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Immunoreactivity of Corticotrophin Releasing Factor (CRF) and Adrenocorticotropic Hormone (ACTH) in the Developing Digestive Tract of the Nile Tilapia, *Oreochromis niloticus*

Mostafa A. Mousa*, Mohamed F. Kora, Doaa M. El-Sisy, Noha A. Khalil
Aquaculture Division, National Institute of Oceanography and Fisheries, Cairo, Egypt

***Corresponding Author: mostafa_mousa2002@yahoo.com**

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ABSTRACT

Ancient roles of CRF and ACTH in immune–endocrine interactions were obtained early in the development process of fish alimentary canal. The immunolocalization of corticotrophin releasing factor (CRF) and adrenocorticotropic hormone (ACTH) was inspected in the developing gut of *Oreochromis niloticus* larvae. The aim was to investigate a possible involvement of these molecules early in the integration of immunological and endocrine systems. Immediately after hatching, the gut of *O. niloticus* is observed as a straight undifferentiated tube, and with the rapid development, it differentiates into four segments: buccopharinx, esophagus, presumptive stomach and intestine. The immunohistochemical investigation showed the immunolocalization of CRF in the growing digestive tract at all stages (from hatching to 42 days post-hatching). Immunoreaction of CRF was detected in the mucosal epithelium of both the undifferentiated gut and the developing esophagus, stomach, and intestine. Furthermore, CRF immunoreactivity was found in the gastric glands of the stomach. The number of CRF-immunoreactive (ir) cells and the strength of immunoreaction gradually increased as the larvae developed, particularly after the exogenous feeding began; 21 days after hatching. Only the goblet cells of the developing intestine exhibited an ACTH immunoreactivity, which increased at 7dph during the yolk sac resorbtion period. A dramatic decrease was recoded in the number and size of ACTH-ir cells associated with the beginning of the exogenous feeding, and at 28 days post hatching, a very weak immunoreaction was produced. The widespread anatomic distribution and early onset of CRF and ACTH activities, in the developing gut, indicate that these molecules play a functional role in food intake, growth, immunological response, and osmoregulation during *O. niloticus* development, particularly with the start of the exogenous feeding.

INTRODUCTION

The Nile tilapia, or *O. niloticus*, is a quickly growing fish that is found all over the world. As it is widely known, this species makes a substantial contribution to Egypt's freshwater fish polyculture **(Ali *et al.*, 2020)**. As a result, there has been an increased demand among fishpond operators for a sufficient supply of fry and fingerlings of this

species **(Nasr-Allah *et al*., 2021)**. Raising larvae has proven to be very challenging, with significant mortality rates typically occurring in the second and third weeks following hatching **(Cuevas-Rodríguez *et al*., 2017; Ali *et al.,* 2020)**. Given that starvation results from the physical inability to eat after endogenous reserves are exhausted, the cause of these deaths may be physiological in nature **(Ranjan *et al*., 2018; Sakyi *et al*., 2021)**.

Due to their small size and the challenges associated with rearing them, the functional life history of larval stages for many teleost species is poorly understood **(Timmermans, 1987)**. Fish larvae have been difficult to rear, with relatively low survival rates during the larval and juvenile stages, and high mortality has been detected during the early yolk sac stage in hatcheries **(Harboe *et al*., 1994; Ottesen & Bolla, 1998; Santos *et al*., 2021)**. Although the causes are frequently complicated and unknown, microbes have been linked to epizootic fatalities **(Ottesen & Olafsen, 2000; Taha *et al*., 2020; Deng *et al*., 2021)**. Fish take at least a few weeks after hatching to fully develop their unique immune systems **(Ellis, 1988; Chantanachookhin *et al*., 1991; Elkatatny *et al*., 2020)**. Therefore, fish larvae may have to rely primarily on non-specific defense until the appearance of lymphoid cells and the production of immunoglobulins **(Bly *et al*., 1986; Kanlis *et al*., 1995; Williams & Bernier, 2020)**.

