Immunocytochemical identification and ontogeny of adenohypophyseal cells in a cave fish, *Phreatichthys andruzzii* (Cypriniformes: Cyprinidae)

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Running title: Pituitary cell types in *P. andruzzii*

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ABSTRACT

The morphogenesis of the pituitary gland and the chronological appearance of adenohypophyseal cells were investigated for the first time in a blind cave fish, *Phreatichthys andruzzii*, by immunocytochemistry. The adult adenohypophysis contained: a rostral pars distalis, with prolactin (PRL) cells arranged in follicles and adrenocorticotropic (ACTH) cells; a proximal pars distalis with somatotropic (GH), β-thyrotropic (TSH), β-gonadotropic type I (GtH I) and type II (GtH II) cells; and a pars intermedia with α-somatolactin (SL), α-melanotropic (MSH) and β-endorphin (END) cells. All regions were deeply penetrated by neurohypophyseal branches. At hatching (24 hours post-fertilization) the pituitary was an oval cell mass, close to the ventral margin of diencephalon. The first immunoreactive cells appeared as follows: PRL at 0.5 days after hatching (dah), GH and SL at 1.5 dah, END at 2 dah, TSH, ACTH and MSH at 2.5 dah, GtH I at 28 dah, GtH II at 90 dah. The neurohypophysis appeared at 5 dah and extensively branched inside the adenohypophysis at 130 dah, but no boundary was found between rostral pars distalis and proximal pars distalis at this stage. The potential indexes of prolactin and growth hormone production respectively increased until 28 and 60 dah. Activity of PRL and GH cells, measured as ratio of cell area to nucleus area, was significantly higher in juveniles than in larvae. These results are a premise to physiological investigations on the hypothalamus-pituitary axis of *P. andruzzii*, relevant for conservation of this endangered species.

Key Words: hypogean fish; pituitary gland; immunostaining; pituitary hormones; early development

INTRODUCTION

Phreatichthys andruzzii Vinciguerra, 1924 (Cypriniformes: Cyprinidae) is a blind tropical cave fish inhabiting underground waters in a rather restricted area of central Somalia and showing marked morphological and behavioural adaptations to subterranean life (Berti & Messana, 2010). Molecular clock calculations supported the hypothesis that the colonization of hypogean habitats by *P. andruzzii* was induced by deep climate changes in the Afro-Arabian region during Plio-Pleistocene and confirm a close phylogenetic relationship between the genera *Phreatichthys* and *Garra* (Colli *et al.*, 2009).

P. andruzzii reaches up to 106 mm in total length and 87 mm in standard length (Berti & Messana, 2010). Scales and pigmentation are completely absent; the eyes reach the maximum differentiation 36 hours after egg laying and rapidly degenerate, leaving only a rudimentary cyst after a month (Berti *et al.*, 2001). In the adult anophthalmia is complete and accompanied by the loss of optic nerves; the entire encephalon undergoes a marked reduction, mostly involving the optic lobes (Ercolini & Berti, 1982). Retinal development in *P. andruzzii* was recently studied by expression of marker genes in eye morphogenesis and differentiation. The initial steps were apparently similar to those of other teleosts, but the ensuing eye degeneration was due to a block of differentiation of retinal cell types after the first generation of retinal ganglion cells, followed by an apoptotic wave in cells failing to reach terminal differentiation (Stemmer et al. 2015).

The species has been inserted since 1996 in the IUCN Red List of Threatened Species and classified as “Vulnerable D2” (Getahun, 2010), because any change in water quality or depth in the few isolated wells where it lives may have dramatic effects on its population. For purposes of conservation, a detailed knowledge of its life cycle is a relevant issue.

In Vertebrates, the pituitary is the main endocrine gland controlling key processes such as growth, development, immunological and stress responses, reproduction and adaptation to the environment. Wingstrand (1966) first described the morphological features of the gland and its phylogenesis from Agnata to Mammalia, and later Henderson (1997) described the physiology of the hypothalamus-

hyphophyseal complex. In fishes, the anatomy and histophysiology of the pituitary were reviewed by Ball and Baker (1969) and Doerr-Schott (1976). The pituitary of teleosts is divided in two major regions, the neurohypophysis and the adenohypophysis (Follénius *et al.*, 1978; Agulleiro *et al.*, 2006). The neurohypophysis, derived from a projection of the diencephalon, is composed of peptidergic, aminergic and GABAergic fibres mostly derived from the hypothalamus. Since the pituitary of teleosts has no portal system and median eminence, the neurosecretory terminals of the hypothalamus exert a direct control on the secretory cells of the adenohypophysis (Holmqvist & Ekstrom, 1995).

The hormone-producing cell types in fish adenohypophysis were studied by histochemical, cytophysiological, ultrastructural and immunocytochemical techniques (Doerr-Schott, 1976; Follénius *et al.*, 1978; García Ayala *et al.*, 1997; Agulleiro *et al.*, 2006; Borella *et al.*, 2009). Eight (Agulleiro *et al.*, 2006) or nine (Pogoda & Hammerschmidt, 2007; Grandi *et al.*, 2014) adenohypophyseal cell types were identified and characterized, based on their morphology and secreted hormones. In teleosts the secretory cells of the adenohypophysis are generally distributed in three well-defined regions, deeply penetrated by neurohypophyseal branches (Agulleiro *et al.*, 2006). The prolactin and corticotropic cells are usually detected in the rostral pars distalis (RPD), the somatotropic, tireotropic and gonadotropic (GtH I and GtH II) cells in the proximal pars distalis (PPD), the somatolactin, melanotropic and endorphin cells in the pars intermedia (PI). Somatolactin is the only hormone produced by adenohypophyseal cells which is not found in other vertebrates (Fukamachi *et al.*, 2005).

Pituitary hormones, especially the growth hormone, the prolactin and somatolactin, are known to play a relevant role in regulation of development and growth in fish embryos and larvae (Majumdar & Elsholtz, 1994). Studies on morphogenesis of the pituitary gland and ontogenesis of its cell types in embryos and larvae have been reported in teleosts (de Jesus *et al.*, 2014) and Chondrostei (Grandi et al., 2004). The onset of secretory activity in adenohypophyseal cell types changes according to systematic group and developmental stage, reflecting the involvement and the relevance of the

hormones in a given life stage (Yadav, 1995). Recent studies in embryos showed that all secretory cells of the adenohypophysis originate from the anterior region of the neural ridge (Pogoda & Hammerschmidt, 2009).

Studies on pituitary cell identification in cave fish are very limited and the only data available are on *Anoptichthys jordani* (Characiformes), a Mexican cave fish (Mattheij, 1968). Concerning *P. andruzzii*, many details of its life cycle are still poorly understood: for example it is unclear whether the extensive morphophysiological modifications involved in adaptation to subterranean life are related to changes in structure and development of the pituitary gland.

In this study we report for the first time the anatomical distribution of the different adenohypophyseal cells in adults of *P. andruzzii* and the morphogenetic changes during the development of the pituitary gland, including chronological appearance of different endocrine cell types. These data may contribute to increase knowledge about endocrinology and biology of this endangered cave fish species.

MATERIALS AND METHODS

Source of fish

Larvae and juveniles of the blind cave fish *Phreatichthys andruzzii* were raised from eggs obtained in July 2013 after hormone stimulation from male and female individuals belonging to the sixth generation raised at the animal facility of the Department of Life Sciences and Biotechnology, University of Ferrara (Italy). The original fish were collected in 1982 in the wild from wells of Somalia (locality Gheriale) (04°08’22’’N, 46°29’05”E) and bred at the Department of Evolutionary Biology, University of Florence (Italy) (Frangioni *et al.*, 1997). Adult cave fish were held in 60-litre tanks with a recirculating system supplied with biologically filtered tap water. The fish were kept in darkness at a constant temperature of 27 °C except during feeding and aquaria maintenance. Fish were fed three times a week with frozen chironomid larvae.

126 Fertilized eggs were collected two hours after laying, cleaned and placed into E3 medium for
127 zebrafish embryos (5 mM NaCl, 0.17 mM KCl, 0.33 mM $CaCl_2$ - $2H_2O$, 0.33 mM $MgSO_4$ - $6H_2O$,
128 0.0002% methylene blue). They were incubated in 200 mm Petri dishes kept in darkness at 28° C in
129 a thermostat and hatched after 24 hours. The embryos hatched as larvae that reabsorbed the yolk sac
130 in 4-6 days, becoming actively feeding. At 60 days after hatching (dah) they assumed the aspect of
131 an adult, although of smaller size, and were considered as juveniles. The larvae were reared in a
132 small glass aquarium (20x30x15 cm) filled with about 4 litres of decanted tapwater (changed every
133 other day) and kept in darkness at 28°C. From 1.5 dah onwards, the larvae were fed daily with
134 freshly hatched nauplii of *Artemia* sp. (Crustacea Anostraca) supplemented with fine granular dry
135 food (Sera GmbH, Heinsberg, Germany). From day 28 onwards, the fish were fed daily *ad libitum*
136 with TetraMin Hauptfutter (Tetra Werke, Melle, Germany) and with *Artemia* sp. nauplii. The
137 average water temperature was 27.87±0.18 °C and the dissolved oxygen concentration, monitored
138 by a WTW Oxi 330i oximeter (WTW-GmbH, Weilheim, Germany), was 7.98±0.47 mg/L.
139 Larvae were collected at the time of hatching and at 0.5, 1, 1.5, 2, 2.5, 5, 14, 21, 28 dah, and
140 juveniles at 60, 90, 110, 130 dah. For each age class, seven animals were collected and five of them
141 were randomly chosen for examination. Among all laboratory reared individuals, only two adults
142 were available for this study. All animal handling and research protocols were approved by the
143 Institutional Animal Care and Use Committee of the University of Ferrara (Ferrara, Italy). Fish
144 rearing and care conformed to the European directive 2010/63/UE on the protection of animals used
145 for scientific purposes.
146
147 Tissue processing
148 The animals were anesthetized by immersion in a solution of chloretone (β,β,β-trichloro-tert-butyl
149 alcohol in tapwater, 0.5 g/L). Total body length (TL) was measured to the nearest 0.1 mm using a
150 stereomicroscope Nikon C-DSS230 (Nikon Corp., Tokyo, Japan) provided with a Nikon Digital
151 Sight DS-5 M camera and the acquired images were analyzed by Kontron KS300 Imaging System

Program (Kontron, Milan, Italy). Afterwards, the whole head of each individual was detached from body at the opercular rim and fixed by immersion in sublimated Bouin-Holland's solution. In adults it was necessary to separate the hypophysis from the highly ossified cranial bones, thus it was not possible to preserve the continuity of the hypophysean stalk with the hypothalamus.

