Vlaams Instituut voor de Zee
Flanders Marine Institute

ELSEVIER

Comparative Biochemistry and Physiology Part B 122 (1999) 423–431

CBP

Immunohistochemically detected ontogeny of prolactin and growth hormone cells in the African catfish *Clarias gariepinus*

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Received 14 September 1998; received in revised form 14 January 1999; accepted 27 January 1999

Abstract

The chronological appearance of selected endocrine cells in the pituitary of African catfish *Clarias gariepinus* (Burchell, 1822) was studied morphologically, histologically and immunohistochemically by using antisera raised against catfish growth hormone (cgGH) and recombinant tilapia prolactin I (tPRL). cgGH- and tPRL-like immunoreactive cells were visible from day 1 post fertilisation (hatching) throughout the juvenile and the adult stage. From 1 to 90 days after hatching, the larval pituitary is oval in shape with a distinctly shaped rostral pars distalis, proximal pars distalis and pars intermedia. From day 120 onwards allometric growth of the rostral and proximal pars distalis extended the prolactin and growth hormone cells anteriorily and posteriorily, respectively. Size and activity of the prolactin and growth hormone cells, measured by the ratio of cell surface to nuclear surface remained constant until day 40 and showed a growth spurt thereafter. Growth hormone content, measured with a catfish-specific radio-immunoassay from hatching until 60 h post hatching, increased exponentially between 30 and 60 h.

Keywords: African catfish; *Clarias gariepinus*; Growth hormone; Immunohistochemistry; Ontogeny; Pituitary gland; Prolactin; RIA; Teleostei

1. Introduction

While classical techniques such as experimental endocrinology, histology and immunohistochemistry have taught us much about the location and possible functions of the growth hormone (GH)–prolactin (PRL) family [11,18], more recent studies on gene regulation provide evidence for specific and defined roles of hormones [33,39]. GH and PRL, originating from the same stem cells [7], are expressed in specific cells located in the pituitary. GH plays a role in anabolic processes, energy mobilisation (including energy repartitioning and lipid mobilisation) and osmoregulation in fish [9]. PRL has multiple effects in fish, ranging from osmoregulation, ion transport across gill epithelia, growth, reproduction and metabolism to behaviour [11,32]. The regulation of these hormones, together with somatolactin (SL) which belongs to the same GH-PRL gene family [42], has several common features at the molecular level. For example the pituitary-specific Pit-1 protein regulates the temporal and spatial transcription of PRL, GH and SL [43]. Insulin-like growth factor (IGF)-I mediates the action of GH and PRL [34,39]. In addition, it has become clear that the molecular evolution of fish GH, PRL and IGF-I largely follows the morphological evolution in several sustained bursts of evolution [34,48]. It has been hypothesised that the ancestral gene of GH, PRL and SL played a role in regulating the flux of metabolites necessary to maintain

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0305-0491/99/$ - see front matter
PII: S0305-0491(99)00017-6

cell volume in response to changes in environmental salinity in the early deuterostome ancestors of the chordates [9].

The African catfish *Clarias gariepinus* (Burchell, 1822) (Teleostei, Siluroidei) has been used as an endocrinological model, especially for the regulation and functioning of the gonadotrophic hormone (GtH) [38]. The genes GH and PRL have been studied to a lesser degree, but they have been cloned [16], the growth hormone protein has been purified [6] and a radioimmunoassay has been developed [26]. GH release in catfish is affected by growth hormone-releasing hormone and somatostatin [26]. The embryonic and larval development of African catfish follows the typical pattern of bony fish [27].

The embryonic and larval expression of GH and PRL is indicative of the time-specific onset of their activity. In addition, the endocrine actions of GH and PRL may vary among species. Thus far the ontogeny of GH- and PRL-expressing-cells has been studied by light microscopy or electron microscopy combined with histochemical and later on immunohistochemical identification in teleosts such as *Anguilla anguilla* [2], *Cynolebias whitei* [35], *Dicentrarchus labrax* [8], *Hexogrammos otakii* [20], *Oncorhynchus mykiss* [36], *Oncorhynchus kisutch* [24], *Oreochromis mossambicus* [19], *Oryzias latipes* [37], *Pleuronectes platessa* [12], *Poecilia reticulata* [21] and *Sparus aurata* [31]. A single histochemical study of development has been reported for a catfish, *Clarias batrachus* [5]. More recently the embryonic and larval appearance of GH, SL and PRL transcripts in lower vertebrates has been shown to trace the appearance of these peptides [14,22].