Daily adaptation increases occur in teleost larvae, and numerous organs differentiate and become active. The neuro-immune-endocrine integration must occur early in these coordinated processes. Many physiological processes and several known signal molecule families are involved in the communication between the nervous, endocrine, and immune systems. According to reports, CRF controls the thyroid function, food intake, body temperature, and growth **(De Pedro *et al*., 1985, 1993; Watanabe *et al*., 2016; Rousseau *et al*., 2021; Maugars *et al*., 2022)** and the reproductive system **(Rivier & Rivest, 1991; Lovejoy & Hogg, 2021)**. Furthermore, CRF is believed to be the main stimulatory factor and is essential for the secretion of ACTH during the reaction to stress **(Rotllant *et al*., 2000; Van Enckevort *et al*., 2000; Flik *et al*., 2006; Lai *et al*., 2021)**. Moreover, during times of stress, CRF controls cardiac output and the secretion of ACTH from catfish leukocytes in circulation **(Arnold & Rice, 2000; Arai *et al*., 2001; Pohl *et al*., 2001; Pepels *et al*., 2004)**. Thus, more details on modifications to the CRF and ACTH systems in various organs during the Nile tilapia's, *O. niloticus*, larval development may improve our comprehension of the physiological roles of CRF and ACTH in immune response, food intake, and growth during this species' development. This study aimed to explore the anatomical distribution of CRF- and ACTH-immunoreactivity in the digestive system during the larval development of *O. niloticus*.

MATERIALS AND METHODS

Spawning and rearing of larvae

To ensure a high-quality and sufficient number of eggs, brood fish were reared in two ponds prior to spawning. The males and females were kept apart in separate ponds

since January and were fed a 40% protein diet every day. 150– 250g medium-sized tilapia brood fish were utilized. In hapas designated for spawning, semi-natural spawning **(Khalil & Mousa, 2013)** took place on May 1st (temperature: 23– 25°C). 30 fine-mesh 1-m^2 spawning hapas were stocked with brood *O. niloticus* at a ratio of two males and four females per hapa. Every day, breeding activity was checked.

Following the breeding process, the fertilized eggs were gathered, placed in plastic funnels for hatching, and given access to a running water stream. Larvae that had just hatched were placed in glass aquariums and kept at 25ºC. Every aquarium has roughly 500 larvae per aquarium; 10 larvae per liter. Every day, the water in the aquariums was replaced, and the latters were cleaned, and the dead larvae were removed. Pressurized air was used for a gentle aeration. Moreover, ambient photoperiod was used to maintain all aquariums. The larvae were naturally fed with fresh plankton collected from a pond that was previously fertilized using a plankton net.

Sampling and handling of larvae

Eight distinct post-hatched ages of tilapia larvae were sampled. Ten animal groups were anesthetized in a solution of clove oil (40mg/ l) from Sigma on days 0, 1, 2, 3, 4, 7, 21, 35 and 42 after hatching. The animals were then fixed in toto in Bouin's fluid at room temperature for 48 hours. After fixation, the samples were moved to 70% alcohol and dehydrated using a sequence of graded ethanol solutions. They were then cleaned in xylene and embedded in paraplast (M.P. 56– 58˚C). Serial transverse and longitudinal sections, each measuring 5µm in thickness, were then cut and placed on glass slides. Serial transverse and sagittal sections, each 5µm thick, were cut, stained with Harris's hematoxylin **(Conn, 1953)**, and counter-stained in an aqueous solution of eosin for microscopical inspection.

Method of immunohistochemistry

Antibodies

The National Institutes of Health provided a rabbit antiserum against human ACTH. Dr. Nigel Brooks generously donated rabbit anti-ovine CRF (MRC Reproductive Biology Unit, Centre for Reproductive Biology, Edinburgh, Scotland).

Immunohistochemical reactions

As previously mentioned, vectastain ABC (Avidin-biotin peroxidase complex) Kit (Vector Laboratories) was typically used for immunohistochemical staining **(Mousa & Mousa, 1999)**. To summarize, the sections were deparaffinized in xylene and then rehydrated using graded ethanol, and two 10-minute washings in phosphate-buffered saline (PBS; pH 7.4). Except as noted, all incubations were carried out at room temperature, and PBS was used to wash the samples three times for a total of twenty minutes following each step. The sections were incubated for 45 minutes with 10% methanol, 0.3% H2O2, and PBS in order to inhibit endogenous peroxidase. The sections were incubated for 60 minutes in PBS containing 0.3% Triton X-100, 1% BSA, 4% goat serum (GS), and 4% horse serum (block solution) in order to prevent nonspecific binding.