After 24 h fixation, the specimens were dehydrated through a series of graded ethanol (50-99%). During dehydration the deposits of mercury chloride were removed by treatment with iodine-potassium iodide in 90% ethanol for 24 h. Tissue pieces were then cleared in xylene, embedded in Paraplast (melting point 56°C) and 5 μm serial sagittal sections were mounted on glass slides. Some tissue pieces were fixed in 2% glutaraldehyde solution buffered at pH 7.2 with 0.1 M sodium cacodylate, dehydrated and embedded in Epon-Araldite, sectioned at 1.5 μm with a Reichert Ultracut E ultramicrotome (Reichert-Jung, Wien, Austria) and stained with methylene blue – azur A. In order to evaluate the reaction to different antisera, adjacent sagittal sections of the hypophysis from each individual were divided into 6 batches, separately mounting each consecutive section on glass slides. One or more sets of 6 slides were prepared for each individual. Adjacent sections from each batch were treated with twelve different antisera and the immunoreactive cells were visualized by an immunoperoxidase avidin-biotin complex (ABC) reagent, with diaminobenzidine as a substrate. Some sections were stained with Herlant's tetrachrome. The sections were analysed and acquired with a Nikon Eclipse 80*i* light microscope equipped with a Nikon Digital Sight DS-Fi1 camera (Nikon Corp., Tokyo, Japan).

Primary antibodies

The following antisera were kindly provided by Dr P.M. Ingleton (Sheffield University, Sheffield, UK): chum salmon growth hormone (GH) and anti-chum salmon GH (diluted 1:2500), chum salmon prolactin (PRL) and anti-chum salmon PRL (diluted 1:2000). Dr. H. Kawauchi (Kitasato University, Iwate, Japan) kindly donated the purified chum salmon gonadotropin I and II β-subunits (GtH I and GtH II), anti-chum salmon GtH I β-subunit (lot. No. 8707, diluted 1:250-1:500), and

178 anti-chum salmon GtH II β-subunit (lot No. 8506, diluted 1:1000). Dr. A. Takahashi (Kitasato
179 University, Iwate, Japan) kindly provided the antiserum to chum salmon N-acetyl-β-endorphin II
180 (END) (lot No. 8202, diluted 1:5000). Dr. P. Thomas (University of Texas at Austin, Texas, USA)
181 kindly donated the anti-red drum α-subunit somatolactin (SL) (diluted 1:1000) and anti-croaker
182 gonadotropin (GtH) (diluted 1:500). All human antigens and antisera were obtained from
183 Biogenesis (Poole, UK): anti-growth hormone (GH) (diluted 1:200-1:1000), anti-thyroid
184 stimulating hormone β-subunit (TSH) (diluted 1:500), anti-follicle stimulating hormone β-subunit
185 (FSH) (diluted 1:800), anti-melanocyte stimulating hormone α-subunit (MSH) (diluted 1:500), and
186 anti-adrenocorticotrophic hormone (1-24) (ACTH) (diluted 1:2500). All antisera were polyclonal,
187 raised in the rabbit and diluted in phosphate-buffered saline solution (PBS, 10 mM sodium
188 phosphate, 0.15 M sodium chloride, pH 7.6).
189
190 Immunostaining
191 Sections dewaxed in xylene were rehydrated in a graded ethanol series and rinsed in PBS. The
192 tissue sections were incubated in sealed humid chambers for 30 min in PBS with 0.1% Triton X-
193 100 and 0.3% hydrogen peroxide, treated with normal goat serum for 30 min, then rinsed in PBS.
194 Sections were exposed to primary antisera for 18-24 h at 4°C, then to biotinylated anti-rabbit IgG
195 for 1 h and to the ABC reagent (Vectastain "ABC" Elite Kit, Vector Labs., Burlingame, California,
196 USA) for 1h. Visualization of immunostaining was obtained by 3,3'-diaminobenzidine
197 tetrahydrochloride (DAB, Vector Labs) in 50 mM Tris-HCl (pH 7.6) with 0.003% hydrogen
198 peroxide. The sections were lightly counter-stained with Harris's haematoxylin, soaked in tapwater
199 for 10 minutes and mounted for observation. In sections of adults, nickel chloride was added to the
200 DAB solution to enhance the immunoreactions, according to the manufacturer's suggestion.
201 Adjacent sections were incubated in absence of the primary antiserum, or a non-immune rabbit
202 serum in place of the primary one, in order to verify the specificity of immunoreactions. For GH,
203 PRL, GtH I, GtH II, ACTH, MSH, TSH and FSH, the specificity was also validated by treating

some sections with antisera preincubated for 24 h at 4°C with an excess of the appropriate antigen. No immunostaining was observed in these sections. The human antisera against ACTH, MSH, TSH and FSH were claimed by the producer to be specific for their antigens. The specificity of the antiserum against SL was determined by Zhu and Thomas (1995) before use. Primary antisera against GtH and END were respectively validated in *Odontesthes bonariensis* (Atheriniformes) (Vissio *et al.*, 1997) and in *Acipenser transmontanus* (Acipenseriformes) (Joss *et al.*, 1990).

Morphometric analyses

Analysis of pituitary volumes and PRL and GH cell masses. The volumes of whole pituitary were determined in 5 µm thick serial paraffin sections examined with a Nikon Eclipse 80*i* light microscope equipped with a Nikon Digital Sight DS-Fi1 camera, using the software NIS Elements AR 2.30 (Nikon Instruments Europe) for computerised image analysis. Area values were multiplied by the section thickness to yield the volume of pituitary in each section: these volumes were added to obtain the total pituitary volume. The volume of immunoreactive (ir) PRL and ir-GH cell masses were measured by the technique previously applied to the whole pituitary, on serial sections at 20-25 µm intervals. The volumes of ir-PRL and ir-GH cell masses over the pituitary volume (% PRL and % GH) represented the potential index of the production of prolactin and growth hormone (Tanaka *et al.*, 1995).

Analysis of pituitary cell area and nucleus area. Cell and nucleus areas of ir-PRLand ir-GH cells were also measured by the previous method on 10 cells from each individual. At 1.5-5 dah a minor number of cells were available for measurement, thus 5 or more cells were analysed for each individual. For each interval of development expressed in days, at least five individuals were analyzed. Only cells showing an entire nucleus on the slide were measured. The ratio of cell area to nucleus area was used as a measure of cell activity (Volckaert *et al.*, 1999).

228 *Statistical analyses:* The percentage values of ir-PRL and GH cell masses were verified for
229 normality by the Kolmogorov-Smirnov test and the homoscedasticity by the Levene test.
230 Significant differences were assessed by one-way ANOVA, followed by a post hoc Tukey's test or
231 Student's t test, selecting the significance level $p < 0.05$. All analyses were performed by the
232 STATISTICA 7.1 program (StatSoft, Tulsa, Okhlahoma, USA). The data pertaining to cell and
233 nucleus areas, and to the ratio of cell area to nucleus area of the ir-GH and ir-PRL cells were
234 analysed in the same way.
235
236 **RESULTS**
237 Adult pituitary
238 Macroscopically, the pituitary of adult *Phreatichthys andruzzii* was ovoid, approximately 800 μm in
239 length, 650 μm in width and 300 μm in thickness, with a more enlarged posterior region. A thin
240 median fold, giving it the appearance of a two-lobed gland when observed from the ventral side,
241 crossed the gland longitudinally.
242 The morphology of the pituitary gland did not differ from the basic model found in other teleosts,
243 consisting of an adenohypophysis (AD) and a neurohypophysis (NH). On histological basis, the AD
244 could be divided in a pars intermedia (PI) and a pars distalis (PD), in turn divided into a rostral pars
245 distalis (RPD) and proximal pars distalis (PPD) [Fig. 1(a)]. The RPD was located on the anterior
246 ventral side of the pituitary, while the PPD occupied the central region. The PI extended in the
247 posterior side of the gland and included about half of the pituitary area at the mid-sagittal section. A
248 narrow irregular cavity, lined by a monolayered epithelium over a neurohypophyseal tissue,
249 appeared in PI [Fig. 1(b)]. Several NH processes penetrated the three AD regions.
250 The RPD was characterized by the presence of cells immunoreactive to PRL (ir-PRL) and to ACTH
251 (ir-ACTH). The ir-PRL cells, strongly immunoreactive and located in RPD, were clearly organized
252 in follicles with an enlarged lumen [Fig. 1(c)] composed of tall columnar cells with abundant
253 cytoplasm and relatively large nuclei located in the opposite pole to the non-immunoreactive lumen.

The polyhedral somatotropic cells, immunoreactive to GH (ir-GH), were restricted to PPD [Fig. 1(d)], where they represented the main component, arranged in irregular rows surrounding the neural tissue or in small groups in the central and caudal PPD. Some isolated ir-GH cells on the boundary between PPD and RPD appeared more intensely immunostained. None of the ir-GH cells reacted with the antiserum to human growth hormone at any dilution tested.

Among ir-GH cells, discrete groups of oval or elongated cells immunoreactive to TSH (ir-TSH) were detected in PPD [Fig. 1(e)]. They were arranged in discontinuous strands and small groups, often bordering the penetrating neurohypophyseal tissue, and were more numerous in the dorsal area of the gland. Some isolated and intensely immunostained ir-TSH cells were also found in RPD, adjacent to ir-PRL follicles and sometimes among ir-PRL cells in the follicle walls.

Many cells located in PPD simultaneously immunoreacted to human FSH (ir-FSH) and croaker GtH (ir-GtH) antisera [Fig. 1(f)], but no immunoreaction was detected with antiserum against chum salmon gonadotropin I β-subunit. These cells, isolated or in small groups, and roundish or elongated with a central nucleus, were scattered within ir-GH cords and usually found in central-anterior PPD. A few isolated ir-FSH cells, strongly immunoreactive, were also located in the same PPD region on the boundary with RPD.

Cells immunoreactive to chum salmon GtH II (ir-GtH II), isolated or in small groups, were found intermingled among ir-GH, ir-TSH and ir-FSH cells in the central anterior PPD [Fig. 1(g)]. Some roundish ir-GtH II cells appeared more intensely immunostained.

Based on all observations, the ir-FSH cells were identified as GtH I: these cells were much more numerous in comparison to ir-GtH II ones and had a different distribution.

Several slightly stained ir-ACTH cells were distributed in RPD in small islets close to ir-PRL follicles or in short cords near the neural processes [Fig. 1(h)], or in the PPD side adjacent to RPD, either isolated or in small clusters. These cells were not immunoreactive to melanocyte stimulating hormone α-subunit (ir-MSH), thus were considered adrenocorticotrophic ones. A more intense immunoreactivity by ir-ACTH cells was observed in the outer border of PI and in an irregular layer

lining NH, which deeply branched in central PI [Fig. 1(i)], in the same positions where MSH cells were expected to be found. Actually, many fusiform ir-MSH cells were found in PI in contact with neurohypophyseal tissue and intermingled with non-immunoreactive cells [Fig. 1(j)], but they did not completely overlap with ir-ACTH ones.

Most PI cells were immunoreactive to chum salmon N-acetyl-β-endorphin II (ir-END) [Fig. 1(k)]. The ir-END cells most intensely stained were located in the external edge of the region and along the NH border, isolated or in small clusters. The other slightly stained ir-END cells, organized in rows or in irregular thick layers, were found among PI folds protruding in the neurohypophyseal tissue, mostly on the dorsal side.