Our study monitors the chronological appearance of immunoreactive GH and PRL cells in the pituitary of African catfish from hatching until adulthood and compares the adult pituitary with the larval pituitary. In addition, morphometric traits of the PRL and GH cells are measured simultaneously and the GH content of the youngest stages is related to larval age.

2. Materials and methods

2.1. Larviculture

African catfish *C. gariepinus* were spawned and the larvae hatched according to the procedures detailed previously [47]. Fish were raised at 28°C in a fresh water flow-through system. They were fed Artemia cysts from day 4 (yolk sac resorption completed) till day 8. From day 8 till day 12, the larvae were weaned to a dry commercial catfish diet (Trouvit, Belgium). Thereafter the fish were fed progressively larger dry food pellets ad libitum (Bio Meerval, Trouvit, Belgium).

2.2. Tissue preparation, histology and immunohistochemistry

At least ten fish were sampled shortly after hatching (day 1), at day 6, 10, 15, 22, 30, 60, 90 and 270 (adult). Complete catfish larvae were fixed in Bouin–Hollande sublimate [10] from days 1 to 15. After fixation, the heads of 22- and 30-day-old juveniles were decalcified in formic acid (4 N) for 2 days, while the pituitaries of older fish were removed from freshly killed specimen and fixed in toto in Bouin–Hollande sublimate. Tissues were dehydrated through an ethanol–dioxane series and embedded in Paraplast. Sections of 6 μm thick were put on gelatin subbed slides.

Polyclonal rabbit antiserum directed against African catfish GH (cgGH) was obtained as specified previously [26] and used at a dilution of 1:10 000. Polyclonal rabbit antiserum directed against recombinant tilapia *Oreochromis niloticus* prolactin I ($tPRL_I$) [41] was donated by F. Rentier-Delrue and used at a dilution of 1:1,500. Its cross-reactivity to catfish PRL has been documented previously [26].

The tissue sections were processed for immunohistochemistry as described previously [44]. After an overnight incubation with the primary antibodies, sections were stained using HRP-labelled secondary antibodies (DAKO) and diaminobenzidine as chromogen. Adjacent sections were stained with the GH and PRL antisera and a modified trichrome Masson staining [30].

Controls were carried out to test efficiency, accuracy and precision, sensitivity and specificity of both antisera [28]. All steps in the staining procedure were standardised. Efficiency was tested by progressive elimination of a step in the staining procedure. Sensitivity was tested by using initially decreasing dilutions until immunostaining disappeared (1:100 000, 1:50 000, 1:10 000, 1:5000 and 1:1000 for cgGH and 1:3000, 1:1500, 1:1000 and 1:500 for $tPRL_I$). Cells from both tilapia and African catfish were used to test specificity. The cgGH antiserum did not cross react with tilapia GH (*O. niloticus*), while the $tPRL_I$ antiserum reacted with PRL cells in tilapia and African catfish, but did not cross react with any other cell type. cgGH and $tPRL_I$ specifically stained GH and PRL cells, respectively, without background.

2.3. Radioimmunoassay

Larvae were sampled every 6 h from fertilisation (0 h) until 72 h post fertilisation. Growth hormone was quantified in the early life stages by radioimmunoassay (RIA) [26]. Ten larvae were pooled and homogenised in assay buffer (50 mM Tris, 1% BSA, 0.5% NaN_3, 0.1% Triton-X, pH 7.8) and measured with an antibody specific to African catfish GH. The interference of yolk in the assay was checked with a dilution curve of larval

extracts. Final results are expressed as picogram GH per individual. Larvae older than 72 h were assayed individually. PRL was not quantified since no catfish specific RIA was available.

2.4. Morphometry

Various morphometric parameters of the GH and PRL immunoreactive cells were measured with a Video Image Analysis System (Kontron, Germany). The absolute cell diameter, cell surface, nuclear diameter and nuclear surface were measured on 15 cells from several individuals (except at days 1–4 when less cells were available for measurement). Only cells with a complete nucleus on the slide were measured. The ratio of cell area to nuclear area was used as a measure of cell activity. Statistical analysis was performed with the Statistica v. 4.5 (STATSOFT) software package.