After that, the sections were incubated with the subsequent antibodies for an entire night at 4°C: a polyclonal antibody of rabbit (1:500) against human ACTH or a polyclonal antibody of rabbit (1:1000) against ovine CRF. Next, the sections were incubated for one hour with Vector Laboratories' biotinylated secondary antibody and for forty-five minutes with avidin-biotin-conjugated peroxidase. Afterward, the sections were cleaned and stained for 3- 5 minutes using 3, 3-diaminobenzidine tetrahydrochloride (DAB) (Sigma) with 0.01% H2O2 in 0.05M Tris-buffered saline (pH 7.6). The sections were cleaned using tap water, dehydrated in alcohol, cleared in xylene, and mounted in DPX following the enzyme reaction.

RESULTS

Histological changes during alimentary tract development

During the early life stage, after hatching, the alimentary canal was observed as a straight tube, and histologically undifferentiated and positioned dorsally in relation to the yolk sac. *O. niloticus* larvae underwent fast developmental changes within 7 days of hatching, resulting in the distinguishing of the gut into 4 segments: the buccopharinx, esophagus, presumptive stomach, and intestine (Fig. 1). The last organ in the digestive system to differentiate was the stomach. Following yolk sac resorbtion at 7dph, clusters of cuboidal cells were visible in the future fundic region, which would develop into gastric glands. (Fig. 1a, b). At 7dph, a dilation of the posterior esophagus, lined by a basic cuboidal epithelium, began to differentiate as the prospective stomach (Fig. 1a, b). A primitive pyloric sphincter, which divides the future stomach from the anterior section of the intestine, began to form at 21dph (Fig. 1c, d). The stomach developed a pouch at 28dph, and its epithelium started to separate, allowing the fundic and pyloric regions to be identified (Fig. 1c- f). The stomach's folded mucosa in the pyloric region was kept apart from the anterior intestine by the pyloric sphincter, which was lined by a short ciliated columnar epithelium (Fig. 1c). The stomach wall, which is thicker in the pyloric region, was made up of circular muscle fibers, some blood vessels, a thin serosa with squamous cells, submucosa, and mucosa (Fig. 1c, d). At 28dph, submucosal acinar cell aggregates, primarily centered in the fundic area, giving rise to gastric glands (Fig. 1e, f).

A basic ciliated columnar epithelium with median to basal nuclei lined the newly hatched larvae's primitive gut (Fig. 1g). At the onset of exogenous feeding, the intestinal lumen expanded. but there was no intestinal mucosa folding, and from 7dph onward, tiny, non-staining vacuoles were visible in the cytoplasm of intestinal enterocytes (Fig. 1h). Primordial mucosal folding started at 21dph and by 28dph, the anterior intestine looked well developed (Fig. 1i). From 28dph, goblet cells were seen scattered among the intestinal enterocytes (Fig. 1i).