Several cells strongly immunoreactive to red drum α-somatolactin (ir-SL) were found in close proximity to NH interdigitations, mainly on the PI dorsal area [Fig. 1(l)]. Other ir-SL cells, either strongly or slightly stained, were scattered in ventral anterior PI. No cross-reaction between ir-SL cells and ir-GH or ir-PRL ones was ever observed.

Larval and juvenile pituitary

In newly hatched larvae (about 24 hours post-fertilization) and 3.5 ± 0.1 mm long), the brain arched around the ventral part of the body and small ventricles surrounded by actively proliferating cells could be recognized. A large yolk sac was visible below the abdomen. The pituitary gland anlage was first recognized in the ventral region of the third ventricle, between the diencephalon and the primitive oral epithelium [Fig. 2(a)]. It appeared as a small ovoid mass of relatively large, roundish cells containing a round euchromatic nucleus with one or two nucleoli: no immunoreactivity was detected in any of these cells.

Larvae at 0.5 dah were 3.7 ± 0.2 mm long and showed only the mandibular arch of the aorta. The mouth was on the lower side and unopened. At this stage, the pituitary consisted of a small ovoid cell mass, adhering to the diencephalon floor and near the chordal cartilage: the oral cavity epithelium was in contact with the ventral side of the gland. Only few ir-PRL cells, slightly stained

306 and located in the central region, could be detected: they had rather large nuclei surrounded by a
307 thin cytoplasmic layer [Fig. 2(b)].
308 One day after hatching the larvae had a well-developed yolk sac and were 3.9 ± 0.3 mm long. The
309 mouth was still unopened and the blood circulation was well established; cell divisions were
310 frequently observed in the pituitary. The ir-PRL cells were more numerous and more intensely
311 stained, but no other immunoreactivity was detected [Fig. 2(c)].
312 In larvae about 1.5 dah (4.3 ± 0.3 mm long) the pituitary, more roundish and located in front of the
313 chord, emerged from the diencephalon floor, separated by a thin connective lamina. On its ventral
314 side the gland was attached to the oral epithelium by a cellular connection [Fig. 2(d)]. A few ir-SL
315 cells (one per section), weakly immunoreactive, appeared in the dorsal-central region of the gland
316 [Fig. 2(e)]. A very weak GH immunoreactivity was also detected for the first time in a few cells of
317 the posterior-central region [Fig. 2(f)].
318 The larvae at 2 dah (4.9 ± 0.4 mm long) still exhibited a yolk sac, although smaller than in the
319 previous stage: the mouth was open, the jaws were mobile and the digestive tract was in place. The
320 pituitary slightly increased in size but its shape was more elongated. The NH was not yet
321 recognizable and in AD there were no clear boundaries between PI and PD: all cells had a similar
322 structure. A few ir-END cells, intensely stained, rather large and roundish, appeared for the first
323 time in the posterior part of the gland, although no ir-ACTH or ir-MSH cells were detectable [Fig.
324 2(g)]. The ir-PRL cells began to increase in number and size, forming a compact group in the
325 anterior region: some of them were also found in the posterior region [Fig. 2(h)]. Some ir-GH cells,
326 intensely immunostained, were visible in the central region of the gland [Fig. 2(i)]. The ir-SL cells,
327 located in the caudal region, were more numerous and showed a more intense immunoreactivity
328 than in the previous stage [Fig. 2(j)].
329 In larvae 2.5 dah (5.3 ± 0.4 mm long) other three types of immunoreactive cells (ir-TSH, ir-ACTH
330 and ir-MSH) simultaneously appeared in the gland. The few isolated ir-TSH cells were located in
331 the central-dorsal region [Fig. 2(k)]. Some ir-ACTH cells were identifiable in the anterior and

posterior regions [Fig. 2(l)]: the latter ones, located in the presumptive PI, also reacted to human α-MSH antiserum [Fig. 2(m)]. The cells in the anterior region were thus considered adrenocorticotropic ones. A few round ir-END cells were scattered in the ventral PI while some ir-SL cells, strongly immunoreactive, were located in the dorsal region. The ir-GH and ir-PRL cells increased in number and immunoreactivity, respectively occupying most of the posterior and anterior region of the presumptive PD.

Larvae about 5 dah (6.6 ± 0.4 mm long) had absorbed much of the yolk sac and initiated exogenous feeding. The open mouth was terminally located and the swimming bladder was filled with air. The pituitary, partially protruding from the diencephalon, was flat and stretched in anterior-posterior direction: its dorsal side was connected to the diencephalon by nervous fibres surrounded by connective tissue: these fibres represented the first appearance of NH entering the adenohypophyseal tissue by short and thin nerve processes [Fig. 3(a)]. At this stage, a topographic segregation of the immunoreactive cell types was observed along the anterior-posterior axis, thus a boundary between PD and PI could be established. The ir-GH and ir-PRL cells increased both in number and immunostaining intensity and began to form compact groups in PD, respectively in the central [Fig. 3(b)] and ventral anterior [Fig. 3(c)] parts. The ir-TSH cells were more numerous and intensely stained, mostly distributed in the central PD region. The ir-ACTH cells were detected in PD, mostly in the ventral anterior region and some in the dorsal one, and in PI along the rear edge [Fig. 3(d)]. In PI some of these cells were also immunoreactive to human α-MSH (ir-MSH) and some others to chum salmon N-acetyl-β-endorphin II (ir-END) [Fig. 3(e)]. The central area of PI was occupied by ir-SL cells, located near the thin neurohypophyseal branches [Fig. 3(f)].

In larvae from 14 (about 7.9 ± 0.5 mm long) to 21 (about 9.1 ± 0.6 mm long) dah the pituitary slightly increased but maintained its flat shape and its close contact with the floor diencephalon. In the dorsal region, the NH was slightly enlarged and had thin nerve processes penetrating into adenohypophyseal tissue. All secretory cell types characterizing the previous developmental stages were detectable, although in variable number, size and immunoreactivity.

In larvae 28 dah (about 10.3 ± 0.9 mm long) the pituitary, enlarged and thicker, showed some blood capillaries in the peripheral region and was ventrally limited by a thin cartilage plaque. The NH, clearly distinguishable in the dorsal region, penetrated PI and PD with thin branches [Fig. 3(g)]. The PD, thicker and slightly elongated, had no clear separation between the proximal region (PPD) and the rostral one (RPD). A few ir-FSH cells, strongly immunoreactive, first appeared in the central region of PD [Fig. 3(h)] interspersed among ir-GH and near ir-TSH cells [Fig. 3(i)]. As in the adults, the ir-FSH cells also reacted with antisera against GtH but not with those against GtH II, therefore they were identified as GtH I.

The ir-TSH cells, more numerous and irregular, had slightly immunostained cytoplasm and a rather small nucleus [Fig. 3(i)]. The ir-PRL cells, strongly immunoreactive, occupied most of the anterior PD. All other adenohypophyseal cell types observed in the previous stages gradually increased in number, size and staining intensity, but maintained their distribution.

In juveniles 60 dah (14.8 ± 1.3 mm long) the pituitary increased in size and maintained its shape and close contact with the diencephalon. The gland was connected to the brain floor by a short neurohypophyseal stalk and penetrated by extended NH branches [Fig. 3(j)]. Blood vessels were visible in ventral PI and posterior PD among cell cords. Many large ir-GH cells with hypertrophic cytoplasm, organized in rows, were present in the medial and dorsal regions of PD [Fig. 3(k)]. Other ir-GH cells, smaller and polyhedric, were visible in the outermost part of anterior PD. Most strongly immunoreactive ir-PRL cells were located in a compact group in the ventral PD, under the ir-GH cells [Fig. 3(l)]. The ir-ACTH cells, either isolated or in short rows, were mostly distributed among ir-GH and ir-PRL cells in PD, but some were visible in PI. Some ir-TSH and ir-FSH (GtH I) cells (the latter ones isolated and polymorphic) were scattered in PD. The ir-MSH and ir-END cells occupied the posterior region of PI, mostly along the external border: the two types of immunoreactive cells only partially overlapped and the ir-END cells were fewer in number. The inner part of PI was mostly occupied by ir-SL cells located near the neurohypophyseal branches.

In juveniles 90 dah (17.8 ± 1.7 mm long) the pituitary significantly increased in size, enlarging along the dorsal-ventral axis and leaning backwards. A thick infundibular stalk separated the gland from the ventral surface of diencephalon and connected it to the hypothalamus [Fig. 4(a-e)]. The gland was deeply penetrated by neurohypophyseal branches and some dilated capillaries appeared among rows of cell clusters. The central PD region was almost entirely composed of a compact mass of roundish ir-GH cells [Fig. 4(a)]. Rows of ir-PRL cells occupied the ventral PD [Fig. 4(b)] and some ir-ACTH cells were found in ventral anterior PD and along the outer PI border [Fig. 4(c)]. Some ir-TSH were interspersed among ir-GH cells in the central PD, where some ir-FSH (GtH I) cells were also detected [Fig. 4(d)]. In the same region, a few ir-GtH II cells, small and irregular, appeared for the first time, close to the ir-GH cells and not overlapping the ir-FSH cells [Fig. 4(e)]. Some ir-MSH, slightly immunoreactive, and ir-END cells, intensely stained, were detected along the PI border. The more numerous ir-SL cells were extensively distributed in PI around the neurohypophyseal tissue, intermingled with some non ir-SL cells.

In juveniles 110 dah (20.1 ± 1.5 mm long) the pituitary further enlarged and the central PD was occupied by the intensely stained and more numerous ir-GH cells. The ir-FSH (GtH I) and ir-GtH II cells, together with all other secretory cell types, increased their number in their respective pituitary regions.

