicated that PAS (+) were SL-producing cells and PAS (−) were MSH-producing cells (Rand-Weaver et al., 1991; Kaneko, 1996). In salmonids, PAS (−) SL-ir cells were reported (Rand-Weaver et al., 1991). Conversely, in the gilthead sea bream, SL-ir cells appear to be PAS (+) in adult specimens, while they are PAS (−) in larval stages (Villaplana et al., 1997). This observation was in agreement with previous reports on the presence of a glycosylated

and a nonglycosylated form of this hormone in *Sparus aurata* (Cavari et al., 1995) and *C. dimerus* (Pandolfi et al., 2001). Juveniles of *A. sapidissima* also showed two cell types in PI: SL- and MSH-producing cells, however, both were PAS (−) cells (results not showed). Thus, it seems that, at least in salmonids (Rand-Weaver et al., 1991), larval stages of *S. aurata* (Villaplana et al., 1997) and *C. dimerus* (Pandolfi et al., 2001), and in *A. sapidissima* (present results) SL protein is not glycosylated.

There are few reports on the ontogeny of SL cells. The onset of SL-ir was 42 days before hatching in the salmonid *Oncorhynchus nerka* (Parhar et al., 1995) and at hatching in the non-salmonid *S. aurata* (Villaplana et al., 1997, 2000). In the ayu, raised in brackishwater, SL cells were detected just after hatching (Saga et al., 1999). In *C. dimerus*, SL-ir cells were detected 2 days after hatching (Pandolfi et al., 2001). In *A. sapidissima* embryos SL-ir cells first appeared at 3 days after fertilization and, at hatching, a strong immunoreactivity was observed. Several physiological roles have been suggested for SL (see Kaneko, 1996). In larvae, it has been suggested that SL could play a role in the early development (Majumdar and Elsholtz, 1994). In *A. sapidissima* the early appearance of SL also suggests a role of this hormone in the embryonic development.

4.4. ACTH cells and MSH cells

Adrenocorticotropic hormone (ACTH) and melanotropic hormone (MSH) belong to the propiomelanocortin (POMC) family. This precursor molecule is processed in a different way and originates ACTH in corticotropic cells and MSH in melanotropic cells (Dores, 1990). Several studies have demonstrated that ACTH cells are located in the RPD and MSH cells in the PI (Farbridge and Leatherland, 1986; Quesada et al., 1988). Our results in *A. sapidissima* agree with the previous reports in other teleosts including the clupeid *A. pseudoharengus* (Cook et al., 1973).

In *A. sapidissima*, ACTH cells were detected 2 days before hatching in the rostral region of the adenohypophysis. Other populations of ACTH immunoreactive cells were detected in the caudal portion 1 day before hatching. This population could represent MSH cells since they were detected 1 day after hatching using the antiserum anti-α-bMSH. In marine species, immunoreactivity of ACTH and MSH cells has been observed always after hatching (Cambré et al., 1990; Power and Canario, 1992; Saga et al., 1999). However, freshwater fish showed immunoreactivity before hatching (Mal et al., 1989; Naito et al., 1993; Ruijter et al., 1984; Saga et al., 1993). In the ayu, raised in brackishwater, ACTH and MSH cells were detected at hatching (Saga et al., 1999). In *A. sapidissima*, the presence of ACTH and MSH cells before hatching suggests a functional interrenal axis during embryonic stages.

In teleost, ACTH stimulates interrenal gland for the production of cortisol. This hormone is involved in several physiological processes: stress, metabolism, osmoregulation, etc. (Wendelaar Bonga, 1997). On the other hand, α-MSH is involved in the adaptation to background color and also in the stimulation of ACTH release during stress (Bagnara and Hadley, 1973; Lamers et al., 1992). The physiological role of ACTH and α-MSH in embryos and larvae is not well known. In *Cyprinus carpio*, ACTH, α-MSH, and cortisol are present in unfertilized eggs and their levels decreased until 24 h after fertilization, when an endogenous production of these hormones starts. This indicates the need of these hormones of material origin in the earliest development stages and the early activation of a pituitary-interrenal axis in the embryos. Also it has been suggested about a relationship of these hormones with the stress associated to hatching (Stouthart et al., 1998). In the same way, in *A. sapidissima* a functional pituitary-interrenal axis, with active ACTH cells and interrenal secretion of cortisol, could be necessary for stress associated to hatching and for the adaptation to the new hypoosmotic environment.