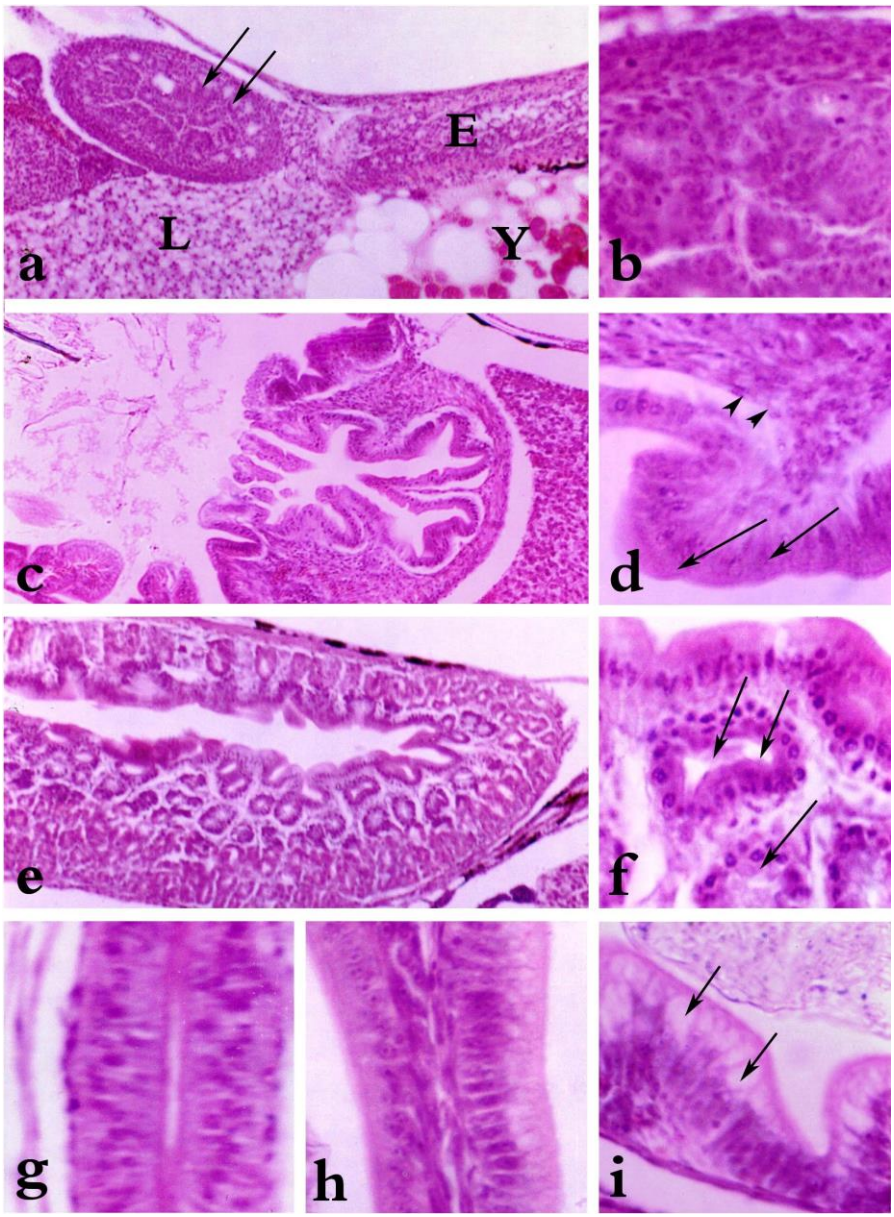

Fig. 1. Parts of sagittal sections of *O. niloticus* larvae stained with hematoxylin and eosin showing: **a)** Larvae at 7 days post-hatching showing presumptive stomach (arrows), beside (E) esophagus, (L) liver, and (Y) yolky material. X100; **b)** A magnified portion of **(a)** showing clusters of cuboidal cells (future fundic region) that would develop into gastric glands. X400; **c)** Larvae at 21dph showing primordial pyloric sphincter, separating the future stomach from the anterior portion of the intestine; **d)** A magnified portion of **(c)** showing mucosa (columnar epithelium) (arrows) and submucosa (arrowheads) of the wall of the pyloric region. X400; **e)** Larvae at 28dph showing fundic region of developed stomach. X100; **f)** A magnified portion of **(e)** showing gastric glands (arrows) appeared as submucosal acinar cell aggregates. X400. **g)** newly hatched larvae showing undifferentiated intestine lined by a simple ciliated columnar epithelium. X400; **h)** Larvae at 7 days post-hatching showing the intestinal wall with no folding of the mucosa. X400. **i)**, Larvae at 21dph showing folding of the mucosa and the appearance of goblet cells (arrows). X400

Immunoreactivity of CRF and ACTH during alimentary tract development

Immunohistochemical analysis was used to examine the distribution of CRF and ACTH in developing *O. niloticus* larvae between 0 and 35dph.

CRF immunoreactivity

According to the immunohistochemical analysis, CRF was found in the growing digestive tract (Figs. 2, 3). The immunoreaction of CRF was found in the esophagus, stomach, and intestine, three distinct areas of the developing digestive tract (Figs. 2-3). Moderately, CRF immunoreactivity was found in the mucosal layer of the undifferentiated digestive tube at hatching (0dph) (Fig. 2a). After the yolk sac resorbs at 7dph, the digestive tract differentiates into distinct regions, and strong CRF immunoreactivity is limited to the mucosal epithelium of the esophagus, stomach, and intestine (Fig. 2b, c). Furthermore, CRF immunoreactivity was detected in the gastric glands of the stomach (Fig. 2b, c, d, f). There was a relative decrease in CRF immunoreactivity at 12dph, following the completion of yolk sac resorbtion but prior to the full development of the stomach (Fig. 3a, b). CRF immunoreactivity was elevated in the pyloric region of the developing stomach at 21dph, following the start of exogenous feeding and the differentiation of the fundic and pyloric portions of the stomach (Figs. 3c-f).