In juveniles 130 dah (22.5 ± 1.7 mm long) the pituitary reached about 240 μm in length, 155 μm in width and 120 μm in thickness. A thick neurohypophyseal stalk connected the gland to the hypothalamus. The NH extended along the dorsal PD and extensively branched inside the gland upon reaching the PI [Fig. 4(f)]. Even at this late stage, no boundary was visible between RPD and PPD. The ir-GH cells, more scattered at this stage, were mostly located in two separate groups in the dorsal-central and anterior-ventral PD [Fig. 4(g)]. The ir-PRL cells were found near the ventral border of PD, posterior to ir-GH cells, and in the central PD [Fig. 4(h)]: they were not yet organized in follicles as in the adult. The numerous ir-ACTH cells were scattered in PD, intermingled with ir-PRL cells, and along the outer PI border, either isolated or in small clusters [Fig. 4(i)]. The ir-TSH

409 cells were interspersed in PD, some in contact with neurohypophyseal branches. A higher number
410 of ir-FSH (GtH I) cells, isolated or in small groups, were detected among ir-GH ones in PD, mostly
411 along the dorsal region. Some ir-GtH II cells, smaller and sometimes strongly immunoreactive,
412 were distributed along the dorsal PD [Fig. 4(j)]. Some strongly immunoreactive ir-MSH [Fig. 4(k)]
413 and ir-END cells [Fig. 4(l)] were located near the outer PI border. Adjacent to the intensely stained
414 cells, other ir-END cells appeared slightly immunostained, probably because of cross-reactivity
415 with ir-MSH cells. The ir-SL cells were found in the central PI, close to neurohypophyseal
416 branches. No hypophyseal cavity such as that observed in the adult was yet found at this stage.
417
418 Morphometric analyses
419 The total volume of the pituitary from larvae 0.5 dah to juveniles 130 dah increased about 67.2 fold
420 (44.2 vs. 2969.3 x 10^3 μm^3). The differences in morphometric parameters of all immunoreactive
421 cells were difficult to assess in relation to developmental stages, but since ir-PRL and ir-GH cells
422 were mostly arranged in compact groups it was possible to measure the volume of cell masses from
423 immunostained serial paraffin sections.
424 The volume of ir-PRL and ir-GH cell masses from larvae 2.5 dah to juveniles 130 dah respectively
425 increased 54.6-fold (7.1 vs. 388.2 x 10^3 μm^3) and 93.1-fold (6.5 vs. 605.8 x 10^3 μm^3).
426 The changes of potential indexes of PRL and GH production, expressed as percentage of volume of
427 ir-PRL and ir-GH cell masses over the whole pituitary volume (respectively, %PRL and %GH),
428 measured during the examined developmental stages, are reported in Table I. The %PRL
429 continuously and significantly ($P < 0.05$) increased, reaching a peak in larvae 28 dah, then sharply
430 decreased, remaining low until juveniles 130 dah. The %GH increased until juveniles 60 dah, with
431 significant ($P < 0.05$) differences from day 14 onwards, and decreased thereafter.
432 In ir-PRL cells the areas of cells and nuclei progressively increased along stages, reaching the
433 maximum value in juveniles 90 dah, and then decreasing in juveniles 110 and 130 dah, respectively
434 to values of juveniles 60 dah for cell areas and of larvae 28 dah for nucleus areas. The ratio of cell

435 area to nucleus area remained more or less constant until larvae 28 dah and progressively increased,
436 reaching the maximum value in juveniles 130 dah. The cell and nucleus areas, and the ratio of cell
437 area to nucleus area, were significantly higher in juveniles than in larvae ($P < 0.05$) [Fig. 5 (a), (c),
438 (e)]. In ir-GH cells the areas of cells and nuclei also increased, reaching the maximum values
439 respectively in juveniles 110 dah and 90 dah. Cell and nucleus areas slightly decreased thereafter
440 but never returned to the values detected in larvae 28 dah. The ratio of cell area to nucleus area was
441 more or less constant along larval stages but progressively increased in juveniles, reaching a
442 maximum in juveniles 130 dah. As for ir-PRL cells, also for ir-GH cells the cell and nucleus areas
443 and the ratios of cell area to nucleus area were always significantly higher in juveniles than in
444 larvae ($P < 0.05$) [Fig. 5 (b), (d), (f)].

445

446 **DISCUSSION**

447 The distribution of different adenohypophyseal cell types are reported for the first time in adults of
448 the cave fish *Phreathychtys andruzzii*, together with the pituitary development and the
449 chronological differentiation of hormonal cells in larvae and juveniles.
450 In *P. andruzzii* adults the pituitary was divided in three regions and the distribution of
451 adenohypophyseal cell types appeared in good agreement with that of other teleosts (Agulleiro *et*
452 *al.*, 2006; Borella *et al.*, 2009). Prolactin (PRL) and adenocorticotropic (ACTH) cells were
453 identified in RPD and somatotropic (STH), thyrotropic (TSH) and gonadotropic (GtH I and GtH II)
454 cells in PPD. Somatolactin (SL), melanotropic (MSH) and endorphin (END) cells were localized in
455 PI. The adult pituitary was characterized by an hypophyseal cavity burrowed in the
456 neurohypophyseal tissue and lined by a non-immunoreactive cubic cell monolayer. This cavity
457 should be identified as an extension of the third ventricle of the brain into the infundibulum, the
458 infundibular recess. In teleosts the presence of a fully developed infundibular recess was previously
459 reported in cichlids (Pandolfi *et al.* 2001).

460 The ontogeny of pituitary cells has been studied in several teleost species, but with different
461 duration of embryonic and post-embryonic stages and different time course of appearance of each
462 endocrine cell types (Cambré *et al.*, 1990; de Jesus *et al.*, 2014).
463 In *P. andruzzii* all hormone-producing cells were identified in the adenohypophysis from juveniles
464 90 dah onwards, by immunocytochemistry with twelve different antisera. The pituitary anlage was
465 detected in newly hatched larvae as a small cell group, adhering to the ventral side of the
466 diencephalon and attached to the oral epithelium by a cellular connection, which disappeared after
467 1.5 dah. This cellular connection could correspond to the primordium of the buccohypophyseal
468 canal previously described in Clupeiformes (Laiz-Carrión *et al.* 2003).
469 The gland increased in size in larvae 2.5 dah, but a neurohypophysis was not yet identifiable,
470 suggesting the absence at this stage of a functional hypothalamic-pituitary system for
471 neuroendocrine control of early larval functions, as in the golden dorado *Salminus brasiliensis*
472 (Characiformes) (de Jesus *et al.*, 2014). A neurohypophysis with short and thin branches extending
473 towards the posterior region was first observed in larvae 5 dah in the dorsal region of the gland. A
474 similar condition was reported in *S. brasiliensis*: the first neurohypophysis anlage was visible in
475 larvae 3 dah and it expanded by branching at 5 dah (de Jesus *et al.*, 2014). In other Cypriniformes
476 the neurohypophysis appeared in larvae about 2 dah (96 hours post fertilization) of the zebrafish
477 *Danio rerio* (Chapman *et al.*, 2005) and in larvae 2.5 dah (3.5 days post fertilization) of the silver
478 carp *Hypophthalmichthys molitrix* (Burlakov *et al.*, 2006).
479 In *P. andruzzii* the neurohypophysis developed into a long pituitary stalk and extensively branched
480 into the adenohypophysis only in juveniles 90 dah, when the gland clearly detached from the
481 diencephalon. From 110 to 130 dah the pituitary stalk increased in size and the neurohypophyseal
482 branches became thicker, but the infundibular recess characterizing the adult gland was still absent
483 at these late stages.
484 The somatolactin, prolactin and growth hormones all belong to the GH/PRL gene family and their
485 genes are thought to have evolved from a common ancestral gene by duplication and divergence

(Rand-Weaver & Kawauchi, 1993). In *P. andruzzii* these secretory cells specifically reacted only with the corresponding antisera and showed no cross-reactivity. In adults most of the RPD was occupied by PRL cells, organized in follicles as in *Oncorhynchus keta* (Salmoniformes) (Naito *et al.*, 1993) and in the Japanese eel *Anguilla japonica* (Anguilliformes) (Arakawa *et al.*, 1992) and the European eel *A. anguilla* (Grandi *et al.*, 2003). However, in *P. andruzzii* no organization in follicles was ever observed in any juvenile stage examined.

Some weakly stained ir-PRL cells were first observed in the gland centre in larvae 0.5 dah: the immunoreactivity intensified at 1 dah and these cells later became a significant component of the anterior ventral PD, probably reflecting the PRL cell migration in progressively anterior positions. This hypothesis is supported by the presence of some ir-PRL cells in posterior PD during early larval stages. In teleosts PRL plays an osmoregulatory role during the life cycle of freshwater, brackish and euryhaline species (Manzon, 2002), being essential for ion uptake and reduction of ion and water permeability (Sakamoto & McCormick, 2006). An osmoregolatory role has been shown in embryo and larval stages of the euryhaline black sea bream, *Acanthopagrus schlegeli* (Perciformes) (Kimura & Tanaka, 1991). The early appearance of PRL in larvae 0.5 dah of *P. andruzzii* is consistent with its key role in adaptation to hypoosmotic environments. The PRL is also involved in larval growth and differentiation (Majumdar & Elsholtz, 1994) and through cortisol synthesis may stimulate intestinal cell proliferation and apoptosis during salinity acclimatization (Sakamoto &McCormick, 2006). Besides its osmoregulatory role, in *P. andruzzii* the early appearance of ir-PRL cells suggests a role in eye degeneration. The eyes attain their maximum differentiation 36 hours after egg laying, that is 0.5 dah according to the time scale of the present study: they rapidly degenerate through genetically controlled apoptosis and about a month later only a rudimentary cyst is left (Berti *et al.*, 2001). Interestingly, the ir-PRL cells appeared 0.5 dah, simultaneous to the first signs of eye degeneration (Berti *et al.*, 2001) and the potential index of hormone production, calculated as percentage of the pituitary volume occupied by ir-PRL (%PRL),

511 increased from 1.5 dah onwards, reaching a peak in larvae 28 dah, apparently synchronous with eye
512 degeneration. This interesting point is presently under investigation for further details.
513 From larvae 28 dah to juveniles 130 dah the total volume of ir-PRL cell mass increased although
514 there was a percentage decrease: this could be explained by the enlargement of the total volume of
515 the pituitary due to the rapid increase of activity in other hormone-producing cells. Moreover, the
516 ir-PRL cells were significantly larger in juveniles than in larvae, and the ratio of cell area to nucleus
517 area was the highest in juveniles 130 dah. These results suggest an increased synthetic activity and
518 hormone release by ir-PRL cells in juvenile stages, related to the role of this hormone in freshwater
519 adaptation processes. In the cichlid fish *Cichlasoma dimerus* (Perciformes) the activity of PRL cells
520 was significantly higher in individuals exposed to long photoperiod then in individuals exposed to a
521 short one (Fiszbein *et al.*, 2010). However, in the adult cavefish (raised in total darkness as it occurs
522 in the wild) the PRL cells were hypertrophic and strongly immunoreactive, suggesting an intense
523 PRL production unrelated to photoperiod. This interesting point requires further investigation.
524 In *P. andruzzi* the ir-GH cells were specifically recognized by chum salmon GH antiserum and
525 undetected by human GH antiserum, as found in most teleosts (Agulleiro *et al.*, 2006). However, in
526 *Barbus barbus* (Cypriniformes) the use of antisera to porcine GH resulted in selective
527 immunostaining of GH cells (Toubeau e*t al.,* 1991); as previously suggested (Grandi *et al.*, 2014)
528 this variable immunoreactivity could be due the difference in GH aminoacid sequence between
529 teleosts and mammals. In adults the ir-GH cells, either clustered or isolated, were restricted to PPD
530 as in other teleosts (Agulleiro *et al.*, 2006). The ir-GH cells appeared simultaneously to ir-SL cells:
531 they were scanty and weakly stained at 1.5 dah, but more numerous and intensely stained at 2.5 dah.
532 However, in larvae 5dah they become a significant component of the central PD and showed a
533 strong immunoreactivity, suggesting a high hormone production. The early and almost
534 simultaneous appearance of ir-GH, ir-PRL and ir-SL cells, together with the presence of some ir-
535 PRL cells in the posterior region of the gland suggests a common hormone origin from a primordial
536 stem cell, as hypothesized in the sea bream *Sparus aurata* (Perciformes) (Villaplana *et al.*, 1997). In