4.5. GTH cells

Gonadotrophins (I and II) and TSH are included in the family of adenohypophyseal glycoprotein hormones. These hormones present identical α-subunits but different β-subunits (Pierce and Parsons, 1981). So, the use of antisera against β-subunit permits the identification of GTH and TSH cells.

In *A. sapidissima* GTH cells with different sizes and shapes were detected at dorsal and ventral regions of PPD, while fusiform GTH cells were observed around the cavities of the RPD. The localization of GTH cells in the PPD is similar to those reported in other teleosts (García-Hernández et al., 1996; Quesada et al., 1988; Segura-Noguera et al., 2000). In addition, the existence of GTH cells at RPD also agrees with some reports (Dubourg et al., 1985; Olivereau and Nagahama, 1983). However, at variance with *A. sapidissima*, in the clupeid *A. pseudoharengus* only PRL and ACTH cells have been described in the RPD (Cook et al., 1973).

Two types of GTHs (GTH I and GTH II) in two different gonadotropic cells have been reported in several species (Gen et al., 2000; Naito et al., 1991; Nozaki et al., 1990). In *Alosa sapidissima*, the existence of two morphologically different populations of GTH cells suggests the existence of two types of GTHs. However, GTH cells showed immunoreactivity against anti-carp β-GTH II but not anti-salmon β-GTH I (data not showed). It is known that β-subunit of GTH I presents a poorly conserved amino acid sequence (Kawauchi et al., 1989), which could explain the lack of immunoreactivity.

Further studies using homologous antisera to GTH I and GTH II will elucidate this question.

The ontogeny of GTH cells has been studied in several teleost species and it never found GTH-ir cells before hatching (Cambré et al., 1990; Naito et al., 1993; Power and Canario, 1992; Saga et al., 1999). In salmonids, GTH II cells were not present at any stage of embryonic or larval development (Mal et al., 1989; Naito et al., 1993; Nozaki et al., 1990; Saga et al., 1993). In *P. altivelis*, using antisera against sGTH II and sGTH I, only GTH II-ir cells were observed at hatching time and during larval development (Saga et al., 1999). Our results obtained in *A. sapidissima* agree with these results and showed, using an antiserum anti-β-cGTH II, weakly GTH-ir cells at hatching time. In general, teleosts showed a delay in the appearance of GTH-ir cells. This suggests that this hormone is not involved in normal embryonic and larval development.

4.6. TSH cells

In a first attempt, we used an antiserum against β-subunit of the human TSH in order to localize TSH cells. However, we did not detect any reactivity (not shown). Thus, since the direct immunocytochemical identification of TSH cells was not possible, we used an alternative strategy.

In teleost, the antiserum against α, β-GTH cross-reacts with GTH and TSH cells, since the α chain of GTH displays homology with the α chain of TSH. On the other hand, the antiserum raised against β-GTH binds only to GTH cells (Pierce and Parsons, 1981). Thus, we stained the same cells in consecutive sections with the two antisera and we identified as putative TSH cells those that were anti-α, β-cGTH II positive and anti-β-cGTH II negative. These cells are located, as for other teleosts, in the dorsal part of the PPD (Farbridge and Leatherland, 1986; Follénius et al., 1978; Segura-Noguera et al., 2000). However, since we do not know whether there are one or two GTHs in *A. sapidissima* (sea above), the possibility exists that the cells that we identified as putative TSH cells were indeed GTH cells and not TSH cells.

The ontogenetic study of TSH was not made in *A. sapidissima* since the small size of pituitary in embryos and larvae precludes the use of the strategy described above. Ontogenetic studies about TSH cells are scarce in teleost. In *Dicentrarchus labrax*, TSH-ir cells were detected 1 day after hatching (Cambré et al., 1990). In *O. mykiss*, TSH cells were detected at the last stage before hatching (42 days post-fertilization) (Saga et al., 1993). However, in the other salmonid, *Oncorhynchus keta*, TSH cells were observed 2 weeks before hatching (5 weeks after fertilization) (Naito et al., 1993). In *P. altivelis*, TSH-ir cells were detected for the first time 50 days after hatching (Saga et al., 1999).