3. Results

3.1. General morphology, histology and immunohistochemistry of the adult pituitary

Macroscopically, the pituitary of adult *C. gariepinus* is an ovoid organ with a slight median fissure giving rise to the appearance of a two-lobed gland, when observed from the ventral side. Midsagittal sections show an irregularly shaped oval pituitary connected to the hypothalamus, and an infundibular recess. Trichrome staining differentiated the adenohypophysis of the adult African catfish into three areas: the rostral pars distalis (RPD), the proximal pars distalis (PPD) and the pars intermedia (PI; Fig. 1). Acidophilic cells staining by Pollak and reacting for GH, PRL and SL were observed in the pars distalis (PD) and PI. Cells reacting with the anti-GH serum were located in the PPD adjacent to the gonadotrophs; they were somewhat more loosely packed and occupied a larger area dorsally. Their shape was triangular, the cell diameter was 8.7 μm ($\pm$0.5) and they tended to be organised in chains. Cells reacting with the anti-PRL serum were confined to the RPD, stained purple with trichrome and occupied a rather large area. They were densely packed, had a rounded shape and measured 2.9 μm ($\pm$0.7). Double staining with Pollak's method and PRL/GH immunohistochemical techniques corroborated these results. Adrenocorticotropic-like cells (ACTH), identified with Trichrome Pollak in pale blue, were located throughout the RPD intermingled with the PRL cells and the neurohypophysis. Their size and shape was similar to the PRL cells. Gonadotropin-like cells (GtH) stained blue with trichrome and could only be identified ventrally of the proximal pars distalis. Since the fish sampled had not spawned, the GtH cells were not activated yet [29]. Cells probably secreting thyroid stimulating hormone (TSH) stained blue and were located in between the GH cells.

3.2. Morphology of the larval pituitary

The general morphology of the larval and juvenile pituitary differs from the adult (Figs. 1–3). One- to 6-day-old larvae have a pituitary gland shaped triangularly which is in intimate contact with the hypothalamus. We could not find evidence for an infundibular stalk after inspecting serial sections. The infundibular recess/third ventricle of the hypothalamus is visible from day 6 onwards. The pituitary contains a dense mass of cells without clear boundaries between the cells of the RPD, PPD and PI. The RPD is located in the anterior corner of the triangular pituitary. At day 6 the neurohypophyseal fibres are visible; the pituitary gland

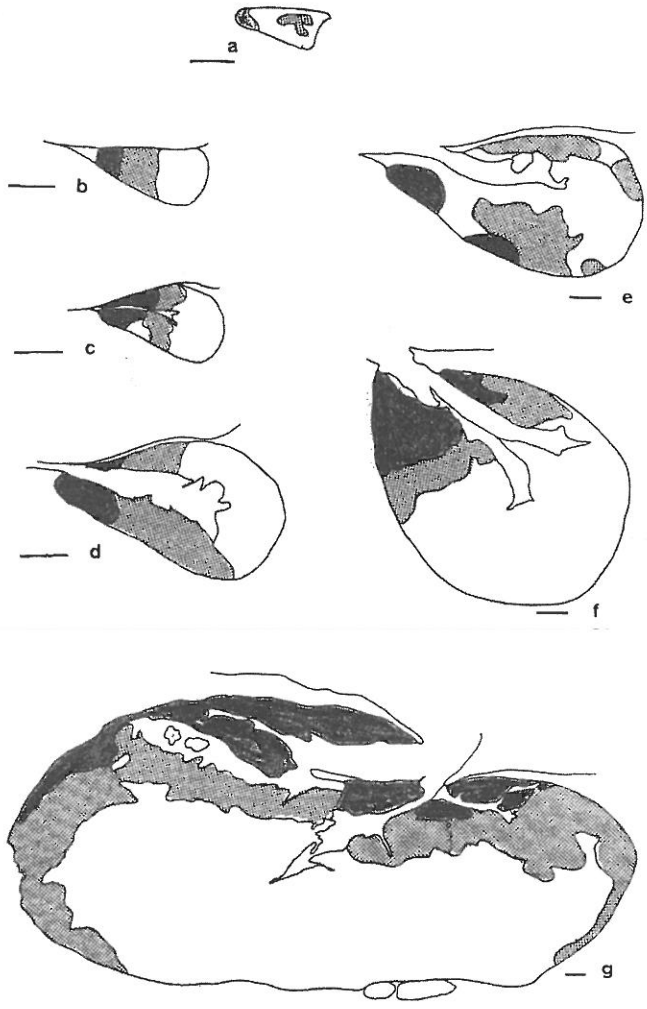

Fig. 1. Diagram of a midsagittal section through the pituitary gland of larval, juvenile and adult African catfish showing GH (dotted) and PRL (gray) cells as obtained from trichrome Pollak and immunohistochemical staining: (a) day 1; (b) day 6; (c) day 10; (d) day 22; (e) day 30; (f) day 90; and (g) day 270 (adult). The bar indicates 50 μm.