ACTH immunoreactivity

According to the immunohistochemical analysis, ACTH was only restricted to the intestine (Fig. 4). Following hatching, certain cells in the mucosal layer of the undifferentiated alimentary canal exhibited an ACTH immunoreactivity during the early life stage (Fig. 4a). The goblet cells of the developing intestine were found to exhibit strong ACTH immunoreactivity at 7dph during the period of yolk sac resorbtion (Fisg. 4b, c). When exogenous feeding began, ACTH immunoreactivity demonstrated a marked decline in ACTH-ir cell quantity and size, resulting in extremely low immunoreactivity at 28dph (Figs. 4d, f).

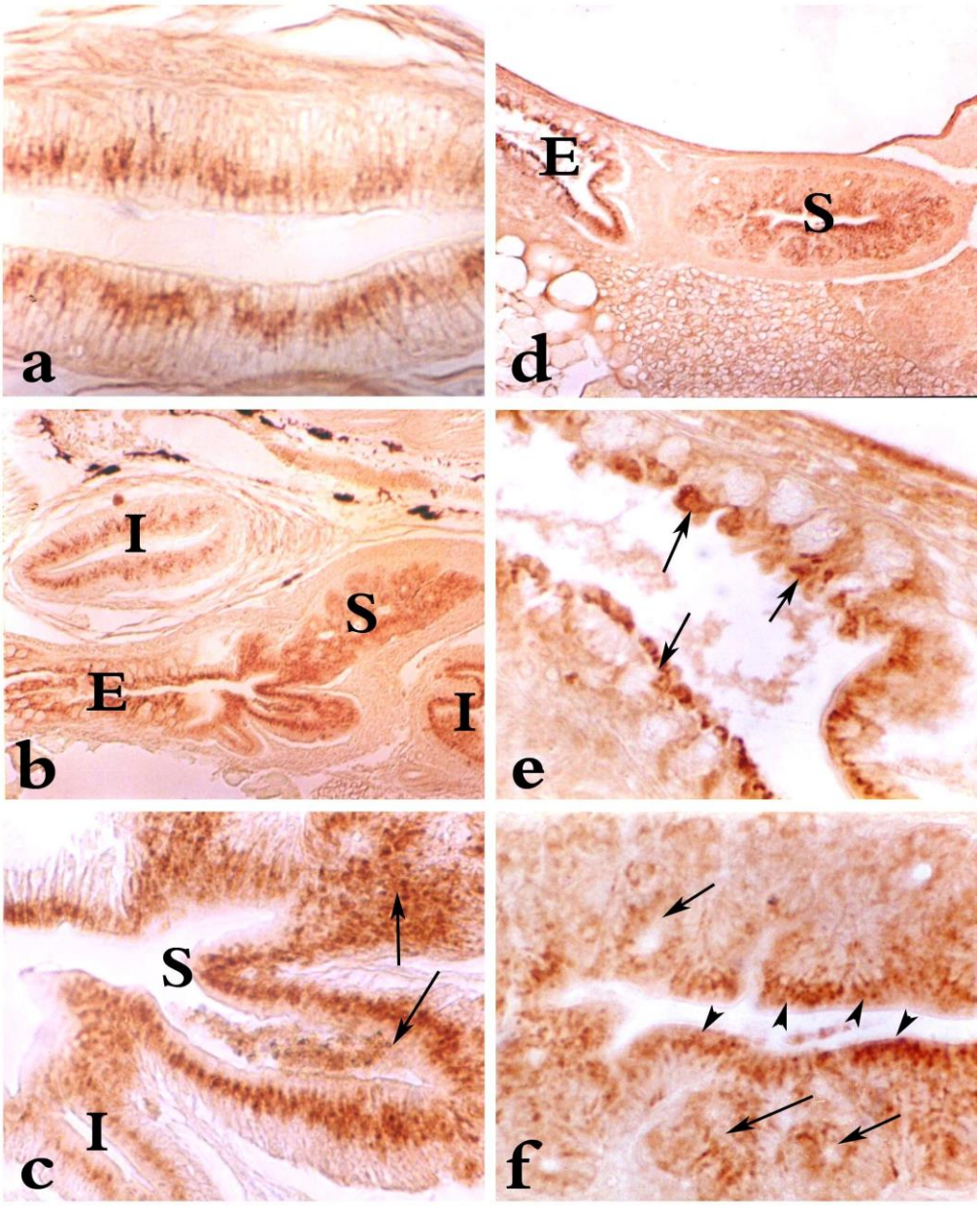

Fig. 2. Sagittal sections of *O. niloticus* larvae immunostained with rabbit polyclonal antibody against ovine CRF. **a)** Newly hatched larvae showing undifferentiated intestine exhibiting moderately immunoreactivity in mucosal layer; X400. **b)** Larva at 7dph showing strong immunoreactivity in the (E) esophagus, (S) stomach and (I) intestine; X100. **c)** A magnified portion of **(b)** showing CRF strong immunoreactivity in the gastric glands (arrows) of the stomach; X400. **d)** Larva at 10dph showing CRF immunoreactivity in the (E) esophagus and (S) stomach. **e)** A magnified portion of **(d)** showing CRF strong immunoreactivity in the mucosal epithelium of esophagus (arrows); X400. **f)** A magnified portion of **(d)** showing CRF immunoreactivity in the mucosal epithelium (arrowheads) and the gastric glands (arrows) of the stomach; X400

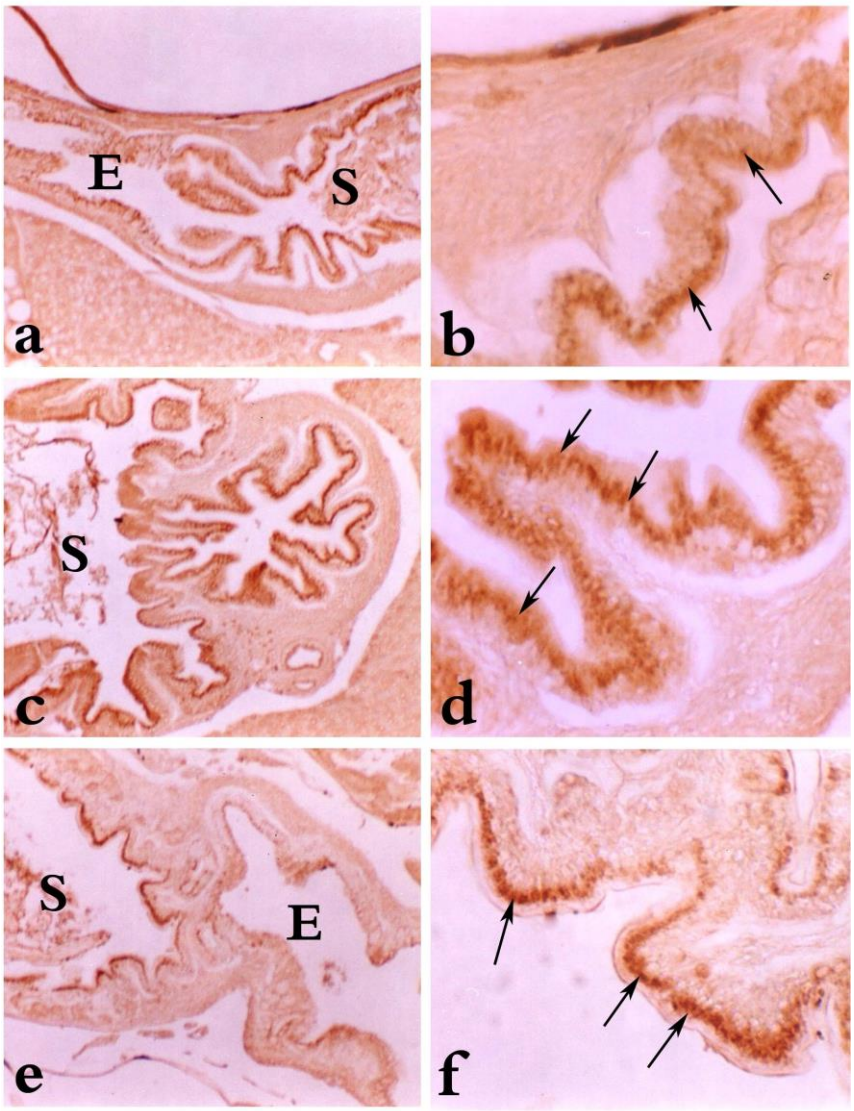

Fig. 3. Sagittal sections of *O. niloticus* larvae immunostained with rabbit polyclonal antibody against ovine CRF; **a)** Larva at 12dph showing CRF immunoreactivity in the (E) esophagus and the pyloric portion of the (S) stomach ;X100; **b)** A magnified portion of **(a)** showing CRF moderately immunoreactivity in the mucosal epithelium of the pyloric portion of the stomach (arrows); X400. **c)** Larva at 21dph showing CRF immunoreactivity in the (S) stomach; X100. **d)** A magnified portion of **(c)** showing CRF strong immunoreactivity in the mucosal epithelium of the pyloric portion of the stomach (arrows); X400. **e)** Larva at 35dph showing strong CRF immunoreactivity in the (S) stomach and weak immunoreactivity in the (E) esophagus; X100. **f)** A magnified portion of **(e)** showing CRF strong immunoreactivity in the mucosal epithelium of the pyloric portion of the stomach (arrows); X400