TSH, though thyroid hormones, has a role during the larval development (Brown and Bern, 1989). In addition, these hormones also present an important role during the metamorphosis in pleuronectiforms (Schreiber and Specker, 1998). In *A. sapidissima*, the larval development is associated to a process of metamorphosis (Johnson and Loesch, 1983; Shardo, 1995). In this way, and similar to other teleosts, it is logical to suppose an important physiological role of TSH in this process.

In conclusion, this study shows the chronological appearance of PRL, ACTH, GH, GTH, SL, and MSH cells in the pituitary of the clupeid fish *A. sapidissima*. PRL, ACTH, and SL cells were observed 3 days before hatching, MSH cells were present 1 day pre-hatching, while GH and GTH cells were observed at hatching time. This and other studies demonstrate that, in freshwater teleost, adenohypophyseal hormones play important roles in embryonic development and that these roles are more relevant than in seawater teleosts. Further research should be necessary to clarify the functional role of each hormones during the ontogenetic development of *A. sapidissima*.

Acknowledgments

This research was supported in part by a pre-doctoral fellowship to R.L.C. (Ministerio de Educación y Ciencia, Madrid). This work was supported by Grant BOS2001-4031-C02-01 (Ministerio de Ciencia y Tecnología, Spain) to J.M.M. and M.P.M.R. The authors are grateful to the staff of S.O. Conte Anadromous Fish Research Center, Turners Falls, USA, for helping during sampling. We are grateful to Dr. Kawauchi for the gift of chum salmon PRL, GH, SL, α, β-GTH II, β-GTH I, and β-GTH II antisera; Dr. M. Valdivia for the gilthead seabream GH and SL antisera; Dr. S.E. Wendelaar Bonga for the α-MSH antiserum; Dr. Burzawa-Gerard for the carp α, β-GTH II and carp β-GTH II antisera; to NIDDK and NHPP for the human β-TSH antiserum; and Dr. P. Fernández-LLebrez for the second antiserum. We also thank Dr. P. Fernández-LLebrez and the two anonymous reviewers for a critical reading of the manuscript.

References

Adams, C.W.M., Swettenham, K.V., 1958. The histochemical identification of two types of basophil cell in the normal human adenohypophysis. J. Pathol. Bacteriol. 75, 95–103.

Astola, A., Pendón, C., Ortiz, M., Valdivia, M.M., 1996. Cloning and expression of somatolactin, a pituitary hormone related to growth hormone and prolactin from gilthead seabream, *Sparus aurata*. Gen. Comp. Endocrinol. 104, 330–336.

Bagnara, J.T., Hadley, M.E., 1973. In: N.J. Englewood Cliff (Ed.), Chromatophores and Color Change: the Comparative Physiology of Animal Pigmentation. Prentice-Hall, Englewood Cliffs, NJ.

Ball, J.N., Baker, B.I., 1969. The pituitary gland: anatomy and histophysiology. In: Hoar, W.S., Randall, D.J. (Eds.), Fish Physiology, vol. 2. Academic Press, New York, pp. 1–205.

Balon, E.K., 1981. Saltatory processes and altricial to precocial forms in the ontogeny of fishes. Am. Zool. 21, 573–596.

Brown, C.L., Bern, H.A., 1989. Thyroid hormones in early development, with special reference to teleost fish. In: Schreibman, M.P., Scanes, C.G. (Eds.), Hormones in Development, Maturation and Senescence of Neuroendocrine Systems. A Comparative Aproach. Academic Press, New York, pp. 289–306.

Cambré, M., Mareels, G., Corneille, S., Moons, L., Ollevier, F., Vandesande, F., 1990. Chronological appearance of the different hypophysial hormones in the pituitary of sea bass larvae (*Dicentrarchus labrax*) during the early development: an immunocytochemical demonstration. Gen. Comp. Endocrinol. 77, 408.