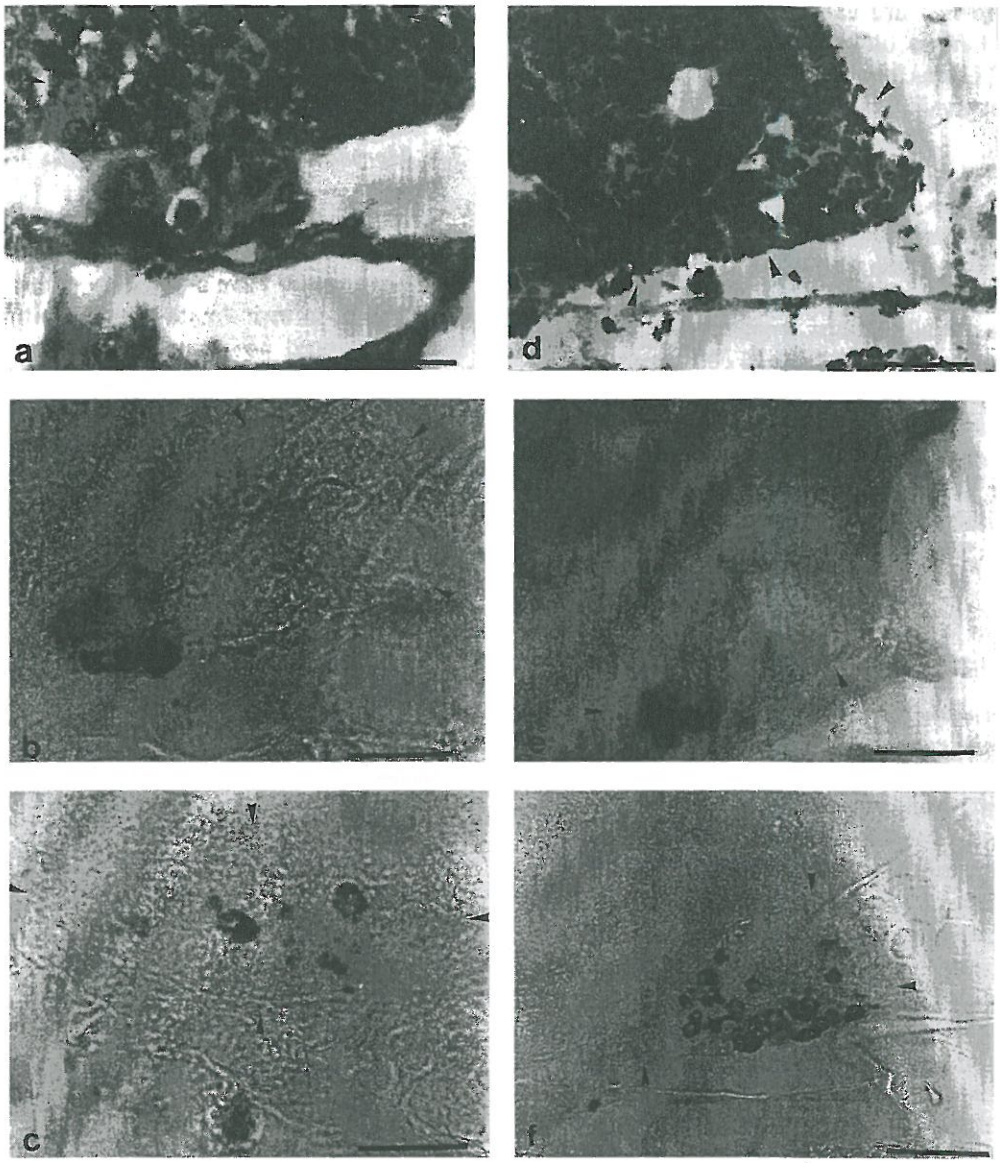

Fig. 2. Midsagittal section through the pituitary of African catfish on days 1 and 6. The actual size is indicated with a bar representing 20 (panels a–c) and 40 μm (panels d–f). Upper panel (a, d): section stained with trichrome Pollak; middle panel (b, e): section immunostained for PRL; lower panel (c, f): section immunostained for GH. Arrows indicate the position of the pituitary. Note that the adenohypophysis is connected to the buccal epithelium.

remains located close to the hypothalamus and is triangular in cross-section. The adenohypophysis is connected to the epithelium of the buccal cavity. At day 10 the infundibular stalk becomes visible and a clear distinction is seen between the RPD, PPD and PI. At day 22 the neurohypophyseal fibres deeply penetrate in the pituitary and the pituitary becomes pear-shaped in cross-section. From day 90 onwards, the pituitary gland resembled the adult type with clear separations between the RPD, PPD, PI and neurohypophysis, although proportionally still smaller than the adult. The shape is oval in cross-section.