537 some freshwater and brackish water teleosts the ir-GH cells appeared before hatching (Naito *et al.*,
538 1993; Saga *et al.*, 1993, 1999), and in others around or immediately after hatching. For example,
539 they appeared within 1 dah in the African catfish *Clarias gariepinus* (Siluriformes) (Volckaert *et*
540 *al.*, 1999), 1.5 dah in *S. brasiliensis* (de Jesus *et al.*, 2014), and 2 dah in the Mozambique tilapia
541 *Oreochromis mossambicus* (Perciformes) (Parhar, 1997), in *C. dimerus (*Pandolfi *et al.*, 2001) and
542 in *D. rerio* (Herzog *et al.*, 2003). In some marine teleosts the ir-GH cells were detected at hatching
543 or upon yolk absorption (Cambré *et al.*, 1990; Power & Canario, 1992; Tanaka *et al.,* 1995; Laiz-
544 Carrión *et al.*, 2003). In *P. andruzzii* the %GH increased from larvae 2.5 dah to juveniles 60 dah
545 and then decreased, in a similar way to %PRL. Since the main role of GH is to promote body
546 growth, it is possible that the growing potential in *P. andruzzii* is higher in early stages then in later
547 ones. This point requires further investigation because no data on body growth are presently
548 available in this species. In ir-GH cells the ratio of cell area to nucleus area was the highest in
549 juveniles 130 dah. In teleosts GH is known to play a role in osmoregulation (Sakamoto &
550 McCormick, 2006), energy mobilization, immune response and reproduction (Björnsson *et al.*,
551 2002). Similar roles could also be hypothesized in *P. andruzzii* juveniles.
552 In *P. andruzzi* adults, the ir-SL cells, intermingled with ir-MSH and ir-END cells were scattered in
553 the central PI, around the interdigitating branches of the neurohypophysis, as described in other
554 teleosts, (Agulleiro *et al.*, 2006). In *P. andruzzii* the weakly stained ir-SL cells first appeared at 1.5
555 dah and became strongly immunoreactive at 2.5 dah; later they appeared located near the
556 neurohypophyseal branches intermingled with ir-MSH cells. A similar localization was observed in
557 larvae and juveniles of other teleosts (Villaplana *et al.*, 1997; Saga *et al.,* 1999; Pandolfi *et al.,*
558 2001). The ir-SL cells have been reported in teleosts from 14 days before hatching to 23 dah (de
559 Jesus *et al.*, 2014). In *P. andruzzii* the timing of the appearance of SL cells corresponds to what
560 observed in *S. brasiliensis* (de Jesus *et al.*, 2014), while in *D. rerio* the SL cells appeared at the
561 embryo stage 30 hours after fertilization (Herzog *et al.*, 2003). Somatolactin is the last pituitary
562 hormone identified within the GH/PRL family (Rand-Weaver & Kawauchi, 1993) and in teleosts its

563 synthesis and secretion have been reported in calcium regulation, acid-base balance, phosphate and
564 fat metabolism (Company *et al.*, 2001), reproductive physiology, stress response (Rand-Weaver *et*
565 *al.*, 1993), yolk reabsorption and reserve tissue mobilization (Villaplana *et al.*, 1997). Somatolactin
566 has been suggested to play a role in embryo and larval development (Majumdar & Elsholtz, 1994;
567 Laiz-Carrión *et al.*, 2003): the early appearance of ir-SL cells hints to a similar role in the cave fish.
568 In our study, the ir-TSH cells were identified by antiserum against human β-TSH and were clearly
569 separated from GtH cells in central dorsal PPD, as in other teleosts (Agulleiro *et al.*, 2006). Some
570 ir-TSH cells were also found in RPD near the ir-PRL follicles, as previously reported in the grass
571 carp *Ctenopharyngodon idella* (Cypriniformes) (Grandi et al., 2014), in *A. japonica* (Ueda *et al.*,
572 1983) and in *A. anguilla* (Grandi *et al.*, 2003). In the cave fish larvae the weakly immunoreactive ir-
573 TSH cells were detected for the first time at 2.5 dah and later they slightly increased in number and
574 immunoreactivity. There are few studies on ontogenesis of ir-TSH cells in teleosts: their first
575 appearance is highly variable, from 14 days before hatching to 50 dah (de Jesus *et al.*, 2014). In *D.*
576 *rerio* embryos, ir-TSH were observed 42 hours after fertilization (Herzog *et al.*, 2003). The ir-TSH
577 cells act during embryonic and larval development, up to organogenesis, through thyroid hormones
578 (Brown & Bern, 1989) and in Pleuronectiformes play a relevant role during inshore migration and
579 metamorphosis (Einarsdóttir *et al.*, 2006). The data on TSH cells in the cave fish suggest that the
580 pituitary-thyroid axis plays a key role in the first larval stages and in juvenile development.
581 In adult *P. andruzzii* the presence of two immunologically different populations of GtH cells
582 suggests the existence of two types of gonadotropins (GtH I and GtH II), as in other teleost species
583 (Swanson *et al.*, 1991). The GtH I cells were immunodetected in the cave fish by human β-FSH and
584 croaker GtH antisera but not by chum salmon β-GtH I antiserum. A high divergence in amino acid
585 sequence of the β subunit of GtH I, previously reported in teleosts (Kawauchi *et al.*, 1989), could
586 explain the lack of immunoreactivity in the cave fish.
587 The distribution and localization of GtH I and ir-GtH II cells were similar to that of other teleosts
588 (Agulleiro *et al.*, 2006). The ir-FSH (GtH I) cells were mostly found in PPD, but some were near

589 PRL follicles at the boundary between RPD and PPD. The ir-GtH II cells were generally less
590 numerous than GtH I ones, but mostly gathered in dorsal anterior PPD where GtH I cells were
591 scarce. It is difficult to ascertain whether these cells separately synthesize in adults the two
592 gonadotropins, both gonadotropins simultaneously or the two hormones at different time intervals:
593 all three conditions have been found in teleosts (Nozaki *et al.*, 1990; Miranda *et al.*, 2001).
594 In *P andruzzi* the GtH I cells first appeared in larvae 28 dah, thus their activity preceded that of ir-
595 GtH II cells, which appeared in juveniles 90 dah. Preliminary data on cave fish gonad development
596 suggest that the appearance of GtH I and ir-GtH II cells respectively mark the onset of proliferation
597 of primordial germ cells within the gonad and the first morphological evidence of gonad sex
598 differentiation. A similar condition was observed in *C. idella*, in which the ir-FSH cells were
599 detected during the initial developmental stages of the gonad and ir-GtH II only at the onset of sex
600 differentiation (Grandi *et al.*, 2014).) In the mummichog *Fundulus heteroclitus*
601 (Cyprinodontiformes) the ir-FSH cells appeared 1-2 weeks after hatching, about at the time of
602 morphological sex differentiation, and the cells immunoreactive to luteinizing hormone (LH),
603 corresponding to ir-GtH II cells, appeared 6-12 weeks after hatching, when gonad differentiation
604 was completed (Shimizu *et al.*, 2008). In this species GtH I was considered involved in sex and
605 gonad differentiation and both gonadotropins in vitellogenesis and active spermatogenesis (Shimizu
606 *et al.*, 2008). In *D. rerio* the ir-FSH cells also appeared before LH cells (Herzog *et al.*, 2003), but in
607 the pejerrey *Odonthestes bonariensis* (Atheriniformes) the ir-GtH II cells appeared before ir-GtH I
608 ones and both cell types were detected before the onset of gonad differentiation (Miranda *et al.*,
609 2001). The functional role of the two gonadotropins in gonad differentiation and maturation is still
610 highly debated in teleosts (Levavi-Sivan *et al.*, 2010).
611 The ACTH, α-MSH, β-endorphin and β-lipotropic hormones all belong to the family of pro-
612 opiomelanocortin (POMC) hormones, derived from a different processing of the common precursor
613 molecule, POMC. The cross-reactivity of ir-ACTH and ir-MSH cells may be due to the sequence
614 identity of the first 13 aminoacids in α-MSH and ACTH (Dores & Baron, 2011). As observed in

615 other teleosts (Agulleiro *et al.*, 2006), a combined use of human ACTH (1-24) and MSH antisera
616 allowed to identify the two cell types in *P. andruzzii*.
617 In the adult cave fish the ir-ACTH cells, weakly immunoreactive and arranged in rows, were
618 observed in the RPD around the prolactin follicles, close to neurohypophyseal branches, and
619 isolated or clustered in PPD. This arrangement is uncommon among teleosts, in which ACTH cells
620 typically form a palisade border in RPD between the neurohypophysis and the ir-PRL cells
621 (Agulleiro et al., 2006). The ir-ACTH cells appeared for the first time 2.5 dah in the anterior and
622 posterior part of the pituitary in cave fish larvae. In juvenile stages the ir-ACTH cell staining was
623 intense: the weak immunoreaction in adults could therefore indicate a reduced hormone storage
624 related to high release because of stress condition of adults in laboratory rearing, rather than to a
625 reduced production. In teleosts this hormone regulates the synthesis and release of cortisol by the
626 interrenal glands and is involved in several physiological processes, including stress response,
627 adaptation to hypoosmotic environments, metabolism and immunocompetence (Stouthart *et al.*,
628 1998).
629 In adult *P. andruzzii* the ir-MSH cells were largely distributed in PI, in contact with
630 neurohypophyseal tissue and intermingled with ir-SL cells: such regular distribution was reported in
631 most teleosts, either larvae (de Jesus et al., 2014) or adults (Agulleiro *et al.*, 2006). In the cave fish
632 the weakly stained ir-MSH cells first appeared at 2.5 dah in the posterior part of the gland,
633 simultaneously to ir-ACTH ones, suggesting a synchronized activation of these two hormones as in
634 other teleosts (Cambré *et al.*, 1990; Naito *et al.*, 1993; Saga *et al.*, 1999; de Jesus *et al.*, 2014), and a
635 common developing mechanism in two endocrine cell populations belonging to the same lineage. In
636 the cave fish juveniles, the ir-MSH cells increased in number and were always confined to PI: their
637 distribution only partially overlapped that of ir-ACTH ones. The MSH is involved in
638 melanogenesis, colour adaptation and stimulation of ACTH release under stress conditions (Lamers
639 *et al.*, 1992). Since the cave fish are totally depigmented, it is likely that the role of MSH is to
640 regulate ACTH release under stress. The simultaneous appearance of ir-ACTH and ir-MSH cells in