Cavari, B., Noso, T., Kawauchi, H., 1995. Isolation and characterization of somatolactin from pituitary glands of gilthead sea bream *Sparus aurata*. Aquaculture 137, 171–178.

Cook, H., Rusthoven, J.J., Vogelzang, N.J., 1973. The rostral *pars distalis* of the pituitary gland of the freshwater and marine alewife (*Alosa pseudoharengus*). Z. Zelforsh. 141, 145–159.

Dores, R.M., 1990. The proopiomelanocortin family. In: Epple, A., Scanes, C.G., Stetson, M.H. (Eds.), Progress in Comparative Endocrinology. Wiley–Liss, New York, pp. 22–27.

Dubourg, P., Burzawa-Gerard, E., Chambolle, P., Kah, O., 1985. Light and electron microscopic identification of gonadotrophic cells in the pituitary gland of the goldfish by means of immunocytochemistry. Gen. Comp. Endocrinol. 59, 472–481.

Farbridge, K.J., Leatherland, J.F., 1986. A comparative immunohystochemical study of the pars distalis in six species of teleost fishes. Fish Physiol. Biochem. 1, 63–74.

Follénius, E., Doerr-Schott, J., Dubois, M.P., 1978. Immunocytology of pituitary cells from teleost fishes. Int. Rev. Cytol. 54, 193–223.

García-Hernández, M.P., García-Ayala, A., Elbal, M.T., Agulleiro, B., 1996. The adenohypophysis of Mediterranean yellowtail, *Seriola dumerilii* (Risso, 1810): an immunocytochemical study. Tissue Cell 28, 577–585.

Gen, K., Okuzawa, K., Senthilkumaran, B., Tanaka, H., Moriyama, S., Kagawa, H., 2000. Unique expression of gonadotropin-I and -II subunit genes in male and female red seabream (*Pagrus major*) during sexual maturation. Biol. Reprod. 63, 308–319.

Huang, L., Specker, J.L., 1994. Growth hormone- and prolactin-producing cells in the pituitary gland of striped bass (*Morone saxatilis*): immunocytochemical characterization at different life stages. Gen. Comp. Endocrinol. 94, 225–236.

Johnson, J.R., Loesch, J.G., 1983. Morphology and development of hatchery-cultured American shad, *Alosa sapidissima* (Wilson). Fish. Bull. 81, 323–339.

Kaneko, T., 1996. Cell biology of somatolactin. Int. Rev. Cytol. 169, 1–24.

Kaneko, T., Kakizawa, S., Yada, T., Hirano, T., 1993. Gene expression and intracellular localization of somatostatin in the pituitary of rainbow trout. Cell Tissue Res. 272, 11–16.

Kawauchi, H., Abe, K.I., Takahasi, A., Hirano, T., Hasegawa, S., Naito, N., Nakai, Y., 1983. Isolation and properties of chum almon prolactin. Gen. Comp. Endocrinol. 49, 446–458.

Kawauchi, H., Suzuki, K., Itoh, H., Swanson, P., Naito, N., Nagahama, Y., Nozaki, M., 1989. The duality of teleost gonadotropins. Fish Physiol. Biochem. 7, 29–38.

Kimura, R., Tanaka, M., 1991. Prolactin production during larval and early juvenile periods of euryhaline marine fish, black sea bream *Acanthopagrus Schlegeli*. Nippon Suisan Gakkaishi. 57, 1833–1837.

Lamers, A.E., Flik, G., Atsma, W., Wendelaar Bonga, S.E., 1992. A role for diacetyl α-melanocyte-stimulating hormone in the control of cortisol release in the teleost *Oreochromis mossambicus*. J. Endocrinol. 135, 285–292.

Leach, S.D., Houde, E.D., 1999. Effects of environmental factors on survival, growth, and production of American shad larvae. J. Fish Biol. 54, 767–786.

Leonard, J.B.K., McCormick, S.D., 1999. The effect of migration distance and timing on metabolic enzyme activity in an andromous clupeid, the American shad (*Alosa sapidissima*). Fish Physiol. Biochem. 20, 163–179.

Majumdar, S., Elsholtz, H.P., 1994. Comparative aspects of pituitary development and Pit-1 function. In: Farrel, A.P., Randall, D.J., Sherwood, N.M., Hew, C.L. (Eds.), Fish Physiology, vol. 13. Academic Press, San Diego, pp. 309–330.