3.3. Histology, morphometry and immunohistochemistry of the larval adenohypophysis

$tPRL_I$-like immunoreactive cells were observed in the rostral part of the pituitary gland immediately after hatching (day 1; Fig. 2(b)). The cells stained rather weakly and were round in shape. Cell surface and the ratio of cell surface to cell nucleus remained constant until day 40 and increased thereafter (Fig. 4). PRL cells tended to occur in the rostral part close to the infundibular stalk; a clear allometric growth with extension to the anterior and posterior part was observed

from day 120 onwards. The PRL cells observed in adult African catfish extend dorsally, anteriorily and laterally in the rostral pars distalis.

cgGH-like immunoreactive cells were observed in the mid part of a dense mass of pituitary cells immediately after hatching (day 1; Fig. 2(c)). The cells reacted strongly and had an angular shape. Cell surface and the ratio of cell surface to cell nucleus remained constant until day 40 and increased thereafter with a concomitant change in shape from round to triangular (Fig. 4). GH cells tended to occur in the middle part of the pituitary posterio-ventrally from the PRL cells till day 90 (Fig. 3(f)). Until that time the position of the PPD relative to the RPD was most unambiguous. At 120 days allometric growth extended the position anteriorily and posteriorily such that they envelope the pars intermedia. The GH cells observed in adult African catfish were located in the PPD.

3.4. GH content of the larvae

The GH content stayed below detection levels until 36 h post fertilisation (Fig. 5). It was only at 42 h that measurable amounts were detected; thereafter the content increased exponentially. At 72 h GH was detectable in homogenates of individual larvae. GH content correlates linearly with body mass up to 60 h post hatch: GH content (pg ind^{-1}) = $-33.33 + 453.87$*Dry Weight (mg ind^{-1}) ($r = 0.95$; $P < 0.001$).