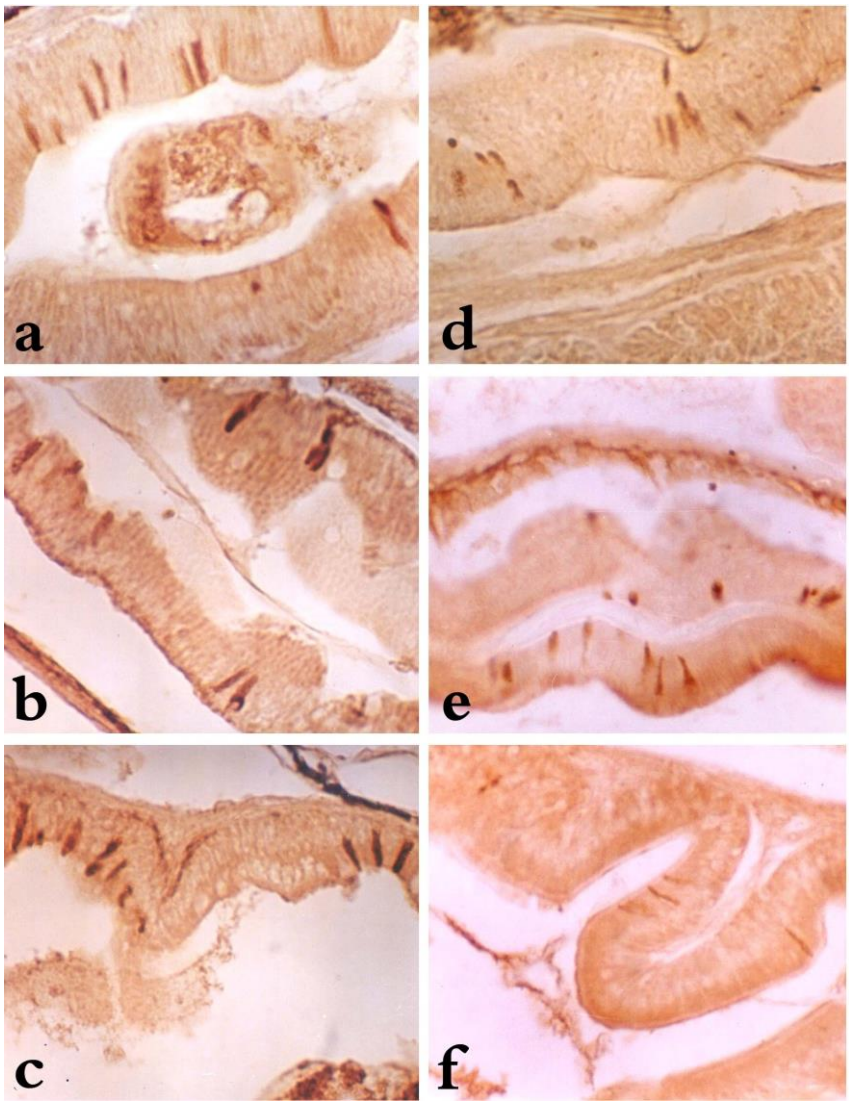

Fig. 4. Sagittal sections of *O. niloticus* larvae immunostained with rabbit antibody against human ACTH. X400. Immunostaining is observed mainly in some cells of mucosal layer of the undifferentiated alimentary canal immediately after **(a)** hatching, and in the **(b- f)** goblet cells of the developing intestine. **a)** 0dph larva. **b)** 7dph larva. **c)** 10dph larva. **d)** 15dph larva. **e)** 21dph larva. **f)** 28dph larva. Note, that the beginning of external nutrition, ACTH immunoreactivity showed significantly decrease in both size and number of ACTH-ir cells, which gave very weak immunoreaction at 28dph