Mal, A.O., Swanson, P., Dickhoff, W.W., 1989. Immunocytochemistry of the developing salmon pituitary gland. Am. Zool. 29, 94A.

Manzon, L.A., 2002. The role of prolactin in fish osmoregulation: a review. Gen. Comp. Endocrinol. 125, 291–310.

Martínez-Barberá, J.P., Pendón, C., Rodríguez, R.B., Pérez-Sánchez, J., Valdivia, M.M., 1994. Cloning expression and characterization of a recombinant gilthead seabream, *Sparus aurata*. Gen. Comp. Endocrinol. 96, 179–188.

McManus, J.F.A., Mowry, R.W., 1968. Técnica Histológica. Atica (Ed.), Madrid.

Naito, N., Hyodo, S., Okumoto, N., Urano, A., Nakai, Y., 1991. Differential production and regulation of gonadotropins (GTH I and GTH II) in the pituitary gland of rainbow trout *Oncorhynchus mykiss*, during ovarian development. Cell Tissue Res. 266, 457–467.

Naito, N., De Jesus, G., Nakai, Y., Hirano, T., 1993. Ontogeny of pituitary cell-types and the hypothalamo-hypophysial relationship during early development of chum salmon, *Oncorhynchus keta*. Cell Tissue Res. 272, 429–437.

Nozaki, M., Swanson, P., Dickhoff, W.W., Nakai, Y., Suzuki, K., Kawauchi, H., 1990. Salmonid pituitary gonadotrophs: II. Ontogeny of GTH I and GTH II cells in the rainbow trout (*Salmo gairdneri irideus*). Gen. Comp. Endocrinol. 77, 358–367.

Olivereau, M., Nagahama, Y., 1983. Immunocytochemistry of gonadotrophic cells in the pituitary of some teleost species. Gen. Comp. Endocrinol. 50, 252–260.

Pandolfi, M., Dante, A., Maggese, C., Ravaglia, M., Vissio, P., 2001. Ontogeny of immunoreactive somatolactin, prolactin, and growth hormone secretory cells in the developing pituitary gland of *Cichlasoma dimerus* (Teleostei, Perciformes). Anat. Embryol. 203, 461–468.

Parhar, I.S., Iwato, M., Pfaff, D.W., Schwanzel-Fukuda, M., 1995. Embryonic development of gonadotropin-releasing hormone neurons in the sockeye salmon. J. Comp. Endocrinol. 302, 256–270.

Pérez-Sánchez, J., Le Bail, P.-Y., 1999. Growth hormone axis as marker of nutritional status and growth performance in fish. Aquaculture 177, 117–128.

Pierce, J.G., Parsons, T.F., 1981. Glycoprotein hormones: structure and function. Annu. Rev. Biochem. 50, 127–139.

Power, D.M., Canario, A.V.M., 1992. Immunocytochemistry of somatotrophs, gonadotrophs, prolactin, and adrenocorticotropin cells in larval sea bream (*Sparus aurata*) pituitaries. Cell Tissue Res. 269, 341–346.

Quesada, J., Lozano, M.T, Ortega, A., Agulleiro, B., 1988. Immunocytochemical and ultrastructural characterisation of the cell types in the adenohypophysis of *Sparus aurata* L. (Teleost). Gen. Comp. Endocrinol. 72, 209–225.

Rand-Weaver, M., Baker, B.J., Kawauchi, H., 1991. Cellular localization of somatolactin in the pars intermedia of some teleost fishes. Cell Tissue Res. 263, 207–215.

Ruijter, J.M., van Keemenade, J.A.M., Wendelaar Bonga, S.E., 1984. Environmental influences on prolactin cell development in the cyprinodont fish, *Cynolebias whitei*. Cell Tissue Res. 238, 595–600.

Saga, T., Oota, Y., Nozaki, M., Swanson, P., 1993. Salmonid pituitary gonadotrophs. III. Chronological appareance of GTH I and other

adenohypophyseal hormones in the pituitary of the developing rainbow trout (*Oncorhynchus mykiss irideus*). Gen. Comp. Endocrinol. 92, 233–241.