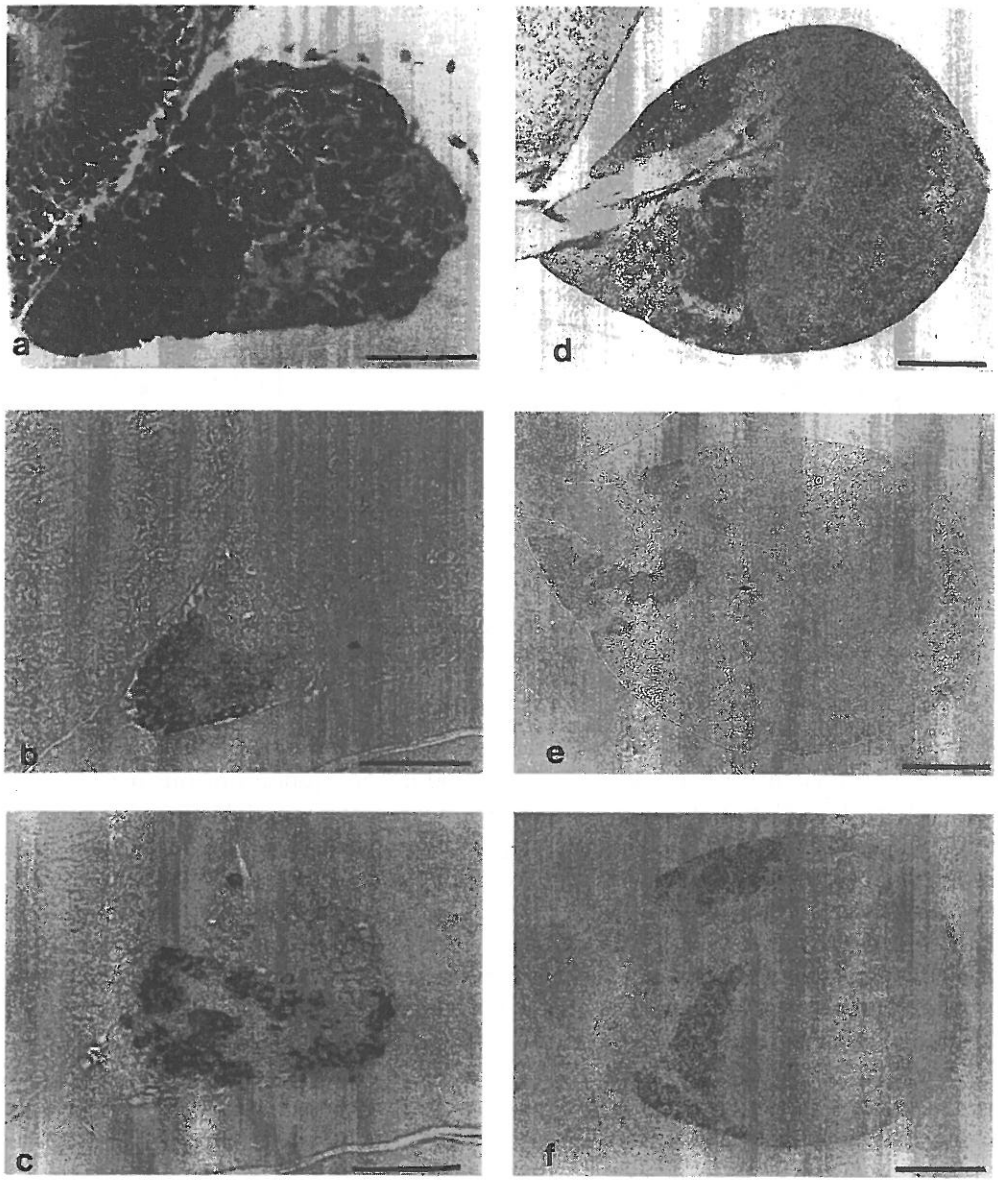

Fig. 3. Midsagittal section through the pituitary of African catfish on days 22 and 90. The actual size is indicated with a bar representing 40 (panels a–c) and 200 μm (panels d–f). Upper panel (a, d): section stained with trichrome Pollak; middle panel (b, e): section immunostained for PRL; lower panel (c, f): section immunostained for GH.