DISCUSSION

The digestive system of *O. niloticus* larvae underwent ontogeny in a manner comparable to that of most teleost fish species that have been characterized thus far. After seven days of hatching, or the initial feeding, the digestive tract of *O. niloticus* differentiated into four morphologically distinct regions: the buccopharynx, esophagus,

presumed stomach, and intestine. Before *O. niloticus* transitioned to exotrophic feeding, the immunocytochemical results demonstrate that immunoreactivity to antibodies against CRF and ACTH, which are well-known for being involved in stress response **(Stefano *et al*., 1996; Ottaviani *et al*., 1997; Mola *et al*., 2004; Pepels & Balm, 2004; Flik *et al*., 2006; Lai *et al*., 2021)**, existed in the gut of the larva from an early stage (0dph). In *Dicentrarchus labrax*, immunostaining for CRF is localized in nerve fibers of the gut wall from the pharynx to the former gut at eight days following hatching, while the larvae are still feeding on yolk. A similar pattern of immunolocalization is observed in 24-day-old larvae, with the addition of large cells immunopositive to CRF found in the wall of the midgut and hindgut **(Mola *et al*., 2011)**.

Only the goblet cells of the developing intestine exhibited ACTH immunoreactivity in the current study, and these cells demonstrated a strong immunoreaction between 7 and 10dph. When exogenous feeding began, ACTH-ir cells' size and number dramatically decreased, and at 28dph, they produced a very weak immunoreaction. Similar immunolocalization of ACTH was demonstrated in the digestive tract of *Dicentrarchus labrax* **(Mola *et al*., 2004)**. CRF and ACTH are major players in the stress response **(Ottaviani *et al*., 1997; Mola *et al*., 2004; Pepels & Balm, 2004; Flik *et al*., 2006; Lai *et al*., 2021)**. The finding of CRF- and ACTH-like materials by immunohistochemistry in the same digestive tract regions of *O. niloticus*, where the gut-associated lymphoid tissue (GALT) will differentiate, raises the possibility that CRF and ACTH are involved in early defense mechanisms in *O. niloticus* before the growth of immune responses mediated by cells in GALT.

The early immunoreaction of CRF in the developed *O. niloticus* along with its dissemination into the esophagus, stomach, and intestine, additionally its elevated immunoreactivity during larval development, imply that CRF may participate in the processes of immune defense. Furthermore, CRF-related peptides may mediate the appetite-suppressing effects of subordination stress on fish, which may have a physiological function in controlling food consumption **(Bernier, 2006; Conde-Sieira *et al*., 2018; Rupia *et al*., 2023)**. Additionally, central nervous system endogenous CRF plays a function in promoting fish larvae's locomotor activity **(Clements *et al*., 2002; Faught & Vijayan, 2022)**.

Primary neurohormone CRF is accountable for controlling the release of ACTH in mammals **(Vale *et al*., 1997)**. Additionally, it works in fish and other vertebrates as an effective ACTH secretagogue **(Ando *et al*., 1999; Bernier *et al*., 1999; Flik *et al*., 2006; Lai *et al*., 2021)**. According to research results, this peptide may have a primary function in vertebrates as a dual hypophysiotropic agent acting on both the interrenal (adrenal) and thyroid axes **(Denver, 1999; Boorse & Denver, 2004; Kaneko *et al*., 2005; Watanabe *et al*., 2016; Rousseau *et al*., 2021)**. Thyroid hormones are known to increase the survival rates of teleost larvae and hasten the immune system's growth and development

(Power *et al*., 2001; Gavlik *et al*., 2002; Lam *et al*., 2005; Rousseau *et al*., 2021; Lazcano *et al*., 2023).

The distribution of ACTH in *O. niloticus* during its early larval stages points to a paracrine/autocrine mode of action. The localization of extra-pituitary ACTH has been reported in both mammalian and non-mammalian species, and its function in immune-regulation has been studied **(Ottaviani *et al*., 1997; Feng *et al*., 2022)**. Regarding chemotaxis and phagocytosis, human peripheral blood mononuclear cells and invertebrate immunocytes exhibit an enhanced bacterial phagocytosis upon stimulation of cell migration by ACTH (1-24) and other fragments **(Genedani *et al*., 1990, 1994; Ottaviani *et al*., 1990, 1994; Feng *et al*., 2022)**. Given that *O. niloticus*'s gut exhibits ACTH-like immunoreactivity, its function in osmoregulation regarding salinity fluctuations over the first 28 days of life may also be explained. The results presented here highlight the significance of both CRF and ACTH in osmoregulation, immunological response, feeding behavior, and growth during *O. niloticus* development.

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Conflict of Interest

According to the author, there are no conflicts of interest.

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