Saga, T., Yamaki, K., Doi, Y., Yoshizuka, M., 1999. Chronological study of the appearance of adenohypophysial cells in the ayu (*Plecoglossus altivelis*). Anat. Embryol. 200, 469–475.

Sathyanesan, A.G., 1963. On the structural peculiarities of the pituitary gland in some clupeid fishes with a note on their probable evolutionary significance. Anat. Rec. 146, 109–115.

Schoots, A.F.M., De Bort, R.G., Van Eijs, G.J.J.M., Denucé, J.M., 1982. Evidence for a stimulating effect of prolactin on teleostean hatching enzyme secretion. J. Exp. Zool. 219, 129–132.

Schoots, A.F.M., Ruijter, J.M., van Kemenade, J.A.M., Denucé, J.M., 1983. Immunoreactive prolactin in the pituitary gland of cyprinodont fish at the time of hatching. Cell Tissue Res. 233, 611–618.

Schreiber, A.M., Specker, J.L., 1998. Early larval development and metamorphosis in the summer flounder: changes in per cent whole-body water content and effects of altered thyroid status. J. Fish Biol. 55, 148–157.

Segura-Noguera, M.M., Laiz-Carrión, R., Martín del Río, M.P., Mancera, J.M., 2000. Immunocytochemical study of the pituitary gland of the white seabream (*Diplodus sargus*). Histochem. J. 32, 733–742.

Shardo, J.D., 1995. Comparative embriology of teleostean fishes. I. Development and staging of the American shad, *Alosa sapidissima*. J. Morphol. 225, 125–167.

Sternberger, L.A., 1986. Immunocytochemistry. Wiley–Liss, USA.

Stouthart, A.J.H.X., Lucassen, E.C.H.E.T., van Strien, F.J.C., Balm, P.H.M., Lock, R.C., Wendelaar Bonga, S.E., 1998. Stress responsiveness of the pituitary-interrenal axis during early life stages of common carp (*Cyprinus carpio*). J. Endocrinol. 157, 127–137.

Tanaka, M., Tanangonan, J.B., Tagawa, M., de Jesús, E.G., Nishida, H., Isaka, M., Kimura, R., Hirano, T., 1995. Development of the pituitary, thyroid, and interrenal glands and applications of endocrinology to the improved rearing of marine fish larvae. Aquaculture 135, 111–126.

Vacca, L.L., 1982. Double bridge techniques of immunocytochemistry. In: Bullock, G.R., Petrusz, P. (Eds.), Techniques in Immunocytochemistry, vol. 1. Academic Press, London, pp. 155–182.

van Zoest, I.D., Heijmen, P.S., Cruijsen, P.M.J.M., Jenks, B.G., 1989. Dynamics of background adaptation in *Xenopus laevis*: role of catecholamines and melanophore stimulating hormone. Gen. Comp. Endocrinol. 76, 19–28.

Villaplana, M., García-Ayala, A., García-Hernández, M.P., Agulleiro, B., 1997. Ontogeny of the immunoreactive somatolactin cells in the pituitary of gilthead sea bream (*Sparus aurata* L., teleostei). Anat. Embryol. 196, 227–234.

Villaplana, M., García Ayala, A., Chaves Pozo, E., Agulleiro, B., 2000. Identification of mammosomatotropes, growth hormone cells, and prolactin cells in the pituitary gland of the gilthead seabream (*Sparus aurata* L., Teleostei) using light immunocytochemical methods: an ontogenetic study. Anat. Embryol. 202, 421–429.

Volckaert, F.A.M., Mugoyo, J.W.M., Lescroart, O., Grisez, L., Ollevier, F., 1999. Immunohistochemically detected ontogeny of prolactin and growth hormone cells in the African catfish *Clarias gariepinus*. Comp. Biochem. Physiol. 122, 423–431.

Wendelaar Bonga, S.E., 1997. The stress response in fish. Physiol. Rev. 77, 591–625.

Zydlewski, J., McCormick, S.D., 1997. The ontogeny of salinity tolerance in the American shad, *Alosa sapidissima*. Can. J. Fish Aquat. Sci. 54, 182–189.