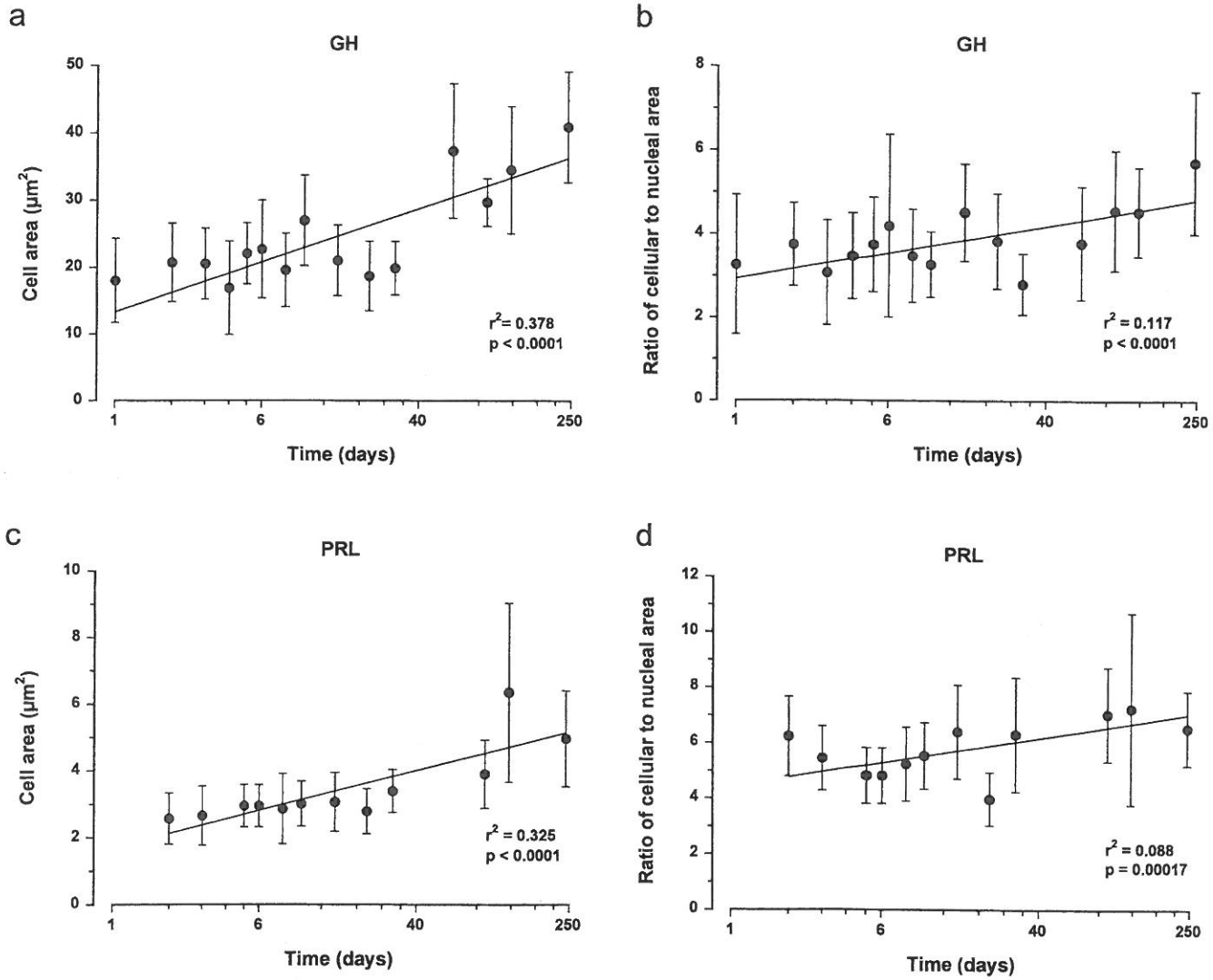

Fig. 4. Linear regression of cell surface (a, c) ($\mu m^2 \pm SD$) and ratio of cell surface to nuclear surface (b, d) ($\pm SD$) of GH (a, b) and PRL cells (c, d) in function of time (from hatching till adulthood) of African catfish. Note the $\log_{10}$ scale on the x-axis.

4. Discussion

The physiological actions of prolactin and growth hormone cover a broad spectrum [11,18] and are likely of relevance during larval development. Their spatial and temporal dynamics as observed in other vertebrate taxa point to physiologically variable actions [15,22]. However, the ontogeny of hormones of the GH-PRL group have been studied in only a limited number of fish. African catfish is a suitable model for such studies because of the short duration of embryonic development, the external embryonal development, the large size of the adults (which facilitates sampling), the detailed knowledge of the biology and the ease to culture.

Most significantly, very shortly after hatching (eleuthero-embryo) cells immunoreactive to GH and PRL were detected. GH cells appear active during the early stages of development, although few cells are present at that time. The absolute amount of GH in the larvae increases measurably and exponentially between 42 and 60 h (the endpoint of our measurements). Both observations prove that GH is active at the very early stages of development. GH expression is thought to be linked to motion; the first movements of catfish embryos are registered at about 18 h post fertilisation at 28°C (pers. ob.). Our attempts to detect GH in unhatched embryos immunohistochemically or by radioimmunoassay failed. In the first case this is probably due to the low sensitivity of the method while in the latter case we noticed interference with vitelline substances when we tried to measure GH in a large batch of larvae. Partial purification of the tissue extracts might improve the RIA while PCR titration of mRNA could provide a more sensitive detection method for younger stages. GH mRNA was detected in larvae of sea bream from day 3 post hatch [14]. Thus the pres-

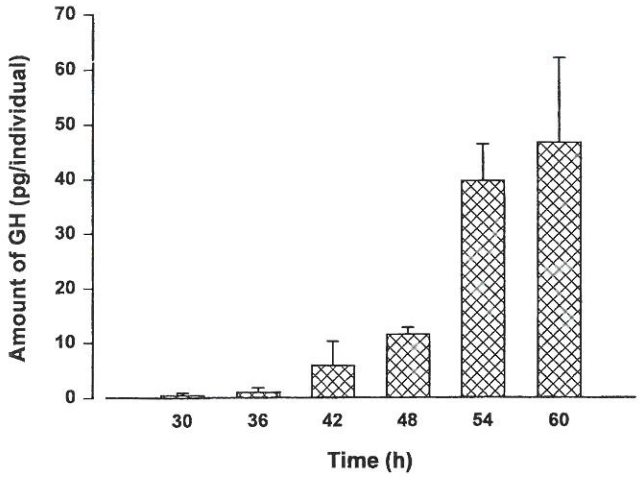

Fig. 5. Total average GH content (pg ind^{-1}) ($\pm$SD) in the body of larval African catfish from hatching until 60 h.

ence of GH (and PRL) and/or their mRNAs in unfertilised fish eggs and in embryos remains an open question. Lipophilic substances such as L-thyroxine (T_4) and triiodo-L-thyronine (T_3), which are influenced by GH and originate from the mother, are present in fish eggs, their levels decreasing with development [25]. The growth regulator IGF-I, which affects PRL and GH expression, is present as mRNA in unfertilised eggs and embryos [34].

PRL cells appeared active during the early stages of development. A weaker signal was detected shortly after hatching, which was also reported in medaka and *C. whitei* [37]. Most important is the osmoregulatory action of PRL in freshwater species, including catfish [11]. The differences in the appearance of GH and PRL merely reflect species-specific differences in patterns of development [4]. Development is saltatory due to the passing of thresholds and can be modified by an altered time of appearance of structures and functions. The distribution and appearance of PRL and GH cells among species shows elements of this process (see later). It might also be argued that freshwater species have in comparison to marine species a higher need to regulate osmotic pressure. Indeed, PRL expression has been detected in sea bass [8] and sea bream [31] at a later stage in development than trout [36] and catfish.