641 larvae 2.5 dah and the increase in number and immunoreactivity of these cells in the next stages
642 also suggests a functional pituitary-interrenal-axis related to adaptation to hypoosmotic environment
643 during early larval development, as found in the common carp, *Cyprinus carpio* (Cypriniformes), at
644 hatching (Stouthart *et al*., 1998).
645 In the adult cavefish the ir-END cells were localized in the PI region along the external edge and the
646 neurohypophysis border. The weak immunoreactivity to chum salmon N-acetyl-β-endorphin II
647 detected in cell rows inside PI folds could be due to a cross reactivity of this antiserum with
648 precursors and/or products containing the β-endorphin sequence in ir-ACTH and ir-MSH cells.
649 The strongly immunostained ir-END cells were observed for the first time in larvae 2 dah in the
650 posterior region, where at a later stage the ir-MSH cells also appeared, and increased in number
651 during development. In all stages examined, the ir-END and ir-MSH cells were restricted to the
652 same region of pituitary, but it was difficult to establish whether the immunoreactivity to END and
653 MSH was located in different cells or there was partial or complete co-localization. A complete co-
654 localization between ir-END and ir-MSH cells was reported in *C. carpio* (van den Burg *et al.*, 2001)
655 and in the Atlantic halibut *Hippoglossus hippoglossus* (Pleuronectiformes) (Weltzein *et al.*, 2003).
656 However, recent immunocytochemical and ultrastructural studies on the pituitary of juveniles of *C.*
657 *idella* showed that there were cells interspersed among ir-MSH and ir-SL cells which synthesized
658 only N-acetylated β-endorphin II (Grandi *et al.*, 2014).
659 The literature on ir-END cell ontogenesis in fish is very limited. A study on the Adriatic sturgeon
660 *Acipenser naccarii* (Acipenseriformes) reported that the first appearance of cells immunoreactive to
661 chum salmon N-acetylated β-endorphin II occurred at 4 dah and in all larval and juvenile stages the
662 same immunoreactivity appeared in ir-MSH cells located only in PI (Grandi & Chicca, 2004). In
663 teleosts the only data available concerned *C. idella*, where the ir-END cells were identified in the PI
664 region in juveniles before gonad sex differentiation. After gonad differentiation, these cells were
665 detected in higher number in the same region (Grandi *et al.*, 2014). In the cave fish the ir-END cells
666 are detected at 2 dah while ir-ACTH and ir-MSH cells at 2.5 dah: a possible explanation for the lack

667 of immunoreactivity for ACTH and MSH at 2 dah is the low aminoacid sequence identity, since the
668 END antiserum was from *O. keta* while the other two were human ones.
669 Besides their opioid activity, endorphins in vertebrates are involved in stress response. In *C. carpio*
670 the corticotropin-releasing factor is known to stimulate ACTH and MSH secretion and induce
671 endorphin release under stress condition (Van den Burg *et al.*, 2001). In *P. andruzzii* the high
672 number of ir-END cells in the adult is most likely related to stress conditions due to laboratory
673 rearing.
674 Overall, these results provide for the first time a complete map of all adenohypophyseal cells in a
675 cave fish, useful for further studies on the hypothalamus-pituitary axis. A detailed knowledge of
676 hypothalamic and hypophyseal control of key physiological processes such as growth, reproduction
677 and environmental adaptation in *P. andruzzii* could be a relevant issue in the conservation of this
678 threatened species.
679

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Table I. Average and standard deviation of potential index of PRL and GH production, expressed as percentage of the pituitary volume occupied by ir-PRL and ir-GH cells (respectively, %PRL and %GH) in individuals of *P. andruzzii* of different developmental stages, expressed as days after hatching (dah). From 1.5 to 28 dah, larvae; from 60 to 130 dah, juveniles. For each developmental stage 5 individuals were examined. LT: range of total length. Values followed by different letters are significantly different ($P < 0.05$).

Developmental stage (dah)	LT (mm)	% PRL	% GH
1.5	3.8-4.5	8.51 ± 0.74^{a}	-
2.5	4.9-5.9	10.17 ± 0.43^{b}	9.34 ± 0.44^{a}
5	6.1-7.1	12.44 ± 0.77^{c}	11.21 ± 0.60^{a}
14	7.4-8.7	15.66 ± 0.54^{d}	14.72 ± 0.64^{b}
21	8.0-9.9	17.53 ± 0.64^{e}	18.28 ± 0.68^{c}
28	9.1-11.4	21.23 ± 0.78^{f}	21.75 ± 1.08^{d}
60	12.9-16.2	16.26 ± 0.88^{de}	26.34 ± 1.04^{e}
90	15.3-19.4	14.22 ± 0.91^{cd}	24.15 ± 0.95^{f}
110	17.9-21.4	12.56 ± 0.87^{c}	22.08 ± 0.99^{d}
130	20.1-24.1	13.07 ± 1.31^{c}	20.38 ± 1.41^{d}

FIGURE LEGENDS

Fig. 1. Pituitary gland of adults of *Phreatichthys andruzzii*. (a) Sagittal section of the pituitary in a 57.5-mm adult male showing the rostral pars distalis (RPD), the proximal pars distalis (PPD), the pars intermedia (PI) and the neurohypophysis (Nh) with deep interdigitations protruding into the adenohypophysis. A narrow irregular cavity is present in PI. Herlant's tetrachrome staining. Scale bar = 200 μm. (b) Magnification of Fig. 1(a), showing the monolayered epithelium outlining the irregular cavity within PI (arrow). The neurohypophyseal processes are deeply branched. Scale bar = 70 μm.

[(c)-(l)] Consecutive sagittal sections of the pituitary gland in a 59.5-mm adult male, each one immunostained with a different antiserum. All sections were stained with avidin-biotin-peroxidase complex (ABC), adding nickel chloride to the 3,3'-diaminobenzidine tetrahydrochloride for enhanced immunoreaction. Scale bars = 70 μm. (c) Cells immunoreactive to chum salmon prolactin (ir-PRL) in RPD, organized in follicles. (d) Cells immunoreactive to chum salmon growth hormone (ir-GH) in PPD. (e) Cells immunoreactive to human thyroid stimulating hormone β-subunit (ir-TSH) interspersed between ir-GH cells in PPD and near to PRL follicles in RPD. (f) Cells immunoreactive to human follicle stimulating hormone β-subunit (ir-FSH) in central-anterior PPD. (g) Cells immunoreactive to chum salmon gonadotropin II β-subunit (ir-GtH II) in central-anterior PPD. (h) Cells immunoreactive to human adrenocorticotrophic hormone (1-24) (ir-ACTH) interspersed among ir-PRL follicles in RPD and in small rows near the neurohypophysis branches in PPD. (i) Other ir-ACTH cells along the PI edge and the neurohypophysis (Nh) border. (j) Cells immunoreactive to human melanocyte stimulating hormone α-subunit (ir-MSH) in PI. (k) Cells immunoreactive to chum salmon N-acetyl-β-endorphin II (ir-END) in PI. (l) Cells immunoreactive to red drum somatolactin α-subunit (ir-SL) in PI.

Fig. 2. Sagittal sections of the brain and pituitary in *P. andruzzii* larvae, with the anterior side at right. (a) Newly hatched larva stained with Herlant's tetrachrome. The pituitary anlage (dashed outline) surrounded by a thin layer of connective tissue is located between the diencephalon and the oral epithelium (arrow). Scale bar = 30 μm. (b) Larva 0.5 days after hatching (dah) showing the first appearance of weakly immunoreactive ir-PRL cells in the central region of the pituitary. Scale bar = 30 μm. (c) Larva one day after hatching, showing some intensely stained ir-PRL cells. Scale bar = 30 μm.

(d) Semithin section from a resin included tissue piece of a larva 1.5 dah, stained with methylene blue – azur A. The pituitary, anterior to the notochord (Nc), adheres above to the diencephalon floor (arrowheads) and is connected below to the oral epithelium (arrow). Scale bar = 30 μm. [(e), (f)] Consecutive sections from a larva 1.5 dah. Nc, notochord. Scale bars = 30 μm. (e) First appearance of weakly immunoreactive ir-SL cells (arrow) in the dorsal central region. The inlay shows details of an ir-SL cell in Fig. 2(e). Scale bar = 7.5 μm. (f) First appearance of weakly immunoreactive ir-GH cells (arrow) in the posterior central region. The inlay shows details of an ir-GH cell in Fig. 2(f). Scale bar = 7.5 μm.

[(g)-(j)] Consecutive sections from a larva 2 dah, respectively immunostained with anti-END, anti-PRL, anti-GH and anti-SL. Nc, notochord. Scale bars = 30 μm. (g) First appearance of ir-END cells in the posterior region of pituitary. (h) Some ir-PRL cells clustered in the anterior region and another one isolated in the posterior region. (i) A small group of ir-GH cells in the central region. (j) A small group of ir-SL cells in the posterior-dorsal region.

[(k)-(m)] Consecutive sections from a larva 2.5 dah, respectively showing the first appearance of ir-TSH, ir-ACTH and ir-MSH cells. Scale bars = 30 μm. (k) The ir-TSH cells are interspersed in the central region (arrow). (l) The ir-ACTH cells are found in the anterior and posterior regions (arrows). (m) The ir-MSH cells are located in the posterior region, in the same position of the ir-ACTH cells (arrows).

Fig. 3. Sagittal sections of the brain and pituitary in *P. andruzzii* larvae and juveniles, with the anterior side at right. (a) Larva 5 dah stained with Herlant's tetrachrome. The pituitary, more elongated in the anterior axis, protrudes from the diencephalon and shows a pars intermedia (PI) and a pars distalis (PD). A small neurohypophyseal region (arrow) is found on the dorsal side. Scale bar = 30 μm.

[(b)-(h)] Consecutive sections of a pituitary from another larva 5 dah, respectively immunostained with anti-GH (b), anti-PRL (c), anti-ACTH (d), anti-END (e) and anti-SL (f). All cell types are more numerous and intensely stained. Scale bars = 30 μm.

(g) Larva 28 dah stained with Herlant's tetrachrome. The enlarged pituitary maintains its flat shape and its close contact with the diencephalon. The neurohypophysis (Nh) with short branches appears slightly enlarged. Scale bar = 30 μm.

[(h), (i)] Consecutive sections of the pituitary of the same larva 28 dah, respectively immunostained with anti-FSH (h) and anti-TSH (i). Scale bars = 30 μm. (h) First appearance of ir-FSH cells in the central region of PD. (i) The ir-TSH cells are also located in the central PD, but they do not overlap with ir-FSH cells.

(j) Juvenile 60 dah stained with Herlant's tetrachrome. The pituitary, connected to the brain through a short neurohypophyseal stalk and thin neurohypophyseal branches (Nh), shows a increase of all its components and blood vessels in the ventral-region (arrow). Scale bar = 30 μm.

[(k). (l)] Consecutive sections of the pituitary of the same juvenile 60 dah, respectively immunostained with anti-GH and anti-PRL. Scale bars = 30 μm. (k) The ir-GH cells occupy most of the PD. (l) The ir-PRL cells are located in the ventral anterior PD.

Fig.4**.** Sagittal sections of the brain and pituitary in *P. andruzzii* juveniles, with the anterior side at right. The gland, enlarged along the dorso-ventral axis and clearly separated from the diencephalon,

is connected to the brain by a thick neurohypophyseal stalk emerging from the dorsal-anterior region. [(a)-(e)] Consecutive sections of the pituitary from a juvenile 90 dah, respectively immunostained with anti-GH (a), anti-PRL (b), anti-ACTH (c), anti-FSH (d) and anti-GtH II (e). Scale bars = 50 μm. (a) The ir-GH cells form a compact mass in posterior PD. (b) The ir-PRL cells are located in the ventral PD. (c) The ir-ACTH cells are grouped in the ventral anterior PD and in the outer PI border. (d) The ir-FSH cells are interspersed in the posterior PD, among ir-GH cells. (e) The ir-GtH II cells appear for the first time in the central region of PD.
[(f)-(l)] Consecutive sections of the pituitary from a juvenile 130 dah. The gland greatly enlarges in all its components. Scale bars = 80 μm. (f) Section stained with Herlant's tetrachrome. A small PI, an enlarged PD and blood vessels (arrow) are visible in the gland. No structural differences are detectable in PD between the proximal and the rostral region. The neurohypophysis (Nh) has a thicker neurohypophyseal stalk and processes invading both PD and PI.
[(g)-(l)] Consecutive sections of the pituitary of the same juvenile 130 dah, respectively immunostained with anti-GH (g), anti-PRL (h), anti-ACTH (i), anti-GtH II (j), anti-MSH (k) and anti-END (l). (g) The ir-GH cells are located in two distinct groups in the PD. (h) The ir-PRL cells are arranged in two groups near the ir-GH cells. (i) The numerous ir-ACTH cells are distributed in ventral PD and posterior PI. (j) The few ir-GtH II are mostly located in dorsal PD. [(k), (l)] The ir-MSH and ir-END cells are both located in PI, partially co-localized.

Fig. 5. Average of cell area [(a), (b)], nucleus area [(c), (d)] ($\mu m^2 \pm$S.D.) and ratio of cell area to nucleus area [(e), (f)] (±S.D.) of ir-PRL [(a), (c), (e)] and ir-GH cells [(b), (d), (f)] in larvae (L) and juveniles (J) of *P. andruzzii*. Averages are always significantly different between larvae and juveniles ($P < 0.05$).

Table I. Average and standard deviation of potential index of PRL and GH production, expressed as percentage of the pituitary volume occupied by ir-PRL and ir-GH cells (respectively, %PRL and %GH) in individuals of *P. andruzzii* of different developmental stages, expressed as days after hatching (dah). From 1.5 to 28 dah, larvae; from 60 to 130 dah, juveniles. For each developmental stage 5 individuals were examined. LT: range of total length. Values followed by different letters are significantly different ($P < 0.05$).

Figure 1

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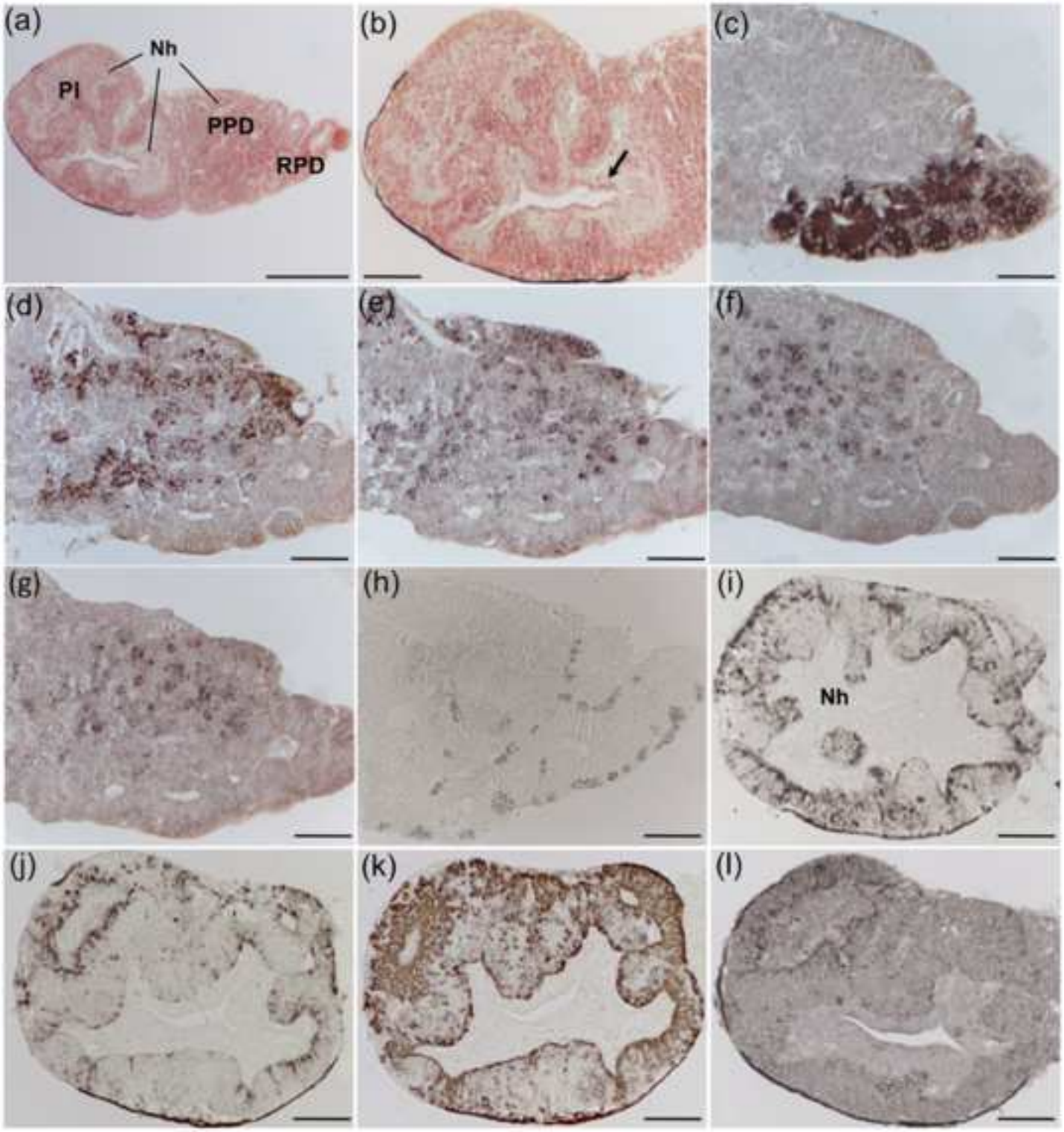

Figure 2

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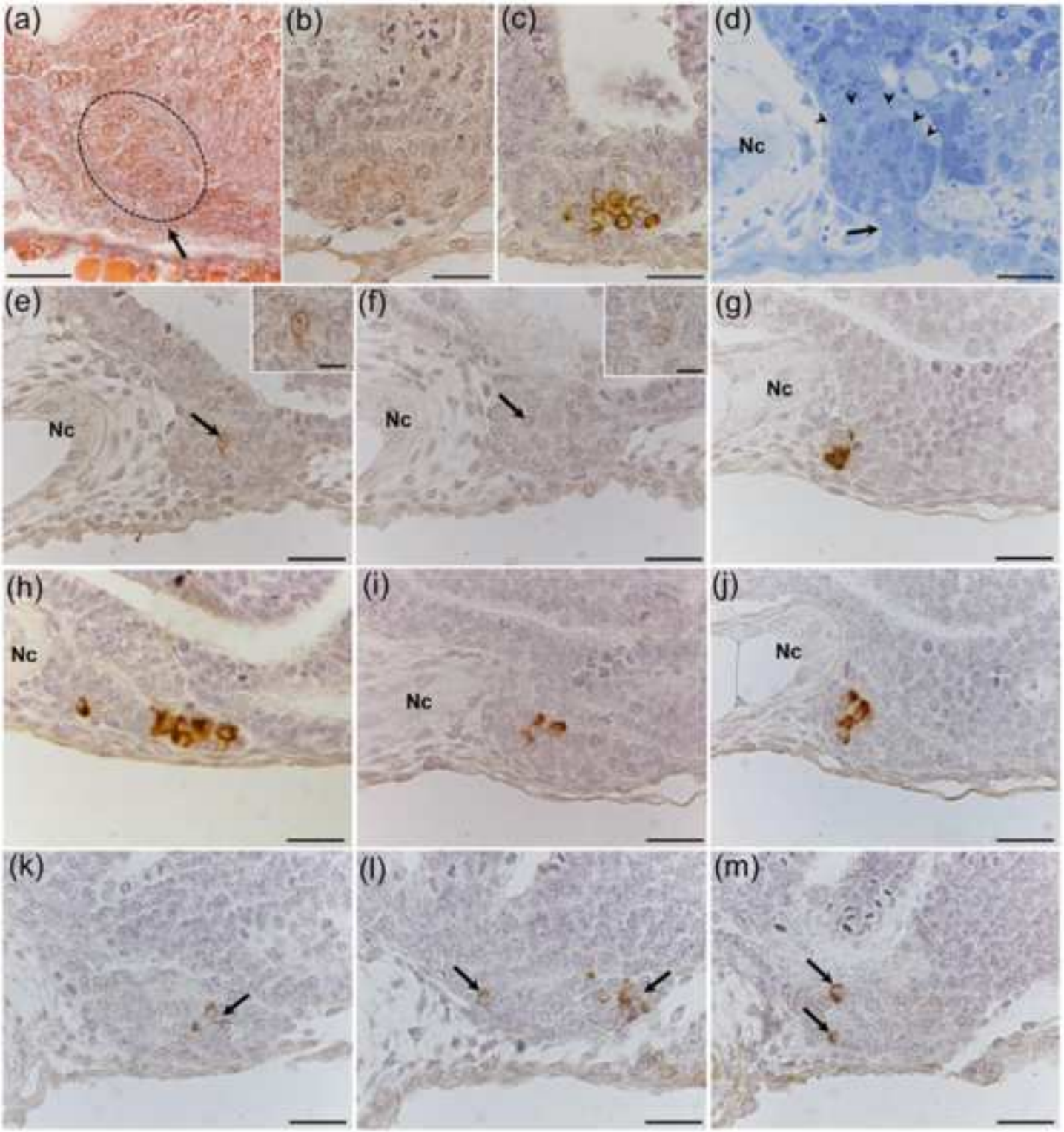