The pituitary of African catfish, sea bass and sea bream grow allometrically but the expression of African catfish GH follows a linear pattern in relation to body mass up to 60 h post hatch. At some stage, either earlier (catfish) or later (sea bass) in development, the relative position of the GH and PRL cells is conserved, but the final three-dimensional distribution pattern may show major modifications anteriorily, posteriorily and laterally. This raises the question whether the differential developmental rate of the various species is a crucial factor. African catfish embryos develop relatively fast at warm temperatures; the threshold temperature for development is 14.5°C [17]. Larvae hatch 24 h post fertilization at 28°C and resorb their lipid-rich yolk during the following 2 days, only to start feeding at day 3 post hatch. The digestive system is limited to an 'Anlage' which develops very fast during the first 4 days of exogenous feeding [46]. The ontogeny of the chondrocranium reflects the premature development of the African catfish at hatching [1]. This study confirms that the development of the nervous system is premature at hatching. The hypothalamo–pituitary–gonadal axis fully develops during puberty.

We compared the ontogeny of GH and PRL among a few well studied teleosts (Table 1). In general the development of catfish larvae follows that of other teleosts such as rainbow trout [45], sea bass [8] and zebrafish [23] although the order of appearance of the hormones considered may change. During embryonic development GH and PRL appear only in salmonids before hatching; other fish produce GH and PRL shortly after hatching. The reduced need to osmoregulate in sea water (with its high Ca content) delays the expression of PRL in marine fish. However, a detailed interspecific comparison among stages is hampered by the absence of a standardised staging terminology.

Table 1
Comparative developmental biology of GH and PRL in African catfish (this study), rainbow trout [36], sea bass [8] and sea bream [31]

Timing of characteristics	African catfish (*Clarias gariepinus*)	rainbow trout (*Oncorhynchus mykiss*)	sea bass (*Dicentrarchus labrax*)	sea bream (*Sparus aurata*)
Days to hatch from fertilisation	1 day at 28°C	40 days at 12°C	4 days at 16°C	2 days at 16°C
Days to first feeding from fertilisation	3 days at 28°C	45 days at 12°C (stage 37)	6 days at 19°C	4 days at 16°C
Size or age to adult[a]	35 cm (240 days at 26°C)	24 months	25 (M)–32 (F) cm	12 months (M)–24 months (F)
Days pre/post-hatch; appearance of:				
GH	0 days	−14 days (stage 21)	+1 days	+1 days
PRL	0 days	−27 days (stage 28)	+9 days	+4 days

[a] F: female; M: male.

Another aspect is the observed increase in cell size of the PRL and GH cells at 40 days of age. Anurans show various patterns of decreasing and increasing cell size during metamorphosis [15]. It was also shown that the volume and activity of PRL cells is a function of environmental osmolarity [35]. Whether our observations can be interpreted in view of an increase in activity or a species-specific effect related to age remains to be verified experimentally, preferably by the measurement of receptor density [32].

We used the tilapia PRL_I antiserum (the form which is the most similar to other fish PRL genes, contains 188 amino acids and shows 69% sequence identity to $tPRL_{II}$) to screen for PRL in African catfish. Recombinant $tPRL_I$ is more sensitive to the transfer from fresh to salt water in comparison to recombinant $tPRL_{II}$ [3,13] while it may possess somatotropic actions similar to GH [39]. The tilapia PRL_{II} hormone comprises 177 amino acids and is known to be present in the same granules as PRL_I [40]. The $tPRL_{II}$ antiserum turned out to express antigenic epitopes equally efficient as $tPRL_I$ without any obvious difference between the PRL cells stained. The presence of more than one form of the hormones PRL and GH in catfish remains to be elucidated; so far, there is evolutionary no indication of gene duplication.

In conclusion, GH and PRL immunoreactive cells have been identified at all stages of free living larvae and adults of African catfish. Their activity appears to be changing through life time. The question remains when exactly these hormones are expressed for the first time during embryonic development; immunoreactive sites are present shortly after hatching.

Acknowledgements

F.A.M. Volckaert is a research associate of the Fund for Scientific Research–Vlaanderen (Belgium); J.W.M. Mugoyo acknowledges a scholarship of the General Direction for Overseas Development (Belgium). We thank F. Rentier-Delrue for PRL antibodies, K. Mol for tilapia pituitaries, F. Vandesande for general advise, E. Holsters for animal care and L. Moons together with two anonymous reviewers for critically reading the manuscript. Research was supported by an equipment grant and project grant G.2014.94 of the Fund for Scientific Research–Vlaanderen (Belgium).

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