Figure 3

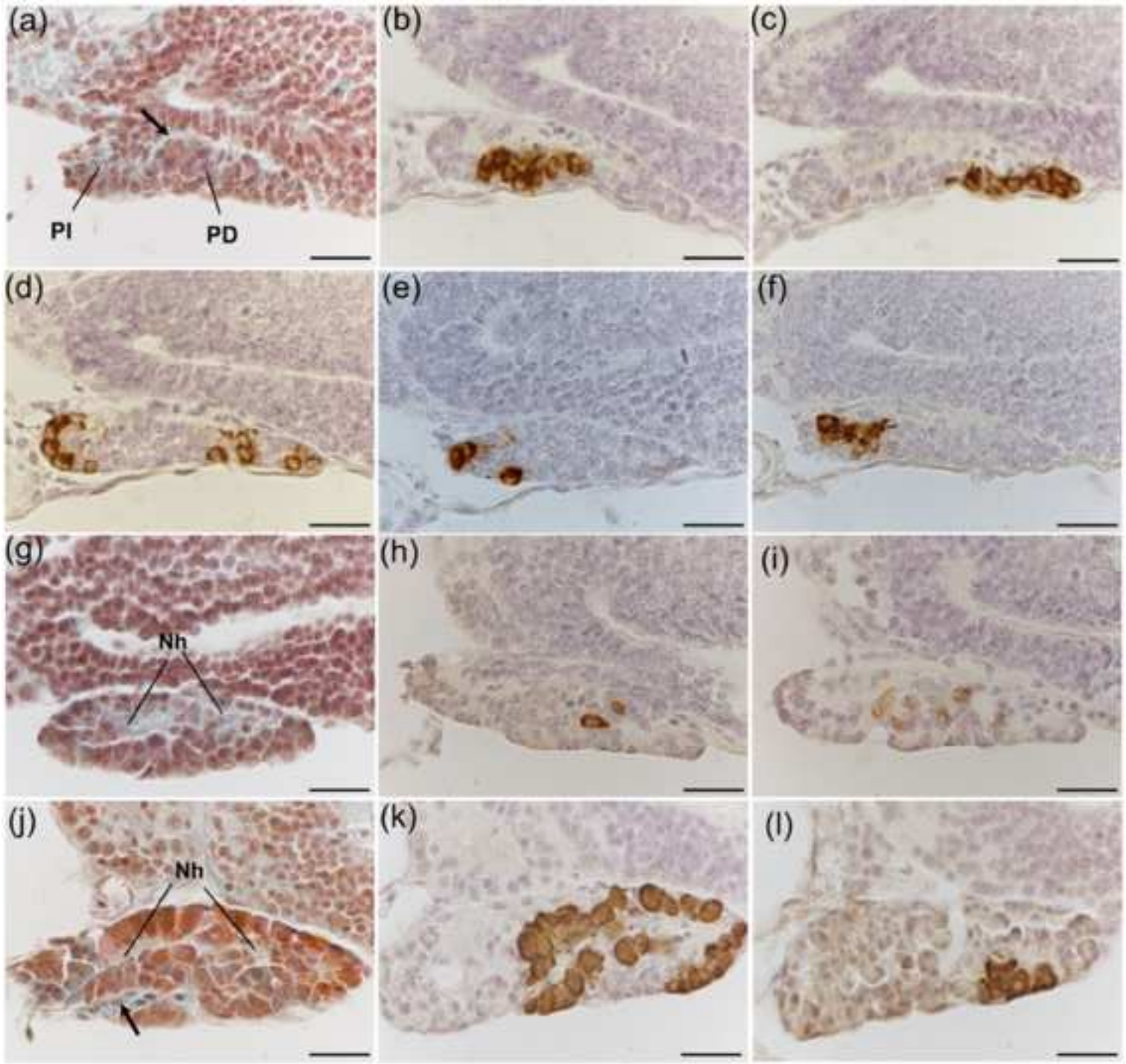

Figure 4

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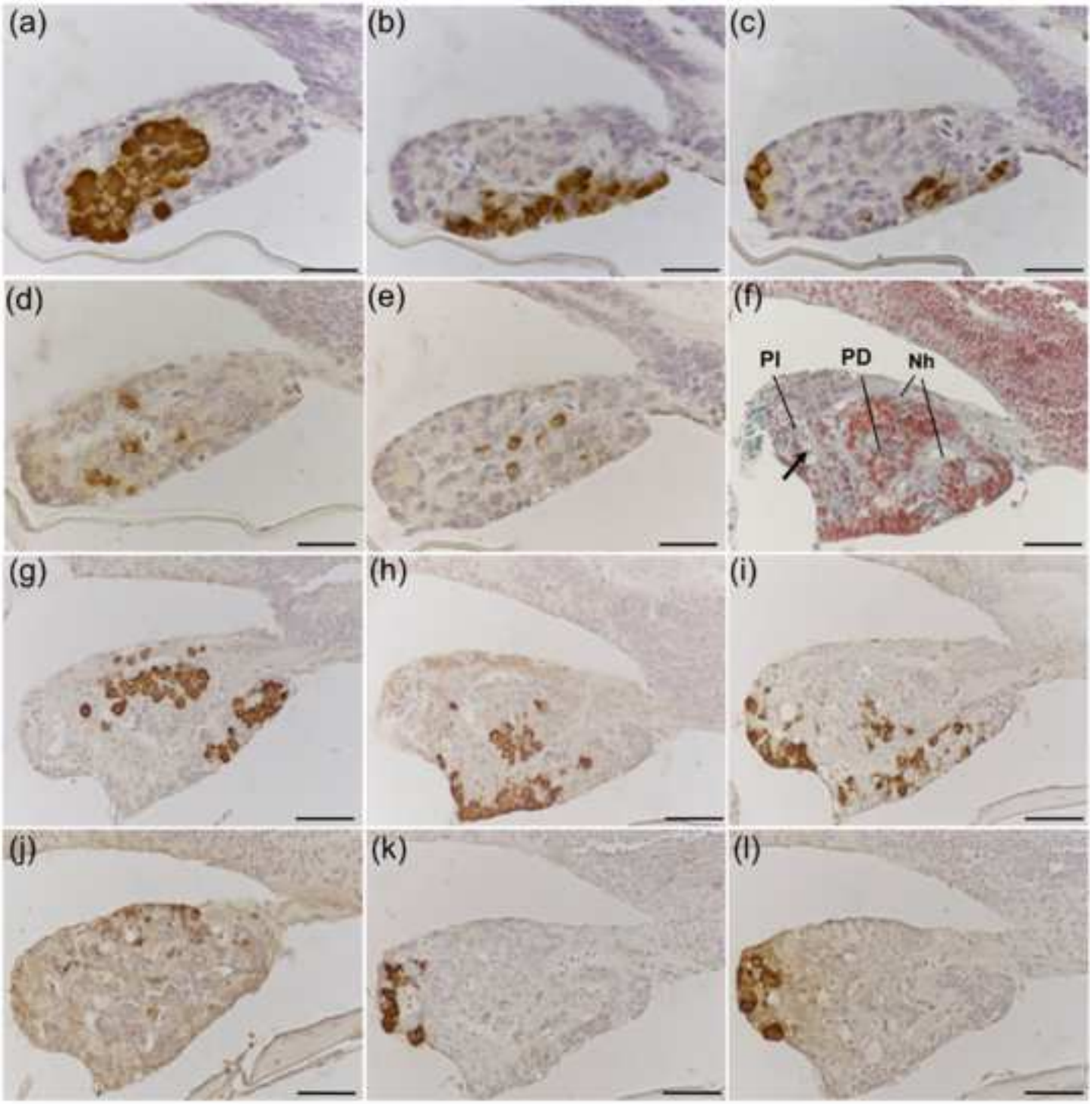

Figure 5

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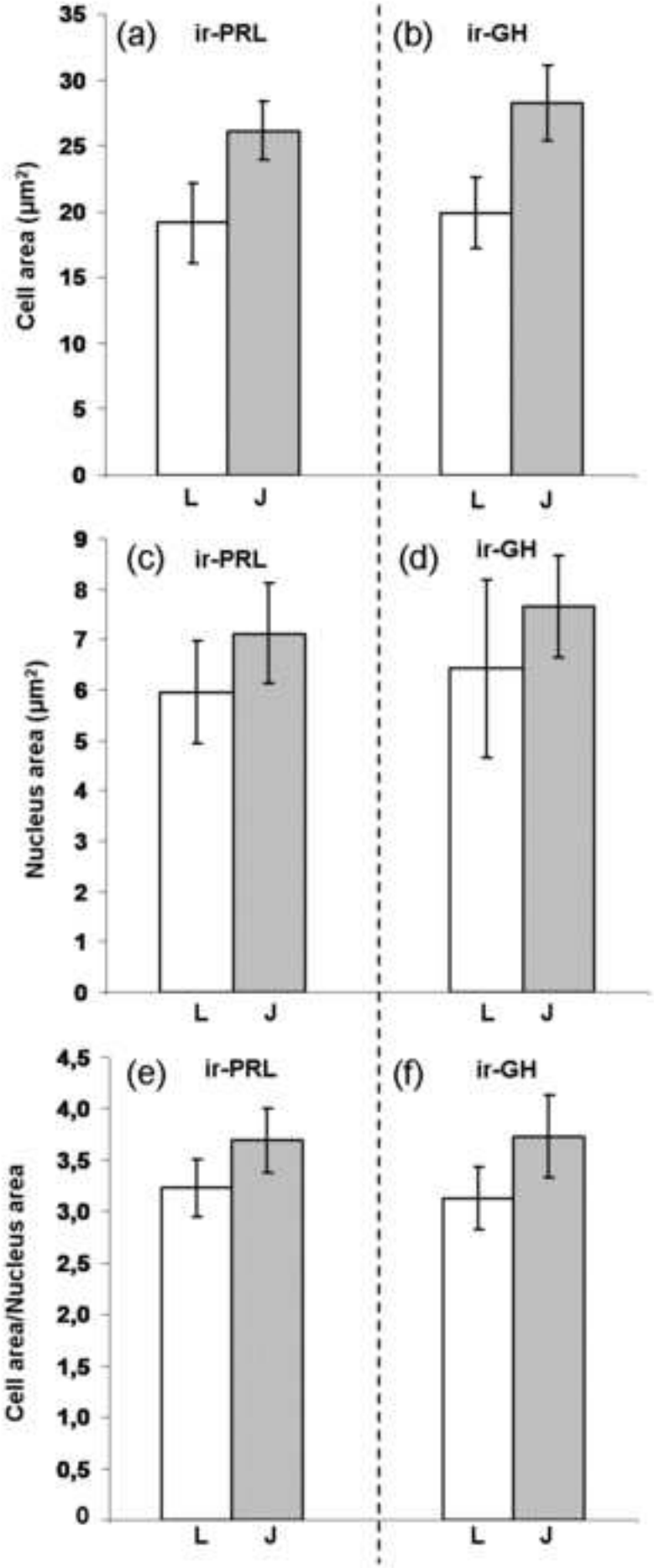

To

Editorial Manager

Journal of Fish Biology

Dear Sirs,

Following the suggestions from Dr David J. McKenzie, Associate Editor, and Dr Neel Aluru, Assistant Editor, we extensively rewrote and reorganized our manuscript, focusing on the ontogeny of the pituitary gland and shortening the Introduction and Discussion sections. Also, we had the manuscript completely revised by one of the authors, an English native speaker.

Therefore we would like to submit our extensively modified manuscript to JFB, under the new title "Immunocytochemical identification and ontogeny of adenohypophyseal cells in a cave fish, *Phreatichthys andruzzii* (Cypriniformes: Cyprinidae)".

Thank you very much for all your suggestions and best wishes.

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